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Methionine challenge paradoxically induces a greater activation of the antioxidant defence in subjects with hyper- vs. normohomocysteinemia

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Abstract

To determine whether hyperhomocysteinemia induced post-methionine loading (PML) is associated with different response in the aminothiol redox state and oxidative stress vs. normohomocysteinemia, we assessed PML plasma thiols, vitamins, free malondialdehyde (MDA), and blood reduced glutathione (GSH) in 120 consecutive subjects (50 [35–56] years, 83 males), divided into two groups according to PML plasma total Hcy $< 35\,\mu M$ (Group 1, $n = 65$) or $\geq 35\,\mu M$ (Group 2, $n = 55$).

In the group as a whole, plasma reduced cysteine and cysteinylglycine, blood reduced GSH (all p for time $= 0.0001$) and plasma total GSH (p for time $= 0.001$) increased from baseline to PML. MDA values were unchanged. Group 1 and 2 differed in blood reduced GSH (p for group $= 0.004$, higher in Group 2), and MDA levels (p for group $= 0.024$, lower in Group 2).

The oxidative stress induced by methionine challenge seems to be opposed by scavenger molecules activation, namely GSH, and lipid peroxidation does not increase. This mechanism paradoxically appears to be more efficient in hyperhomocysteinemic subjects.

Keywords: *Methionine loading, oxidative stress, aminothiols, glutathione, antioxidants, homocysteine*

Introduction

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical may be generated by several pathways, including auto-oxidation of aminothiols [1]. Aminothiols are intracellular and extracellular redox buffers, but excess plasma levels may have a pro-oxidant function. Previous studies have shown that high levels of cysteine (Cys) and homocysteine (Hcy) are associated with vascular disease and this link is strongly plasma level-related [2,3]. Hcy causes injury of the vascular endothelium [4], smooth muscle cell proliferation [5], and thrombosis [6]. Cys promotes detachment of human arterial endothelial cells in culture [7], and in the presence of metal ions undergoes autoxidation that results in free radical generation [8]. Conversely, the aminothiol glutathione (GSH) and its metabolite cysteinylglycine (Cys–Gly) have important antioxidative properties [9]. GSH acts as cosubstrate for GSH peroxidase, which catalyzes the reduction of both hydrogen and lipid peroxides to their corresponding alcohols by a reaction involving GSH oxidation. To maintain the cellular redox balance, the oxidized form

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of GSH is quickly reduced by the NADPH-dependent GSH reductase or excreted from intra to extracellular space, when excessive amounts are formed [10].

Oxidative stress determines an imbalance between free radical production and endogenous antioxidant defences and can induce tissue damage through oxidative modification of cellular biomolecules such as lipids, proteins and nucleic acids [11].

The methionine loading test was originally developed to detect heterozygosity for the deficiency of cystathionine-β-synthase, a key enzyme in the transsulfuration pathway of homocysteine [12], and has also been shown to identify subjects at risk for thrombosis [13]. Acute hyperhomocysteinemia induced by methionine loading is associated with endothelial dysfunction through a mechanism which may or may not involve increased oxidative stress in healthy adults [14,15]. The net balance between the pro-oxidant effects of a transient Hcy load and the activation of the antioxidant defence in relation to the individual aminothiols status has not yet been clarified. As a matter of fact, the complex interplay between high levels of Hcy and changes in other aminothiols may modulate or even mediate atherogenesis [16].

The aim of our study was to evaluate the changes in aminothiols and vitamins and the increment of oxidative stress, expressed as free malondialdehyde (MDA) plasma levels, using a potentially pro-oxidant stimulus such as experimental hyperhomocysteinemia post-methionine loading (PML).

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Materials and methods

Study population

One-hundred twenty consecutive patients (50 [35–56] years, 83 males) who underwent peripheral venous blood sampling under fasting conditions and after a methionine loading were enrolled in the study in order to evaluate their redox state. Exclusion criteria were as follows: >75 years, previous history of immunological or neoplastic diseases, ongoing infection, impaired renal function, surgery or trauma within the previous month, vitamin supplements within 2 weeks before the study. Thirty-seven (31%) patients had had previous (>6 months) atherothrombotic events which included myocardial infarction in 20, transient ischemic attacks in 11, stroke in 3, peripheral artery occlusion in 2, and retinic thrombosis in 1. Thrombo-embolism at venous side occurred in 23 (19%) patients as follows: pulmonary embolism in 4, deep venous thrombosis in 12, and superficial recurrent thrombosis in 7 patients.

