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ORIGINAL CONTRIBUTION

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No evidence for prooxidative effects of homocysteine in vascular endothelial cells

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Abstract Many epidemiological studies predict a role for homocysteine (HCys) in cardiovascular disease occurrence, progression, and risk factors. In vitro studies demonstrated that HCys is an atherogenic determinant that promotes oxidant stress, inflammation, endothelial dysfunction and cell proliferation. This study originally attempted to examine the mechanism by which exposure of endothelial cells to HCys (0–250 μM) initiates inflammatory reaction and oxidative stress, by (i) investigating whether physiological and pathophysiological concentrations of HCys exhibit a prooxidative activity in vitro, (ii) examining the interaction of monocyte adhesion (Mono Mac 6) to monolayers of human microvascular endothelial cells (HMEC-1) exposed to different HCys concentrations, and (iii) examining if adherent monocytes increase reactive oxygen species either in endothelial cells or in monocytes themselves. However, our results demonstrate that HCys had neither prooxidative nor cytotoxic effects on endothelial cells. Only a moderate time- and concentration-dependent increase in monocyte adhesion up to 28.3 ± 5.5% was achieved relative to control after 4 h of HCys stimulation. This effect was accompanied by an increased VCAM and ICAM-1 mRNA expression. This "proinflammatory" effect appeared also when HMEC-1 cells were incubated with cysteine or glutathione at the concentration range 0–250 μM, demonstrating a non-specific rather than a specific HCys effect. In addition, adherent monocytes did not increase ROS formation neither in endothelial cells nor in monocytes themselves, indicating no direct or indirect cytotoxic or prooxidative effects of HCys.

Key words monocyte adhesion – homocysteine – reactive oxygen species – cell adhesion molecules

Introduction

A moderate increase in blood homocysteine (HCys) levels has been proposed to be an independent risk factor for myocardial infarction, stroke, and peripheral vascular disease [6, 32, 33]. However, the pathophysiological basis for increased risk of atherosclerotic diseases in homocyst(e)inemia is uncertain although there is evidence that suggests HCys may have direct cytotoxic effects that could result in endothelial damage and dysfunction [2, 11, 25, 26, 28]. However, it is not clear why excess HCys is harmful although many in vitro studies hypothesize that HCys may predispose to vascular disease by altering the normal antithrombotic

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and vasoprotective phenotype of vascular endothelium, perhaps by a mechanism involving generation of peroxides and other reactive oxygen species [4, 8, 15, 21, 24]. Oxidative stress is strongly associated with the pathophysiology of cardiovascular disease (CVD) [9, 22]. As a consequence of endothelial damage and dysfunction, an increased adherence of monocytes, neutrophils and lymphocytes to endothelial cells may occur, which is considered as one of the initial factors leading to the development of atherosclerosis in the long term. Monocytes play a key regulatory step in the progression of atherosclerosis by the building of foam cells, which accumulate and form plaques in arterial walls. The effect of HCys on monocyte–endothelial cell interaction is still largely unknown. This study evaluated whether the physiologically or pathophysiologically relevant concentrations of HCys increase adhesion of endothelial cells and result in enhanced monocyte binding. In addition, the ability of HCys to induce oxidative stress and cell damage in endothelial cells and HCys induced monocyte–endothelial interaction on the formation of ROS in endothelial cells or in monocytes was assessed.

Materials and Methods

Cell culture and HCys treatment

The human microvascular endothelial cell line (HMEC-1) [1] which shows features of small- and large-vessel endothelial cells [34] was cultured as described in detail by Ades et al. [1]. Homocysteine is easily oxidized to homocystine and can form mixed disulfides with other thiols. The formation of homocystine and mixed disulfides occurs not only in vivo but also in vitro so that the indicated concentrations of HCys (0–250 μM) refer to the total HCys (oxidized plus reduced) although the reduced form of HCys was added to the culture medium.

