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PHOTOSTABILITY OF LIPID COMPONENTS OF HUMAN BLOOD PLASMA LIPOPROTEINS DURING EXPOSURE TO LONG WAVE ULTRAVIOLET RADIATION (UV-A) ALONE AND IN THE PRESENCE OF 8-METHOXYPSORALEN

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In this work, photooxidation of lipid components isolated from human blood plasma lipoproteins (VLDL, LDL and HDL) after irradiation with UV-A alone and in the presence of 210 μg×l^{-1} 8-methoxypsoralen (8-MOP), called PUVA method (psoralen+UV-A), was studied. No significant changes in the lipids isolated from lipoproteins irradiated without 8-MOP were found. In the case of lipoprotein irradiated in the presence of 8-MOP the ESR fluidity study using methyl-5-doxylstearate as spin label showed that photostability of lipid components of lipoproteins decreased in order: VLDL>LDL>HDL. The FT-IR and ^{1}H-NMR study revealed that lipid components of LDL and especially of HDL fraction during PUVA exposition undergo photodegradation, probably due to oxidation of unsaturated phospholipids by reactive oxygen species (mainly singlet oxygen) produces by 8-MOP sensitization. In contrast, lipids from PUVA treated VLDL fractions were practically intact. These results may be explained by the various antioxidant concentrations in individual lipoprotein fractions or by difference in diameter of VLDL, LDL and HDL particles influencing on accessibility of reactive oxygen species to lipoprotein lipid components. Whereas, it is not clear whether in conditions of experiment the cyclobutane photoadducts of 8-MOP with unsaturated lipids were formed.

INTRODUCTION

Extracorporeal photopheresis (ECP) is a modality of PUVA-therapy, which involves exposure of leukocyte-enriched blood in the mixture supplemented with 30% of blood plasma (Edelson, Berger, Gasparro, Jegasothy, Heald, Wintroub, Vonderheid, Knobler, Wolff, Plewig *et al.* 1987) by UV-A radiation (λ = 320-400nm) after pretreatment with 8-methoxypsoralen (8-MOP) leading to cell apoptosis. The mechanism of ECP involves photoaddition of 8-MOP to cell components (mainly DNA) as well as photooxidation of cell structures (particularly proteins and lipids) *via* reactive oxygen species sensitized by 8-MOP, similarly to action of photodynamic therapy. ECP demonstrated clinical efficacy in cutaneous T-cell lymphoma (CTCL) in Sezary syndrome, in scleroderma, in patients with acute and chronic graft-versus-host-disease (GVHD) after bone marrow transplantation and in other autoimmune diseases (Pawlaczyk, Żmudzińska & Jenerowicz 2003, Martino, Console, Pucci, Irrera, Messina, Bresolin, Morabito & Iacopino 2004). Since 1988 this method has been approved by US Food and Drug Administration for the treatment of CTCL.

According to Fossel, Fletcher, McDonagh & Hui (1991), beneficial effect of ECP in the Sezary syndrome may be connected with 8-MOP sensitized photooxidation of low density lipoproteins which form peroxide products cytotoxic towards lymphocytes. These authors predicted that the target of LDL photoperoxidation are unsaturated fatty acids from lipid core of lipoproteins; analogically to effect of ionizing radiation on cell membranes leading to peroxidation of polyunsaturated fatty acid residues forming their phosphorlipid components. (Grzelińska, Bartosz, Gwoździński & Leyko 1979). On the other hand, Patsch, Sailer, Kostner, Sandhofer, Holasek & Braunsteiner (1974) have indicated that amount of phospholipids in the individual lipoprotein fractions is rather similar (19.9, 25.5 and 28.6% in VLDL, LDL and HDL, respectively).

Abbreviations: ^{1}H-NMR – proton nucleic magnetic resonance, 8-MOP – 8-methoxypsoralen, CTCL – cutaneous T-cell lymphoma, ECP – extracorporeal photopheresis, ESR – electron spin resonance, FT-IR – Fourier transformation infrared spectroscopy, GVHD – graft versus host disease, HDL – high density lipoproteins, HPLC – high performance liquid chromatography, LDL – low density lipoproteins, M5NS – methyl 5-doxylstearate, PBS – phosphate buffer saline, PUVA – psoralen + ultraviolet A, UV-A – ultraviolet A, VLDL – very low density lipoproteins

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This suggests that PUVA exposition of VLDL and HDL should produce, likewise as in the case of LDL, lipid peroxides which could reveal cytoxic activity toward T-lymphocytes.

