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AGE-RELATED CHANGES IN LIPID PEROXIDATION PRODUCTS IN RAT ADRENAL GLAND

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ABSTRACT

Chloroform-methanol extracts from rat adrenals at five different ages (2, 6, 12, 18 and 24 months), were studied by fluorescence. After obtaining excitation and emission spectra, fluorescence intensity was measured at 365 nm excitation and 455 emission for all time points of aging. An additional study of lipid peroxidation employing a thiobarbituric acid reaction was made.

Fluorescence intensity increased during aging from 16.39 x 10^3 arbitrary units of fluorescence per gram of tissue at 2 months, to 34.33 x 10^3 units at 24 months. Thiobarbituric acid reaction products expressed in nmol of malondialdehyde per gram of adrenal increased from 172.97 at 2 months to 640.83 at 24 months. One way analysis of variance revealed a statistically significant difference ($p<0.05$ and $p<0.01$ respectively).

The results show an age-related steady increase in lipid peroxidation products in rat adrenals and suggest their accumulation in lipofuscin granules.

INTRODUCTION

Free radicals acting on polyunsaturated fatty acids of tissues lead to the formation of lipid hydroperoxydes (1) which decompose into carbonyls such as malondialdehyde (MDA). Carbonyls can react with free amino groups of proteins, aminophospholipids and nucleic acids, thereby resulting N,N´-disubstituted 1-amino-3-iminopropenes, which are fluorescent conjugated Schiff bases (2). These fluorescent properties can thus be used in the detection of lipid peroxidation (3).

Due to a similarity noted in the spectral properties of Schiff bases and the age pigment lipofuscin (2,4) spectrofluorometric methods may also be applied in aging studies. In fact, in extracts obtained from aged *Drosophila melanogaster* (5), testes of aged mice (6) and other tissues of aged rats (7) an increase in fluorescence intensity was observed when compared with extracts from young animals.

In the adrenal cortex of the rat, it has been known since long that fluorescent lipopigment accumulates in aged animals (8). In ultrastructural studies, there is considerable evidence for an age-related increase in lipofuscin granules in all three zones of the cortex of the rat: the zona glomerulosa (9), zona fasciculata (10,11) and zona reticularis (12). Despite the consistency of the data, it was not evaluated if such structural age-related changes were associated with modifications related to free radical effects. Taking several time points during aging as recommended (13), we decided thus to perform a study in the adrenal extracts of rats to evaluate changes which might indicate the occurrence of lipid peroxidation.

MATERIAL AND METHODS

Male Wistar rats from the colony of the Gulbenkian Institute of Science (Oeiras, Portugal) were allowed to grow with free access to water and laboratory diet and were kept at room temperature with natural day-night light cycles. At 2, 6, 12, 18 and 24 months (mo.), 7 randomly selected and apparently healthy animals were sacrificed after intraperitoneal administration of 50 mg/Kg of pentobarbital. The left adrenal was dissected from surrounding fat and frozen at -20°C until assay. After sacrifice, all the animals were necropsied. Some rats, aged 18 and 24 months, exhibited gross evidence of disease, particularly in the lungs, which showed irregular round nodules, some of them containing jelly or cheese-like material. Frequently, tumors of subcutaneous tissue of the shoulders and limbs were observed. Occasional tumors of the viscera with apparent metastases to the liver were also seen. When such pathological changes were evident, the animals were put off the study.

Processing of the adrenals: After decapsulation, each adrenal was homogenized in 1 ml of 0.1 M phosphate buffer, pH 7.2 for 90 sec with a Teflon-glass homogenizer. The extraction was made on 0.5 ml aliquots, adding 10 ml of a 2:1 mixture of chloroform-methanol (E. Merck, Germany), 100 µl of butylated hydroxytoluene (Aldrich Chemical Co.) and vortexing for 2 min. The remaining extraction procedure and removal of interfering compounds continued according to a previous method (6).

Spectrofluorometry: The fluorometric study of the samples was made with a Jasco FP 770 spectrofluorometer (Japanese Spectroscopic Co, Tokyo) equipped with a Xenon arc lamp 150 W, a photomultiplier R376 and set with a sensitivity of 100. The standard used was quinine sulfate, 1 µg/ml of 0.1 N H_2SO_4, which had a relative fluorescence intensity of 22. Excitation and emission spectra of the samples of each animal at 2, 12 and 24 mo. were obtained and the values averaged for the number of animals studied. The fluorescence intensity of the samples at all ages, was measured with the

excitation and emission wave lengths set at 365 and 455 nm respectively and calculated in arbitrary units of fluorescence per gram of tissue (AUF/g) (6).

Thiobarbituric acid (TBA) reaction: A preliminary study of the TBA reaction for the detection of lipid peroxidation products was made on adrenal samples of the same animals, employing a spectrophotometric method (14). As standard, MDA (1,1,3,3 - Tetramethoxypropane, Sigma Chemical Co.) was used and the final values were expressed as nmol of MDA/g of tissue.

Statistical Analysis: The means of the measurements at the different times were compared using ANOVA. To assess statistically significant differences between individual pairs, the Student´s *t* test was used.

RESULTS

The excitation and emission spectra obtained as described above are depicted in Figure 1. A notorious increase in the fluorescence intensity with the increase of age was observed with excitation maxima at 320-325 nm and emission maxima at 450-455 nm.

The intensity of fluorescence whose results are shown in Table 1, also increased in the 5 time points studied from 16.39 x 10^3 AUF/g of tissue at 2 mo. to 34.33 x 10^3 AUF/g of tissue at 24 mo.

