Alcohol and Alcoholism Vol. 47, No. 5, pp. 497–500, 2012
Advance Access Publication 29 May 2012
doi: 10.1093/alcalc/ags058

PHARMACOLOGY AND CELL METABOLISM

Alterations of Homocysteine Serum Levels during Alcohol Withdrawal Are Influenced by Folate and Riboflavin: Results from the German Investigation on Neurobiology in Alcoholism (GINA)

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(*Received* 5 *January* 2012; *first review notified* 24 *February* 2012; *in revised form* 5 *April* 2012; *accepted* 16 *April* 2012)

Abstract — **Aims**: Various studies have shown that plasma homocysteine (HCY) serum levels are elevated in actively drinking alcohol-dependent patients a during alcohol withdrawal, while rapidly declining during abstinence. Hyperhomocysteinemia has been associated not only with blood alcohol concentration (BAC), but also with deficiency of different B-vitamins, particularly folate, pyridoxine and cobalamin. **Methods**: Our study included 168 inpatients (110 men, 58 women) after admission for detoxification treatment. BAC, folate, cobalamin, pyridoxine, thiamine and riboflavin were obtained on admission (Day 1). HCY was assessed on Days 1, 7 and 11. **Results**: HCY levels significantly declined during withdrawal. General linear models and linear regression analysis showed an influence of BAC, folate and riboflavin on the HCY levels on admission as well as on HCY changes occurring during alcohol withdrawal. No significant influence was found for thiamine, cobalamin and pyridoxine. **Conclusions**: These findings show that not only BAC and plasma folate levels, but also plasma levels of riboflavin influence HCY plasma levels in alcohol-dependent patients.

INTRODUCTION

Homocysteine (HCY) metabolism is known to be fundamentally altered during alcohol intoxication and withdrawal (Bleich *et al*., 2000b, 2004, 2005). This has been shown for alcohol-dependent patients, but also—to a lesser extent—for social drinkers (Bleich *et al*., 2001). HCY levels found in alcohol-dependent patients tend to be much higher than the common cut-off levels for HCY, defined in a study of Ubbink *et al*. (1995) as 11.7 μmol/l or in a different investigation as 11.4 μmol/l for male and 10.4 μmol/l for female subjects (Selhub *et al*., 1999).

The amino acid HCY is known to increase glutamatergic neurotransmission via activation of the *N*-methyl-D-aspartate (NMDA)-receptor of the glutamatergic system (Bleich *et al*., 2004; Bleich and Hillemacher, 2009). Elevated HCY levels lead to neurodegeneration and have been associated—inter alia—with vascular diseases and brain atrophy (Sachdev, 2004; Wilhelm *et al*., 2008). During alcohol withdrawal neurotransmission via the (up-regulated) NMDA-receptors is not longer blocked by ethanol, resulting in an extensive overstimulation by HCY-associated NMDA-receptor agonism. This extensive glutamatergic reaction has been assumed to be the neurobiological background of the association between elevated HCY levels and the higher risk of alcohol withdrawal seizures (Bleich *et al*., 2000a, 2006; Bayerlein *et al*., 2005; Hillemacher *et al*., 2007). Furthermore, elevated HCY levels have been found to correlate with short-term cognition deficits during alcohol withdrawal (Wilhelm *et al*., 2006) as well as long-term cerebral atrophy (Bleich *et al*., 2003).

This increase in HCY levels in alcohol-dependent patients is based on the direct effects of ethanol (Bleich *et al*., 2000b, 2005) as well as an alcohol-associated deficiency of folate (vitamin B9), cobalamin (vitamin B12) and pyridoxine (vitamin B6) (Majumdar *et al*., 1982; Cravo and Camilo, 2000; Bleich *et al*., 2004; Chen *et al*., 2011). Folate and 5-methyltetrahydrofolate are substrates for the remethylation of HCY to methionine via the action of the methionine synthase (Moat *et al*., 2003; Bleich *et al*., 2004), cobalamin acts as a cofactor for methionine synthase. Riboflavin is a precursor of flavin adenine dinucleotide (FAD), which is a coenzyme of methylenetetrahydrofolate reductase (MTHFR) (Moat *et al*., 2003). Pyridoxine acts as a cofactor for cystathionine-β-synthase (CBS), responsible for transsulphuration of HCY into cystathionine (Bleich *et al*., 2004). Thiamine has been supposed to act on the HCY metabolism via enhancing the oxidative decarboxylation in the transamination of methionine (Franken *et al*., 1996). The relevance of B-vitamine intake on HCY levels has been shown in numerous studies (Konstantinova *et al*., 2007).

