Homocysteine disulphides and vascular disease

Mauro Iuliano\textsuperscript{a}, Gaetano De Tommaso\textsuperscript{a} and Raffaele Ragone\textsuperscript{b,}\textsuperscript{*}
\textsuperscript{a}Dipartimento di Chimica, Universit`a Federico II, Naples, Italy
\textsuperscript{b}Dipartimento delle Scienze Biologiche, Universit`a Federico II, Naples, Italy

The final draft of this manuscript was ready on occasion of the retirement of Professor Liberato Ciavatta, who has been an inspiration to a generation of students. M.I., G.D.T. and R.R. wish to dedicate this paper to his integrity and rigor in scientific research.

Abstract. The total plasma concentration of homocysteine is a marker of this amino acid’s atherogenic potential. However, the homocysteine pool exists almost entirely as oxidized homocysteine equivalents (OHcyE), composed of homocystine and cysteine-homocysteine disulphides (20–30%), and protein-bound disulphide (70–80%). We have noticed that the total concentration of OHcyE in injured coronary artery tissue is higher than the aqueous solubility of homocystine (∼1.4–1.5 × 10^{-3} mol kg^{-1} versus ∼0.6 mol kg^{-1}). Based on the measurement of the solubility of homocystine in a plasma-mimetic condition (0.17 mol kg^{-1} NaCl at 37°C), we have estimated that OHcyE may really reach their saturation limit in the vascular tissue (∼0.93–1.02 × 10^{-3} mol kg^{-1}), above which their deposition as solid phase may occur. This means that significant leakage of intracellular fluid can promote OHcyE crystallization in tissue fluids, which may serve to initiate inflammation. 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, homocysteine solubility, risk factors, vascular dysfunction

Abbreviations: OHcyE, oxidized homocysteine equivalents; DPP, differential pulse polarography

1. 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 [2–4]. Even though a causal role of homocysteine in cardiovascular disease remains to be established [5–7], it is believed that homocysteine excess may damage vascular tissue, so that blood homocysteine is currently considered as an independent index of vascular risk [8–10]. Mechanisms underlying homocysteine-associated vascular injury are under investigation [11,12]. 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-disulphide (homocystine) and cysteine-homocysteine hetero-disulphide. 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 disulphide (70–80%) [13,14]. 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 –CH₂– 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.) [15]. Nevertheless, there is no evidence that cysteine causes vascular damage, but a toxic effect of homocysteine on endothelial cells has been widely claimed [12,16–18], possibly through thrombosis-promoting inflammatory pathways [19].

Recently, it has been pointed out that homocysteine is the upper structural homologue of cysteine (the homodisulphide of cysteine) [20], which is known to be scarcely soluble [21] and therefore capable to form kidney stones in genetically-disposed patients [22]. By analogy, it has been hypothesized [20] that vascular injury could be mechanically primed by the formation of homocysteine 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 is worth mentioning that, in a population of patients with heart disease, levels of OHcyE were close to 1.4–1.5 × 10⁻³ mol kg⁻¹, which is ~15 fold higher than in normal coronary tissue [23] (see Discussion for more details). At a glance, this elevation corresponds to a concentration of OHcyE higher than the aqueous solubility of homocysteine at room temperature (unpublished data from our lab), and is affected by a statistical variability significantly lower than that observed in plasma. Indeed, the saturation concentration of OHcyE in the occluded coronary artery tissue can be reasonably placed between ~5 × 10⁻⁴ and ~0.9–1.0 × 10⁻⁵ mol kg⁻¹ at the plasma ionic strength, depending on the relative abundance of cysteine-homocysteine and homocysteine (see Discussion for more details). The equilibrium relationship involving cystine, homocysteine and mixed disulphide under physiological concentrations of cysteine and homocysteine has been previously investigated [14,24,25].

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 [26]. Pursuing this idea, we have measured the solubility of homocysteine in aqueous sodium chloride solutions at physiological temperature.

2. Materials and methods

2.1. 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. HClO₄ from Merck, respectively.

2.2. 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 (m₁, mol kg⁻¹) of homocysteine was evaluated by this technique, based on the semireaction

\[
\text{O} \quad \text{OH} \quad \text{NH}_2 \quad \text{O} \quad + 2H^+ + 2e^- \rightarrow 2 \text{HO} \quad \text{NH}_3 \quad \text{SH}
\]

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 homocysteine 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% NaN₃ 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 homocysteine 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 homocysteine solution was added to 0.1 mol kg⁻¹
HClO₄ 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 746 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 were as rated Ag/AgCl and Pt as reference and working electronion structurally homologous to cystine, the depen-
dence 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 [27]. Indeed, at NaCl concentrations covering the physiological range, 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 (see Table 1). Solubility values were therefore fitted either to a parabolic function:

\[
\ln m_I = \ln m_0 + a C_S + b C_S^2
\]

