

## Prostaglandin E<sub>2</sub> and leukotriene B<sub>4</sub> synthesis by peripheral leucocytes in alcoholics

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**SUMMARY** Alcohol inhibits phospholipase (PL) activity in a number of animal models. We have therefore measured prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>), liberated by stimulated peripheral blood mononuclear cells (PBMC) and neutrophils respectively in chronic alcoholics and in control subjects. Peripheral blood mononuclear cells from alcoholics produced less PGE<sub>2</sub> ( $p < 0.01$ ) and neutrophils produced less LTB<sub>4</sub> ( $p < 0.025$ ). Reduced PGE<sub>2</sub> production by PBMC of alcoholics was corrected by the addition of exogenous arachidonic acid ( $p < 0.005$ ) whilst neutrophil LTB<sub>4</sub> production remained lower in the alcoholics ( $p < 0.01$ ). Percutaneous liver biopsies were undertaken in the 20 alcoholics having abnormal liver function tests. Prostaglandin E<sub>2</sub> biosynthesis was lower in PBMC from patients with alcoholic hepatitis than with alcoholic cirrhosis ( $p < 0.05$ ). Analysis of PBMC fatty acid composition demonstrated that endogenous arachidonate and linoleate contents were not significantly different in alcoholics and controls. Cells from controls and alcoholics were incubated with 0, 50 and 150 mmol/l ethanol for two hours but there was no alteration in PGE<sub>2</sub> or LTB<sub>4</sub> biosynthesis. In summary, we found reduced eicosanoid production by peripheral leucocytes in alcoholics, supporting the hypothesis that chronic alcohol consumption either inhibits membrane bound phospholipase activity or enhances, alternatively, catabolism of eicosanoids. This phenomenon is more marked in alcoholic patients with hepatitis than in those with cirrhosis alone.

Acute or chronic alcohol consumption may damage the liver of susceptible individuals. This effect is dose related and may also be dependent upon nutritional status and genetic determinants in individual patients.<sup>1</sup>

Animal experiments have shown that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) protects the hepatocyte against D-galactosamine and carbon tetrachloride induced toxicity.<sup>2</sup> Furthermore, inhibition of leukotriene B<sub>4</sub> (LTB<sub>4</sub>) synthesis decreases the inflammatory response in immune hepatitis.<sup>3</sup> The Kupffer cell is thought to originate from the same stem cell as the peripheral blood monocyte<sup>4</sup> and may be the major source of hepatic prostaglandin biosynthesis.

Endogenous arachidonic acid (AA), present in the

cell membrane, is released from the triglycerides phosphatidyl choline and phosphatidyl inositol by phospholipases A<sub>2</sub> and C respectively. The predominant origin of free intracellular AA is phosphatidyl choline.<sup>5</sup> Phosphatidyl inositol is only present in small quantities in leucocyte membranes<sup>6-8</sup> and is thus a minor source of free AA. Both phospholipases are calcium dependent.

Because of methodological problems related to isolation of Kupffer cells and neutrophils from percutaneous liver biopsy specimens, we have used the peripheral blood mononuclear cell (PBMC) and neutrophil to investigate the effects of alcohol on eicosanoid secretion by stimulated cells *in vitro*.

### Methods

#### PATIENTS

Forty three patients (aged 35-60 years) who met

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internationally agreed criteria for the diagnosis of alcoholism<sup>9</sup> were studied. This group consisted of hospital inpatients who had not taken alcohol or any drugs known to alter eicosanoid metabolism in the seven days before phlebotomy. Twenty four healthy volunteers (age and sex matched), recruited from the clinical and laboratory staff of the hospital, served as a control group. The alcohol abusers who consumed in excess of 80 g alcohol daily for a period of at least five years were divided into two groups – those with normal haematological and biochemical parameters (n=23) and those with one or more abnormal results (MCV=97 (1.1) fl; alk phos 155 (19) IU/l; GGT 169 32 IU/l; AST 87 (13) IU/l; total bilirubin 33 (9)  $\mu$ mol/l; mean (SEM)).

