Immunohistochemical detection of N-homocysteinylated proteins in humans and mice

Joanna Perła-Kaján a,1, Olaf Stanger b, Michał Łuczk a, Agnieszka Ziółkowska d, Ludwik K. Malendowicz d, Tomasz Twardowski a, Sárka Lhotak e,f, Richard C. Austin e,f, Hieronim Jakubowski a,g,*

a Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland
b University Hospital for Cardiac Surgery, Salzburg, Austria
c Department of Biochemistry and Molecular Biology, Poznań University of Medical Sciences, Poland
d Department of Histology and Embriology, Poznań University of Medical Sciences, Poland
e Department of Medicine, McMaster University, Hamilton, Ontario, Canada
f Henderson Research Centre, Hamilton, Ontario, Canada
g Department of Microbiology & Molecular Genetics, UMDNJ-New Jersey Medical School, International Center for Public Health, Newark, NJ 07101, USA

Received 24 March 2008; accepted 8 April 2008

Abstract

N-homocysteinylation of ε-amino group of protein lysine residues by homocysteine (Hcy) thiolactone has been implicated in vascular disease in humans. We have previously generated polyclonal rabbit anti-N-Hcy-protein IgG antibodies that specifically recognize the Nε-Hcy-Lys epitope on N-homocysteinylated proteins. The present work was undertaken to examine the utility of these antibodies for the immunohistochemical detection of N-homocysteinylated proteins in biological samples. We found that the rabbit antibody specifically detected N-Hcy-protein in a dot-blot assay, that the signal resulting from the reaction of the antibody with N-Hcy-protein depended on the amount of the antigen, and that the sensitivity of the assay was protein-dependent. The rabbit anti-N-Hcy-protein IgG also specifically detected Nε-Hcy-Lys epitopes in human tissues, as shown by positive immunohistochemical staining of myocardium and aorta samples from cardiac surgery patients, and a lack of staining when the antibody was pre-adsorbed with N-Hcy-albumin. We also observed increased immunohistochemical staining for N-Hcy-proteins in aortic lesions from ApoE−/− mice with hyperhomocysteinemia induced by a high methionine diet, relative to ApoE+/− mice fed a control chow diet. In conclusion, polyclonal rabbit anti-N-Hcy-protein antibody can detect and monitor N-homocysteinylated proteins in human and mouse tissues with good sensitivity and specificity.

Keywords: Homocysteine thiolactone; N-homocysteinylated protein; Antibodies; Atherosclerosis

1. Introduction

The nonprotein amino acid homocysteine (Hcy) is a universal intermediate in methionine metabolism. In mammals, dietary methionine, an essential amino acid, is the only source of Hcy. However, although it is a normal metabolite, Hcy excess can be harmful. For example, severe hyperhomocysteinemia observed in genetic disorders of Hcy metabolism, causes pathologies in multiple organs and leads to premature death from vascular complications [1]. Even mild

0753-3322/S - see front matter © 2008 Elsevier Masson SAS. All rights reserved.

Please cite this article in press as: Perła-Kaján J et al., Immunohistochemical detection of N-homocysteinylated proteins in humans and mice, Biomed Pharmacother (2008), doi:10.1016/j.biopha.2008.04.001
hyperhomocysteinemia, which is prevalent in the general population, is associated with increased risk of cardiovascular [2] and neurodegenerative disorders such as dementia and Alzheimer’s disease [3]. Many studies, but not all, suggest that Hcy plays a causal role in atherothrombosis and brain disease. For example, Hcy-lowering by vitamin-B supplementation improves vascular outcomes in cystathionine-β-synthase–deficient patients [4], prevents brain disease in methylenetetrahydrofolate reductase–deficient patients [5], and improves cognitive function in the general population [6]. Patients at high risk for stroke [7,8], but not myocardial infarction [8,9], benefit from lowering of plasma Hcy by supplementation with folic acid and B-vitamin. Furthermore, studies of genetic and nutritional hyperhomocysteinemia in animal models provide consistent support for a causal role of Hcy in atherothrombosis [10].