(1)
or with a two-parametric solubility dependence that is suitable to describe the activity coefficients of zwitterionic amino acids [28]:

\[
\ln m_I = \ln m_0 - \log \gamma_{\pm} = \ln m_0 - 2 \chi_{AB} C_S - \xi_{ABB} m_I C_S^2 - 4 \omega_{AAB} m_I C_S
\]

(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 molar activity coefficient, \( \chi_{AB}, \xi_{ABB} \) and \( \omega_{AAB} \) are Pitzer-type interaction parameters [29,30], 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. Use of Eq. (1) gave \( a = 2.63 \pm 0.17 \text{ kg mol}^{-1} \) (mean ± s.d.) and \( b = -2.11 \pm 0.21 \text{ kg mol}^{-2} \) (mean ± s.d.) \( (R^2 = 0.895) \), whereas equation 2 resulted in \( \chi_{AB} = -2.51 \pm 1.60 \text{ kg mol}^{-1} \) (mean ± s.d.), \( \xi_{ABB} = 1837 \pm 552 \text{ kg}^3 \text{ mol}^{-3} \) (mean ± s.d.), and \( \omega_{AAB} = 475 \pm 689 \text{ kg}^2 \text{ mol}^{-2} \) (mean ± s.d.) \( (R^2 = 0.902) \). A discussion on physical meaning and values of these coefficients is outside the scope of this study. At present, it is most important to check whether homocystine can achieve such levels in the body that precipitation in the tissue may occur. In general, ionic

| \( I \)   | \( m_I \pm \text{s.e.m.} \) | \( (10^{-2} \text{ mol kg}^{-1}) \) |
|---------|----------------|----------------|
| 0.0000  | 0.633 ± 0.003 |                 |
| 0.1501  | 1.067 ± 0.003 |                 |
| 0.2485  | 1.060 ± 0.003 |                 |
| 0.2991  | 1.191 ± 0.003 |                 |
| 0.3574  | 1.115 ± 0.002 |                 |
| 0.4010  | 1.316 ± 0.002 |                 |
| 0.6822  | 1.381 ± 0.002 |                 |
| 0.8515  | 1.293 ± 0.002 |                 |
| 1.0034  | 1.082 ± 0.002 |                 |

*measurements performed at 37°C.

\(^b\)standard error of the mean.
Fig. 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).

Fig. 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).

strength is the physicochemical factor that largely dominates the solubility of charged solutes. Thus, the use of NaCl alone is sufficient to mimic effects that may occur in vivo, whenever caused by the contribution of salts of different nature. It can be easily estimated by equations 1 and 2, respectively, that the solubility of homocystine in 0.17 mol kg^{-1} NaCl, which mimics the plasma ionic strength, lies somewhere between 0.93 \times 10^{-3} and 1.02 \times 10^{-3} mol kg^{-1}. 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 [23], according to the view that intracellular homocysteine is likely the form that triggers adverse cellular events [14].

4. Discussion

In assessing a comparison between normal vascular tissue and atherogenic tissue obtained from occluded coronary artery, Tyagi and co-workers [23] 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 respon-
Fig. 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 \( \ln m_I \) is the natural logarithm of the solubility and \( I \) the ionic strength expressed in mol kg\(^{-1}\).

Possible for the development of atherosclerotic lesions and vascular dysfunction. Taking into account that the mass of water in the adult human heart is \( \sim 4 \)-fold the protein content [31], we have converted these values into the molar concentration units used here and estimated that the levels of OHcyE previously reported [23] amount to \( \sim 0.9–1 \times 10^{-4} \) and \( \sim 1.4–1.5 \times 10^{-3} \) 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 homocysteine at the plasma ionic strength (\( \sim 0.93–1.02 \times 10^{-3} \) mol kg\(^{-1}\) in 0.17 mol kg\(^{-1}\) NaCl at 37°C, see Results).

As the solubility of the cysteine-homocysteine disulphide is expected to be intermediate between the solubilities of cystine (\( \sim 5 \times 10^{-4} \) mol kg\(^{-1}\) in 0.2 mol kg\(^{-1}\) NaCl at 25°C [27]) and homocysteine (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, which implies that OHcyE levels in the injured tissue are saturating. This conclusion is strengthened by two further observations. First, the statistical variability of the OHcyE elevation in the atherogenic tissue, as previously reported [23], is significantly lower than that usually observed from population studies on plasma levels. In general, saturating conditions are characterized by reduced dispersion of measurements, because the concentration of the solute in equilibrium with the solid phase is constant and not anymore affected by linkage with other equilibria. Second, the saturation concentration of OHcyE in the tissue is expected to be even lower compared to blood levels, owing to the lower ionic strength of tissue fluids.

