Homocysteine induced impairment of nitric oxide-dependent vasorelaxation is reversible by the superoxide dismutase mimetic TEMPOL

Davy Hucks, Raj C. Thuraisingham, Martin J. Raftery and Magdi M. Yaqoob

Experimental Medicine, Nephrology and Critical Care, William Harvey Research Institute, Charterhouse Square, St Bartholomew’s and the Royal London Hospitals, Queen Mary, University of London, London EC1M 6BQ, UK

Abstract

Background. Elevated plasma homocysteine concentrations in renal patients are associated with accelerated cardiovascular disease. The mechanism(s) by which homocysteine acts remains unclear however, evidence implicates a role involving endothelial dysfunction.

Methods. Rat femoral arteries after acute or 4-h pre-incubation with racemic D,L-homocysteine (100 μM) were mounted on a myograph, pre-constricted with phenylephrine (10 μM) and responses to acetylcholine-dependent vasorelaxation examined. The incubations were repeated in the presence of indomethacin (10 μM), ω-nitro-γ-arginine methyl ester (100 μM), L-arginine (100 μM), tetrahydrobiopterin (1 μM), catalase (1200 U/ml), ebselen, a peroxynitrite chelator (20 μM) and TEMPOL, a superoxide dismutase mimetic (1 mM).

Results are shown as means±standard error, expressed as per cent relaxation to acetylcholine added (nmol/l).

Results. Increasing concentrations of homocysteine had no affect when added directly to basally relaxed or pre-constricted freshly isolated vessels. However, 4-h pre-incubation with or without homocysteine significantly shifted the acetylcholine EC50 (EC50 was defined as the concentration of acetylcholine that caused relaxation of the phenylephrine contracted tissue by 50%), control(4 h)=74.7 nmol/l±10.5 vs 100 μM D,L-homocysteine(4 h)=159.9 nmol/l±20.6; P<0.05) without affecting maximal relaxation. Response to endothelial independent relaxation was unaffected. Indomethacin, indomethacin and ω-nitro-γ-arginine methyl ester, L-arginine and tetrahydrobiopterin, catalase and ebselen had no effect on the EC50 in homocysteine-exposed arteries. However, TEMPOL normalized vasorelaxation in homocysteine-treated arteries (75.2 nmol/l±14.6) but had no effect on the 4-h control group. Moreover, washing TEMPOL from the treated vessels restored endothelial dysfunction in D,L-homocysteine-treated vessels (163.9 nmol/l±34.1).

Conclusions. We conclude that homocysteine causes endothelial dysfunction by up-regulating a potential superoxide generating system resulting in reduced nitric oxide bio-availability.

Keywords: endothelial dysfunction; D,L-homocysteine; oxidation; TEMPOL

Introduction

Homocysteinaemia, a common feature in dialysis patients, has been identified as an important risk factor in the development of cardiovascular disease (CVD). It has been identified in up to 10–40% of patients with symptomatic atherosclerosis [1]. A clear link between homocysteine and CVD was found in monozygotic diseases, called as a whole, homocysteinuria, in which plasma homocysteine levels are several fold higher (400–500 μmol/l). More recently mild (<106 μmol/l) hyperhomocysteinaemia has been recognized as a CVD risk in the general population, and in a number of acquired conditions, such as chronic renal failure where homocysteine levels can range from moderate (16–30 μmol/l) to intermediate (30–100 μmol/l). In a recent prospective observational study of 175 chronic renal failure, high baseline homocysteine levels were
associated with an increase in mortality rates [2]. Circulating homocysteine comprises of two major components, a bound component where the homocysteine is bound to plasma protein, principally albumin and a free unbound fraction. Both free and bound fractions are collectively measured and given to the clinician as a value of total homocysteine. Nutritional (particularly vitamin B6, B12 and folate levels), environmental and genetic status (polymorphism in methyl-tetra-hydro-folate-reductase) all play an important role in the regulation of homocysteine in the blood [3].