In our work, the study of lipid component photooxidation in VLDL, LDL and HDL lipoproteins under PUVA exposition in vitro was performed.

MATERIALS AND METHODS

Lipoproteins (VLDL, LDL and HDL fractions) were processed by sequential ultracentrifugation of human blood (Havel, Eder & Bragdon 1955), collected from 10 healthy donors in Poznań Blood-Donation Center. In several fractions total protein concentration was determined using a colorimetric method described by Lowry, Rosebrough, Farr & Randall (1951), and then all these fractions were diluted by PBS (Biomed, Lublin, Poland) to a final concentration of 10.0 mg×l^{-1} *vs.* total protein. Triglyceride concentration in each examined lipoprotein fraction was also determined with use of commercial BioMérieux test and clinical chemistry analyzer with read-easy performance VISUAL (BioMérieux, Marcy d'Etoile, France).

The PUVA-exposition was performed according to Fossell, Fletcher, McDonagh & Hui (1991). 1.93 ml of each diluted non-deoxygenated fraction was mixed with 70 μl of 8-methoxypsoralen (Sigma, St. Louis, MO, USA) 6 mg×l^{-1} stock solutions to the both concentration of 210 μg×l^{-1}. The obtained reaction mixtures were incubated for 15 min and subsequently irradiated with UV-A radiation (λ = 365 nm) from distance 5 cm for 20 min (fluence rate 8.3 mW×cm^{-2}, fluence 10 J×cm^{-2}) using lamp Emita-VP60 (Famed, Łódź, Poland). Fluence rate was measured using E_e-meter luminometer, constructed in the Department of Light Technology at University of Technology in Poznań, Poland. The excess of 8-MOP after exposition was removed by dialysis. Lipoprotein fractions untreated with 8-MOP were samples of reference.

To examine photooxidation process undergoing during PUVA exposition, the fluidity and chemical structure of lipoprotein lipid components before and after UV-A irradiation were studied. The lipid components were isolated from non-exposed und UV-A exposed lipoproteins using extraction by 2:1 chloroform-methanol mixture, according to Folch, Less & Sloane Stanley (1957). 1 ml of each fraction were shaked 15 min with 20 ml of in the proportion, and then kept for 15 min in dark to obtain two layers. The upper layer was removed and to each sample 16 ml of 0.05 M NaCl was added to separate protein components of lipoproteins from their lipid components. The obtained solutions containing lipid components of lipoproteins were evaporated under low pressure to dryness and the obtained dry residues were further analyzed.

To determine fluidity changes of lipids, a spin labeling method with methyl-5-doxylstearate (M5NS) as spin label was applied. M5NS was synthesized in Department of Biophysics of University of Łódź according to Hubbell & McConnell (1971). The ethanolic solution of M5NS was added to the suspension 6.0 mg lipid in 0.6 ml of PBS (Biomed Lublin, Poland) to final concentration of spin label 5×10^{-6} M. The ESR spectra were recorded at the temperature 21°C in a Bruker ESP-300E spectrometer operating at X-band, at the modulation frequency 100 kHz. The modulation amplitude was 1.01 G, the receiver gain 2×10^{6}, the time constant 5.12 ms, the scan time 10.5s and the microwave power 6.38 mW, in the range of field intensity from 3430 to 3530 G.

Generally, the fluidity of extracted lipids was estimated on the base of h_0/h_{+1} and h_0/h_{-1} ratios where h_0, h_{+1} and h_{-1} were the peak height of the central, low-field and high-field lines, respectively (Fig. 1).

