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Age-related changes in antioxidant defence mechanisms and peroxidation in isolated hepatocytes from spontaneously hypertensive and normotensive rats

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Abstract

The effects of age and hypertension on the antioxidant defence systems and the lipid peroxidation in rat isolated hepatocytes were studied. Four different age groups (1, 3, 6 and 12 months) were considered in spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats. Age-associated changes were observed on vitamin E status, glutathione (GSH) level, MDA formation and glutathione peroxidase (GSH-Px) activity in both strains. Maximal levels or activities of these parameters were found at 3 and 6 months, except for MDA which was low at 3 months. Then, a fall was observed at 12-month-old compared to 6-month values. In addition, GSH-Px activity was significantly lower in SHR than in WKY rats, except at the age of one month. The decrease of this enzyme activity could induce an increased cellular generation of radical species and lipid peroxidation, which might be link to hypertension. (Mol Cell Biochem **132:** 25–29, 1994)

Key words: hepatocytes, Hypertension, age, GSH-Px

Introduction

The free radical theory of aging [1, 2] is a widely accepted explanation for the progressive accumulation of age-related cell constituent damages. Some studies have shown an age-dependent increase of lipid peroxidation in liver and other tissues of rat and mouse [3, 4], and the hepatic dysfunction caused by products of lipid peroxidation [5]. In addition, lipid peroxidation is a causal contributor to the pathophysiology of many diseases [6], including vascular diseases [7], among which hypertension represents a major cardiovascular risk factor [8, 9].

Under physiological conditions, there is a continuous production of reactive oxygen species, controlled by a number of defensive enzymatic and non-enzymatic systems. Among the antioxidant enzymes, glutathione per-

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oxidase (GSH-Px) protects the cell from the damaging effects of oxidizing species like organic hydroperoxides and hydrogen peroxide. Non enzymatic defences are provided by antioxidant molecules, the most important being vitamin E and reduced glutathione (GSH) which act as a radical scavenger and a reductant, respectively.

The purpose of this report is twofold, first to investigate the effects of age on the antioxidant defence systems and the lipid peroxidation in rat isolated hepatocytes and second to investigate the effects of hypertension on these parameters.

Materials and methods

Animals

Male spontaneously hypertensive rats (SHR) and male normotensive Wistar-Kyoto (WKY) rats were purchased from IFFA-CREDO (L'Arbresle, France). Animals were fed a commercial standard pellet (Sourriffarat, UAR, Epinay sur Orge, France) and received tap water *ad libitum*. The composition of the diet was 21% proteins, 53.5% glucides, 4% fats, 4.5% cellulose, 11.5% water, 5.5% ashes, mineral mix (27.8 g/kg), vitamin mix (16,000 IU/kg vitamin A, 2,000 IU/kg vitamin D_3 and 170 mg/kg vitamin E). The systolic blood pressure was measured by the tail cuff method [10]. Experiments were performed at different ages (1, 3, 6 and 12 months).

Cells

Isolated hepatocytes were prepared according to the method of Seglen [11] as modified by Skrede *et al.* [12]. The liver was first perfused *in situ* through veina porta with perfusion buffer pH 7.4. While still being perfused, the liver is cut from the carcass, and the perfusion is then switched over to collagenase buffer, according to the method of Seglen [11]. The liver is then transferred to a Petri dish and gently dispersed in Krebs-Henseleit solution with a stainless steel comb. The suspension is then purified by a succession of filtration and centrifugation [11] in order to remove the non-parenchymal cells, damaged cells, subcellular debris and small clumps of non perfused tissue. The cells are then resuspended in Krebs-Henseleit buffer as described [12]. Counting and viability of the cells (measured by exclusion of trypan blue) were performed in a hemocytometer and revealed that 90–95% of cells were viable. The final suspension was frozen before analysis in presence of ascorbic acid (0.5%).

Determination of GSH-Px

The enzyme activity was measured on hepatocytes solubilized with lubrol by the method of Paglia and Valentine [13] as modified by Chaudière and Gérard [14]. The measurement was performed at 37° C in Tris-HCl buffer (50 mM), containing reduced glutathione (2 mM), glutathione-reductase (1 U/ml), NADPH (0.14 mM) and tert-butyl hydroperoxide (0.2 mM) as the hydroperoxide substrate. GSH-Px activity was spectrophotometrically determined in following the oxidation of NADPH at 340 nm (spectrophotometer Beckman UV-DU8, Fullerton, USA).