Forty-seven subjects (39%) were mild smokers, but all refrained from smoking a day before the study. Sixty-six patients (55%), who were taking cardioactive drugs, omitted these medications for at least 24 h before the study.

The protocol was approved by the Local Ethics Committee. All subjects submitted consent forms to participate in the study.

Study design

After an overnight fast, an antecubital vein was cannulated and blood was drawn into different pre-chilled Vacutainer tubes to measure plasma reduced and total aminothiols (Hcy, Cys, GSH, Cys–Gly), blood reduced GSH, α-tocopherol, ascorbic acid, MDA, vitamin B_{12}, folate, glucose, creatinine, fibrinogen, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and γ-glutamyl transpeptidase (γ-GT). Blood sampling and the above quoted determinations were repeated 4 hours after oral methionine, (L-methionine 3.8 g/m^2 body surface in 100 ml of water). Patients are required to lay at rest in a quite room during the 4 h-test with the permission to drink some water only.

Chemical analysis

Immediately after blood collection, sample preparation and analyses for plasma reduced and total aminothiols, α-tocopherol, and ascorbic acid were performed; analyte concentrations were determined by high pressure liquid chromatography (HPLC; ProStar, Varian, Surrey, UK), according to methods previously described [17,18]. MDA levels were determined in stored plasma by gas chromatography–mass spectrometry with isotope dilution technique [19]. Blood reduced GSH, an index of GSH concentrations into circulating cells, was determined as previously reported [20]. Values are expressed in μM.

Vitamin B_{12} and folates were measured by competitive immunoassay using direct chemiluminescence, while glucose, γ-GT, creatinine, fibrinogen, total cholesterol and triglycerides were determined using standard laboratory methods. HDL cholesterol was measured after precipitation with dextran sulfate–magnesium and LDL cholesterol was calculated using the Friedewald's method.

The cut-off values of baseline and PML hyperhomocysteinemia were determined according to data obtained from a large population of healthy subjects with a wide age range, without risk factors or vascular events, using the 90th percentile of control distribution. Plasma total Hcy ≥ 15 μM at baseline and ≥35 μM PML were considered indicative of hyperhomocysteinemia; these cut-offs were similar to those reported in the literature [21,22].

Genetic analysis

DNA was extracted from aliquots of blood cellular fraction and stored at −80°C. The methylenetetrahydrofolate reductase (MTHFR) 677C → T genotype was identified by PCR amplification, followed by

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HinfI restriction digestion, while cystathionine β-synthase 833T → C genotype was identified by PCR amplification, followed by BsrI restriction digestion. Digestion products were separated by 3% agarose gel electrophoresis, stained with ethidium bromide. The MTHFR wild type allele gave 198 bp fragment, and insertion variant gave two fragments of 175 and 23 bp. The cystathionine β-synthase wild type allele gave 174 bp fragment, and insertion variant gave two fragments of 132 bp and 42 bp [23].

Genotype characterization was carried out in 109/120 subjects.

Statistical analysis

Continuous variables are expressed as median and interquartile ranges [I–III]. Baseline between-group differences were assessed by unpaired Student's t test for continuous variables or by Mann Whitney's *U*-test for non-normally distributed variables, and by Chi-square or Fisher exact test for categorical variables. ANOVA with repeated measures was used to test redox state changes from baseline to PML between groups; a logarithmic transformation was applied if a non-parametric distribution was found.

A two-tailed p value <0.05 was considered statistically significant. The statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) release 10.0 for Windows.

Results

Baseline clinical, biochemical and genetic characteristics of the overall population are reported in Table I. No mutation was found for cystathionine β-synthase.

