Homocysteine Increases Nitric Oxide Synthesis in Cytokine-Stimulated Vascular Smooth Muscle Cells

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Background—Elevated plasma homocysteine levels have been reported to be an independent risk factor for vascular disease. However, there have been no reports concerning the effects of homocysteine on the production of nitric oxide (NO), another modulator of vascular function and proliferation, by the vascular smooth muscle.

Methods and Results—We investigated the effects of homocysteine on NO synthesis by measuring the production of nitrite, a stable metabolite of NO, in cultured rat vascular smooth muscle cells (VSMCs). Incubation of cultures with interleukin (IL)-1β 10 ng/mL for 24 hours caused a significant increase in nitrite generation. The IL-1β–induced nitrite production by VSMCs was significantly increased by homocysteine in a dose-dependent manner. This effect of homocysteine was significantly inhibited in the presence of N\textsubscript{G}-monomethyl-L-arginine or actinomycin D. The homocysteine-induced nitrite production was accompanied by increased inducible NO synthase mRNA and protein accumulation. Cysteine, glutathione, or hydrogen peroxide also increased nitrite accumulation in IL-1β–stimulated VSMCs. Coincubation with the radical scavenger catalase or superoxide dismutase markedly reduced homocysteine-induced nitrite accumulation.

Conclusions—Homocysteine enhances NO synthesis in IL-1β–stimulated VSMCs, and oxidative products are involved in the effect of homocysteine. (Circulation. 1999;99:1230-1235.)

Key Words: homocysteine ■ interleukins ■ nitric oxide ■ muscle, smooth ■ free radicals
was obtained from Transduction Laboratory. Homocysteine, cysteine, glutathione, and hydrogen peroxide were purchased from Nacalai Tesque Inc. N°4-Monomethyl-L-arginine (L-NMMA) and actinomycin D were from Sigma Chemical Co. Superoxide dismutase and catalase were from Seikagaku Kogyo. All other chemicals used were of the highest grade commercially available.

**Cell Culture**

Primary cultures of VSMCs were obtained from the media of thoracic aortas of Sprague-Dawley rats (200 to 250 g), as described previously. The cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were harvested twice a week by treatment with 0.125% trypsin and passaged at a 1:3 ratio in 100-mm culture dishes. A typical experiment was performed with cultured cells at passage levels 5 to 10. Cells (3×10⁶/mL) were plated in 24-well or 100-mm culture dishes in DMEM, supplemented as described above, and allowed to grow to subconfluence for 24 to 48 hours, after which they were preincubated in DMEM containing 0.5% FCS and supplemented with insulin (5 μg/mL) and transferrin (5 μg/mL) for 24 hours and used for the experiments described below.

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1985).

**Measurements of Nitrite**

NO production by the cultured cells was determined by measurement of the nitrite contents of the culture medium. VSMCs plated in 24-well dishes were incubated in DMEM containing 0.5% FCS at 37°C. The nitrite contents of culture media were determined by the modification of the method, and 30-μg aliquots were subjected to electrophoresis on 1% agarose gels. After electrophoretic separation, RNA was transferred onto nitrocellulose membranes, and the resultant blots were incubated with the anti-iNOS antibody as described previously. The separated proteins were electrophoretically transferred onto nitrocellulose membranes, and the resultant blots were incubated with the anti-iNOS antibody for 2 hours followed by horseradish peroxidase-labeled donkey anti-rabbit IgG for 1 hour. Peroxidase-labeled proteins were detected with the ECL detection system (Amersham) on x-ray film; the results were quantified by densitometric scanning.

**Statistical Analysis**

Data are expressed as mean±SEM of 4 samples, which represented ≥3 separate experiments. Differences were analyzed by 1-way ANOVA combined with Scheffé’s test, and a value of P<0.05 was considered to be statistically significant.

**Results**

**Effects of Homocysteine on Nitrite Production**

First, we investigated the effects of IL-1β on nitrite production by VSMCs. As shown in Figure 1, addition of IL-1β 10 ng/mL stimulated nitrite production by VSMCs in a time-dependent manner. This IL-1β-stimulated nitrite accumulation was significantly increased by simultaneous treatment of the cells with homocysteine 1 mmol/L. After a 24-hour incubation, the level of IL-1β-stimulated nitrite accumulation in the presence of homocysteine was 1.5- to 2-fold that in the absence of homocysteine.

The effect of IL-1β on nitrite production by VSMCs was dose-dependent (Figure 2). Addition of homocysteine 1 mmol/L to the culture further enhanced IL-1β-induced nitrite production.

![Figure 1](image1.png)

**Figure 1.** Time course of nitrite accumulation stimulated by IL-1β in presence or absence of homocysteine in VSMCs. Cells were unstimulated (●) or stimulated with 10 ng/mL IL-1β in presence (○) or absence (△) of 1 mmol/L homocysteine for various periods as indicated. Nitrite accumulation in culture medium was measured as described in Methods, and values were normalized to protein content per well. Points and bars represent mean±SEM (n=4). *P<0.05 vs IL-1β-stimulated cells.

