NeuroToxicology ®9 (2):173-180 (1988)

Brain Alterations Induced by Vitamin E Deficiency and Intoxication with Methyl Ethyl Ketone Peroxide

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ABSTRACT: *Rats fed a vitamin E-deficient diet from age 3-10 weeks were either maintained on a vitamin E-deficient diet or fed a vitamin E-enriched diet for 8 subsequent weeks. The content of vitamin E, endoperoxide-derived malonaldehyde, lipofluorescent material and polyunsaturated fatty acids, and the activities of catalase, glutathione reductase, and glutathione peroxidase were then measured in cerebral tissues, with or without intoxication with methyl ethyl ketone peroxide (MEKP). For this purpose, one half of the animals in each vitamin E group received an ip injection of 5 mg MEKP per kg of body weight, which was followed 44 hours later, i.e., 4 hours before sample collection, by a second ip injection of 15 mg MEKP per kg of body weight. Despite the fact that the vitamin E concentration was 12-times lower in the brain of vitamin E-deficient rats, no significant change in other cerebral parameters was found between the two groups of animals. In contrast, the activity of selenium-glutathione peroxidase was markedly decreased in the liver of 10-week old vitamin E-deficient rats. Unexpectedly, acute systemic intoxication with MEKP caused only a small, albeit significant, decrease in glutathione reductase activity in the brain of vitamin E-sufficient rats, while no significant change in other cerebral parameters was observed in either group of animals. These results suggest that the central nervous system (CNS) is still substantially protected when its vitamin E content has been decreased to 3 μg/g fresh weight, and that systemic intoxication with MEKP may not cause lipid peroxidation in the CNS.*

Key Words: Brain, Vitamin E, Selenium, Glutathione Peroxidase, Methyl Ethyl Ketone Peroxide

INTRODUCTION

Peroxidation of membranes which are rich in polyunsaturated fatty acids, such as those of the central nervous system (NS) (Sastry, 1985), is an important factor in tissue damage and cell death. As a highly efficient scavenger of peroxyl radicals in phospholipid bilayers,

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Accepted: December 8, 1987

vitamin E, *i.e.*, alpha-tocopherol, affords major protection against *in vivo* lipid peroxidation (Tappel, 1978; Witting, 1980).

Methyl ethyl ketone peroxide (MEKP) is a polymerizing agent which spontaneously decomposes into free radicals in the presence of trace metal ions. Systemic injection of MEKP in vitamin E-deficient rats causes severe lipid peroxidation, as measured by the production of exhaled pentane (Litov *et al.*, 1981; Herschberger and Tappel, 1982), and has also been reported to cause molecular alteration of brain DNA (Summerfield and Tappel, 1984).

Our initial aim was to induce a cerebral oxidative stress by means of systemic MEKP intoxication, to study the role of cerebral antioxidant systems other than vitamin E, especially hydroperoxide-detoxifying enzymes. Because of the low degree of vitamin E heterogeneity in the brain (Vatassery and Younoszai, 1978), it was expected that systemic intoxication of vitamin E-deficient rats with MEKP would trigger a relatively homogeneous oxidative stress in the brain.