In the overall population, the temporal trend in aminothiols from baseline to PML (Figure 1) was consistent with the effects of an acute methionine load; vitamins and MDA did not change PML.

The study population was divided into 2 Groups according to normal or elevated plasma Hcy levels following methionine challenge, in accordance with the previously reported cut-off value. There were 65 normohomocysteinemic (Group 1) and 55 hyperhomocysteinemic (Group 2) subjects. Twelve patients in Group 2 had normal baseline Hcy value, but abnormal PML Hcy. Fasting Hcy values in the 43 patients with hyperhomocysteinemia at baseline ranged between 15.43 and 49.67 μM [19.44–32.74]. Significant differences between the two Groups among variables reported in Table I were observed in vitamin B_{12} ($p = 0.001$), folates ($p = 0.01$), and MTHFR genotype ($p = 0.001$).

Table I. Demographic, clinical, biochemical parameters, and genetic characteristics in the overall population, normo- (group 1), and hyperhomocysteinemic (group 2) patients.

	All cases ($n = 120$)	Group 1 ($n = 65$)	Group 2 ($n = 55$)	p
Age (years)	50 [35–56]	51 [34–55]	49 [35–57]	0.70
Male gender, *n* (%)	83 (69%)	45 (69%)	38 (69%)	1.00
Body mass index, (kg/m^2)	25.3 [22.3–27.8]	25.6 [21.8–27.3]	25.2 [23.4–28.3]	0.38
Smokers, *n* (%)	47 (39%)	20 (31%)	27 (49%)	0.06
Diabetes, *n* (%)	5 (4%)	3 (5%)	2 (4%)	1.00
Hypertension, *n* (%)	52 (43%)	29 (45%)	23 (42%)	0.85
Hyperlipidemia, *n* (%)	50 (42%)	24 (37%)	26 (47%)	0.27
ACE-inhibitors, *n* (%)	26 (22%)	13 (20%)	13 (24%)	0.66
β-blockers, *n* (%)	17 (14%)	10 (16%)	7 (13%)	0.79
Calcium-channel blockers, *n* (%)	23 (19%)	14 (21%)	9 (16%)	0.50
Statins, *n* (%)	25 (21%)	12 (18%)	13 (24%)	0.51
Antiplatelet agents, *n* (%)	51 (43%)	29 (45%)	22 (41%)	0.71
Vitamin B_{12}, pg/mL	385 [260–502]	429 [340–548]	307 [223–435]	0.001
Total cholesterol, mg/dl	188 [161–214]	183 [161–206]	195 [162–225]	0.16
HDL cholesterol, mg/dl	52 [42–62]	52 [44–64]	50 [40–61]	0.22
LDL cholesterol, mg/dl	108 [91–140]	106 [90–130]	115 [97–150]	0.17
Triglycerides, mg/dl	91 [66–137]	90 [64–126]	95 [69–149]	0.11
Creatinine, mg/dl	0.88 [0.77–1.00]	0.87 [0.76–1.00]	0.90 [0.78–1.03]	0.22
Folates, ng/ml	4.80 [3.50–6.40]	5.40 [4.45–7.72]	3.90 [3.20–5.55]	0.01
γ-GT, U/l	25 [14–35]	25 [12–37]	25 [15–35]	0.84
Glucose, mg/dl	96 [88–103]	94 [87–104]	98 [89–103]	0.69
Fibrinogen, mg/dl	290 [249–331]	294 [237–348]	287 [251–326]	0.77
MTHFR genotype, *n* (%)				0.001
Wild-type	34 (31%)	25 (44%)	9 (17%)	
Homozygote	49 (45%)	16 (28%)	33 (63%)	
Heterozygote	26 (24%)	16 (28%)	10 (19%)	
Atherothrombotic events, *n* (%)	37 (31%)	20 (31%)	17 (31%)	1.00
Venous events, *n* (%)	23 (19%)	10 (16%)	13 (24%)	0.35

Data are represented as median and interquartile ranges [I–III] or number (percentage). ACE, angiotensin-converting enzyme; γ-GT, γ-glutamyl transpeptidase; MTHFR, methylenetetrahydrofolate reductase.

