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Homocysteine is a potent modulator of plasma membrane electron transport systems

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Abstract The deregulation of homocysteine metabolism leads to hyperhomocysteinemia, a condition described as an independent cardiovascular disease risk factor. Ubiquitous plasma membrane redox systems can play a dual pro-oxidant and anti-oxidant role in defense. In this study, we test the hypothesis that homocysteine, as a redox active compound, could modulate the endothelial plasma membrane redox system. We show that homocysteine behaves as a very potent stimulator of this activity. Furthermore, we show that this inducing effect is also produced on tumor cells and that it can be observed at both the activity and protein levels. On the other hand, homocysteine treatment decreases the activity of the specific ectocellular tumor NADH oxidase. Taken together, these results underscore a potential antitumoral action of homocysteine.

Keywords Plasma membrane redox · Ferricyanide reductase · Homocysteine · Endothelial cell · Cancer · NADH oxidase

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Introduction

Hyperhomocysteinemia has been associated with an increased risk of cardiovascular disease, including atherosclerosis, thrombosis, stroke and peripherial arterial occlusive disease (Hankey and Eikelboom 1999; Medina et al. 2001; Medina and Amores-Sánchez 2000; Perla-Kajan et al. 2007; Refsum et al. 1998). However, there is no clear consensus as to the potential mechanisms whereby excess homocysteine could contribute to vascular disease (Medina and Amores-Sánchez 2000; Perla-Kajan et al. 2007), although endothelial cell damage and/or injury should be expected. At the molecular level, several potential mechanisms have been proposed, including induction of endoplasmic reticulum stress, protein *N*-homocysteinylation and epigenetic effects concerning methylation status (Hultberg et al. 2000; Jakubowski 1999, 2004; Medina et al. 2001; Perla-Kajan et al. 2007). Furthermore, since homocysteine is a redox active compound, induction of oxidative stress is one of the most favored postulated mechanisms of homocysteine toxicity (Medina et al. 2001; Perna et al. 2003; Zou and Banerjee 2005). On the contrary, potential protective effects of homocysteine have also been reported. It has been shown that extracellular superoxide dismutase, an important antioxidant in vascular tissues, is positively associated with homocysteine levels (Wilcken et al. 2000). At least under certain conditions, homocysteine behaves as an anti-oxidant (Zappacosta et al. 2001). Furthermore, homocysteine reduces the expression levels of endothelin-1, a well-known potent vasoconstrictor (Drunat et al. 2001). Our group has previously reported potent anti-angiogenic and anti-invasive effects of homocysteine (Chavarría et al. 2003a, b; Rodríguez-Nieto et al. 2002).

Redox reactions are essential for the function of cell membranes. It should be stressed that every bioenergeti-

cally competent cell membrane does contain redox systems (Medina et al. 1997). In fact, plasma membrane electron transport systems or plasma membrane redox systems (PMRS) have been found in every tested living cell. PMRS are not a simple curiosity, an evolutive relic. On the contrary, there is increasing, experimental evidence for their direct involvement in several vital functions, including bioenergetics, iron uptake and cell growth and proliferation (Medina et al. 1997). Cell defense is another function in which PMRS are involved, and in this function it can play a double key role. A tightly controlled and localized production of reactive oxygen species by a plasma membrane NADPH oxidase can be used as a potent microbicidal weapon. On the other hand, ubiquitous PMRS play a role in defense against oxidative stress damage (del Castillo-Olivares et al. 2000).

We postulated the hypothesis that, being homocysteine a redox-active compound, it should modulate the activity of endothelial cell PMRS. To test this hypothesis, we used cultured bovine aorta endothelial cells (BAEC) and determined the effects of homocysteine on BAEC ferricyanide reductase activity under different culture conditions. In this report, we show for the first time that, indeed, homocysteine behaves as a very potent inducer of endothelial cell PMRS activity. Furthermore, we show that this inducing effect of homocysteine is also produced in tumor cells on the levels of one of the protein components of PMRS and on tumoral ferricyanide reductase activity. On the contrary, homocysteine treatment inhibits the specific ectocellular, tumoral NADH oxidase (tNOX) activity of cancer cells.

Material and methods

Material

Cell culture media were purchased from Cambrex (Walkersville, MD, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, UK). Homocysteine and analytical reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA). Plasticware for cell culture was supplied by NUNC (Roskilde, Denmark).