Analysis of cytotoxicity by the evaluation of plasma membrane damage

The entry of the fluorescent dye ethidium homodimer-1 (EthD-1) (Molecular Probes, UK) into endothelial cells was used as a marker of membrane damage and cell viability. HMEC-1 cells were grown to confluence in a Falcon 48 multiwell tissue culture plate (Becton Dickinson, Heidelberg, Germany) and stimulated with doses of HCys ranging 0–250 μM. One hour prior to measurement, 100 μM EthD-1 was added to each well. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids. EthD-1 is excluded by the intact plasma membrane of living cells. Sham-treated cells with the addition of 100 μM EthD-1 served as a negative control (baseline). Cells treated 1 h prior to measurement with 0.08% digitonin served as a positive control of the test system and used as the reference for 100% membrane damage. The fluorescence (ex.: 485 ± 20 nm; em.: 645 ± 40 nm) in the wells was measured at various time points (0–72 h) using a fluorescence multiwell reader (BIO-TEK FL600, Vermont, USA). The percentage of cells with membrane damage was calculated from the fluorescence readings as: % membrane damage = $(F_{\text{HCys treated cells}} - F_{\text{digitonin-treated cells}}) \div (F_{\text{digitonin-treated cells}} - F_{\text{sham-treated cells}})$.

Biochemical assessment of lipid peroxidation (TBARS-assay)

The analysis of 2-thiobarbituric acid reactive substances (TBARS) as a marker of lipid peroxidation was carried out as described previously [7, 12].

Measurement of intracellular formation of reactive oxygen species (ROS) in living cells

To detect HCys-induced intracellular reactive oxygen species (ROS) in living endothelial cells, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, bis (acetoxy-methyl) ester (C-H_2DCF-DA/AM) was used as a probe according to the method described by Royall et al. [23]. HMEC-1 cells were grown to confluence in 24-well tissue culture plates, and stained for 30 min in complete medium with 5 μM C-H_2DCF-DA/AM. After four washes in HBSS (Hanks balanced salt solution)/20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), cells were exposed to various HCys concentrations (0–250 μM). At various time points fluorescence (ex.: 485 ± 20 nm; em.: 530 ± 25 nm) of the cells in HBSS/20 mM HEPES-buffer was measured using a fluorescence multiwell reader (BIO-TEK FL600). Fluorescence of untreated cells served as a negative control and was equalized to 100% (baseline), whereas cells treated with 200 μM H_2O_2 in combination with 10 μM Fe(III)/100 μM ADP served as a positive control for the test system.

Assay for monocytic cell adhesion to endothelial cells

HMEC-1 cells were grown to confluence in 48-well tissue culture plates. After 3 days of confluence, the cells were stimulated with various concentrations of HCys, cysteine and glutathione (0–250 μM).

Mono Mac 6 cells [35] originally derived from a human acute monocytic leukemia, were grown in suspension culture in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, non-essential amino acids, 1mM sodium pyruvate and 9 μg/ml bovine insulin at 37°C in a 5% CO_2 atmosphere. Prior to the adhesion assay the Mono Mac 6 cells were labeled with the fluorescent dye Calcein-AM (0.5 μM) for 60 min at 37°C (Molecular Probes). Stained cells were washed twice with medium and centrifuged at 300*g* for 5 min. 2.5×10^5 viable cells suspended in 500 μl medium were added to each well of endothelial cells after removal of the incubation medium. The binding of Mono Mac 6 to HMEC-1 cells was performed at 37°C for 2 h. Unbound cells were removed by replacing medium. Non-specific cell adhesion was prevented by washing the cells at 420 rpm for 3 min using a horizontal shaker at room temperature. After replacing the medium, fluorescence (ex.: 485 ± 20, em.: 530 ± 25) of the wells was measured. HMEC-1 cells treated overnight with 20 ng/ml TNF-α (4,000 U/ml) served as a positive control for the test system. TNF-treated cells incubated with unstained Mono Mac 6 cells served as a blank and corresponding fluorescence values were subtracted.

