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LIPID PEROXIDATION IN HYPERLIPIDAEMIC PATIENTS. A STUDY OF PLASMA USING AN HPLC-BASED THIOBARBITURIC ACID TEST

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The question of "increased lipid peroxidation" in plasma from hyperlipidaemic patients was investigated using an improved HPLC-based assay for thiobarbituric acid-reactive material. Levels of TBARS in healthy human controls were at or close to zero, provided that butylated hydroxytoluene was added to the sample with the TBA reagents. Levels of plasma TBARS in hyperlipidaemic patients were elevated, although the absolute levels were much lower than those reported previously in the literature.

KEY WORDS: Lipid peroxidation, atherosclerosis, hyperlipidaemia, TBARS, malondialdehyde, HPLC.

INTRODUCTION

There is considerable current interest in the role played by lipid peroxidation in the development of atherosclerosis[1,2]. Indeed, the interior of advanced human arteriosclerotic lesions is a highly pro-oxidant environment[3]. Hyperlipidaemia predisposes to atherosclerosis, and extensive studies in Japan (reviewed by Yagi[4]) have led to the suggestion that hyperlipidaemic patients have elevated levels of lipid hydroperoxides in their blood, which might damage vascular endothelium[4] and initiate atherosclerosis[1]. Unfortunately, these studies were carried out using the thiobarbituric acid (TBA) test to measure malondialdehyde (MDA) and it is now appreciated that the TBA test can generate many artefacts when applied to human body fluids[5,6].

First, non-lipid constituents of plasma, and aldehydes other than MDA, react with TBA to give chromogens which contribute to absorbance measurements at 532 nm[5,7]. Indeed, the bulk of the apparent MDA detected in the TBA test is not present in the sample being assayed, but is generated by decomposition of lipid hydroperoxides during the acid-heating stage of the assay[5,6]. This peroxide decomposition is accelerated by traces of contaminating iron salts in the TBA reagents and it can be inhibited by chelating agents[6,8]. Second, peroxide decomposition produces radicals that can attack other lipid molecules during the assay, amplifying the response. Thus the greater the lipid content of an aliquot of body fluid tested, the greater will be the TBA reactivity, because of increased "amplification" during the test. This artefact could obviously influence the results of determinations on

hyperlipidaemic samples[9]. Human plasma contains chain-breaking antioxidants[2], variation in the concentrations of which could also affect the results of the TBA test[9].

In the present paper, we have re-examined the question of whether or not hyperlipidaemic patients show elevated plasma lipid peroxidation. First, samples were treated with an excess of the chain-breaking antioxidant butylated hydroxytoluene, BHT[10,11], in order to suppress peroxidation during the assay and eliminate artefacts due to the effect of variations in endogenous lipid and antioxidant content on events taking place during the assay itself. Second, the authentic MDA-$(TBA)_2$ chromogen was separated from other chromogens using high-performance liquid chromatography. HPLC greatly enhances the specificity of the TBA test[12–14] and we have developed an improved method for this purpose.

MATERIALS AND METHODS

Reagents

All chemicals, including tetraethoxypropane, were obtained from Sigma Chemical Co Ltd, Poole, Dorset, UK. Hyperlipidaemic plasma samples were obtained from blood drawn for authentic clinical purposes from patients attending the Lipid Clinic at Addenbrooke's Hospital, Cambridge, UK. Most patients were being treated with the lipid-lowering drug Simvastatin: preliminary experiments showed that this did not generate chromogens in the TBA test. Control samples were from volunteer laboratory staff. In all cases, blood was drawn into heparinized containers and centrifuged immediately to obtain plasma, which was frozen at −70°C for a maximum period of 7 days before analysis. Storage for such a short period has been shown not to cause increases in TBA-reactive material. Plasma total cholesterol levels were measured as described in Carpenter *et al*[15]. Rat liver microsomes were prepared by differential centrifugation as described by Quinlan *et al*[16].

Microsomal Lipid Peroxidation

Rat liver microsomes (0.25 mg/ml microsomal protein) were incubated at 37°C for various times in 20 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.4) in the presence of 0.1 mM $FeCl_3$ and 0.1 mM ascorbic acid in a total volume of 8 ml. At each time point, 4 ml of each 8 ml reaction mixture were added to 0.4 ml 0.2% (w/v) BHT in ethanol and the other 4 ml were added to 0.4 ml ethanol. From each 4 ml volume 0.25 ml was added to 1.5 ml phosphoric acid (H_3PO_4) for the HPLC method. After 10 min at room temperature 0.5 ml freshly prepared TBA reagent (0.6% w/v TBA in water, heated gently to 60°C to dissolve the TBA) was added to each tube, followed by mixing and heating at 90°C for 30 minutes.