BAEC isolation and culture

Bovine aortic archs were isolated from calfs immediately after their sacrifice at the local slaughterhouse Famadesa (Málaga), transported to the lab immersed in PBS containing penicillin-streptomycin and amphotericin at standard cell culture concentrations, and used immediately upon arrival for isolation of primary bovine aortic endothelial cells (BAEC) by a collagenase treatment, as first described by Gospodarowicz et al. (Gospodarowicz et al. 1976). BAEC were cultured as maintained as we described elsewhere (Martinez-Poveda et al. 2005). In brief, they were cultured in 90 mm culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) 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% FBS. Cultures were carried out in cell incubators under controlled temperature (37 °C) and atmosphere (5% of CO_2).

Human tumor cell culture

Human U2-OS osteosarcoma and Hep G2 hepatocellular carcinoma cells were supplied by ATCC and maintained according to supplier's suggestions.

Treatments

Confluent cultures ($4–5\times10^6$ cells per dish) were used. Medium was removed and cell were washed 3 times with cold PBS. Afterwards, 5 ml of fresh medium in the presence or absence of the mentioned concentrations of homocysteine were added and cells were incubated under standard culture conditions for an additional 4 or 24 h period of time.

Ferricyanide reductase assay

Trans-plasma membrane electron transport activity of endothelial and tumor cell PMRS was measured as reduction of potassium ferricyanide, by using the ferrocyanide determination assay described by Avron and Shavit (Avron and Shavit 1963), as applied for culture cells (Baoutina et al. 2001), with slights modifications. After treatments, cells were washed 3 times with cold PBS. Afterwards, PBS supplemented with 5 M glucose and 0.1 mM ferricyanide was added as to have 1×10^6 cells/ml and cells were reincubated at 37 °C. At the indicated times (0. 15, 30 and 60 min), 0.1 ml supernatants were removed and mixed with 0.05 ml (*v*/*v*/*v* = 2:2:1) of a pre-mix containing 3 M sodium acetate (pH 6.5), 0.2 M citric acid and 3.3 mM ferric chloride (in 0.1 M acetic acid). Finally, 10 μl of 3.3% (*w*/*v* in water) bathophenanthroline disulphonic acid (BPS) was added. After 10 min of incubation at room temperature, absorbance was measured at 535 and an experimentally determined absorption coefficient of 21.6 mM^{-1} cm^{-1} was used.

Western blot

BAEC and U2-OS cells were grown in cell culture dishes (diameter, 10 cm) up to 80–90% confluency were incubated for 4 and 24 h in the presence or absence of 5 mM homocysteine. After three washes with cold PBS, cells

were lysed with 0.3 ml of RIPA + CHAPS (30 mM HEPES–NaOH, pH 7.3, 150 mM NaCl, 1% (*w*/*v*) Triton X-100, 1% (*w*/*v*) deoxycholate, 0.1% (*w*/*v*) SDS and 2% (*w*/*v*; CHAPS) in the presence of a cocktail of protease inhibitors (Sigma reference P8340, containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin and 1.4 mM E-64). Extracts were centrifuged for 15 min at 13,000 rpm and 4 °C. Supernatants were heated at 100 °C for 5 min and used to load 25 μg protein per well in a 10% polyacryamide gel. After PAGE, semiwet electroblotting to nitrocellulose membranes was carried out using 20% methanol. Membranes were blocked overnight with fat-skimmed milk (10%). A specific polyclonal antisuerum against pig liver cytocrome b5 reductase (Navarro et al. 1995) was kindly provided by Dr. José M. Villalba (University of Córdoba, Spain) and it was used at a 1:2,500 dilution. After incubation with this primary antibody for 1 h at room temperature, nitrocellulose membranes were washed three times with blocking buffer and then incubated 45 min at room temperature with a secondary antibody (provided by GE Healthcare) at 1:5,000 dilution and revealed by using Bio-Rad's Immun-Star detection kit according supplier's instructions. Some controls were carried out by using Laemmli buffer in the extraction procedure instead of RIPA + CHAPS.