Liver biopsies were carried out on the 20 patients with abnormal liver function tests. Of these, 10 patients had features of alcoholic hepatitis with (fatty changes in seven and Mallory's hyaline in four and 10 had micronodular cirrhosis (active hepatitis in three and cirrhosis without inflammation in seven).

#### ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND NEUTROPHILS:

Twenty millilitres of venous blood was drawn into sodium heparin vacutainers (Becton-Dickinson, Rutherford, New Jersey, USA) and 10 ml into vacutainers without anticoagulant for isolation of cell and serum respectively. Leucocyte rich plasma obtained after dextran sedimentation was layered on Hypaque-Ficoll gradients (Pharmacia) and centrifuged at 400 g for 20 minutes before harvesting PBMC from the interface. The neutrophils and residual erythrocytes sedimented to the base of the gradient. Identification and counting of PBMC and neutrophils were performed by fluorescent microscopy using ethidium bromide and acridine orange staining. As lymphocytes do not produce appreciable amounts of PGE<sub>2</sub>,<sup>10</sup> secretion was assumed entirely due to monocytes. Esterase staining was used to determine the percentage of monocytes in the mononuclear cell fraction.<sup>11</sup> Cells were resuspended in Dulbecco's phosphate buffered saline (PBS) containing 1 mmol/l glucose to concentration of  $1 \times 10^6$  cells/ml. Using these techniques no neutrophils were found in the PBMC fractions and *vice versa*. In addition, freshly isolated cells were in excess of 98% of viable as assessed using fluorescent microscopy with ethidium bromide and acridine orange staining.

#### OPSONISATION OF ZYMOSAN

One hundred milligrams zymosan A (Sigma) was suspended in 10 ml PBS, allowed to swell overnight, centrifuged to remove fines and resuspended in 10 ml PBS. One millilitre samples were incubated with 1 ml autologous serum for 30 minutes at 37°C. The

samples were centrifuged to sediment the opsonised zymosan and washed with PBS to remove residual serum. The opsonised zymosan was resuspended in 5 ml PBS with a final concentration of 2 mg/ml.

#### PROSTAGLANDIN E<sub>2</sub> AND LEUKOTRIENE B<sub>4</sub> SECRETION

Half a millilitre of the mononuclear cell suspension was incubated with 0.5 ml of opsonised zymosan for 30 minutes at 37°C in the presence and absence of 10 nmol arachidonic acid (AA). This time period was found on preliminary experiments to be the most appropriate for optimal secretion of PGE<sub>2</sub>. Briefly, secretion of PGE<sub>2</sub> was shown to increase with time up to 10 minutes, after which the level of secretion remained constant for up to 45 minutes; 0.5 ml of neutrophil suspension was incubated with 0.5 ml 4  $\mu$ mol/l calcium ionophore A23187 (Sigma) for 10 minutes at 37°C in the presence and absence of 10 nmol AA.

#### PROSTAGLANDIN E<sub>2</sub> AND LEUKOTRIENE B<sub>4</sub> DETERMINATION

After incubation samples were placed on ice. Cells and zymosan were removed by centrifugation at 400 g and 4°C for 25 minutes. Prostaglandin E<sub>2</sub> was determined by radioimmunoassay using PGE<sub>2</sub> antiserum (Sigma) according to the method of Bauminger *et al.*<sup>12</sup> Leukotriene B<sub>4</sub> content was assessed using LTB<sub>4</sub> antiserum (Wellcome) according to the method of Salmon.<sup>13</sup> Antisera were found to have only 3% crossreactivity with arachidonic acid and other eicosanoids. PGE<sub>2</sub> estimation were validated using thin layer chromatography and LTB<sub>4</sub> using high performance liquid chromatography. Intra and inter-assay variation was less than 15%.

