Hyperhomocysteinaemia induced by dietary folate restriction causes kidney oxidative stress in rats

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Diet is the most common cause of mild hyperhomocysteinaemia (HHcy), which occurs in approximately 5–7 % of the general population. Since HHcy causes endothelial damage by oxidative stress in different organs, the present study was designed to examine whether HHcy might be involved in renal oxidative stress. Twenty-five male Wistar rats were randomly divided into two groups: one (n 13) was fed ad libitum a folate-free diet (FF) and the other (n 12) was fed the same diet supplemented with folic acid (control, CO). After 8 weeks the animals were killed and kidneys removed. Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured in plasma and kidney homogenates. Renal tissue sections were analysed by indirect immunostaining with the primary antibody against oxidatively modified LDL receptor (LOX-1). A marked HHcy was confirmed in the FF group. As compared with CO animals, MDA levels in plasma and kidney homogenate were significantly higher in FF rats (P<0.05). Similarly, renal GPx and SOD activities were significantly higher in the FF group (P<0.001). No differences were found in LOX-1 immunohistochemical expression, which in the two groups was displayed in tubular cells. The present study provides evidence that HHcy does produce renal oxidative stress mediated by lipid peroxidation, and that the increased kidney MDA displayed by FF animals may enhance kidney antioxidant activity and thereby attenuate both kidney damage and expression of LOX-1.

Homocysteine: Folic acid: Oxidative stress: Kidney

Homocysteine (Hcy) is a sulphhydryl-containing amino acid derived from the demethylation of methionine. Hcy may be further metabolized by the sulphuration pathway to cysteine, or remethylated using either methyltetrahydrofolate or betaine, which is confined to the liver (Konukoglu et al. 2003). Mild hyperhomocysteinaemia (HHcy), most commonly caused by diets poor in homocysteine-lowering vitamins, i.e. folate, vitamin B\(_6\) and/or vitamin B\(_12\) (Selhub et al. 1993; Most et al. 2004), occurs in approximately 5–7 % of the general population (McCully, 1996). Recent studies have suggested that HHcy is a major independent risk factor for CVD (Konukoglu et al. 2003; Zhou et al. 2003), with mild HHcy found in approximately 20–30 % of patients with coronary, cerebrovascular and peripheral vascular disease (Malinow, 1990; Brattström & Wilcken, 2000; Stanger et al. 2001; Aamir et al. 2004; Fruchart et al. 2004).

Some investigators have postulated that Hcy might cause atherosclerosis by damaging the endothelium either directly or by altering oxidative status. Endothelial Hcy-mediated cytotoxicity is, in part, attributable to the generation of reactive oxygen species, such as hydrogen peroxide (H\(_2\)O\(_2\)), superoxide anion (O\(_2^-\)) and hydroxyl radical (HO\(^•\)) (Misra, 1974), during the auto-oxidation of Hcy to homocysteine or other mixed disulphides (Starkebaum & Harlan, 1986; Welch et al. 1997; Heydrick et al. 2004). The effect of HHcy on organ oxidative status has been investigated in endothelium (McCully, 1996; Morita et al. 2001), liver (Huang et al. 2001), heart (Young et al. 1997) and brain (Sachdev et al. 2004), but little is known on the effect of this amino acid derivative on renal oxidative status. Some investigators have found that HHcy induced through different approaches did produce renal damage in both man and animals. Chen et al. (2002) and Li et al. (2002) reported the development of glomerulosclerosis in HHcy rats. Kumagai et al. (2002) found numerous renal lesions including tubular atrophy and interstitial fibrosis in rats with HHcy induced by feeding the animals a folate-deficient diet. While Fischer et al. (2003) reported an increase in lipid peroxidation in HHcy rats, the effect of HHcy on renal oxidative status and activity of antioxidant enzymes has not been investigated.

The goal of the present work was therefore first to induce HHcy status in rats by feeding the animals a folate-depleted diet, and second, to study the impact of this HHcy on renal oxidative status and antioxidant enzymatic competence.