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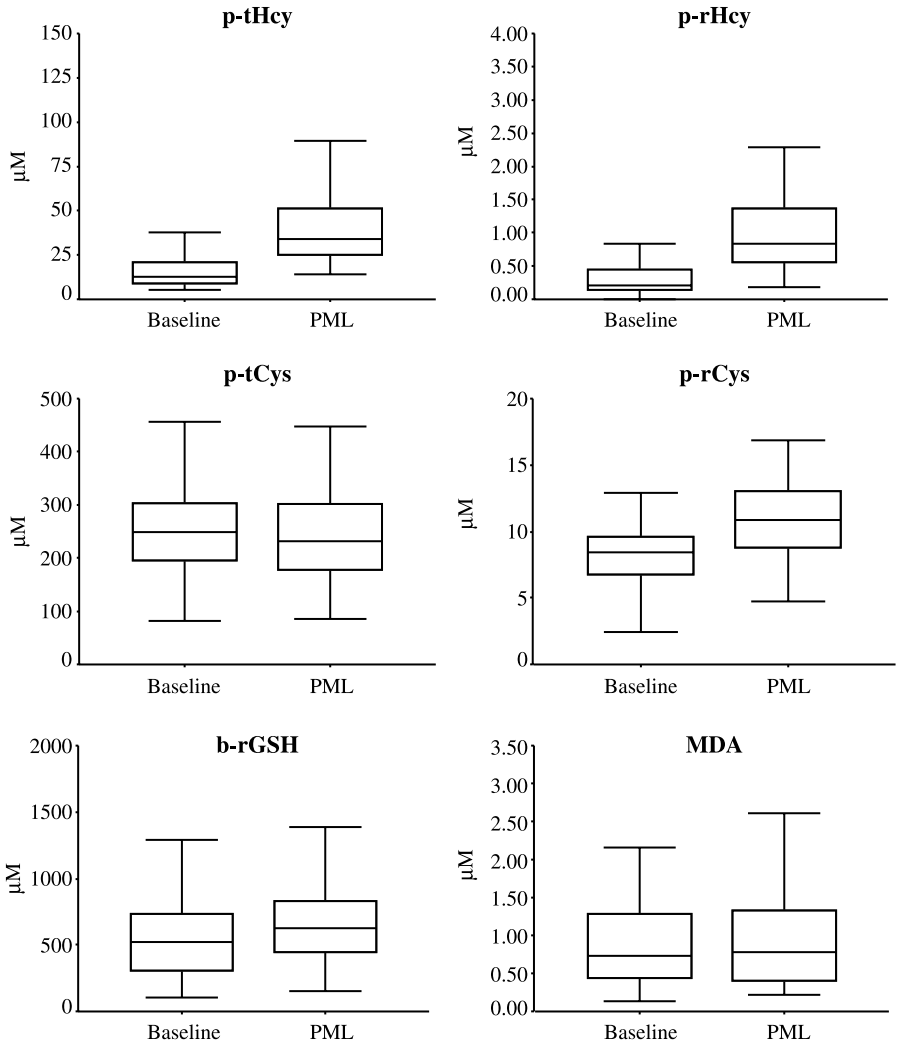

Figure 1. Temporal trend in aminothiols and MDA from baseline to PML in the overall population. p-tHcy, plasma total homocysteine; p-rHcy, plasma reduced homocysteine; p-tCys, plasma total cysteine; p-rCys, plasma reduced cysteine; b-rGSH, blood reduced glutathione; MDA, free malondialdehyde.

Plasma total and reduced Hcy was higher and increased more in Group 2 than in Group 1 (p for time = 0.0001; p for time*group interaction group = 0.0001; p for group = 0.0001). Plasma reduced Cys increased in both Groups (p for time = 0.0001), indicating an activation of the methionine transsulfuration pathway. Blood reduced GSH levels, significantly increased PML with respect to baseline (p for time = 0.0001), were higher in Group 2 than in Group 1 (p for group = 0.004) at both time points. Increased concentrations of plasma reduced Cys–Gly, a catabolite of GSH metabolism, were found PML (p for time = 0.0001). Although MDA did not change in the overall population PML, plasma concentrations were lower in Group 2 with respect to Group 1, both at baseline and PML (p for group = 0.024) (Table II).

