Homocysteine Altered ROS Generation and NO Accumulation in Endothelial Cells

Chih-Mei Tsen[1], Chien-Cheng Hsieh[2], Chia-Hung Yen[1] and Ying-Tung Lau[1]

[1]*Department of Physiology and Pharmacology*
Chang Gung University College of Medicine
Taoyuan, Taiwan, R.O.C.
[2]*Department of Food and Nutrition*
Chinese Culture University
Taipei, Taiwan, R.O.C.

Abstract

Mild hyperhomocysteinemia (HHcy) is a risk factor for vascular disease and is closely associated with endothelial dysfunction. Oxidative stress and decreased nitric oxide (NO) bioavailability were reported in HHcy-induced vascular injury; however, the exact relationship is not understood. We thus directly determine the production of reactive oxygen species (ROS) and NO in cultured endothelial cells (HUVECs) to demonstrate the correlated variation between ROS and NO induced by Hcy (homocysteine), Cys (cysteine), another thiol compound, and Met (methionine), precursor of HHcy in animal study. HUVECs were treated with Hcy, Cys, or Met for 0.5 or 22-24 h; ROS generation was detected by DCF fluorescence with flow cytometry and NO by chemiluminescence. In non-cytotoxic (<1.0 mM) concentration ranges, Met exerted no effects on either ROS production or NO concentration, Cys decreased ROS production and increased NO in both short-term (0.5 h) and long-term (22-24 h) treatments; Hcy, however, induced a biphasic effect on ROS production, i.e., inhibitory at 0.5 h but stimulatory at 24 h. The maximal stimulation by Hcy (0.25 mM) was significantly reduced by co-incubation (12 h) with estrogen (1 μM). Hcy caused an early (0.5 h) increase of medium NO which was absent in long-term Hcy treatment. The oxidative stress caused by long-term Hcy incubation could be ameliorated by estrogen, consistent with earlier *in vivo* observations that estrogen prevents HHcy-induced injury.

Key Words: cysteine (cys), homocysteine (Hcy), nitric oxide (NO), reactive oxygen species (ROS)

Introduction

Hyperhomocysteinemia (HHcy) is an independent risk factor for premature atherosclerosis and thrombosis (1,2). Evidence indicate that endothelial injury induced by elevated levels of homocyst(e)ine (Hcy) occurs in both HHcy animal models (7, 16) and cultured endothelial cells (17). The underlying mechanism responsible for the cytotoxic effects on endothelial cells by Hcy is not clear and may involve the reactivity of the sulfhydryl group of Hcy (1) that eventually lead to an imbalance in local nitric oxide (NO) as well as between pro- and anti-oxidative forces (28). Stamler *et al*. (25) found that while brief exposure (15 min) of endothelial cells to Hcy stimulates the formation of S-NO-homocysteine (S-NO-Hcy), a compound possessing vasoactive properties similar to NO; prolonged (>3 h) exposure to Hcy, however, leads to a failure to sustain S-NO-Hcy formation and a decreased bioavailability of NO. Furthermore, recent findings indicate that Hcy induces production of superoxide anion in permeabilized porcine aortic endothelial cells which may account for most of the observed inhibition of endothelium-dependent relaxation in rabbit aorta (14). Evidence also suggests that Hcy exerts important effects on cellular NO production including the activation of endothelial NO synthase (29) and

Corresponding author: Dr. Ying-Tung Lau, Department of Physiology and Pharmacology, Chang Gung University College of Medicine, 259 Wen Hwa 1Rd., Kwei-Shan, Tao-Yuan, Taiwan, R.O.C. TEL: 011-886-3-2118800 ext. 5095, Fax: 011-886-3-2118700, E-mail: ytlau@mail.cgu.edu.tw
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enhancement of inducible NO synthase (11, 30) under stimulation. The endothelial production of reactive oxygen species (ROS) and NO, including the balance between them, are thus likely the candidate for the observed complex vascular effects of Hcy.

We and others have previously shown that under oxidative stress such as in the presence of oxidized low-density lipoprotein (oxLDL) exposure (32), HUVECs are stimulated to generate ROS. Therefore, one approach to test whether Hcy causes biphasic oxidative stress as suggested by Stamler et al. (25) is to investigate whether Hcy can alter cellular ROS production in a time-dependent manner, and whether if NO varies in a correlated manner. With the successful application of 2',7'-dichlorofluorescin diacetate (DCFH-DA) in the detection of intracellular ROS production in endothelial cells (3,32), it has become possible to directly evaluate the effect of Hcy on ROS production and the NO status in HUVECs. It is not known whether other thiol, such as cysteine, would have similar effects. We thus also tested the effects of cysteine and methionine (precursor of Hcy) on ROS production and NO accumulation following two different treatment durations (0.5 h or 22-24 h).