The results of the assay for TBA reaction are depicted in Table 1. From 2 mo. untill 24 mo. there is a steady increase from 172.97 to 640.83 nmol of MDA/g of adrenal.

In the statistical analysis, an age-related significant increase of both the fluorescence intensity and TBA reactive substances values was found ($p<0.05$ and $p<0.01$ respectively). When individual pairs were contrasted using Student´s *t* test, a significant difference in fluorescence intensity ($p<0.05$) was found at 24 mo. when compared to 2, 6 and 18 mo.; similar significant differences ocurred when comparing MDA level at 24 months with levels at all other ages.

Table 1: Fluorescence intensity, TBA reactive substances and rat adrenal weights at different ages

	2 mo.	*6 mo.*	*12 mo.*	*18 mo.*	*24 mo.*
F[a]	16.390±3.38	16.397±5.41	18.656±4.31	18.476±1.31	34.327±6.76
TBA[b]	172.97±35.0	182.94±46.7	223.89±12.1	234.58±42.5	640.83±27.4
Ad. Weight[c]	16.29±0.61	16.57±0.81	16.86±1.14	16.86±0.94	16.72±1.55

[a] Fluorescence intensity in AUF/g of tissue x 10^3 ± Standard error x10^3. n= 7.

[b] TBA reactive substances in nmol of MDA/g of tissue ± Standard error. n=3.

[c] Decapsulated left adrenal weight in mg ± Standard error. n= 7.

DISCUSSION

N,N´-disubstituted 1-amino-3-iminopropenes, conjugated Schiff bases, are products of lipid peroxidation with fluorescent properties which exhibit maximum intensity when the excitation and emission wave lengths are set at 340-370 nm and 420-470 nm respectively (6).

The use of fluorescence measurement has high sensitivity (15). For the study of lipid peroxidation, this method has been applied to pure chemical systems

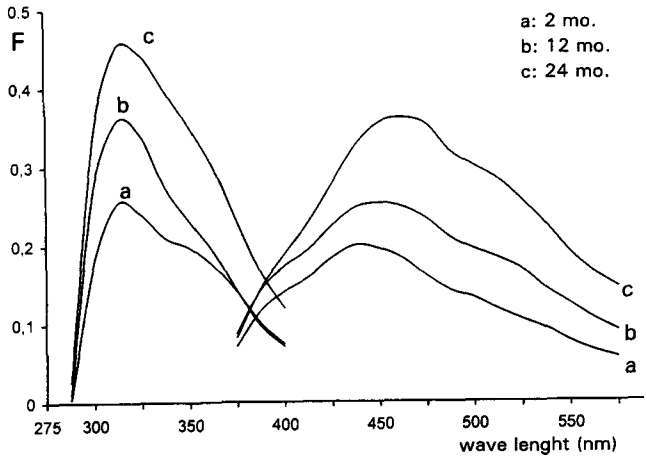

Figure 1: Excitation and emission spectra of chloroform-methanol extracts of rat adrenal glands at 2, 12 and 24 mo. F: fluorescence intensity in arbitrary units of fluorescence.

(2,16,17) but also to extracts of red blood cells (18), adipose tissue (19) and mitochondria and microsomes (15). In these studies, not only the presence of conditions of enhanced peroxidation was associated with an increase in the intensity of fluorescence, but also, in some of them, the introduction of antioxidants prevented such increase.

The similarities in spectral properties of Schiff bases and extracts of tissues from old subjects led to the assumption that those products are probably responsible for the fluorescent properties of lipofuscin, the age pigment (2). In this setting, an increased age related fluorescence was detected in lipid extracts from *Drosophila melanogaster* (5), rat testes (6,7) and other tissues (7).

The data we found in the adrenal extracts show a significant increase in fluorescence intensity per unit of weight in the five time points from 2 months until 24 months. It indicates that during aging, fluorescent material similar to conjugated Schiff bases is possibly accumulating in the rat adrenal. Fluorescent steroid hormones may be present in the extracts and interfere with the intensity of fluorescence. However, either corticosterone, the main steroid synthesized in the rat adrenal (20) or cortisol, have excitation and emission maxima at 470 nm and 520 nm respectively (21) and so their contribution is probably not significant. In addition, in another method to detect products of lipid peroxidation, an increase in TBA reactive substances in extracts from the old rats adrenals was also observed.

A relation between these adrenal fluorescent products and the lipofuscin granules is likely. Lipopigments have since long been known as fluorescent. Excitation and emission maxima range from 360-395 nm and 430-460 nm respectively (22), therefore very similar to the fluorescent products obtained from lipid peroxidation. Also, in a previous histological study (23), sections of rat heart muscle, kidney cortex, interstitial cells of testes and adrenal cortex displayed enhanced fluorescence when compared to similar sections from young animals.

Moreover, in spectrofluorometric studies made in extracts of rat heart, kidney (7) and testes (6,7), it was shown an increase in fluorescence in old animals compared to young ones. No data was presented however concerning the adrenals.

The results of the present study, showing an age-related increase in fluorescent products similar to conjugated Schiff bases are in agreement with previous morphologic quantitative data obtained with the fluorescence (23) and the electron microscope (9,10,11,12) studies reporting an age-related increase in lipofuscin granules in all three zones of the adrenal cortex. Such data make lipofuscin granule the likely cellular structure where the fluorescent substances accumulate. The evidence here reported is also highly suggestive of enhanced free radical activity in the adrenals of aged rats.

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