The present study was performed to validate previous findings investigating alterations of HCY serum levels during alcohol withdrawal in a large population of alcohol-dependent patients and to study the role of the B-vitamins involved in HCY metabolism in the pathogenesis of alcohol-associated hyperhomocysteinemia.

SUBJECTS AND METHODS

The present study was part of the German Investigation on Neurobiology in Alcoholism (GINA). All patients were recruited from the Department of Addiction and Psychotherapy of the hospital 'Rheinische Kliniken' in Bonn, Germany. Written informed consent was obtained from all 363 patients (251 men, 112 women). In a subsample of serial patients, we performed a prospective approach, obtaining data on Days 1, Day 7 and Day 11 after admission for detoxification treatment. This subsample was used in the present analysis, consisting of 168 patients (110 men, 58

women). All procedures were approved by the local ethics committee of the University of Bonn, Germany (Nr. 024/07), and were in accordance with the Helsinki Declaration of 1975, as revised in 1983. All participants suffered from alcohol dependency according to ICD-10 and were included in the study on admission for alcohol detoxification. Patients were mainly detoxified using clomethiazole following a symptom-triggered regime using the Banger-Score (Banger *et al*., 1992). If, for clinical reasons, clomethiazole could not be used, benzodiazepines were administered. Patients suffering from dependence of other substances except alcohol and nicotine were excluded from the study.

Laboratory measurements

Fasting blood samples were obtained on Day 1 (admission), 7 and 11 of detoxification treatment. Blood samples were centrifuged and consecutive serum and lithium heparin plasma samples were stored at −80°C directly after collection. HCY was assessed at all three time points while vitamin serum levels were obtained at admission.

Plasma total HCY concentrations were measured by means of particle-enhanced immunonephelometry with a Dimension Vista™ system according to the manufacturer's instructions (Siemens Healthcare Diagnostics, Eschborn, Germany). The reference interval given by Siemens Healthcare Diagnostics was 4.9–15.0 µmol/l. Serum vitamin B12 and folate concentrations were measured by means of a homogenous, competitive chemiluminescent immunoassay based on the LOCI™ technology with a Dimension Vista™ system (Siemens Healthcare Diagnostics). Reference intervals given by Siemens Healthcare Diagnostics were 3.1–17.5 ng/ml for folate and 254–1320 pg/ml for cobalamin.

Serum alcohol concentration was measured by means of an enzymatic test (alcohol dehydrogenase method) with a Dimension Vista™ system (Siemens Healthcare Diagnostics).

Blood concentrations of thiamine, riboflavin and pyridoxine were analyzed using commercially available high-performance liquid chromatography (HPLC) assays (Chromsystems Instruments & Chemicals GmbH, Munich, Germany) on an HPLC system with a fluorescence detector (Agilent, Series 1200, Agilent, Waldbronn, Germany). Reference intervals given by Chromsystems were 66.5–200 nnmol/l for thiamine, 174–471 nmol/l for riboflavin and 8.7–27.3 µg/l for pyridoxine.

Statistical assessment

Results are presented as mean + SD or SE. Variables were normally distributed as tested using the Kolmogorov–Smirnov test. Linear regression models and general linear models for repeat measurements were used to test the influence of different variables on HCY levels. Significance level (*P*-value) was set <0.05.

Data were analyzed using IBM SPSS Statistics 19.0 and Graph Pad Prism™ 5.0 (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

Demographic characteristics of the study population are given in Table 1. Very few patients suffered from a definitive B-vitamin deficiency (two patient presented folate deficiency, one patient thiamine deficiency, yet none of the other included B-vitamins).

Pearson's correlation analysis showed significant results for HCY and folate ($r = -0.48$, $P < 0.001$), HCY and riboflavin ($r = -0.28$, $P < 0.001$) as well as for HCY and cobalamin ($r = -0.21$, $P = 0.005$). HCY and thiamine as well as HCY and pyridoxine were not significantly correlated.