Furthermore, estrogen has been demonstrated to reduce the stimulated ROS production in HUVECs (32) and that methionine-loaded female rats exhibits an improved endothelium-dependent relaxation (33), we therefore also investigated whether estrogen could prevent the putative Hcy-induced ROS production.

Materials and Methods

Isolation and Culture of Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were harvested as described previously (5, 32) and were grown in MCDB 107 medium supplemented with 10% fetal calf serum (FCS), endothelial cell growth factor (ECGF, 40 μg/ml), heparin (100 μg/ml) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml). Near-confluent cell monolayer (2-5 passage) in 75t-flask was used.

MTT Test

HUVECs (6×10^4 cell/well) were subcultured onto 24 well plates and maintained in 2% FCS phenol red-free MCDB 107. The cells then underwent treatments of Hcy, Cys, or Met for 24 h (37°C). Cell viability was evaluated by assessing mitochondrial dehydrogenase activity as previously reported (10, 22) and the O.D. value of untreated HUVECs (control) was taken as 1.0.

Determination of Reactive Oxygen Species (ROS) Generation

The intracellular ROS measurement of HUVECs was performed by flow cytometry as described previously (3, 32) with some modifications. DCFH-DA is a lipid permeable nonfluorescent compound and is oxidized by intracellular ROS to form the lipid impermeable and fluorescent compound DCF (24). Organic tert-butylhydroperoxide (TBH), a model of a hydroperoxide compound, was also used (15, 32) in addition to Hcy to serve as a positive control for ROS generation in activated HUVECs. For experiments, HUVECs were pre-incubated in phenol red-free MCDB107 medium (to avoid the interference of phenol red) containing 2% FCS, ECGF, and heparin with or without amino acid (Hcy, Met, or Cys) range from 0.05 to 5 mM for 0.5 h or 22-24 h.

In the last 30 min-period, DCFH-DA was added (final concentration, 1 μM) with or without amino acid (Hcy, Met, or Cys) to HUVECs and cells were trypsinized (0.05% trypsin) and then resuspended in phosphate buffer (1 ml) to a final concentration of 2×10^5 cells/ml. ROS generation of these cells were determined by flow cytometry (FACscan, Becton-Dickinson, CA) using 488 nm for excitation and 525 nm for emission.

For the preventative study, the cells were preincubated with 17β-estrodiol (E_2, 1 μM) together with Hcy (0.25 mM) for 0.5, 12 or 22-24 h. Intracellular ROS was then analyzed as described before.

Determination of Nitric Oxide (NO)

NO release from cultured monolayers of HUVECs, similar to other cells/tissues, could induce signaling and responses (27, 31). It was determined by first converting the nitrite and nitrate, the stable product of NO in aqueous media, into NO using vanadium (III) chloride in HCl at 90°C. NO is then detected by a gas-phase chemiluminescent reaction between NO and ozone with NOA 280i (Sievers, Boulder, CO, USA). Samples were first deproteined by adding 2 volume of ethanol and aliquot (10 μl) were used with the calibration curve (10 nM-500 nM, correlation coefficient better than 0.997) constructed each time. Results were calculated with NO Analysis software (version 3.2). Medium samples without cells were taken as blank and their NO values were subtracted from cell culture media samples.

Statistical Analysis

Differences among the groups were analyzed by using one-way analysis of variance combined with