Although the cytotoxic effects of Hcy excess have been confirmed in many biological model systems, the underlying mechanisms are not entirely clear. One hypothesis suggests that metabolic conversion to Hcy-thiolactone contributes to the pathophysiology of Hcy excess [11,12] and is involved in atherothrombotic disease in humans [13,14]. Indeed, Hcy-thiolactone is elevated in cystathionine-β-synthase- or methylenetetrahydrofolate reductase–deficient patients and in mice fed with a high methionine diet [15], and is more toxic to human cells than Hcy itself [reviewed in refs. [16,17]].

Hcy-thiolactone is detrimental because of its ability to modify proteins by forming adducts in which Hcy is N-linked to the ε-amino group of protein lysine residues [11,18]. N-homocysteinylated by Hcy-thiolactone affects the protein structure, impairs or alters their physiological function, and is directly cytotoxic [reviewed in refs. [13,14,16,17]]. Protein N-homocysteinylated leads to pathophysiological responses, including increased thrombogenesis (caused by N-Hcy-fibrinogen) [19,20] and an autoimmune response elicited by N-Hcy-proteins, associated with stroke and coronary artery disease [14,21,22].

Chemical and immunological methods for monitoring and quantification of N-Hcy-proteins are being developed to study the role of protein N-homocysteinylation in pathophysiology. The chemical methods depend on measurements of Hcy liberated from proteins subjected to acid hydrolysis, after the conversion to Hcy-thiolactone [12,37] or derivatization [23,24]. Immunological methods rely on ELISA assays to detect the binding of an anti-N-Hcy-protein antibody to N-Hcy-protein [21,25–27]. Here, we describe the application of rabbit polyclonal anti-N-Hcy-protein antibody for immunohistochemical monitoring of N-Hcy-proteins in situ in human and mouse tissues.

2. Materials and methods

2.1. Reagents

Keyhole limpet hemocyanine (KLH), complete Freund’s adjuvant, Protein A immobilized on 6% fast-flow bead Agarose, i-Hcy-thiolactone, anti-rabbit IgG alkaline phosphatase conjugate, p-nitrophenyl phosphate, diethanolamine, human hemoglobin, albumin, horse myoglobin and cytochrome c, bovine serum albumin, EDTA, potassium phosphate, Tween 20, Trypan Blue, glycine, and DTT were obtained from Sigma. Reagents for immunostaining of human tissues were purchased from Dako. Biomax-30K NMWL membranes were from Millipore. Nunc Maxi-Sorp 96-well plates were purchased from Fisher.

2.2. Preparation of modified proteins

Proteins (10 mg/ml), dissolved in 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, were modified with i-Hcy-thiolactone at a molar ratio 1:10 at 25 °C for 22 h. To prepare an antigen having blocked thiol groups, N-Hcy-proteins were treated with 2.5 mM DTT and alkylated at room temperature with 10 mM iodoacetamide for 1 h in the dark.

2.3. Cell culture

Human breast cancer cells MCF-7 (American Type Culture Collection) were grown in DMEM medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C, 5% CO2. About 106 cells were plated per 3-cm well and 24 h later the medium was removed, cell layer rinsed with phosphate-buffered saline (PBS), fresh medium without antibiotics supplemented with Hcy-thiolactone (0.01 mM–2 mM) was added to the wells. Incubation with Hcy-thiolactone was carried for 24 h at 37 °C, 5% CO2. The medium was removed, the cell layer rinsed twice with PBS, the cells detached from the plate by an 8-min incubation with 50 mM EDTA at 37 °C and resuspended gently in 5 mL of PBS. Cell viability, determined by mixing 25 µL of cell suspension with 25 µL Trypan blue was 84, 63, 57, 33, 34, and 36% for cultures supplemented with 0, 0.01, 0.1, 0.25, 0.5, 1, and 2 mM Hcy-thiolactone, respectively. Cells were collected by centrifugation and lysed on ice for 1 h with 50 µL of RIPA buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 7.5), 1% Nonidet P-40, 0.25 sodium deoxycholate and protease inhibitors (Complete Tablet, Roche A/S). The lysate was clarified by centrifugation and protein concentration measured with the Bradford reagent (Bio-Rad).

