

## Short communication

# Folate protects against oxidative modification of human LDL

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(Received 13 March 2001 – Revised 28 June 2001 – Accepted 24 August 2001)

Elevated plasma total homocysteine is considered to be a graded risk factor for cardiovascular disease. Folate, through its homocysteine-lowering potential, may therefore be protective. Folate, however, may have protective effects independent of homocysteine-lowering. We have measured the effects of folate on Cu-catalysed oxidative damage to the unsaturated lipids in human LDL. Experiments were carried out in the presence of citrate, and followed increases in absorption at 234 nm, which measures the amount of conjugated diene produced. There is a lag time during which endogenous antioxidants are oxidised, followed by rapid oxidation of lipid. Addition of 0–6  $\mu\text{M}$ -5-methyltetrahydrofolate produced a dose-dependent increase in the lag time, suggesting that folate may have a direct anti-oxidant role *in vivo*, which is independent of any indirect effects through lowering of homocysteine levels.

### Folate: LDL: Oxidation: Homocysteine

There are numerous factors involved in the aetiology of cardiovascular disease, many of which are associated with impaired endothelial function in advance of more overt effects. Endothelial dysfunction can arise from a direct effect on smooth muscle tone, or by formation of oxidised plaques derived from foam cells on the endothelial wall. One compound currently high on the list of possible intervention strategies is homocysteine, a known risk factor for cardiovascular disease (Boushey *et al.* 1995; Nygard *et al.* 1997). The mechanism of action of homocysteine is unclear, although it has been suggested that this could be related to a reduction in nitric oxide activity.

Folate reduces the concentration of plasma homocysteine (Bellamy *et al.* 1999) and it is through this homocysteine-lowering potential that folate has come to be regarded as potentially protective against cardiovascular disease. However, recent work has suggested that folate may have protective effects independent of homocysteine lowering.

A reduction in flow-mediated arterial dilatation induced by oral administration of methionine to healthy volunteers could be completely prevented by pre-treatment with high-dose folate (Usui *et al.* 1999). This effect was independent of homocysteine, and was thought to be due to enhanced

vascular nitric oxide activity. A similar conclusion was reached by Verhaar *et al.* (1998), who demonstrated that intravenous folate could restore endothelial function in familial hypercholesterolaemia patients, possibly by reduced catabolism of nitric oxide. Wilmink *et al.* (2000) showed that high-dose oral folate administration could prevent endothelial dysfunction associated with a fat load.

Folate may have other functions, in particular by reducing oxidative damage to LDL lipid, through free-radical scavenging activity. In this way folate would act independently of effects on nitric oxide and smooth muscle tone but rather influence foam cell formation. We therefore set out to study the effect of folate (in its most active circulating form, 5-methyltetrahydrofolate, 5-MTHF) on LDL oxidation *ex vivo*. The results demonstrate a direct protective effect of folate on LDL oxidation.

### Materials and methods

#### Preparation of LDL

Blood was collected from a healthy volunteer into lithium heparin, and centrifuged at 4°C at 1000 g for 15 min to

**Abbreviation:** 5-MTHF, 5-methyltetrahydrofolate.

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remove cells. LDL was isolated from the plasma by centrifugation through a self-generating density gradient of iodixanol (Liposep; Lipotek Ltd, Liverpool, UK) (Graham *et al.* 1996). Briefly, plasma was mixed with iodixanol in Hepes-buffered saline (0.14 M-NaCl, 10 mM-Hepes, pH 7.4) to make a 12% iodixanol solution. This was layered underneath a 9% iodixanol solution and centrifuged for 2.5 h at 16°C at 100 000 rpm in an Optima Max-E centrifuge (Beckman Instruments Ltd., High Wycombe, UK). The fractions containing LDL were separated using a Gilson FC 203B fraction collector (Gilsen Inc., Middleton, WI, USA) and pooled. To remove iodixanol and water-soluble endogenous antioxidants, the pooled LDL fraction was passed through a 1.0 × 30 cm column of Sephadex G-25 (Sigma Chemical Co., Poole, Dorset, UK), and eluted with Hepes-buffered saline. Eluates were assayed for cholesterol content using a cholesterol oxidase assay (Sigma) and adjusted to a cholesterol concentration of 162 µg/ml, equivalent to an LDL protein concentration of 100 µg/ml. This method produced LDL preparations ready for incubation within 5 h of obtaining the plasma, and within 1 h of removal of endogenous antioxidants.

#### LDL oxidation

Oxidation was initiated by the addition of CuSO<sub>4</sub> (final Cu concentration 30 µM) and sodium citrate (final concentration 360 µM; Schnitzer *et al.* 1995), and carried out at 37°C, pH 7.4. Oxidation of LDL lipids to conjugated dienes was measured by following the absorbance at 234 nm (Buege & Aust, 1978). The LDL preparation was incubated in a quartz cuvette, and the absorbance measured at 234 nm against a blank of Hepes-buffered saline, using a Camspec (Camspec Ltd., Cambridge, UK) M330 single beam u.v.-visible spectrophotometer operating at 37°C. LDL lipid oxidation was plotted as a function of incubation time. A stock solution of 5-MTHF (1.0 mM) was prepared and stored for no more than 24 h before use. 5-MTHF was added to LDL preparations, prior to incubation, to a final concentration of 0, 1, 4, and 6 µM. The kinetics of LDL

lipid oxidation were analysed in terms of the lag time prior to oxidation, using a graphical method (McDowell *et al.* 1995). The experiment was repeated on three consecutive days using the same batch of LDL stored under N<sub>2</sub> at 4°C, and control experiments were always carried out on identical samples.

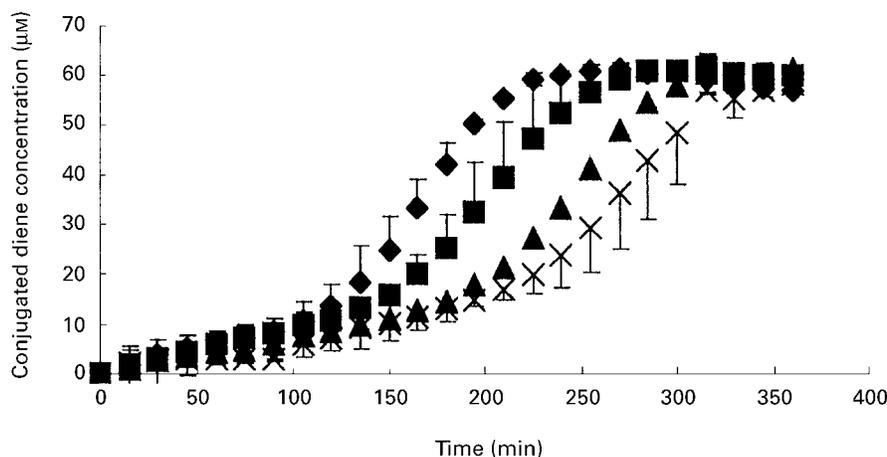
#### Results

After initiation of LDL lipid oxidation by addition of Cu<sup>2+</sup>, there was a slow rise in the absorbance at 234 nm, due to oxidation of polyunsaturated lipid to conjugated dienes (Fig. 1). After a lag time of about 125 min, the oxidation rate increased sharply, and then the concentration of conjugated dienes reached a plateau at about 250 min. This is typical behaviour that has been seen in many studies of LDL lipid oxidation (Buege & Aust, 1978). The lag time is thought to be due to the oxidation of endogenous antioxidants (Esterbauer *et al.* 1989).

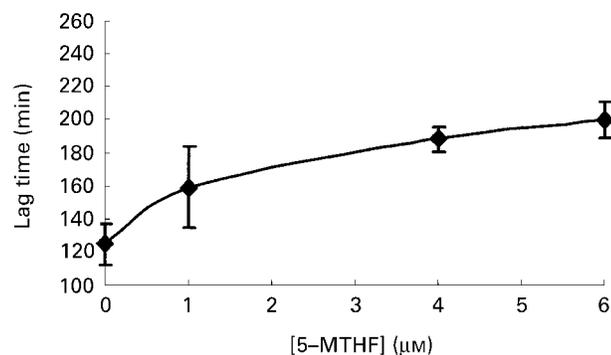
On addition of folate, the lag times increased (Fig. 1). The lengthening of the lag time by folate in the range 0–6 µM was dose-dependent (Fig. 2). Further increases in folate concentration to 50 µM had little further effect (results not shown).

#### Discussion

The results demonstrate a marked protective effect of folate on human LDL lipid *ex vivo*. The only components present in the experimental system are LDL (plus anything that copurifies with LDL, which might include fat-soluble substances such as α-tocopherol), and the added buffer, Cu and citrate. Citrate was added because it has been shown by others, and we have confirmed this, that kinetic studies of metal-catalysed lipid oxidation are much more reproducible and interpretable in the presence of citrate (Schnitzer *et al.* 1995). The effects of folate in this system cannot be attributed to nitric oxide availability or to indirect effects via homocysteine, and must therefore be due to a direct effect of folate on free-radical-induced oxidation of LDL lipid. The



**Fig. 1.** Concentration of conjugated dienes resulting from LDL oxidation as a function of time, in the presence of increasing amounts of 5-methyltetrahydrofolate (5-MTHF). For details of procedures, see p. 637. ♦, control; ■, in the presence of 1 µM-5-MTHF; ▲, in the presence of 4 µM-5-MTHF; ×, in the presence of 6 µM-5-MTHF. Values are means for two or three separate experiments, carried out on the same batch of LDL on successive days. 95% CI are shown by vertical bars: error bars are symmetrical, but only one side is shown to avoid confusion.



**Fig. 2.** Lag time for oxidation of LDL lipid to conjugated dienes (measured by taking the tangent to the curve shown in Fig. 1 at the steepest part of the slope, and extrapolating back to the baseline absorbance), as a function of 5-methyltetrahydrofolate (5-MTHF) concentration. Values are means with 95% CI shown by vertical bars.

most obvious rationale is a free radical scavenging activity of folate (Verhaar *et al.* 1998).

The results presented here suggest a further mechanism by which folate could act to reduce the risk of cardiovascular disease, and therefore add additional weight to suggestions that folate supplementation in the general population may have vasculoprotective effects (Wilmink *et al.* 2000). The concentrations of 5-MTHF that have been shown to have a beneficial effect are high but may be achievable through folate supplementation (Lobo *et al.* 1999; Bostom *et al.* 2000).

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