Hypercholesterolaemia and lipid lowering treatment do not affect the acute endogenous fibrinolytic capacity in vivo

D E Newby, F N Witherow, R A Wright, P Bloomfield, C A Ludlam, N A Boon, K A A Fox, D J Webb

Objective: To assess acute tissue plasminogen activator (t-PA) release in vivo in patients with hypercholesterolaemia in the presence and absence of lipid lowering treatment and in matched normocholesterolaemic controls.

Design: Parallel group comparison and double blind randomised crossover.

Setting: University hospital.

Patients: Eight patients with hypercholesterolaemia (> 7.8 mmol/l) and eight matched normocholesterolaemic controls (< 5.5 mmol/l).

Methods: Blood flow and plasma fibrinolytic factors were measured in both forearms during unilateral brachial artery infusions of the endothelium dependent vasodilator substance P (2–8 pmol/min) and the endothelium independent vasodilator sodium nitroprusside (1–4 µg/min).

Interventions: In patients, measurements were made on three occasions: at baseline and after six weeks of placebo or pravastatin 40 mg daily administered in a double blind randomised crossover design.

Main outcome measures: Acute release of t-PA.

Results: Compared with patients, in normocholesterolaemic control subjects substance P caused greater dose dependent increases in forearm blood flow (p < 0.05) but similar increases in plasma t-PA antigen and activity concentrations. During pravastatin treatment in patients, total serum cholesterol fell by 22% from a mean (SEM) of 8.1 (0.3) to 6.4 (0.4) mmol/l (p = 0.002) and substance P induced vasodilatation was no longer significantly impaired in comparison with controls. However, despite reproducible responses, pravastatin treatment was not associated with significant changes in basal or substance P induced t-PA release.

Conclusions: Hypercholesterolaemia and lipid lowering treatment cause no demonstrable effects on acute substance P induced t-PA release in vivo. This suggests that the preventative benefits of lipid lowering treatment are unlikely to be mediated by improvements in endogenous fibrinolysis.
hypercholesterolaemia, the aims of the present study were to determine whether there is also an impairment of t-PA release in patients with hypercholesterolaemia and whether treatment with pravastatin could enhance t-PA release in these patients.

METHODS

Patients and control subjects

Eight patients with primary hypercholesterolaemia were recruited from the clinic if their serum cholesterol concentrations exceeded 7.8 mmol/l (> 296 mg/dl). Following screening with clinical examination, repeated questioning for symptoms, clinical chemistry screen (liver enzymes, electrolytes, urea, and creatine), haematology screen (full blood and differential counts), urinalysis, and 12 lead ECG, patients were excluded if they had diabetes mellitus, hypertension, ischaemic heart disease, peripheral vascular disease, an abnormal resting ECG, or other clinically significant disease. Patients were matched with normcholesterolaemic (< 5.5 mmol/l; < 209 mg/dl) healthy control subjects for age, sex, and smoking habit. All studies were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects was receiving vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours and from food, tobacco, and drinks containing caffeine for at least nine hours before each study. All studies were performed in a quiet temperature controlled room maintained at 23.5–24.5°C.

Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27 standard wire gauge steel needle (Cooker’s Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16 gauge epidural catheter (Portex Ltd, Hythe, UK) and patency was maintained by infusing of saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) through an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min. Pharmaceutical grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (David Bull Laboratories, Warwick, UK) were administered following dissolution in saline.

Forearm blood flow and blood pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury in silastic strain gauges applied to the widest part of the forearm.22 During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mm Hg using E20 rapid cuff inflators (DE Hokanson Inc, Bellevue, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mm Hg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (DE Hokanson) was processed by a MacLab analogue to digital converter and Chart version 3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded on to a MacIntosh Classic II computer (Apple Computers Inc, Cupertino, California USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semiautomated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).23

Venous sampling and assays

Venous cannulae (17 gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten millilitres of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden, for t-PA assays) and citrate (Monovette, Sarstedt, Nümbrecht, Germany, for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 minutes at 4°C. Platelet free plasma was decanted and stored at −80°C before assay.

Plasma t-PA and PAI-1 antigen concentrations were determined using an enzyme linked immunosorbent assay (ELISA): Coaliza t-PA24 and Coaliza PAI-1” (Chromogenix AB, Mölndal, Sweden), respectively. Plasma t-PA and PAI-1 activities were determined by a photometric method: Coaet t-PA and Coaest PAI-1 (Chromogenix AB), respectively.25 Plasma PA-1 activity is determined by a back titration method and represents the total t-PA inhibitory activity of the plasma.26 All fibrinolytic assays were performed in duplicate and the mean value was taken. Intra-assay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen and 2.4% for activity, respectively. Interassay coefficients of variability were 4.0%, 7.3%, 4.0% and 7.6%, respectively. The limits of sensitivity of the assays were 0.5 ng/ml, 2.5 ng/ml, 0.10 IU/ml, and 5 AU/ml, respectively. Haemtocrit (Hct) was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetra-acetic acid and was obtained from the infused forearm at baseline and at 8 pmol/min of substance P. Serum cholesterol and triglyceride concentrations were determined by an enzymatic colorimetric method (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany). Low density lipoprotein (LDL) cholesterol was determined by the method of Friedewald and colleagues.27

Study design

All patients attended on each of the three study days: baseline and following six weeks’ treatment with placebo and six weeks’ treatment with pravastatin 40 mg daily. Placebo and pravastatin treatments were given in a randomised double blind crossover design. Control subjects attended on one occasion only.

On each study day, subjects attended fasted at 0900 and rested recumbent throughout. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm was cannulated. Throughout all protocols, forearm blood flow (FBF) was measured every 10 minutes. Before administration of substance P and sodium nitroprusside, saline was infused for 30 minutes to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal FBF. Substance P was infused at 2, 4, and 8 pmol/min for 10 minutes at each dose28 and sodium nitroprusside was infused at 1, 2, and 4 µg/min for 10 minutes at each dose.29 The order of administration of substance P and sodium nitroprusside was randomised.

Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and FBFs were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel version 5.0; Microsoft Corporation, Redmond, Washington, USA). Recordings from the first 60 seconds after wrist cuff inflations were not used because of the variability in blood flow that this causes.22 Usually the last five flow recordings in each three minute measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point, in effect using the non-infused arm as a contemporaneous control for the infused arm.22 Percentage changes in the infused FBF were calculated22 as follows:

$$\% \text{ change in blood flow} = 100 \times \left(\frac{l/Ni - l/Ni}{l/Ni}\right)$$

where I, and Ni, are the infused and non-infused FBFs at baseline (time 0), respectively, and I, and NI, are the infused and non-infused FBFs at a given time point, respectively.

www.heartjnl.com
Estimated net release of t-PA activity and antigen was defined previously as the product of the infused forearm plasma flow (based on the mean Hct and the infused FBF) and the concentration difference between the infused ([t-PA]_{inf}) and non-infused arms ([t-PA]_{non-inf}):

estimated net t-PA release = FBF × (1 − Hct) × ([t-PA]_{inf} − [t-PA]_{non-inf})

Data were examined, where appropriate, by two way analysis of variance with repeated measures and two tailed paired Student’s t test using Excel version 5.0. Reproducibility of the responses to substance P infusion was assessed by comparing the baseline data with placebo treatment study days using the method of Bland and Altman. Coefficients of reproducibility were determined for 95% confidence intervals using the Student’s t distribution. All results are expressed as mean (SEM). Significance was taken at the 5% level. On the basis of previous data the study had 90% power to detect a 20% change in plasma t-PA concentrations between treatment periods at the 5% level.

RESULTS

Table 1 shows patient and control subject characteristics. The groups were well matched for age, sex, body mass index, smoking habit, blood pressure, heart rate, Hct, and FBF. Total cholesterol and LDL cholesterol concentrations were significantly higher in the patient group and fell during pravastatin but not placebo treatment (table 1). On each of the study days in the patient and control groups, blood pressure, heart rate, Hct, blood flow, and t-PA concentrations of the non-infused arm ([t-PA]_{non-inf}) did not change.

Blood flow responses

Substance P caused dose dependent increases in blood flow of the infused forearm in both patients and controls (p < 0.001 for both, analysis of variance; fig 1). In comparison to the control group, the substance P induced increases in blood flow were significantly less in the patient group (p < 0.05, two way analysis of variance; fig 1). During pravastatin treatment, substance P appeared to cause a slightly greater increase in blood flow but this was not significantly different from baseline responses (p = 0.30, two way analysis of variance, baseline versus pravastatin) although it was no longer significantly different from control group responses (p = 0.24, two way analysis of variance).

Sodium nitroprusside also caused dose dependent increases in blood flow of the infused forearm (p < 0.001, analysis of variance).
variance; fig 1), which were similar in patient and control groups. Neither pravastatin nor placebo treatment influenced the response to sodium nitroprusside infusion.

Fibrinolytic factor responses
Substance P caused dose dependent increases in plasma t-PA antigen and activity concentrations in the infused forearm of both patients and controls (p < 0.001 for both, analysis of variance; table 2). Substance P induced increases in plasma t-PA concentrations were similar in both groups and, in the patient group, were unaffected by pravastatin or placebo treatment. The concentration differences between the forearms and the estimated net release of t-PA antigen and activity also increased dose dependently (p < 0.001, analysis of variance; fig 2), which did not differ between the patient and control groups or during pravastatin and placebo treatment. There were no significant differences in plasma PAI-1 antigen and activity concentrations at baseline, although there appeared to be a trend for plasma PAI-1 antigen concentrations to be lower in the control group (p > 0.12, unpaired t test; table 3). Plasma PAI-1 concentrations were unaffected by substance P infusion or pravastatin treatment (table 3).

Reproducibility of substance P responses
Comparison of responses at baseline and during placebo treatment shows good reproducibility for substance P induced increases in plasma t-PA concentrations and FBF (table 4).

DISCUSSION
Despite impaired endothelium dependent forearm vasodilatation, we have shown that in patients with hypercholesterolaemia intrabrachial substance P infusions are associated with a normal capacity to release t-PA acutely. Moreover, pravastatin treatment, sufficient to reduce cholesterol concentrations by 22%, had no significant effects on acute t-PA release. This suggests that, despite the presence of endothelial dysfunction, hypercholesterolaemia does not influence the acute fibrinolytic capacity of the endothelium and that the preventative benefits of lipid lowering treatment are unlikely to be mediated by improvements in endogenous fibrinolysis.

Consistent with previous findings, we have shown that in patients with hypercholesterolaemia, endothelium dependent vasodilatation in response to substance P infusion is impaired. However, in contrast to the pronounced impairment of t-PA release that we have recently described in cigarette smokers, hypercholesterolaemia and endogenous fibrinolysis 51...
smokers, but not in patients with hypercholesterolaemia, and that cigarette smoking is associated with thrombotic occlusion whereas hypercholesterolaemia is linked to atherogenesis and plaque rupture. This would also indicate that endothelial dysfunction can be manifest in separate distinct pathways depending on the nature of the insult.

The WOSCOPS (West of Scotland Coronary Prevention Study) was the first major randomised controlled trial to show the primary preventative benefits of lipid lowering therapy. We therefore chose to examine the effects of pravastatin 40 mg daily on endothelial and fibrinolytic function in a well defined and otherwise healthy hypercholesterolaemic population. Although the total cholesterol was reduced by 22% and pravastatin was used at doses that have been shown to confer major preventative benefits in several large scale clinical trials, the total serum cholesterol concentration remained significantly higher than that in the normocholesterolaemic population. It may be that a greater reduction in cholesterol concentrations would have facilitated a significant improvement in endothelium dependent vasodilatation. However, the mean cholesterol concentrations of the patients in WOSCOPS were similar to those in the present study and the relative risk reduction in ischaemic events is the same across a broad range of cholesterol concentrations. Moreover, it appears that statins do not just lower cholesterol and may have many ancillary vascular actions. Finally, since we did not observe a significant difference in t-PA release between hypercholesterolaemic and normocholesterolaemic subjects, it is unlikely that additional reductions in lipid concentrations, brought about by higher doses of pravastatin or more potent statins, would influence the acute release of t-PA.

The influence of lipid lowering therapy on endothelial dysfunction was initially studied in patients with hypercholesterolaemia following treatment for 3–6 months. However, more recent studies have shown that endothelial dysfunction can be reversed by statin treatment for six or even four weeks. Indeed, two hour treatment with plasma LDL aphaeresis is associated with rapid and immediate reversal of endothelial dysfunction. It is therefore unlikely that the absence of an effect of pravastatin treatment is caused by the length of treatment and is consistent with a recent preliminary report from the WOSCOPS that showed no

### Table 3

<table>
<thead>
<tr>
<th>Substance P dose (pmol/min)</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infused arm</td>
<td>Non-infused arm</td>
<td></td>
</tr>
<tr>
<td>PAI-1 antigen (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1 activity (AU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infused arm</td>
<td>Non-infused arm</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean (SEM).

### Table 4

<table>
<thead>
<tr>
<th>Substance P dose (pmol/min)</th>
<th>Baseline mean</th>
<th>Placebo mean</th>
<th>Mean of differences</th>
<th>Coefficient of repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA antigen (ng/ml)</td>
<td>4.6 (0.8)</td>
<td>4.7 (0.6)</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>5.4 (0.8)</td>
<td>5.4 (0.6)</td>
<td>0.1</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>5.7 (0.9)</td>
<td>6.0 (0.9)</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>8</td>
<td>7.4 (1.1)</td>
<td>7.5 (1.3)</td>
<td>0.1</td>
<td>1.6</td>
</tr>
<tr>
<td>t-PA activity (IU/ml)</td>
<td>0.5 (0.1)</td>
<td>0.6 (0.1)</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>1.3 (0.5)</td>
<td>1.1 (0.4)</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>2.0 (0.6)</td>
<td>1.7 (0.9)</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>8</td>
<td>3.4 (1.1)</td>
<td>3.9 (1.4)</td>
<td>0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Absolute forearm blood flow (ml/100 ml/min)</td>
<td>3.5 (0.3)</td>
<td>3.2 (0.3)</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Change in forearm blood flow (%)</td>
<td>11.2 (0.7)</td>
<td>10.3 (1.3)</td>
<td>0.9</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>14.4 (1.8)</td>
<td>12.6 (1.5)</td>
<td>1.7</td>
<td>4.6</td>
</tr>
<tr>
<td>8</td>
<td>15.7 (1.1)</td>
<td>14.9 (1.5)</td>
<td>0.8</td>
<td>3.4</td>
</tr>
<tr>
<td>t-PA activity (IU/ml)</td>
<td>206 (20)</td>
<td>240 (30)</td>
<td>35</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>300 (39)</td>
<td>300 (36)</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>8</td>
<td>402 (49)</td>
<td>384 (58)</td>
<td>17</td>
<td>76</td>
</tr>
</tbody>
</table>

Data are mean (SEM).
changes in basal plasma t-PA concentrations despite 12 months’ treatment with pravastatin.

Moreover, in contrast to previous studies, we have used a randomised double blind crossover study design which provides a greater sensitivity to detect potential differences in responses. The coefficients of variation of different methods of clinical measurement.

ACKNOWLEDGEMENT

This work was supported by a grant from the British Heart Foundation (PG/96149). Dr Fraser Withrow was the recipient of a British Heart Foundation Junior Research Fellowship (FS/2000/005). Professor David Webb was supported by a Research Leave Fellowship from the Wellcome Trust. We wish to thank Linda Flint, Pamela Dawson and Margaret Millar for their assistance with the preparation of this manuscript.

REFERENCES


25 Klotz C, Verheijen JH. Leiden fibrinolysis working party: blood collection and handling in performed as an in vivo model for tissue plasminogen activator (PA) and plasminogen activator inhibitor-1 (PAI-1). Fibrinolysis 1990;4(suppl 2):155–61.


38 Vaughan CJ, Murphy MB, Buckley BM. Statins do more than just lower cholesterol. Lancet 1996;348:1079–82.


www.heartjnl.com