Compared to the prediction of homocysteine solubility and to the hypothesis that the bloodstream could be the possible location of near-saturation levels of OHcyE [20], the measurements that we have now performed substantiate the view that significant leakage of intracellular fluid can promote OHcyE crystallization in vascular tissue. On this basis, we speculate that deposition of homocysteine 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 could result in the formation of microcrystals in the tissue, but not in massive accumulations, thus being hard to recognize with the naked eye or even with usual diagnostic tools, and demanding investigation taking adequately aim at their identification.

Understanding the physicochemical properties of OHcyE deposition in vitro is of foremost importance,
in the light of the differences previously observed between normal vascular tissue and atherogenic tissue obtained from occluded coronary artery [23]. As stated above, in all cases of biocrystallization, the transition to the solid phase may be influenced by other species dissolved in intracellular water, such as amino acids, glucose, uric acid, proteins, lipids, polysaccharides, etc. However, alteration of the OHcyE solubility most likely depends on ionic strength modifications, which in turn depend on the concentration of ionic species. As such, NaCl alone, which is by far the most populated ionic species, and therefore the major determinant of ionic strength in the body fluids, should be sufficient to mimic major effects that may occur in vivo.

As a matter of fact, our measurements show that the saturation limit of homocysteine dissolved in a NaCl solution of concentration close to the plasma ionic strength is roughly two orders of magnitude higher than the levels of total plasma homocysteine present in both cardiovascular disease and normal patients [23, 32]. As a consequence, OHcyE may achieve saturation in the atherogenic tissue. Based on the concept that plasma homocysteine may be released from damaged tissues, this seems to support the view that elevated levels of total plasma homocysteine may represent, at least in part, the effect, and not solely the cause, of vascular dysfunction [33], since repair of DNA, RNA, and protein involves methylation and increased generation of S-adenosylhomocysteine and homocysteine within the cell [34]. This also means that total plasma homocysteine might not be the most appropriate index for cardiovascular risk [14], and most clinical applications should adopt OHcyE as a more direct marker, also considering that, based on a thorough review of the published literature, homocysteine has not met all of the stated criteria required for acceptance as a biomarker for risk assessment in primary prevention [35]. We also believe that prevention and treatment of vascular disease would be greatly improved by the comprehension of mechanisms causing OHcyE saturation in the atherogenic tissue.

Acknowledgements

Funding sources supporting this work were from the Council for Scientific Research of the Regione Campania, Italy.

References

[1] L.J. Langman and D.E.C. Cole, Homocysteine: cholesterol of the 90s? Clin Chim Acta 286 (1999), 63–80.
[2] G.J. Hankey and J.W. Eikelboom, Homocysteine and vascular disease, Lancet 354 (1999), 407–413.
[3] S. Seshadri, A. Beiser, J. Selhub, P.F. Jacques, I.H. Rosenberg, R.B. D’Agostino, P.W. Wilson and P.A. Wolf, Plasma homocysteine as a risk factor for dementia and Alzheimer’s disease, N Engl J Med 346 (2002), 476–483.
[4] J. Selhub, Public health significance of elevated homocysteine, Food Nutr Bull 29 (2008), S116–S125.
[5] J.D. Finkelstein and J.J. Martin, Homocysteine, Int J Biochem Cell Biol 32 (2000), 385–389.
[6] E. Falk, J. Zhou and J. Møller, Homocysteine and atherothrombosis, Lipids 36 (2001), S3–S11.
[7] J. Selhub, The many facets of hyperhomocysteinemia: studies from the Framingham cohorts, J Nutr 136 (2006), 1726S–1730S.
[8] O. Nygård, S.E. Vollset, H. Refsum, L. Brattström and P.M. Ueland, Total homocysteine and cardiovascular disease, J Intern Med 246 (1999), 425–454.
[9] R. Castro, I. Rivera, H.J. Blom, C. Jakobs and I. Tavares de Almeida, Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview, J Inherit Metab Dis 29 (2006), 3–20.
[10] L.L. Humphrey, R. Fu, K. Rogers, M. Freeman and M. Helfand, Homocysteine level and coronary heart disease incidence: a systematic review and meta-analysis, Mayo Clin Proc 83 (2008), 1203–1212.
[11] A. D’Angelo and J. Selhub, Homocysteine and thrombotic disease, Blood 90 (1997), 1–11.
[12] S.R. Lentz, Mechanisms of homocysteine-induced atherothrombosis, J Thromb Haemost 3 (2005), 1646–1654.
[13] D.W. Jacobson, Homocysteine and vitamins in cardiovascular disease, Clin Chem 44 (1998), 1833–1843.
[14] E.B. Stamm and R.D. Reynolds, Plasma total homocyst(e)ine may not be the most appropriate index for cardiovascular disease risk, J Nutr 129 (1999), 1927–1930.
[15] M.A. Mansoor, A.M. Svardal, J. Schneede and P.M. Ueland, Dynamic relation between reduced, oxidized, and protein-bound homocysteine and other thiol components in plasma during methionine loading in healthy men, Clin Chem 38 (1992), 1316–1321.
[16] N. Weiss, C. Keller, U. Hoffmann and J. Loscalzo, Endothelial dysfunction and atherothrombosis in mild hyperhomocysteinemia, Vasc Med 7 (2002), 227–239.
[17] N. Weiss, S.J. Heydrick, O. Postea, C. Keller, J.F. Jr. Keaney and J. Loscalzo, Influence of hyperhomocysteinemia on the cellular redox state – impact on homocysteine-induced endothelial dysfunction, Clin Chem Lab Med 41 (2003), 1455–1461.
[18] R.C. Austin, S.R. Lentz and G.H. Werstuck, Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease, Cell Death Differ 11 (2004), S56–S64.
[19] P. Libby, Inflammation in atherosclerosis, Nature 420 (2002), 868–874.
[20] R. Ragone, Homocysteine solubility and vascular disease, FASEB J 16 (2002), 401–404.
[21] E. Königberger, Z. Wang and L.-C. Königberger, Solubility of L-cysteine in NaCl and artificial urine solutions, Monatsh Chem 130 (1999), 39–45.
[22] T. Knoll, A. Zöllner, G. Wendt-Nordahl, M.S. Michel and P. Alken, Cystinuria in childhood and adolescence: recom
mendations for diagnosis, treatment, and follow-up, *Pediatr Nephrol* **20** (2005), 19–24.

