Downstream effects on human low density lipoprotein of homocysteine exported from endothelial cells in an in vitro system

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Abstract   A model system is presented using human umbilical vein endothelial cells (HUVECs) to investigate the role of homocysteine (Hcy) in atherosclerosis. HUVECs are shown to export Hcy at a rate determined by the flux through the methionine/Hcy pathway. Additional methionine increases intracellular methionine, decreases intracellular folate, and increases Hcy export, whereas additional folate inhibits export. An inverse relationship exists between intracellular folate and Hcy export. Hcy export may be regulated by intracellular *S*-adenosyl methionine rather than by Hcy. Human LDLs exposed to HUVECs exporting Hcy undergo time-related lipid oxidation, a process inhibited by the thiol trap dithionitrobenzoate. This is likely to be related to the generation of hydroxyl radicals, which we show are associated with Hcy export. Although Hcy is the major oxidant, cysteine also contributes, as shown by the effect of glutamate. Finally, the LDL oxidized in this system showed a time-dependent increase in uptake by human macrophages, implying an upregulation of the scavenger receptor.   These results suggest that continuous export of Hcy from endothelial cells contributes to the generation of extracellular hydroxyl radicals, with associated oxidative modification of LDL and incorporation into macrophages, a key step in atherosclerosis. Factors that regulate intracellular Hcy metabolism modulate these effects.—Nakano, E., F. A. Taiwo, D. Nugent, H. R. Griffiths, S. Aldred, M. Paisi, M. Kwok, P. Bhatt, M. H. E. Hill, S. Moat, and H. J. Powers. **Downstream effects on human low density lipoprotein of homocysteine exported from endothelial cells in an in vitro system.** *J. Lipid Res.* 2005. 46: 484–493.

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Homocysteine (Hcy) is the demethylated derivative of methionine (1), and there is a well-established epidemiological relationship between total plasma homocyst(e)ine concentration (tHcy) and cardiovascular disease risk (2–5). It has been estimated that 10% of the population risk for cardiovascular disease in the United States is associated with increased tHcy (6). Increased plasma tHcy is linked to increased risk of cardiovascular disease to a similar extent to that of smoking and hyperlipidemia, which strengthens the link between smoking and hypertension (7). However, although the epidemiological evidence strongly supports a connection, it does not provide strong support for increased tHcy being a cause of cardiovascular disease. Most prospective studies support a causative link (8), but there remains considerable doubt (9). There has thus been considerable research into the association between plasma tHcy and cardiovascular disease, and it has been suggested that the reduced form, Hcy, in the presence of suitable catalysts such as transition metals, is responsible for the oxidation of LDL, which is subsequently taken up by macrophages, converting them into foam cells and leading to the formation of arterial plaques (10). Although this theory has received general support, a number of problems remain, not least the fact that Hcy in plasma is rapidly oxidized, so that Hcy forms only approximately 1% of the tHcy in plasma (11). Some authors have suggested that tHcy in vivo may be an antioxidant and not a prooxidant at all (12–14). We have explored the putative antioxidant and prooxidant activities of Hcy and have demonstrated that prooxidant activity occurs across the physiological range of concentrations of Hcy (15).

Previous groups have shown the oxidative potential of extracellular thiols in cellular systems, presumably through thiol export and subsequent oxidation (16–18). Hcy in the plasma has to be a product of export from cells; therefore, the oxidative potential of Hcy toward LDL is likely to be determined by the rate of Hcy export from cells and the subsequent rate of its oxidation. A variety of cells in culture can evidently export Hcy, and the rate of export appears to reflect flux through the methionine/Hcy pathway (**Fig. 1**) (19–22). Different cell types have very different export rates: hepatocytes have been reported to have a high rate of Hcy export, whereas quiescent lymphocytes and fibroblasts have a much slower export (19). High cell density has been shown to decrease Hcy export (19, 23). One group (24) has also shown that additional folate in the medium reduces Hcy export, although those workers did not use a folate-free medium as a test control.

We have therefore undertaken a methodical analysis of Hcy export and its effect on LDL oxidation. We have previously shown that Hcy is a prooxidant at physiological concentration and that it most likely acts by generating free radicals on oxidation (15). Here, we investigate the hypothesis that it is the slow continuous production of Hcy by endothelial cells that is responsible for the atherogenic effect of tHcy. We demonstrate the involvement of the hydroxyl radical in the oxidation. We further show that the oxidized LDL produced in this way is internalized by macrophages, indicating that continuous production of Hcy by endothelial cells is likely to be a major oxidant for LDL.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma Chemical Co. (London, UK) unless otherwise stated.

Cell culture media

Basal medium for the maintenance of freshly isolated human umbilical vein endothelial cells (HUVECs) and for the experimental systems was prepared specially by Gibco as the standard MCDB131 medium but without any methionine or folate in any form. To this medium, glutamine (2 mM), epidermal growth fac-

tor (10 ng/ml), holotransferrin (5 μg/ml), insulin (5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) were added. For the growth medium, fetal calf serum (20%; Gibco), methionine (100 μM), and folinic acid (25 nM) were added. For the test medium, instead of these we added endothelial cell growth supplement (50 μg/ml; Firstlink) and Albumax (20 mg/ml; Gibco). For experiments examining the effect of additional folate, folinic acid was also added at 10, 100, or 1,000 nM.

