Homocysteine-Induced Inhibition of Endothelium-Dependent Relaxation in Rabbit Aorta
Role for Superoxide Anions

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Abstract—Hyperhomocysteinemia is associated with endothelial dysfunction, although its mechanism is unknown. Isometric tension recordings and lucigenin chemiluminescence were used to assess the effects of homocysteine exposure on endothelium-dependent and -independent relaxation in isolated rabbit aortic rings and superoxide anion (O$_2^-$) production by cultured porcine aortic endothelial cells, respectively. Homocysteine (0.1 to 10 mmol/L) produced a significant ($P<0.001$) concentration- and time-dependent inhibition of endothelium-dependent relaxation in response to both acetylcholine and the calcium ionophore A23187. Only the intracellular O$_2^-$ scavenger 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron, 10 mmol/L) significantly ($P<0.001$) inhibited the effect of homocysteine on acetylcholine- and A23187-induced relaxation. Incubation of porcine aortic endothelial cells with homocysteine (0.03 to 1 mmol/L for up to 72 hours) caused a significant ($P<0.001$) time-dependent increase in the O$_2^-$ released by these cells on the addition of Triton X-100 (1% [vol/vol]), with levels returning to values comparable to those of control cells at the 72-hour time point. These changes in O$_2^-$ levels were associated with a time-dependent increase in endothelial cell superoxide dismutase activity, becoming significant ($P<0.001$) after 72 hours. Furthermore, the homocysteine-induced increase in endothelial cell O$_2^-$ levels was completely inhibited ($P<0.001$) by the concomitant incubation with either Tiron (10 mmol/L), vitamin C (10 μmol/L), or vitamin E (10 μmol/L). These data suggest that the inhibitory effect of homocysteine on endothelium-dependent relaxation is due to an increase in the endothelial cell intracellular levels of O$_2^-$ and provide a possible mechanism for the endothelial dysfunction associated with hyperhomocysteinemia. (Arterioscler Thromb Vasc Biol. 2000;20:422-427.)

Key Words: homocysteine ■ endothelial function ■ nitric oxide ■ oxygen-derived free radicals

D eficiencies in the enzymes cystathionine β synthase and methylenetetrahydrofolate reductase lead to elevated plasma homocyst(e)ine levels (free and bound homocysteine plus homocysteine and homocysteine-cysteine mixed disulfide). Patients with these disorders have much higher plasma plus homocystine and homocysteine-cysteine mixed disulfide (free and bound homocysteine 30 mmol/L) than do normal subjects (≥80 μmol/L compared with ≤10 μmol/L, respectively) and are prone to premature cardiovascular disease. Severe hyperhomocysteinemia, arising, for example, from cystathionine β synthase deficiency, is rare among the general population, whereas mild hyperhomocysteinemia (15 to 30 μmol/L) is common. A recent epidemiological study confirms that even such modestly elevated homocysteine levels are associated with an increased risk of atherosclerosis and can theoretically be caused by reduced activity of the enzyme methylenetetrahydrofolate reductase or deficiencies in folic acid and vitamin B$_6$. The mechanism of the vascular injury seen in hyperhomocysteinemia is not known, although growing evidence suggests that endothelial dysfunction plays a major role. Indeed, homocysteine has been shown to produce endothelial injury in animal models and cell culture studies.

In addition to hyperhomocysteinemia, other diseases that affect the cardiovascular system are also characterized by endothelial dysfunction. It is becoming widely accepted that the endothelial dysfunction associated with conditions such as hypercholesterolemia, diabetes, reperfusion injury, hypertension, possibly Alzheimer’s disease, and smoking is due to an increase in superoxide anion (O$_2^-$) production. The reaction between O$_2^-$ and endothelium-derived nitric oxide (NO) could lead to a decreased bioavailability of NO. The pathophysiological consequences of such a reaction include alterations in vascular function and the possible premature development of atherosclerosis. Whether hyperhomocysteinemia results in overproduction of oxygen-derived free radicals by endothelium remains undetermined.

