Amino Acids (2021) 53:739–744
https://doi.org/10.1007/s00726-021-02979-9

ORIGINAL ARTICLE

Identifying a role for the interaction of homocysteine and copper in promoting cardiovascular-related damage

Megha Gupta[1] · Jiries Meehan-Atrash[1] · Robert M. Strongin[1]

Received: 15 January 2021 / Accepted: 3 April 2021 / Published online: 22 April 2021

Abstract

Observations that copper and homocysteine levels are simultaneously elevated in patients with cardiovascular disease has generated interest in investigating the interactions between copper and homocysteine. Several prior studies have shown that complexes of copper and homocysteine are toxic, leading to cardiovascular damage in vitro. It is not clear, however, why related effects do not occur with other structurally similar, more abundant cellular thiols such as glutathione and cysteine. Herein, a mechanism for a selective redox interaction between copper and homocysteine is demonstrated. It involves a kinetically favored intramolecular hydrogen atom transfer that results in an alpha-amino carbon-centered radical known to promote biomolecular damage.

Keywords Homocysteine · Diketopiperazine · Hydrogen atom transfer · Alpha carbon radical · Cardiovascular disease · Copper · Thiyl radical

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, accounting for approximately 17.9 million deaths in 2016 according to the World Health Organization. In addition, by 2030, CVD-related deaths are projected to reach an annual rate of 23.6 million globally (Vittorini and Clerico 2008). There is a need to better understand the biological mechanisms related to CVD, particularly those not clearly associated with the traditional factors such as smoking, obesity, hypertension and diabetes (Fonseca et al. 2004; Aje 2009; Balagopal et al. 2011). To this end, alternative risk factors and biomarkers for CVD are of significant interest (Vittorini and Clerico 2008).

Elevated circulating levels (> 12–15 μM) of homocysteine (Hcy) are associated with CVD. Aberrant levels of Hcy are also linked to Alzheimer's disease, stroke, birth defects, osteoporosis, cancer and many other major illnesses (Schalinske and Smazal 2012). Hcy, however, is still not widely accepted as a CVD biomarker, despite intensive study since 1991 (North American Symptomatic Carotid Endarterectomy Trial Collaborators 1991; Codoñer-Franch and Alonso-Iglesias 2016). Biomarkers can be classified as either risk factors that play causal roles in disease or as risk markers that are associated with a disease but do not have a clear role. The identification of new risk factors enables the understanding of mechanisms that allow for the development of novel treatment strategies (Tamura et al. 1996; DeGoma et al. 2012). Biomarkers used by clinicians typically are required to play a causal role in a disease (Selleck et al. 2017). Therefore, defining the role of Hcy in CVD is essential for it to be optimally used as a non-traditional prognostic and diagnostic tool.

Large clinical trials involving vitamin B and folate supplementation therapy to lower Hcy levels have not led to clear evidence showing a reduction in CVD (Smulders and Blom 2011; Baggott and Tamura 2015). and the putative role of Hcy in disease continues to be explored (Al Mutairi 2020). Baggot and Tamura (2015) investigated the iron-promoted demethylation of methionine (Met) to Hcy. They concluded that iron may be the actual CVD causative agent, with elevated levels of Hcy simply serving as a surrogate measure of non-protein-bound iron. More recently, Jakubowski and co-workers (Borowczyk et al. 2018) showed that copper promotes relatively more

Handling editor: H. Jakubowski.

✉ Robert M. Strongin
strongin@pdx.edu

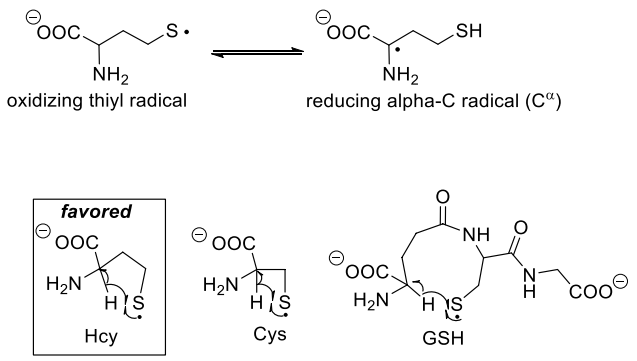
[1] Department of Chemistry, Portland State University, Portland, OR 97207, USA

efficient demethylation of Met to Hcy. Copper and iron are well-known to mediate Fenton reactions. Schöneich (Hong and Schöneich 2001; Mozziconacci et al. 2013) has postulated that a Fenton reaction involving Met protein residues leads to a sulfur-centered radical cation intermediate that aids the initiation of the demethylation mechanism of Met to Hcy.

