Age-related changes in an antioxidant defense system in elderly patients with essential hypertension compared with healthy controls

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Background and aims: Oxidative stress has been reported to increase with aging. Oxidative stress is also associated with hypertension, and antioxidant treatment has been shown to enhance antioxidant defense system. We therefore aimed to analyze the relationship between aging and some markers of oxidative stress in elderly patients with essential hypertension compared with healthy controls.

Material and Methods: Blood was collected from 18 patients with essential hypertension and 21 age- and sex-matched healthy controls aged over 65. Patients were on their usual medications while participating in the study. Oxidative stress parameters were investigated by measuring the concentration of glutathione (GSH) in whole blood and activities of glutathione peroxidase (GPx-1), glutathione reductase (GR), catalase (CAT), and Cu–Zn superoxide dismutase (CuZn SOD, SOD-1) in erythrocytes. GSH, GPx-1, GR, CAT, and CuZn SOD correlations with age were expressed as Pearson product-moment correlation coefficient *r*. Independent-samples *T* test was used to compare mean values of parameters between groups.

Results: (1) Among all parameters analyzed herein, the activity of SOD-1 showed the most explicit decrease in relation to age, both in healthy controls and hypertensive subjects with *r* values of -0.54 ($P = 0.05$) and -0.68 ($P < 0.01$), respectively. (2) Age-related changes in parameters of oxidative stress did not differ significantly between groups. (3) Mean activity of SOD-1 was significantly higher ($P < 0.05$) in elderly hypertensives (2341.7 ± 213.71 U/g Hb) when compared with healthy controls (2199.7 ± 213.66 U/g Hb). (4) Mean GSH level was significantly higher ($P < 0.01$) in patients (3.1 ± 0.29 mmol/l) than in controls (2.8 ± 0.37 mmol/l). (5) Increased level of GSH in hypertension was followed by significantly ($P < 0.01$) higher activity of GR in this group when compared with controls (83.4 ± 15.25 and 64.1 ± 9.40 U/g Hb, respectively).

Conclusions: (1) The antioxidant barrier changes in elderly subjects with senescence. (2) CuZn SOD activity is negatively correlated with age and this association is not altered by factors that modulate the enzyme activity, such as hypertension and antihypertensive treatment. (3) Significantly higher concentration of GSH and significantly higher GR activity in patients may suggest a significant role of GSH metabolism in the pathogenesis of hypertension, as well as its contribution to the effect of antihypertensive treatment.

Keywords: Antioxidant enzymes, Glutathione, Aging, Hypertension

Introduction

Considering that aging and hypertension are both associated with increased oxidative stress and that antihypertensive treatment enhances antioxidant defense systems, we analyzed age-related changes in

antioxidant parameters in elderly patients treated for hypertension compared to healthy controls.

The oxidative hypothesis of senescence has become one of the most prolific theories of aging since its origin in 1956.¹ Age-related oxidative stress is generated by a combination of increased production of free radicals, decreased antioxidant levels, diminished activities of antioxidant enzymes, and impaired repairs of

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oxidative damage. There are specific enzymes and low molecular weight substances for removing reactive oxygen species (ROS) superoxide dismutase (SOD) catalyzes dismutation of superoxide ($O_2^{\bullet-}$), catalase (CAT), scavenges hydrogen peroxide (H_2O_2), glutathione peroxidase (GPx) converts H_2O_2 to water, and neutralizes lipid peroxyl radicals. Glutathione (GSH) serves as a major thiol-disulfide redox buffer of the cell, and its reduced form is maintained by glutathione reductase (GR). When the production of oxidants overwhelms the cellular antioxidant capacity, the state of oxidative stress occurs, resulting in molecular and cellular tissue damage and severe metabolic malfunctions.²

Oxidative stress and hypertension are independent risk factors for cardiovascular events and the latter occur more frequently in old age. It has been suggested that adverse cardiovascular changes in aging may be altered by angiotensin-converting enzyme (ACE) inhibitors. Angiotensin II (AngII) is a principal effector of the renin-angiotensin system (RAS) and has been shown to promote aging and cellular senescence.³ The proposed mechanism of angiotensin II-mediated oxidative stress assumes increased $O_2^{\bullet-}$ generation by activated nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH) oxidases, whose role in cardiovascular disease has already been established.⁴ Overproduction of superoxide reduces nitric oxide (NO) bioavailability, as $O_2^{\bullet-}$ readily reacts with NO, to produce peroxynitrite ($ONOO^-$). NO is well known for its vasodilatory properties.⁵ When the production of NO is impaired, endothelium-dependent vasodilatation is blunted, which has been observed both in aged subjects and hypertensive patients.⁶