The aims of the present study were to assess the effects of homocysteine exposure on endothelium-dependent and
-independent vascular smooth muscle relaxation in an isolated in vitro arterial preparation and to study the effect of homocysteine on O$_2^-$ levels in cultured endothelial cells.

**Methods**

**Preparation of Free L-Homocysteine**

Free reduced L-homocysteine was prepared, as described previously, by incubating L-homocysteine thiocionate for 5 minutes in 5 mol/L NaOH at 40°C before it was neutralized with 0.1 mol/L phosphate buffer (pH 7.8) and 2.5 mol/L HCl and diluted to the appropriate concentration with Krebs buffer. This reaction has previously been demonstrated to facilitate the complete conversion of L-homocysteine thiocionate to free reduced L-homocysteine. The vehicle was prepared in the same way, omitting L-homocysteine thiocionate.

**Isometric Tension Recordings**

The thoracic aortas of male New Zealand White rabbits (2 to 2.5 kg) were removed into fresh Krebs buffer composed of (mmol/L) NaCl 138, KCl 5.3, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, glucose 15, NaHCO$_3$ 24, CaCl$_2$ 1.5, and indomethacin 0.01 and gassed with 95% O$_2$/5% CO$_2$ at 37°C. For isometric tension recording, 2- to 3-mm-wide endothelium-intact or -denuded rings were mounted in tissue baths containing fresh Krebs buffer with a resting tension set at 2 g. After a 1-hour equilibration period, tissues were repeatedly exposed to phenylephrine (PE, 1 μmol/L) until a stable and repeatable level of constriction was obtained. Experiments were then carried out as outlined below, and all data were expressed as percentage relaxation of the appropriate PE-induced constriction.

**Endothelium-Intact Tissues**

In the experiments using endothelium-intact tissues (except those using either the calcium ionophore A23187 or sodium nitroprusside (SNP)), all rings were initially preconstricted with a submaximal concentration of PE (1 μmol/L) followed by exposure to increasing concentrations of acetylcholine (ACh, 1 nmol/L to 10 μmol/L). After they were washed and reequilibrated, some tissues were incubated for 3 hours with either vehicle (see above), homocysteine (0.1 to 10 mmol/L), cysteine (1 mmol/L), glutathione (1 mmol/L), or L-homocysteine thiocionate (1 mmol/L). Other tissues were incubated for 1, 3, and 5 hours with vehicle or homocysteine (1 mmol/L) or L-homocysteine thiocionate (1 mmol/L). Another group of tissues was incubated for 3 hours with either homocysteine alone (1 mmol/L) or in the presence of either superoxide dismutase (SOD, 60 U/mL), catalase (CAT, 120 U/mL), SOD plus CAT (60 and 120 U/mL, respectively), superoxide scavenger, or intracellular O$_2^-$ scavenger 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron, 10 mmol/L). After these various incubations, all rings were reconstricted with PE (1 μmol/L), and the concentration responses to ACh were repeated.

For experiments with A23187, tissues were first incubated for 3 hours with either homocysteine alone (1 mmol/L) or homocysteine in the presence of either SOD, CAT, SOD plus CAT, vitamin C, or Tiron as described above. They were then constricted with PE (1 μmol/L), and concentration responses to ACh were repeated. Finally, tissues were exposed to PE and SNP (1 nmol/L to 10 μmol/L, respectively) and then washed, followed by a 3-hour incubation with either vehicle or homocysteine (1 mmol/L). After reconstruction with PE, concentration responses to SNP were again measured.

**Endothelium-Denuded Tissues**

In the first series of experiments, tissues were exposed to PE as described above, followed by SNP (1 nmol/L to 10 μmol/L). After they were washed, the tissues were incubated for 3 hours with either vehicle or homocysteine alone (1 mmol/L). After reconstruction with PE, concentration responses to SNP were repeated at the end of the incubation period.