In addition to promoting the production of Hcy, copper also forms complexes with Hcy that are detrimental to endothelial cells (Kang 2011) and the cardiovascular system (Apostolova et al. 2003; Carrasco-Pozo et al. 2006). Kang (2011) has attributed the enhanced toxicity of Cu-Hcy complexes to changes in the redox status of copper by Hcy. For example, Hcy significantly enhances the low-density lipoprotein (LDL) oxidase activity of ceruloplasmin via Hcy-induced conversion of Cu (II)-ceruloplasmin to Cu (I)-ceruloplasmin (Exner et al. 2002). In addition, the Cu (I) chelator, bathocuproide disulphonate, inhibits Cu-Hcy toxicity to cultured primary neurons (White et al. 2001).

Although studies support the fact that the reduction of Cu (II) to Cu (I) via Hcy plays a role in cellular toxicity and tissue damage, (Hill et al. 1999; Mansoor et al. 2000; White et al. 2001; Jeremy et al. 2002; Koupparis et al. 2006; Shukla et al. 2007) it is currently not clear why Cu-Hcy, but not copper complexes with cysteine (Cys) or glutathione (GSH), is so strongly linked to cardiovascular damage via redox interactions. In prior investigations, we have shown that Hcy exhibits distinctive redox chemistry compared to other biothiols (Sibrian-Vazquez et al. 2010). This is because the Hcy thiyl radical can undergo a kinetically favored hydrogen atom transfer (HAT) reaction to afford a captodatively stabilized C^{α}-radical (Scheme 1). This property of Hcy was first proposed by Zhao and co-workers (1994) to occur under basic conditions (wherein the amino group is predominantly neutral to promote captodative stabilization), and subsequently shown by us to occur under physiological conditions and in human blood plasma (Wang et al. 2004, 2005). The HAT mechanism is kinetically favored for Hcy compared to other biological thiols due to the 5-membered ring transition state. This unique property of Hcy, for example, allows its selective detection over Cys and GSH in neutral buffer and in human blood plasma using mildly oxidizing chromogens such as methyl viologen (Wang et al. 2004, 2005, 2009; Sibrian-Vazquez et al. 2010). This is because the C^{α}-radical is a relatively potent reducing agent whereas the thiyl radical is oxidizing (Zhao et al. 1994). C^{α}-radical and the thiyl radical reduction potentials have been reported as E^0 ($NH_2=CHR^+/NH_2CHR^{\cdot}$) = −1.9 V and E^0 ($RS^{\cdot}$, H^+/RSH) = +1.3 V vs NHE, respectively (Wardman 1989; Zhao et al. 1994).

The hypothesis driving the study described herein is that a redox interaction between Hcy and copper leads to HAT from sulfur to form the alpha-amino acid carbon radical. Since the HAT process is less favorable for Cys and GSH thiyl radicals, this hypothesis potentially explains the unique redox behavior and toxicity of Cu-Hcy complexes (Kang, 2011). Importantly, C^{α}-radicals are well-known to lead to free radical and oxidative biomolecule damage, including protein carbonyl formation and peptide fragmentation (Sibrian-Vazquez et al. 2010; Schöneich 2012).



Scheme 1 The Hcy thiyl radical undergoes a kinetically favored intramolecular hydrogen atom transfer (HAT) to form a reducing Cα-radical

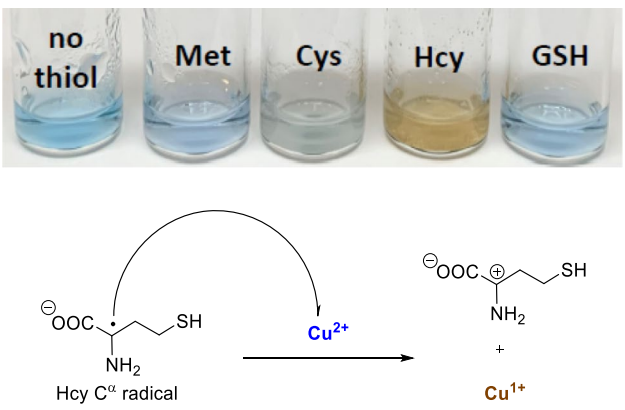


Fig. 1 Cu2+as a selective oxidant of Hcy. Conditions: solutions heated at reflux for 2 min containing, 0.1 ml of 0.5 M Tris buffer pH=7.5, 0.5 ml of 5 mM thiols and CuCl2 (1:1)