![Figure 2](image2.png)

**Figure 2.** Dose-dependent effects of IL-1β on nitrite production by VSMCs. Cells were exposed to various concentrations of IL-1β (0.1 to 100 ng/mL) for 24 hours with (solid columns) or without (hatched columns) homocysteine (1 mmol/L). Nitrite accumulation in culture medium was measured, and values were normalized to protein content per well. Data represent mean±SEM (n=4). *P<0.05.
Figure 3. Dose-dependent effects of homocysteine on nitrite accumulation. VSMCs were incubated for 24 hours with (solid columns) or without (hatched columns) 10 ng/mL IL-1β in presence of various concentrations of homocysteine (10⁻⁶ to 10⁻³ mol/L) as indicated. Nitrite accumulation in culture medium was measured, and values were normalized to protein content per well. Data represent mean±SEM (n=4). *P<0.05 vs control cells without homocysteine.

Figure 3 shows the dose-response relationship of the effect of homocysteine on NO synthesis. Homocysteine increased IL-1β–stimulated nitrite production by VSMCs in a dose-dependent manner. Homocysteine by itself did not affect the basal level of nitrite production.

As shown in Figure 4, in the presence of the NOS inhibitor L-NMMA 1 mmol/L or the RNA synthesis inhibitor actinomycin D 5 μg/mL, the effects of homocysteine and IL-1β were completely abolished.

Effects of Homocysteine on iNOS mRNA and Protein Levels

We then examined whether homocysteine induced an increase in iNOS mRNA accumulation in VSMCs. As shown in Figure 5, unstimulated cells did not express iNOS mRNA. Incubation with IL-1β for 24 hours resulted in an induction of iNOS mRNA expression, and its expression was further increased in the presence of homocysteine at >0.1 mmol/L.

The expression of iNOS protein by homocysteine was also analyzed by immunoblotting with the anti-iNOS antibody (Figure 6). No immunoreactive band of iNOS was detected in unstimulated VSMCs. The iNOS protein band with a molecular mass of ~125 kDa appeared clearly after exposure to homocysteine.
IL-1β for 24 hours, and its accumulation was further increased in the presence of homocysteine.

**Involvement of Oxidants in the Action of Homocysteine**

We investigated the mechanism of the stimulatory effect of homocysteine on nitrite production. Oxygen-derived molecules such as superoxide and hydrogen peroxide generated by way of the reactive sulfhydryl (SH) group are thought to account for the endothelial cytotoxicity of homocysteine.28,29 We thus investigated the involvement of these molecules in homocysteine-induced nitrite production. First we investigated the effect of other molecules containing the SH group, cysteine, and glutathione. As shown in Figure 7, both cysteine and glutathione also enhanced nitrite production by IL-1β–stimulated VSMCs. Furthermore, the effect of homocysteine was not maintained in the presence of glutathione (Figure 8).

We next examined the effect of hydrogen peroxide on nitrite production. Addition of hydrogen peroxide dose-dependently (0.01 to 0.1 mmol/L) increased nitrite production by IL-1β–stimulated cells, whereas it did not affect the basal level of nitrite production (Figure 9). We then investigated whether radical scavengers could inhibit the effect of homocysteine. As shown in Figure 10, addition of the radical scavenger catalase 1000 U/mL or superoxide dismutase 1000 U/mL markedly reduced homocysteine-induced nitrite accumulation.

**Discussion**

Despite considerable epidemiological evidence identifying homocysteine as a risk factor for arteriosclerosis, the molecular mechanisms underlying homocysteine-induced arteriosclerosis have not been fully elucidated. Injury of vascular endothelial cells has been implicated in the pathogenesis of arteriosclerosis, and previous studies on homocysteine-induced atherosclerosis and thrombosis have focused on endothelial cells.18,30–32 However, Tsai et al22,33 showed that homocysteine increased DNA synthesis in cultured VSMCs, suggesting that homocysteine also acts directly on the vascular smooth muscle. In this study, we investigated whether homocysteine modulated NO synthesis in VSMCs. Although

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**Figure 7.** Effects of cysteine and glutathione on nitrite accumulation. VSMCs were incubated for 24 hours with (solid columns) or without (hatched columns) 10 ng/mL IL-1β in presence of 1 mmol/L cysteine (CYS) or 1 mmol/L glutathione (GSH). Nitrite accumulation in culture medium was measured, and values were normalized to protein content per well. Data represent mean±SEM (n=4). *P<0.05.

