Applied Medical Informatics
Original Research Vol. 28, No. 2 /2011, pp: 15-21

Inflammation and Oxidative Stress in Young and Aged Rats after Acute Homocysteine Administration

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Received: 17 May 2011/Accepted: 8 June 2011/Published online: 15 June 2011

Abstract
Introduction: Hyperhomocysteinemia plays an etiologic role in homocystinuria, neurodegenerative and cardiovascular diseases. Potential mechanisms involved in the degenerative diseases of aging include: oxidative stress, endothelial dysfunction and inflammation. *Aim:* In the present study we evaluated the effect of acute administration of homocysteine (Hcy), at a level similar to that found in homocystinuria, on biochemical markers of inflammation (such as IL-6) and of oxidative stress (such as gluthathione peroxidase - GPx). *Material and Method:* The study was performed on 40 young and older Wistar rats. IL-6 serum level was quantified by a high-sensitivity enzyme-linked immunoabsorbent assay (ELISA) method and the activity of whole blood GPx was measured using a commercially available Randox kit. *Results:* Our results showed that Hcy administration increased the pro-inflammatory cytokine IL-6 in young rats ($p<0.3$) and decreased IL-6 level in older rats ($p<0.008$), when compared to the control group. GPx activity was found to increase with age (587.07 U/gHb versus 847.5 U/gHb, $p<0.001$). Two hours after Hcy administration, GPx activity was found to decrease, but not in a statistically significant manner. The difference between GPx activities in Hcy treated groups remains statistically significant ($p<0.01$) in the younger group, compared to older group (556.62 U/gHb versus 748.38 U/gHb). *Conclusion:* Our results indicate the existence of a correlation between hyperhomocysteinemia, proinflammatory state and oxidative stress, illustrated by the direct dependence of whole blood GPx activities on the increasing age.

Keywords*:* Hyper-homocysteinemia; Aging; Cytokines; GPx activity; Oxidative stress.

Introduction

Increased understanding of the key role of homocysteine (Hcy) in atherogenesis, carcinogenesis and degenerative diseases of aging has led to new insights in the acute inflammatory response, oxidative stress, endothelial dysfunction, and cytotoxicity of homocysteine (Hcy) [1]. Inflammation represents a generalized sequence of events, known as the acute phase response, which is mediated by the generation of early response cytokines, such as interleukins IL-1β, IL-6, tumor necrosis factor-alpha (TNF-α), acute-phase proteins, the expression of cell-surface adhesion molecules and the synthesis of chemotactic molecules [2, 3].

Interleukin 6 (IL-6) is a multifunctional cytokine that plays important roles in host defense, acute phase reactions, immune responses, nerve cell functions and hematopoiesis [1-5]. It is expressed by a variety of normal and transformed lymphoid and non-lymphoid cells. The production of IL-6 is up-regulated by numerous signals, such as mitogenic or antigenic stimulation, lipopolysaccharides, calcium ionophores, cytokines and viruses. IL-4, IL-10 and IL-13 inhibit the expression of IL-6 in monocytes [4, 5].

Elevated IL-6 serum levels have been observed in a number of pathological conditions, including bacterial and viral infections, trauma, autoimmune diseases, inflammation and malignancies. It also plays a key role in the signaling pathways modulating the complex relationship between aging and chronic morbidity [6].

The high-affinity IL-6 receptor (IL-6 R), which mediates IL-6 bioactivity, consists of two membrane glycoproteins: an 80 kDa low-affinity IL-6-binding receptor (IL-6 R) and a 130 kDa signal transducing protein (gp130) that lacks IL-6 binding ability [4]. The binding of IL-6 to IL-6 R recruits gp130 to form a trimeric complex that dimerizes into a hexameric complex, which finally transduces the IL-6 signal. Soluble forms of both IL-6 R and gp130 have been detected in blood. Soluble IL-6 R is capable of binding IL-6 and to induce homodimerization of membrane gp130 and subsequent signal transduction [7-10].

Inflammation creates an oxidative stress environment [11, 12]. The current view of the origin of oxidative stress in cells exposed to increased levels of Hcy is that auto-oxidation of thiol groups generates hydrogen peroxide and reactive oxygen species, such as superoxide anion and hydroxyl radical [1].