RT-PCR for ICAM-1, VCAM and E-selectin

Changes in the expression of cell adhesion molecules due to HCys were measured by RT-PCR. For quantifying specific PCR-products, *β*-actin was co-amplified and served as an internal control. At the indicated time points and HCys concentrations, mRNA was isolated from confluent HMEC-1 cells, reverse transcribed and subjected to PCR analysis for VCAM, E-selectin and ICAM-1. Specific primers for VCAM were: 5′-CTGAG CGGGAAGGTGAGGAGT-3′ and 5′-ATCTCTGGGGGC AACATTGACA-3′, E-selectin: 5′-AATGTGTGGGTCT GGGTAGGAA-3′ and 5′-TGCAGGATGATTTGAAGGT GAAC-3′, ICAM-1: 5′-CGTGCCGCACTGAACTGGAC-3′ and 5′-CCTCA-CACTTCACTGT CACCT-3′, *β*-actin: 5′-CACGAAACTACC-TTCAACTCCA-3′ and 5′-ACTC GTCATACTCCTGCTTGCT-3′. For PCR amplification a 50 μl reaction contained 2 μl of ss cDNA solution, 3 units of Vent DNA polymerase (Biolabs, Schwalbach, Germany), 1 mM of each of the four nucleotide triphosphates, and the appropriate amount of *β*-actin and adhesion primers. After denaturing at 94.5°C for 5 min the thermocycling was carried out at 94.5°C for 1 min, 62°C for VCAM or 60°C for E-selectin and ICAM for 2 min and 72°C for 3 min. Typically, 35 cycles were employed, followed by a final extension step at 72°C for 10 min. For analysis of the PCR products, the samples (5 μl) were mixed with 1 μl of DNA-buffer (20% ficoll, 0.25% bromphenol blue, 0.1 M EDTA pH 8.0, 1%SDS) and electrophorezed at 60 V for 1.5 h in 1% agarose gel (SeaKem GTG; Biozym, Freiburg, Germany). DNA bands were visualized after ethidium bromide staining under long-wavelength ultraviolet light and quantified by densitometric scanning of the gel with the Herolab E.A.S.Y enhanced analysis system (Herolab, Wiesloch, Germany). To correct for differences during PCR, integrated optical densities were normalized to the co-amplified ß-actin band. Results of densitometric analysis are given as means ± SD of three independent experiments.

Assay for ROS formation after monocytic cell adhesion to endothelial cells

HMEC-1 were grown to confluence in 48 multiwell tissue culture plates. Homocysteine (0–250 μM/l) stimulation was carried out in phenol-red free growth medium for 4 h at 37°C and 5% CO_2. Each assay was performed in quadruplicate. Prior to the assay, Mono Mac 6 were washed once with HBSS (Hanks balanced salt solution) and adjusted to a concentration of 1×10^6 cells/ml in HBSS. After HCys incubation, medium was removed and 5×10^5 Mono Mac 6 cells/well were added. Incubating cells with 0.66 μg/μl zymosan (Sigma, Deisenhofen, Germany) served as a positive control of the test system. It is used to induce experimental inflammation and monocyte activation. Thereafter, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, bis (acetoxy-methyl) ester (C-H_2DCF-DA/AM) was added to the final concentration of 10 μM to detect the formation of reactive oxygen species. At various time points fluorescence (ex.: 485 ± 20 nm; em.: 530 ± 25 nm) of the cells was measured using a fluorescence multiwell reader (BIO-TEK FL600). Fluorescence of untreated cells served as a negative control and was equalized to 100% (baseline).

Statistical analysis

Results are expressed as means ± SEM unless stated otherwise. Differences between groups were assessed by the two-tailed Wilcoxon test for unpaired samples. The significance level was set at $\alpha = 5\%$ for all comparisons.