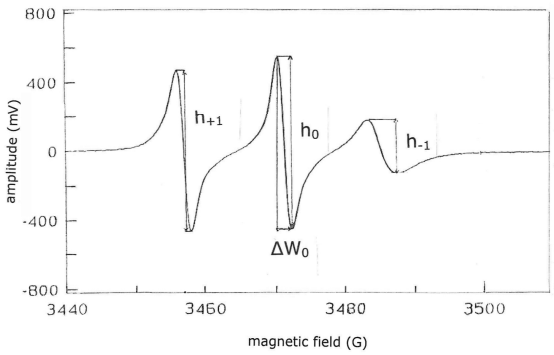

Fig. 1. ESR spectrum of methyl 5-doxylstearate labeled lipoprotein lipids. On the plot the spectral amplitudes of low, central and high field peak as well as the peak linewidth of the central line, signified as h_{-1}, h_0, h_{+1} and ΔW_0 were indicated.

The rotational correlation time for a rod-like molecule was calculated using the formula of Hemminga (1975):

$$\tau_c = K \times \Delta W_0 \times [(h_0/ h_{-1}-1)^{1/2}]$$

where K = 6.5×10^{-10} s×G and ΔW_0 is peak-to-peak linewidth of the central line.

For investigation of chemical changes undergoing during UV-A exposition, Fourier transformation – infrared spectrophotometry (FT-IR) and proton nuclear magnetic resonance spectrometry (^{1}H-

NMR) were applied. FT-IR spectra were performed for about 3-10 mg of substance in photoacoustic cuvette MTEC 100 (Ames, IA, USA) using FT-IR Ramspec 152 spectrophotometer (Bomem Québec, Canada). All recorded spectra were normalized *vs.* the spectrum of powdered coal.

In the case of ^{1}H-NMR measurement, about 6.0 mg of substance were dissolved in $CDCl_3$ (Sigma, St. Louis, MO, USA) and the spectra were recorded on Varian Unity 300 spectrometer at the field frequency 4000 Hz, the spectral width 20 ms and acquisition time 3.7 s, using tetramethylsilan (Sigma, St. Louis, MO, USA) as an internal standard.

RESULTS AND DISCUSSION

The total protein and determination in VLDL, LDL and HDL before and after UV-A exposition has indicated no significant changes of protein amount in the course of UV-A irradiation both alone and in the presence of 8-MOP. These results are similar to those obtained by Beijersbergen van Henegouven, Wijn and Schoonderwoerd (1989) obtained for PUVA exposed skin cells. According to these authors a principal cellular target of 8-MOP during PUVA exposition are lipids. Thus in the further study the lipids extracted from intact and UVA irradiated lipoproteins were investigated

Table 1. Protein and triglyceride concentration ($g \times dl^{-1}$) in intact, UVA and PUVA treated lipoprotein fractions. The results were expressed as a mean from 10 samples ±s.d.

lipoprotein fraction	total protein			Triglycerides		
	intact	after UVA exposition	after PUVA exposition	intact	after UVA exposition	after PUVA exposition
VLDL	11.0±6.0	9.8±3.0	9.0±1.0	55.6±6.3	54.5±7.2	54.7±4.5
LDL	17.3±4.5	17.0±2.5	17.5±5.5	7.4±0.5	7.4±0.8	7.6±0.7
HDL	30.7±2.2	29.6±2.0	25.1±1.8	4.5±0.4	4.3±0.5	4.0±0.2

The concentration of triglycerides in all lipoprotein fractions was also constant during UV-A irradiation both in the absence and in the presence of 8-MOP (Table 1). Similarly, investigation of lipid fluidity changes performed by determination of h_0/h_{-1} and h_0/h_{+1} ratios and rotational correlation time τ_c of M5NS spin labeled lipids isolated from lipoproteins intact and irradiated by UV-A in the absence of 8-MOP has indicated no significant changes of these spectral parameters (Table 2). However, in the case of lipid components UV-A irradiated in the presence of 8-MOP the changes of parameters were appeared. The lipids from PUVA-irradiated HDL fractions showed a significant (about twice) decrease of all measured ESR parameters indicating increase in fluidity during PUVA exposition while in the case of LDL lipids only h_0/h_{-1} and h_0/h_{+1} ratios were diminished while τ_c was not changed. In contrast, VLDL lipid all measured spectral parameters before and after PUVA exposition was constant (Table 2).