Tumoral-NADH oxidase activity

Hep G2 and U2-OS tumor cells were pre-treated for 4 h with 5 mM homocysteine. After treatment, cells were washed with PBS and tNOX activity was determined spectrophotometrically as the disappearance of NADH measured at 340 nm in a reaction mixture containing phosphate buffered saline (pH 7.4), 5 mM glucose, 2 mM KCN (to inhibit mitochondrial oxidase activity) and 0.15 mM NADH at 37 °C.

Results and discussion

BAEC PMRS reduces extracellular ferricyanide

Our group has a wide previous experience in the characterization of PMRS activities in tumor cells (Medina et al. 1992; Medina and Núñez de Castro 1995; Medina et al. 1988). In fact, we have previously carried out a deep characterization of Ehrlich ascites tumor cell ferricyanide reductase activity (del Castillo-Olivares et al. 1996a, b, c; del Castillo-Olivares et al. 1995, 1996a, b, c; Del Castillo-Olivares et al. 1994; del Castillo-Olivares et al. 1996a, b, c; Medina et al. 1994; Medina and Núñez de Castro 1994; Medina et al. 1988; Rodríguez-Caso et al. 1997). The use of ferricyanide as an exogenously added, non-permeant extracellular electron acceptor is widely used to determine the trans-plasma membrane electron transport activity of PMRS (Baoutina et al. 2001; Crane et al. 1985; Medina et al. 1997; Medina et al. 1988). When this activity has to be determined in enriched plasma membrane fractions, in proteoliposomes or in very dense whole cell populations (10^7–10^8 cells/ml), the direct determination of ferricyanide reduction provides enough sensitivity for accurate measurements. Ferricyanide in buffered solution yields an intense yellow color, with a maximum of absorbance at 420 nm. On the other hand, ferrocyanide (the product of ferricyanide reduction) is colorless in solution. Therefore, direct ferricyanide reduction is easily determined by following the decrease in absorbance at 420 nm of buffered solutions supplemented with ferricyanide in the presence of cells. An absorption coefficient of 1 mM^{-1} cm^{-1} is reported for ferricyanide (Medina et al. 1988). However, cell cultures do not provide such high densities required for accurate direct measurement of ferricyanide reduction. For such cases, the indirect determination of total reduced ferrocyanide by the Avron and Shavit method (Avron and Shavit 1963) can be applied, since it provides a 21.6-fold increase in sensitivity. This procedure has been successfully applied to culture cells at densities of 10^6 cells/ml (Baoutina et al. 2001).

To our knowledge, ferricyanide reductase activity has not been previously determined in BAEC. We used the Avron and Shavit method (Avron and Shavit 1963) with the modifications described in Material and methods, after a test phase of optimization of the procedure. In our hands, the most reliable results were obtained by using the described pre-mix (with a ferric chloride concentration that is 50% lower than that used in the original method) and measuring absorbance at 535 nm exactly 10 min after BPS addition. We supplemented the ferricyanide reductase buffered medium with 5 mM glucose in order to warrant cytosolic NADH recycling for up to a hour of incubation (Medina et al. 1988). In such a way, the intracellular electron donor for PMRS activity does not become limiting under our experimental conditions.

Figure 1a shows that BAEC have an active ferricyanide reductase activity, which linearly increases the levels of reduced ferrocyanide in the buffer for at least a hour. The accumulated reduced ferrocyanide in the supernatants after 1 h-incubation was 3.80±0.97 μM. Therefore, BAEC have an active PMRS exhibiting a ferricyanide reductase activity at similar levels to those reported for murine and human monocytes and macrophages (Baoutina et al. 2000, 2001).

Homocysteine treatment induces BAEC ferricyanide reductase activity

To study the effects of homocysteine on BAEC ferricyanide reductase activity, we initially selected the following