#### FATTY ACID ANALYSIS

Lipids from 500  $\mu$ l of the cell fractions and from 110  $\mu$ l of serum were extracted with 2.5 ml chloroform:methanol (2:1). Heptadecanoic acid (C17:0) was used as an internal standard. The lower layer was dried down and transesterified as follows: 0.5 ml boron trifluoride methanol (Sigma, Poole, Dorset, UK) was added and incubated at 75°C for 30 minutes. After incubation the samples were extracted three times with 1 ml petroleum ether (40-60°), the petroleum ether was dried down and resuspended in 20  $\mu$ l n-hexane. A two  $\mu$ l sample was injected for each run using a 1.5 m $\times$ 4 mm column containing 10% Silar 10 C on 100-120 mesh Chromosorb WHP (Pye Unicam, UK). The running temperature was 150-230°C at 4°/min. Fatty acid identification was confirmed by comparison of retention times with authentic standards (Sigma, UK).

Table 1 Total arachidonic and linoleic acid content of peripheral blood mononuclear cells

	Arachidonic	Linoleic
Control (n=20)	32 (4) [11-57]	17.0 (1.5) [9-47]
Alcohol (n=21)	24 (6) [7.5-57.3]	13.5 (1.7) [1.3-29.2]
	p=NS	p=NS

EFA concentrations are expressed in ng/mg DNA; mean (SEM) [range]

#### IN VITRO ETHANOL INCUBATION STUDY

Peripheral blood mononuclear cells were isolated from 10 control subjects and 15 alcoholics by the method described above. Cells were suspended in PBS alone and with 50 and 150 mmol/l ethanol. After incubation for two hours, the cells were stimulated with zymosan in the presence and absence of 10 nmol arachidonic acid. Prostaglandin E<sub>2</sub> and leukotriene B<sub>4</sub> concentrations of the supernatant were measured by radioimmunoassay as already described.

#### STATISTICAL ANALYSIS

Data on eicosanoid and fatty acid concentrations are expressed as mean and standard error. Differences found between the patients and controls were analysed by Student's two-tailed *t* test for parametric data and the Mann-Whitney U-test for non-parametric data. A *p* value of less than 0.05 was considered significant.

#### Results

Endogenous arachidonic and linoleic acid content of PBMC was similar in both alcoholics and control subjects (Table 1). PGE<sub>2</sub> production was significantly

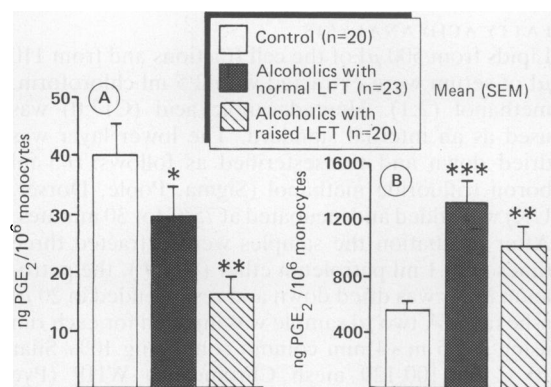


Fig. 1 Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production by PBMC in the absence (A) and presence (B) of added arachidonic acid. \*Represents *p* < 0.05; \*\*represents *p* < 0.001 and \*\*\**p* < 0.005.

Table 2 Prostaglandin E<sub>2</sub> production by PBMC from patients with histologically assessed alcoholic liver disease

	PGE <sub>2</sub> -AA	PGE <sub>2</sub> +AA
Cirrhosis (n=10)	25.4 (4.8) [5-52]	1162 (237) [566-3000]
Hepatitis (n=10)	11.8 (2.4) [3-22]	506 (143) [75-1400]
	<i>p</i> < 0.005	<i>p</i> < 0.005

PGE<sub>2</sub> concentrations are expressed as ng/10<sup>6</sup> cells. AA=arachidonic acid Mean (SEM) [range]

lower in PBMC from patients abusing alcohol compared with control subjects, the effect being more marked in cells from patients with biochemical and histological evidence of liver damage (*p* < 0.05) (Fig. 1). Biosynthesis of LTB<sub>4</sub> by neutrophils was significantly depressed in alcoholics compared with control subjects (Fig. 2). Addition of arachidonic acid (AA) to stimulated PBMC resulted in an enhanced PGE<sub>2</sub> production by cells from alcoholics (Fig. 1) whereas LTB<sub>4</sub> secretion by neutrophils remained significantly lower in the alcoholic groups in the presence of added arachidonic acid (Fig. 2).