Isolation and culture of HUVECs

Human umbilical cords from normal deliveries were collected from the Maternity Unit, Jessop Hospital for Women, Sheffield, into a bottle containing 40 ml of DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 100 μg/ml gentamycin, and 20 mM HEPES and stored at 4°C. Ethical approval to collect umbilical cords was obtained from the North Sheffield Research Ethics Committee. Cords were processed within 48 h of birth, and all procedures were carried out in a class II cabinet. Cords were washed with DMEM and treated with 1,200 U/ml collagenase type IV for 10 min at room temperature. The digest was centrifuged, resuspended in growth medium, and seeded onto 0.2% gelatin-coated T75 flasks (Costar, Inc.) and incubated at 37°C in a 5% CO_2 atmosphere. Typically, one 15 cm cord was seeded into one T75 flask. When the cells reached confluence (5–7 days), they were passaged by treatment with trypsin/EDTA (0.5 g/l trypsin and 0.02% EDTA in Hank's buffered saline solution) and split to produce 8×10^5 cells per T75 flask (typically a 1:3 split). After 4 days, cells were passaged onto gelatin-coated 6-well (2×10^5 cells/well) or 24-well (7.5×10^4 cells/well) plates depending on the experiment. Medium was renewed every 2–3 days throughout. At confluence (3–4 days), the medium was changed to test medium for experimental work.

HUVECs were characterized by positive immunofluorescence for von Willebrand factor, CD31, and *Ulex europeaeus* agglutinin. Cells were fixed using cold methanol/acetone, washed in PBS, permeabilized by 0.5% Triton X-100, and washed in PBS. For von Willebrand factor and CD31 (antibodies from Novocastra Labs and R&D Systems, respectively), the primary antibody was applied as a 1:50 or 1:100 dilution, respectively, in phosphate-buffered saline plus 1% skimmed milk powder. Cells were left for 1 h, washed, treated with a secondary FITC-conjugated goat anti-mouse antibody (Sigma) at a 1:150 dilution, incubated for 1 h, washed, and stained with Hoechst 33342. For *U. europeaeus* agglutinin, a FITC-conjugated antibody was used directly (Vector Laboratories), diluted to 20 μg/ml. Fluorescence was observed using a Leica fluorescence microscope.

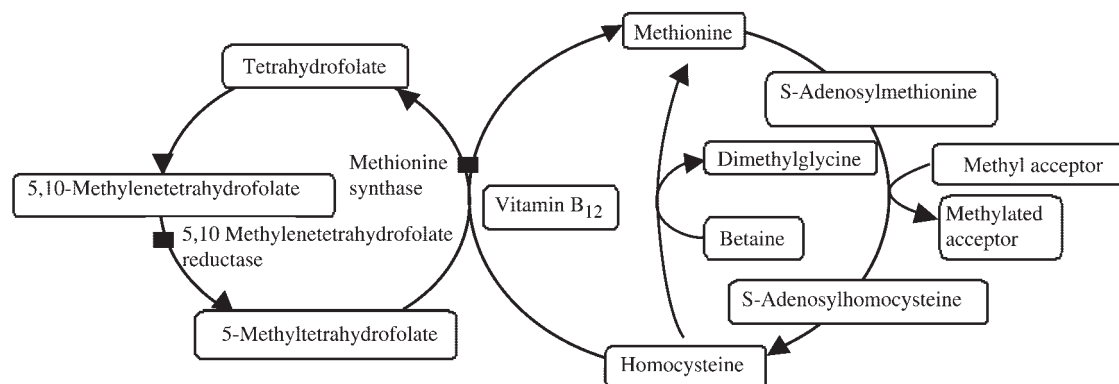All experiments were performed with at least three different HUVEC isolates.

Fig. 1. The remethylation pathway of homocysteine (Hcy).

Cell viability

Cell viability was measured at the end of each experiment by staining with 10 μM Hoechst 33342 and 10 μM propidium iodide (Hcy export experiments) or 0.05% trypan blue (all other experiments).

LDL preparation

LDL was prepared as described (25). Briefly, freshly obtained human plasma was centrifuged in an iodixanol solution (2.5 h, 100,000 rpm). The LDL-rich fraction was collected and assayed for cholesterol using a Sigma kit (401-25P). All experiments were carried out at a LDL protein concentration of 100 μg/ml. LDL in suspension was stored at 4°C under nitrogen and used within 3 days. All experiments were carried out using LDL isolated from a single volunteer, for consistency.

Measurement of extracellular homocyst(e)ine

tHcy in the medium was measured by a fully automated fluorescence polarization immunoassay using an IMX analyzer (Abbott Diagnostics), and each set of measurements was calibrated and checked against standards. The assay has a coefficient of variation of ~2% within runs and 4.5% between runs.

Measurement of intracellular homocyst(e)ine and intracellular and extracellular methionine

Cells were detached from T75 flasks using trypsin/EDTA, washed, counted, weighed, and resuspended in MCDB131 before storage at −80°C for later use. For preparation of the final cell extract, the suspension was thawed and cells were drawn up into a 20 gauge needle several times to break up any intact cells. tHcy and methionine were measured in supernatants after centrifugation.

tHcy in cell lysates was measured by HPLC with fluorescence detection as previously described, with modifications (26). Lysate (50 μl) was added to 100 μl of cysteamine (internal standard). The sample was then treated with 30 μl of 10% tri-butylphosphine in dimethylformamide to reduce disulfide bonds between Hcy and proteins and between other thiols. Plasma proteins were removed by deproteinization with 100 μl of 10% trichloroacetic acid, and samples were then centrifuged at 3,000 g for 10 min. Fifty microliters of the resulting supernatant was added to 100 μl of borate buffer (2.5 M, pH 10.5) and 30 μl of 7-fluoro-2,1,3-benzoxadiole-4-sulfonamide (1 mg/ml in borate buffer) to permit subsequent fluorimetric detection. This mixture was then incubated for 1 h at 60°C. tHcy was separated on a Nucleosil 120 C18 (4.6 × 250 mm, 5 μm) column (Jones Chromatography) with isocratic elution at a flow rate of 1.0 ml/min with a 0.1 M potassium phosphate buffer, pH 2.1, containing 4.5% acetonitrile.