Analysis of Plasma Samples

Where indicated, BHT was added to the freshly-drawn plasma and 0.25 ml of plasma was added to 1.5 ml H_3PO_4 and the analysis continued as described above.

Tetraethoxypropane standards (dissolved in ethanol) at concentrations of 2.5, 5.0 and 10 μM were included in parallel with all samples. Absorbances and peak heights were converted into μM MDA equivalents from the results of these standards.

HPLC Analysis

Sample (20 μl) was injected onto a Spherisorb 5 ODS_2 (C_{18}) column (HPLC Technology, Macclesfield, Cheshire, UK) fitted with a guard (Hiber C_8, HPLC Technology). HPLC was carried out using a Waters Millipore (Watford, Hertfordshire, UK) Model A6000 HPLC system. Sample is eluted with 65% (v/v) 50 mM KH_2PO_4-KOH buffer pH 7.0 and 35% (v/v) methanol at a flow rate of 1 ml/minute. A sharp peak corresponding to the $(TBA)_2$-MDA adduct elutes at a retention time of approximately 4.8 minutes, as detected by a uv/visible detector set at 532 nm (see Figure 1).

Using these conditions the column requires washing with methanol:HPLC grade

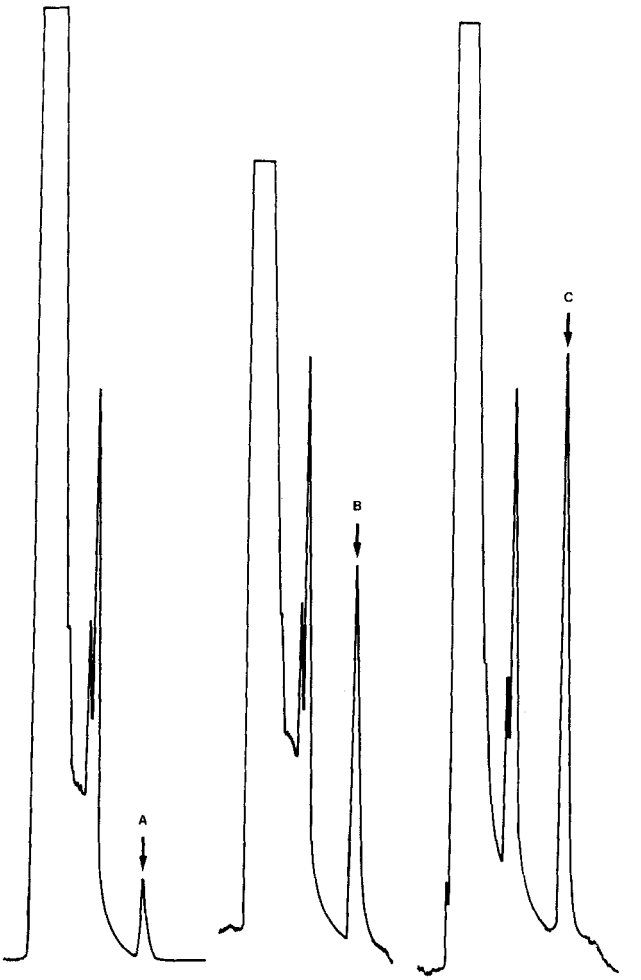

FIGURE 1 *Typical HPLC chromatogram showing separation of the $(TBA)_2$-MDA adduct.* The TEP (MDA) standards were prepared and the assay performed as in Section 2.9. 20 μl of each reaction mixture were injected onto the HPLC column. The retention time of the adduct in this experiment was 4.2 minutes. A = blank (ethanol) peak, B = 2.5 μM MDA peak, C = 5 μM MDA peak. Full scale deflection = 0.01 absorbance.

H_2O (80:20) at the end of each run for 30 minutes, and the guard column cartridge should be replaced every 60 determinations. With constant use for analysis of samples of body fluids, a column should be repacked after 300 determinations.

RESULTS

The HPLC method described in this paper gives a clear separation of the $(TBA)_2$-MDA adduct (Figure 1). Peak identity is confirmed by spiking the reaction mixture with authentic $(TBA)_2$-MDA produced from tetraethoxypropane added to the reaction mixture to generate MDA during the acid hydrolysis stage of the assay and checking for co-elution, and by checking the fluorescence maximum of the chromogen at 553 nm (when excited at 515 nm.).

Studies on Microsomal Lipid Peroxidation

The amount of free MDA in most peroxidizing lipid systems is low, but rat liver microsomes are claimed to be an exception[17]. Table 1 shows the time course of TBARS formation, as measured by HPLC, in rat liver microsomes whose peroxidation was induced by adding $FeCl_3$ and ascorbate[18]. It may be seen that, even in this system, addition of BHT with the TBA reagents decreased the amount of TBARS at all time points except when peroxidation was essentially complete, again illustrating the role that is played by further peroxidation of lipids during the assay itself. Addition of larger quantities of BHT did not alter the results obtained (data not shown).

