

DYNAMIC ACTIVITY OF SOME ANTIOXIDANT ENZYMES IN PRIMARY CULTURES OF NEURONS UNDER HYPOXIA AND ISCHEMIA

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Abstract

Models of oxidative stress are important in elucidating pathologies associated with increased levels of reactive oxygen species, including stroke and neurodegenerative diseases, such as Alzheimer and Parkinson diseases. Cerebellar granule neurons primary cultures became the most popular model for the *in vitro* studies, bringing information about the development functionality and pathology. In this paper, we present the results of the antioxidant enzymes activities (catalase, superoxide dismutase, glutathione peroxidase) under oxidative stress conditions induced by hypoxia and ischemia. The spectrophotometric methods used, allow the quantification of the production of reactive oxygen species after hypoxic incubation at 37°C and ischemia, within one hour, two hours, and three hours by monitoring the activity of antioxidant enzymes.

Rezumat

Modelele prin care se induce stresul oxidativ sunt importante în elucidarea patologiilor asociate cu nivele crescute ale speciilor reactive de oxigen, inclusiv accidentul vascular cerebral și bolile neurodegenerative precum Alzheimer și Parkinson. Culturile primare de neuroni granulari cerebelari au devenit un model comun acceptat pentru studiile *in vitro*, și aduc informații privind dezvoltarea, funcționalitatea și patologia lor. În lucrarea de față, sunt prezentate rezultatele activității unor enzime antioxidante (catalaza, superoxid dismutaza, glutatation peroxidaza) în condițiile inducerii stresului oxidativ prin hipoxie și ischemie. Metodele spectrofotometrice folosite permit cuantificarea producerii de radicali liberi la o oră, două ore și trei ore de incubare hipoxica și ischemică la 37°C monitorizarea activității enzimelor antioxidante.

Keywords: oxidative stress, cerebellar granule neurons, free radicals, catalase, superoxide dismutase, glutathione peroxidase.

Introduction

Reactive oxygen species (ROS) induce oxidative stress in biological systems, leading to alterations within the structure and function of lipids, proteins and nucleic acids and, subsequently, to cell damage and death [1]. These ROS include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2),

and hydroxyl radical ($\text{OH}^\bullet$), which are all produced under a wide variety of pathological conditions (including hypoxia/ischemia), although some may also be produced under physiological conditions, usually as products of oxidative metabolism in cells [2]. The brain is exceptionally at risk for oxidative damage because it consumes far more oxygen than any other body tissue, it has low levels of antioxidants and possesses a tendency to accumulate metals, which are highly potent in generating ROS [3,15].

Neurons involute following different routes (apoptosis, necrosis) during hypoxia/ ischemia, wide phenomenon reproduced *in vitro* by exposing cell cultures to oxygen deprivation (OD) and oxygen-glucose deprivation (OGD) [4]. Assessing the viability after OD and OGD exposure is therefore a challenge due to the complex pathways initiated by this metabolic stress and the important implications for the neuroprotective mechanisms research [5].

Antioxidant enzymes activity inversely varies with the evolution of oxidative stress under mechanisms hypoxia/ischemia [6,7]. This paper intends to determine which enzyme is more effective regarding the neuroprotective mechanisms and which is the status where neurons can not return to the pre-injury metabolic status.

Materials and Methods

All animal procedures were carried out with the approval of the Ethics Committee for animal research "Carol Davila" University of Medicine and Pharmacy (Bucharest, Romania), and in accordance with the European Communities Council Directive 86 609 EEC.

We used 20 male white Wistar rats, five days old (P_5), healthy, weighing 10 to 15 grams.

Cell culture

We performed our experiments on primary DIV8 (7-8 days of *in vitro* growth) cultures of cerebellar granule neurons (CGN), after which CGN acquire a differentiated phenotype. Cerebella were collected from 5-days old (P_5) Wistar rat pups after cervical dislocation, dissected free of meninges, diced and incubated in the presence of papain (7 U/mL, Worthington). After a further incubation in the presence of DNA-ase (500U/mL, Sigma) and MgCl_2 (4mM), cells were dissociated by trituration and were resuspended in culture medium BME (Basal Medium Eagle GIBCO). Neurons were plated on poly-D-lysine (25 $\mu\text{g/mL}$, Sigma) coated 96-wells plates (Falcon) at density of 0.2×10^6 cells/well and maintained in culture medium supplemented with 25 mM KCl, 2 mM L-glutamine, 32mM glucose, antibiotic/antimycotic (AB/AM) and 10% heat inactivated horse

serum (Sigma). Cultures were maintained at 37°C in humidified 5% CO₂ incubator. At 24 hours after plating, cytosine arabinoside (10µM, Sigma) was added to prevent glial proliferation. The percentage of glial cells was no more than 1% as assessed by glial fibrillary acidic protein (GFAP) and β -tubulin immunostaining [8].