Methionine concentrations in cell lysates and medium were measured by HPLC with fluorescence detection according to Potgieter et al. (27), with slight modifications. Fifty microliters of lysate was added to 50 μl of the internal standard (D,L-hydroxynorvaline, 100 μM). Proteins were removed by the addition of 500 μl of acetonitrile while mixing vigorously. The resulting supernatant was removed, evaporated to dryness under nitrogen, and then reconstituted in HPLC buffer. For precolumn derivatization with o-phthaldialdehyde, 20 μl of the eluate was mixed with 20 μl of o-phthaldialdehyde and then injected onto the HPLC column [Phenomenex Prodigy C18, reverse phase (RP), 150 × 4.6 mm]. Methionine was separated using a two-solvent gradient system at a flow rate of 1 ml/min.

Measurement of intracellular folate

Cells were detached using trypsin/EDTA, washed, counted, suspended in lysis buffer (Abbott Laboratories), and disrupted by freezing at −80°C for 3 days and thawing. The resulting suspension was centrifuged at 150,000 g to produce a clear supernatant, and the total folate concentration was measured using a commercial Ion Capture Assay System (Abbot IMX; Abbott Laboratories). Results were obtained as folate per cell (counted using a hemocytometer). The assay has a coefficient of variation of ~4.7% within runs and 5.2% between runs.

Measurement of free radicals in the medium

Confluent HUVECs were cultured on six-well plates in test medium containing 100 μM methionine plus 1 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap and 30 μM copper. At various times, the medium was collected, centrifuged to remove suspended cells, and frozen until required for measurement of free radicals. Thawed samples were placed in an electron paramagnetic resonance (EPR) quartz flat cell and mounted in the instrument cavity. First derivative EPR spectra were obtained on a Bruker EMX 6/1 EPR spectrometer operating at X-Band frequency range (9.7 GHz) and a modulation frequency of 100 kHz. Instrument settings were as follows: field set, 3,488 Gauss (G); scan width, 100 G; microwave power, 5 mW; modulation amplitude, 1.5 G; time constant, 10.24 ms; scan time, 83.89 s. For purposes of comparison, the hydroxyl radical was independently generated by the Fenton reaction and the superoxide radical anion, $O_2^{\cdot-}$, was generated by the xanthine oxidase enzyme-catalyzed oxidation of xanthine to uric acid in the presence of DMPO. Spectral simulations were performed using the SimFonia program from Bruker.

Measurement of lipoprotein oxidation

To HUVECs in test medium on six-well plates, LDL was added to a concentration of 100 μg LDL protein/ml. Cuprous sulfate was also added at 30 μM. When required, the thiol-blocking agent DTNB or glutamate, which inhibits cystine uptake into cells, was added at 500 μM or 10 mM, respectively. The plates were incubated for up to 72 h at 37°C. Butylated hydroxytoluene (final concentration 500 μM) and diethylenetriaminepentaacetic acid (final concentration 1 mM) were added to prevent further oxidation. LDL lipid oxidation was measured by monitoring the product of the reaction with thiobarbituric acid, using the thiobarbituric acid reactive substances (TBARS) assay (13, 28) as previously described (15).

Preparation of macrophages

All containers and pipettes were washed in Sigmacote and allowed to dry. Blood was collected from a healthy human volunteer into 0.4% sodium citrate. Blood was diluted 2:5 with sterile PBS containing 0.1% BSA. This was layered over Lymphoprep and centrifuged for the removal of platelets (160 g, 15 min, 20°C). Further centrifugation (350 g, 20 min, 20°C) allowed mononuclear cells to be collected (29). Mononuclear cells were washed and resuspended in RPMI 1640 medium (Gibco) containing 10% fetal calf serum and pipetted onto 96-well plates at 2×10^6 cells/ml. After overnight culture at 37°C in 5% CO_2, nonadherent cells were removed by washing, and RPMI medium containing 10% fetal calf serum and 50 ng/ml macrophage colony-stimulating factor was added. Medium was replaced every 2 days. After 5 days, the cells had differentiated into macrophages, as shown by visual inspection and by positive immunofluorescence for CD206 antibody.

Determination of macrophage uptake of oxidized LDL

A standard fluorescent fully oxidized LDL sample was prepared. LDL was oxidized (15) over 5 h at 37°C in the presence of copper because a TBARS assay showed that oxidation was complete by this time. The sample was partially buffer-exchanged

and concentrated to 1 mg/ml LDL protein in HBS (HEPES-buffered saline: 0.14 M NaCl, 10 mM HEPES, pH 7.4) using a 10 kDa molecular mass cutoff Vivaspin ultrafiltrator (Vivascience). The purified LDL was allowed to react (4:1, v/v) overnight at 37°C with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; as a 1.07 mM solution in dimethyl sulfoxide). After the reaction, the buffer was exchanged using a second Vivaspin ultrafiltrator. The DiI-labeled LDL was stored at −80°C until needed.