Discussion

The main finding of the present study is that hyperhomocysteinemia induced by oral methionine loading is associated, with respect to normohomocysteinemia, with a greater activation of the antioxidant defence as expressed by higher intracellular GSH content, which in turn results in lower lipid peroxidation.

Time course of aminothiols PML

Methionine challenge determined significant increases in plasma total and reduced Hcy over time. Furthermore, concurrent variations in plasma reduced Cys, blood reduced GSH, plasma total GSH and plasma reduced Cys–Gly were observed in accordance with aminothiol metabolism. These findings express an activation of the antioxidant defense. Hcy is, through transmethylation of S-adenosylmethionine, the first substrate of the transsulfuration pathway, that produces intracellular Cys, one of the precursor aminoacids of GSH; Cys is the rate-limiting substrate for the synthesis of GSH [24]. Consistently with previous reports, the slight decrease over time of total Cys in our series is to be interpreted

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Table II. Aminothiols, vitamins and MDA concentrations at baseline and PML in the normo- (Group 1), and hyperhomocysteinemic (Group 2) patients

	Group 1 ($n = 65$)		Group 2 ($n = 55$)		p		
	Baseline	PML	Baseline	PML	*T*	*I*	*G*
p-tHcy	9.0 [7.6–11.7]	26.1 [21.5–30.8]	22.5 [15.6–31.8]	55.2 [42.9–67.8]	0.0001	0.0001	0.0001
p-rHcy	0.16 [0.11–0.22]	0.61 [0.46–0.83]	0.35 [0.22–0.37]	1.34 [0.92–2.29]	0.0001	0.0001	0.0001
p-tCys–Gly	31.5 [21.6–44.4]	29.8 [20.7–39.1]	32.1 [24.0–54.5]	36.4 [24.7–50.9]	0.12	0.08	0.098
p-rCys–Gly	3.64 [2.35–5.24]	4.27 [2.85–5.93]	3.51 [2.15–5.90]	5.19 [2.93–7.23]	0.0001	0.30	0.57
p-tCys	238 [189–301]	231 [175–292]	267 [202–321]	235 [193–330]	0.049	0.58	0.21
p-rCys	7.95 [6.87–9.28]	10.61 [8.83–12.09]	8.89 [5.89–10.91]	11.33 [8.79–13.86]	0.0001	0.09	0.096
b-rGSH	465 [272–664]	541 [420–745]	591 [380–794]	705 [475–981]	0.0001	0.81	0.004
p-tGSH	5.36 [4.01–7.29]	6.47 [4.10–7.47]	6.19 [4.52–7.82]	6.83 [5.09–9.86]	0.001	0.09	0.13
p-rGSH	2.35 [1.61–4.25]	2.28 [1.69–3.62]	1.87 [1.37–3.74]	2.52 [1.39–4.96]	0.71	0.13	0.71
Ascorbic acid	54.1 [30.4–68.9]	52.9 [32.1–66.4]	46.1 [31.3–62.9]	43.8 [29.9–61.6]	0.28	0.78	0.59
α-tocopherol	22.2 [17.1–30.9]	22.7 [16.4–28.7]	22.9 [16.7–28.0]	21.8 [15.7–28.3]	0.13	0.93	0.40
MDA	0.78 [0.47–1.55]	0.89 [0.50–1.75]	0.68 [0.36–1.20]	0.68 [0.36–1.14]	0.14	0.68	0.024

Data are presented as median and interquartile ranges [I–III]. Analyte levels are expressed in μM. p-tHcy, plasma total homocysteine; p-rHcy, plasma reduced homocysteine; Cys–Gly, cysteinylglycine; Cys, cysteine; b-rGSH, blood reduced glutathione; MDA, free malondialdehyde. *T*, indicates *P* for time; *I*, indicates *P* for time*group interaction; *G*, indicates *P* for group; in ANOVA with repeated measures.

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as an expression of Cys displacement by elevated Hcy levels from plasma protein binding sites and was associated with an increment of reduced Cys [25].