Results

Formation of reactive oxygen species (ROS) by HCys

To examine the hypothesis that HCys per se contributes to atherogenesis and endothelial dysfunction

by a mechanism that involves the generation of ROS [3], various concentrations of HCys were examined for their ability to induce the production of endogenous ROS and subsequent lipid peroxidation in endothelial cells. The formation of ROS was monitored by the fluorescent dye 2′,7′-dichlorodihydrofluorescein (DCF) which can be used as an indicator of increased reactive oxygen species formation or generally as a marker of oxidative stress in living cells [14, 23]. Lipid peroxidation was determined by measuring the levels of TBARS. As shown in Fig. 1, HCys did not induce the formation of ROS (Fig. 1A) or TBARS (Fig. 1B) in endothelial cells. In addition, no considerable increase of damaged cells was found within the first 24 h of HCys stimulation (0–250 μM) (Fig. 1C). In contrast, we observed a protective effect at higher concentrations of HCys. Cytotoxic effects of HCys were measured up to 72 h with no positive result (data not shown). The assumption that HCys may have prooxidative effects or may have direct cytotoxic properties could not be confirmed.

Effect of HCys on monocytic cell adhesion

We did not detect any direct cytotoxic effects of HCys on confluent layers of HMEC-1 cells. Therefore, the possibility that HCys modifies the adhesive properties of endothelium [5, 19] was investigated. Pre-incubation of HMEC-1 with HCys (0–250 μM) resulted in an increased adhesion of Mono Mac 6 cells to endothelial cells (Fig. 2A). The effect of HCys was time and dose dependent. Maximal binding of up to 28.3 ± 5.5% was achieved relative to control values (0% baseline) after 4 h. The highest values of adhesion were seen over a HCys concentration range of 16–64 μM and declined after 24 h. The effect on cell adhesion could no longer be observed in cells stimulated for 72 h with HCys. This effect was found to be nonspecific since other thiol compounds like cysteine and glutathione did mimic homocysteine activity (Fig. 2B, C). Increased monocyte adhesion to endothelial cells may therefore be a "non-specific" rather than a specific HCys effect. Endothelial cells treated for 16 h with TNF-α served as a positive control for the test system and increased the adhesion of Mono Mac 6 to HMEC-1 cells by an average of 126.5 ± 27.9%.

Because the adhesion of neutrophils to endothelium can trigger the release of ROS [5], we investigated whether monocytic cell adhesion changes the oxidative metabolism in endothelial cells using the DCF-assay. However, the adhesion of monocytes to HCys-stimulated HMEC-1 cells (0–250 μM) did not increase the formation of ROS within endothelial cells. Moreover, monocytes adherent to HCys-treated endothelial cells were not able to produce sufficient endogenous ROS to give a positive signal in the DCF-assay (Table 1).