The method used to determine macrophage uptake of oxidized LDL was based on that described by Griffiths et al. (30), with important modifications. In preliminary experiments, incubation of macrophages with different amounts of DiI-labeled LDL produced a linear increase in fluorescence up to an addition of 25 μg of LDL protein per well (210 μl). Therefore, a standard protocol was adopted, in which 10 μg/well DiI-labeled LDL was added to the cells, together with 20 μg/well partially oxidized LDL, obtained by incubating LDL in our standard experimental system (100 μM methionine, 30 μM copper) for different periods of time and stored at −80°C until needed. Cells were incubated with the LDL mixture for 16 h at 37°C, washed twice with PBS, and lysed with 5% Triton X-100. The fluorescence of each well was measured using a plate reader, with an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

Statistical tests

Statistical tests were carried out using SPSS 10.0 for Windows. For comparisons of the effects of different treatments, the numbers of data points were not considered sufficient for parametric tests; therefore, the Mann-Whitney test was used. For analysis of time-dependent changes, a Kruskal-Wallis H test was used. Association between intracellular folate and exported Hcy was investigated using linear regression analysis.

RESULTS

Hcy export from HUVECs

Except where indicated otherwise, at least 80% of cells were viable after incubation for up 72 h in test medium. The cells were of normal appearance and stained positive with antibodies against von Willebrand factor, *U. europeaeus* agglutinin, and CD31, demonstrating that they still retained the characteristics of endothelial cells.

Confluent HUVECs were incubated in media to which methionine had been added at 0, 100, or 200 μM and to which folinic acid had been added at concentrations between 0 and 1,000 nM. Folinic acid was used as a more stable precursor of 5-methyl tetrahydrofolate, the main circulating form of folate in the body. Cells exported Hcy at a constant rate for at least 72 h (**Fig. 2**). Addition of 100 μM methionine to the test medium led to a marked and statistically significant increase in the rate of Hcy export from the cells, which was further increased, but not significantly, on further increasing the concentration of methionine (Fig. 2). By contrast, addition of folate (in the form of folinic acid) led to a dose-dependent decrease in the rate of Hcy export, which reached significance at 72 h (**Fig. 3**).

These results are consistent with the export of Hcy reflecting an imbalance between the production of Hcy and its metabolism by means of remethylation (Fig. 1). We
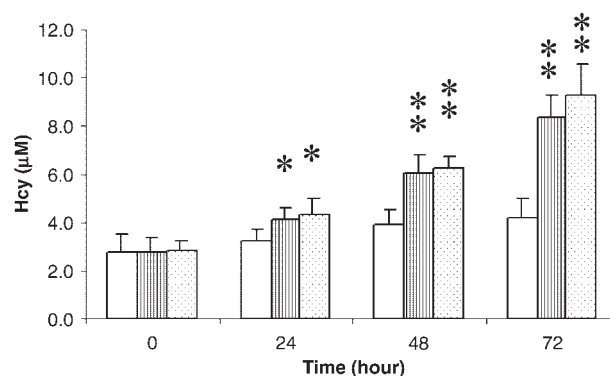

Fig. 2. Export of Hcy from human umbilical vein endothelial cells (HUVECs). The effect of the addition of methionine (100 and 200 μM) to the medium is indicated. Bars indicate the addition of 0, 100, and 200 μM methionine (open, striped, and dotted bars, respectively). Results are derived from five measurements on three independent HUVEC cultures. Error bars denote standard deviations. Asterisks show significant differences compared with time 0: * $P < 0.05$, ** $P < 0.01$. For each set of conditions, the increase in Hcy with time is significant ($P < 0.05$).

therefore investigated the relationship between Hcy export and the intracellular concentration of folate, a key determinant of the remethylation rate. The concentration of intracellular total folate decreased slowly over the course of 72 h in culture. Addition of methionine to the culture medium markedly enhanced the observed decrease in intracellular folate (**Fig. 4**). Intracellular folate was responsive to additional extracellular folate in a dose-response manner but increased only by a factor of 2 in response to a 10-fold increase in extracellular folate (**Fig. 5**), suggesting that transport of folate across the cell membrane is tightly regulated. In the absence of additional folinic acid in the medium, a strong negative correlation was shown between intracellular folate and extracellular Hcy ($R = -0.71$, $P < 0.005$) (**Fig. 6**).