Table 2. Variation of h_0/h_{-1}, h_0/h_{+1} and correlation time (τ_c) for methyl 5-doxylstearate labeled lipids isolated from intact, UV-A and PUVA treated lipoprotein fractions, as a measure of their fluidity changes. The results were expressed as a mean from 10 samples ±s.d.

parameter	VLDL			LDL			HDL		
	intact	after UVA exposition	after PUVA exposition	intact	after UVA exposition	after PUVA exposition	intact	after PUVA exposition	after PUVA exposition
h_0/h_{-1}	1.10±0.11	1.11±0.10	1.09±0.13	1.09±0.12	1.10±0.13	1.07±0.10	1.15±0.13	1.16±0.20	1.04±0.10
h_0/h_{+1}	3.43±0.24	3.41±0.15	3.40±0.17	3.33±0.12	3.31±0.11	3.25±0.10	3.46±0.21	3.44±0.18	3.11±0.19
τ_c(ns)	1.13±0.14	1.12±0.20	1.13±0.10	1.19±0.11	1.20±0.12	1.13±0.10	1.13±0.10	1.12±0.11	0.97±0.08

These results suggest that lipids of lipoproteins fractions are not *per se* photolabile but undergo photodegradation under the sensitization by 8-MOP. The observed increase of HDL and LDL lipid fluidity, similar to that indicated for erythrocytes membranes under exposition on the red light

in the presence of hematoporphyrin (Lakos & Berki 1995), suggests that unsaturated fatty acids of HDL and LDL fraction during PUVA exposition undergo peroxidation, as reported by Fossel *et al.* (1991) for LDL fractions.

This prediction was confirmed by IR analysis which in the case of VLDL lipids showed no significant changes during PUVA exposition (Fig. 2A) while for LDL lipid components a small increase of 3400 cm^{-1} band assigned to –OH group was observed (Fig. 2B) and under irradiation of HDL lipids the 3400 cm^{-1} band increased 6-fold comparing to analogical enhancing of LDL lipids with parallel decrease of 3000 cm^{-1} band (Fig. 2C) assigned to C=C double bond (Arrondo & Goñi 1998, Yang, Li, Xu & Kuang 2000).

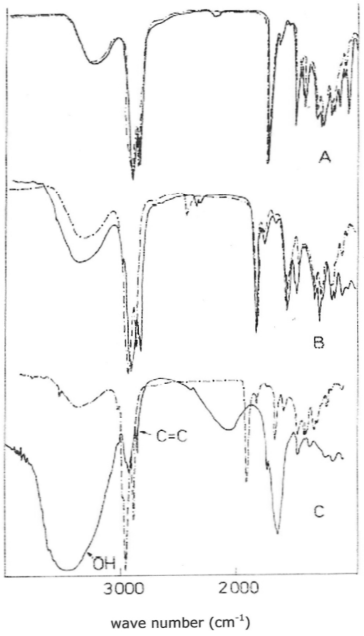

Fig. 2. FT-IR spectra of total lipids isolated from intact (.-.-) and PUVA exposed (−) lipoprotein fractions. A-VLDL, B-LDL, C-HDL

At the same time, in the IR spectra of LDL and HDL lipids the band at 2077 cm^{-1} was appeared, probably connected with associated –OH groups and in the HDL lipid spectra the absorption band at 1645 cm^{-1} was appeared which may be assigned to C=O stretching band characteristic for carboxylic groups (Nara, Okazaki & Kagi 2002).

As a next step of our study, the NMR analysis of lipid mixture extracted from lipoproteins was performed to better recognize the chemical structure of obtained photoproducts. For LDL and especially for HDL lipids (Fig. 3) the ^{1}H-NMR spectra revealed a decrease of peaks at 4.2-4.5 ppm which may be assigned to =CH protons in triglyceride and phospholipid compounds (Tugnoli, Poerio & Tosi 2004).

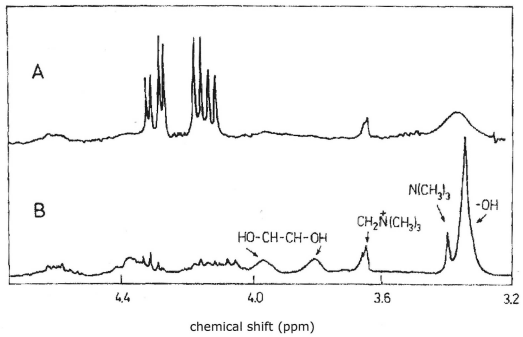

Fig. 3. ^{1}H-NMR spectra of total lipids isolated from intact (A) and PUVA exposed (B) HDL fractions.