In the second series of experiments, tissues were first incubated for 3 hours with homocysteine (1 mmol/L). They were then constricted with PE (1 μmol/L), and concentration responses to atrial natriuretic peptide (0.01 nmol/L to 0.1 μmol/L) were established.

**Porcine Aortic Endothelial Cells**

Porcine aortic endothelial cells (PAEs) were isolated and cultured as previously described. These experiments were designed to investigate the effect of lower concentrations of homocysteine and longer incubation times on endothelial function. All experiments were carried out on first-passage cells.

**Superoxide Anion Production by Lucigenin Chemiluminescence**

In these experiments, PAEs were incubated for 24, 48, and 72 hours with homocysteine (0.01 to 1 mmol/L). Fresh homocysteine was added to the cells after each 24-hour period to prevent loss of homocysteine due to endothelial cell metabolism. Other cells were incubated for 24 hours with either cysteine (1 mmol/L) or glutathione (1 mmol/L). A further group of PAEs was incubated for 24 hours with either homocysteine alone (1 mmol/L), homocysteine in the presence of Tiron (10 mmol/L), Tiron alone, homocysteine in the presence of either vitamin C (10 μmol/L) or vitamin E (10 μmol/L), vitamin C alone, or vitamin E alone. At the appropriate end point, O$_2^-$ levels were measured as described below.

In another series of experiments, PAEs were incubated for 24, 48, and 72 hours with homocysteine (1 mmol/L, only) described above. Again, at the appropriate end point, some cells were taken for O$_2^-$ measurements. The remaining cells were used for the measurement of SOD activity as described below.

For the measurement of PAE O$_2^-$ production, cells were taken at the appropriate end point and washed with sterile saline (0.9% [wt/vol]) before being trypsinized (0.05% [wt/vol])--digested. They were then isolated by centrifugation at 200g for 10 minutes at room temperature. The resulting cell pellet was resuspended in HEPES (20 mmol/L)–buffered physiological saline (pH 7.3) composed of (mmol/L) NaCl 138, KCl 5.3, NaHPO$_4$ 1.2, MgSO$_4$ 1.2, glucose 15, and CaCl$_2$ 1.5, and the cell number was measured with a Coulter Counter. The O$_2^-$ production by these cells was measured immediately by use of lucigenin chemiluminescence.

PAEs were added to an aliquot of the HEPES buffer (at 37°C), and lucigenin was added to a final concentration of 500 μmol/L. The cells were then placed into the warmed (37°C) chamber of a custom-built luminometer, with the output (in millivolts) displayed on a Macintosh computer via a Maclab apparatus. The O$_2^-$ production by these cells was measured after the addition of 1% (vol/vol) Triton X-100. In some cases, Triton (10 mmol/L) was also added at the same time as Triton X-100. The integral for the response represents the total O$_2^-$ produced and was normalized to cell number. All data are expressed as millivolt · seconds per million cells, i.e., (mV · s)/10$^6$ cells.

**SOD Activity**

PAE cell pellets, prepared as described above, were resuspended in 50 mmol/L Tris buffer containing 0.5% Triton X-100, pH 8.0, and lysed by freeze-thawing. Lysates were centrifuged at 13 000g for 10 minutes at 4°C. Determination of SOD activity in the cell lysates was performed as previously described; the method was based on the ability of SOD to inhibit the auto-oxidation of pyrogallol and was adapted for use on the Cobas Bio Autoanalyser (Roche Diagnostic). Purified SOD from bovine erythrocytes (Sigma Chemical Co) was used to construct a standard curve for this assay. Lysate protein content was measured by using a BCA protein kit (BCA assay, Pierce). All data are expressed as units of SOD activity per milligram protein.

**Statistical Analysis**

For aortic ring experiments, maximum relaxation response (Rmax) values for each concentration-response curve were calculated by use of Kaleidagraph software for Macintosh and either Student unpaired t tests or ANOVA, followed by an appropriate multiple range test that compared these values when appropriate. For the experiments that used cultured PAEs, data were compared by ANOVA, followed by an appropriate multiple range test. All differences were considered significant at $P<0.05$. 