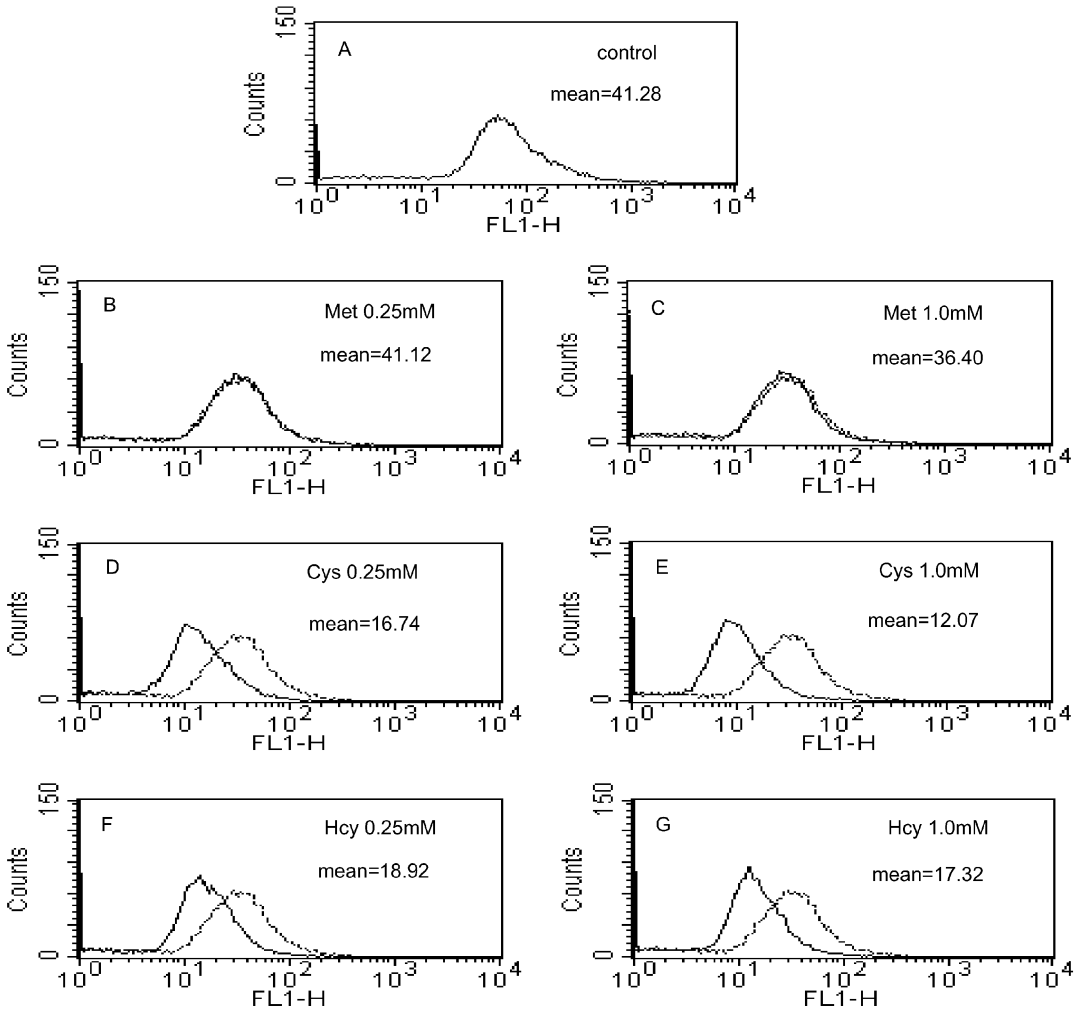

Fig. 1 ROS production in HUVECs for 0.5 h-treatment of amino acids. Control (dye only, panel A), Met (0.25, 1.0 mM)-treated cells (panel B and C, bold curve), Cys (0.25, 1.0 mM)-treated cells (panel, D and E, bold curves), and Hcy (0.25, 1.0 mM) treated cells (panel F and G, bold curves). At the end of the incubation period (0. 5 h), ROS production was determined by flow cytometry. FL1-H: the fluorescence intensity of DCF.

the Bonferroni test and Student's *t*-test. Values were as mean ± SEM (n=number of observations). Differences between groups were considered to be significant at $P<0.05$.

Results

Cell viability was examined after 24 h-incubation with Hcy, Met and Cys over a concentration range from 0.05 to 5.0 mM. There was no significant cell death up to 1.0 mM for any of these amino acids. However, at a concentration of 5.0 mM, Hcy was cytotoxic and viability was reduced to 0.73 ± 0.03 (P <0.01 compared with control); Met and Cys did not significantly affect the viability of HUVECs (data not shown). We therefore examined both the short-term (or acute, 0.5 h) and long-term (or chronic, 22-24 h) effects of these amino acids on basal ROS production by determining DCF fluorescence of HUVECs in a non-cytotoxic range (≤ 1.0 mM). A typical observation of an acute study was shown in Fig. 1. Under basal conditions, addition of DCF-DA to HUVECs exhibited a (control) response (Panel A), Met (0.25 or 1.0 mM) did not affect this response at all (Panel B and C). Cys (0.25 or 1.0 mM) significantly reduced the fluorescence (left shift, Panel D and E). Hcy (0.25 or 1.0 mM) also caused similar reduction (Panel F and G). The results of 3-5 acute experiments were summarized in Fig. 2. Met exerted no effects while Cys and Hcy inhibited basal ROS production of HUVECs significantly after 0.5 h incubation.