It has been reported that chronic long-term inhibition of the RAS can prevent most of the deleterious effects induced by aging in the cardiovascular system of normal rats.⁷ The effect of interference with the RAS is believed to be related to the reduction of intracellular oxidative stress produced by angiotensin II and activated AT_1 angiotensin receptors.^{8–10} Furthermore, treatment with ACE inhibitors and AT_1 receptor antagonists has been found to increase the activity of endothelial cell-SOD, the major antioxidant enzyme of the human arterial wall, in mice,¹¹ rats,¹² and humans.¹³ Moreover, it has been demonstrated that different antihypertensive drugs possess direct and indirect antioxidant activity and are able to increase both enzymatic and non-enzymatic antioxidant defenses through several mechanisms.^{10,11,14–16}

In this study we compared the relationship between age and selected parameters of oxidative stress in two groups of elderly people: treated hypertensives and healthy controls. First, we addressed the question how did antioxidant defense parameters change with age in both groups of subjects and, second, whether

age-related changes in antioxidant defense systems were altered by antihypertensive medication.

Materials and methods

Subjects

According to their health conditions and medical record, 39 elderly subjects were recruited through the Department of Gerontology and Clinic of Geriatrics, Nicolaus Copernicus University Collegium Medicum in Bydgoszcz.

Table 1 shows the clinical characteristics of the control and experimental subjects. Persons addicted to alcohol and/or tobacco, patients undergoing antioxidant supplementation and patients with ischemic heart disease, history of stroke, renal failure, cancer, dementia (Alzheimer disease), or other conditions of known or suspected free radical etiology were excluded from the study.

The hypertension group consisted of 18 established hypertensive patients (6 men and 12 women) mean age 81.9 ± 6.73 . Patients included in this group were already diagnosed for hypertension and were on their usual antihypertensive medication at the time of blood collection. Seven hypertensives were treated with combinations of statins, β -blockers, and ACE inhibitors, five patients were treated with combinations of statins and β -blockers, four patients were treated with combinations of statins and ACE-inhibitors, one patient was treated with a combination of β -blocker and ACE-inhibitor, and one patient was

Table 1 Clinical characteristics of the subjects included in the study

Parameters	Healthy elderly controls	Elderly hypertensive patients
Number of subjects	21	18
Age (year)	77.2 ± 8.91	81.9 ± 6.73
Body mass index (kg/m ²)	27.1 ± 4.91	28.2 ± 4.88
Systolic blood pressure (mmHg)	130.2 ± 13.56	129.4 ± 7.37
Diastolic blood pressure (mmHg)	74.9 ± 10.42	70.6 ± 11.80
Plasma glucose (mmol/l)	5.00 ± 0.42	5.07 ± 0.64
Plasma HDL cholesterol (mmol/l)	1.1 ± 0.22	1.2 ± 0.31
Plasma LDL cholesterol (mmol/l)	2.1 ± 0.88	2.4 ± 0.40
Triglycerides (mmol/l)	1.6 ± 0.72	1.8 ± 0.68
C-reactive protein sCRP (mg/l)	1.2 ± 1.14	2.2 ± 2.08
WBC ^{10⁹}	7.3 ± 1.65	6.7 ± 1.48
RBC ^{10¹²/l}	4.5 ± 0.70	4.5 ± 0.32
Hb (g/dl)	13.6 ± 1.97	13.8 ± 1.18

Note: Values are expressed as mean $\pm$ SD. There were no statistically significant differences between groups; $P < 0.05$ was considered statistically significant.

treated with sartan only. We considered treated hypertensives to be those with regulated blood pressure, i.e. below 140/90 for the past 6 months.

The control group consisted of 21 subjects (7 men and 14 women), mean age 77.2 ± 8.9 . Controls were age- and sex-matched with the patients ($P = 0.13$ and 0.73 , respectively).

All subjects gave informed consent for participating in the research. The local ethics committee of Medical University in Bydgoszcz approved the study protocol.

We did not include a drug-free hypertensive group in our study. We decided not to withdraw hypertensive patients from their usual medication for ethical reasons, with advanced age being the most important concern and risk factor for possible complications, associated with treatment cessation. Moreover, the average age for diagnosis of hypertension is far below the mean age of the two groups included in this study and we were not able to find a sufficient number of age-matched elderly people with newly diagnosed hypertension.

