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# Anti-angiogenic effects of homocysteine on cultured endothelial cells

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**Abstract**

High levels of homocysteine induce a sustained injury on arterial endothelial cells which accelerates the development of thrombosis and atherosclerosis. Some of the described effects of homocysteine on endothelial cells are features shared with an anti-angiogenic response. Therefore, we studied the effects of homocysteine on key steps of angiogenesis using bovine aorta endothelial cells as a model. Homocysteine decreased proliferation and induced differentiation. Furthermore, 5 mM homocysteine produced strong inhibitions of matrix metalloproteinase-2 and urokinase, two proteolytic activities that play a key role in extracellular matrix re-modeling, and decreased migration and invasion, other two key steps of angiogenesis. This study demonstrates that homocysteine can inhibit several steps of the angiogenic process. 

*Keywords:* Homocysteine; Angiogenesis; Matrix metalloproteinase-2; Urokinase

Homocysteine is a sulfur-containing, non-proteinogenic amino acid which takes a key place in between folatecycle and activated methyl cycle [1]. Hyperhomocysteinemia has been associated with an increased risk of cardiovascular disease, including atherosclerosis and thrombosis [2–4].

Substantial evidence indicates that progression of atherosclerosis is related to enhanced pro-oxidant activity [5]. Homocysteine can contribute to this, since it modulates glutathione peroxidase expression, nitric oxide bioavailability, and endothelin-1 production [6,7]. Furthermore, homocysteine produces reactive oxygen species with the catalytic help of copper ion in serum, injuring and inducing apoptosis in endothelial cells [8,9]. However, homocysteine also can induce apoptosis in human endothelial cells in the absence of oxidative stress [10].

On the other hand, atherosclerosis is also related to changes towards a pro-coagulant phenotype of endothelium. High levels of homocysteine induce pro-coagulant effects, including inhibition of protein-C activation, antithrombin III, and thrombomodulin [1]. In addition, homocysteine increases the expression of endothelial cell surface molecules linked to vascular disease, such as ICAM-1 and PAI-1 [11].

Angiogenesis is the generation of new capillaries by a process of sprouting of pre-existing microvessels. Vessel proliferation is under stringent control, but in many pathological conditions (i.e., solid tumor progression, metastasis, diabetic retinopathy, hemangiomas, arthritis, psoriasis, and atherosclerosis) the disease appears to be driven by persistent upregulated angiogenesis [12]. When resting endothelial cells are activated by an angiogenic signal, they are stimulated to release degrading enzymes allowing endothelial cells to migrate, proliferate, and finally differentiate to form new vessels. Any of these steps may be a potential target for pharmacological intervention. Some of the described effects of homocysteine on endothelial cells, namely, reduction of proliferation, induction of ICAM-1 and PAI-1 are features shared with an anti-angiogenic response.

The purpose of the present study was to examine the effects of homocysteine on key steps of angiogenesis using bovine aorta endothelial (BAE) cells as a model. Herein, we demonstrate that homocysteine modulates several of these key steps towards anti-angiogenesis.

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## Materials and methods

*Cell culture and proliferation assay*. BAE cells were maintained as previously described [13] in Dulbecco's modified Eagle's medium containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 μg/mL), and amphoterycin (1.25 μg/mL) supplemented with 10% fetal bovine serum (FBS).

In experiments with proliferating BAE cells, $2.5 \times 10^4$ cells in a total volume of 625 μL medium were incubated in each well of 24-well plates with serial dilutions of homocysteine. For non-proliferating cell experiments, 0.5 mL cell suspension (approximately, $2.5 \times 10^5$ cells) coming from a highly confluent cell culture dish was plated per well in 24-well plates; after 24 h, medium was removed, cells were washed and new medium containing the different tested concentrations of homocysteine was added. In both cases, after 3 days of incubation (37 °C, 5% $CO_2$ in a humid atmosphere), cells were detached and counted by using a Coulter counter.

*Apoptosis assays*. BAE cells were grown to 75% confluency on 8-well Falcon culture slides and incubated for 14 h with or without 5 mM homocysteine. The TUNEL assay was performed according to the manufacturer's instructions (Roche Diagnostics, Barcelona, Spain).

The assay of activation of caspase-3 was carried out using the fluorogenic substrate Ac-DEVD-AMC according to the published standard procedure [14].

*Conditioned media and zymographic assays*. BAE cells at 75% confluency were changed to medium in the absence of FBS and maintained in the presence or absence of 5 mM homocysteine for 24 h. Cells were photographed under phase contrast in a Nikon Diaphot-TMD microscope. Conditioned media, cell extracts, and zymographies were carried out as described [13]. Quantitative analysis was performed with the NIH-Image (1.6) Program.