Oxygen and oxygen-glucose deprivation

In order to achieve OGD (oxygen-glucose deprivation) the culture media were replaced with a HEPES-basal experimental media containing: 120mM NaCl, 25mM KCl, 0.62mM MgSO₄, 1.8mM CaCl₂, 11.1mM glucose, 10mM HEPES (adjusted to pH=7.4 with NaOH if necessary, at 37°C). Oxygen-glucose deprivation (OGD) was induced by gently washing the cells twice (effective dilution 1: 1000) with glucose-free EM before switching them into a glucose-free, deoxygenated EM. Cultures were then immediately placed into a humidified hypoxia chamber (Figure 1) (Billups-Rothenberg Inc., Del Mar, CA), which was further flushed with 100% N₂ for 10 min and then sealed. Using this protocol cells are exposed to an oxygen concentration of about 4% during OGD. After a three-hour hypoxic incubation at 37°C, the cultures were removed from the incubator chamber and cell injury was assessed immediately after exposure. Some assessments were also carried after different periods of OGD exposure, at 1 hour and 2 hours.



Figure 1

Hypoxia and ischemia chamber, Billups-Rothenberg Inc., Del Mar, CA

Catalase activity determination

Method adapted after Abei H (1984) which measures spectrophotometrically the catalase activity by substrate (hydrogen peroxide) consumption. Results are expressed as U/mg prot/min [9].

Superoxid dismutase (SOD) activity determination

SOD determination is based on the method first introduced by Beauchamp and Fridovich (1971) which implies the NBT (nitro blue tetrazolium) reduction to formazan by the superoxide anion in the case that SOD inhibits this reaction. Results are expressed as U/mg prot/min [10].

Glutathione peroxidase activity determination

Method adapted after Paglia and Valentine (1967) which measures the decrease in absorbance at 340 nm by the consumption of substrate under the action of glutathione peroxidase. Results are expressed as mU/mg prot/min [11].

The total proteins were assessed according to Lowry's method [14].

The data were analyzed using Microsoft Office Excel 2010, GraphPad Prism 5.01, MedCalc 11.4.2 software. The statistical analysis was performed using the ANOVA tests (Repeated Measures, Single-Factor, and Two-Factor without Replication Analysis of Variance).

Results and Discussion

To express the activity of antioxidant enzymes (CAT, SOD, GPx) we developed a standard curve for BSA (bovine serum albumin).

We induced hypoxia and ischemia in primary cultures of neurons and determined the enzyme activity spectrophotometrically (Table I and Table II).

Table I

The antioxidant enzymes activities: catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) after 1 hour, 2 hours, 3 hours of hypoxic incubation at 37°C.

	Hypoxia		
	CAT activity	SOD activity	GPx activity
	U/mg prot./ min	U/mg prot./ min	mU/ mg prot/ min
Control	15.765 ± 0.316	1.765 ± 0.029	3.734 ± 0.054
1h	11.872 ± 0.213	1.391 ± 0.024	3.691 ± 0.054
2h	9.977 ± 0.175	1.204 ± 0.025	3.633 ± 0.054
3h	7.636 ± 0.131	0.912 ± 0.016	3.605 ± 0.053