Statistical analysis using general linear models for repeat measurements showed that HCY levels decline rapidly during alcohol withdrawal ($F = 45.28$, $P < 0.001$), with significant differences between all time points (Day 1/7: $P < 0.001$; Day 1/11: $P < 0.001$; Day 7/11: $P < 0.001$; Fig. 1). A multivariate linear regression model showed that HCY serum levels on Day 1 of alcohol withdrawal are significantly influenced by blood alcohol concentration (BAC) on admission ($B = 1.60$,

Table 1. Demographic characteristics of the study population

	Mean	SD	Median	Range
Age (years)	48.1	8.7	48.0	21–66
BAC (per mille)	1.46	1.1	1.43	0–4.2
HCY Day 1 (µmol/l)	19.6	12.9	15.3	5.5–94.9
HCY Day 7 (µmol/l)	12.9	5.9	11.6	5.1–55.9
HCY Day 11 (µmol/l)	11.9	5.1	10.7	0.2–50.4
Folate (ng/ml)	9.8	5.7	9.1	1.5–20.0
Riboflavin (nmol/l)	295.1	51.0	288.6	192.7–480.1
Thiamine (nmol/l)	164.4	47.1	160.9	16.8–338.6
Pyridoxine (µg/l)	31.4	48.9	25.1	10.6–522.6
Cobalamin (pg/ml)	705.7	293.8	650.0	228.0–1500.0
Sex	male: $n = 110$		female: $n = 58$	
Nicotine consumption (males: $n = 87$)	24.1	10.2	20.0	5–60
Nicotine consumption (females: $n = 42$)	21.7	9.2	20.0	6–40

SD, standard deviation; BAC, blood alcohol concentration at admission; HCY, homocysteine serum levels, measured at Day 1, 7 or 11 after admission for in-patients detoxification treatment.
Vitamin levels were measured at admission. Nicotine consumption given as smoked cigarettes per day.

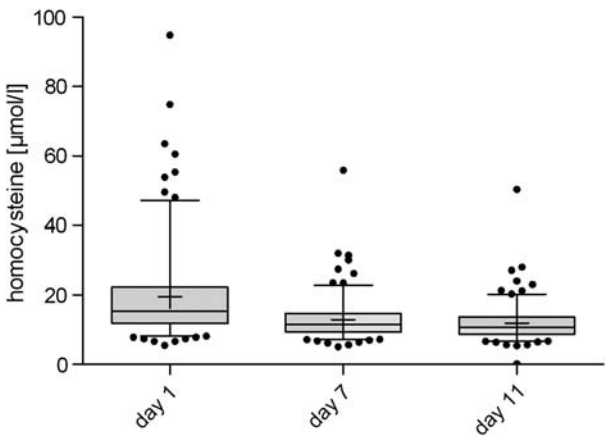

Fig. 1. Decrease of homocysteine serum levels during alcohol withdrawal. Box plot showing the changes of homocysteine plasma levels during withdrawal. Box represents 25–75 percentile and whiskers represent 5–95 percentile. Bar represents median and cross represents mean. Homocysteine plasma levels decreased significantly during the course of withdrawal. Statistical details are summarized in Results section.

$T = 2.30$, $P = 0.022$), folate ($B = -0.75$, $T = -5.47$, $P < 0.001$) and riboflavin ($B = -0.06$, $T = -3.44$, $P = 0.001$), but not by thiamine, pyridoxine or cobalamin serum levels. Correlation analysis revealed no significant association between folate and riboflavin levels ($r = 0.119$, $P = 0.121$). To test which factors influence the changes of HCY levels over time (Days 1, 7 and 11), we performed a general linear model computing BAC and vitamin levels as covariates. We found a significant interaction between HCY changes over time and BAC on admission (F = 4.80, $P = 0.009$), folate serum levels on admission ($F = 7.02$, $P = 0.001$) and riboflavin serum levels on admission ($F = 3.228$, $P = 0.042$), but not with other tested variables (thiamine, pyridoxine or cobalamin). Including the interaction between folate and riboflavin and omitting thiamine, pyridoxine and cobalamin results did not relevantly differ regarding the influence of thiamine, riboflavin and BAC on changes of HCY over time without showing a significant effect of the interaction (folate/riboflavin: $F = 1.56$, $P = 0.214$).

Comparing the subgroups of patients with high (HCY >15.35 µmol/l, $n = 84$) and low (HCY ≤15.36 µmol/l, $n = 84$) HCY serum levels at admission, *t*-test for independent samples revealed that the high-HCY group also showed significantly elevated HCY levels on Day 7 ($T = 6.4$, $P < 0.001$) and elevated HCY levels on Day 11 ($T = 6.0$, $P < 0.0001$) as well as reduced folate ($T = -6.3$, $P < 0.001$), riboflavin ($T = -2.7$, $P = 0.008$) and pyridoxine ($T = -2.1$, $P = 0.039$) serum levels at admission.