MATERIALS AND METHODS

From the 16th day of gestation, eight breeding female rats of the sprague-Dawley strain (Iffa Credo, L'Arbresle, France) were fed a casein-based semi-synthetic diet that contained less than 1 ppm of vitamin E (UAR, Villemoisson, France). The resulting 90 pups of either sex were maintained with their mothers on the vitamin E-deficient diet until weaning, *i.e.*, day 21 after birth. From the day of weaning, the young rats were fed the vitamin E-deficient diet *ad libitum*. On day 75 after birth, the rats were divided into two subgroups, one that was maintained on the vitamin E-deficient diet (Vit. E^- group) and the other that was fed a vitamin E-enriched diet obtained by the addition of 170 mg of d,1-alphatocopherolacetate per kg of diet (Vit. E^+ group). All rats were killed between day 136 and day 147 after birth. Forty-eight hours before being killed, one half of the animals in each group received an ip injection of 5 mg MEKP per kg of body weight. This was followed by a second ip injection of 15 mg MEKP per kg of body weight, 4 hours before sample collection. All the animals ($MEKP^-$/Vit. E^-, $MEKP^+$/Vit. E^-, $MEKP^-$/Vit. E^+ and $MEKP^+$/Vit. E^+) were then killed by decapitation, following diethylether anesthesia and cerebral perfusion through the left heart with 50 ml physiological saline containing 10 units/ml heparin and 0.05% pyrogallol as an antioxidant. The brain and liver were immediately excised. Aqueous homogenates were made with a Teflon® pestle homogenizer in 50 mM Tris-HC1, 0.1 mM ethylene-diamine tetracetic acid (EDTA), 0.1% lubrol, pH 7.6-(1:7, W:V). Similarly, cerebral lipid extracts (Radin, 1981) were made with a Teflon® pestle homogenizer (1:19, W:V) in hexane/isopropanol 2:3, W:V) containing 0.5% butylated hydroxytoluene (BHT) as an antioxidant. Aliquots of low-speed supernatants of the aqueous homogenates were used for measurements of enzyme activities as well as malonaldehyde and total proteins. The lipid extracts were used for measurement of vitamin E and lipid fluorescence (Tappel, 1975), and for acidic transmethanolysis of total lipids (Rogozinski, 1964). Analysis of the fatty acid profile of the resulting methyl esters was performed by Gas-Liquid Chromatography (GLC) on a fused-silica capillary column impregnated with Carbowax 20 M and using heptadecanoic acid as an internal standard. Lipofluorescence was measured at 370 exc./450 em., using a Farrand MK1 spectrofluorimeter calibrated with a standard solution of quinine sulfate (Tappel, 1975); vitamin E was measured by a modification of a published procedure (Hatam and Kayden, 1979) that used reverse-phase high pressure liquid chromatography (HPLC) on a 10 µ-C18 column (250 x 4 mm), with elution by 95% methanol and absorbance detection at 295 nm; alpha-tocopherolacetate was used as an internal standard. Glutathione peroxidase activity was measured by a modification of the method of Paglia and Valentine (1967), at 0.5 mM GSH, pH 7.6, 37 ° C, and using 0.2 mM hydrogen peroxide (selenium-dependent activity) and cumene hydroperoxide (total activity) as substrates in two separate assays. Catalase activity was measured by monitoring the disappearance of 10 mM H_2O_2 at 240 mn (Aebi, 1974) in 50 mM Tris-HC1, 0.1 mM EDTA, 0.1% lubrol, pH 7.6 and 37 °C. Endoperoxide-derived malonaldehyde was measured as the absorbance of the thiobarbituric acid-adduct at 535 nm, on aliquots of tissue aqueous homogenate after a

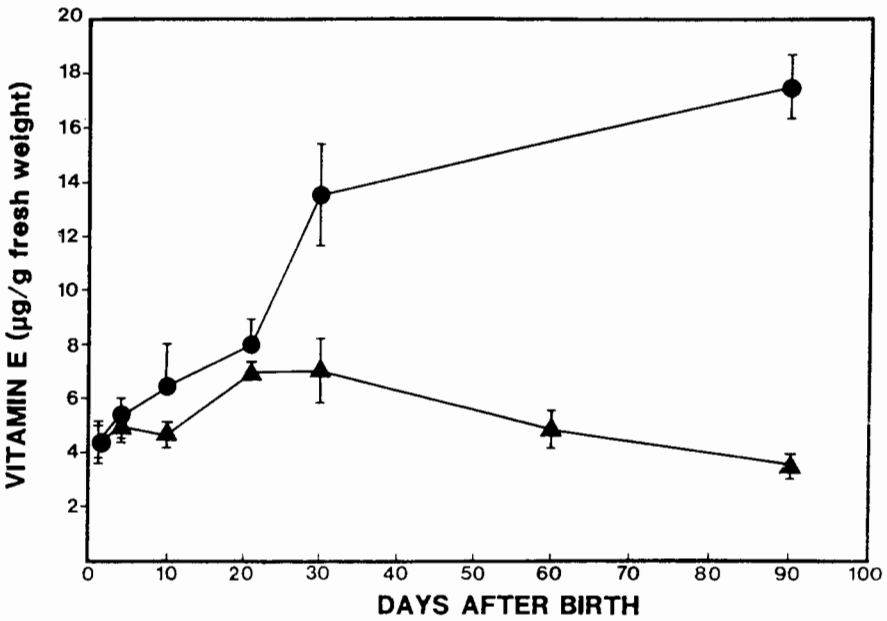

FIG. 1 Time-course of vitamin E concentration[a] in the brain of developing rats. Rats were either fed a casein-based vitamin E-deficient diet (- ▲ -), or a chow based vitamin E-sufficient diet (- ● -). Note that the chow-based diet was only used here to assess the efficiency of vitamin E depletion with the casein-based deficient diet. The Vit. E^+ group described in this study was fed a casein-based diet which had been enriched with alpha-tocopherolacetate, as described in the Materials and Methods Section. This resulted in a final vitamin E concentration in the brain of approximately 30 µg/g fresh weight, i.e., outside the scale of the figure. [a]Mean ± SD, N ≥ 4.

10-fold dilution in 50 mM KCl (Mihara *et al.*, 1980). Total proteins were measured by the dye-binding assay of Bradford (1976), using bovine serum albumin as a standard. Total phosphorus of lipid extracts was measured by the method of Chen *et al.* (1956). For statistical evaluation of the data, paired comparisons were made using Student's t-test.

RESULTS AND DISCUSSION

On the day of weaning, the vitamin E content of the brain of the eight suckling mothers was 12.2 ± 2.6 µg of alphatocopherol per g of fresh tissue, which is approximately two-thirds of the observed value in non-deficient female rats of the same age.

The time-course of vitamin E concentration in the brains of the young rats is shown in Fig. 1. Clearly, until weaning, the maternal milk provided a substantial source of vitamin E for the brain of these rats, even though their mothers were fed a vitamin E-deficient diet from the third week of gestation.

The effect of weaning on the efficiency of the deficient diet was very important since on day 30, vitamin E concentration in the brain of vitamin E-deficient rats was already twice as low as that of vitamin E-sufficient rats (rats that were fed on a normal rat chow diet). It appears, however, that the brain is protected compared to other tissues against vitamin E loss, since after 4 months of vitamin E-deficient diet, its vitamin E content by weight was only 2.8 times lower than that measured on the day of weaning, in spite of a 6- to 8-fold increase in body weight.

In 3-month old rats, the vitamin E content of vitamin E-deficient animals was less than 20% of that typically observed in rats fed a vitamin E-sufficient chow-based diet (Fig. 1). For subsequent intoxication experiments, control rats (Vit. E^+ group) were obtained by addition of alpha-tocopherolacetate to the vitamin E-deficient diet of rats that had already been maintained on the deficient diet until the age of 2.5 months. This was designed to make

sure that the direct influence of vitamin E would be studied rather that the consequences of vitamin E deficiency during the period of development. A high level of alpha-tocopherolacetate was used to allow a quick vitamin E replenishment in the Vit. E^+ group. One will note the remarkable efficiency of vitamin E re-incorporation into the brain of these future control animals (Vit. E^+): as shown in Table 1, on the day of intoxication, *i.e.*, between days 136 and 146, the brains of Vit. E^+ rats contained approximately 12 times more vitamin E than the brains of Vit. E^- rats. This was approximately 50% higher than the vitamin E content of rats fed the chow-based diet more commonly used in this laboratory (Fig. 1).

Effect of Vitamin E Deficiency Before Intoxication

The concentrations of vitamin E, malonaldehyde and fluorescent lipopigments are reported in Table 1. In the brain, the vitamin E concentrations observed in females were slightly higher than those observed in males, but this difference was not significant. As for the contents of malonaldehyde and fluorescent pigments, there were no significant differences between vitamin E^+ and vitamin E^- groups.

It is noteworthy that the content in fluorescent pigments was higher in the cerebellum, this difference being significant in the vitamin E^+ group ($p < 0.05$). Autofluorescence in the cerebellum was independent of the vitamin E content. Apparently, an increase in cerebral tissue damage may require a much longer-lasting vitamin E deficiency (Manwaring and Csallany, 1981).

The activities of glutathione peroxidase and glutathione reductase are reported in Table 2. Glutathione reductase activity was significantly higher in females of the vitamin E^+ group, but other sex differences were not significant. There were significant differences between the vitamin E^+ and the vitamin E^- rats. Neither catalase (data not shown) nor glutathione peroxidase activities varied with the vitamin E content of the brain.

As shown by the substrate specificity, glutathione peroxidase activity was exclusively due to the selenoenzyme in the brain. More unexpectedly, this was also the case in the liver (Table 2) with the casein-based diet that was used in this study. This conclusion was checked on a limited number of samples by the use of the selenium-specific inhibitor

TABLE 1. Vitamin E, Malonaldehyde and Lipofluorescence in Brain and Cerebellum of Vit. E^+ and Vit. E^- Rats, Before and After Intoxication with MEKP[a].

		Before Intoxication		After Intoxication	
		Vit. E^+	Vit. E^-	Vit. E^+	Vit. E^-
Brain Vitamin E[b]	(M)	26.8 ± 2.9 (4)[a]	2.50 ± 0.37 (6)	25.8 ± 8.1 (5)	2.47 ± 0.30 (6)
	(F)	32.2 ± 5.1 (4)	2.84 ± 0.48 (4)		2.96 ± 0.32 (5)
Brain Malonaldehyde[c]	(M)	0.45 ± 0.03 (4)	0.37 ± 0.17 (6)	0.50 ± 0.07 (4)	0.40 ± 0.17 (6)
	(F)	0.46 ± 0.03 (4)	0.40 ± 0.20 (5)	0.33 (2)	0.42 ± 0.18 (6)
Brain Fluorescence no sex discrimination[d]		2.4 ± 0.5 (8)	2.4 ± 1.0 (11)	2.0 ± 0.9 (12)	1.8 ± 0.5 (6)
Cerebellum Fluorescence no sex discrimination[c]		4.0 ± 1.2 (8)	4.3 ± 2.3 (11)	3.4 ± 2.9 (11)	3.4 ± 2.9 (6)

(M) = Male rats. (F) = Female rats.

[a] Number of rats are indicated within parentheses.

[b] µg alpha-tocopherol/g fresh weight.

[c] Thiobarbituric acid adduct measured as absorbance 535/50 mg fresh weight.

[d] Fluorescence units/mg phosphorus (370 exc./450 em.); no significant sex difference.

TABLE 2. Enzyme Activities of Brain and Liver of Vit. E^+ and Vit. E^- Rats Before and After Intoxication with MEKP[a].

Enzyme activity		Before Intoxication				After Intoxication			
		Vit. E^+		Vit. E^-		Vit. E^+		Vit. E^-	
Glutathione peroxidase[b] (Brain)	(M)	27.9 ± 0.3	(4)[a]	24.5 ± 3.2	(5)	23.3 ± 3.4	(4)	28.2 ± 4.2	(5)
	(F)	34.1 ± 6.2	(4)	30.1 ± 5.9	(5)	24.9 ± 7.7	(4)	28.6 ± 6.0	(5)
Glutathione reductase[c] (Brain)	(M)	26.0 ± 2.1	(4)	19.6 ± 4.6	(5)	16.7 ± 3.5	(4)	23.4 ± 5.1	(5)
	(F)	31.8 ± 3.1	(4)	22.2 ± 4.0	(5)	15.6 ± 0.5	(3)	16.8 ± 3.2	(5)
Glutathione peroxidase[b] Liver	(M)	249 ± 47	(4)	107 ± 24	(5)	179 ± 143	(4)	105 ± 19	(5)
	(F)	825 ± 180	(4)	204 ± 113	(5)	695 ± 124	(3)	760 ± 125	(5)

(M) = Male rats. (F) = Female rats.

[a] Number of rats are indicated within parentheses.

[b] Nanomoles hydrogen peroxide reduced/min/mg protein; no selenium-independent activity was detected in brain.

[c] Nanomoles NADPH oxidized/min/mg protein.

mercaptosuccinate (Chaudiere *et al.*, 1984). This suggests that selenium-independent activity, which is believed to reflect a side-reactivity of a glutathione-S-transferase, is essentially induced by food additives. Among the later, phenolic antioxidants such as butylated hydroxyanisole (BHA) and BHT probably play a major role (Pearson *et al.*, 1983).

Enzyme activities of the liver are shown in Table 2. In the vitamin E^+ group, selenium-glutathione peroxidase activity is approximately 3.6 times higher in females ($p < 0.01$). It decreases very markedly in both males and females of the vitamin E^- group ($p < 0.01$), this decrease being more important in females. Such a large decrease in hepatic glutathione peroxidase may be due to an impairment of selenium absorption during prolonged vitamin E deficiency (Witting, 1980). A more moderate decrease in glutathione peroxidase activity had been previously reported following a 3-month long vitamin E-deficient diet (Scott *et al.*, 1977).

TABLE 3. Polyunsatured Fatty Acid (PUFA) Composition of Total Lipids, in the Brain of Vit. E^+ and Vit. E^- Rats, Before and After Intoxication with MEKP.

Fatty Acid (% of Total PUFA)	Before Intoxication		After Intoxication	
	Vit. E^+ (N = 8)	Vit. E^- (N = 11)	Vit. E^+ (N = 11)	Vit. E^- (N = 6)
18 : 2 n-6	1.5 ± 0.4	0.4 ± 0.7	1.5 ± 0.2	0.5 ± 0.7
20 : 4 n-6	36.2 ± 1.2	33.3 ± 2.8	35.6 ± 2.3	33.8 ± 2.5
22 : 4 n-6	2.1 ± 1.0	9.5 ± 6.8	8.5 ± 4.1	8.7 ± 4.6
22 : 5 n-6	11.6 ± 0.8	9.6 ± 0.9	10.5 ± 0.5	9.8 ± 0.6
22 : 5 n-3	5.5 ± 0.9	10.1 ± 2.7	4.9 ± 1.4	8.3 ± 2.4
22 : 6 n-3	43.2 ± 1.1	37.1 ± 4.3	39.1 ± 2.4	38.8 ± 3.2
Total (% of Total Fatty Acids)	34.9	31.5	32.0	32.8

The fatty acid composition of brain total lipids is shown in Table 3. Here, no significant sex differences were observed, which explains why no discrimination was made in the Table. One should note that the content in linoleic acid 18:2 n-6 decreased significantly ($p < 0.01$) in the vitamin E$^-$ group. Other differences were not significant. In the cerebellum, the situation was essentially identical, although the content of polyunsaturated fatty acids was higher (data not shown) because of a higher content in docosahexaenoic acid 22:6 n-3, *i.e.*, 20% of total fatty acids instead of 12-15% in brain ($p < 0.01$).

Effect of Intoxication with MEKP

Unexpectedly, intoxication with MEKP did not alter most of the above-discussed parameters in the brains of either Vit. E$^+$ or Vit. E$^-$ rats, including vitamin E itself, malonaldehyde, lipofluorescence, catalase and glutathione peroxidase activities, and polyunsaturated fatty acids (see Tables 1-3). Only a slight but significant decrease of glutathione reductase activity ($p < 0.05$) was observed in the vitamin E$^+$ group.

This is in contrast with the alterations of DNA that were previously observed in the brain of MEKP-treated rats (Summerfield and Tappel, 1984). Such alterations, which included an increase in protein-DNA as well as interstrand-cross-links, may therefore be unrelated to an increase in lipid peroxidation in cerebral tissues. In our hands, while systemic administration of MEKP does cause a strong oxidative stress in the liver, it does not afford a clear-cut model of oxidative stress in the brain, even with high sub-lethal doses such as those used in this study. One should consider the possibility that the increase in DNA cross-links reported by Summerfield and Tappel (1984) was not a consequence of lipid peroxidation. Indeed, the absence of brain lipid peroxidation following systemic intoxication suggests that the blood-brain barrier may prevent MEKP from efficiently entering into the brain.

Alternatively, a certain amount of oxidative damage could result in an increase in DNA cross-linking without any requirement for lipid peroxidation (Lesko *et al.*, 1982). This could be consistent with the slight decrease in glutathione reductase activity which was observed in this study following intoxication with MEKP, although it is difficult to interpret the absence of such alterations in the vitamin E-deficient rats.

In conclusion, our 4-month long vitamin E deficient-diet resulted in a marked decrease of the vitamin E content of cerebral tissues, but only induced very moderate changes in other cerebral parameters. In contrast, a marked decrease of selenium-glutathione peroxidase was observed in the liver of vitamin E-deficient rats. These results suggest that the CNS is still adequately protected against membrane oxidative damage when its vitamin E content is maintained around 3 μg/g fresh weight. Whether this would still be true in a situation of acute cerebral oxidative stress cannot be assessed by systemic intoxication with MEKP. The enhancement of the acute toxicity of MEKP in vitamin E-deficient rats primarily reflects the inability of peripheral tissues to maintain a sufficient level of protection against peroxide-induced oxidative stress.

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