Values are mean ± SE

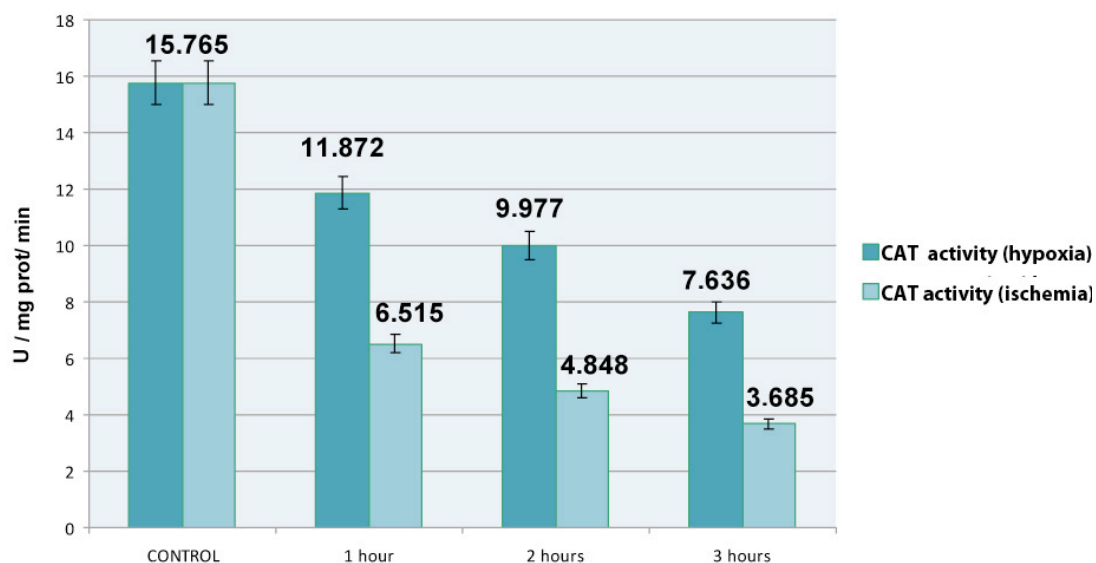
Table II

The antioxidant enzyme activities: catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) after 1 hour, 2 hours, 3 hours of ischemic incubation, at 37°C.

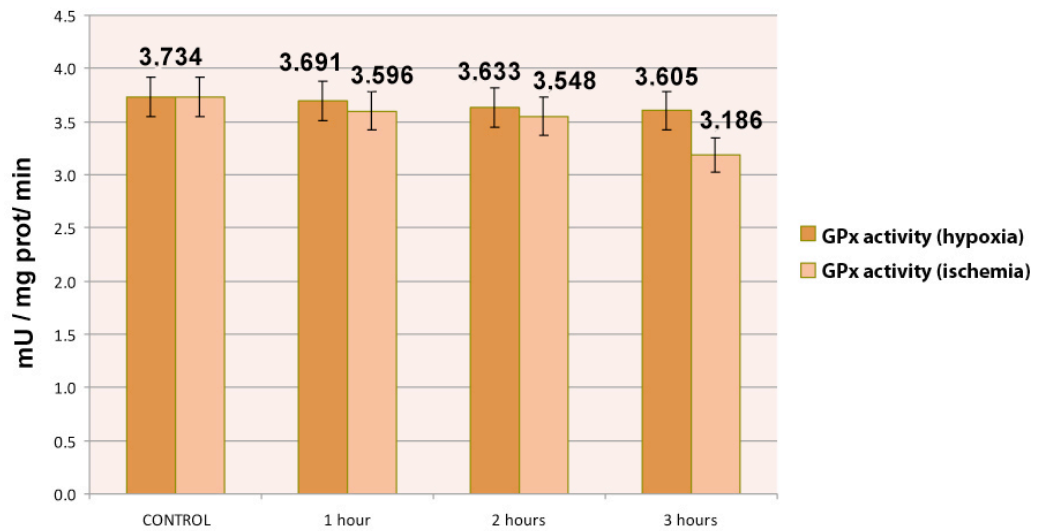
	Ischemia		
	CAT activity	SOD activity	GPx activity
	U/mg prot./ min	U/mg prot./ min	mU/mg prot./ min
Control	15.765 ± 0.316	1.769 ± 0.028	3.734 ± 0.054
1h	6.515 ± 0.103	0.819 ± 0.013	3.596 ± 0.053
2h	4.828 ± 0.098	0.622 ± 0.010	3.548 ± 0.054
3h	3.685 ± 0.081	0.482 ± 0.008	3.186 ± 0.051