Additionally, we performed the described linear regression analysis separately for both genders. This analysis showed a significant influence on HCY serum levels in male patients for folate ($B = -0.78$, $T = -5.16$, $P < 0.001$) and riboflavin ($B = -0.05$, $T = -3.01$, $P = 0.003$) but not for BAC, thiamine, pyridoxine and cobalamin. In female patients, the analysis showed a significant influence on HCY for folate ($B = -0.74$, $T = -2.61$, $P = 0.012$) and BAC ($B = 3.52$, $T = 2.27$, $P = 0.028$), not for any of the other included variables.

Furthermore, we compared smoking and non-smoking patients at the time of inclusion into the study. Using the previously described linear regression analysis, the results show a significant influence on HCY levels at Day 1 for folate ($B = -0.67$, $T = -2.41$, $P = 0.22$), thiamine ($B = 0.07$, $T = 2.14$, $P = 0.40$) and riboflavine ($B = -0.09$, $T = -2.25$, $P = 0.032$) in the group of non-smoking patients ($n = 39$) and a significant finding for folate ($B = -0.76$, $T = 0–4.54$, $P < 0.001$), riboflavine ($B = -0.5$, $T = -2.5$, $P = 0.013$) and BAC ($B = 2.04$, $T = 2.33$, $P = 0.021$) in the smokers' group ($n = 129$)

DISCUSSION

The present investigation confirms previous observations that reported elevated HCY levels in patients with alcohol dependence undergoing detoxification treatment. As shown previously, on the time of admission the extent of the HCY serum levels depends significantly on BAC (Bleich *et al*., 2000b, 2005). However, apart from BAC, serum levels of riboflavin and folate also showed a significant influence on HCY serum levels at admission (Day 1), at least in the male subgroup. The same result was found when general linear model analysis was performed, analyzing the effect of BAC and B-vitamins on the changes of HCY during withdrawal. Interestingly, cobalamin and pyridoxine showed no significant influence on HCY. The same results were found when comparing patients according to nicotine consumption and gender. This result is in line with previous studies showing a specific importance of folate and riboflavin supplementation on HCY serum levels in healthy volunteers (Ganji and Kafai, 2004; Araki *et al*., 2006), which may also depend on the MTHFR genotype (Chuang *et al*., 2006). Recently, riboflavin has also been studied as dietary supplement during pregnancy showing an effect on maternal HCY levels but not on orofacial clefts in the offspring (Vujkovic *et al*., 2010). However, riboflavin, which, to our knowledge, has not been studied in the context of alcohol-associated hyperhomocysteinemia before, showed a significant impact on HCY levels, which did not depend on its interaction with folate. Riboflavin is a precursor of various flavin coenzymes, particularly of FAD, which is a coenzyme of MTHFR (Moat *et al*., 2003). Substituted by FAD MTHFR catalyses 5,10-methylenetetrafolate to 5-methylenetetrafolate, which is of importance as a donor of methyl groups for remethylation of HCY (Moat *et al*., 2003). Accordingly, MTHFR activity is reduced in ribloflavin-deficient rats (Bates and Fuller, 1986).

It is surprising and difficult to interpret that we found no interaction between riboflavin and folate in our patient group. In further studies, it may be interesting to include analysis of FAD, which we did not perform in our study but which is the active coenzyme in the folate-dependent MTHFR metabolism (Hustad *et al*., 2002; Jacques *et al*., 2002).

However, the present work suffers from several limitations: first, an influence of withdrawal medication with benzodiazepines and possibly also with clomethiazole on HCY levels cannot be ruled out and was recently stated in a clinical study (Pompeia *et al*., 2009). The lack measurements of vitamin serum level on Day 7 and 11 must also be considered a limitation. Furthermore, the lack of an association between cobalamin and pyridoxine and HCY in the regression analysis is surprising. This may be explained at least partially by the wide variations in both vitamin serum levels.

Overall, our results show that, apart from individual BAC, folate and riboflavin plasma levels on admission contribute to HCY elevation in alcohol-dependent patients. This effect was not only shown at the beginning of in-patient treatment but also regarding the decline of HCY during the withdrawal period. Noteworthy, only a negligible number of patients suffered from a definite B-vitamin deficiency. Thus, the present findings support the hypothesis that a supplementation of folate and riboflavin may be useful in alcohol-dependent patients during active drinking as well as detoxification and that currently used reference values for B-vitamins may need to be adjusted for alcohol-dependent patients.

Funding — We thank ERAB (European Foundation for Alcohol Research; former European Research Advisory Board) for supporting this study (grant number EA 07 04) and the Support of the Center for Clinical Research ('Zentrum für Klinische Forschung', M.L.).

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