Endothelial dysfunction plays a major role in CVD. Several human and animal studies have demonstrated that elevated serum concentrations of homocysteine impairs vascular responsiveness by an unknown mechanism [4–7]. Using an endothelial cell culture model Upchurch et al. [8] demonstrated that homocysteine caused a decrease in nitric oxide production, as measured by nitric oxide meter, after 4h. Also, using endothelial cells in culture, Zhang et al. [9] has demonstrated a dose-dependent reduction in nitric oxide production when using concentrations up to 50 μM homocysteine. Emsley et al. [10] using rat aortic rings demonstrated that endothelial dysfunction induced by homocysteine after 3h could be enhanced by the addition of Cu²⁺ ions and reversed by the addition of a copper chelator. Proposed toxicity mechanisms of hyperhomocysteinemia either acting directly or in synergy with other risk factors are oxidation (thiol auto-oxidation), nitrosylation (reducing nitric oxide bioavailability by chelation), homocysteinylolation (post-biosynthetic acylation of free amino groups in proteins by homocysteine thiolactone resulting in structural and functional alterations at molecular and cellular level) and hypomethylation (inhibition of transmethylation reactions, a key process involved tissue repair). However, no mechanism has been established, so far, which can provide an exhaustive biological basis for its toxic effects [11].

Nitric oxide is a potent vasodilator generated from the enzyme endothelial nitric oxide synthase (eNOS) in the presence of L-arginine, tetrahydrobiopterin and other co-factors. Nitric oxide in the vasculature causes relaxation in smooth muscle cells and inhibits the activation of platelets in coagulation. Factors that interfere with its generation would lead to endothelial dysfunction. Activation of eNOS occurs when there is a rise in intracellular calcium; shear stress and drugs such as acetylcholine and bradykinin are all able to mediate their effects through nitric oxide release. We speculated that clinically relevant high homocysteine could directly interfere with the nitric oxide pathway and cause endothelial dysfunction. We developed a method of prolonged arterial organ culture of resistance femoral arteries using the Mulvaney–Halpern small vessel myograph to study the effects of clinically relevant concentrations of L-homocysteine on endothelial-dependent and -independent vascular reactivity.

Subjects and methods

Tissue preparation

Male Wistar Kyoto rats (200–250 g) were rapidly killed by cervical dislocation and the hind limbs were swabbed in 70% ethanol and removed at the groin. The femoral arteries were taken from an area at the top of the thigh to the point above the first bifurcation; side branches that form the gracilis and cremaster arteries that are found in this section were not used in this study. Vessels between the order of 350 and 500 μm were dissected free of surrounding fascia and cut into 2 mm segments. Some arteries were used directly but most were placed in Dulbecco’s modified eagle’s medium media (DMEM) at a temperature of 37°C in an atmosphere of 5% CO₂ and incubated for 4h either in the absence of homocysteine (control4h) or with 100 μmol/l D,L-homocysteine (homocysteine4h). On occasion the DMEM was supplemented for the 4h with 100 μmol/l D,L-cysteine as a thiol containing amino acid control, co-factors of nitric oxide synthesis or homocysteine metabolism.

Freshly isolated vessels or after prolonged incubation the arteries were placed in physiological salt solution (PSS) and mounted on a Mulvaney–Halpern small vessel wire myograph (400A series, Danishmyotechnology). The vessels were stretched to an equivalent transmural pressure of 100 mmHg (L100). Following a 60-min normalization period, which included contracting the tissue three times with a second PSS containing 80 mM KCl (equi-substitution for NaCl) for 3 min (KPSS), the integrity of the endothelium was tested by constricting with 10 μM phenylephrine and after steady contraction obtained, relaxed with 10 μM acetylcholine. After washing with PSS the muscle tone relaxed back to baseline levels.

Experimental protocols

To determine whether homocysteine had a direct effect on vessels, up to 1 mM homocysteine was added to freshly isolated, pre-contracted arteries.