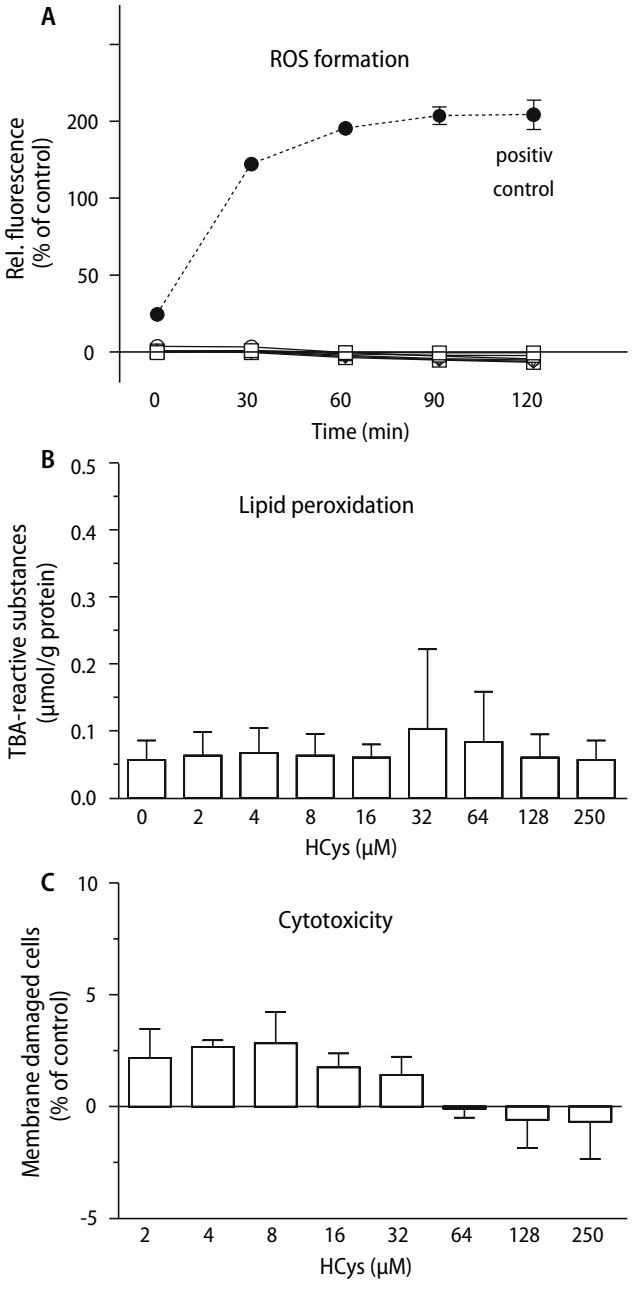

Fig. 1 (**A**) Effect of different concentrations of HCys (0–250 μM) on the formation of reactive oxygen species in living endothelial cells. Cells treated with 200 μM H_2O_2/10 μM Fe(III)/100 μM ADP (•) served as a positive control of the test system. Data are means ± SEM of three independent experiments ($n = 3$). 8 μM HCys (□); 16 μM HCys (Δ), 32 μM HCys (▽); 64 μM HCys (◇); 128 μM HCys (○); 250 μM HCys (◁). (**B**) Effect of HCys (0–250 μM) on the formation of TBARS (marker of lipid peroxidation) 4 h after treatment. Data are means ± SD of three independent experiments ($n = 3$). (**C**) Effect of HCys (0–250 μM) on the induction of plasma membrane damage. The percentage of damaged cells after an incubation time of 24 h HCys is shown. Data are means ± SEM of three independent experiments ($n = 3$)

