The 6-hydroxydopamine induces changes in rat brain glutathione S-transferase

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RESUMEN. Los parámetros técnicos que influyen en la eficacia de la lesión con 6-hidroxiadopamina (6-OHDA) están normalizados, sin embargo, sólo una parte de los animales lesionados alcanzan un elevado grado de reducción dopaminérgica estriatal (94-99 %) con una conducta rotatoria de más de 620 vueltas/90 min inducidas por D-amfetamina. Animales con estas características se denominaron como totalmente lesionados (ATL). Con el objetivo de estudiar las diferencias entre los diferentes grupos, se determinaron las concentraciones de dopamina (DA) y sus catabolitos en el estriado de animales no lesionados, parcialmente lesionados (APL). Menos de 620 vueltas/90 min) y ATL. Además, se determinó la actividad específica de la glutatión S-transferasa (GST) y los parámetros cinéticos para la enzima purificada del cerebro de animales no tratados, APL y ATL. La reducción dopaminérgica estriatal fue mucho mayor en los animales a ATL que en los APL. La actividad de la GST mostró un significativo incremento bilateral en el grupo APL con respecto a los no tratados. En los TLA el incremento de la actividad de la GST fue menor y sólo significativamente mayor en el estadio lesionado. Estas diferencias también se ver manifiestas en los parámetros cinéticos de la enzima purificada del cerebro en los diferentes grupos. Estos resultados pudieran evidenciar una participación de la GST sobre la eficacia de la lesión en los APL, sugiriendo por tanto una participación de las enzimas de la GST en la neuroprotección y recuperación del tejido dopaminérgico.

INTRODUCTION

The oxidative stress hypothesis is one of the most accepted to explain the etiology and progression of idiopathic Parkinson's disease. This theory points to an increase in free radical generation by dopamine (DA) metabolism, accompanied with insufficient scavenging mechanisms, as key factors contributing to the neuronal loss in the substantia nigra pars compacta. Low levels of reduced glutathione (GSH), ascorbate, peroxidase and catalase activities, as well as, increments in iron, lipoperoxides, gamma-glutamyl transpeptidase and superoxide dismutase have been found in the postmortem parkinsonian brain.

The 6-hydroxydopamine (6-OHDA) lesion in the nigrostriatal pathway is a widely used animal model of Parkinson's disease, since this dopamine analog specifically destroys dopaminergic and noradrenergic afferents. The molecular events linked to the neurotoxicity also involved oxygen active species, besides cytotoxic quinones non-enzymatically generated.

The technical parameters which influence lesion (injection flow rate, animal age, anesthetic, etc.) are standardized, but in spite of that, only a part of the animals subject to the procedure becomes totally lesioned (TLA) with 94-99 % of striatal dopamine (DA) depletion and more than 620 turns/90 min. In amphetamine-induced circling behavior test, this fact could be related to anatomic differences and inaccuracies in the...
Injection placement, but it is also possible the influence of inter-individual dissimilarities with respect to the sensitivity of the dopaminergic neurons. The group of animals, suffering a lesser extent of damage, could be relying on a more capable molecular defensive machinery.

Conjugation with GSH is considered to be an important detoxifying reaction for electrophilic compounds, both endogenous and exogenous. In general this reaction is catalyzed by the glutathione S-transferase (GST), a multigene family of isoenzymes widely distributed in animal tissues. Brain GST is mainly found in the glial component, but it is also present in neurons. GST activity is heterogeneous distributed in rat brain. This distribution is altered both in animals with lesions in the septohippocampal pathway and in aged animals.

The objective of this work was to study the involvement of GST on the putative interindividual lesion response differences. For that, it was first measured the levels of DA and its catabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striata of untreated animals, partially lesioned animals (PLA), less than 620 turns/90 min) and TLA; then the authors assayed GST specific activity in the same brain region. Besides, it was purified GST from the whole brain of untreated animals, PLA and TLA, so as to know the kinetic parameters of the enzyme family in the experimental groups.

MATERIALS AND METHODS

Chemicals

In the assays it were employed 1-chloro-2,4-dinitrobenzene (CDNB) and bovine serum albumin (BDH, Analar); GSH and HClO4 70 % (Merek); coomassie brilliant blue G-250, 6-OHDA (SIGMA); epoxy-activated Sepharose 6B (Pharmacia LKB). All other chemicals used were of analytical degree.

Animals

Sixty-six male Wistar rats (Centro Nacional de Producción de Animales para Laboratorio, Ciudad de La Habana), weighing 200-250 g at the start of the experiment, were used. These animals were housed in a room with controlled temperature, under a 12 h light/dark period and with free access to food and water.

Thirty-nine rats were anesthetized with chloral hydrate (420 mg/kg body weight), injected in a stereotaxic apparatus, and injected on the right nigrostriatal pathway with 3 mL 6-OHDA (8 mg dissolved in saline with ascorbic acid 0.2 mg/mL) at a flow rate of 1 mL/min. After the injection the syringe was allowed to remain in the brain for 5 min. The stereotaxic coordinates were: posterior to bregma, 4.4 mm; lateral, 1.2 mm; ventral to the skull surface, 7.8 mm.