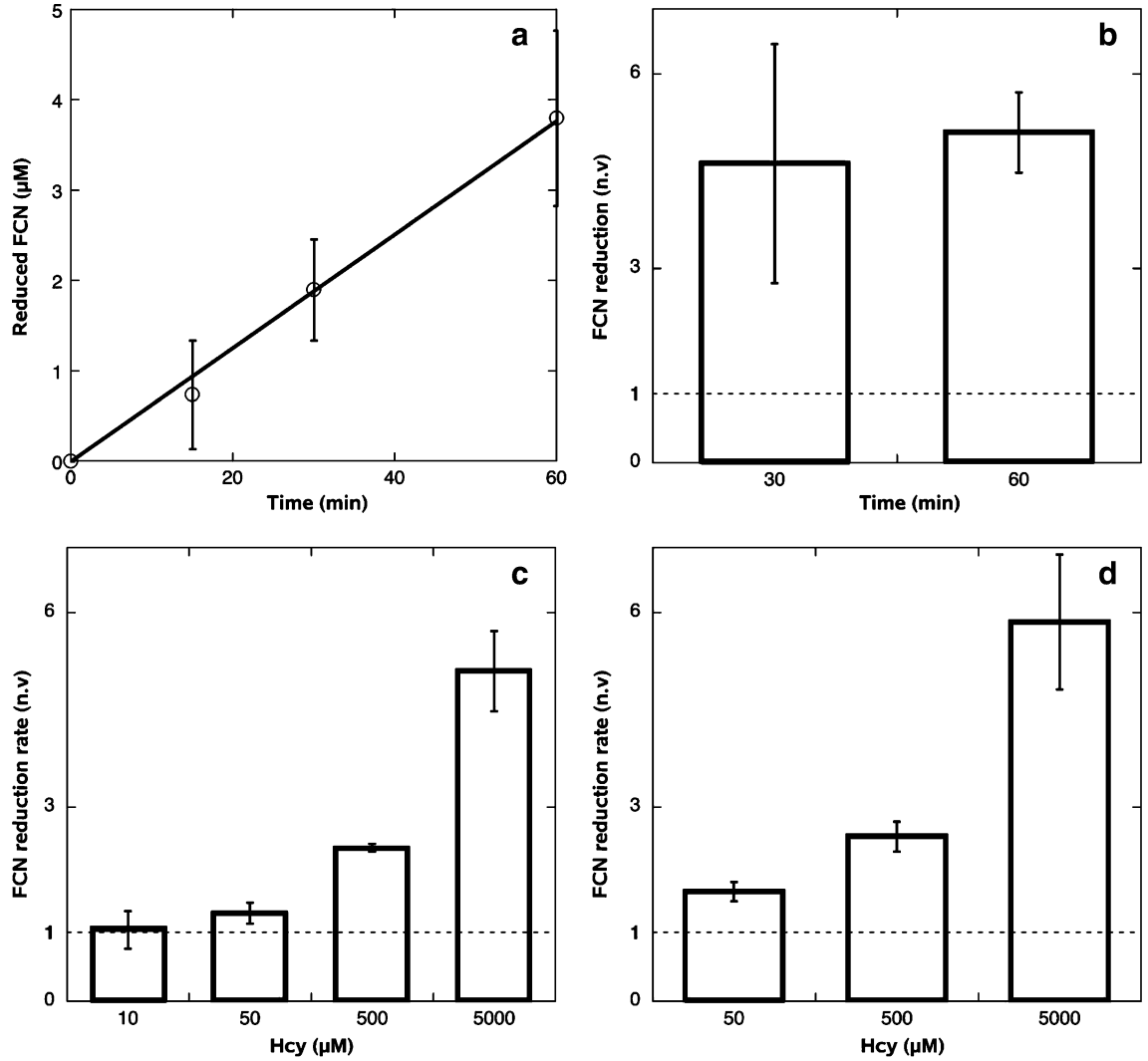

Fig. 1 **a** Time course of BAEC ferricyanide reductase activity. The assay was carried out as described in "Material and methods." The accumulation of ferrocyanide is depicted. **b** Stimulatory effect of 5 mM homocysteine pretreatment for 4 h on BAEC ferricyanide reductase activity. Data are represented as normalized values (*n.v.*), taking the values of control, untreated BAEC as 1 (*dash line*). **c** Dose-response of the stimulatory effect of homocysteine pretreatment for 4 h (in the presence of serum) on BAEC ferricyanide reductase activity. Data are represented as normalized values (*n.v.*), taking the values of control, untreated BAEC as 1 (*dash line*). Ferricyanide reduction rates at 0.5 and 5 mM homocysteine are significantly greater than that exhibited by control, untreated cells ($p<0.05$, according to a Student's *t* test). **d** Dose-response of the stimulatory effect of homocysteine pretreatment for 4 h (in the absence of serum) on BAEC ferricyanide reductase activity. Data are represented as normalized values (*n.v.*), taking the values of control, untreated BAEC as 1 (*dash line*). Ferricyanide reduction rates at the three tested concentrations of homocysteine are significantly greater than that exhibited by control, untreated cells ($p<0.05$, according to a Student's *t* test). In all the cases (**a** through **d**), data are means ± SD of, at least, three different, independent experiments (each of them with duplicated determinations)