2.4. Mice

Female ApoE−/− mice on a C57BL/6 background at 6 weeks of age were fed a standard chow diet (Altromin c1057-157d). One group of four mice (high-Met group) received drinking water supplemented with 0.5% Met for 18 weeks. Control group of four mice received plain drinking water. Plasma total Hcy levels were 25.3 ± 9.9 µM and 6.1 ± 1.3 µM in the high-Met and control groups, respectively [28].

2.5. Human samples

Samples of cardiac tissues were obtained from 60–85 (mean 64.6) years old patients undergoing routine cardiac surgery (coronary artery bypass grafting and valve replacement...
operations) at the University Hospital for Cardiac Surgery, Salzburg, Austria. All patients gave written informed consent. Standard medications (statins, β-blockers, ACE inhibitors, aspirin) were discontinued before surgery. Right atrium myocardium was removed for cannulation and installation of extracorporeal circulation. Pieces from left mammary arteries, saphenous veins and explanted mitral and aortic valves were harvested for fixation and histological processing. Preoperative mean plasma total Hcy concentrations were 14.6 ± 3.7 μM.

2.6. Polyclonal rabbit anti-N-Hcy-protein antibody

The rabbit antibody was prepared by a modification of our previous procedure [21,26]. KLH was dissolved at 20 mg/mL in 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, 0.9 M NaCl, and modified with 50 mM l-Hcy-thiolactone HCl for 16 h at 37 °C. The extent of modification was 131 mol Hcy/mol KLH (72% lysine residues modified), as determined from parallel reactions with d,L-[35S]Hcy-thiolactone [29,30]. 500 μg of N-Hcy-KLH adduct was dissolved in 0.5 mL phosphate-buffered saline and emulsified with 0.5 mL complete Freund’s adjuvant and injected intradermally into several sites in male Californian White rabbits at 0, 2, 5, 9, 15, and 19 weeks. Blood was collected before and 7, 15, 18, and 20 weeks after inoculation. After clotting, serum was separated by centrifugation for 15 min at 1000×g, and stored at −80 °C. Titers of anti-N-Hcy-protein antibodies were determined by ELISA with N-Hcy-albumin as an antigen, as described previously [21]. The IgG fraction of the rabbit antibodies was isolated by affinity chromatography on a 1-mL protein A-Sepharose column (Sigma-Aldrich) as previously described [26].

2.7. ELISA

Solid phase indirect antibody capture technique was used to assay anti-N-Hcy-protein antibodies. Microtiter plate wells were coated with N-Hcy-albumin, iodoacetamide-treated N-Hcy-albumin, N-Hcy-hemoglobin, or native hemoglobin (200 μL, 20 μg/mL) [21]. For antibody titer determinations and for competitive ELISA experiments, rabbit sera or purified antibodies were diluted 100-fold. Alkaline phosphatase—conjugated goat anti-rabbit IgG diluted 1:2000 was used as a secondary antibody. p-Nitrophenyl phosphate was used as a substrate for alkaline phosphatase. After a 30-min incubation, the absorbance was measured at 405 nm on a 96-well plate reader [26].

2.8. Dot blots

N-Hcy-myoglobin, N-Hcy-hemoglobin, N-Hcy-cytochrome c, and N-Hcy-Lys-albumin were spotted onto nitrocellulose membrane and incubated with antibodies diluted 1:100 at room temperature for 3 h. Horseradish peroxidase—conjugated goat anti-rabbit IgG diluted 1:10,000 was used as a secondary antibody. 4-chloro-1-naphthol was used as a substrate for horseradish peroxidase. After a 30-min incubation, the membrane was scanned and the signal intensity quantified by using Image Quant 5.1.

