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The influence of the chain length of aldehydes on the fluorescence of chromolipids

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Incubation of phosphatidylethanolamine containing liposomes with malondialdehyde and other aldehydes with different chain lengths results in the fluorescence of chromolipids. Relative to malondialdehyde, the fluorescence was greatly enhanced with increasing chain length upon incubation of 2-alkenals with phospholipids. Similar results were found using the total lipid extracted from erythrocyte ghosts. It seems that the hydrophobic character of the aldehydes is important for the amount of fluorescence detected in lipid bilayers.

Chromolipid; Malondialdehyde; Aldehyde; Chain length

1. INTRODUCTION

As a result of lipid peroxidation in biological membranes malondialdehyde [1] and other aldehydic products are formed such as *n*-alkanals, 2-alkenals, 2,4-alkadienals and 4-hydroxyalkenals [2]. Next to malondialdehyde, 4-hydroxynonenal has been identified as one of the major compounds formed during the peroxidation of polyunsaturated fatty acids, cells and particulate fractions [2-4].

During lipid peroxidation of biological materials fluorescent chromolipids are formed [5-9] with spectral characteristics similar but not always identical [8], to lipofuscin [1,7,10]. These pigments accumulate with age and are regarded as end products of the peroxidation process. It is generally believed that fluorescent chromolipids result from the condensation of malondialdehyde (MDA) with the amino group of phospholipids [1], e.g. phosphatidylethanolamine (PE) and phosphatidylserine (PS), proteins [6,11], nucleic acids [9] and amino acids [7,10] to form Schiff bases. However, recently Kikugawa [8] reported dihydropyridine derivatives of high fluorescence in reactions of primary amines with MDA under physiological conditions.

Polymerisation of membrane proteins during the process of lipid peroxidation has been reported by Koster et al. [6,12,13] and others [8,14] and was thought to be closely related to MDA production. Recently the involvement of 4-hydroxynonenal in the formation of fluorescent chromolipids during the peroxidation of microsomes, mitochondria and liposomes has been reported [4,7] and was suggested to be of more importance for the formation of chromolipids than malondialdehyde. The involvement of aldehydic products of lipid peroxidation other than malondialdehyde and 4-hydroxynonenal in the formation of fluorescent chromolipids has not yet been studied. However, fluorescence spectra of erythrocyte proteins modified with monofunctional aldehydes have been described [14].

In this paper we report the formation of fluorescent chromolipids in the reaction of aldehydes with

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phosphatidylethanolamine containing liposomes and with lipids extracted from erythrocyte membranes.

2. MATERIALS AND METHODS

2.1. *Lipids and aldehydes*

Phosphatidylcholine (PC) was purchased from Brunschweig Chemie. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) were purchased from Supelco. The fatty acid composition of PC and PE were determined by GLC as described before [15]. PC consisted of di-18^2PC (>99% pure) and PE of 16^0(14%), 18^0(36%), 18^1(7%), 18^2(8%), 20^4(15%), 22^5(3%), 22^6(17%). The total lipid from erythrocyte membranes was extracted as described by De Gier et al. [16].

n-Alkanals and 2-alkenals were purchased from Janssen Chemica, 4-hydroxynonenal was a gift from Dr H. Esterbauer (University of Graz, Austria). MDA was freshly prepared from 1,1,3,3-tetraethoxypropane [5,8]. The purity of the aldehydes was estimated by capillary gas-liquid chromatography, after converting them into dimethylacetals by heating with methanol-HCl as described for fatty acid methyl esters [15].

Liposomes containing 0.4 mg PC, 0.4 mg PE and 0.06 mg PS per ml Tris-HCl buffer (0.03 M, pH 8.0) were prepared according to the method of Pick [17] in a nitrogen atmosphere. A predominant unilamellar structure of the liposomes was confirmed by electronmicroscopy.

2.2. *Erythrocyte ghosts*

Erythrocytes from healthy donors were separated from the plasma and the cells were washed three times with phosphate buffered saline (pH 7.4). Subsequently the erythrocytes were lysed in 10 mM phosphate buffer (pH 7.4). The membranes were collected by centrifugation at 20000 × *g* for 40 min and washed repeatedly with 10 mM phosphate buffer (pH 7.4) until the washings were colourless. The preparations were stored at −70°C and used within a few days.