Blood reduced GSH significantly increased after methionine challenge in our series. GSH is one of the most important antioxidant compounds in the blood that eliminates free radicals and organic peroxides, and maintains α-tocopherol and ascorbic acid in their reduced and radical-scavenging forms [26]. GSH status is a highly sensitive indicator of cell function and viability; in fact, blood GSH depletion is associated to many pathological conditions both in human [20] and animal models [27]. GSH is exported from the cell by a specific transport protein and its breakdown through γ-GT leads to the production of Cys–Gly. Plasma total GSH increased in the overall population PML, in accordance with an increased export of GSH and Cys–Gly production in order to maintain cellular GSH homoeostasis.

We also assessed α-tocopherol and ascorbic acid for their important role as intracellular and extracellular antioxidants. In accordance with previous reports [12], vitamin concentrations were unchanged PML, suggesting that methionine loading did not promote lipid peroxidation. Free MDA, a product of lipid peroxidation after exposure to ROS, an established marker of cell membrane injury [28], was unchanged after methionine loading, as previously reported in healthy adults [29,30]. This finding suggests that acute plasma Hcy elevations do not induce major oxidative stress. However, we observed wide variations in PML MDA concentrations that stimulated our search for possible mechanisms underlying different reactions to increased oxidative stress. So we tested the impact of hyperhomocysteinemia on the aminothiol, vitamin and MDA changes in response to the methionine challenge.

Hyperhomocysteinemia and activation of antioxidant defence

Group 2 hyperhomocysteinemic subjects showed significantly higher blood reduced GSH levels than Group 1, both at baseline and following methionine challenge. Therefore, hyperhomocysteinemia seems to be associated with a greater availability of this key antioxidant molecule. Increased intracellular GSH levels in our hyperhomocysteinemic patients may be explained by at least two different mechanisms. While high-grade oxidative stress may induce cellular suppression of GSH antioxidant defence and depress Hcy-derived GSH synthesis [31], mild to moderate increases in oxidant agents may up-regulate the transcription of gamma-glutamylcysteine synthase genes, that will counteract oxidative stress via GSH increase [32]. In addition, higher intracellular GSH levels may depend on greater availability of the substrates involved in its synthesis, especially cysteine [33,34] as observed in PML acute hyperhomocysteinemia. In fact, high Hcy levels are associated with an increase in Cys [35], which is then quickly transported into the cell and metabolized to GSH. Previous works showed that factors stimulating L-cysteine or L-cystine uptake typically increase cell or tissue GSH levels [36,37].

Lower MDA levels in hyperhomocysteinemic patients than in normohomocysteinemic subjects together with increased intracellular GSH content provide evidence of a greater activation in the antioxidant defence in this group.

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The majority of Group 2 patients had chronic hyperhomocysteinemia, with elevated Hcy levels both at baseline and PML, while 22% of this population had normal baseline Hcy. We reasoned that this patient subset may be daily exposed to increased oxidative stress anyway, as a consequence of dietary intake. Actually, when between-group differences were tested excluding the 12 patients with normal baseline Hcy levels from Group 2, the same results of increased blood reduced GSH and lower MDA levels with respect to Group 1 were obtained.

The C677T variant of MTHFR has been long recognized to carry an increased risk of atherothrombotic events and is associated with higher Hcy levels after methionine loading.[38] Consistently with these findings, in our cohort homozygosis for MTHFR was more prevalent in Group 2 than in Group 1 subjects. Since the aim of our investigation was to assess the pro-oxidant effect of methionine challenge, we used Hcy levels and not MTHFR genotype as a selection criterion.

In conclusion, our study demonstrates that oxidative stress, potentially elicited by experimental hyperhomocysteinemia, may be prevented by a concomitant increase in intracellular and extracellular GSH, resulting in lower lipid peroxidation. On the other hand, the difference in GSH and MDA between normohomocysteinemic and hyperhomocysteinemic subjects suggests that chronic mild–moderate hyperhomocysteinemia is associated with increased availability of scavenger molecules that contrast oxidative stress.

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