2.9. Immunohistochemistry

Human cardiac tissue sections (myocardium, arterial and venous vessels, heart valves) from cardiac surgery or mice aortic sections were fixed in formalin and embedded in paraffin. Tissue sections were mounted on poly-l-Lys-coated slides. Paraffin sections were de-waxed and re-hydrated in xylene and ethanol, followed by rinsing with water. Endogenous peroxidase activity was blocked by 30-min incubation with 1% H2O2 and nonspecific binding was suppressed by 30-min incubation with goat serum diluted 1:20 in PBS. The sections were then incubated with rabbit N-Hcy-protein antibody diluted 1:100 in PBS containing 2 mM DTT for 3 h. The primary antibody binding was detected by incubation with biotinylated secondary goat anti-rabbit IgG antibody followed by the streptavidin-conjugated horseradish peroxidase; 3,3’-diaminobenzidine or Nova Red chromogen was used to visualize antibody binding to human or mouse tissues, respectively. To visualize the nuclei and cytoplasm, consecutive tissue sections were stained with hematoxylin and eosin.

The following controls were used: tissues preincubated with 50 mM Hcy-thiolactone for 3 h at 37 °C, tissue preincubated with 2 mM DTT for 15 min followed by 20 mM iodoacetamide in the dark for 1 h at 37 °C, and the rabbit antibody preincubated with N-Hcy-albumin, native albumin, or iodoacetamide-treated N-Hcy-albumin (5 mg/mL) for 3 h at room temperature.

3. Results and discussion

3.1. Rabbit polyclonal anti-N-Hcy-protein antibodies

We used intradermal inoculations with N-Hcy-KHL to induce anti-N-Hcy-protein antibodies in rabbits. Maximal titers of anti-N-Hcy-protein antibodies, determined by ELISA using N-Hcy-hemoglobin, a coating antigen, were observed after 15 weeks in one rabbit and after 20 weeks in another rabbit. Competitive ELISA experiments demonstrated that the rabbit antibody specifically recognizes the Nc-Hcy-Lys epitope on N-homocysteinylated proteins. For example, antibody binding was competed out by N-Hcy-LDL, N-Hcy-hemoglobin, N-Hcy-albumin, N-Hcy-transferrin, and N-Hcy-antitrypsin, but not by corresponding native proteins or N-Hcy-proteins in which the N-linked Hcy thiol was blocked by the treatment with iodoacetate [21,26].

3.2. Dot-blot assays

Myoglobin modified with Hcy-thiolactone at a 1:10 molar ratio incorporates about 1 mol N-linked Hcy per mole myoglobin. As illustrated in Fig. 1, as little as 0.02 μg of N-Hcy-myoglobin could be detected on dot blots using the rabbit anti-N-Hcy antibody, whereas unmodified native myoglobin was not recognized. The antibody bound N-Hcy-myoglobin...
in a dose dependent manner. Similar results were obtained with N-Hcy-hemoglobin, N-Hcy-cytochrome c, and N-Hcy-albumin (Fig. 1B). Antibody reactivity was eliminated by treating N-Hcy-proteins with iodoacetamide, which reacts with the sulfhydryl group of protein N-linked Hcy (not shown).

Human breast cancer cells MCF-7 were grown in the absence and presence of exogenous Hcy-thiolactone as described in Section 2. Cellular proteins were extracted and subjected to dot-blot analysis with the rabbit anti-N-Hcy-protein antibody. As illustrated in Fig. 2, N-Hcy-protein can be detected on dot blots of cellular proteins from the MCF-7 cells. The intensity of basal immunostaining with anti-N-Hcy-protein antibody substantially increased in cellular protein samples from Hcy-thiolactone supplemented cultures (Fig. 2).

Overall, these analyses indicate that N-Hcy-protein-directed antibodies provide a uniquely useful marker for N-Hcy-protein. To apply this new immunological tool to the analysis of N-Hcy-proteins in situ, we employed immunohistochemistry to examine N-Hcy-proteins in tissue sections.

3.3. Immunohistochemistry

Fixed sections of human myocardium and aortic valve exhibited immunoreactivity with anti-N-Hcy antibody (Fig. 3A, and C). The immunostaining was largely eliminated by treatment with iodoacetamide (which destroys the Nc-Hcy-Lys epitope) before incubation with the antibody (Fig. 3E). The immunostaining was also eliminated when the antibody was pre-adsorbed with N-Hcy-albumin (Fig. 3B, and D). Treatments of myocardium and aortic valve tissue sections with Hcy-thiolactone, which increase tissue levels of N-Hcy-protein, resulted in substantial increases in immunostaining (not shown). Mouse cardiac muscle also showed positive staining for N-Hcy-protein (in fact, it can be clearly seen in the sections from the ApoE−/− mice in Fig. 4 that mouse cardiac muscle is positively stained). However, no staining was observed for mouse liver sections (not shown), consistent with efficient Hcy metabolism in the liver and a limited metabolic capacity for Hcy in the cardiovascular tissues [31]. These findings extend the results with membrane-bound N-Hcy-proteins to demonstrate that N-Hcy-proteins can be visualized specifically by immunohistochemistry.

