The concentration of homocysteine-derived disulfides in human coronary artery

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Abstract

Background Based on previous findings, we have estimated that, in injured coronary artery tissue, the low molecular weight disulfides homocystine and cysteine-homocysteine, otherwise identified as oxidized homocysteine equivalents (OHcyE), may achieve a total concentration that is higher than the aqueous solubility of homocystine at room temperature. In order to verify whether or not OHcyE could reach their saturation limit in the vascular tissue, we have measured the solubility of homocystine in physiological-like condition.

Materials and methods The solubility of homocystine has been measured in aqueous sodium chloride solutions at 37 °C by differential pulse polarography based on the reduction of homocystine to homocysteine.

Results We have estimated that the concentration achieved by OHcyE in injured coronary artery tissue is at least near-saturating, because the solubility of homocystine in physiological-like condition, above which deposition of homocystine and/or cysteine-homocysteine as solid phase occurs, almost exactly matches its value. Near-saturation levels of OHcyE within the vascular tissue means that significant leakage of intracellular fluid can promote OHcyE crystallization in tissue fluids, which may serve to initiate inflammation.

Conclusions We speculate that deposition of OHcyE crystals could damage blood vessels and act as a primer of homocysteine-triggered inflammation, thus being along the causal pathway that leads to vascular dysfunction.

Keywords Atherosclerosis, cardiovascular disease, homocysteine, homocystine solubility, risk factors, vascular dysfunction.

Introduction

Demethylation of the essential amino acid methionine produces the thiolic non-proteinogenic amino acid homocysteine [1]. Mild to moderate elevations of blood homocysteine have been associated with high risk of coronary heart disease and other vascular alterations [2,3]. Even though a causal role of homocysteine in cardiovascular disease remains to be established [4,5], it is believed that homocysteine excess may damage vascular tissue, so that blood homocysteine is currently considered as an independent index of vascular risk [6]. Mechanisms underlying homocysteine-associated vascular injury are under investigation [7,8]. Till now, almost all homocysteine-concerned research has assumed that elevation of the total plasma concentration of this amino acid (free and protein-bound) is a marker of its atherogenic potential. However, free homocysteine comprises three distinct fractions, i.e., reduced homocysteine, homocysteine-homocysteine homo-disulfide (homocystine) and cysteine-homocysteine hetero-disulfide. Since the reduced form barely amounts to 1-2% of the body's total homocysteine, the homocysteine pool exists almost entirely as homocystine and cysteine-homocysteine (20-30%), also referred to as 'oxidized homocysteine equivalents' (OHcyE), and protein-bound disulfide (70-80%) [9,10]. It is also worth considering that the total plasma concentration of cysteine, a proteinogenic amino acid that is the lower structural homologue of homocysteine because of one less $–CH_2–$ group in the side-chain, is 20- to 30-fold higher than that of homocysteine, and the concentration of reduced cysteine is ~70-fold higher than that of reduced homocysteine (5.0 ± 3.6 versus 0.07 ± 0.02 μmol/L, mean ± s.d.) [11]. Nevertheless, there is no evidence that cysteine causes vascular damage, but a toxic effect of homocysteine on endothelial cells has been widely claimed [8,12–14], possibly through thrombosis-promoting inflammatory pathways [15].

Recently, it has been pointed out that homocystine is the upper structural homologue of cystine (the homo-disulfide of cysteine) [16], which is known to be scarcely soluble [17] and