2.3. *Measurement of fluorescence*

For fluorescent chromolipid detection the liposomes (0.3 ml) were incubated for 20 h with 0.2 ml of different aldehydes (2 mM final concentration) in 0.9% (w/v) NaCl solution at room temperature. Subsequently the reaction mixture was extracted with 1.9 ml of a mixture of chloroform/methanol (1:2, v/v) and then, after 0.63 ml chloroform and 0.63 ml water were added, centrifuged for 10 min at 2500 × *g*. 1 ml was taken from the chloroform layer and after addition of 0.1 ml methanol the fluorescence was measured at the maximal excitation wavelength between 300 and 420 nm and at the maximal emission wavelength between 380 and 500 nm, respectively (Perkin Elmer fluorescence spectrophotometer, type MPF-3). Quinine sulphate (0.1 μg/ml in 0.05 M H_2SO_4) was used for calibration. Controls consisting of liposomes alone and aldehydes without liposomes were incubated for 20 h and did not reveal any fluorescence.

3. RESULTS AND DISCUSSION

The amount of fluorescence of chromolipids in liposomes induced by the Fe^{2+}-ascorbate system is dependent on the concentration of PE (fig.1). It is shown that the PE/PC ratio correlates well with the fluorescence. The spectral characteristics are an excitation wavelength near 360 nm and an emission wavelength near 430 nm. During the time course of the peroxidation process a shift in emission wavelength from 460 nm towards lower wavelengths was noted. It is generally assumed [1,10] that the increase in fluorescence is the result of the formation of conjugated Schiff bases between aldehydes and the amine group in phospholipids, e.g. PE and PS, of which malondialdehyde was suggested to be the most important second product of lipid peroxidation in this respect. Kikugawa [8] reported fluorescent compounds of more complicated structure e.g. 1-substituted-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes as a result of the reaction of ethanolamine or other primary amines with MDA or with monofunctional aldehydes.

Maxima of 386–403 nm and 444–465 nm were reported for respectively the excitation and emission spectra.

Esterbauer and co-workers [7] suggested that aldehydes other than MDA can be responsible for the chromophore formation in lipid extracts of biological materials which is supported by other investigators [8,14]. In particular 4-hydroxynonenal was mentioned by Esterbauer et al. [7]. These authors also noted differences in the spectral

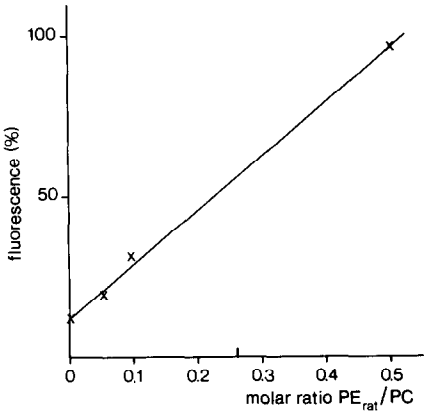

Fig.1. Fluorescent chromolipid formation in liposomes containing different amounts of phosphatidylethanolamine. Liposomes containing 0.4 mg phospholipid per ml Tris-HCl buffer (0.03 M, pH 8.0) were incubated for 120 min with 0.2 mM $FeSO_4$ and 1 mM Na-ascorbate (final concentration). The fluorescence was measured at an excitation/emission of 360/430 nm.

characteristics between fluorescent chromolipids formed by MDA and those found after peroxidation of biological materials. They reported excitation/emission wavelengths of 400 /475 nm for MDA and 350–360/430 nm for lipid extracts from biological materials. For 4-hydroxynonenal the same excitation/emission wavelength was found as for biological material. The shift in excitation/emission maxima from 400/460 nm to 360/430 nm which we noted during the time course of peroxidation suggests an increasing contribution of aldehydic products other than MDA in fluorescent chromolipid formation during peroxidation.

Fig.2 shows the fluorescence of chromolipids upon incubation of PE containing liposomes with respectively MDA, *n*-alkanals and 2-alkenals. Taking MDA as a reference (1.00) a lower fluorescence was found for the *n*-alkanals, without any influence of the chain length. However, the 2-alkenals showed a much stronger fluorescence than MDA when the same amounts of aldehydes were incubated with the lipids. It was found that the double bond in these aldehydes strongly enhances the fluorescence. In contrast to the *n*-alkanals the fluorescence is increased by increasing chain length which is probably caused by the increasing lipophilic character of the aldehydes.