**Figure 8.** Effects of homocysteine on nitrite accumulation in presence of glutathione. Cells were incubated with 10 ng/mL IL-1β for 24 hours in presence of 1 mmol/L homocysteine (HCY) and/or 5 mmol/L glutathione (GSH). Nitrite accumulation in culture medium was measured, and values were normalized to protein content per well. Data represent mean±SEM (n=4). *P<0.05.

**Figure 9.** Effects of hydrogen peroxide on nitrite accumulation. VSMCs were incubated for 24 hours with (solid columns) or without (hatched columns) 10 ng/mL IL-1β in presence of hydrogen peroxide (0.01 to 0.1 mmol/L). Nitrite accumulation in culture medium was measured, and values were normalized to protein content per well. Data represent mean±SEM (n=4). *P<0.05 vs control cells without hydrogen peroxide.

**Figure 10.** Effects of radical scavengers on effect of homocysteine. Cells were incubated for 24 hours with 10 ng/mL IL-1β, 1000 U/mL catalase (CAT), and/or 1000 U/mL superoxide dismutase (SOD) in presence (solid columns) or absence (hatched columns) of 1 mmol/L homocysteine. Nitrite accumulation in culture medium was measured, and values were normalized to protein content per well. (−) indicates control cells. Data represent mean±SEM (n=4). *P<0.05.
Homocysteine by itself showed no effect on NO production, it augmented IL-1β-induced NO production in a dose-dependent manner. The stimulatory effect of homocysteine was abolished when actinomycin D was present, suggesting that the augmentation of nitrite production by homocysteine is due to induction of iNOS. Indeed, we observed that homocysteine increased IL-1β-induced iNOS mRNA and protein accumulation in VSMCs. The concentrations of homocysteine used did not exhibit any cytotoxic effects on VSMCs, judging from 51Cr release from the cells (data not shown).

Damage to the vascular endothelium by homocysteine is believed to be secondary to oxidative product formation accompanying oxidation of the SH group of the amino acid.

We previously reported that the radical scavenger catalase blocked the effect of homocysteine on endothelial glycaminoglycan metabolism.

We thus investigated the involvement of oxygen-derived molecules in homocysteine-induced NO production by VSMCs and found that hydrogen peroxide as well as cysteine and glutathione also enhanced NO production, and the radical scavenger catalase and superoxide dismutase significantly inhibited the effect of homocysteine as well as those of cysteine and glutathione (data not shown). Furthermore, the effect of homocysteine was not maintained in the presence of glutathione. These results suggest that oxidative products are involved in the action of homocysteine, although the precise mechanism remains obscure. Welch et al.

Recently reported that homocysteine induced NO synthesis in VSMCs by NF-κB–dependent transcriptional activation of iNOS.

In the present study, homocysteine significantly increased IL-1β–stimulated NO production at 0.1 to 1 mmol/L. The effect of homocysteine occurred at the same concentrations as used in previous experiments.

Atherosclerosis appears to be associated with homocysteine concentrations of 0.2 to 0.25 mmol/L in hyperhomocysteinemic patients, although levels of homocysteine found in the plasma of patients with vascular diseases are considerably lower than those in homozymous homocysteinemic patients. For example, the Physicians’ Health Study demonstrated that patients with plasma homocysteine levels >15.8 μmol/L had a 3-fold increase in the risk of myocardial infarction. Whether such low levels of homocysteine could affect NO synthesis in vivo is unknown.

iNOS activity is induced in blood vessel wall and cultured VSMCs by endotoxins and cytokines.

Joly et al. demonstrated that in vivo balloon injury induced NO activity in rat carotid arteries, even in the absence of endothelium. Hannah et al. reported that arterial smooth muscle cells in the neointima formed after deendothelializing balloon injury of the rat carotid artery expressed the cytokine-inducible isoform of NOS. button et al. also reported that iNOS mRNA and protein were present within human atherosclerotic lesions. NO production by VSMCs may in part compensate for the absence of endothelial NO synthesis by inhibiting smooth muscle cell proliferation, as well as by limiting thrombus formation by preventing platelet adhesion and aggregation.

This hypothesis is supported by the observation in animals that L-arginine attenuates neointimal formation after balloon injury or that iNOS suppresses the development of allograft atherosclerosis. Thus, homocysteine-induced NO may suppress the atherothrombotic risk of hyperhomocysteinemic states. However, the potential role of excess NO derived from iNOS in the vascular tissue under pathological conditions has not been fully characterized and is still controversial. NO also has toxic and cytolytic effects, and increased expression of iNOS may promote the process of atherogenesis by increasing cell death and necrosis.

In conclusion, homocysteine enhances NO synthesis in IL-1β–stimulated VSMCs, and oxidative products are involved in the effect of homocysteine. However, further studies are necessary to clarify the role of homocysteine in NO production by the vascular tissue and its involvement in the pathogenesis of vascular lesions.

Acknowledgments

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References


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