As our earlier study of triglyceride amount in the intact and UV-A treated lipoproteins has indicated that its concentration does not change during UV-A exposition (Table 1), thus in condition of our experiment, the target of 8-MOP attack were probably unsaturated phospholipids.

The ^{1}H-NMR spectra may confirm this predict-tion: formation of peaks at 3.41 and 3.61 ppm during PUVA exposition may be connected with destruction of tertiary amino group and ammonia group (Sze & Jardetzky 1990) which are both characteristic for phospholipids. The appearance of two peaks in the region 3.8-4.0 ppm can be assign-ned to formation of interesting group HO-CH-CH-OH under photooxidation of unsaturated bonds (Potapenko 1991). The increase of the peak at 3.4 ppm may also be attributed to the forming of –OH. However, analysis of peak heights in the NMR spectra did not demonstrate any simple correlation between decrease of =CH protons and appear of –OH groups as potential result of photo-oxidation (data not shown).

The peaks appeared in the 3.8-4.0 ppm region may be also assigned to protons of cyclobutane ring which form during photocycloaddition to unsaturated phospholipids (Zarębska, Waszkow-ska, Caffieri & Dall'Acqua 1998), which play an important role in PUVA therapy. However, lack of peaks in the region 6.8-7.6 ppm, attributed to protons of psoralen compound, suggests that such photoadducts were not present among the products of 8-MOP photoreaction with lipoprotein lipids. In the presence of oxygen, these photo-adducts are very unstable (Caffieri 2002) and, on the other hand, they easily undergo degradation during extraction (Zarębska *et al.* 1998). Thus in the studied conditions, 8-MOP acts rather as pho-tosensitizer of peroxidation, *via* formation of reactive oxygen intermediates (mainly singlet

oxygen) just as in the photodynamic therapy (Potapenko 1991, Caffieri 2002).

No changes in the range 0.99-1.01ppm, attributed to proton cholesterol ring (Sze & Jardetzky 1990, Tugnoli *et al.* 2004) during PUVA exposition of studied lipoproteins were also observed. This suggests that cholesterol and its esters which may be also a target of photooxidation (Bowry, Stanley & Stocker 1992) do not probably participate in the observed photooxidation sensitized by 8-MOP.

CONCLUSIONS

This *in vitro* study has indicated that unsaturated phospholipids of LDL and HDL may be the target of 8-MOP during UVA irradiation to form hydroxylic and carboxylic products. Significantly higher photolability of HDL lipid components comparing to these of LDL, despite a similar phospholipids and unsaturated fatty acid amounts in both these fractions (Patsch, Sailer, Kostner, Sandhofer, Holasek & Braunsteiner 1974, Dimitriadis, Griffin, Collins, Johnson, Owens & Tomkin 1996) may be explained by lack of antioxidants in most HDL particles, in contrast to LDL which contain α-tocopherol and ubiquinol-10 (Bowry *et al.* 1992). Similarly, a relatively high photostability of VLDL may be due to the carrying of α-tocopherol (Fechner, Schlame, Guthmann, Stevens & Rüstow 1998). An alternative explanation of observed phenomenon is the difference in size of the LDL and HDL particles. The diameter of HDL particles (8-10 nm) is about two times lower as much as that of LDL particles (19-21 nm) and about five times lower as much as that of VLDL particles (30-80 nm), which may also affect the accessibility of generated by 8-MOP singlet oxygen to attack lipid components in lipoprotein fractions (Jerlich, Hammel, Nigon, Chapman & Schaur 2000, Kostner & Laggner 1989, Wasan, Ramaswamy, Kwong & Boulanger 2002).

For study of kinetics and mechanism of observed photooxidation processes the separation and isolation of photoproducts for different points of time reaction will be necessary. This method (*e.g.* HPLC) should enable the structural analysis of photoproducts as well as determination of photophysic parameters of this reaction such as quantum yield or lifetime of 8-MOP excited states. It is also necessary to examine in animal model (*e.g.* in mice) the potential cytotoxicity of HDL peroxide photoproducts toward T-lymphocytes. The results of such research will enable to determine potential therapeutic efficacy of these peroxides in the diseases treated with ECP.

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