Chronic effects of amino acids on ROS production were similar to acute effects for Met and Cys (Fig. 3, Panel A-E), but different for Hcy; i.e., at the concentration of 0.25 mM (Panel F), there was an increase of ROS production (right shift) while at the concentration of 1.0 mM (Panel G), no significant change occurred. In 4-6 chronic experiments, Met

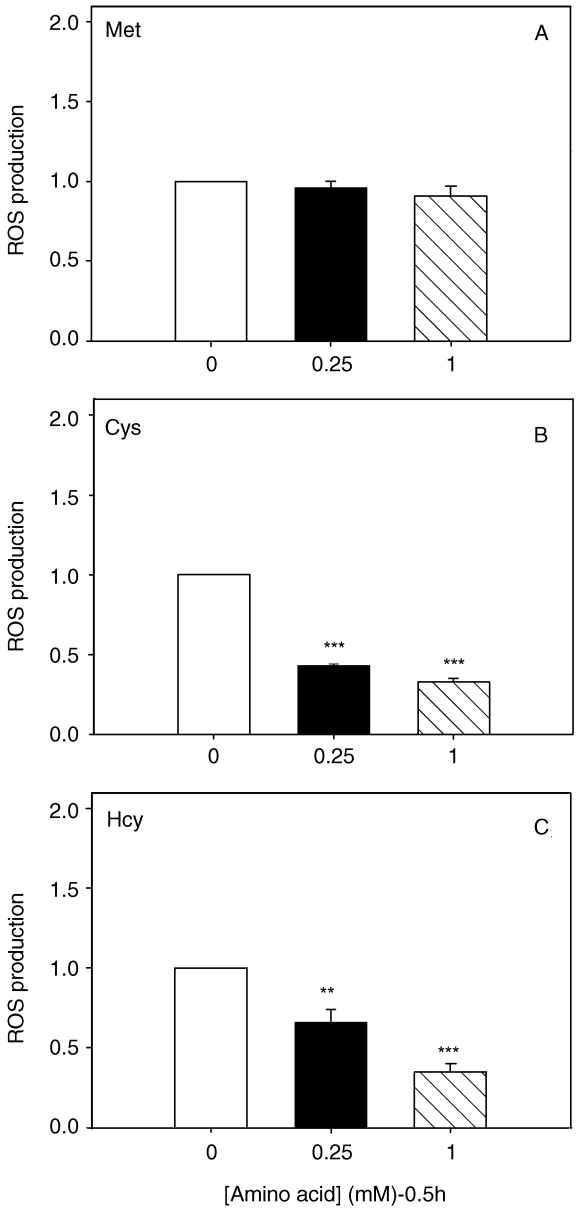

Fig. 2 Summary of short-term (0.5 h) effects of amino acids on ROS production in HUVECs. Cells were exposed to Met (A), Cys (B) or Hcy (C) at concentrations of 0.25 and 1.0 mM. *P<0.05; **P <0.01; ***P<0.001 between control (open column) and treated.

(0.25 or 1.0 mM) did not affect ROS production (Fig. 4A); Cys significantly reduced ROS production in HUVECs (Fig. 4B), similar to that of acute studies (Fig. 2B). Hcy, however, enhanced ROS production in HUVECs and that significant increase was found at concentration of 0.1 and 0.25 mM (Fig. 4C). These findings were consistent with previous report (29) where superoxide anion production (determined by lucigenin chemiluminesence) was found to be increased by Hcy in permeabilized porcine aortic endothelial cells. Because 0.25 mM Hcy appeared to stimulate ROS production the strongest (near 2-fold, Fig. 4C), we further tested the pro-oxidative effect at this concentration in the absence or presence of estrogen (E_2). We have previously shown that the oxidized low-density lipoprotein (oxLDL)-induced ROS production in HUVECs is ameliorated by 1 μM E_2 (32).