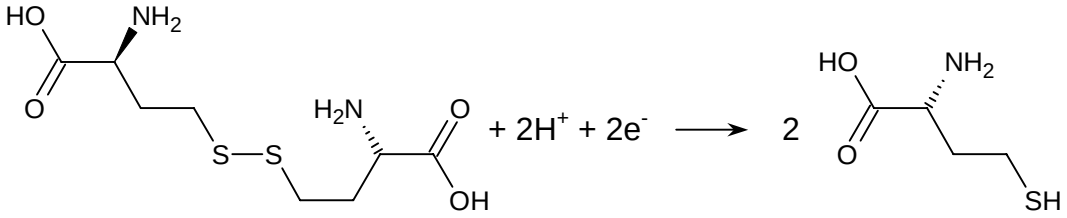
therefore capable to form kidney stones in genetically-disposed patients [18]. By analogy, it has been hypothesized [16] that vascular injury could be mechanically primed by the formation of homocystine crystals in the bloodstream, which could transiently grow after methionine intake and then dissolve during the time lag needed to reach basal conditions. In spite of the fact that, likely for the above reason, homocystine precipitates have never been found in endothelial cells or other tissues, it must be noticed that, in a population of patients with heart disease, levels of OHcyE were ~15 fold higher in the occluded coronary artery than in normal coronary artery [19]. We have estimated that, as a result of this elevation, the concentration of OHcyE in the atherosclerotic tissue is definitely higher than the aqueous solubility of homocystine at room temperature (unpublished data from our lab) and is affected by a statistical variability significantly lower than that observed in plasma. This has suggested that the concentration of OHcyE in the occluded coronary artery tissue could be close to saturation. Although intracellular water also contains proteins, lipids, polysaccharides, and other species that may influence phase transitions, prevention and treatment of solid deposition in the human body, such as crystals of xanthine, uric acid and urates, cystine, oxalates, etc, are based on the understanding of the physicochemical properties underlying the precipitation of the substances involved [20]. Pursuing this idea, we have measured the solubility of homocystine in aqueous sodium chloride solutions at physiological temperature.

Materials and Methods

Chemicals. Purissimum grade (≥99.0%) *DL*-homocystine [meso-4,4’-dithio-bis(2-aminobutanoic acid)] was purchased from Fluka. Ultra-pure (≥99.99%) sodium chloride and sodium azide from Baker and Aldrich, respectively, were dried at 120 °C and stored in a dryer before use. Surfactant and perchloric acid solutions were prepared by diluting Triton X-

100 as obtained from LKB Bromma and purum p.a. $HClO_4$ from Merck, respectively.

Solubility measurements. Differential pulse polarography (DPP) has become and remains an appreciated and trusted method in the study of solutions, thanks to the high reproducibility of the experimental curves. Therefore, the solubility (m_I, mol kg^{-1}) of homocystine was evaluated by this technique, based on the semireaction



Saturated homocystine solutions were prepared with a leaching apparatus suitable to prevent solid particles from coming into contact with the magnetic stirrer. In fact, preliminary measurements have showed an increase of solubility over periods of weeks when the solid was in mechanical contact with the stirrer, because the solid was transformed into a phase made of smaller particles. To avoid grinding by the stirrer, solid homocystine was wrapped up in a highly retentive filter paper (Whatman 42) bag. This in turn was kept immersed in a glass cylinder containing sodium chloride solution, to which 0.1% NaN_3 was added to prevent decomposition, while continuously stirring with a magnetic bar (Fig. 1). The cylinder was then placed in a thermostated water bath at 37.00±0.05 °C and the homocystine concentration was monitored in the time, until it reached a constant value, which usually took place in about 10-15 days. Finally, polarographic assays were performed when an exactly weighed aliquot of the saturated homocystine solution was added to 0.1 mol kg^{-1} $HClO_4$ and 0.003% Triton X-100 solution to remove the signal drift caused by the sudden intensity increase of the diffusion current. Air-free samples were obtained by bubbling with nitrogen for 15 min and DPP traces were recorded with a Metrohm 747 VA model dropping mercury electrode apparatus interfaced with a Metrohm 746 VA model trace analyzer, using saturated Ag/AgCl and Pt as reference and working electrodes, respectively. The instrumental settings

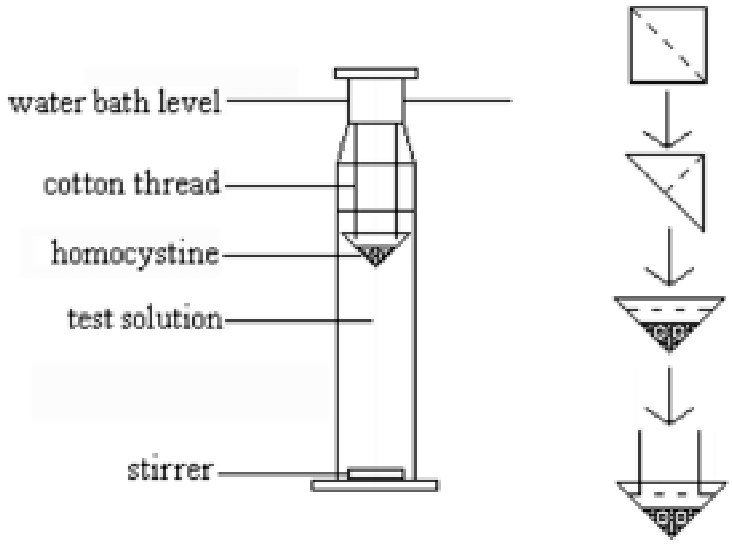


Figure 1. The apparatus for preparation of saturated homocystine solutions.

were as follows: 4 mV/s, scan rate; 50 mV, pulse amplitude; 60 ms, pulse time; 500 ms, drop lifetime. The potential sweep was from -1200 to -50 mV and the peak position around E_p = -710 mV with a half-peak width $w_{1/2}$ = 160 mV (Fig. 2). As no substantial solubility difference was found among *D*-, *L*- and *DL*-homocystine, the less expensive *DL*-isomer was employed,

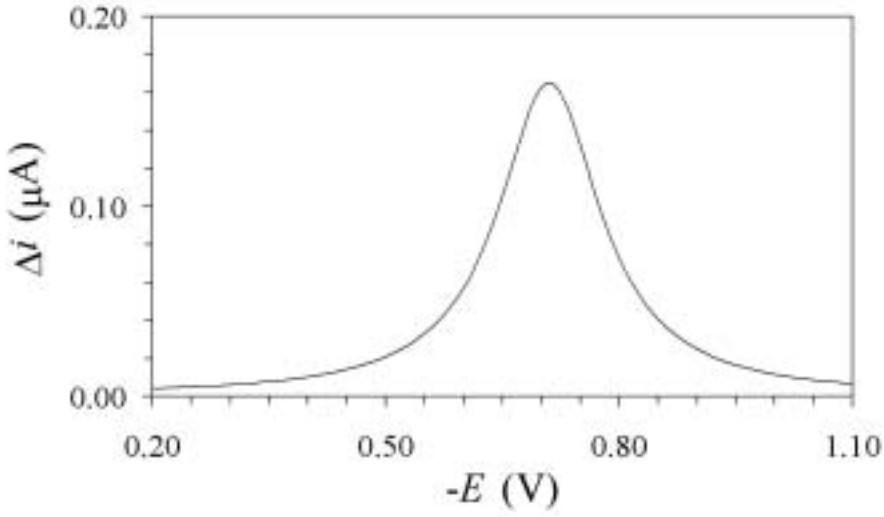


Figure 2. Differential pulse polarogram for *DL*-homocystine reduction. The curve represents the dependence of the current passing through the system (Δi, μA) on the electrochemical potential of the dropping electrode (*E*, V).

and its concentration was calculated using a calibration curve based on standard solutions (Fig. 3). Three replicates were run for each point. All calculations were performed with the program Scientist version 2.0 from Micromath, Saint Louis, Missouri.

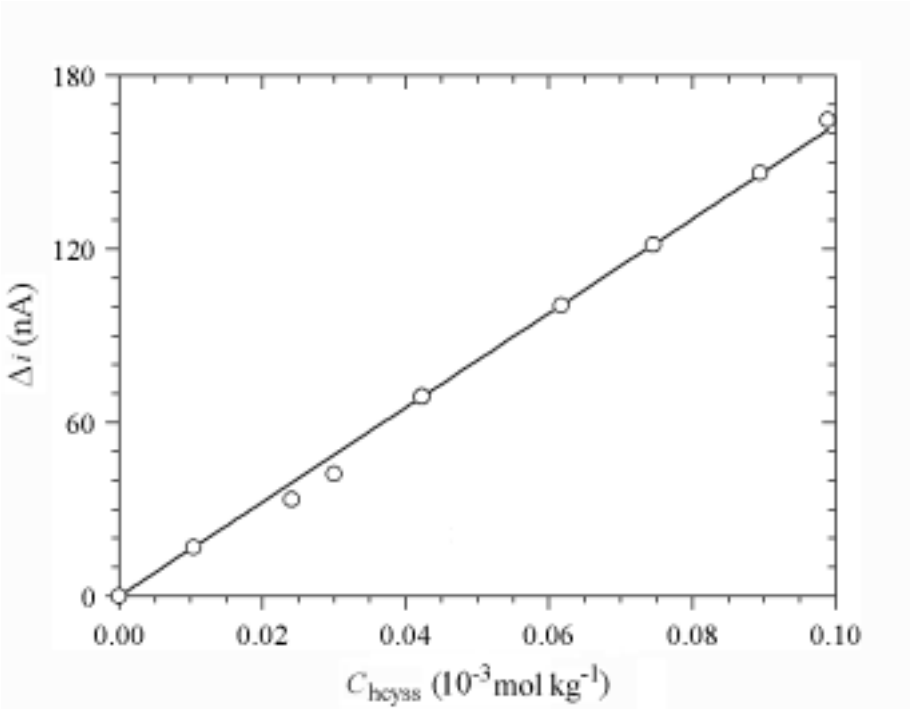


Figure 3. Calibration curve for *DL*-homocystine. The equation resulting from the linear regression analysis of the capacity current (Δi, nA) versus the concentration of standard solutions (C_{hCyss}, 10^{-3} mol kg^{-1}) was y = 1631.09x (R^2 = 0.999).

Results

Despite the fact that homocystine is a double zwitterion structurally homologous to cystine, the dependence of its solubility on the NaCl concentration is non-monotonic, whereas the solubility of cystine has been found to increase monotonically up to 4.4 mol kg^{-1} NaCl [21]. In fact, at very low NaCl concentrations, homocystine exhibits 'salting-in' behavior, with the solubility increasing with increasing ionic strength. Instead, higher salt concentrations have a 'salting-out' effect, and the solubility decreases with increased ionic strength. Solubility values were therefore fitted either to a parabolic function:

$$\ln m_{\mathrm{I}} = \ln m_0 + aC_{\mathrm{S}} + bC_{\mathrm{S}}^2 \qquad (1)$$

or with a two-parametric solubility dependence that is suitable to describe the activity

coefficients of zwitterionic amino acids [22]:

$$\ln m_I = \ln m_0 - \log\gamma_{\pm} = \ln m_0 - 2\chi_{AB}C_S - \xi_{ABB}m_I C_S^2 - 4\omega_{AAB}m_I C_S \qquad (2)$$

In these equations, m_0 is the solubility at zero ionic strength, *a* and *b* are empirical fitting parameters, $\gamma_{\pm}$ is the mean molal activity coefficient, $\chi_{AB,}$ ξ_{ABB} and ω_{AAB} are Pitzer-type interaction parameters [23,24], and C_S is the salt concentration, which equals the ionic strength (*I*) for a monovalent salt like NaCl.

Best fitting of data is graphically represented in Fig. 4. At present, it is most important to check whether homocystine can achieve such levels in the body that precipitation in the tissue

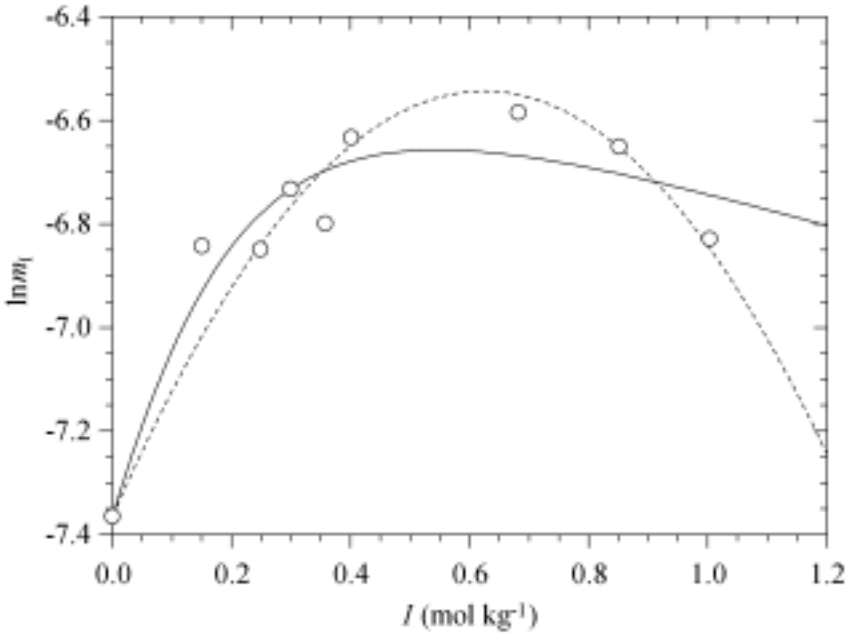


Figure 4. Solubility of *DL*-homocystine at 37 °C as a function of the ionic strength. The curves represent best fitting of experimental data to equation 1 (dashed) or 2 (solid), where lnm_I is the natural logarithm of the solubility and *I* the ionic strength expressed in mol kg^{-1}.

may occur. We notice therefore that its solubility in 0.17mol kg^{-1} NaCl lies somewhere between 0.93x10^{-3} and 1.02x10^{-3} mol kg^{-1}, as results from equations 1 and 2, respectively. This represents the saturation limit in a condition corresponding to a physiological environment and is best compared with homocystine levels that were previously measured in the vascular tissue [19], according to the view that intracellular homocysteine is likely the

form that triggers adverse cellular events [10].

Discussion

In assessing a comparison between normal vascular tissue and atherogenic tissue obtained from occluded coronary artery, Tyagi and co-workers [19] provided evidence that elevation of OHcyE in the pathological tissue (1.5 ± 0.3 μg per mg of total protein versus a ~15-fold lower concentration in normal controls) is much larger than the pathologically significant increase reported for plasma homocysteine and is likely responsible for the development of atherosclerotic lesions and vascular dysfunction. Taking into account that the mass of water in the adult human heart is ~4-fold the protein content [25], we have converted these values into the molal concentration units used here and estimated that the levels of OHcyE reported in [19] amount to ~0.9-1x10^{-4} and ~1.4-1.5x10^{-3} mol kg^{-1} in normal and atherogenic tissue, respectively. To substantiate this datum, we have then compared the OHcyE levels in the atherogenic tissue with the solubility of homocystine in a condition that models the plasma ionic strength (~0.93-1.02x10^{-3} mol kg^{-1} in 0.17 mol kg^{-1} NaCl at 37 °C, see Results).

As the solubility of the cysteine-homocysteine disulfide is expected to be intermediate between the solubilities of cystine (~5x10^{-4} mol kg^{-1} in 0.2 mol kg^{-1} NaCl at 25 °C, see [21]) and homocystine (as measured by us), it is apparent that the concentration reached by OHcyE in the atherogenic tissue almost exactly matches their saturation limit *in vitro*. We have also noticed that the statistical variability of the OHcyE elevation in the atherogenic tissue, as reported in [19], is significantly lower than that usually observed from population studies on plasma levels. This is evidence in support of the hypothesis that OHcyE levels in the injured tissue are close to saturation, a condition that is characterized by constancy of their concentration and, therefore, by reduced dispersion of measurements. Finally, consideration that the saturation concentration of OHcyE in the tissue is expected to be even lower

compared to blood levels, because tissue fluids have lower ionic strength, strengthens this hypothesis.

The present analysis identifies the vascular tissue, instead of the bloodstream [16], as the possible location of near-saturation levels of OHcyE. This implies that significant leakage of intracellular fluid can promote OHcyE crystallization in tissue fluids. On this basis, we speculate that deposition of homocystine and/or cysteine-homocysteine crystals could be along the causal pathway that leads to vascular dysfunction, in much the same manner as a concentration of sodium urate above the solubility level is most likely the culprit responsible for gout. In the case of gout, however, chronic cumulative urate crystal formation in tissue fluids leads to urate deposition in tissues as tophi, but no evidence has ever been presented in the scientific literature in the 75 years since the discovery of homocystine to support our conclusion about deposition of OHcyE crystals. To this we reply that deposition of OHcyE microcrystals in the tissue could not necessarily result in massive accumulations and be hard to recognize with the naked eye or even with usual diagnostic tools, thus demanding investigation adequately taking aim at their identification.

As a matter of fact, our measurements permit to evaluate that the levels of total plasma homocysteine present in coronary heart disease and in normal patients are ~96-fold [$(11.5 \pm 5.8)\text{x}10^{-6}$ M] and ~113-fold [$(9.7 \pm 4.9)\text{x}10^{-6}$ M] (mean ± s.d.) [19] lower than the presumed saturation limit of homocystine, respectively. On this aspect, it has been reported that elevated levels of plasma homocysteine may represent, at least in part, the effect of vascular dysfunction [26], based on the concept that plasma homocysteine may be released from damaged tissues, since repair of DNA, RNA, and protein involves methylation and increased generation of S-adenosylhomocysteine and homocysteine within the cell. Whether or not OHcyE levels are saturating, further studies are necessary to clarify what makes their concentration achieve those values in the atherogenic tissue.

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