After establishing the viability after a 4-h incubation, cumulative concentration–response relationships were obtained for acetylcholine in freshly isolated arteries and in those arteries incubated for 4 h in the presence or absence of homocysteine. In order to establish the specific effects of homocysteine, we also used another thiol containing amino acid, cysteine. The constricting agent was 10 μM phenylephrine in all cases. After relaxing the tissue with acetylcholine, the vessel was thoroughly washed in PSS and left until a stable baseline obtained. There was then a short incubation with either 10 μM indomethacin, 1200 U/ml bovine catalase, the peroxynitrite scavenger, 2-phenyl-1,2-benzisoleselenazol-3(2H)-one called ebselen (20 μM) and the superoxide dismutase (SOD) mimetic TEMPOL (1 mM). These compounds have been shown to have effects on several aspects of vascular reactivity when in prolonged incubation. Therefore, to limit their action to the immediate effects of the contraction/relaxation cycle they were added for 20 min prior to constricting with 10 μM phenylephrine and subsequent relaxation to acetylcholine. The experiments that involved increasing L-arginine and tetrahydrobiopterin, co-factors of nitric oxide synthesis and increasing folate status were performed by adding these supplements alongside homocysteine at the start of the 4-h incubation period.
Chemicals and solutions

Tissue culture materials were obtained from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK). Other chemicals were obtained from VWR (Lutterworth, UK) or Sigma and were of AnaRA grade or Sigma-ultra grade with the exception of TEMPOL, Calbiochem, (CN Bioscience, UK). All drugs were prepared as stock solutions on the day of experiment and dissolved in freshly prepared PSS of the following composition. In mM: NaCl 118, NaHCO3 24, MgSO4 1, NaH2PO4 0.435, glucose 5.56, Na-pyruvate 5, CaCl2 1.8 and KCl 4. The high potassium containing PSS had similar constituents with the exception that 80 mM KCl was equimolar substituted for NaCl. Water was supplied by VWR at AnaRA grade. Where solubility in aqueous solution could not be achieved, 50% DMSO v/v solution was used.

Data analysis

The myograph was linked to a computer running Myodaq 2.0 for data acquisition at a sampling rate of one point per second and Myodata 2.0 for the subsequent data analysis (Danishmyotechnology).

Initial tensions developed after constriction with 10 μM phenylephrine are given in mN/mm² of artery length. The results of acetylcholine relaxation are expressed as percentage relaxation of initial tone after 10 μM phenylephrine. EC50 was defined as the concentration of acetylcholine that caused relaxation of the phenylephrine contracted tissue by 50%. EC50 values were obtained from individual concentration–response curves that achieved >80% relaxation after the addition of acetylcholine. Those that failed to reach this relaxation were deemed to have had the endothelial cell layer damaged whilst mounting. Curves were fitted utilizing a single rectangular three parameter curve fit using regression wizard from Sigma Plot 5 (Jandel Scientific, USA). All other values were given as mean ± standard error of the mean. Data were compared using a one-way Student’s t-test. Differences were considered significant at P < 0.05.

Results

Direct effect of homocysteine on basal and pre-constricted vessel

In freshly isolated intact arteries, homocysteine did not cause an increase in the basal level of tone over a concentration range 10–1000 μmol/l, nor when the same artery was contracted with phenylephrine did homocysteine cause any detectable increase or decrease in tone (Figure 1). A 20-min pre-incubation with homocysteine (100 μM) prior to contraction and subsequent relaxation with acetylcholine showed no difference in either maximum force generated by phenylephrine nor was there a change in the maximum relaxation caused by acetylcholine (data not shown).

Effect of 4-h pre-incubation in the presence of 100 μM homocysteine

Following a 4-h incubation either in the presence or absence of 100 μM D,L-homocysteine the arteries showed no impairment in contraction as indicated by maximum force generated to 10 μM phenylephrine induced tone (Table 1). However, there was a significant rightward shift in the concentration–response curves to acetylcholine in homocysteine exposed [EC50 between control(4h)] and the homocysteine-treated(4h) arteries [74.7 nmol/l ± 10.5 (n = 17) vs 159.9 nmol/l ± 20.6 (n = 11; P < 0.05)], respectively (Figure 2). The maximum amount of relaxation (Rmax) was unaffected by homocysteine indicating a lack of endothelial cell toxicity. 100 μmol/l D,L-cysteine had no effect on vasodilation when compared with control. Similarly lower concentrations of D,L-homocysteine (30 μmol/l) had no significant effect on vasodilation.
Effect of homocysteine on endothelial-independent vasorelaxation

The nitric oxide donor sodium nitroprusside, which causes relaxation in smooth muscle independently of the endothelial cell, was substituted for acetylcholine. After 10 μmol/l phenylephrine contraction, the subsequent responses were identical in the homocysteine-treated (4 h) artery to that of control (4 h) (Figure 3).