Percutaneous liver biopsies were performed in the 20 alcoholics with abnormal liver function tests; 10 having alcoholic hepatitis and 10 alcoholic cirrhosis. Prostaglandin E<sub>2</sub> biosynthesis was significantly lower in patients with hepatitis and with cirrhosis alone (Table 2). To determine whether reduced eicosanoid production in cells from alcoholics resulted from the effects of acute or chronic alcohol exposure, cells

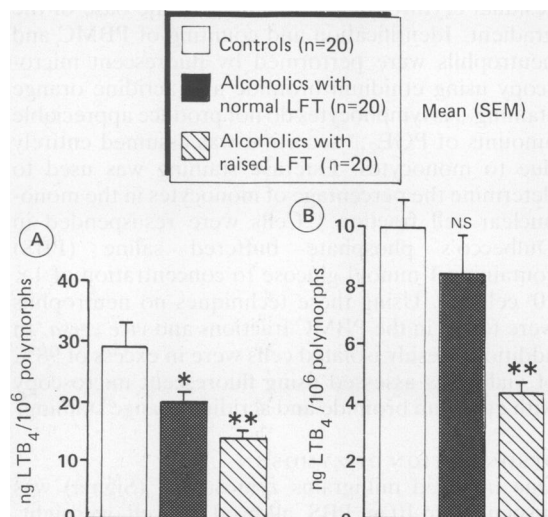


Fig. 2 Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) production by neutrophils in the absence (A) and presence (B) of added arachidonic acid. \*Represents *p* < 0.05 and \*\*represents *p* < 0.01.

Table 3 *In vitro* incubation of peripheral blood mononuclear cells (PBMC) and neutrophils with ethanol

		Mean (SEM) [Number Studied] Range No Alcohol	Mean (SEM) [Number Studied] Range 50 mM Ethanol	Mean (SEM) [Number Studied] Range 150 mM Ethanol
Monocytes Control PGE <sub>2</sub>	Zymosan	40.0 (7) [10] 5.6-35	56.0 (15) [5] 6.8-91	58 (26) [5] 15-70
	Zymosan+AA	723 (99) [10] 580-1200	820 (117) [5] 580-1100	768 (102) [5] 510-1000
Monocytes Alcoholic PGE <sub>2</sub>	Zymosan	22.8 (4) [11] 9.4-51	15.0 (3) [7] 9.4-35	20.2 (5) [7] 11.5-38
	Zymosan+AA	599 (112) [11] 130-1300	644.3 (142) [7] 400-900	760 (171.5) [7] 500-900
Neutrophils Control LTB <sub>4</sub>	Ionophore	6.8 (0.7) [4] 4.8-8	8.4 (1.0) [4] 6.5-10	7.8 (0.3) [4] 7-8
	Ionophore+AA	3.2 (1.0) [4] 1.0-7.2	1.2 (0.5) [4] 1.0-2	1.0 (0.4) [4] 1.0-2.1
Neutrophils Alcoholic LTB <sub>4</sub>	Ionophore	7.6 (0.8) [9] 5-12	8.5 (1.0) [9] 3.4-14	6.9 (0.7) [9] 4.6-10.0
	Ionophore+AA	2.9 (0.5) [9] 1.2-6.0	4.8 (1.5) [9] 1.0-14	4.2 (0.7) [9] 3.8-10.0

Eicosanoid values are expressed in ng/10<sup>6</sup> cells. PBMC were stimulated with opsonised zymosan, neutrophils with calcium ionophore, in the presence and absence of arachidonic acid (AA).

from controls and alcoholics were incubated with 0, 50 and 150 nmol/l ethanol for two hours. Ethanol in these concentrations had no effect on PGE<sub>2</sub> synthesis by PBMC or LTB<sub>4</sub> release by neutrophils from either alcoholics or control subjects (Table 3).