We also investigated the concentration of intracellular Hcy in the presence and absence of extracellular me-
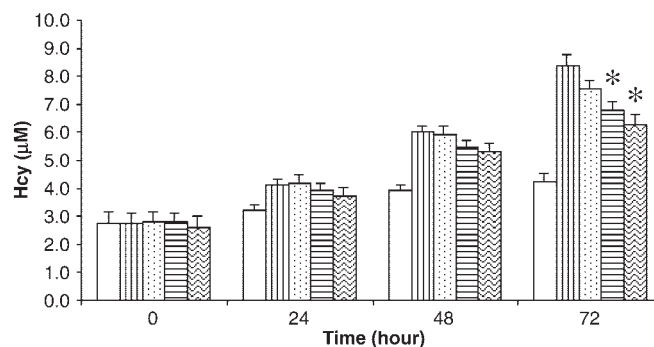
Fig. 3. Effect of adding folinic acid on Hcy export. Bars indicate control (no methionine) and solutions with 100 μM methionine plus the addition of 0, 10, 100, and 1,000 nM folinic acid (open, vertical striped, dotted, horizontal striped, and wavy bars, respectively). Results are derived from five experiments on three independent HUVEC cultures. Error bars denote standard deviations. Asterisks show significant differences from the 100 μM methionine, 0 nM folinic acid value at $P < 0.05$.
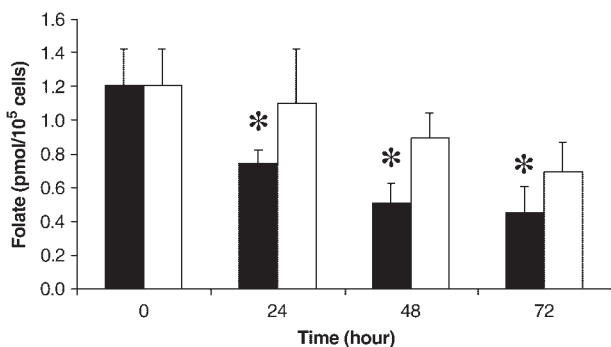
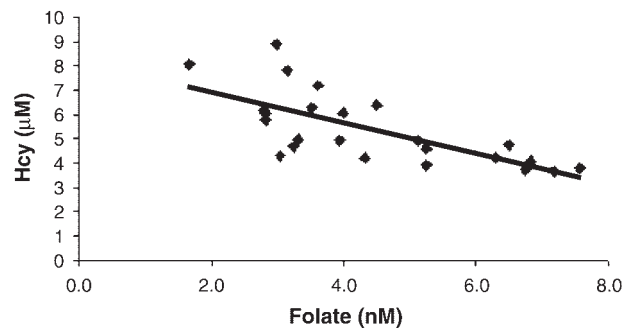**Fig. 4.** Intracellular folate concentration in HUVECs. The results are from 12 experiments using four independent HUVEC cultures and are presented as means $\pm$ SD. The effect of the addition of 100 μM methionine to the medium is shown (closed bars) and compared with results in the absence of methionine (open bars). Asterisks denote significant differences at the $P < 0.005$ level.

thionine (**Fig. 7**). Unexpectedly, the results showed an increase in intracellular Hcy in the absence of extracellular methionine but relatively little change in the presence of methionine. Correcting for cell number made no difference to the time/concentration profile. This result was unexpected because we had already demonstrated that additional methionine increases Hcy export and had presumed this to occur via increased flux from increased intracellular methionine. We therefore measured intracellular methionine in cell extracts collected in duplicate from a single experiment. Methionine concentration changed from a baseline 3.1 nmol/10^6 cells through 3.7 at 24 h, 4.8 at 48 h, and 5.3 at 72 h, confirming the anticipated increase. In contrast, the concentration of methionine in the medium decreased from 107 μM at baseline, through 97, 82, and 78 μM at 24, 48, and 72 h, respectively. We suggest that the reason for this observation lies in the well-established link between S-adenosyl methionine (SAM) and Hcy metabolism. Increase in extracellular methionine leads to an increase of intracellular methionine, which in turn should lead to an increase in SAM. SAM inhibits $N^{5,10}$-methylenetetrahydrofolate reductase (31) and also

stimulates the transsulfuration pathway, which is a metabolic route to remove Hcy in liver (32). Endothelial cells have little or no transsulfuration activity and can only remove excess Hcy by export from the cell (33). Therefore, we suggest that an increase in the concentration of intracellular SAM may stimulate Hcy export, regulating intracellular Hcy and acting as a major determinant of the plasma concentration of tHcy. This is in contrast with an earlier suggestion (34) that the intracellular Hcy/SAM ratio might regulate Hcy export.

Generation of free radicals

HUVECs were cultured in the presence of DMPO, which acts as a spin trap. EPR spectra of the cell supernatants were recorded after various growth periods. The major signal recorded gave Hamiltonian parameters determined to be $A_N = 14.84$ G and $A_H = 14.84$ G. These measurements were confirmed by fitting simulations to be characteristic of the hydroxyl radical, HO$^\bullet$, and are for example quite distinct from sulfhydryl radicals (**Fig. 8**) (35). In the absence of cells, a weak signal was recorded. This is thought to arise from the medium composition, which might comprise hydroxyl radical-generating factors like amino acids together with metal ions, which are capable of Fenton-type reactions. A slight time-dependent in-
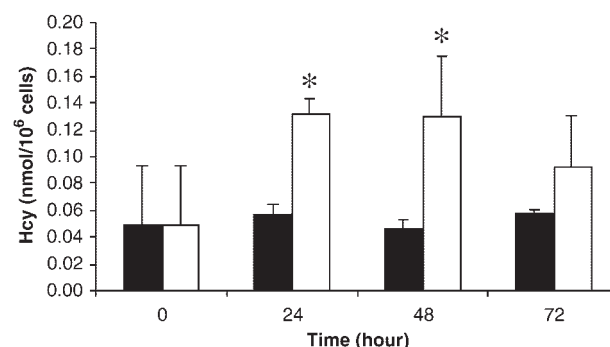

Fig. 6. Association between intracellular folate and extracellular Hcy. Measurements were made on three different HUVEC preparations after various periods of growth up to 72 h ($R = -0.71$).

Fig. 5. Effect of extracellular folinic acid on intracellular folate concentrations. Bars indicate the addition of 0, 100, and 1,000 nM folinic acid (open, striped, and dotted bars, respectively). The results are from seven experiments using four independent HUVEC cultures and are presented as means $\pm$ SD. Asterisks show significant differences from the 0 nM folinic acid values ($P < 0.005$).