Results of 3 experiments where Hcy (0.25 mM) with or without E_2 (1 μM) were added for 0.5 h, 12 h, or 24 h are shown in Fig. 5. Similar to Fig. 2C, Hcy (0.5 h) inhibited the ROS generation significantly and E_2 alone or with Hcy exerted no additional effects (Fig. 5A). However, for 12 h-treatment (Fig. 5B), Hcy caused a significant increase in ROS production; co-incubation of E_2 with Hcy (solid column) reduced the stimulated ROS production to a level below the untreated HUVECs (open column). Similarly, E_2 reduced the Hcy-induced increase of ROS production to a level not different (18% lower) to that of control after treatment for 22-24 h (Fig. 5C).

We have also determined the NO concentrations ([NO]) of the culture media incubating the HUVECs used in some of the experiments. To test the sensitivity of the system for the detection of potential interaction of ROS and NO, we employed known ROS generating agent TBH and antioxidant NAC (N-Acetyl-L-cysteine). In control media, [NO] was determined to be 0.49 ± 0.10 μM (n=12), TBH (0.1 mM) treatment (30 min) decreased [NO], albeit not significantly (0.32 ± 0.07 μM). NAC (1 and 5 mM) caused significant increase of [NO] to 0.89 ± 0.09 μM and 1.20 ± 0.13 μM, respectively. The system to detect [NO] was thus able to respond to altered oxidative status of the media. In 2-3 experiments, the values of [NO] of incubation media in the presence of various amino acids were also determined (Fig. 6). For short-term incubation, Met exerted no effect on [NO] whereas Cys and Hcy (both 0.25 and 1.0 mM) caused significant enhancement (upper panel). For long-term incubation, only Cys (both 0.25 and 1.0 mM) stimulated [NO] (lower panel). Because activation of iNOS by cytokines in HUVECs take a long time and that Hcy alone does not induce either mRNA or protein level of iNOS in smooth muscle cell cultures (11), the NO detected in our culture media was unlikely derived from iNOS.

Discussion

Our major findings are that Hcy (0.25 or 1.0 mM) could induce a biphasic effect on the production of ROS in HUVECs: inhibitory for acute (0.5 h) treatment (Fig. 2C) similar to that of Cys (Fig. 2B), but stimulatory for chronic (22-24 h) treatment (Fig. 4C), opposite to that of Cys (Fig. 4B). Methionine (Met), an amino acid often used to induce hyperhomocysteinemia and thus accumulates *in vivo*