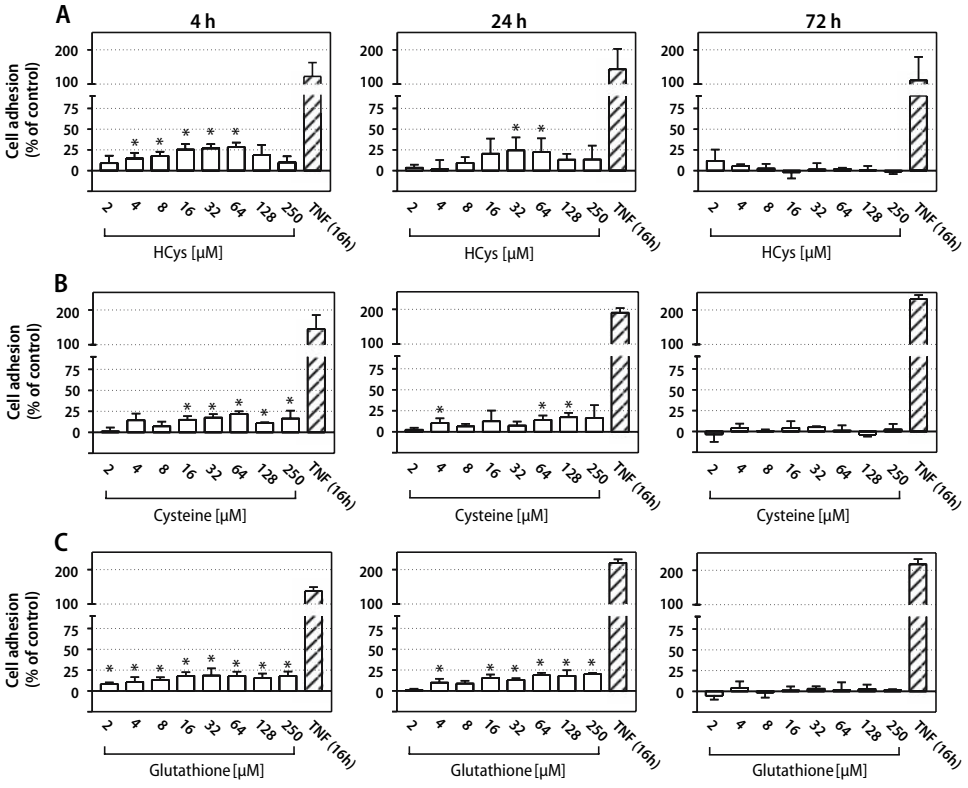

Fig. 2 Effect of different amounts of HCys (**A**), cysteine (**B**), and glutathione (**C**) on monocyte adhesion to endothelial cells. Results are given as means ± SEM of three independent experiments. All variables were normalized to control measurements and expressed as relative changes to controls. Endothelial cells treated for 16 h with 20 ng/ml TNF-α (4,000 U/ml) served as a positive control of the test system. *$P < 0.05$

Table 1 Effect of monocyte–endothelial cell interaction on ROS formation

HCys (µM)	0 (control)	2	4	8	16	32	64	128	250	Zymosan
ROS-formation (10 min)	0	2.3 ± 2.2	−4.6 ± 1.1	−8.8 ± 3.5	−6.0 ± 1.7	−7.5 ± 1.1	−12.9 ± 2.7	−7.4 ± 2.9	−12.8 ± 3.4	78.2 ± 13.6
ROS-formation (30 min)	0	−0.8 ± 3.3	4.9 ± 1.1	3.2 ± 5.6	3.6 ± 1.4	4.7 ± 4.6	6.2 ± 3.5	4.8 ± 5.0	6.0 ± 2.0	91.6 ± 34.2
ROS-formation (60 min)	0	−6.8 ± 2.8	−2.2 ± 1.4	−3.6 ± 3.6	0.8 ± 3.4	0.5 ± 4.8	−0.8 ± 2.9	0.5 ± 4.2	−3.8 ± 2.7	130.6 ± 70.9

At different time points, the formation of ROS in endothelial and monocytic cells was measured by the DCF-assay. Fluorescence of untreated cells (control) served as a negative control and was equalized to 100% (baseline). Changes in fluorescence are indicated as % of control. Cells incubated with 0.66 µg/µl zymosan served as a positive control of the test system. Zymosan is prepared from yeast cell wall and consists of protein carbohydrate complexes. It is able to induce experimental inflammation and monocyte activation and should therefore result in an increased ROS-formation. Data are means ± SEM of four independent experiments (n=4)

Table 2 Effect of HCys (4 h) on VCAM, E-selectin, and ICAM-1 mRNA expression

HCys (µM)	0 (control)	2	4	8	16	32	64	128	250	TNF-α
VCAM	0	15.4 ± 25.1	30.5 ± 48.1	13.5 ± 18.5	28.4 ± 42.5	17.9 ± 34.4	30.6 ± 43.4	29.9 ± 34.2	−1.3 ± 18.4	242.3 ± 91.0
E-selectin	0	−11.8 ± 12.0	4.3 ± 17.6	3.6 ± 13.0	−14.3 ± 17.1	4.2 ± 9.4	−6.5 ± 8.5	−35.5 ± 24.3	−26.5 ± 19.4	707.9 ± 180.3
ICAM-1	0	8.3 ± 11.4	21.4 ± 25.9	27.1 ± 22.5	−5.6 ± 22.6	28.2 ± 25.9	28.1 ± 25.9	18.2 ± 33.8	26.7 ± 33.4	129.1 ± 41.5

mRNA expression of untreated cells (control) was equalized 100% (baseline). Changes in expression are indicated as % of control. Endothelial cells treated for 4 h with 20 ng/ml TNF-α (4,000 U/ml) served as a positive control of the test system. Values are means ± SEM of three independent experiments (n = 3)

Effect of HCys on VCAM, E-selectin and ICAM-1 mRNA expression

To elucidate the reason for the increased adhesion of monocytes to HCys treated HMEC-1 cells, expression of endothelial adhesion molecules was analyzed by semi-quantitative RT-PCR. The incubation of HMEC-1 with HCys (0–250 µM) resulted in a small increase of VCAM and ICAM-1 mRNA expression 4 h after HCys treatment (Table 2). After 72 h, the expression of VCAM and ICAM-1mRNA returned to the base line or untreated control values (data not shown). Our

findings indicate that HCys is not able to promote oxidant stress, inflammation, or endothelial dysfunction in our cell system.