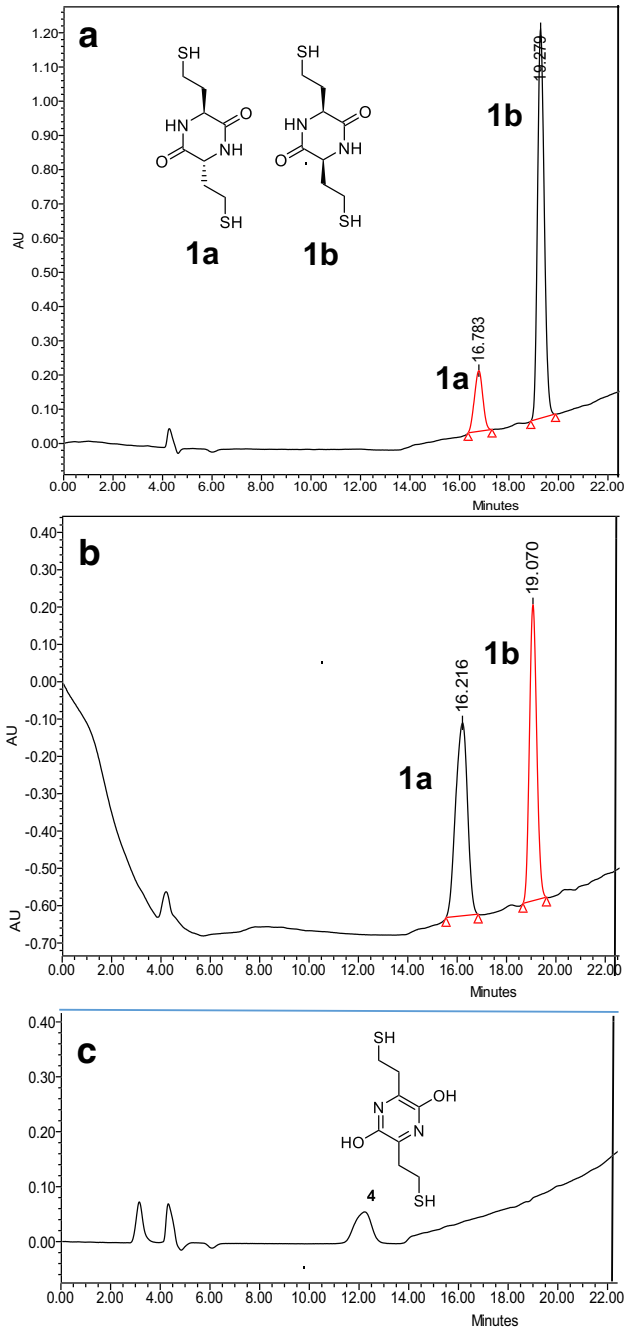


Fig. 2 HPLC chromatograms (200 nm) showing: **A**, Peaks corresponding to diketopiperazine stereoisomers **1a** and **1b**; **B**, chromatogram of **1a** and **1b** solution after gentle reflux for 2 min; **C**, chromatogram of a solution of **1a** and **1b** in the presence of CuCl2 (1 equiv) after 10 min at room temperature, showing the disappearance of **1a** and **1b** and the formation of **4**. The formation of **4** is strong evidence for the HAT mechanism (Scheme 2)

Results and discussion

To investigate the unique redox interaction between Hcy and copper, $CuCl_2$ was dissolved in neutral buffer with either Met, Cys, Hcy or GSH. Figure 1 shows that a selective colorimetric reaction occurs between Cu (II) and Hcy, as compared to Cu (II) and Met, Cys or GSH. The Hcy-containing solution turned yellowish-brown from blue, indicative of Cu (I) formation, as observed previously (Apostolova et al. 2003).

To demonstrate that the Hcy selectivity shown in Fig. 1 is due to the kinetically favored HAT process, analogous to what was previously observed for Hcy and peptide-bound Hcy (Sibrian-Vazquez et al. 2010), the effect of copper on the oxidation of the Hcy diketopiperazine **1** was investigated. Chiral diketopiperazines are excellent models for studying the oxidation and racemization of peptides occurring via the generation of C^{α} radicals since they have similar bond angles to larger naturally occurring peptides. In addition, their ready formation of diastereomers upon racemization as a result of C^{α} formation obviates the need for chiral analytical columns to follow the transformation (Mieden and Von Sonntag 1989; Sibrian-Vazquez et al. 2010).

Diketopiperazine **1** (1 mM, as a mixture of **1a** and **1b**, Fig. 2) was synthesized as described previously (Vigneaud et al 1938) and dissolved in MeOH: H_2O (7:3). Figure 2 shows the HPLC chromatogram of the diastereoisomers **1a** and **1b** before (Panel A) and after heating for 2 min at a gentle reflux (Panel B).