Fig. 7. Intracellular Hcy concentration. The results are from two experiments using two independent HUVEC cultures and are presented as means $\pm$ SD. The effect of the addition of 100 μM methionine to the medium is shown (closed bars) and compared with results in the absence of methionine (open bars). Asterisks show significant differences with methionine ($P = 0.05$).
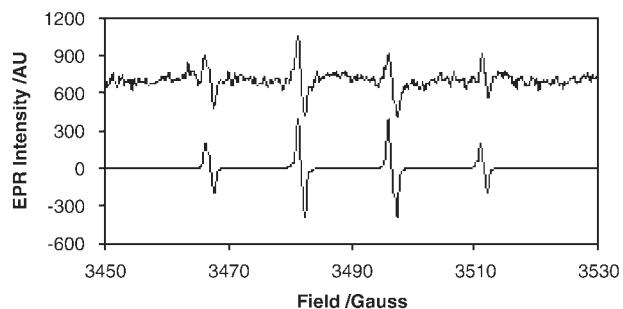
Fig. 8. Electron paramagnetic resonance (EPR) spectrum of HU-VECs incubated with 5,5-dimethyl-1-pyrroline *N*-oxide. Top: Experimental spectrum. Bottom: Simulation showing correspondence of the four-line features ascribed to the hydroxyl radical, using A_N = 14.87 Gauss (G), A_H = 14.87 G. AU, arbitrary units.

crease in intensity was also recorded. However, on incubation with HUVECs, a higher level of hydroxyl radical was recorded, with a much higher rate of increase over time compared with samples in the absence of cells (**Fig. 9**). The higher rate of increase in hydroxyl radical production in the presence of cells was continuous over the time of the experiment (48 h). We have thus shown that the major reactive oxygen species (ROS) in our Hcy-mediated cellular oxidative reactions is specifically the hydroxyl radical. Incubation with the thiol blocker DTNB reduced the concentration of the hydroxyl radical generated in the cellular system (Fig. 9). Incubations in the presence of a range of copper concentrations showed a concentration-dependent increase in hydroxyl radical (F. A. Taiwo, D. Nugent, E. Nakano, H. Griffiths, and H. J. Powers, unpublished data), consistent with the expected importance of copper in the free radical generation process.
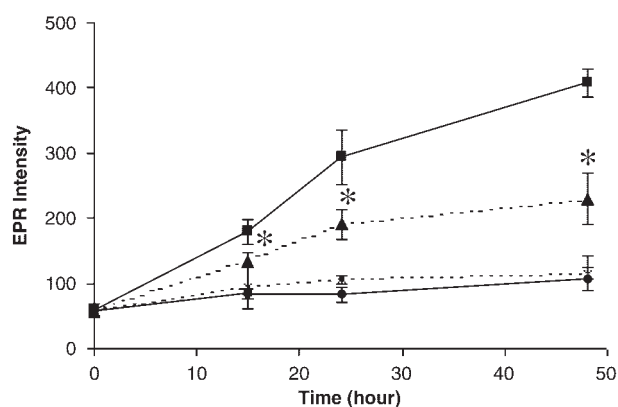
Fig. 9. Intensity of the free radical signal observed in EPR spectra as a function of incubation time. Diamond, no cells; square, with cells; × (dashed line), no cells plus DTNB; triangle (dashed line), with cells plus DTNB. Results were generated from three separate experiments carried out on cells isolated from a single umbilical cord. Experiments carried out on cells from another two umbilical cords showed identical results. Error bars denote standard deviation. The increase in EPR intensity with time is significant in all cases ($P < 0.05$), except in the absence of cells with DTNB. EPR intensity (arbitrary units) is significantly higher in the presence of cells. Asterisks show significantly lower EPR intensity than in the absence of DTNB ($P = 0.05$).

LDL oxidation

LDL was oxidized more rapidly in the presence of HU-VECs than in their absence (**Table 1**). Addition of DTNB to the medium led to a significant reduction in the extent of LDL oxidation (Table 1). Control experiments carried out in the absence of copper produced very slow oxidation of LDL, as expected (15).

It has been suggested that DTNB (or more specifically the TNB⁻ anion) acts independently as an antioxidant (18). It is therefore important to establish whether the effect of DTNB on LDL oxidation is simply a direct antioxidant effect or whether it is achieved through prevention of oxidation of the thiol groups on Hcy. The above experiment was also carried out in the absence of cells. There was a small reduction in the amount of oxidized LDL generated (Table 1), but the change was much less significant than in the presence of HUVECs. Thus, we conclude that although DTNB may have some antioxidant activity, most of the effect seen here is attributable to the blocking of thiol groups.

Cysteine can be exported from endothelial cells and may contribute to the oxidation of LDL in our system. To minimize this contribution, glutamate was added to the incubation medium to inhibit cystine uptake and thereby reduce cysteine export (18). Addition of 15 mM glutamate to the medium had no significant effect on the concentration of extracellular Hcy (data not shown) but led to a modest but significant reduction in the extent of LDL oxidation (**Table 2**). These values suggest that the export of cysteine may have contributed ∼50% to the overall oxidation of LDL. The results in Table 2 are shown at 24 h only, because by 48 h in the presence of glutamate and LDL, cells had begun to change shape from the typical elongated shape of HUVECs to smaller and rounder cells, and viability counts decreased.