Materials and methods

Animals, diets and experimental design

The animal protocol was approved by the Committee of Animal Care established at the Applied Pharmacobiology Research

Abbreviations: CO, control; FF, folate-free; GPx, glutathione peroxidase; Hcy, homocysteine; HHcy, hyperhomocysteinaemia; LOX-1, oxidatively modified LDL receptor; MCP-1, monocyte chemoattractant protein 1; MDA, malondialdehyde; PCC, protein carbonyl groups; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

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Center (APRC, University of Navarra, Pamplona, Spain) and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission on Life Sciences, 1996). Twenty-five male Wistar albino rats (Rattus norvegicus), bred at the APRC and weighing between 221 and 246 g, were housed in plastic-bottomed cages (PANLAB, Barcelona, Spain) in a room with controlled temperature (20 ± 2°C), relative humidity (45%), laminar air flow and light–dark cycle (light from 08.30 to 20.30 hours). Water was freely available at all times. After being adapted to diets and cages for 3 d with free access to control diet, the rats were randomly divided into two groups of equal average weight. One group (FF, n 13) was fed a folate-free diet (Diets Inc., Bethlehem, PA, USA) following the criteria of Clifford and Kuory (Clifford et al. 1989) related to L-amino-acid-defined diets. The control group (CO, n 12) was fed the same diet but supplemented with 8 mg folic acid/kg, and was taken as control. Both diets contained 1% succinyl sulphathiazole (w/w) to prevent folate deficiency. On the first day and at the end of the 8-week feeding period, rats were lightly anaesthetized by intraperitoneal injection of 0·1 mg ketamine/g (Warner-Lambert Company, Morris Plains, NJ, USA) and blood samples were drawn from the retro-ocular venous plexus from all rats, with capillary tubes (Marienfeld, Mergenheim, Germany). Blood was centrifuged for 10 min at 3500 g and plasma was stored at −80°C until analyses.

At the end of the 8-week experiment, anaesthetized rats were killed by decapitation and kidneys were removed, weighed, and a portion was snap-frozen in liquid N2 and kept frozen at −80°C until analysis. The remaining tissue was embedded in formaldehyde for 24 h and then stored in alcohol. To prepare kidney homogenates, kidneys were thawed on ice and homogenized (1:10, w/v) in 20 mm-Tris–HCl containing 5 mm butylated hydroxytoluene and 0·01% EDTA (Sigma, St. Louis, MO, USA) in an Ultraturrax blender. The homogenates were centrifuged at 3000 g for 10 min. Supernatants were then collected for malondialdehyde (MDA) analysis, and centrifuged at 20 000 g prior to determination of antioxidant enzyme activities.

**Analytical procedures**

Creatinine, cholesterol, glucose, triacylglycerols and NEFA were measured in plasma using an automatic autoanalyzer (Cobas Mira ABX; Roche, Walpole, MA, USA). Plasma Hep was measured as total Hcy by HPLC using a Hewlett Packard HP series 1050 instrument, model 79855A (ABX, Newark, CA, USA), according to Pfeiffer et al. (1999). The inter- and intra-assay CV were <10%. Plasma folate concentrations were determined by RIA, with the aid of a commercially available kit (ABX; DPC, Madrid, Spain). The inter- and intra-assay CV were <5%.

**Assessment of plasma and renal markers of oxidative stress.** Levels of MDA, a validated index of lipid peroxidation (Esteban and Cheeseman, 1990), were measured in plasma and kidney after heating samples at 45°C for 60 min in acidic medium and quantified by colorimetric assay (Lipid Peroxidation Assay kit; Calbiochem, San Diego, CA, USA). The inter- and intra-assay CV were <5%. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured in plasma and kidney homogenates at 37°C in the Cobas Mira automatic analyser with the aid of commercially available kits (Ransel; Randox ABX, Barcelona, Spain). Total SOD activity, measured in diluted samples of kidney homogenate (1:100) and serum (1:50), was determined by a slightly modified method (McCord & Fridovich, 1987) where xanthine and xanthine oxidase were employed to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium. Data are expressed as U/mg protein using a standard provided in the kit and the inter- and intra-assay CV were <5%. GPx activities were measured in diluted samples of kidney (1:20) and serum (1:50) according to a previously described method (Paglia & Valentine, 1967). Briefly, GPx catalyses the oxidation of glutathione by cumene hydroperoxide and in the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. Values are expressed as U/mg protein. Inter- and intra-assay CV were <6%.