Glutathione measurement

Total glutathione was determined according to the method of Neuschwander-Tetri and Roll [15]. Disulfides were reduced with dithiothreitol and derivatized with ortho-phthaldialdehyde (OPT), and the GSH-OPT complex was quantified by reverse phase high-performance liquid chromatography (rp-HPLC). HPLC was performed using a Kontron LC pump T-414 equipped with a Nucleosil C_{18} 15 cm × 4.6 mm (5 μm particle size) column (SFCC/Shandon, Eragny, France), a Kontron Uvikon 735 LC detector (Kontron, Zurich, Switzerland), connected with a Shimadzu GR3A integrator (Kyoto, Japan). Chromatographic conditions are those reported in [16].

Vitamin E status

Vitamin E determinations were done according to a method previously reported [17]. Briefly, isolated hepatocyte suspensions were mixed with two volumes of ethanol/water (1:1, v/v) containing tocol as an internal standard. After extraction twice with three volumes of hexane, the vitamin E extract was analyzed by rp-HPLC.

Thiobarbituric acid (TBA)-malondialdehyde (MDA) complex determination

Isolated hepatocytes suspensions were treated by

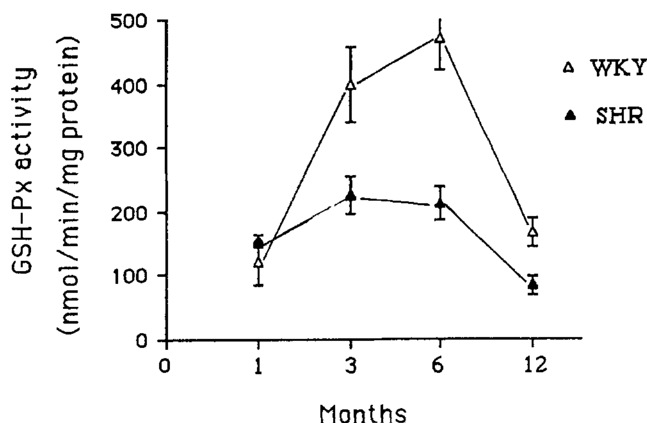

Fig. 1. Effect of age on glutathione peroxidase activity in isolated hepatocytes from normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Values represent the means ± S.E.M. (n = 4).

10 mM TBA for one hour at 95° C. After extraction by ethylacetate, the TBA-MDA complex was quantified by rp-HPLC according to the method of Therasse and Lemonnier [18].

Statistical analysis

Results are expressed as the mean with its standard error (mean ± SEM) for each group. Data were analyzed using the STATVIEW SE program for Macintosh according to two-way analysis of variance with balanced mixed models. Differences of means were evaluated for significance at 5% probability by Newman-Keul's test.

Results

The systolic blood pressures in SHR were significantly higher than those observed in WKY rats at the different ages (135 ±4, 221 ±8, 232 ±10 and 237 ±8 mmHg at 1-, 3-, 6- and 12 month-old versus 107 ± 6, 141 ± 7, 144 ± 8 and 148 ± 7 mmHg, respectively). The hypertension was established at the age of 3 months and remained constant at 6 and 12 months.

GSH-Px activity in isolated hepatocytes from WKY rats and SHR as a function of age is shown in Fig. 1. In WKY rats, a marked increase of GSH-Px activity was found until the age of 6 months and this activity felt in the 12 month-old group. On the other hand, apart from the 1 month-aged rats, GSH-Px activity was significantly (as judged by the t-test applied to paired groups for each age) lower in SHR than in WKY rats. The P values of two-way analysis of variance are 0.001 for strain, 0.001 for age and 0.0035 for interaction. Significant interaction term could mean that the GSH-Px activity of 3 month-old SHR was about the same as the activity of 12 month-old WKY rats. Hypertension and aging would then synergize for the lowering of the GSH-Px activity.

Concerning TBA-MDA formation, age-related differences were reported in Table 1.Principally, an important increased TBA-MDA level was detected at 6 months compared to 1-, 3-, and 12-month-old groups in both WKY animals and SHR. The amounts of TBA-MDA showed a trend to be impaired in SHR as compared to WKY rats, nevertheless the decreased values were not statistically significant (F for strain: 2.7, N.S.).

'Age-dependent variations of vitamin E in isolated hepatocytes from WKY animals and SHR were shown in Table 1. Vitamin E levels were higher in the hepatocytes from 3-month-old rats as compared to the levels in 1- and 12-month-old animals in both strains. No significant changes appear between WKY rats and SHR (F for strain: 0.09, N.S.).