In the present study, we evaluated the effect of homocysteine administration, mimicking the condition observed in homocystinuria [12], on the level of serum IL-6, a marker of acute inflammation. In addition, blood gluthathione peroxidase (GPx) activity, a parameter of oxidative stress, was also assayed in young and older Wistar rats.

Material and Methods

Animals and Reagents

In our experiment we used 40 female Wistar rats, weighting between 50-70 grams (young rats) and between 260-280 grams (aged rats). They were provided by the Central Animal House of the "Ion Chiricuţă" Cancer Institute, Cluj-Napoca, Romania.

Animals were maintained on a 12/12 h light/dark cycle, in standard laboratory conditions. Twelve hours before the experiment a fasting period was induced with water *ad libitum*.

Solution of saline Hcy (20.27 mg/mL), was obtained by using D,L-Hcy (TCI Europe nv Belgium) and 0.9% NaCl solution.

Acute Homocysteine Administration

The animals were distributed among four separate groups, as follows: two groups, both containing 10 young rats (1 month old) and two additional groups, both represented by 10 mature rats (12 months old). Within each age class, the animals were distributed using a random number

generation program. They received a single intraperitoneal (i.p.) injection of saline (control) or Hcy (0.6 μmol/g body weight), according to the following criteria:

1. Group I - control rats aged 1 month and treated with 0.9 % NaCl solution
2. Group II – rats aged 1 month treated with i.p. Hcy (0.6 μmol/g body weight)
3. Group III - control rats aged 12 months and treated with 0.9 % NaCl solution
4. Group IV – rats aged 12 months treated with i.p. Hcy (0.6 μmol/g body weight)

Two hours after the injection, blood and plasma were collected and the animals were sacrificed using an ethylic ether overdose.

The experiments were performed accordingly to the Declaration of Helsinki regarding animal experiments.

GPx Activity Assay

Venous blood samples were collected into heparinised tubes. GPx activity was measured using a RANSEL kit (Randox Labs., UK) at 37°C, on a Cobas Mira Plus (Roche) analyzer.

The assay was based on previously described methods [13], in which GPx catalyzed the oxidation of glutathione (GSH) by cumene hydroperoxide (ROOH). In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) was immediately converted to the reduced form (GSH), with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was subsequently measured.

$$2GSH + ROOH \xrightarrow{GPx} ROH + GSSH + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

The activity of GPx was expressed in U/L for the absolute activity and U/g Hb for the specific activity.

Hemoglobin assay (Drabkin's method)

Hemoglobin (Hb) concentration was determined according to the Drabkin method [13].Venous blood samples were added to the Drabkin's reagent, a mixture of sodium bicarbonate, potassium ferricyanide and potassium cyanide. The iron atom of hemoglobin (Hb), released by hypotonic lysis, was oxidized to Fe^{III} by potassium ferricyanide. The resulted MetHb reacted with potassium cyanide to form CNMetHb with a maximum absorbance at 540 nm. The color intensity was proportional to the total hemoglobin concentration. The concentration of Hb was expressed as grams/dL, assuming a molar absorption coefficient of 11000 L/mol/cm.

Measurement of serum IL-6 level

IL-6 level in rat serum was quantified by a high-sensitivity enzyme-linked immunoabsorbent assay (ELISA), using the Quantikine commercially-available kit. A monoclonal antibody targeted against rat IL-6 has been pre-coated on a microplate. After the samples were pipetted into the wells, IL-6 molecules were bound by the immobilized antibody. After washing away any unbound substrate, an enzyme-linked polyclonal antibody specific for rat IL-6 was added to the microplate wells. Following a second wash to remove any unbound antibody-enzyme complex, a substrate solution was finally added. The enzyme reaction yielded a colored product, whose absorbance was directly dependent on the amount of rat IL-6 bound during the initial step. The sample values were subsequently read from the calibration curve, obtained by using 6 rat IL-6 standards (2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml) [9, 10].

The Quantikine IL-6 immunoassay, designed to measure IL-6 in rat serum, contained an E. coli-expressed recombinant rat IL-6 and antibodies targeted against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant protein. Results obtained using natural rat IL-6 showed dose-response curves that were parallel to the standard curves obtained using the Quantikine standards [9, 10].