incubation conditions: a 4 h treatment of BAEC with 5 mM homocysteine. Although 5 mM homocysteine is a concentration far higher than those found under hyperhomocysteinemia (15–150 μM) and even hyperhomocysteinuria (up to 0.5 mM), this concentration is very frequently used in acute, short incubations of endothelial cells to simulate endothelial cell responses to homocysteine under conditions of hyperhomocysteinemia (Medina et al. 2001). Many times, this is unavoidable because endothelial cells in culture upregulate the metabolism of homocysteine considerably. In fact, similar high concentrations of homocysteine have been previously used in recent relevant studies involving cultured endothelial cells (Bessede et al. 2001; Rodríguez-Nieto et al. 2002; Zhang et al. 2001). On the other hand, a remarkable functional genomics study has previously revealed that a 4 h treatment with 5 mM homocysteine is enough to induce relevant regulatory responses in endothelial cells (Outinen et al. 1999).

Figure 1b shows that a 4 h treatment of BAEC with 5 mM homocysteine induces a extremely potent inducing

effect on ferricyanide reductase activity, which increases to levels fivefold those exhibited by control, untreated cells. This means that homocysteine is one of the most potent inducers of PMRS ever described. In fact, this stimulatory effect of homocysteine is as high as that previously reported for ascorbate and dehydroascorbate pretreatment of J774 macrophages (Baoutina et al. 2001). This effect was not significantly modified by increasing the treatment time up to 24 h (results not shown).

Figure 1c shows that the effect of homocysteine pretreatment on BAEC ferricyanide reductase activity is dose-dependent, exhibiting significant activating effects from 0.5 mM homocysteine on.

Since the presence of serum during homocysteine treatment of BAEC could decrease the actual effective concentration of homocysteine in the medium, we also studied the dose response in the absence of FBS. Figure 1d shows that, indeed, the stimulatory effect of homocysteine under these conditions is greater than that observed in the presence of FBS for all the tested homocysteine concentrations. It should be underscored that in the absence of FBS even as low homocysteine concentrations as 50 μM-corresponding to moderate to medium hyperhomocysteinemia (Medina et al. 2001)-induce significant increases of BAEC ferricyanide reductase activity.

Homocysteine stimulates ferricyanide reductase activity of tumor cells

To test whether this potent inducing effect of homocysteine on ferricyanide reductase activity was endothelial cell specific or not, we carried out those experiments described in Fig. 1c also using human Hep G2 hepatocellular carcinoma and U2-OS osteosarcoma cells. Figure 2 shows that, indeed, also human tumor cell ferricyanide reductase activity is potently stimulated by homocysteine treatment. These results rule out the possibility of an endothelial specific response and points to a putative protective, antitumoral effect of homocysteine, since increased ferricyanide reductase activity is found in less proliferant and less malignant tumor cells (del Valle et al. 1998).

Homocysteine induces the protein expression levels of a cytochrome b reductase component of PMRS

The availability of a specific antiserum against a cytochrome b5 reductase component of PMRS made it possible for us to test whether the stimulatory effect of homocysteine on ferricyanide reductase activity was also observable as an inducing effect on protein levels of PMRS. In our hands, when RIPA + CHAPS buffer is used to make cell extracts this cytochrome b5 reductase is revealed in a Western blot as a main band at an apparent 27 kDa and a minor band at the expected 34 kDa height corresponding to its reported size; when Laemli buffer is used, the most prominent band is that of an apparent 34 kDa (Fig. 3a). Figure 3b shows that in BAEC both bands are clearly increased after 24 h of treatment with 5 mM homocysteine. On the other hand, for U2-OS cells a 4 h treatment with