ApoE−/− mice develop atherosclerosis spontaneously on a normal chow, but the process is accelerated by hyperhomocysteinemia caused by a high-Met diet [32]. Mean aortic lesion size in the aortic root of the hyperhomocysteinemic mice is significantly larger than in control mice [28]. In addition to causing hyperhomocysteinemia, high-Met diet also causes significant elevations in Hcy-thiolactone [15] and N-Hcy-protein levels in mice (H. Jakubowski, unpublished data). As shown in Fig. 4A and B, N-Hcy-protein immunostaining was observed within atherosclerotic lesions in sections prepared from aortas of ApoE−/− mice fed with a normal chow diet. The N-Hcy-protein immunostaining was increased in aortic lesions form ApoE−/− mice fed with a high-Met diet (Fig. 4C and D).
The staining for N-Hcy-proteins is similar to immunohistochemical staining for markers of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) observed previously in ApoE−/− mice [28]: both are localized in lesions and both are increased by a high-Met diet. ER stress, caused by the appearance of the misfolded/aggregated proteins in the ER, activates a signaling pathway, the UPR, that when overwhelmed can lead to apoptotic cell death. Treatments with Hcy-thiolactone or Hcy cause protein N-homocysteinyla-
tion (Fig. 2) [11,33] and induce ER stress and UPR [ref. [34]; R.C. Austin, unpublished data] and apoptotic death in cultured cells [35,36]. N-Hcy-proteins are known to have a propensity to form aggregates [18]. In this scenario, the appearance of aggregated N-Hcy-proteins in the ER is responsible for cell

![Fig. 3. Immunohistochemical detection of N-Hcy-protein in human cardiac tissues. Reaction product (diaminobenzidine) is brown. Human myocardium (panels A, B) and aortic valve (panels C–E) immunostained with rabbit anti-N-Hcy-protein antibody (panels A, C). Staining is largely eliminated in negative controls: antibody preincubated with N-Hcy-albumin (panels B, D) and tissue preincubated with iodoacetamide before immunostaining (panel E).](image)

![Fig. 4. Immunohistochemical detection of N-Hcy-protein in aortic lesions from ApoE−/− mice. Immunostaining for N-Hcy-protein in aortic root lesions (indicated by arrows) is increased in ApoE−/− mice fed with a high-Met diet (panels C, D), compared to a control diet (panels A, B).](image)
damage and atherothrombosis observed in hyperhomocysteinemia according to the following scheme:

\[
\text{Hcy} \rightarrow \text{Hcy} - \text{thiolactone} \rightarrow \text{protein modification} \rightarrow \text{protein misfolding/aggregation} \rightarrow \text{ER stress} \rightarrow \text{UPR activation} \rightarrow \text{cell dysfunction or death} \rightarrow \text{atherothrombosis.}
\]

Our immunohistochemical findings, which are consistent with the results of assays demonstrating the presence of N-linked Hcy in mammalian proteins [12,37], indicate that protein N-homocysteinylatation is an important process linked to human disease. In particular, our findings in human cardiac tissues and mouse atherosclerotic lesions emphasize the role of N-Hcy-proteins in vascular disease and support the emerging view that N-homocysteinylatation is a ubiquitous protein modification, which has important pathophysiological consequences.

In conclusion, our data show that the rabbit anti-N-Hcy-protein IgG antibody is a useful tool for monitoring N-Hcy-protein in human and mouse tissues. The availability of such antibody will facilitate studies of the role of Hcy-thiolactone-modified proteins in human disease.

Acknowledgment

This work was supported in part by a grant No. N401 065 32/1504 from the Ministry of Science and Higher Education, Poland.

References