Results

Whole blood GPx enzymatic activities and IL-6 levels in rat serum, determined before and after Hcy exposure, are presented in Figures 1 and 2.

It was noticed that GPx activity increased with age (587.07 U/g Hb in younger rats, versus 847.5 U/g Hb, in the older animals, $p<0.001$).

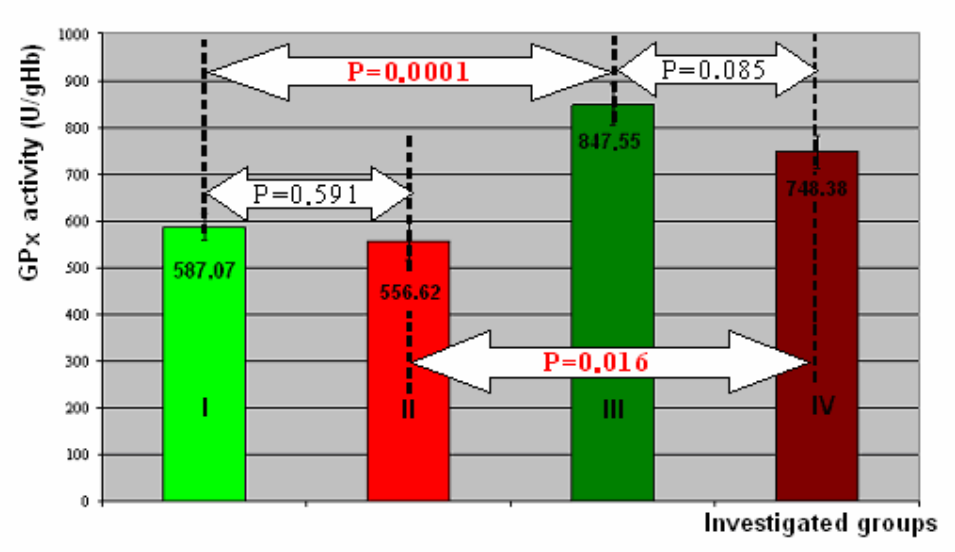

Figure 1. The effects of hyperhomocysteinemia on GPx activity

Two hours after Hcy administration, GPx activity decreased, but not in a statistically significant manner. The difference between GPx activities in Hcy treated rats remains statistically significant ($p<0.01$) in the younger group, as compared to the older group (556.62 U/g Hb, versus 748.38 U/g Hb).

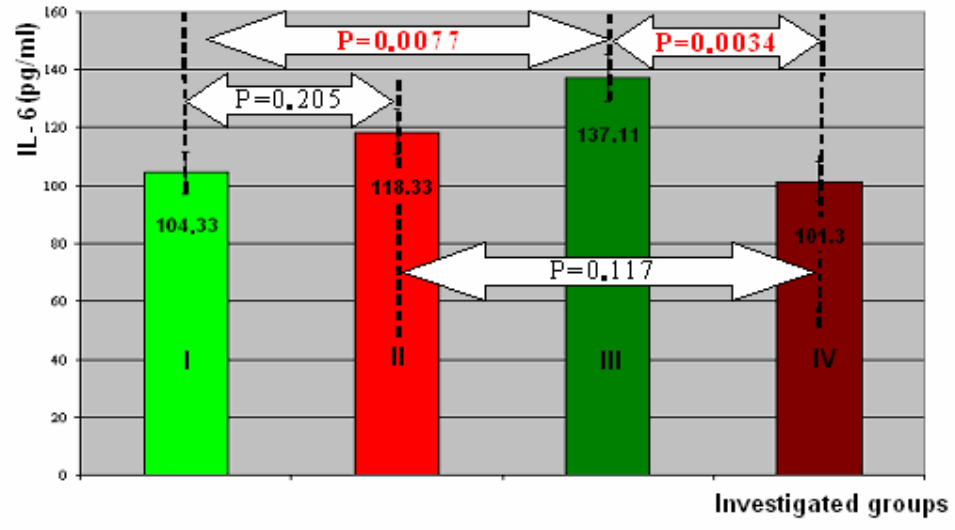

Figure 2. The effects of hyperhomocysteinemia on serum IL-6 level

We also noticed that acute Hcy administration induced a significant decrease of IL-6 in older

rats ($p<0.008$), when compared to the control group. However, in younger animals, IL-6 levels displayed a statistically insignificant increase ($p<0.3$), after Hcy administration.