Studies on Human Plasma

Freshly-prepared human plasma from hyperlipidaemic patients and apparently-healthy approximately age-matched controls were treated with BHT and subjected to the HPLC-based TBA test. Parallel samples were also tested in the absence of BHT. In the presence of BHT, the TBARS of freshly-prepared plasma from healthy human subjects is very low, the mean value being much less than 1 μM (Table 2). Four of the twelve control subjects showed no detectable TBARS. Omission of BHT

TABLE 1
Formation of TBARS in peroxidizing rat liver microsomes
Results are mean ± SE and are expressed as μM MDA equivalents from a standard curve using tetraethoxypropane. Peroxidation was started at time zero by adding ascorbate and $FeCl_3$ and samples withdrawn for analysis at the times stated. Some samples were treated with BHT before adding the TBA reagents and others were not

Time (min)	TBARS (μM MDA equivalents)	
	−BHT	+BHT
0		
2	3.7 ± 1.9	2.7 ± 1.5
5	12.0 ± 0.8	5.8 ± 0.9
10	14.3 ± 0.5	7.6 ± 0.8
15	14.6 ± 0.5	9.6 ± 0.6
20	14.7 ± 1.0	12.8 ± 1.0

TABLE 2
TBARS in plasma from hyperlipidaemic patients and normolipidaemic controls

Control subjects
Number 12 (5 males, 7 females)
Mean age 43 Age range 23–72
Mean plasma cholesterol 4.7 ± 1.2 mM
Cholesterol range 2.8–6.8 mM
TBARS −BHT 0.45 ± 0.35 μM All samples positive
TBARS +BHT 0.10 ± 0.08 μM 4 samples TBARS not detected
Hyperlipidaemic patients
Number 17 (8 males, 9 females)
Mean age 53 Age range 20–67
Mean plasma cholesterol 7.1 ± 1.1 mM
Cholesterol range 5.1–9.0 mM
TBARS −BHT 0.88 ± 0.22 μM All samples positive
TBARS +BHT 0.61 ± 0.25 μM All samples positive

led to higher values, again illustrating the potential for artefact in the TBA assay. By contrast, plasma from all the hyperlipidaemic patients tested contained significantly-elevated TBARS, even in the presence of BHT. Even if the results are standardized for cholesterol (Table 1), the difference is highly significant ($p < 0.002$).

DISCUSSION

Published mean levels of TBARS in human plasma (obtained by the "simple" TBA test) vary widely, e.g. from 47.2 μM MDA equivalents to 4.2 μM (see the reviews in[4,19]). These values are largely artefactual[5,6]. Previous HPLC-based TBA tests have found mean values of 1.4 μM to 0.6 μM (reviewed in[19]). However, specific assays for lipid hydroperoxides find little or none in human plasma, certainly <0.1 μM[20], although one lipid hydroperoxide molecule could, depending on its origin, produce more than one molecule of MDA. Our data show that application of an improved HPLC-based TBA test can give similar low values, provided that BHT is added with the TBA reagents. Four out of 12 control subjects showed no detectable TBARS. Our system thus provided a useful "quick screening" for the presence of lipid peroxidation end products before subjecting samples to more complex and tedious assay procedures[9,20,21].

In theory, our system does not eliminate all potential problems. For example, some molecules other than lipid hydroperoxides, such as certain amino acids and the sugar deoxyribose[22,23] can react in the TBA test to yield an authentic $(TBA)_2$-MDA adduct. In addition MDA can be formed in small amounts during prostaglandin metabolism[24]. Thus even the HPLC-based TBA test can over-estimate the real level of peroxides in human material. However, this does not seem to be a major artefact, since the level of "peroxides" measured by this method in fresh plasma from healthy subjects over a wide age range is extremely low (<1 micromolar) and often zero.

Our results are consistent with earlier claims[4] that plasma from hyperlipidaemic patients does contain elevated lipid hydroperoxides as compared with age-matched controls, even when corrected for lipid and bearing in mind that the patients are being given lipid-lowering drugs. However, the absolute levels of TBARS we obtain are

very much lower. The origin of this plasma TBARS is uncertain. Hydroperoxides could be formed in the plasma (enzymically and non-enzymically), or they could be present in dietary fats and taken into the body from this source. They might also escape from atherosclerotic lesions into plasma. Their elevated levels in hyperlipidaemic patients might simply be a consequence of the greater atherosclerosis in these patients, rather than a cause of it.

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