*Endothelial cell migration and invasion assays*. Real time monitoring of migration and invasion of fluorescence-labeled endothelial cells was assayed by using a 24-well fluorescence-opaque membrane insert as previously described [13].

## Results and discussion

### *Homocysteine induces differentiation, inhibits proliferation but does not induce apoptosis of BAE cells*

BAE cells have been proved to be a useful tool for angiogenesis studies [13,15–17]. Capillary endothelial cells proliferate in response to an angiogenic stimulus during neovascularization. Homocysteine inhibited the growth of proliferating and had much less effect on non-proliferating BAE cells (Fig. 1A). In the presence of serum, homocysteine seems to be a cytostatic agent for BAE cells, with toxic effects only observed at 16 mM homocysteine. Although the anti-proliferant effects of homocysteine on endothelial cells have been related to atherosclerosis [1–4], it should be stressed that proliferation is also a required step in angiogenesis. Furthermore, previous reports on the cytotoxic effects of homocysteine on HUVEC, ECV-304, and other endothelial cells have been carried out under conditions in which control cells are actively proliferating, and not in a quiescent state, as endothelial cells use to be in arteries,

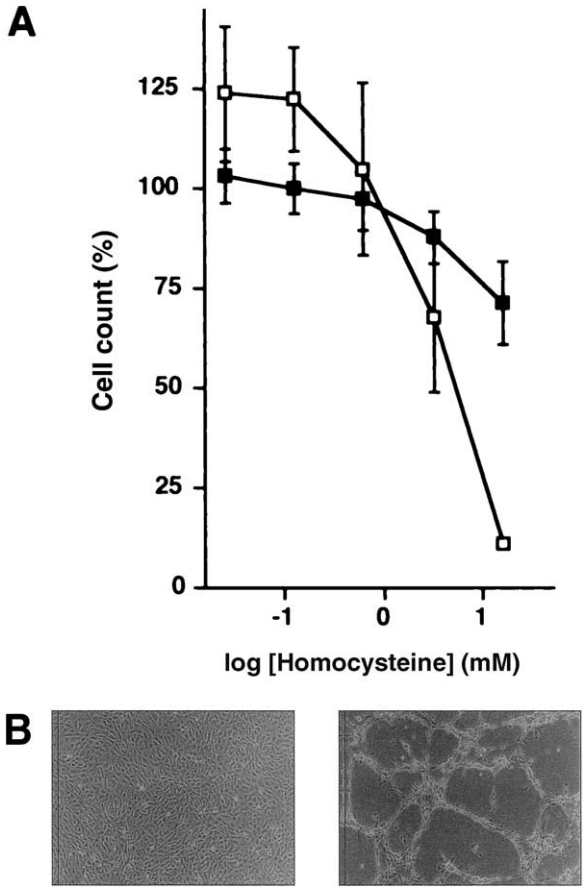


Fig. 1. Homocysteine inhibitis proliferation and induces differentiation of BAE cells. (A) Proliferant (white squares) or non-proliferant (black squares) BAE cells were incubated in the presence of different concentrations of homocysteine for 3 days and then counted as described in Material and methods. Data are given as percentages, taken the count values of control, untreated cells as 100% and they are means ± SD of four different data. (B) Subconfluent (75%) BAE cells were changed to a serum free medium and maintained for 24 h in the absence (left) or presence (right) of 5 mM homocysteine.

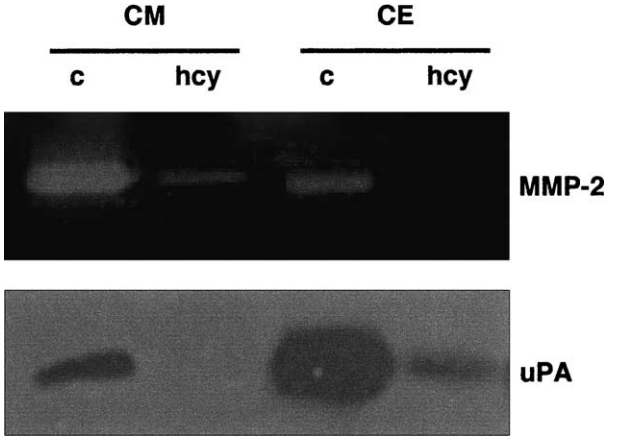


Fig. 2. Homocysteine inhibits extracellular matrix proteases produced by BAE cells. Matrix metalloproteinase-2 (MMP-2) and urokinase (uPA) were detected by zymographic techniques (as described in Material and methods) in both conditioned media (CM) and cell extracts (CE) of control and 5 mM homocysteine-treated BAE cells.