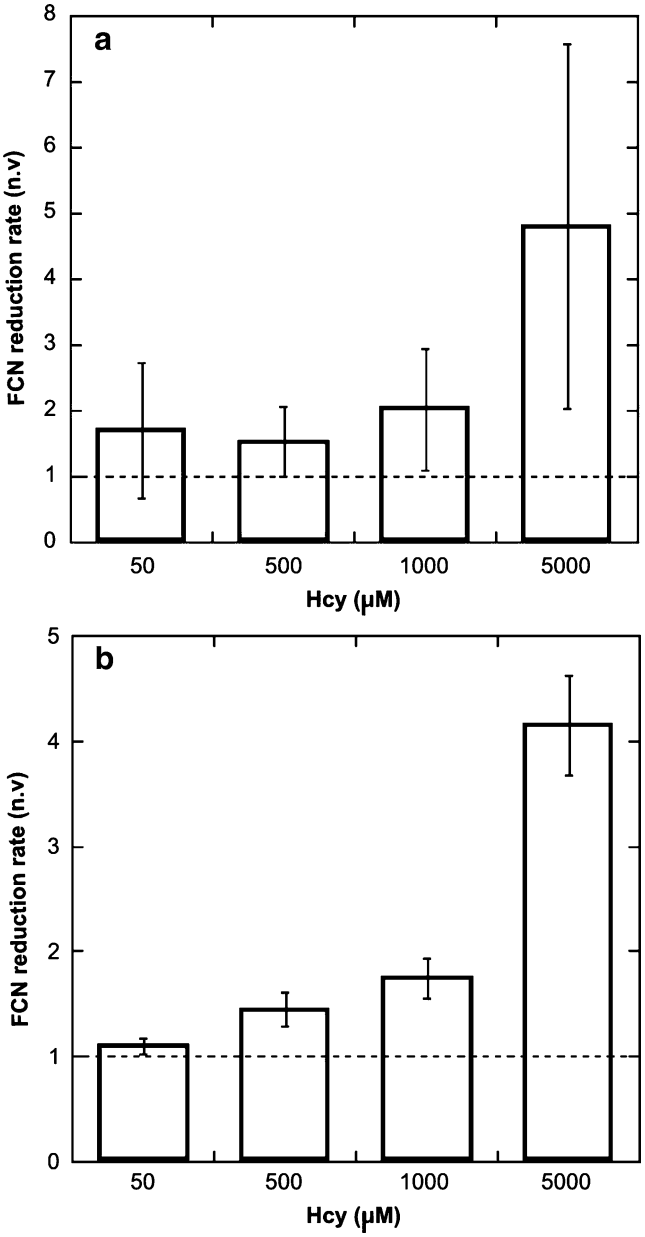

Fig. 2 Dose-response of the stimulatory effect of homocysteine pretreatment for 4 h (in the presence of serum) on Hep G2 hepatocellular carcinoma (**a**) and U2-OS human osteosarcama cell (**b**) ferricyanide reductase activity. Data are represented as normalized values (*n.v.*), taking the values of control, untreated cells as 1 (*dash line*). Ferricyanide reduction rates at 0.5 (only in the case of U2-OS cells), 1 and 5 mM homocysteine are significantly greater than that exhibited by control, untreated cells ($p<0.05$, according to a Student's *t* test)