Biochemical analysis

Venous ethylenediaminetetraacetic acid (EDTA) anti-coagulated fasting blood samples were taken from the subjects. GSH concentration was determined spectrophotometrically in whole blood, by the method of Beutler¹⁷ using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). GSH reacts non-enzymatically with DTNB to generate glutathione disulfide (GSSG) and the highly colored 5-thio-2-nitrobenzoic acid (TNB; peak absorbance at 420 nm).

The remaining blood was centrifuged and, after careful removal of plasma and buffy coat, the red blood cells were washed with cold isotonic saline (0.89% NaCl, pH = 7.4). Afterwards the sediment was lysed by the addition of distilled water.

CuZn SOD, CAT, GPx, and GR activities were determined in hemolyzed blood samples according to the methods of Misra and Fridovich,¹⁸ Beers and Sizer,¹⁹ Paglia and Valentine,²⁰ and Flohe and Gunzler,²¹ respectively. SOD activity was determined in ethanol/chloroform-treated samples, by measuring

the inhibition of autoxidation of adrenaline at pH 10.2 at 37°C. The activity of CAT was determined by following the absorbance of H₂O₂ decomposition at 240 nm, pH 7.0, and 25°C. GR activity was determined by measuring the oxidation of NADPH, as the decrease in absorbance at 340 nm at 37°C in the presence of GSSG. GPx activity was determined by measuring the oxidation of NADPH, as the decrease in absorbance at 340 nm at 25°C in the coupled reaction with *t*-butylhydroperoxide, GSH, and GR.

Statistical analysis

To assess age-associated changes of parameters of oxidative stress in elderly patients with essential hypertension and in healthy elderly controls we performed statistical analysis with Statistica version 9 software. All variables were normally distributed. Results were expressed as the Pearson product-moment correlation coefficient. To examine the significance of differences observed between groups, we applied independent-samples *T* test between groups. Two-tailed significance values are given. *P* values of 0.05 and less were considered to indicate statistical significance in all analyzes performed.

Results

In order to analyse the antioxidant defense in elderly patients with and without hypertension, the concentration of GSH and the activity of the antioxidant enzymes (GPx-1, CAT, GR, and CuZn SOD) were measured. Afterwards we looked at correlations of these measures with age within the hypertension and control groups and compared the Pearson product-moment correlation coefficients between groups. Finally, we examined the differences in mean values of antioxidant defense systems in the hypertension group compared to controls. Table 2 presents the antioxidant defense in relation to age in elderly patients with and without hypertension. Pearson product-moment correlation coefficient values for parameters vs. age are shown. Table 3 provides information on mean values of parameters in treated hypertensives compared with healthy controls.

Table 2 CuZn SOD, GSH, GPx-1, GR, and CAT correlations with age expressed as the Pearson correlation coefficient in healthy controls and treated hypertensives

Parameter	<i>r</i> -Pearson product-moment correlation coefficient values (parameter vs. age)				Significance of differences in the calculated correlation coefficients between controls and hypertensives
	Healthy controls		Treated hypertension		
CuZn SOD (U/g Hb)	−0.54	<i>P</i> = 0.05*	−0.68	<i>P</i> < 0.01*	<i>P</i> = 0.52
GSH (mmol/l)	−0.37	<i>P</i> = 0.26	−0.36	<i>P</i> = 0.16	<i>P</i> = 0.97
GPx-1 (U/g Hb)	−0.15	<i>P</i> = 0.62	−0.13	<i>P</i> = 0.61	<i>P</i> = 0.95
GR (U/g Hb)	0.44	<i>P</i> = 0.56	0.11	<i>P</i> = 0.67	<i>P</i> = 0.31
CAT (BU/g Hb)	−0.34	<i>P</i> = 0.25	−0.00	<i>P</i> = 0.99	<i>P</i> = 0.32

Note: $P < 0.05$ was considered statistically significant, significant values marked with asterisk.

Table 3 CuZn SOD, GSH, GPx-1, GR, and CAT values in treated hypertensives compared with healthy controls

Parameter	Mean values of parameter $\pm$ SD		Significance of differences between the means of controls and hypertensives compared through Independent-Samples T test
	Healthy controls	Treated hypertension	
CuZn SOD (U/g Hb)	2199.7 $\pm$ 213.66	2341.7 $\pm$ 213.71	$P < 0.05^*$
GSH (mmol/l)	2.8 $\pm$ 0.37	3.1 $\pm$ 0.29	$P < 0.01^*$
GPx-1 (U/g Hb)	13.1 $\pm$ 2.01	13.2 $\pm$ 1.95	$P = 0.88$
GR (U/g Hb)	64.1 $\pm$ 9.40	83.4 $\pm$ 15.25	$P < 0.01^*$
CAT (BU/g Hb)	23.7 $\pm$ 1.97	25.1 $\pm$ 1.64	$P = 0.07$

Note: $P < 0.05$ was considered statistically significant, significant values marked with asterisk.