When $CuCl_2$ (1 eq.) is present in the solution containing **1a** and **1b**, the HPLC chromatogram of the mixture (Panel C, Fig. 2) shows rapid disappearance of **1a** and **1b** at room temperature, in < 10 min, along with conversion to **4** and disulfides (Fig. 3). Disulfide formation is well-known as an accompanying, competing process for HAT in the case of Hcy since HAT involves thiyl radicals. Importantly, the production of **4** in the presence of Cu (II) is strong evidence for the formation of a captodatively stabilized Hcy C^{α}-radical intermediate (Sibrian-Vazquez et al. 2010) via the mechanism shown in Scheme 2.

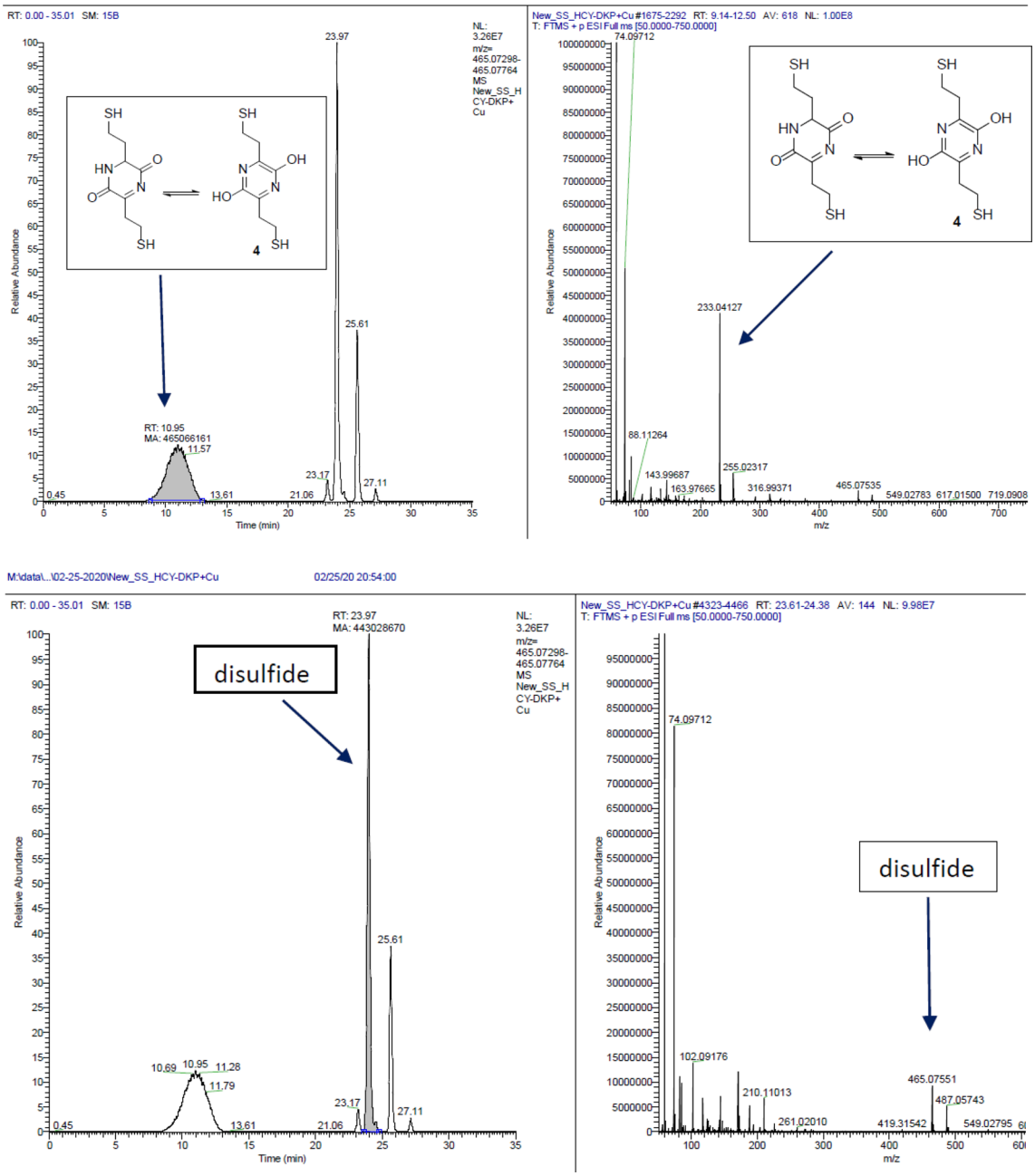
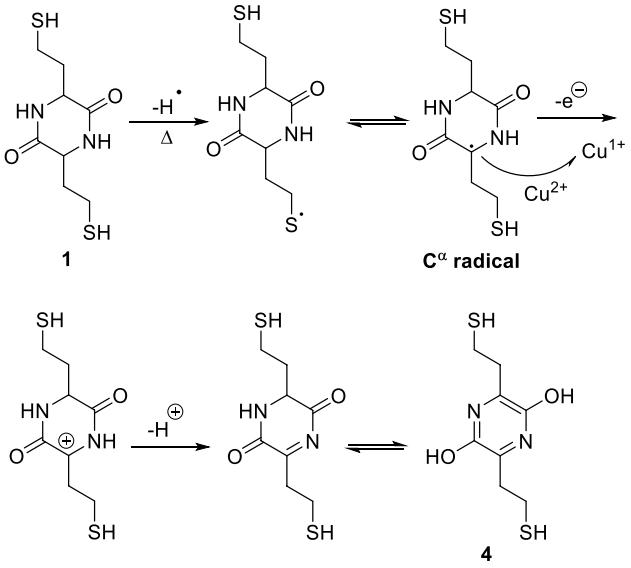