Effect of homocysteine on endothelial-independent vasorelaxation

The nitric oxide donor sodium nitroprusside, which causes relaxation in smooth muscle independently of the endothelial cell, was substituted for acetylcholine. After 10 μmol/l phenylephrine contraction, the subsequent responses were identical in the homocysteine-treated (4 h) artery to that of control (4 h) (Figure 3).

Effect of tetrahydrobiopterin (BH₄) and L-arginine on homocysteine-treated vessel

The possibility of homocysteine sequestrating essential co-factors of NO₃ production or the potential leaching from tissues during the incubation process was addressed by repeating the experiments with key co-factors in excess. Tetrahydrobiopterin (1 μmol/l) and L-arginine (100 μmol/l) were added along with d,l-homocysteine for the 4-h incubation period. The arteries that were supplemented with tetrahydrobiopterin and L-arginine showed a reduced maximal tone following phenylephrine-induced contraction, indicating a possible increase in nitric oxide production (Table 1) but there was no improvement in vascular response to acetylcholine (n = 5). Lack of effect with combination precluded the use of substrate in isolation.

Effect of homocysteine on endothelial-derived hyperpolarizing factors

Endothelial-derived hyperpolarizing factors are a loosely defined group the identity of which is controversial. For this study we defined them as compounds that are neither nitric oxide nor prostanoid in origin. To unmask the endothelial-derived hyperpolarizing factors, nitric oxide and prostanoid production was prevented by using the nitric oxide synthase inhibitors and cyclo-oxygenase inhibitors, o-nitro-L-arginine methyl ester (100 μM) and indomethacin (10 μM), respectively, for 20 min prior to contraction. There was an overall increase in phenylephrine-induced vascular tone after constriction in both control and homocysteine-treated groups. A moderate amount of relaxation was detected over the concentration range of acetylcholine, but there was no significant difference following phenylephrine-induced contraction, indicating a possible increase in nitric oxide production (Table 1) but there was no improvement in vascular response to acetylcholine (n = 5). Lack of effect with combination precluded the use of substrate in isolation.

Table 1. Femoral artery vasoreactivity after homocysteine pre-treatment. Effect of known modulators of vascular tone. SE, standard error.

<table>
<thead>
<tr>
<th></th>
<th>Rₘₐₓ (% relaxation ± SE)</th>
<th>EC₅₀ (nmol/l ± SE)</th>
<th>Max PE contraction (mN/mm² ± SE)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.4±2.7</td>
<td>74.7±10.5*</td>
<td>4.34±0.3</td>
<td>17</td>
</tr>
<tr>
<td>100 μM d,l-homocysteine</td>
<td>89.1±2.2</td>
<td>159.9±20.6</td>
<td>3.92±1.2</td>
<td>11</td>
</tr>
<tr>
<td>100 μM d,l-homocysteine + 10 μM indomethacin</td>
<td>93.5±3.9</td>
<td>154.8±31.5</td>
<td>3.70±0.9</td>
<td>6</td>
</tr>
<tr>
<td>100 μM d,l-homocysteine + 1200 U/ml catalase</td>
<td>87.1±4.7</td>
<td>230.4±26.2</td>
<td>4.10±0.7</td>
<td>7</td>
</tr>
<tr>
<td>100 μM d,l-homocysteine + 20 μM ebselen</td>
<td>89.3±3.1</td>
<td>126.1±19.1</td>
<td>4.77±0.4</td>
<td>6</td>
</tr>
<tr>
<td>100 μM d,l-homocysteine + 1 mM TEMPO</td>
<td>97.4±2.8</td>
<td>67.6±8.7*</td>
<td>3.12±0.6</td>
<td>6</td>
</tr>
<tr>
<td>100 μM d,l-homocysteine + after TEMPO washed off</td>
<td>96.5±4.9</td>
<td>163.9±34.1</td>
<td>2.82±1.6</td>
<td>5</td>
</tr>
<tr>
<td>100 μM d,l-homocysteine + 100 μM L-arginine + 10 mM tetrahydrobiopterin</td>
<td>92.0±1.9</td>
<td>188.4±17.1</td>
<td>2.8±0.3</td>
<td>5</td>
</tr>
</tbody>
</table>

Fig. 2. Following 4-h incubation in the presence of homocysteine there was a clear rightward shift in the acetylcholine-induced vasorelaxation of the phenylephrine-contracted vessel when compared with the 4-h untreated control.