[23] S.C. Tyagi, L.M. Smiley, V.S. Majumdar, B. Clonts and J.L. Parker, Reduction-oxidation (Redox) and vascular tissue level of homocysteine in human coronary atherosclerotic lesions and role in extracellular matrix remodeling and vascular tone. *Mol Cell Biochem* **181** (1998), 107–116.

[24] S. Sengupta, C. Wehbe, A.K. Majors, M.E. Ketterer, P.M. DiBello, D.W. Jacobsen, Relative roles of albumin and ceruloplasmin in the formation of homocysteine, homocysteine-cysteine-mixed disulphide, and cystine in circulation, *J Biol Chem* **276** (2001), 46896–46904.

[25] P.J. Lee and A. Briddon, A rationale for cystine supplementation in severe homocystinuria, *J Inherit Metab Dis* **30** (2007), 35–38.

[26] E. Königsberger and L.-C. Königsberger, Thermodynamic modeling of crystal deposition in humans, *Pure Appl Chem* **73** (2001), 785–797.

[27] E.J. Cohn, T.L. McMeekin and M.H. Blanchard, Studies in the physical chemistry of amino acids, peptides, and related substances: XI. The solubility of cystine in the presence of ions and another dipolar ion, *J Gen Physiol* **21** (1938), 651–663.

[28] E.N. Tsurko and N.V. Bondarev, Interparticle interaction in solutions of beta-alanine, valine and glutamic acid from concentration dependence of activity coefficients of their charged and zwitterionic forms at various ionic strengths, *J Mol Liquids* **113** (2004), 29–36.

[29] K.S. Pitzer, A thermodynamic model for aqueous solutions of liquid-like density, *Rev Mineral Geochem* **17** (1987), 97–142.

[30] K.S. Pitzer, Ion interaction approach: theory and data correlation, in: *Activity coefficients in electrolyte solutions*, (2nd edn), K.S. Pitzer, ed., CRC Press, Boca Raton, 1991, pp. 75–153.

[31] R.M. Forbes, H.H. Mitchell and A.R. Cooper, Further studies on the gross composition and mineral elements of the adult human body, *J Biol Chem* **223** (1956), 969–975.

[32] S.S. Moselhy and S.H. Demerdash, Plasma homocysteine and oxidative stress in cardiovascular disease, *DisMarkers* **19** (2003-2004), 27–31.

[33] N.P. Dudman, An alternative view of homocysteine, *Lancet* **354** (1999), 2072–2074.

[34] M.A. Hofmann, E. Lalla, Y. Lu, M.R. Gleason, B.M. Wolf, N. Tanji, L.J. Jr. Ferran, B. Kohl, V. Rao, W. Kisel, D.M. Stern and A.M. Schmidt, Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model, *J Clin Invest* **107** (2001), 675–683.

[35] G.L. Myers, R.H. Christenson, M. Cushman, C.M. Ballantyne, G.R. Cooper, C.M. Pfeiffer, S.M. Grundy, D.R. Labarthe, D. Levy, N. Rifai and P.W. Wilson, National Academy of Clinical Biochemistry Laboratory Medicine Practice guidelines: emerging biomarkers for primary prevention of cardiovascular disease, *Clin Chem* **55** (2009), 378–384.