GSH content in isolated hepatocytes from WKY rats and SHR was examined (Table 1). In normotensive and hypertensive rats, similar age-related differences were

Table 1. Effect of age on the thiobarbituric acid (TBA)-malondialdehyde (MDA) complex formation, vitamin E status and level of total glutathione (GSH) in isolated hepatocytes from normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR)

	Months	1	3	6	12
TBA-MDA	WKY	30.0 ± 1.5[a]	8.9 ± 2.2[a]	426.8 ± 92.8	36.1 ± 7.0[a]
	SHR	16.7 ± 7.2[a]	6.2 ± 1.2[a]	290.7 ± 34.0	25.7 ± 3.4[a]
Vitamin E	WKY	15.1 ± 6.2[a]	88.0 ± 20.6[b]	52.8 ± 14.4[ab]	33.0 ± 5.6[a]
	SHR	13.6 ± 2.3[a]	88.2 ± 21.3[b]	62.9 ± 6.9[b]	20.7 ± 5.3[a]
GSH	WKY	1.7 ± 0.9[a]	3.4 ± 0.7[ab]	5.3 ± 1.0[b]	0.4 ± 0.1[a]
	SHR	2.5 ± 0.6[a]	3.5 ± 0.6[a]	5.8 ± 0.6	0.3 ± 0.1

Values are expressed as nmol/mg protein and represent the means ± S.E.M. (n = 4). Within rows, means not sharing a common superscript were statistically different (p < 0.05).

observed. Indeed, highest amounts of GSH were found in the 6 month-old groups and lowest amounts in the 12 month-old groups but no differences were noted between WKY rats and SHR (F for strain: 0.39, N.S.).

Discussion

Free radicals and other active compounds have been suggested to be involved in many biological processes including aging and various diseases [19]. Oxidative stress originates as the result of an imbalance between the generation of oxidizing species and cellular antioxidants. Some reports have focused on the possibility that aging may be accelerated by the impair of the antioxidant defences, but the results are still unclear. In the present investigation, the capacity of hepatocytes to control the lipid peroxidation was investigated in normotensive and hypertensive rats of different ages. Among the antioxidant enzymatic defences, GSH-Px protects cells from the damaging effects of oxidizing species like organic hydroperoxides and hydrogen peroxide. In this study, we found an age-related changes in GSH-Px. This activity fits with the parabolic curve observed by Rathbun *et al.* [20] with highest values at middle age. However, age-associated GSH-Px activity in liver was conflicting in the literature. A decrease of the activity was observed by Cand and Verdetti [21] whereas Rikans *et al.* [22] found no age-dependent differences, and Ji *et al.* [23] observed an increase of mitochondrial GSH-Px in old age. These discrepancies presumably reflect some difficulty in evaluating the antioxidant system in the liver and/or could be due to the different experimental procedures used. In addition to the age-dependent GSH-Px activity, we found it lower in the SHR group, except at the age of one month, where the hypertension was not achieved in SHR. The latter finding is consistent with the results of Ito *et al.* [24] found in the myocardium of SHR.

GSH is an ubiquitous cellular component and the most abundant sulfhydryl reducing agent in mammalian tissues. According to the reports of Farooqui *et al.* [25], and Hazelton and Lang [26], significant decreases of GSH contents are observed in aged WKY rats as well as SHR. However, we did not find any changes in hepatic GSH between SHR and WKY rats, meaning that no apparent differences in the GSH metabolism occur between the two strains. This agrees with the assertion that hypertension does not belong to diseases associated with diminished GSH levels [27].

Vitamin E is the major lipophilic chain-breaking antioxidant in cells which protects membrane polyunsaturated fatty acids from lipid peroxidation [28]. The present study shows direct evidence that the vitamin E content is age-dependent, as it has also been shown direct evidence that the vitamin E content is age-dependent, as it has also been reported in humans [17, 29]. Vitamin E is known to relate dynamically with the activity of the GSH-Px enzyme [30]. We did not find a strict correlation between these two parameters but some relationship may be observed in both strains.

Surprisingly, the MDA level was clearly lower in the 12-month-groups, whereas the results of M.Y.H. Farooqui *et al.* [25] suggest that MDA increase with advancing age. However, some reports [21, 23, 31] are consistent with our observation. A possible explanation is that, in aged organs, the lipid peroxidation is followed by an increased rate of lipid and protein copolymerisation due to MDA which is then consumed. It would be then preferable to measure the ability of cells to produce MDA under stimulation instead of measuring its basal level.

In summary, data obtained from the present investigation reveal that the hepatic antioxidant defence system decreased with advancing age and that the MDA level is probably not a good marker of the lipid peroxidation in the liver. In hypertensive rats, glutathione peroxidase activity was drastically decreased. It remains to determine whether such a decrease was due to a genetic strain or to blood pressure elevation, but the result is likely an increase of radical species. Besides considering the decreased GSH-Px activity in the liver as an index of such an activity in other tissues, plasma GSH-Px is assumed to be synthesized by and secreted from hepatic cells [32]. A decreased GSH-Px activity could then lead to decrease both the plasma GSH-Px and the half-life of NO, the potent vasodilating agent [33]. This might have a biological relevance in hypertension [34].

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