Uptake of oxidized LDL into macrophages

Monocytes were seeded onto 96-well plates at a density of 2×10^6 cells/ml. After 5 days, the cells appeared well differentiated. Uptake of oxidized LDL into the macrophages was followed using a competitive assay, in which

TABLE 1. Oxidation of LDL in the presence of HUVECs and the effect of a thiol-blocking reagent

Sample	Concentration of TBARS		
	0 h	24 h	48 h
		μM	
No cells	0.00 ± 0.19	0.57 ± 0.30	0.91 ± 0.36
With cells	0.00 ± 0.19	1.44 ± 0.26[a]	3.17 ± 0.32[b]
No cells + DTNB	0.00 ± 0.19	0.43 ± 0.19	0.48 ± 0.47
With cells + DTNB	0.00 ± 0.19	0.09 ± 0.13[a]	0.09 ± 0.75

HUVEC, human umbilical vein endothelial cell; TBARS, thiobarbituric acid reactive substances. Results (mean ± SD) were corrected by subtraction of the mean TBARS value measured at 0 h. Each result was obtained from experiments carried out in triplicate on three different HUVEC cultures (two for no cells + DTNB), each of which also used a different LDL preparation.
[a] Significantly different on addition of DTNB ($P < 0.005$).
[b] Significantly different from 24 h ($P < 0.005$).

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TABLE 2. Oxidation of LDL in the presence of HUVECs and the effect of glutamate

Sample	Concentration of TBARS	
	0 h	24 h
	μM	
No cells	0.00 ± 0.12	0.56 ± 0.21
With cells	0.00 ± 0.12	2.12 ± 0.70[a]
With cells + glutamate	0.00 ± 0.12	1.41 ± 0.82[a]

Results were corrected as in Table 1. Each was obtained from three or four measurements carried out on three different HUVEC cultures, each of which also used a different LDL preparation.

[a] Significantly different on addition of glutamate ($P < 0.005$).

partially oxidized experimental LDL competed with a fluorescently labeled fully oxidized LDL for uptake into macrophages. The results (**Fig. 10**) demonstrate a time-dependent increase in uptake of experimentally oxidized LDL and suggest that macrophage uptake of LDL occurs as soon as oxidation of LDL starts. LDL oxidized by incubation with HUVECs for 72 h inhibited uptake of the fluorescent oxidized LDL standard by macrophages over 16 h by 59%.

DISCUSSION

Hcy export by HUVECs

We have shown that there is a continuous export of Hcy from HUVECs, which is approximately linear over at least 72 h in culture, and that the export is stimulated by the addition of methionine to the medium but reduced by the addition of folinic acid. Although there have been earlier reports of Hcy export from a variety of cells, this result is significant because the cells used here are primary culture human endothelial cells and because the effect of intracellular folate is clearly evident. Thus, the results reported here are likely to be of relevance to human cardiovascular disease. Furthermore, earlier reports showed a marked reduction in Hcy export when cell density was high, raising doubts about whether a confluent endothelial culture would export much Hcy (19, 23). It is important to demonstrate export from confluent endothelial cells, because this is the most appropriate model for the in vivo vascular endothelium. We have demonstrated that in the presence of methionine a confluent layer produces ∼1.6 μmol/l Hcy/day, which is evidently sufficient to cause significant LDL oxidation in vitro. In vivo, an increase in plasma tHcy of 5 μmol/l was found to have an odds ratio for coronary artery disease or cerebrovascular disease of ∼1.8 (6), implying that the Hcy export seen here is likely to be physiologically relevant. The effects of DTNB on LDL oxidation confirm the role of thiols in this process. The effects of folinic acid on Hcy export from HUVECs support previous results (24). An inhibitory effect was demonstrated at a concentration as low as 10 nM, although it became statistically significant only at 100 nM. Mean plasma folate concentrations in healthy cohorts are reported to lie in the range of 10–25 nM (36, 37), although concentrations can increase severalfold after supplementation (37–39). We therefore conclude that an increase in plasma folate concentration can have a significant impact on the rate of export of Hcy from endothelial cells. We presume that this effect is mediated by changes in intracellular folate, although we have shown that intracellular folate is only moderately responsive to extracellular folate. A 10-fold increase in extracellular folate elicited a 2-fold increase in intracellular folate. This is indicative of a tight control over receptor-mediated transport of folate into cells, regulated by cellular requirements for folate (40).

Flux through the methylation pathway is evidently an important determinant of Hcy export. We confirm that additional methionine in the medium elicits a more rapid rate of export of Hcy from cells, as shown in other cell types (19, 41), and also show that this is associated with a clear increase in intracellular methionine. Additional me-
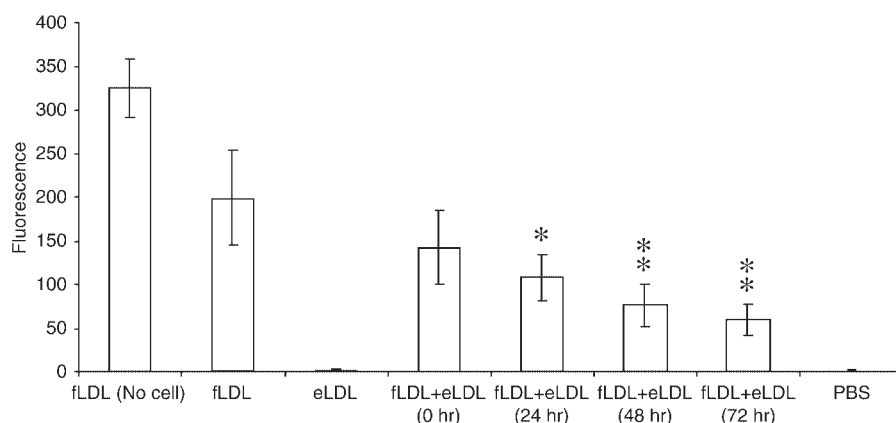