**Protein oxidation, as measured by determination of protein carbonyl groups (PCC),** was assessed by spectrophotometric assay as previously described (Stadtman & Oliver, 1991). The inter- and intra-assay CV were <5%. Kidney concentrations of monocyte chemoattractant protein-1 (MCP-1), an indicator of initiation and progression of renal damage (Viedt & Orth, 2002), were measured with a commercially available ELISA (r-MCP-1 Biokine; Amersham Pharma Biotech, Amersham, Buckinghamshire, UK). The inter- and intra-assay CV were <10%.

Western blots were used for oxidatively modified LDL receptor (LOX-1) was performed in kidney homogenate (Ox-LDLR-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

**Histological examination and immunohistochemistry.** Paraffin-embedded 3 µm thick sections of kidney were stained with periodic acid Schiff, haematoxylin and eosin, and Masson’s monochrome, and optical light microscopy was performed at 10× and 40× magnification. A minimum of ten fields for each sample were examined by two independent observers. Approximately ten sections were examined in each kidney sample.

To explore oxidative damage, an immunohistochemical survey was performed. Paraformaldehyde-fixed renal tissue sections were analysed by indirect immunostaining with the primary antibodies against LOX-1, and with the biotinylated secondary antibody. Specificity of the primary antibody was confirmed by Western blotting. Sections of the antibody-labelled tissue were incubated with a streptavidin–horseradish peroxidase complex (Dako, Glostrup, Denmark) and developed with 3,3′-diaminobenzidine (Merck, Darmstadt, Germany). Negative controls, using PBS in equal amounts for the primary antibody, were used.

**Statistical analysis**

Data are expressed as means with their standard deviations. Statistical significance was estimated with the paired or unpaired t test as appropriate. P<0.05 was considered significant. All analyses were performed using the SPSS statistical package, version 9.0 (SPSS Inc., Chicago, IL, USA). Data were normally distributed for all measured variables. Kolmogorov–Smirnov and Shapiro–Wilk tests were applied in all cases.

**Results**

**Animals**

Fig. 1 shows that at the end of the 8-week experimental period, FF rats had a significantly lower body weight (P<0.001) than CO.
animals. The weight gain was 140.8 (SD 5.3) g in the CO group v. 106.1 (SD 5.3) g in the FF group.

**Folate and homocysteine status and biochemical parameters**

As expected, at the beginning of the 8-week experimental period, plasma Hcy and folate concentrations did not differ between the two groups. After 8 weeks of dietary treatment, plasma folate decreased significantly (P < 0.001) while plasma Hcy concentrations increased significantly (P < 0.001) in FF animals compared with CO rats, corroborating an intermediate HHcy in both groups and found no significant differences among the two experimental groups.

**Lipid peroxidation**

Fig. 2 shows significantly higher (P < 0.05) plasma MDA levels in the FF group (0.41 (SD 0.06) mmol/mg protein) than in CO rats (0.25 (SD 0.03) mmol/mg protein). Similarly, kidney MDA levels were significantly higher (P < 0.05) in FF animals (54.75 (SD 3.75) mmol/g tissue) compared with CO animals (45.31 (SD 2.51) mmol/g tissue).