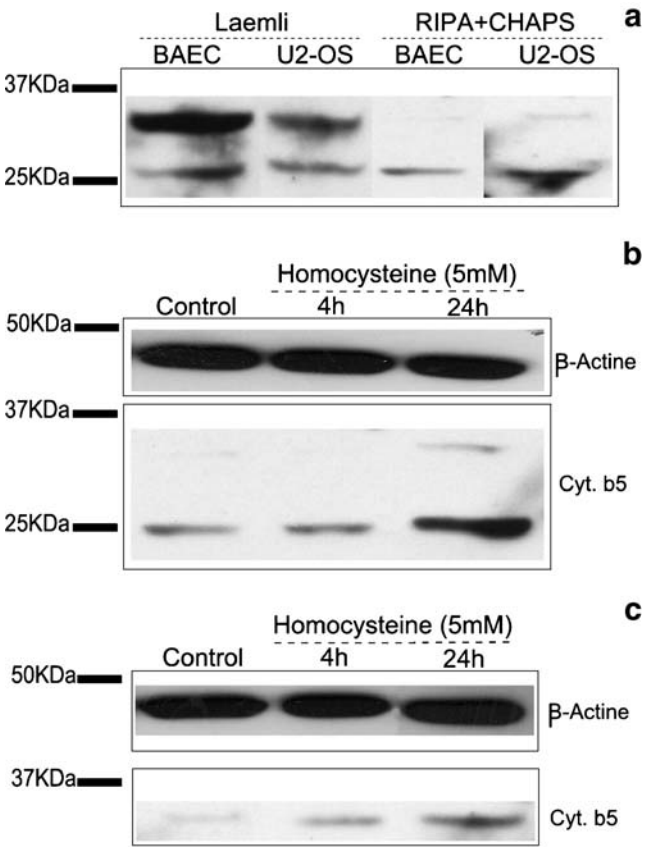

Fig. 3 Inducing effect of 5 mM homocysteine on the protein levels of cytochrome b5 reductase, a component of PMRS. **a** Revealed bands in control extracts by using Laemli or RIPA + CHAPS extracting buffers. **b** Inducing effect observed in BAEC. **c** Inducing effect observed in U2-OS cells. Iv B αvδ C, β-actin is used as an internal loading control

5 mM homocysteine is enough to observe a great increase in the signal corresponding to the 34 kDa band (Fig. 3c).

Homocysteine decreases tumoral-NADH oxidase activity

Morré's group has identified an ectocellular NADH oxidase as an external component of PMRS with specific features in tumor cells (Morré 2004; Morré and Morré 2003; Yagiz et al. 2007; Yantiri and Morré 2001). Furthermore, this tNOX has been shown to be a molecular target for anticancer compounds such as capsaicin, epigallocatechin gallate and phenoxodiol (Chueh et al. 2004; Morré et al. 2007). In contrast to the very potent inducing effects of homocysteine on ferricyanide reductase activity and cytochrome b5 reductase protein levels, we have observed that the specific ectocellular tNOX activity of human Hep G2 hepatocellular carcinoma and U2-OS osteosarcoma cells is strongly inhibited by homocysteine treatment. In fact, Hep G2 cells treated for 4 h with 5 mM homocysteine reduced its tNOX activity a 51±23% of that exhibited by control, untreated cells (means ± SD of four independent experiments). On the other hand, homocysteine treatment inhibited U2-OS cells tNOX activity by 87±15% (means ± SD of 6 determinations in two independent experiments).

The role of homocysteine in cancer is controversial. Some studies point to increased levels of plasma homocysteine as markers for cancer (Chou et al. 2007; Wang et al. 2007), but some others find no usefulness of plasma homocysteine levels as cancer biomarker (Hwang et al. 2005; Ozkan et al. 2007; Schernhammer et al. 2007). Our results on tNOX activity add new insights to the potential antitumoral role of homocysteine.

Concluding remarks

We have previously shown that homocysteine is an inhibitor of angiogenesis and has antitumoral and anti-invasive effects (Chavarría et al. 2003a, b; Rodríguez-Nieto et al. 2002). Based on the redox reactivity of homocysteine, we postulated the hypothesis that this compound should modulate the activity of endothelial cell PMRS. In the present communication, we show that our hypothesis has been fully validated. Furthermore, we demonstrate that homocysteine is, indeed, a very potent inducer of endothelial cell PMRS. Some year ago, a study of macrophage PMRS detected that pretreatment with the antioxidants ascorbate and dehydroascorbate stimulated that activity at similar levels to those observed in our treatments of BAEC with homocysteine (Baoutina et al. 2001). In that previous study, the authors concluded that PMRS could be largely responsible for macrophage-mediated LDL oxidation, a key process in atherosclerosis. Is the extremely potent stimulatory effect of homocysteine on ferricyanide reductase activity a cause or a consequence of its claimed oxidative stress effects? On the other hand, does this stimulated PMRS activity exert any protective role against endothelial cell oxidative stress? These relevant questions related to the pathophysiological roles of homocysteine warrant future studies in this direction.

We have also shown a dual behaviour of homocysteine treatment on tumoral PMRS. On the one hand, homocysteine behaves as a potent inducer of tumoral ferricyanide reductase activity and the cytochrome b5 protein expression. On the other hand, homocysteine inhibits the activity of the ectocellular tNOX, a target of antitumor drugs. Taken together, these inducing effects on the protective antioxidant PMRS and inhibiting effect on the specific antitumoral drug target tNOX underscore a potential antitumoral role of homocysteine. The pharmacological relevance of these findings deserve to be evaluated.

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