We observed that all analyzed parameters were affected by senescence and most of them negatively correlated with age, although the only significant association was a negative correlation between age and CuZn SOD activity.

CuZn SOD activity negatively correlated with age in hypertension as well as in control group, $r = -0.68$ ($P < 0.01$) and $r = -0.54$ ($P = 0.05$), respectively. In both groups the correlation between age and CuZn SOD activity was in the same direction and strength, as confirmed by no significant difference between these two correlation coefficients ($P = 0.52$). Besides the observation that CuZn SOD activity decreased with age in both groups, the mean activity of the enzyme was significantly elevated in hypertensive patients treated for their condition ($P < 0.05$). Addressing a problem of gender associated differences in biological senescence we also compared males and females subgroups, although the number of subjects for each category was not big. When we separated males and females, a significant age-related decrease in SOD activity was observed only in hypertensive women ($P = 0.02$).

With reference to GSH, patients with treated hypertension had significantly higher GSH levels when compared with age- and sex-matched healthy subjects ($P < 0.01$). Moreover, GSH level decreased with age equally in healthy subjects and in patients with hypertension ($P = 0.97$). In order to evaluate whether the increased levels of GSH in elderly patients with hypertension were due to the effect of its metabolic enzymes, the activities of GR and GPx were measured. GR, which restores the reduced form of GSH, exhibited significantly higher activity in patients treated for hypertension, as did GSH concentration, when compared with age- and sex-matched healthy subjects ($P < 0.01$). Concerning the age effect on enzyme activity, no significant correlation was observed. We did not observe clear associations of GPx activity with age, and the mean activity was similar in the examined groups ($P = 0.88$).

In order to further analyze the antioxidant defense in elderly subjects, we also investigated the activity of CAT. This did not correlate with age in elderly subjects

either with or without hypertension. Activity of the enzyme did not differ significantly in the hypertension group when compared with healthy elderly subjects.

Discussion

The most profound result observed herein is the decrease in CuZn SOD activity with age. There are several studies that support this finding,^{22,23} although some did not find the same pattern.²⁴ Significantly, SOD activity has been recently suggested to have an association with lifespan.²⁵ There are many possible mechanisms that can lead to decreased SOD activity with age, i.e. glycation, increased ROS generation, decreased mRNA expression, and deficits of Zn and Cu.

We also observed significantly higher SOD activity in the hypertensive group when compared with healthy controls. Superoxide dismutase deactivates $O_2^{\bullet-}$ radical, which when present in excess, inactivates vasodilator NO. A positive correlation between superoxide production and systolic and diastolic blood pressure has been observed.²⁶ It has been explained by decreased NO bioavailability and concomitant vasoconstriction. Inactivation of $O_2^{\bullet-}$ is crucial for NO bioavailability, as it prevents the formation of peroxynitrite, which has the power to oxidize tetrahydrobiopterin (BH_4), a cofactor for NOS enzymes. The latter are the main source of vascular NO. Oxidation of BH_4 leads to uncoupling of endothelial NOS and, as a consequence, production of $O_2^{\bullet-}$ increases and bioavailability of NO decreases.²⁷ There is a substantial body of evidence for decreased SOD activity in hypertension,²⁸ yet most of the data were gathered from middle-aged patients. These results cannot be related to our finding of increased SOD activity, since we analyzed data from elderly people already treated for hypertension and on their usual medication during the study. Considering this fact, our results are in line with those of other investigators reporting a significant increase in SOD activity under various antihypertensive treatments in humans and in mice.^{11,29} However, although antihypertensive medication was able to elevate SOD activity significantly above that seen in age- and sex-matched healthy controls, the

effect of age on the enzyme activity was not abolished. The older the subject is, the lower the SOD activity both in patients and in healthy controls. This observation suggests a strong association between age and SOD activity. This relationship cannot be easily altered; however, the activity of the enzyme can be modified by pathological conditions and pharmaceutical treatment.