Fig. 3. Endothelial cell-independent vasorelaxation was unaffected by homocysteine pre-treatment.
between control(4 h) and homocysteine-treated(4 h) vessels (Figure 4). We thereby excluded abnormalities in endothelial-derived hyperpolarizing factors as a potential mechanism of homocysteine induced altered vasoreactivity (n = 6).

**Effect of homocysteine on arachadonic metabolism**

During the incubation it is possible that vasoactive prostanoid compounds such as prostacyclin, prostaglandin H2 and leukotriene B4 may have their production altered by the presence of homocysteine. A 20-min incubation with the cyclo-oxygenase inhibitor, indomethacin (10 μM), had no effect on the EC₅₀ when compared with homocysteine treated(4 h) and homocysteine and indomethacin (Table 1), excluding therefore that homocysteine causes impaired vasodilation through a component that is prostanoid mediated (n = 6).

**Effect of homocysteine on hydrogen peroxide formation**

It has been suggested that homocysteine has the ability to auto-oxidize. A potential by-product, hydrogen peroxide may be formed as a consequence of homocysteine treatment. Homocysteine-treated arteries were incubated with 1200 U/ml catalase. No effect on phenylephrine-induced tone was detected. Catalase was supplied as a crude extract at 80% purity and added as a solution directly to the organ bath. The addition of this enzyme to the PSS caused foaming and a darkening of the bathing solution, although pH was unaffected. In both the control and treated vessels the dose–response curve was shifted to the right by the same degree suggesting a non-specific matrix effect (due to contaminates such as endotoxin, thymol and SOD and possibly other proteins containing Fe³⁺ a known scavenger of NO- in the crude enzyme extract), affecting overall assay conditions (n = 7).

**Effect of homocysteine on superoxide**

The water-soluble nitroxide, TEMPOL, is a cell permeable mimic of SOD. A 20-min pre-incubation with 1 mM TEMPOL prior to constricting with 10 μM phenylephrine on the homocysteine-treated(4 h) arteries completely reversed the impaired EC₅₀ induced by the 100 μM D,L-homocysteine insult (Table 1). However, repeated washing of the tissue with phosphate-buffered saline to remove TEMPOL caused the return of the rightward shift in the dose–response curve (Figure 4). TEMPOL had no effect on the acetylcholine response curves in either normal control(4 h) or D,L-cysteine(4 h)-treated control (Figure 5).

**Effect of homocysteine on peroxynitrite**

Peroxynitrite is a product of nitric oxide and superoxide fusion reaction resulting in reduced nitric oxide bio-availability. Ebselen is a seleno-organic compound with several modes of action one of which is to act as a scavenger of peroxynitrite. Ebselen resulted in a slight but insignificant improvement in phenylephrine induced tone (Table 1) and a small decrease in EC₅₀ in the homocysteine treated(4 h) to acetylcholine it, however, did not reach statistical significance (n = 6).

**Discussion**

The main finding of this study is that following a 4-h incubation with a physiologically relevant concentration of homocysteine, it was possible to demonstrate an impaired vasodilation response to acetylcholine in femoral arteries that persisted even after washing homocysteine from the tissue. Furthermore, this impairment was found to be completely reversible by
the SOD mimic, TEMPOL. Interestingly, this beneficial effect disappeared after TEMPOL was removed. These findings are consistent with an up-regulation of superoxide generating pathways.

As a neutral amino acid, homocysteine would freely pass via the B⁰ amino acid transporter into the cell. Once the 4-h incubation period was complete we thoroughly washed the vessels in PSS several times during mounting and normalization prior to the experiment, ensuring no or negligible amounts of homocysteine were present at the time of the phenylephrine contraction. By performing the experiments in the absence of homocysteine we were able to eliminate the possibility of nitric oxide reacting to form s-nitrosohomocysteine, a compound that has vasoactive and thrombogenic properties, which has been suggested as a possible mechanism for the cardiovascular effects of homocysteine [5]. Addition of homocysteine directly, up to a concentration of 1 mmol/l, to a freshly isolated vessel did not demonstrate an effect on either a fully relaxed vessel or constricted artery.