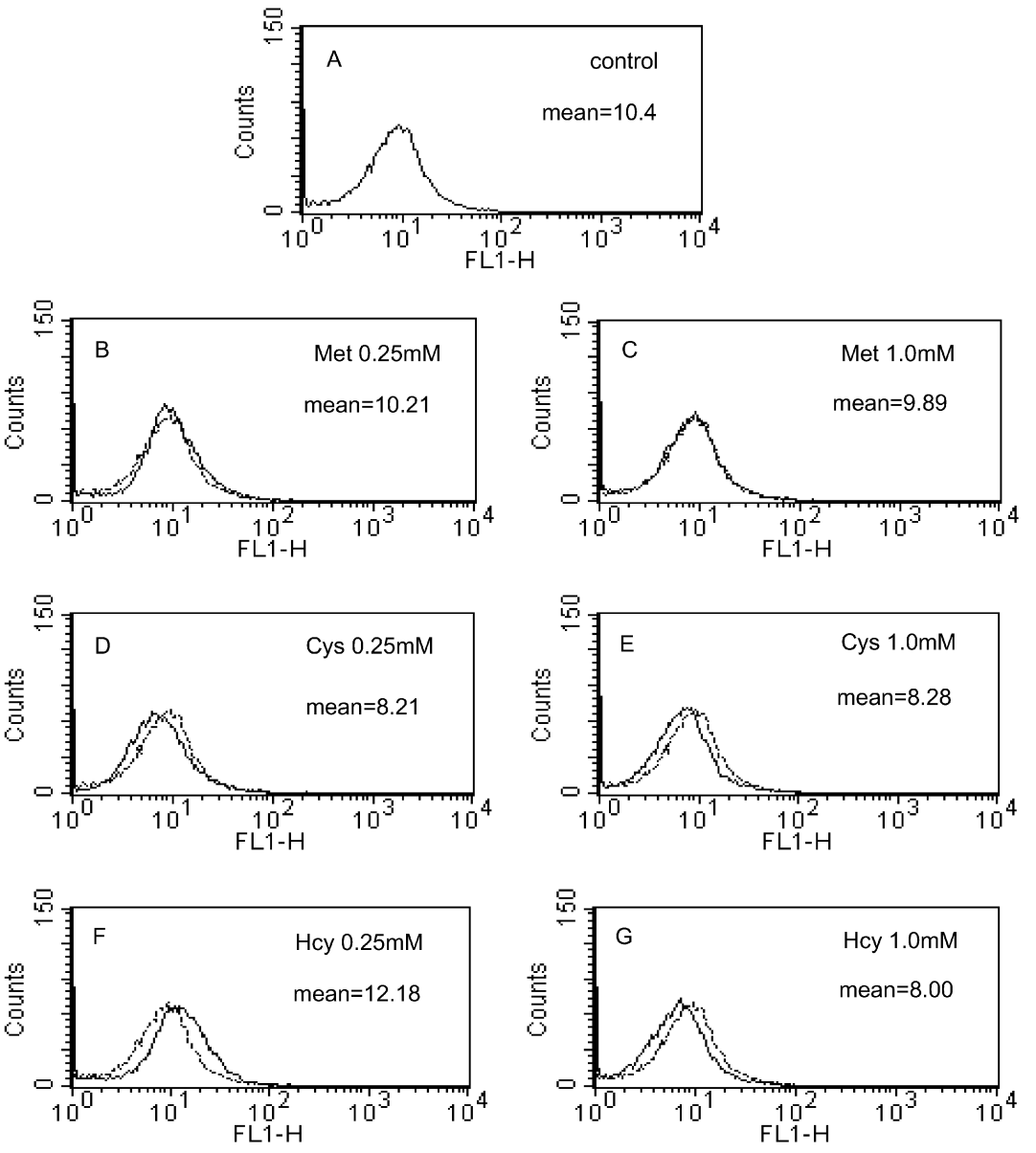

Fig. 3 ROS production in HUVECS for 22-24 h-treatment of amino acids. Control (dye only, panel A), Met (0.25, 1.0 mM)-treated cells (panel B and C, bold curves), Cys (0.25, 1.0 mM)-treated cells (panel D and E, bold curves), Hcy (0.25, 1.0 mM)-treated cell (panel F and G, bold curves). At the end of the incubation period (22-24 h), ROS production was determined by flow cytometry. FL1-H: the fluorescence intensity of DCF.

(33), exerted no effect on ROS production with either acute or chronic incubation (Fig. 2A and Fig. 4A). The stimulatory effect of Hcy on ROS production was detected at 12 h (Fig. 5B) but not 3 h (data not shown), and was eliminated by co-incubation with E_2 (Fig. 5B and 5C). NO concentrations of culture media for HUVECs were also determined and significant enhancements were observed following acute Hcy treatment, but not after 24 h (Fig. 6). Taken together, our results demonstrated that at a concentration effective to cause *in vivo* endothelial dysfunction (14), Hcy induced a biphasic effects on ROS generation and NO accumulation: an early (0.5 h) inhibitory effect on ROS coincided with an increase of media [NO]; whereas the late (24 h) stimulatory effect on ROS was absent of increase of media [NO]. Furthermore, the Hcy-induced stimulation of ROS generation could be reduced by E_2 (Fig. 5). These findings are consistent with the view that Hcy induces endothelial dysfunction accompanied by increased ROS production; and when inhibited, normal function restores (18).

Homocysteine (Hcy) is known to kill endothelial cells at millimolar concentration in the presence of copper or ceruloplasmin (micromolar range), however, homocystine or copper alone is not toxic to these cells