**Activities of antioxidant enzymes**

Results related to oxidative stress parameters in kidney are shown in Fig. 3. Renal activity of GPx in FF animals was significantly higher than in CO rats (P < 0.001, 2.14 (SD 0.03) U/mg protein). The same pattern was observed with SOD (P < 0.001, 23.7 (SD 5.7) U/mg protein). In addition, significant correlations between plasma Hcy and folate concentrations and activity of both kidney antioxidant enzymes were found, with correlation values between renal SOD and plasma Hcy and folate of 0.531 (P < 0.01) and −0.565 (P < 0.01), respectively, and for renal GPx of 0.685 (P < 0.01) and −0.732 (P < 0.01), respectively. Plasma SOD and GPx activities were significantly higher (P < 0.05) in FF rats than in the CO group (Fig. 4).

**Protein oxidation**

Although a slight trend towards increased protein oxidation was observed in the FF animals where 7.26 (SD 1.05) mmol/mg protein compared with 6.67 (SD 0.66) mmol/mg protein in the CO group, these values were not significantly different.

**Monocyte chemoattractant protein 1**

The levels of MCP-1 in kidney measured by ELISA were not different between CO and FF groups, the values being 565.30 (SD 26.9) pg/mg protein, respectively.

**Histological changes**

The histological evaluation showed a regular morphology in both groups and found no significant differences among the two experimental groups.

**Oxidatively modified LDL receptor**

A representative Western blot analysis in kidney homogenate using polyclonal antibody against LOX-1 demonstrated the...
presence of LOX-1 in this tissue, although because the Western assay was carried out under reducing conditions, protein denatur- 
ation most likely occurred creating the second band (Fig. 5).

Immunohistochemical analysis showed that LOX-1 was widely 
expressed in tubular cells, whereas there was almost no staining 
in the glomeruli (Fig. 6).

Discussion

The present study examined the chronic effects of diet-induced HHcy on the rat kidney. The first phase of the experiment 
included the induction of intermediate HHcy by feeding rats a folate-free diet for 8 weeks. Following this depletion phase and 
agreement with previous investigators (Clifford et al. 1990), the parameters measured were sensitive enough to distinguish 
between folate-depleted and folate-repleted animals. Mean plasma folate concentrations in the folate-depleted rats (1·87 
ng/ml) were slightly lower than the values obtained by other authors (O’Leary & Sheehy, 2001). The mean plasma Hcy con-
centrations of depleted rats (75·36 m mol/l) were similar to those 
reported by Miller et al. (1994). The inverse relationship between 
plasma Hcy and folate intake found in the present study was also 
consistent with previous reports in rats (Durand et al. 1996). The 
dietary model used in the present study, as compared with other 
designs (Southern et al. 1998; Morita et al. 2001; Kumagai et al. 2002), may be of relevance because clinically or sub-cli-
nically low folate levels are commonly caused by nutritional folate 
deficiency (Lentz, 1997).

The reduced weight gain displayed by FF rats correlated with 
previous reports (Clifford et al. 1989, 1990; Balaghi et al. 
1993; Huang et al. 2001), but contrasted with others (Durand 
et al. 1996, 1997). These differences in growth response might 
be related to the level of folate restriction and the length of the 
experimental periods.

There were no differences in serum creatinine between groups, 
which echoes the results obtained by Rolland et al. (1995) who 
demonstrated no significant alteration in creatinine clearance in 
hyperhomocysteinaemic minipigs. This may indicate that glomer-
ular filtration rate had not been altered under these experimental 
conditions. The decrease of plasma NEFA and cholesterol 
found in the present study could be attributed to either the low 
folate status (Akesson et al. 1982) or the HHcy condition. The 
fact that plasma cholesterol, triacylglycerols and NEFA levels 
were reduced in FF rats correlates with the findings of Werstuck 
et al. (2001), who demonstrated that Hcy-induced endoplasmic 
reticulum stress causes dysregulation of the endogenous sterol 
response pathway.