## Discussion

Prostaglandin E<sub>2</sub> has potent anti-inflammatory properties including inhibition of T-cell aggregation, histamine release and suppression of macrophage lysosomal proteases.<sup>14</sup> By contrast, LTB<sub>4</sub> is a potent inflammatory mediator, stimulating leucocyte chemotaxis, aggregation and superoxide release.<sup>15</sup>

The isolation of pure viable Kupffer cells from percutaneous liver biopsy specimens is difficult for a number of technical reasons including small sample size, contamination with peripheral blood leucocytes and difficulties separating Kupffer cells from monocytes. Consequently, we chose to use the peripheral blood mononuclear cell as a model of the Kupffer cell (the intrahepatic tissue-fixed macrophage), to which it is ontogenically related.<sup>16</sup> Prostaglandin E<sub>2</sub> is the predominant arachidonic acid metabolite in the

human circulating monocyte whereas the major eicosanoid product of the neutrophil is LTB<sub>4</sub>.

Given the limitation that we used peripheral blood leucocytes in this study, we have shown decreased *in vitro* biosynthesis of cytoprotective PGE<sub>2</sub> by peripheral monocytes, together with decreased LTB<sub>4</sub> production by peripheral neutrophils from patients with alcohol related liver disease. These abnormalities were more marked in those with impaired liver function. PGE<sub>2</sub> released by PBMC was lowest in the group with alcoholic hepatitis.

The reduced eicosanoid production was not the result endogenous substrate depletion as we found no difference in the total cellular arachidonate and linoleate content of PBMC from alcoholics and controls. The depression in PGE<sub>2</sub> biosynthesis by cells from alcoholics was overcome by the addition of exogenous arachidonic acid to PBMC incubates, indicating both the possibility of defective release of endogenous substrate by phospholipases and that the cyclo-oxygenase system was functional. By contrast, LTB<sub>4</sub> production by neutrophils from both controls and alcoholics was further suppressed by the addition of exogenous arachidonic acid. Lipoygenase may

have been inhibited by substrate or by feedback inhibition by 15-hydroxyeicosatetraenoic acids (15-HETEs).<sup>17</sup>

Diminished eicosanoid concentrations in the *in vitro* peripheral leucocyte from chronic alcohol abusers may result from reduced endogenous arachidonate availability caused by decreased phospholipase activity or increased catabolism of the measured products (PGE<sub>2</sub> and LTB<sub>4</sub>).

Ethanol increases calcium binding to mammalian membranes, predominantly to the protein calmodulin.<sup>18,19</sup> The calcium-calmodulin complex associated with the Ca<sup>2+</sup>-ATPase system, increasing calcium efflux across the cell membrane.<sup>20</sup> The consequent reduction in intracellular ionised calcium concentration would in turn reduce calcium dependent phospholipase A2 and C activation. This is supported by the observation that acute ethanol exposure significantly decreases eicosanoid biosynthesis in the isolated perfused rat lung model.<sup>21</sup> By contrast, we did not find any significant change in PBMC, PGE<sub>2</sub> or neutrophil LTB<sub>4</sub> production when these cells from control subjects and alcoholics were incubated in the presence of up to 150 mmol/l ethanol for two hours. This may be related to the relatively long incubation time chosen for our acute ethanol exposure studies.

Peripheral leucocyte eicosanoid production is reduced in patients who abuse alcohol and is most marked in those with active alcoholic hepatitis. It is likely that this alteration in eicosanoid metabolism is the result of reduced release of the substrate arachidonic acid from membrane phospholipids by phospholipase. As neutrophil infiltration is less marked than macrophage and lymphocyte infiltration in alcoholic hepatitis, the findings of reduced PBMC PGE<sub>2</sub> may be more relevant than low LTB<sub>4</sub> production by the peripheral neutrophil. Consequently, reduced biosynthesis of cytoprotective PGE<sub>2</sub> in the liver may predispose to the inflammation of alcoholic hepatitis and the resultant fibrosis so often found in alcohol related liver disease.

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