Following 30 d, the treated rats were kept with amphetamine (5 mg/kg body weight, i.p.-induced circling behavior with a rotometer RotorCIF (Instituto Central de Investigación Digital, Ciudad de La Habana). The rats showed more than 620 turns during a 90 min test time was considered TLA, and the ones turning 200-619 times in the same period formed the group of PLA.

Tissue sampling

After behavioral testing, animals were sacrificed by cervical dislocation and decapitated, their brains were removed and rinsed in cold saline. A group of brains (without the cerebellum) (5 from untreated animals, 5 from PLA and 5 from TLA) were kept at -70 °C for GST purification. The others were used for the dissection of striata which were also kept at -70 °C.

Assays

For the simultaneous determination of DA, DOPAC and HVA, 23 striata (9 from untreated animals, 6 from PLA and 8 from TLA) were homogenized (1:10, W:v) in a glass-teflon potter with HClO4 0.4 mol/LNaHSO3 0.11 % (containing 40 mg/mL dihydroxybenzylamine as internal standard) in an ice-bath. Homogenates were centrifuged at 10000 g for 10 min at 4 °C. The concentrations of DA, DOPAC and HVA were determined in the protein-free supernatant by a High Performance Liquid Chromatography procedure with electrochemical detection using dihydroxybenzylamine as internal standard. GST activity was assayed with CDNB as electrophilic substrate according to the method of Habig. GST activity was assayed with CDNB as electrophilic substrate according to the method of Habig.

GSH and HCLO4 were prepared by varying in the standard assay the concentrations of substrates (GSH in the range 0.5-5.0 mmol/L, CDNB in the range 0.2-2.0 mmol/L). The concentration of purified enzyme was fixed at 1.25 mg/mL. The concentration of GSH in Km (CDNB) testing was fixed at 5.2 mmol/L while CDNB in Km (GSH) testing was fixed at 2.2 mmol/L. The values of Km and Vmax were calculated from double reciprocal plots (Lineweaver-Burk plots) as the negative intercepts of the substrate concentration vs reaction velocity linear regression.

For the in vitro inhibition study, the purified enzyme was exposed to 6-OHDA in an amount range 0.10-1.00 mmol with a final volume of 1 mL at 25 °C. The substrate was added to the concentrations of the standard procedure. The concentration of inhibitor resulting in 50 % inhibition, the IC50 value, was determined from plots of the remaining activity compared with 6-OHDA concentration.

Each point in these kinetic curves represented the means result of a triplicate assay.

Statistical analysis

Group of striatal DA, catabolites and GST mean values were compared by Mann-Whitney's U non-parametric test.

RESULTS

Striatal levels of DA, DOPAC and HVA

Striatal DA content profoundly decreased in PLA and TLA at the side of injection, but the depletion was more pronounced in TLA (p < 0.001) which showed 5.8 % of remaining neurotransmitter levels, unlike PLA.
DOPAC levels also suffered a fall in the striata ipsilateral to the lesion, without noticing statistically significant differences between the lesioned groups. The HVA levels in the lesioned striata of both groups decreased in a lesser extent than the other substances and there were not significant differences between PLA and TLA. Unexpectedly, TLA showed a higher HVA concentration than the one for the untreated group in the contralateral striatum (Fig. 1).

There were no inter-hemispheric differences in the untreated group. In PLA it was found differences between opposite sides for DA and DOPAC levels. TLA exhibited interhemispheric differences for the three detected compounds (Fig. 1).

The decrease of DA catabolites in lesioned striata was less profound than the DA depletion itself, which results in high ratios catabolites/DA. This situation was especially noteworthy in the ratio HVA/DA for the lesioned striata of PLA and TLA with values 3.0 and 12.0 folds respectively higher than untreated group's corresponding striata. These values showed differences (p < 0.01) between these two lesioned groups (Fig. 2).

Striatal GST activity

The unilateral 6-OHDA lesion provoked an increase in striatal GST activity in both lesioned groups (Fig. 3), although PLA exhibited a higher increase (p < 0.01) than TLA (p, 0.05) when they were compared to the untreated rats. There were no significant inter-hemispheric differences for the groups (p > 0.10).

Kinetic studies

Untreated animals and PLA showed similar specific activities and K_m (GSH) values for the GST purified from their brains. It was found a 33 % lower GST specific activity and more than ten-fold higher K_m (GSH) for the enzyme purified from the TLA brains with respect to the enzyme from untreated animals and PLA. Regarding the K_m (CDNB), it was found the highest value for enzyme from PLAs brains. The enzyme from the latter experimental group also exhibited the greatest V_max, followed in decreasing order, by untreated animals and TLA.

It was found that GST purified from TLA brains is three-fold more susceptible to 6-OHDA in vitro inhibitory effect, because of its higher K_m value, when compared to the other groups. The enzyme from untreated animals and PLAs brains behaved similarly in this study (Table 1).

DISCUSSION

It has been widely demonstrated a remarkable depletion of DA, DOPAC and HVA in the striatum lesioned by 6-OHDA, in which a partial restoration can be achieved by the graft of fetal mesencephalic cells. The behavioral dissimilarity between PLA and TLA might be explained by the significant differences (p < 0.001) in striatal DA content found in this work (Fig. 1).