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Chemicals

The majority of drugs and reagents were obtained from Sigma. L-Homocysteine thiolactone was supplied by Calbiochem. Tissue culture reagents were supplied by GIBCO-URL. All were dissolved in distilled water/buffer immediately before use, except in the case of lucigenin and A23187 (both dissolved in dimethyl sulfoxide) and vitamin E (in 0.01% [vol/vol] ethanol).

Results

Isometric Tension Recordings

All data are expressed as mean±SEM (n=4). Preconstriction with PE (1 μmol/L) produced a mean constriction of 4.99±0.08 g (n=148) in endothelium-intact tissues and 5.06±0.14 g (n=52) in endothelium-denuded tissues (P=NS). Relaxation responses are expressed as a percentage of the appropriate PE-induced constriction.

A 3-hour incubation with homocysteine (0.1 to 10 mmol/L) produced a significant (P<0.01 for all concentrations) concentration-dependent inhibition of endothelium-dependent relaxation (Figure 1). Incubation with vehicle at any concentration had no significant effect on the Rmax to ACh (Figure 1, other data not shown).

In the presence of 1 mmol/L homocysteine, the inhibitory effect on endothelium-dependent relaxation was time dependent (Figure 2) and significant (P<0.01) at all time points studied. However, there was no significant difference between the 3- and 5-hour time points (Figure 2). A similar time-dependent effect was seen in the presence of 0.1 mmol/L homocysteine and was significant (P<0.002) at all time points (Rmax 68.04±0.70%, 68.37±1.13%, and 67.68±0.83% in the presence of homocysteine for 3, 5, and 8 hours, respectively, compared with time-matched incubations in the absence of homocysteine (Rmax 77.36±1.78%, 76.59±0.79%, and 76.71±0.72%, respectively). From the results of these initial experiments, the 1 mmol/L homocysteine concentration and 3-hour exposure time were chosen for the rest of the aortic ring experiments.

Of all interventions studied, only Tiron significantly (P<0.0001) inhibited the effect of homocysteine on both ACh-induced (Figure 3a) and A23187-induced (Figure 3b) relaxation. SOD, CAT, SOD plus CAT, l-arginine, and vitamin C were without effect (data not shown). The relaxation response to either ACh or A23187 in the absence of homocysteine was unaffected by Tiron (data not shown).

Cysteine, glutathione, and l-homocysteine thiolactone were all found to be without effect on endothelium-dependent relaxation (Rmax 75.74±1.46%, 74.80±1.55%, and 74.12±1.15% in the presence of homocysteine compared with 76.12±1.04% for control tissues in the presence of cysteine and glutathione and 76.05±0.64% for control tissues in the presence of l-homocysteine thiolactone).

Homocysteine was without effect on the relaxation responses to SNP in both endothelium-intact and -denuded tissues.
homocysteine, none of the other concentrations studied had any effect on PAE O$_2^-$ levels (data not shown). In contrast to these effects on O$_2^-$, the exposure of PAEs to 1 mmol/L homocysteine resulted in a time-dependent increase in SOD activity, reaching significance ($P<0.001$) after 72 hours (Figure 5b).

Exposure of cultured PAEs to either cysteine (1 mmol/L) or glutathione (1 mmol/L) had no effect on control O$_2^-$ levels (26.67±3.52 and 20.55±2.48 mV · s/10$^6$ cells, respectively, compared with 24.05±3.77 mV · s/10$^6$ cells for controls).