Discussion

The GPx activities corresponding to groups I (young rats) and III (aged rats) show a statistically significant difference ($p< 0.0001$), pleading for an increase of GPx activity in an age-dependent manner.

In groups II and IV, GPx activity decreased after 2 hours following Hcy administration, but not significantly, compared to the corresponding control group. However, a statistically significant difference was noticed between younger and older rats after Hcy administration.

On the other hand, serum IL-6 level was also found to increase with age. In this context, we observed a significant difference between younger and older rats, belonging to control groups I and III. This result is in agreement with the statement that inflammation could contribute to both aging and the emergence of degenerative diseases [14, 15].

The short exposure (2 hours) to an increased Hcy concentration induced a decrease of IL-6 level, but only in the aged rats (group III versus group IV), by a mechanism that remains to be deciphered. In younger rats, IL-6 levels displayed a statistically insignificant increase, after Hcy administration.

Hyperhomocysteinemia leads to increased production of reactive oxygen species, by auto-oxidation and by altering the expression of GPx-1.

The oxidative stress induced by Hcy leads to the following consequences:

- oxidative inactivation of endothelium-derived $NO^{\bullet}$ by hydrogen peroxide [16];
- lipid peroxidation [17];
- generation of lipid peroxynitrite [18];
- uncoupling of eNOS (endothelial nitric oxide synthase), mediated by peroxinitrite and by oxidation of its cofactor tetrahydrobiopterin [19].

On the other hand, Hcy induces an increased expression of IL-1β and a decreased vascular synthesis of $NO^{\bullet}$ [20], by activation of N-methyl-D-aspartate (NMDA) glutamate receptor in cultured endothelial cells or rat smooth muscle cells.

IL-1β markedly enhances GPx gene expression. IL-6 has similar effects, but weaker than those induced by IL-1β [21].

Recent research has focused on the correlation between homocysteine and inflammation. A pro-inflammatory state was found to be associated with hyperhomocysteinemia in the elderly [22]. Evidence shows that the concentrations of acute phase proteins, such as fibrinogen, C-reactive protein (CRP) and α-1-antichymotrypsin, correlate with circulating concentrations of homocysteine [23-26].

Preclinical studies indicate that IL-6 may interact with vitamin B_6 metabolism and compromise cystathionine ß-synthase activity, thereby rising plasma homocysteine concentrations [27]. Interestingly, high circulating concentrations of pro-inflammatory cytokines are associated with an elevated risk of medical conditions that have generally been related to hyperhomocysteinemia, such as acute ischemic stroke, myocardial infarction, and more recently, osteoporosis. These manifestations were reported independently of dietary vitamin intake, circulating vitamin concentrations and cardiovascular disease risk factors [28-31].

Recent studies provided evidence that GPx activity decreased after treatment of rat aorta smooth muscle cells with D, L-Hcy (0-500 μmol/L) in a dose-dependent manner, but there was no effect on catalase activity. It was observed that 5 mmol/L Hcy decreased steady-state mRNA for GPx by 90% [32].

According to recent findings, Hcy directly inhibits catalase breakdown of H_2O_2 by conversion of the enzyme into the inactive form [33]. Taking into account the inhibition of GPx activity, Hcy seems to be a major contributor to the neurodegenerative pathology, which has been linked to oxidative stress in most cases [34].

Conclusions

According to our results, the inflammatory process generated by Hcy administration was able to create an oxidative stress condition. We evaluated the effect of acute administration of homocysteine on the serum level of IL-6, a pro-inflammatory cytokine. Another parameter of oxidative stress, glutathione peroxidase, was also evaluated in young and older Wistar rats.

Our data indicate that acute Hcy administration induced a significant decrease of IL-6 in aged rats, by a mechanism that remains to be deciphered. In younger subjects, IL-6 levels displayed an increase, without statistical significance, following Hcy administration.

GPx activity decreased in both groups after Hcy administration. Hcy seems to be a potent pro-inflammatory messenger which can activate different cytokines by enhancing oxidative stress, in young and older rats. These findings could facilitate the identification of new therapeutic approaches to the treatment of homocysteinuric patients.

Conflict of Interest

The authors declared no conflicts of interest.

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