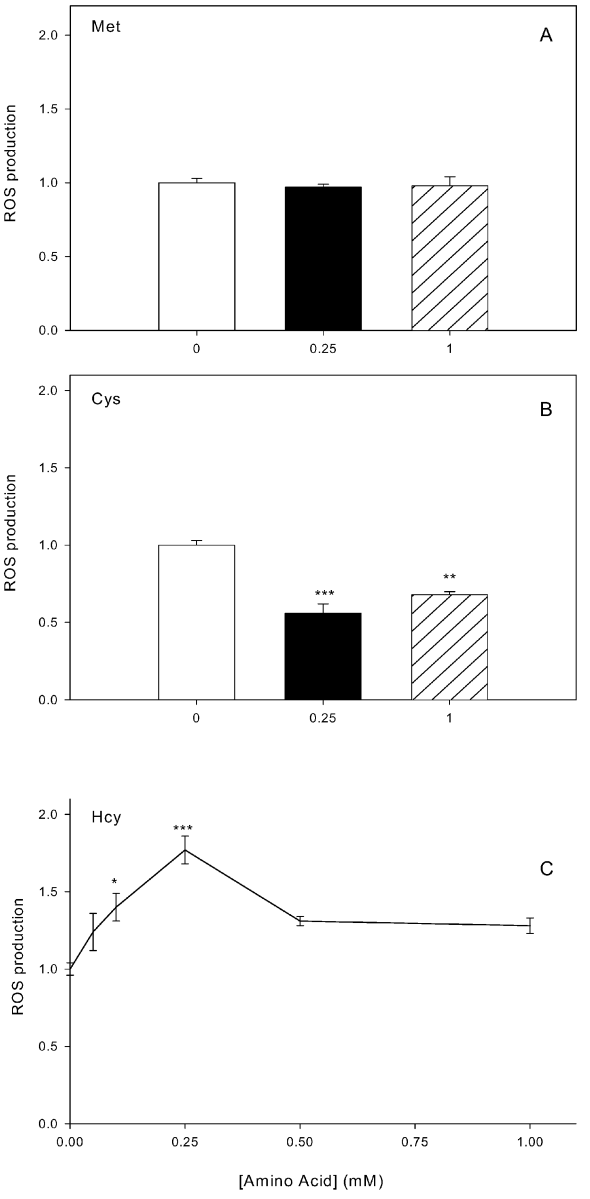

Fig. 4 Summary of long-term (22-24 h) effects of amino acids on ROS production in HUVECs. Cells were exposed to Met (A), Cys (B), or Hcy (C) at concentrations of 0.25 and 1.0 mM. *P<0.05; **P <0.01; *** P<0.001 between control (open column) and treated.

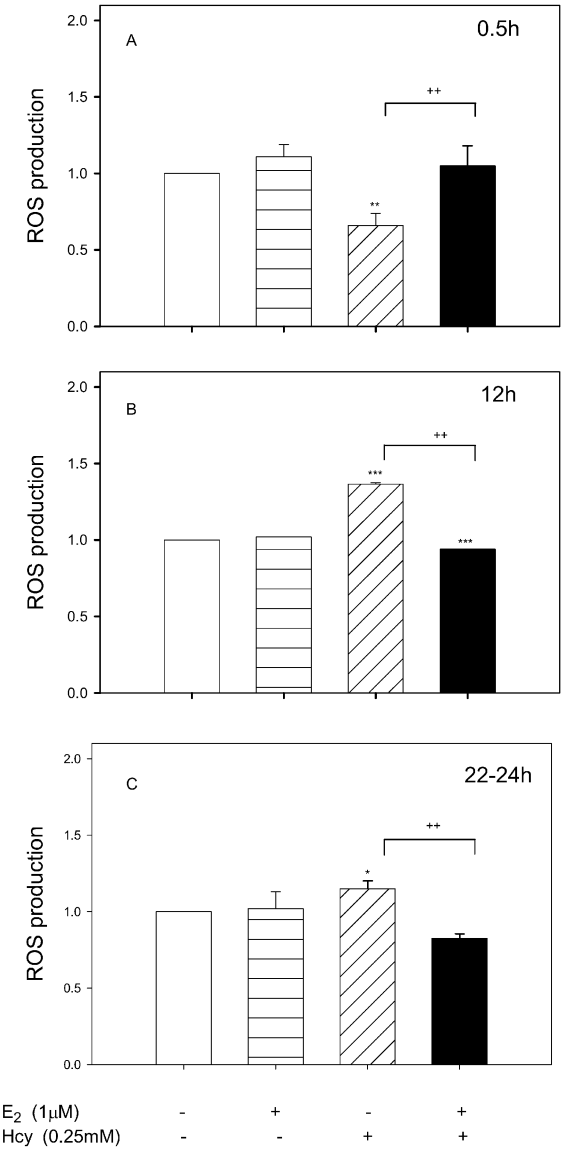

Fig. 5 Summary of the effects of E_2 on Hcy-induced ROS production for different durations in HUVECs. A. 0.5 h, B. 12 h, C. 24 h. *P <0.05; **P<0.01; ***P<0.001 between control (open column) and treated.