Values are mean ± SE

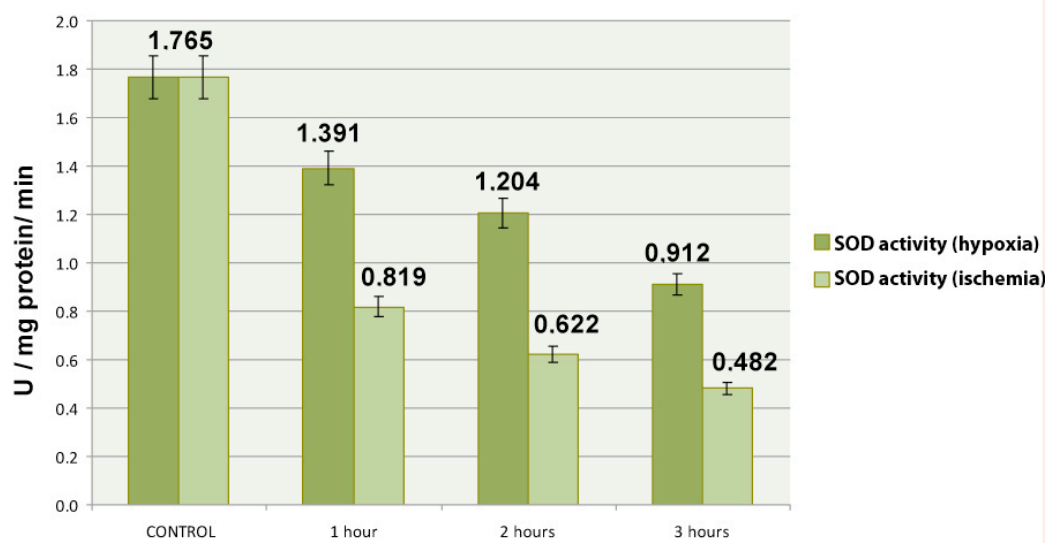
Our study showed that CAT and SOD activities showed significant differences in their specific activity while GPx registered no significant variation [12,13]. Their activity level was lower in ischemia compared with hypoxic state in a rate influenced by their substrate and, therefore by the amount of free radicals formed. The results presented in Table I and Table II are plotted in Figure2, Figure 3 and Figure 4.

**Figure 2**

Dynamic activity of catalase (CAT) activity after hypoxia (H) and after ischemia (I) measured 1 hour, 2 hours, 3 hours later.

**Figure 3**

Dynamic activity of glutathione peroxidase (GPx) activity after hypoxia (H) and after ischemia (I) measured 1 hour, 2 hours, 3 hours later.

**Figure 4**

Dynamic activity of superoxide dismutase (SOD) activity after hypoxia (H) and after ischemia (I) measured 1 hour, 2 hours, 3 hours later.

The activities of the three enzymes taken into study (CAT, SOD, GPx) were compared, being assessed after one, two and three hours of hypoxia/ischemia compared to the control values (Figure 5, Figure 6).

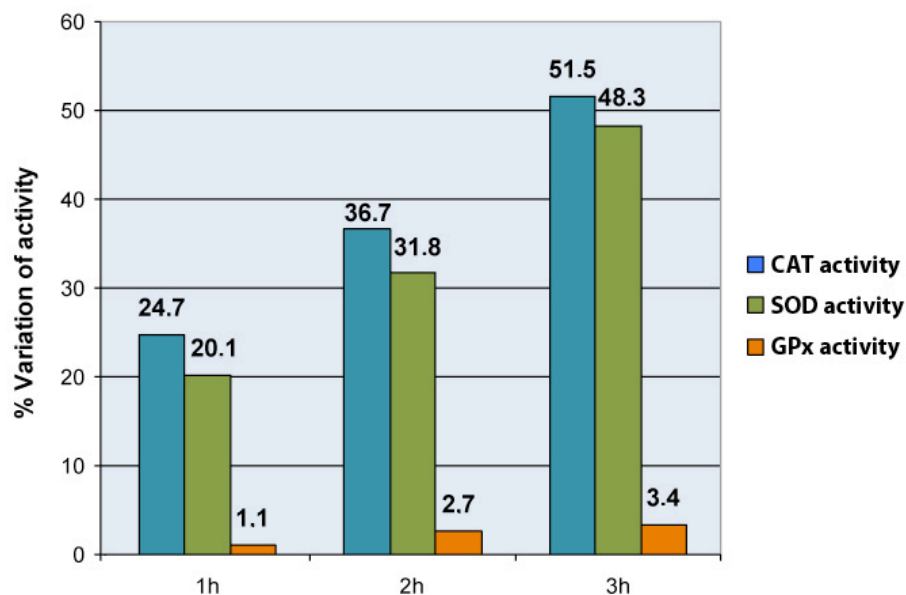


Figure 5

Dynamic activity of antioxidant enzymes after 1 hour, 2 hours, 3 hour of hypoxic incubation, at 37°C.