The HVA/DA ratio is considered as an index of DA turnover, and therefore, it may reflect monoamine...
oxidase activity. It has been proposed that the compensatory increase in DA turnover occurring in the remaining dopaminergic neurons in Parkinson’s disease leads to increased free radical formation and oxidative stress. These results indicate a 4-fold higher striatal DA turnover in TLA than in PLA (Fig. 2), which may itself contribute to a greater overload of scavenging mechanisms, and then, a more premature cellular death.

The highest HVA concentration in the intact striatum of TLA leads to a highest ratio HVA/DA, when it is compared with the corresponding side of untreated and PLA (Fig. 2). At the present time it is hard to explain this intriguing finding.

It was found a bilateral increase of striatal GST activity in PLA group, while TLA exhibited a minor and ipsilateral augment (Fig. 3). This fact suggests an involvement of the enzyme in the antioxidant events that possibly allow a better protection of PLA against neurotoxic damage. This suggestion is according to the transcripational activation effects exerted by oxygen active species on GST expression. Besides, some GST isoenzymes display a selenium-independent glutathione peroxidase activity by conjugating organic peroxides to GST. This activity could be important in neutralizing the cytotoxic products of 6-OHDA metabolism.

Bilateral responses, especially astroglialosis, have been observed before in other experiments involving unilateral treatments. No mechanisms underlying this phenomenon have been proved yet, but several possibilities are considered, including the operation of a diffusible brain-derived substances. Since GST is a mainly glial enzyme, the bilateral response found by authors in this study is supported by the above mentioned reports and might reflect a possible neuro-glial relationship.

Brain GST is a set of about seven cytosolic isozymes belonging to four classes: alpha, mu, pi and theta. Except for the theta class, all these proteins bound to GSH-affinity matrices. Besides these, there are microsomal and nuclear species. Because of this fact, the tested kinetic parameters represent global values of a molecular population, but they are useful to evaluate and compare the change of the enzyme behavior in the experimental groups.

The analysis of these constants (Table 1) reveals differences that tentatively reflect a variation in the isozyme pattern after lesion. The lowest Wmax. and Specific activity, together with the highest Km[GSH] (resulting in a low affinity towards this substrate) of the enzyme purified from TLA brains indicate its lower catalytic efficacy. Besides that, GST from TLA brains is more susceptible to 6-OHDA inhibition. Thus, in the first moments after lesion (before the clearing of the neurotoxin) the brain of TLA were probably less capable of conjugating toxic products from 6-OHDA metabolism due to a stronger uncompetitive inactivation suffered by GST in this group.

On the other hand, the elevated Km[CDNB] of the enzyme from PLA’s brains suggests the induction of the isozyme Aγ, or Yγ, belonging to the alpha class and that is present in rat brain. This isozyme has been reported to have a low affinity towards CDNB and high affinity towards peroxidative products. Moreover, it has also been found that the expression of some isoforms of the alpha class is characterized by low basal levels and high inducibility after exposure to some compounds, including hydrogen peroxide. The induction mechanism is mediated by the protein heterodimeric complex AP-1 (c-fos/c-jun), which enhances the GST transcription by binding to a regulatory AP-1 like binding site located upstream the respective genes. Finally, a recent report demonstrates that following a 6-OHDA lesion there is an elevated striatal c-fos immunoreactivity that is attenuated by the concomitant treatment with an N-methyl-D-aspartate antagonist.

All these evidences are in line with author hypothesis, which concerns to GST a role in the greater spontaneous recovery of PLA, and therefore, in the neuroprotective mechanism of dopaminergic tissue. Molecular characterization studies for the identification of the isoenzymes underlying this phenomenon might be important in neutralizing the cytotoxic products of 6-OHDA metabolism.
zyme(s) putatively involved should be needed to prove this hypothesis.

ACKNOWLEDGMENTS

To doctors A.A. Gomez, J. Bergado and C.M. Diaz for critical review of this manuscript and valuable comments.

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Table 1. Kinetic parameters of GST purified from whole brains of untreated, partially (PLA) and totally (TLA) 6-OHDA-lesioned rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated</th>
<th>PLA</th>
<th>TLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity (µmol min⁻¹ mg⁻¹)</td>
<td>7.33</td>
<td>6.92</td>
<td>4.94</td>
</tr>
<tr>
<td>Km (GSH) (mmol/L)</td>
<td>0.22</td>
<td>0.20</td>
<td>2.59</td>
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<tr>
<td>Km (CDNB) (mmol/L)</td>
<td>0.35</td>
<td>0.31</td>
<td>0.26</td>
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<tr>
<td>Vmax (µmol min⁻¹ mg⁻¹)</td>
<td>8.75</td>
<td>16.77</td>
<td>7.50</td>
</tr>
<tr>
<td>I₅₀, 6-OHDA (mmol/L)</td>
<td>0.66</td>
<td>0.71</td>
<td>0.34</td>
</tr>
</tbody>
</table>