Simultaneous 24-hour incubation of PAEs with homocysteine (1 mmol/L) and the intracellular O$_2^-$ scavenger Tiron (10 mmol/L) resulted in complete inhibition ($P<0.001$) of the O$_2^-$ response to homocysteine alone (17.68±2.50 mV · s/10$^6$ cells compared with 53.33±10.12 mV · s/10$^6$ cells for homocysteine alone). Simultaneous incubations of homocysteine (1 mmol/L) with either vitamin C or E (both 10 µmol/L) produced a significant inhibition ($P<0.001$) of the homocysteine-induced increase in O$_2^-$ (29.93±1.90 and 27.60±2.50 mV · s/10$^6$ cells, respectively, compared with 67.12±4.20 mV · s/10$^6$ cells for homocysteine alone).

In further experiments, the addition of Tiron (10 mmol/L) together with Triton X-100 (1% [vol/vol]) returned the control and homocysteine-induced changes in chemiluminescence to background levels. The latter demonstrates that the chemiluminescent response in the presence of Triton X-100 was due to O$_2^-$ and not other free radicals. Triton X-100 alone had no effect on background chemiluminescence (data not shown).

**Discussion**

The data presented demonstrate that homocysteine inhibits endothelium-dependent relaxation in a concentration- and time-dependent manner. This effect is receptor independent, in view of the fact that homocysteine inhibits endothelium-dependent relaxation to both ACh and A23187 to a similar degree. Free l-homocysteine, prepared from l-homocysteine thiolactone, was used in the present study, because l-homocysteine has previously been described to be the active damaging enantiomer. This method of preparation has previously been demonstrated to facilitate the complete conversion of the l-homocysteine thiolactone to free reduced...
L-homocysteine. Therefore, it is highly unlikely that the thiolactone itself is responsible for the effect on the endothelium-dependent relaxation demonstrated in the present study. This fact is borne out by the complete lack of an inhibitory effect on relaxation after incubation with L-homocysteine thiolactone itself.

Clearly, the concentrations of homocysteine used in the present study are far higher compared with those experienced in vivo. However, they are in the range of the concentrations used in many of the other in vitro studies in the literature. Furthermore, the exposure times used are much shorter than those experienced in hyperhomocysteinemia in humans. Nevertheless, the main aim of the present study was to establish a possible mechanism for homocysteine-induced endothelial dysfunction, and as with other diseases of this nature, the use of higher pharmacological concentrations is sometimes required in vitro to reproduce the in vivo situation. It is interesting to note from the experiments with cultured cells that homocysteine, at a concentration close to that observed in human plasma after an oral methionine load in healthy volunteers and associated with endothelial dysfunction, does indeed produce a significant increase in O$_2^-$ production by the PAEs.

We have already demonstrated in our laboratory that acute treatment with the antioxidant vitamin C can reverse the inhibitory effect of homocysteine on flow-mediated dilatation of the brachial artery in human volunteers (M.F.B. et al, unpublished observations, 1998). Furthermore, a recent study of the brachial artery in human volunteers (M.F.B. et al, unpublished observations, 1998) has shown that treatment with the antioxidant vitamin C can reverse the inhibitory effect of homocysteine; SOD, CAT, and peroxiredoxin (PRX) activities are restored in vitro to reproduce the in vivo situation. It is clear that homocysteine, at a concentration close to that observed in human plasma after an oral methionine load in healthy volunteers and associated with endothelial dysfunction, does indeed produce a significant increase in O$_2^-$ production by the PAEs.

The endothelial specificity of homocysteine was clearly demonstrated in that no effect on endothelium-independent relaxation to either SNP or atrial natriuretic peptide was seen. The fact that homocysteine did not alter the relaxation responses to SNP indicates that it has no effect on NO-induced relaxation per se. Furthermore, the observation that SNP-induced relaxation in endothelium-intact tissues was also unaffected by homocysteine would tend to support the hypothesis that the homocysteine-induced production of O$_2^-$ occurs within endothelial rather than vascular smooth muscle cells. Other researchers have suggested that the reaction of NO with O$_2^-$, generated extracellularly by the auto-oxidation of homocysteine or by the reaction of thiols in the homocysteine molecule with NO, is responsible for the inhibition of NO-mediated relaxation by homocysteine. In the present study, neither cysteine nor glutathione, both containing thiol groups, had any effect on endothelium-dependent relaxation or on cultured endothelial cell O$_2^-$ production, mitigating against a role for thiol groups per se in the inactivation of NO.