Regarding the effect of plasma levels of Hcy on kidney mor-
phology, HHcy induced through different approaches has been 
found to produce renal damage in both human and animal sub-
jects. These include glomerulosclerosis (Chen et al. 2002; Li 
et al. 2002; Yang & Zou, 2003), tubular atrophy and interstitial 
fibrosis (Miller et al. 2000; Kumagai et al. 2002) in rats in 
which HHcy was induced by feeding the animals a diet deficient 
in folate. However, we observed no relevant histological differ-
ences in the FF rats, probably because the 8-week period was

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**Fig. 2.** Effects of hyperhomocysteinaemia on malondialdehyde (MDA) levels in (A) plasma and (B) kidney tissues after 8 weeks for rats in the control (CO, n 12) and folate-free (FF, n 13) groups. Values are means with their standard deviation shown by vertical bars. Mean values were significantly different from those of the CO group: *P*, 0·05.

**Fig. 3.** Renal effects of hyperhomocysteinaemia on activity of (A) glutathione peroxidase (GPx) and (B) superoxide dismutase (SOD) after 8 weeks for rats in the control (CO, n 12) and folate-free (FF, n 13) groups. Values are means with their standard deviation shown by vertical bars. Mean values were significantly different from those of the CO group: ***P*, 0·001.
too short to produce morphological alterations in kidney. Nevertheless, these results correlate with those of Rensma et al. (2003) who found no evidence of significant renal damage in patients displaying both hyperhomocystinuria and strongly elevated plasma Hcy levels (>100 μmol/l).

The present study provided evidence that lack of folate consumption is also associated with increased oxidative damage in kidney. Lipid peroxidation is a well-established mechanism of cellular injury. This reaction leads to the production of lipid peroxides and their by-products, and ultimately to the loss of membrane function and integrity (Turrens et al. 1982). A major secondary oxidation product of PUFA is MDA (Esterbauer & Cheeseman, 1990), a substance that is frequently measured as thiobarbituric acid-reactive substances (TBARS) to assess the degree of peroxidation (Huang et al. 2001; Raimakers et al. 2003). Because the TBARS test is not specific for MDA (Janero, 1990), we employed a new more specific colorimetric assay. As compared with CO animals, FF rats exhibited greater MDA levels in kidney, indicating an increased renal lipid peroxidation, which correlates with the results of other investigators (Fischer et al. 2001). Other studies have also found significantly higher TBARS production in liver of folate-depleted rats as compared with normally fed animals (Huang et al. 2001), and elevated MDA levels have also been found in the heart of pigs with induced HHcy (Young et al. 1997). On the other hand, the results of the present work also correlated with those of Ventura et al. (2000), who demonstrated a significant increase in plasma markers of lipid peroxidation in rats with HHcy induced by an oral methionine loading.

No differences in kidney PCC levels between the two groups were found in the present study. References on this matter are few and contradictory. Assessment of kidney PCC in HHcy subjects has not been found in the literature. Ventura et al. (2000) demonstrated an elevation of plasma PCC in acute HHcy after methionine loading. Nevertheless, Raimakers et al. (2003) were recently unable to detect changes in plasma PCC in similar conditions.

Nagase et al. (2001) demonstrated that Hcy exerts its effects through oxidative stress, and by enhancement of endothelial LOX-1 gene expression. They also observed that this expression was inhibited by antioxidants like Tempol, a SOD mimic, which alleviated LOX-1 augmentation induced by angiotensin II. However, the current immunohistochemical assessment revealed that LOX-1 was expressed in tubular cells but no differences were seen between FF rats and CO animals. Certainly, it could be expected that increased Hcy levels might cause enhanced LOX-1 expression, but it seems likely that the outstanding increase in antioxidant enzymes observed in the present study could inhibit LOX-1 expression.

**Fig. 4.** Effects of hyperhomocysteinaemia on plasma activity of (A) glutathione peroxidase (GPx) and (B) superoxide dismutase (SOD) after 8 weeks for rats in the control (CO, n 12) and folate-free (FF, n 13) groups. Values are means with their standard deviation shown by vertical bars. Mean values were significantly different from those of the CO group: *P<0.05.

**Fig. 5.** Western blot analyses of oxidatively modified LDL receptor in rat kidney under reducing conditions. