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Studies on the effect of iron overload on rat cortex synaptosomal membranes

Katalin Nagy *, Robert A. Floyd, Pál Simon ** and Imre Zs.-Nagy *

Oklahoma Medical Research Foundation, 825 Northeast 13th Street, Oklahoma City, OK 73104 (U.S.A.)

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Iron as ferrous ammonium sulfate was injected into the cerebral spinal fluid of rats. After three consecutive days of injection of 4 μmol of iron, the total iron content of brain cortex synaptosomes from the iron-treated animals was 2-fold higher than that from control animals receiving the saline vehicle only. Spin label studies of the synaptosomal membranes demonstrated that the lipid region of the membranes became more rigid and, in addition, the mobility of labeled SH groups of membrane proteins decreased after the iron treatment. The cholesterol content was significantly higher in iron-treated animals as compared to controls. Centrophenoxine pretreatment (100 mg/kg body weight daily for 6 weeks) diminished the iron effects. Synaptosomal membrane alterations observed after iron treatment were similar to changes observed previously during aging. This lends support to the notion that free-radical induced damage occurs in brain membranes with increasing age.

Introduction

Certain regions of human brain such as globus pallidus, red nucleus and substantia nigra are relatively rich in iron [1,5,10] containing on the order of 180–215 μg/g wet weight [1]. The non-heme iron content of certain areas of human brain increases with age [1–5]. For instance, Hallgren and Sourander [1] demonstrated that globus pallidus of human increased from about 10 μg/g wet weight at birth, to about 130 at age 10, and then to about 200 μg/g wet weight at age 30. The iron content increased only slightly thereafter. It has been shown that age-dependent increases in iron content are less marked in cerebral cortex than in the basal ganglia [4]. The iron content of mouse and rat brain has also been shown to increase with age and that there are regional differences in iron content as in the human brain, even though they are less pronounced [6–11]. Brain non-heme iron is present mostly in the form of ferritin. It is also present as an essential component of specific enzymes, as well as small molecular weight complexes [10]. Certainly, free iron, as well as perhaps some other forms of complexed iron, may be potent catalysts of peroxidative damage [12,13]. Iron in excess is toxic to biological systems. Brain is very susceptible to the toxic action of iron as has been demonstrated by direct iron injections into the brain [26–30].

In general, it has been demonstrated that brain levels of iron do not respond to feeding or intraperitoneal injection of iron. Recent experiments by Gavino et al. [53] and Dillard et al. [31] demon-

* Present address: Verzár International Laboratory for Experimental Gerontology, University Medical School, H-4012 Debrecen, Hungary.

** Central Research Institute for Chemistry, Hungarian Academy of Sciences, Pusztaszeri út 59-67, H-1025 Budapest, Hungary.

strate intraperitoneal injection of 100 to 200 mg into rats caused large increases in total iron of many tissues including heart, testis, kidney and especially liver but did not increase the brain iron level. In contrast, however, it does appear that the intraperitoneal injection of much higher levels of iron dextran does cause selective loading of iron in the basal ganglia but not in the cerebral cortex of rabbits and monkeys [54] and this leads to Parkinsonian-like tremors in the iron-loaded animals [55]. We have developed a new procedure of increasing the iron level in brain. The present paper describes the new method as well as results regarding physicochemical alteration of synaptosomal membranes of rat brain cortex after iron overload was induced by its injection into the spinal fluid.

Materials and Methods

Spin-labels and chemicals. *N*-Oxyl-4',4'-dimethyloxazolidine derivatives of stearic acid with the doxyl groups in C-5 or C-16 positions (5-NS and 16-NS, respectively) were purchased from Syva Co. (Palo Alto, CA) and the sulfhydryl spin-label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (MSL), from Aldrich Chemical Co. (Milwaukee, WI). The iron standard, 1000 ppm in nitric acid 'Baker Instra-Analyzed', as well as Ultrex nitric acid were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Centrophenoxine (Helfergin) was a gift of Promonta (Hamburg, F.R.G.).

Animal treatment. Female Fischer-344 rats, 5 months of age, were used. The rats (body weight 190–230 g) were injected with 4 μmol of ferrous ammonium sulfate which was added as the salt to 0.05 ml of physiological saline and, within seconds, then mixed with 0.2 ml of lidocaine (20 mg/ml) in saline. The iron solution was used as rapidly as possible after its preparation. Animals were subjected to light ether anesthesia prior to cerebral spinal fluid injection of the iron solution. Lidocaine was used to determine the accuracy of iron placement since spinal delivery of lidocaine results in temporary paralysis of hind legs. Control animals were injected with the same volume of physiological salt and lidocaine. Iron injections were given daily for three consecutive days. The iron solution was injected between the 4th and 5th lumbar vertebral of animals draped over a rounded surface to maximize the intravertebral gap. Centrophenoxine pretreatment was carried out as follows: rats of 3.5 months of age were injected intraperitoneally five days a week for six weeks with 100 mg centrophenoxine per kg body weight. Centrophenoxine was dissolved in physiological saline immediately prior to injection. Half of the centrophenoxine-pretreated animals were injected with iron as described above; the other half was injected with physiological salt and lidocaine only.

Synaptosome preparation. Synaptosomes were isolated from the gray matter only of brain cortex. The cortex from two rats was pooled together for each experiment. The synaptosomes were prepared by the method of Gray and Whittaker [14]. This method is used because it is highly reproducible and yields synaptosomes of excellent purity based upon ultrastructural and marker enzyme studies performed earlier using this method [47].

Analytical procedures. Protein content of the synaptosomal fractions was determined by a modified Folin reaction [15].

The total cholesterol content of synaptosomal membranes was measured using an assay based on enzymic oxidation of cholesterol to cholest-4-en-3-one (Total Cholesterol Procedure No. 350, Sigma Diagnostics). Lipid phosphorus levels were analyzed as described by Bartlett [16]. Protein sulfhydryl group content of synaptosomes was estimated by the DTNB method of Sedlak and Lindsay [17]. Total iron content of synaptosomes was determined by atomic absorption spectrophotometry (Perkin-Elmer, Model 2280) following wet ashing with a mixture of nitric and perchloric acid [8]. The internal standard method was utilized to obtain the amount of total iron present in order to eliminate matrix interferences as described previously [8].

Spin-labeling of synaptosomal membranes. Incorporation of the spin-labels was carried out as described earlier [18]. The EPR spectra on 50 μl of spin-labeled synaptosomes were recorded at 37.0 $\pm$ 0.5°C, for the 5-NS and 16-NS labeled membranes, but at 22.0 $\pm$ 0.5°C for the MSL-labeled membranes. A Varian E-9 X-band EPR spectrometer was used. The order parameter for 5-NS was calculated by measuring the hyperfine split-

tings as described by Hubbell and McConnell [19]. The pseudoisotropic rotational correlation time τ_c was calculated for 16-NS according to Eletr and Inesi [20]. For evaluation of the results obtained with MSL label, the ratio of the spectral amplitudes of weakly (w) and strongly (s) immobilized label molecules bound to the SH groups of proteins was used as described by Butterfield et al. [21]. The instrumental parameters were: 9.125 GHz microwave frequency, 0.2 mT modulation amplitude at 100 kHz modulation frequency, response time 0.3 s, sweep time 10 mT/4 min, field set 324 mT, microwave power 4 mW, gain $(1.25–4)\cdot 10^3$. In the case of S determination with the 5-NS spin label, the high-field maximum deflection was obtained by use of much slower sweep speed and higher gain.

Data analysis. Statistical analysis for significance between the means was carried out with the two-tailed Student's *t*-test. Since we used the brain cortex of two rats pooled together to obtain sufficient amount of synaptosomes, the statistical scatter of our data does not represent the real biological scatter but the scatter of technical reproducibility of the experimental results. Two or three parallel measurements were carried out from each sample.

Results

Table I shows the total iron content of rat brain synaptosomes isolated from animals receiving various treatments. The iron content was significantly higher, more than 2-fold greater, in synaptosomes of iron-treated animals than in control animals. Six weeks of centrophenoxine pretreatment of rats considerably decreased the accumulation of iron in the synaptosomes after spinal injection. There was an increase of the iron content of centrophenoxine-pretreated animals, but it remained significantly lower as compared to the iron-treated animals.

The total cholesterol content and the phospholipid content was determined in all the animal groups (Table II). There was a slight but statistically significant increase in the total cholesterol content in the iron-treated animals as compared to the control ones. The phospholipid content between treatments did not vary significantly (Table II) and therefore the differences in total cholesterol content are reflected in the cholesterol to phospholipid ratio (data not shown). Centrophenoxine pretreatment of the rats caused a decrease in the total cholesterol content. This was true for normal as well as for iron-treated animals.

The results presented in Table III demonstrate that the protein sulfhydryl content of brain synaptosomes varied significantly between iron-treated and control animals. The protein-SH content was about 8% higher in synaptosomes of iron-treated animals as compared to controls. In the centrophenoxine-pretreated animals, the iron treatment also increased the protein-SH content, but less markedly. There was no significant change in protein-SH between centrophenoxine-pretreated and control animals.

The data regarding the membrane spin-label parameters are summarized in Tables IV–VI. We have previously observed [18] with the MSL label, that there is slight change with incubation time,

TABLE I

TOTAL IRON CONTENT OF RAT BRAIN CORTEX SYNAPTOSOMES OF CONTROL, IRON-TREATED, CENTROPHENOXINE-PRETREATED AND CENTROPHENOXINE-PRETREATED + IRON-TREATED ANIMAL GROUPS

The iron content is presented as mean ± S.D. *n* indicates the number of animals. *N* indicates the number of measurements. *P* indicates the significance level between the groups as calculated by the Student's *t*-test. n.s., not significant. CPH, centrophenoxine.

Animal group	*n*	*N*	Fe content (ng/mg protein)	Significance level
(A) Control	8	11	178.7 ± 21.6	P(A vs. B) < 0.001
(B) Fe-treated	8	10	435.0 ± 72.6	P(A vs. C) = n.s.
(C) CPH-pretreated	4	6	178.3 ± 20.6	P(A vs. D) < 0.005
(D) CPH-pretreated + Fe-treated	4	6	231.2 ± 39.6	P(B vs. D) < 0.001

TABLE II

TOTAL CHOLESTEROL CONTENT AND PHOSPHOLIPID CONTENT IN RAT BRAIN CORTEX SYNAPTOSOMAL MEMBRANES

Figures are presented as means ± S.D. *n* indicates the number of animals. *N* indicates the number of measurements. *P* indicates the significance level between the groups as calculated by the Student's *t*-test. n.s., not significant. CPH, centrophenoxine.

Animal group	*n*	*N*	Cholesterol content (μmol/mg protein)	Phospholipid content (μmol/mg protein)
(A) Control	8	8	0.197 ± 0.002	0.625 ± 0.005
			P(A vs. B) < 0.001	*P*(A vs. B) = n.s.
(B) Fe-treated	8	8	0.207 ± 0.004	0.630 ± 0.007
			P(A vs. C) < 0.001	*P*(A vs. C) = n.s.
(C) CPH-pretreated	4	4	0.188 ± 0.002	0.620 ± 0.007
			P(A vs. D) = n.s.	*P*(A vs. D) = n.s.
(D) CPH-pretreated + Fe-treated	4	4	0.197 ± 0.001	0.625 ± 0.005
			P(B vs. D) < 0.001	*P*(B vs. D) = n.s.

TABLE III

PROTEIN SULFHYDRYL CONTENT OF RAT BRAIN CORTEX SYNAPTOSOMES

The protein-SH content is presented as mean ± S.D. *n* indicates the number of animals. *N* indicates the number of measurements. *P* indicates the significance level between the groups as calculated by the Student's *t*-test. n.s., not signficant. CPH, centrophenoxine

Animal group	*n*	*N*	Protein-SH (nmol/mg protein)	Significance level
(A) Control	8	8	151.0 ± 2.4	*P*(A vs. B) < 0.001
(B) Fe-treated	8	8	164.1 ± 1.3	*P*(A vs. C) = n.s.
(C) CPH-pretreated	4	4	148.2 ± 4.1	*P*(A vs. D) < 0.001
(D) CPH-pretreated + Fe-treated	4	4	159.2 ± 1.2	*P*(B vs. D) < 0.005

therefore, all of the samples were run in parallel with identical incubation times. The *w/s* ratio is expressed as a percentage of the control values. The results demonstrate that all three parameters, the order parameter for 5-NS spin label, the rational correlation time for 16-NS spin label, and *w/s* ratio for MSL spin label, were modified by iron treatment. The most remarkable change was

TABLE IV

ORDER PARAMETER (*S*) OF 5-NS SPIN LABEL INCORPORATED INTO RAT BRAIN CORTEX SYNAPTOSOMAL MEMBRANES

S is presented as mean ± S.D. *n* indicates the number of animals. *N* indicates the number of measurements. *P* indicates the significance level between the groups as calculated by the Student's *t*-test. n.s., not significant. CPH, centrophenoxine.

Animal group	*n*	*N*	*S*	Significance level
(A) Control	8	12	0.608 ± 0.001	*P*(A vs. B) < 0.001
(B) Fe-treated	8	12	0.613 ± 0.001	*P*(A vs. C) = n.s.
(C) CPH-pretreated	4	6	0.607 ± 0.001	*P*(A vs. D) = 0.001
(D) CPH-pretreated + Fe-treated	4	6	0.610 ± 0.001	*P*(B vs. D) < 0.001

TABLE V

ROTATIONAL CORRELATION TIME (τ_c) OF 16-NS SPIN LABEL INCORPORATED INTO RAT BRAIN CORTEX SYNAPTOSOMAL MEMBRANES

τ_c is presented as mean ± S.D. *n* indicates the number of animals. *N* indicates the number of measurements. *P* indicates the significance level between the groups as calculated by the Student's *t*-test. n.s., not significant. CPH, centrophenoxine.

Animal group	*n*	*N*	τ_c (10^{-10} s)	Significance level
(A) Control	8	12	13.53 ± 0.09	*P*(A vs. B) < 0.001
(B) Fe-treated	8	12	13.92 ± 0.13	*P*(A vs. C) < 0.001
(C) CPH-pretreated	4	6	13.31 ± 0.05	*P*(A vs. D) < 0.001
(D) CPH-pretreated + Fe-treated	4	6	13.82 ± 0.06	*P*(B vs. D) < 0.05

TABLE VI

w/s RATIO CHANGES OF MSL LABELED RAT BRAIN CORTEX SYNAPTOSOMAL MEMBRANES

The *w/s* ratio is presented as mean ± S.D. *n* indicates the number of animals. *N* indicates the number of measurements. *P* indicates the significance level between the groups as calculated by the Student's *t*-test. n.s., not significant. CPH, centrophenoxine.

Animal group	*n*	*N*	*w/s* (%)	Significance level
(A) Control	8	12	100.0 ± 1.4	*P*(A vs. B) < 0.001
(B) Fe-treated	8	15	83.9 ± 6.1	*P*(A vs. C) = n.s.
(C) CPH-pretreated	4	6	99.8 ± 1.2	*P*(A vs. D) < 0.001
(D) CPH-pretreated + Fe-treated	4	7	92.6 ± 1.9	*P*(B vs. D) < 0.005

shown by the MSL spin label. The *w/s* ratio decreased 16% after iron treatment as compared to the control. Centrophenoxine-pretreatment before iron injections prevented the significant increase in the order parameter and in the rotational correlation time. Centrophenoxine-pretreatment also caused an incerase in the *w/s* ratio.

Discussion

Iron is an essential trace metal, being a constituent of many enzymes, electron transfer complexes and oxygen carriers. However, iron in excess is toxic, but the mechanisms involved are still not understood [25]. Excessive Fe accumulation in tissues may result in the formation of oxygen free radicals, hydroxyl radicals and peroxides [22], which can then interact with lipids or proteins [13,23,24]. Jacobs [25] reported evidence that points to lipid peroxidation and membrane damage as a major factor in iron toxicity. Willmore and coworkers [26,27] provided evidence of significant increases in the quantity of fluorescent products during lipid peroxidation after the injection of $FeCl_2$ into rat isocortex. Willmore et al. [28] also showed that iron salts injected into rat isocortex cause transient formation of oxygen free radicals. However, they found that when the rats were pretreated with α-tocopherol, the formation of lipid peroxidation products was significantly inhibited near the iron injection site [29,30]. Synthetic antioxidants have also been shown to be effective in decreasing lipid peroxidation in iron-loaded rats [31]. The potential usefulness of free radical scavengers in iron overload has been discussed [52]. It would have been interesting to obtain values on lipid peroxidation in the present study. We [58] showed previously that malondialdehyde, a commonly used measure of lipid peroxidation, was not metabolized in brain homogenate, but use of this chemical to assess lipid peroxidation in vivo has been shown to present problems [59].

Our results presented here have demonstrated that there is significant total iron accumulation in brain cortex synaptosomes of iron-treated animals. Theoretically, it is possible that the excess iron induces free radical damage to membrane lipids and proteins. Our results using the fatty acid spin labels demonstrate a small but very reproducible and statistically significant increase of the order parameter (S) and rotational correlation time (τ_c) of synaptosomes from iron-treated rats. This indicates a reduced freedom of probe motion and, hence, a decrease in fluidity of the synaptosomal membranes after iron treatment. The lipid layer of the synaptosomal membrane became more rigid in the hydrophobic area of the membrane, as shown by the 16-NS spin label, and near the membrane surface, as shown by 5-NS spin label.

The MSL spin label was used to measure the conformational rigidity of sulfhydryl membrane proteins. The technique of monitoring the motion of spin-labelled sulfhydryl groups of membrane proteins has been used for several years [18,21,22,33]. The weakly (w) immobilized sulfhydryl groups yield a sharp three line spectrum whereas the strongly (s) immobilized sulfhydryl groups yield a spectrum typical of more immobile nitroxyls. The ratio of peak heights of weakly to strongly (*w/s*) MSL spin label measured as described previously [18] was obtained for synaptosomes isolated from control and iron-treated rats. The decreased value of *w/s* in the EPR spectra of synaptosomal membrane proteins of iron-treated animals suggests that membrane organizational or protein conformational changes have occurred. The decreased *w/s* ratio indicates that there is a higher proportion of strongly immobilized sulfhydryl groups allowing only restricted motion of the label, in comparison to weakly immobilized sulfhydryls which allow relatively free motion of the label bound to them. It is known that exposure to

oxidizing conditions causes membrane proteins to become cross-linked into dimers or larger aggregates which is consistent with the changes observed in the present results. The cross-links may be mediated either by interprotein disulfide formation or by reaction between free radical-damaged amino acid residues [23,56]. Peroxidation results in the destruction of sulfhydryl groups in proteins [34]. Thus, peroxidation would be expected to cause a net loss of membrane protein-SH groups. Our results showing a net increase in protein sulfhydryl content in synaptosomes of iron-treated animals as compared to the controls can possibly be explained by the expected higher amount of ferritin formed in the iron-treated animals.

Experimental iron loading in vivo has shown this leads to a rise in intracellular ferritin synthesis [35]. This response of the cells to synthesize ferritin is a protective mechanism. With increasing concentrations of iron, the intracellular ferritin concentration rises to a plateau level about 6–10-times normal as was shown in vivo and in vitro in different tissues [36–39]. Ferritin contains at least 48 cysteine residues [40]. Therefore, the higher protein-SH content found in our study in the iron-treated animals may be due to the possible increase in ferritin synthesis as a result of iron treatment. But, as shown earlier there is a limit to iron-stimulated ferritin synthesis even under continuous overloading [41]; thus, the excess free iron, if present, may induce oxidative damage resulting in the membrane modifications described earlier.

The cholesterol content was slightly but statistically significantly higher in synaptosomal membranes of iron-treated animals as compared to controls. It is known that an increase in the molar ratio of cholesterol to phospholipid causes a decrease in the membrane lipid fluidity [42,43]. The higher cholesterol content may have an enhancing effect, thus further decreasing the lipid fluidity of synaptosomal ' membranes in the iron-treated animals.

Centrophenoxine is a drug that has been found to slow down certain phenomena associated with aging [44]. Its effective portion is dimethylaminoethanol (DMAE), which is incorporated into the nerve cell membranes of experimental animals in the form of phosphatidyl-DMAE [45] and this has proven to be a very efficient free radical scavenger [46,57]. Centrophenoxine treatment has been shown to decrease the microviscosity and the cholesterol content of synaptosomal membranes in old animals [47]. Centrophenoxine also reduced the formation of lipofuscin in experimental animals and increased the activity of superoxide dismutase, glutathione peroxidase and glutathione reductase in different brain regions [48]. Chronic treatment of rats with centrophenoxine resulted in a relatively high concentration of DMAE in brain cell membranes where this compound exerted a protective effect on proteins against oxygen free radicals [49].

In the experiments reported here, centrophenoxine-pretreatment diminished the effect of iron injections. Centrophenoxine significantly prevented the accumulation of iron in the synaptosomes. In addition, after centrophenoxine pretreatment, the cholesterol content of synaptosomal membranes following iron injections was no different from controls. We have shown that both the hydrophobic and hydrophilic regions of the synaptosomal membrane is more fluid following centrophenoxine-pretreatment and iron injection versus animals receiving iron injections. In addition, centrophenoxine exerted a significant protective effect against iron-induced conformational changes of membrane proteins as monitored by MSL spin label.

There is a similarity between the alterations of the synaptosomal membranes observed after iron treatment and that which occurs during aging [18,47]. The common characteristics are: higher cholesterol content, more rigid lipid bilayers, and an increased amount of cross-linked proteins in the membranes. Free radical damage of the membrane components resulting in serious impairment of membrane function is one of the fundamental factors of the membrane hypothesis of aging [50,51]. The present study thus points to an important question: 'Can increased brain iron accelerate the aging phenomenon in brain?'. This question is currently under investigation.

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