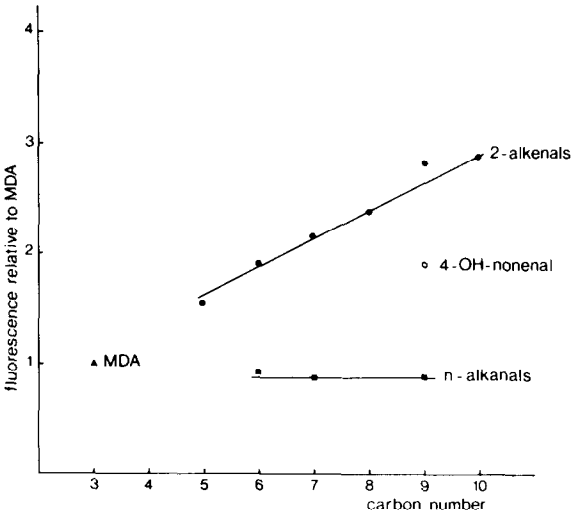

Fig.2. Fluorescence of chloroform/methanol extracts after incubation of liposomes with different aldehydes. Liposomes (0.3 ml) containing 0.4 mg PC, 0.4 mg PE and 0.06 mg PS per ml Tris-HCl buffer (0.03 M, pH 8.0) were incubated for 20 h with different aldehydes, respectively MDA (▲), *n*-alkanals (■), 4-hydroxynonenal (○) and 2-alkenals (●). The fluorescence of the chloroform/methanol-extractable lipid material was measured relative to MDA. The results are plotted against the carbon number of the respective aldehydes.

That the lipophilic character is important is substantiated by the fact that the relative amount of fluorescence formed by the more hydrophilic 4-hydroxynonenal is lower than for the analog 2-nonenal upon incubation with lipids. Though we do not know the quantum yield of the different fluorescent chromolipids in these experiments, it is interesting that the relative amount of fluorescence increases with increasing hydrophobicity of the 2-alkenals. Remarkable is also the fact that a shift occurs in maximal excitation/emission wavelength which we recorded to be respectively 400/460 nm for MDA and 360/430 nm for the 2-alkenals. These results agree with the data of Esterbauer et al. [7] for 4-hydroxynonenal and total lipids from biological membranes.

We selected MDA, 4-hydroxynonenal and 2-octenal as being representative for our further investigations with liposomes prepared from the lipids of erythrocyte ghosts. Incubation of the above mentioned aldehydes with liposomes prepared from lipids of freshly isolated erythrocyte membranes gave similar results to those obtained

with the liposomes prepared from PE and PC (table 1).

These experiments confirm that with increasing chain length (increasing hydrophobic character) of the aldehyde more fluorescence in the lipid matrix of the biomembrane is detected while the more hydrophilic 4-hydroxynonenal contributes less to the fluorescence formation, but compared to MDA this contribution is still quite substantial.

Free radical reactions, ubiquitous in living organisms play a crucial role in the genesis of certain dense and autofluorescent structures [8] found in senescent and injured cells. These age pigments and lipofuscin material are found to be related to aging and pathological conditions [8], and rather tissue specific [18]. Our findings confirm that different aldehydes as well as MDA can contribute to fluorescent chromolipid formation in biomembranes. However, we found that the presence of unsaturated bonds and the influence of chain length strongly influence the amount of fluorescence measured.

Most of the fluorescent material formed was found to be lipid extractable, however also water-soluble fluorescent material was found especially upon incubation with MDA. Disturbance of the molecular organization of biomembranes caused by the interaction of aliphatic aldehydes with specific phospholipids and crosslinking of proteins as a result of lipid peroxidation or enhanced breakdown of plasmalogens might be one of the underlying mechanisms in aging of cells and tissues as well as in pathology e.g. plaque formation in demyelinating diseases such as in multiple sclerosis.

Table 1

Relative fluorescence of liposomes from the total lipid of erythrocytes after incubation with aldehydes

2-Octenal	4.10
4-Hydroxynonenal	2.95
Malondialdehyde	1.00

0.3 ml of liposomes prepared from the total lipid extracted from erythrocyte ghosts (equivalent to 0.21 mg PE) were incubated for 20 h at room temperature with 0.2 ml of the respective aldehydes (2 mM final concentration). The fluorescence of the chloroform/methanol-extractable lipid material was measured relative to malondialdehyde

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