Discussion

McCully [16, 17] proposed the "homocysteine theory of arteriosclerosis" in 1969 on the basis of pathological examinations of autopsy material from children with homocysteinuria. The basis for increased risk of atherogenesis in hyperhomocysteinemia is uncertain. Several mechanisms that may contribute to atherogenesis have been proposed over the last years. Until now, it is not clear why excess HCys is harmful.

Results from a large number of in vitro investigations have implied an adverse role for HCys in different cell types. It has been frequently documented that HCys impair endothelial cell function, oxidize low-density lipids, increase monocyte and leukocyte adhesion, activate the inflammatory pathway, and stimulate smooth-muscle cell proliferation possibly by a mechanism involving the generation of reactive oxygen and nitrogen species [13, 30, 31]. Often, extremely high HCys concentrations, about 100-fold of the levels found in the population at risk had been used and may therefore indicate a lack of specificity. Normal total plasma HCys (combined pool of free, bound, reduced, and oxidized forms of HCys) range between 5 μM and 15 μM with elevations of 16–30 μM, 31–100 μM, and >100 μM classified as mild, moderate, and severe hyperhomocysteinemia [20]. Our in vitro test system uses physiologically and pathophysiologically relevant HCys concentrations. We could not confirm the above mentioned observations that HCys may act as an oxidant or may have direct cytotoxic effects (see Fig. 1). Our results contradict with the most data published to date in respect of HCys and disagree with the universally accepted hypothesis that many effects of HCys in the vasculature may be caused by increased oxidant stress.

Atherosclerosis is viewed as an inflammatory process of the vessel wall that initiates and promotes lesion development. This process involves circulating leucocytes, particularly monocytes, that are recruited to and adhere to the activated endothelium, and that migrate into the subendothelial space where they differentiate into macrophages. HCys has been shown to induce mRNA and protein expression of the proinflammatory cytokines monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) in cultured human aortic endothelial cells [18]. This cascade of events may trigger the recruitment of monocytes to endothelial cells.

We therefore investigated the adhesion of monocytic cells to cultured endothelial cells following HCys treatment. Even at low concentrations (<10 μM) HCys induced the adhesion of monocytes (Mono Mac 6) to human microvascular endothelial cells (HMEC-1). An increased expression of VCAM and ICAM-1 mRNA was observed in endothelial cells after HCys treatment. HCys has been shown to induce the expression of endothelial adhesion molecules like ICAM-1, VCAM, P-selectin and E-selectin in vivo [10, 29]. However, in our in vitro study, similar results were obtained when cysteine and glutathione were used instead of HCys, indicating a "non-specific" rather than a specific HCys effect. Furthermore, Dudman et al. [5] found that following the attachment of neutrophils to endothelial cells, endothelial cell damage appeared which may be induced by reactive oxygen species (ROS) generated by adherent neutrophils and/or endothelial cells. A similar observation was made in 1993 in a rat model, in which enhanced neutrophil adhesion induced temporal changes in the oxidative metabolism of vascular endothelium [27]. In contrast to the above mentioned literature, our findings demonstrate that Mono Mac 6 cells, which adhered to HCys treated endothelial cells, were not able to trigger the release of ROS within these cells. Even under these circumstances, a prooxidative or cytotoxic role of HCys could not be established.

In conclusion, elevated levels of HCys may be a risk factor for CVDs but until now it is not clear why excess HCys may be harmful. Our in vitro results provide evidence against a major effect of HCys on vascular dysfunction due to ROS formation. We could not approve the universally accepted hypothesis that most known forms of damage or injury are due to HCys-mediated oxidative stress. The observed effects of HCys were rather non-specific because other low molecular weight "thiols" like cysteine or glutathione have the similar effects in our in vitro modell. This does not rule out other possibilities how HCys may mediate his deleterious effects.

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