Increased activity of SOD causes a higher turnover of $O_2^{\bullet-}$ and generates a high level of H_2O_2 , overproduction of which is involved in the development of hypertension.³⁰ Two enzymes analyzed in this study are capable of decomposing H_2O_2 , namely GPx and CAT. Neither of them showed a significant correlation with age in either of the two groups analyzed herein. No differences between the two groups of subjects were observed; however, CAT activity was higher in hypertensives than in normotensives, and the difference was close to statistical significance. Increased activities of SOD and CAT are consistent with both $O_2^{\bullet-}$ and H_2O_2 contributing to hypertension, and suggest that the role of SOD and CAT is to decrease the oxidant damage to vascular cells.³¹ Like SOD, CAT activity decreases in non-treated hypertension^{28,32} and can be enhanced by antihypertensive medication.²⁹

With regard to GPx activity, the published data are less consistent and alterations in both directions, as well as no changes, have been reported in hypertension.^{32,33} The effect of age on GPx activity cannot be determined based on the contradictory results available to date.^{22–25,34} With regard to CAT, most studies have reported no change in the enzyme activity during senescence,^{22,23} although a positive correlation with age has been also observed.³⁵ Other parameters with no significant relation to aging in elderly people in our study were GR activity and concentration of GSH. According to the literature, we might expect a decrease in GR activity with age,^{22,36} just as for the GSH level;³⁷ however, studies showing either relatedness,³⁸ or a negative correlation,³⁹ between age and GSH cannot be ignored.

While we were not able to see an effect of age on GSH metabolism, we observed that people treated for hypertension had a significantly higher level of GSH and significantly increased GR activity. These results suggest a significant role for GSH metabolism in maintaining blood pressure in elderly people and, also, that alterations in GSH metabolism contribute to antioxidant properties of antihypertensive treatment. Indeed, antihypertensive medications from different classes have been shown to raise blood²⁹ and renal⁴⁰ GSH levels. We believe that an increase in GSH can increase NO bioavailability and should have a beneficial effect on blood pressure in hypertensive patients. First, it has been established that GSH can react with peroxynitrite

and form S-nitrosoglutathione (GSNO), which subsequently releases NO over prolonged time, thereby extending the half-life of NO manyfold and, in consequence, relaxes vascular tissue.^{41,42} Moreover, GSH can stimulate decomposition of S-nitrosothiols.⁴³ Nitrosothiols reduce vascular tension, partly due to their ability to inhibit NADPH oxidase.⁴⁴ Furthermore, GSNO has been shown to be an efficient NO donor in the presence of CuZn SOD,⁴⁵ the activity of which increased significantly along with GSH concentration in elderly patients with hypertension in our study; this allows to speculate about a potential role of GSNO decomposition in a CuZn SOD-dependent mechanism in lowering blood pressure. The mechanisms associated with GSNO pathways are complex and need to be interrelated to concurrent biochemical processes, but nevertheless the role of GSNO in hypertension can be seen as a protection in response to oxidative stress, in addition to serving as a stabilizer and carrier of NO.⁴⁶

Furthermore, GSH is able to reverse the vasoconstrictive effect related to scavenging of NO by ferrous groups in oxygenated heme.⁴⁷ The activation of the GSH-related antioxidant defense system helps to protect cells from the overproduction of prooxidants and methemoglobinemia.⁴⁸ The effective suppression of metHb formation seems to play a role in the maintenance of good health and successful aging.⁴⁹ As the activity of methemoglobin reductase attenuates with age,⁵⁰ the increasing level of metHb may limit the amount of free nitrite, which is proposed to be the largest intravascular storage pool of vasoactive NO.⁵¹

Altogether, the experimental data gathered herein suggest that age-related changes in an antioxidant defense system in elderly patients treated for hypertension follow the trends observed in healthy controls, despite significant differences in the activity of some antioxidant enzymes and the concentration of GSH between groups. Our results suggest that the antioxidant defense is impaired in elderly subjects as CuZn SOD activity decreases with age. Moreover, the age-dependent impairment in antioxidant defense is at least partly due to the GSH-related defense system. Indeed, it has been already confirmed that a high GSH concentration in blood is associated with excellent physical and mental health in old persons.⁵²

The limitation of the study is the small number of samples, and so these results need to be repeated in a larger study. Furthermore, any interpretation of alterations in antioxidant enzyme activities requires information about the nutritional status of the subjects, which may be of particular significance in the elderly, due to the occurrence of certain forms of malnutrition. However, it is worth noting that, in spite of methodological shortcomings, our study is another²⁵ that has

pointed out that senescence is associated with decreasing CuZn SOD activity. Although antihypertensive medication was able to elevate CuZn SOD activity significantly, the effect of age on the activity of the enzyme was not abolished. The patients with hypertension exhibited a similar pattern of age-related alterations in antioxidative systems to that observed in healthy controls. This finding has methodological implications for projects that study SOD activity in human studies, as well as other parameters of oxidative stress, i.e. that age needs to be considered as a possible confounding factor. Our data provide more information regarding age-related oxidative stress in elderly people who are healthy or treated for hypertension.

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