Fig. 3 Top: HPLC–ESI–MS of product 4 from the copper-promoted oxidation of 1. HPLC chromatogram (left). Mass spectrum (right). Bottom: HPLC–ESI–MS of disulfide formed from the copper-promoted oxidation of 1. HPLC chromatogram (left). Mass spectrum (right). This data supports the mechanism shown in Scheme 2

The corresponding control experiment, using alanine anhydride (**5,** Fig. 4), which does not possess a thiol-containing side chain, led to no significant solution color change or product formation in the presence of copper, even after 2 min at reflux (Fig. 4 and Supporting Information). This result supports the role of the Hcy side chain and the HAT mechanism in promoting the reduction of Cu (II).

Conclusion

Several prior studies have shown that complexes between copper and Hcy are toxic and can promote cardiovascular damage in vitro (Mutari 2020). However, it is not clear why Cu-Hcy, and not Cu-Cys or Cu-GSH, has been specifically linked to CVD since similar chemistry is



Scheme 2 The proposed mechanism of the oxidation of diketopiperazine (**1**) by Cu (II) to **4** via the HAT process

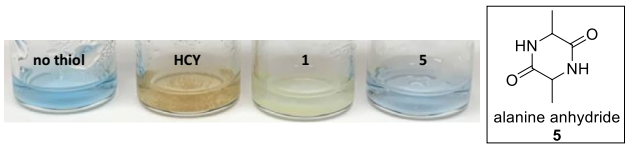


Fig. 4 The solution of alanine anhydride (**5**) and Cu (II) (right) shows no color change in contrast to the solution of **1** or Hcy plus copper. This is consistent with the corresponding HPLC data showing the relative diminished reactivity of **5** compared to **1** or Hcy with copper (Supporting Information). This is strong evidence of the involvement of the side chain of **1** in forming the C^{α} reducing radical via the HAT mechanism. Conditions: solutions heated at reflux for 2 min containing, 0.1 ml of 0.5 M Tris buffer pH = 7.5, 0.5 ml of 5 mM thiols and CuCl2 (1:1). left to right thiols in the copper-buffer solutions are: no thiol, homocysteine (Hcy), homocysteine diketopiperazine (Hcy-DKP) **1** and alanine anhydride **5**

expected to occur between copper and other, relatively more abundant biothiols, such as Cys or GSH. We have shown herein that a redox interaction occurs selectively between copper and Hcy, and that the selectivity is attributable to the kinetically favored formation of the strongly reducing and relatively toxic C^{α} radical of Hcy. Further investigation of the relevance of this mechanism in the pathogenesis of CVD is ongoing.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00726-021-02979-9.

Author contributions MG performed experiments, acquired analytical and chromatographic data, interpreted results and wrote the manuscript. JMA interpreted results, assisted with experiments and proofread the manuscript. RMS conceived of the study, guided the work and contributed to manuscript preparation.

Funding The authors thank Portland State University for its support of this work. The National Science Foundation is acknowledged for support of the BioAnalytical Mass Spectrometry Facility at PSU (MRI 1828573).

Data availability The Supporting Information contains materials and methods as well as NMR spectra and HPLC–UV and HPLC–MS data for compounds described in the text.

Declarations

Conflict of interest The authors have no conflicts of interest or competing interests related to the work described in this manuscript.

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