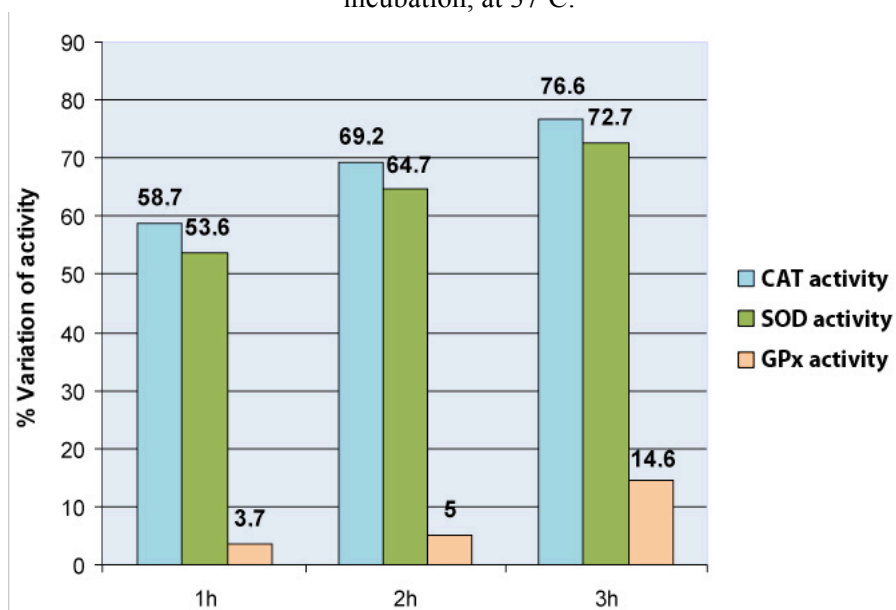


Figure 6

Dynamic activity of antioxidant enzymes after 1 hour, 2 hours, 3 hours of ischemia

It can be noticed a decrease of the mean values of all of the three studied activity enzymes, throughout the four measurements, the changes being statistically significant for SOD and especially for CAT ($p < 0.001$).

Also, for both CAT and SOD, the changes between the two conditions (hypoxia and ischemia) are significant, while for GPx, the changes between the two conditions are statistically not significant.

GPx is the only antioxidant enzyme that showed no statistically significant variations. One possible explanation is that of the intervening catalase levels within the antioxidant defense systems. Catalase converts hydrogen peroxide into oxygen and water, but an important activity amount of this species are formed through superoxide dismutase.

Conclusions

Cultured cerebellar granule neurons involuted, having the characteristics of an apoptotic death in the presence of superoxide anion or singlet oxygen generated by hypoxic and ischemic processes. Enzymatic activity tests showed that the critical time of neuronal morphology and metabolic status was recorded at one hour after exposure. The enzymes studied exerted an effective protection with the possibility to return the neuron to a level compatible with survival ("life"). After that, the antioxidant enzymes activities significantly decrease.

In our study, catalase activity during ischemia had the highest activity, therefore it provides the most effective protection. Superoxide dismutase activity had a similar variation to catalase but with lower values. Glutathione peroxidase is the only enzyme which did not show statistically significant variations. As catalase, GPx has biochemically the same substrate (hydrogen peroxide). While catalase is difficult to saturate the substrate, the GPx uses low amounts of hydrogen peroxide. Another explanation for the reduced activity of GPx is that hypoxia/ischemia, are mechanisms that develop changes within the enzyme's active catalytic center that cause significant functional changes of the enzyme.

In conclusion we can say that under conditions of oxidative stress induced by hypoxia/ischemia developed at the level of cerebellar granule neurons, catalase and superoxide dismutase provides endogenous neuroprotection, while GPx remains in the second line of the enzymatic antioxidant defence.

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