(26). Our finding that 5 mM Hcy induced significant cytotoxicity in the absence of copper is consistent with earlier reports (12). ROS (H_2O_2 or superoxide anion) formation by way of oxidation of Hcy is thought to be the major cause for cytotoxicity (17), however, oxidation product such as homocysteine thiolactone may directly injure endothelial cells (19). Considering the critical role of endothelium-derived relaxing factor (EDRF) in vascular regulation, NO or closely related S-nitrosothiol including S-nitroso-homocysteine (S-NO-Hcy) are found to contribute significantly in modulating the adverse effect of Hcy on vascular functions (25). Hcy reacts with NO or closely related compounds to form S-nitrosothiol, which possesses EDRF-like vasodilatory properties, but this cannot be sustained with continuous presence of Hcy and its potential oxidative influence (25). Moreover, in the presence of bradykinin (an agonist for endothelial NO production), Hcy increases S-nitrosothiol production in a time-and dose-dependent manner that occurs concomitantly with increased eNOS activity and mRNA level (29). This potentially protective effect of Hcy is not shared by cysteine or glutathione (29).

Although thiol-containing substances are considered as antioxidant (20), double-edge effects

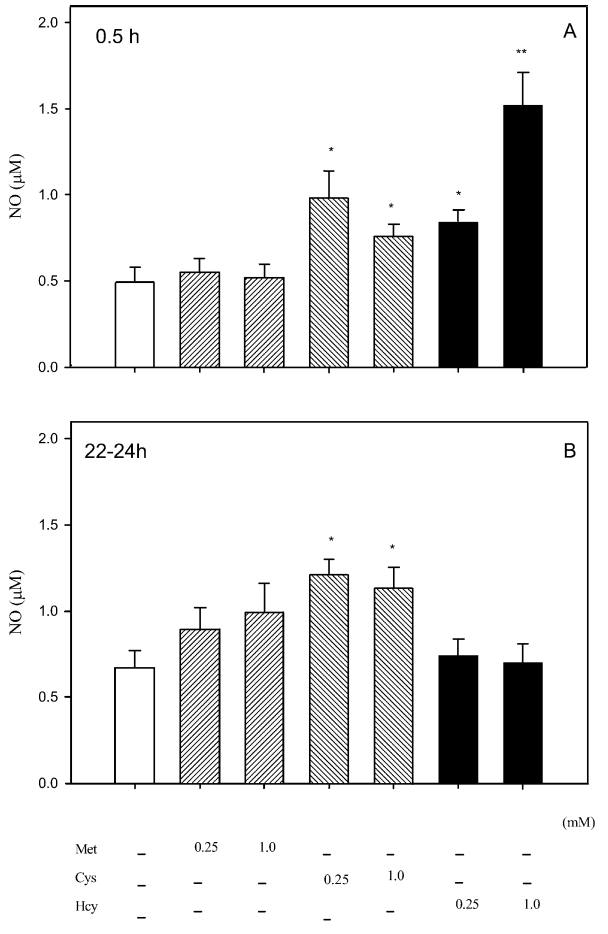

Fig. 6. Summary of the effects of amino acids on NO concentration of the incubation media. Upper panel: acute treatment (0.5 h) of Met, Cys, and Hcy as indicated, Lower panel: chronic treatment (22-24 h). *P<0.05; **P<0.01; ***P<0.001, respectively; when compared with control (open column).

are also reported (8, 23). We found that Cys (up to 1 mM) reduced ROS production in HUVECs in both acute (Fig. 2B) and chronic (Fig. 4B) treatments supporting the notion that Cys may serve as a precursor for cellular glutathione (21). Met was of no effect either in acute (Fig. 2A) or in chronic (Fig. 4A) treatments on ROS generation. Thus, Hcy, not Met is responsible for the associated endothelial dysfunction in Met-treated adults (13).

The view that Hcy may limit NO bioavailability in endothelial cells *via* increased ROS is further supported by randomized crossover study where flow-mediated vasodilatation (endothelium-dependent) of the brachial artery decreased after 4 h post-methionine loading even when Met level was already lowered (1). Moreover, vitamin C ameliorated the endothelial dysfunction after Met loading (4, 13), suggesting that oxidative stress causes the adverse effects on endothelial function. Interestingly, we recently reported that endothelial-dependent relaxation and endothelium suppression were enhanced in female spontaneously hypertensive rats with mild hyperhomocysteinemia (*via* Met loading) and that E_2 could mimic these gender-dependent effects (33). In our present study, E_2 abolished the Hcy-induced ROS generation of HUVECs and thus could exert protection against Hcy-induced endothelial dysfunction *via* an antioxidative mechanism (32). Recent findings also have shown that estrogen exerts an antioxidant effect *in vivo* by reducing superoxide anion bioavailability, further support this theme (6).

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