We were able to show that the impaired vasodilatation was reversed in the presence of TEMPOL, a tissue-soluble SOD mimic that has been used to prevent oxidative damage in several experimental models [12]. A stable cyclic nitroxide, TEMPOL itself may be the source of oxygen free radicals [13]. Its reaction with superoxide may result in the formation of hydroxylamine, which in turn reacts with further superoxide to form hydrogen peroxide. Hydrogen peroxide has been suggested as an endothelial-derived hyperpolarizing factor [14] and it may be the presence of free radicals generated by TEMPOL that causes the improved vasodilation to acetylcholine. However, this seems unlikely as TEMPOL had no detectable effect in control vessels. Furthermore, the lack of biological effects of catalase, and the results of combined L-NAME and indomethacin treatments favours superoxide chelation as the possible mechanism of TEMPOL.

Superoxide and free radical generation are not the only theories that have been put forward to explain the action of homocysteine in vivo. Dimethylamino-hydrolase (DDAH) is an enzyme that prevents the formation of the naturally occurring NOS inhibitor asymmetric dimethylarginine (ADMA) [15]. It has been suggested [16] that homocysteine can inhibit DDAH causing raised ADMA levels and hence greater NOS inhibition. However, L-arginine supplementation in high concentration (enough to reverse ADMA inhibition) had no beneficial effect excluding this intriguing mechanism at least within the in vitro setting. NOS is thought to be a source of free radicals when stimulated under conditions of co-factor depletion [17,18]. Because of the prolonged culture of the arteries may of resulted in leeching or sequestering of essential co-factors of nitric oxide production for other cellular purposes we gave a bolus dose to the incubating media. Addition of excess arginine and the co-factor tetrahydrobiopterin to our system failed to show an effect on the homocysteine(4 h)-treated vessel suggesting neither cofactor depletion nor enzyme inhibition as a reason for microvascular impairment. Nor was homocysteine responsible for any changes in vascular homeostasis as neither cyclo-oxygenase products nor endothelial-derived hyperpolarizing factors were affected by homocysteine incubation. Endothelial cell-independent vasorelaxation was also unaffected (Figure 3).

However, our results must be viewed with caution because of the relative short duration of exposure in our experiments compared to years of exposure in the clinical setting. Moreover, differences between unbound biological active homocysteine levels, different routes of disposal and the possibility of homocysteine acting in synergy with other vascular toxins may at times explain differences seen in animal models and clinical settings with regard to homocysteine toxicity. Nonetheless, our results are very interesting and can partly explain the lack of beneficial effects seen in improvement of endothelial function or overall mortality in randomized trials of therapeutic interventions of homocysteine level reduction. As our experiments were conducted in the absence of homocysteine after the initial incubation period, which consistently revealed TEMPOL-reversible endothelial dysfunction due to a lack of nitric oxide bioavailability, this would suggest that after exposure to elevated homocysteine, a permanent transition occurs in the redox status of vascular tissue. Lowering of homocysteine by folate or...
folinic acid supplementation in such a scenario may not be effective and would require concomitant administration of free radical scavengers. Beneficial effects of free radical scavengers (vitamin E or acetyl cysteine) in patients with chronic failure supports our findings and conclusions [19,20]. In conclusion, our findings suggest that homocysteine, a known cardiovascular risk factor, causes the up-regulation of a source of a superoxide generating system resulting in impaired endothelial-dependent vasorelaxation by reducing nitric oxide bioavailability. Homocysteine has no biological effects on prostanoid pathways or increased endothelial hyperpolarizing factor production. These findings are of clinical relevance in that physiologically relevant homocysteine concentration was used in these experiments. Our results may explain the reasons why therapeutic interventions to lower homocysteine levels alone are ineffective in uraemic populations because of permanent alterations in the redox status of vascular tissue after exposure to homocysteine. The basis of future studies in our laboratories suggest using a combination therapy of free radical scavengers and folate or folinic acid supplementation.

Conflict of interest statement. None declared.

References


Received for publication: 5.11.03
Accepted in revised form: 19.03.04