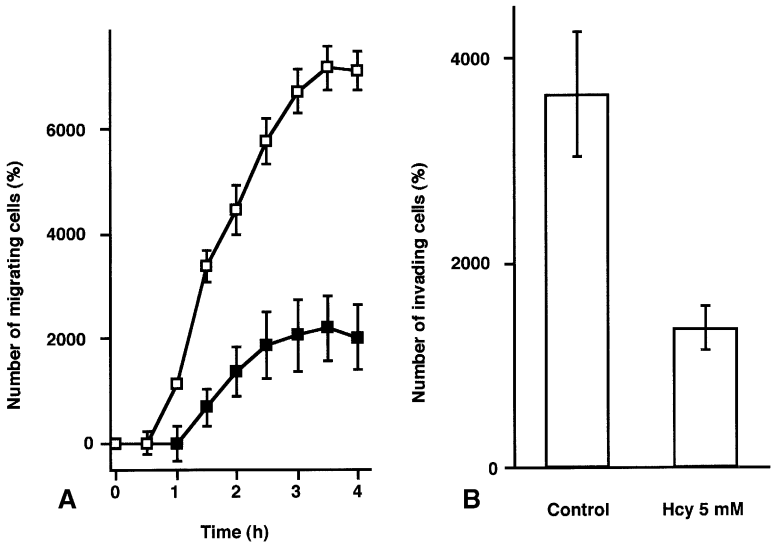


Fig. 3. Homocysteine inhibits BAE cell migration and invasion. (A) Migration of BAE cells either in the absence (white squares) or presence (black squares) of 5 mM homocysteine was determined as described in Material and methods. Data are means ± SD of three different determinations. (B) The invasive capability of BAE cells through a Matrigel layer was determined after 4 h of incubation in the absence or presence of 5 mM homocysteine. Data are means ± SD of three different determinations.

veins, and capillaries [9,10]. In a recent report on endothelial cell apoptosis induced by homocysteine and copper, this cytotoxic effect sharply decreases with increased culture time, that is, decreased proliferation rate [9]. Our results with proliferating and non-proliferating BAE cells do support this observation.

In the absence of serum for 24 h, 5 mM homocysteine did not only preclude BAE cell proliferation, but also produced a re-arrangement of the cells (Fig. 1B), which formed capillary-like structures similar to those produced when these cells are laid onto a Matrigel layer [13]. These morphological data strongly support the concept of homocysteine behaving as a cytostatic compound promoting differentiation of BAE cells. It should be remarked that 5 mM homocysteine had only a weak cytotoxic effect on BAE cells, as revealed both by cell counting (89 ± 7% of cells after 24 h of treatment, taking the initial cell count as 100%) and by the negative results obtained from two apoptosis assays, namely, the TUNEL assay and the assay of activation of caspase-3 (results not shown). This is in contrast with published data for other endothelial cells [9,10].

*Homocysteine affects the capacity to re-model extracellular matrix and inhibits the migratory and invasive capabilities of BAE cells*

A positive proteolytic balance is required for capillary sprout elongation and lumen formation during angiogenesis. Matrix metalloproteinases and serine proteinases play key roles in angiogenesis [18,19]. The proteolytic balance can be modulated by some inhibitors of angiogenesis [20–23]. Both conditioned media and cell extracts from BAE cells exposed for 24 h to 5 mM homocysteine exhibited much lower levels of matrix metalloproteinase-2 and urokinase than control, untreated-cells, as determined by zymographies (Fig. 2). Matrix metalloproteinase-2 contents of both conditioned media and cell extracts from homocysteine-treated BAE cells were less than 10% those of control cells. Similar effects in endothelial cell conditioned media have been described for aeroplysinin-1, halofuginone, and curcumin [13,15,24], three recently described inhibitors of angiogenesis.

On the other hand, there was no detectable urokinase band in conditioned media from homocysteine-treated cells and urokinase in cell extracts from homocysteine-treated cells was only 30% that of control cells (Fig. 2).

Taken together, these data seem to indicate that homocysteine causes a strong shift towards anti-proteolysis in BAE cells. This is in agreement with the observed potent inhibition of the migration and invasive ability of the homocysteine-treated endothelial cells (Fig. 3). Agents as homocysteine, which repress endothelial cell migration and invasion, are potential inhibitors of neovascularization, since both migration and invasion are two essential steps required for angiogenesis to proceed.

## Concluding remarks

Although the concentration of homocysteine used in some experiments (5 mM) is rather high as compared with the concentrations in plasma under physiological and pathological conditions, this is unavoidable because

endothelial cells in culture upregulate the metabolism of homocysteine considerably. In fact, similar high concentrations of homocysteine have been used in recent relevant studies involving cultured endothelial cells [9–11].

In summary, our results demonstrate that homocysteine inhibits certain functions of angiogenic endothelial cells, namely, proliferation, degradation of the basement membrane, migration, and invasion through Matrigel in vitro. These results open new ways to the currently expanding field of homocysteine research [1].

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