Of the other interventions used in the aortic ring studies, only the intracellular O$_2^-$ scavenger Tiron significantly reversed the inhibitory effect of homocysteine; SOD, CAT, and l-arginine were all without effect. SOD and CAT are large molecules and act as extracellular free radical inhibitors. L-Arginine was chosen to exclude an effect of homocysteine on NO synthase (NOS). Taken together, these data suggest that the inhibitory effect of homocysteine is mediated via the increased intracellular production of O$_2^-$ by the endothelium and not by the extracellular auto-oxidation of homocysteine, as previously suggested, or by a direct effect on NOS.

Because cysteine and glutathione were without effect on endothelium-dependent relaxation or cultured endothelial cell O$_2^-$ production and because Tiron alone reversed the inhibitory effect of homocysteine in the ring studies, we chose to use only Tiron in the cultured cell studies.

By using lucigenin chemiluminescence, the present study clearly demonstrates that exposure of cultured endothelial cells to homocysteine causes an increase in the intracellular generation of O$_2^-$ and confirms the findings of the aortic ring studies. The source of these O$_2^-$ radicals is unknown, however, but is likely to be multiple because many cell processes generate O$_2^-$. It has been suggested that sulfur-containing amino acids, such as homocysteine, can themselves spontaneously generate oxygen-derived free radicals. Therefore, it is also possible that in addition homocysteine itself could become a source of intracellularly generated O$_2^-$. The present study demonstrates that continued exposure to homocysteine results in a >3-fold increase in endothelial cell SOD activity. It is possible, indeed likely, that increased SOD activity is a response to the increased O$_2^-$ generation after exposure to homocysteine and probably represents a defense mechanism activated to protect the cell against an oxidative insult.

Many previous studies have alluded to the role played by oxidative stress in the endothelial dysfunction caused by homocysteine. One possible manifestation of this endothelial dysfunction is decreased bioavailability of NO, which has been shown to occur independent of changes in endothelial cell NOS (ecNOS) protein expression and steady-state ecNOS mRNA. This decreased bioavailability of NO, via its reaction with O$_2^-$, could therefore provide a mechanism for the homocysteine-induced inhibition of endothelium-dependent relaxation demonstrated in the present study.

Although oxygen-derived free radicals are themselves directly toxic to endothelial cells, the subsequent formation of peroxynitrite by the reaction of NO with O$_2^-$ could further exacerbate endothelial dysfunction. In some cell types, peroxynitrite has been shown to induce apoptosis, a phenomenon that is both time and concentration dependent and is itself inhibited by NO.

As mentioned previously, the increase in O$_2^-$ is likely derived from more than one source in the endothelial cell. Xanthine oxidase, NADH and NADPH oxidase(s), eicosanoid metabolism, and respiratory chain enzymes are obvious candidates. Even ecNOS itself has been shown to generate O$_2^-$ under certain pathological conditions. The oxygen-derived free radical–generating activities of these enzymes would, under normal conditions, be limited by the antioxidant enzymes present in the endothelium. An imbalance in these antioxidant defenses, due either to overwhelming O$_2^-$ production or inhibition of the antioxidant enzymes themselves, would result in oxidant stress. Given the evidence discussed, it is likely that homocysteine affects O$_2^-$ production and antioxidant defenses. Homocysteine may therefore be seen as initiating a vicious downward spiral to pro-oxidant status, a major manifestation of which is endothelial dysfunction.
In summary, the present study demonstrates that homocysteine inhibits endothelium-dependent relaxation via a mechanism that involves the intracellular generation of oxygen-derived free radicals and provides a possible mechanism for the endothelial dysfunction associated with hyperhomocysteinemia.

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