Fig. 10. Competition of partially oxidized experimental LDL, oxidized by HUVECs for different lengths of time (eLDL), with fluorescently labeled fully oxidized LDL (fLDL), for uptake into macrophages. Results are shown as mean fluorescence values ± SD from three measurements each on three different HUVEC and macrophage preparations. As a positive control, fluorescent LDL was added in the absence of cells to give a maximum signal; as negative controls, experimental oxidized LDL and PBS were added to give a baseline signal. Asterisks show fluorescence significantly lower than 0 h at $P < 0.05$ (*) and $P < 0.005$ (**).

thionine also stimulates a depletion of intracellular folate, which we suggest may occur through inhibition of $N^{5,10}$-methylenetetrahydrofolate reductase (32), by a predicted increase in SAM as methionine increases inside the cells. The factors that regulate Hcy export are not understood, but we suggest that intracellular SAM may play a role. A similar suggestion has also been made by others (19).

Hcy exported from endothelial cells oxidizes LDL via the hydroxyl radical

The results reported for the oxidation of LDL in the presence of DTNB are strong evidence that thiols exported from HUVECs are responsible for much of the LDL oxidation seen in our experimental system. The effect of glutamate on LDL oxidation indicates that cysteine also contributes to LDL oxidation. This requires further investigation, but it is consistent with thiol exchange reactions in plasma being important (42). We have shown that the HUVECs produce a nearly linear increase in the amount of free radical generated in the solution, this free radical being most likely hydroxyl, because the EPR parameters of the predominant radical obtained are characteristic of the hydroxyl radical. We have shown in previous studies that the simultaneous presence of superoxide and hydroxyl radicals in a composite spectrum with carbon-centered radicals is resolvable (43). Therefore, any significant presence of the superoxide radical anion in the copper-Hcy reaction would have been observed by EPR spectrometry; thus, we are confident that the major radical present is hydroxyl. Many authors have suggested that thiols in the presence of copper or iron ions can produce free radicals (44). Although some authors (10, 45, 46) have suggested that the major oxidant species in copper-mediated LDL oxidation is superoxide, others (47, 48) have concluded that the hydroxyl radical may be important. The participation of Hcy in the induction of radical formation also suggests the thiol-derived RS• radical. Our results confirm the generation of hydroxyl radicals. The effect of the thiol blocker DTNB on hydroxyl radical generation in the cellular system is strongly supportive of thiols being the major source of these radicals.

The hydroxyl radical is likely to be generated by the following reactions, in which the copper-catalyzed autoxidation of Hcy in solution is associated with the production of HO•:

$$Cu^{2+} + RSH \rightarrow Cu^+ + RS^\bullet + H^+ \qquad (Eq.\ 1)$$

$$RS^\bullet + RS^\bullet \rightarrow RSSR \qquad (Eq.\ 2)$$

$$Cu^+ + O_2 \rightarrow Cu^{2+} + O_2^{\bullet-} \qquad (Eq.\ 3)$$

$$O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 \qquad (Eq.\ 4)$$

$$H_2O_2 + Cu^+ \rightarrow Cu^{2+} + OH^- + HO^\bullet \qquad (Eq.\ 5)$$
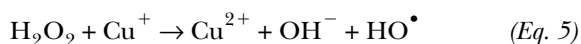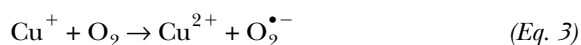
The reactive Cu^+ species is rapidly oxidized to form the superoxide radical anion, which is immediately protonated in a medium of high H^+ concentration to form hydrogen peroxide. In the presence of reactive Cu^+, the Fenton reaction (formula 5) is facilitated to generate hydroxyl radicals. Our observation of the presence of hydroxyl radicals, and the absence of superoxide radicals, indicates that superoxide is rapidly transformed to hydroxyl via hydrogen peroxide. Thus, there is strong circumstantial evidence that the Hcy generated by HUVECs primarily produces hydroxyl radicals and thereby leads to LDL oxidation. This is supported by the observed production of HO• in a cell-free Hcy autoxidation system (our unpublished observations).

As in our previous studies (15), the concentration of copper used in our assays was 30 μM and approximates the in vivo concentration of plasma total copper. Most plasma copper in vivo is bound by albumin and ceruloplasmin, and although tightly bound this copper appears to be redox active (49). In our study, we used a physiological concentration of albumin, thereby binding copper to give approximately the same concentration of free copper as found in plasma.

We have previously demonstrated (15) that Hcy at physiological concentrations can induce the oxidation of LDL through a copper-dependent pathway. Hcy is rapidly oxidized to homocystine both in vitro and in vivo (11, 24), such that only ~1% of the total homocyst(e)ine present in vivo is Hcy (50). The results presented here suggest that it is the continuous export of Hcy from endothelial cells that gives rise to sustained amounts of Hcy in plasma, and thereby to oxidation of LDL in vivo, via free radical mechanisms.

LDL uptake by macrophages

We have also shown that LDL oxidized by our experimental system is taken up by macrophages. This is not unexpected, because many others have shown that oxidized LDL is taken up by macrophages and incorporated to form foam cells (51, 52). The significance of this result is that we have shown in a single system that all of the steps suggested to be responsible for Hcy-dependent atherosclerosis do indeed occur in quantities sufficient to lead to the formation of atherosclerotic plaques: Hcy is exported from endothelial cells, it is oxidized in the presence of copper ions to form free radicals, LDL is oxidized by the free radicals, and the oxidized LDL is taken up by macrophages. These results lend powerful support to this suggested mechanism for Hcy involvement in atherosclerosis (10).

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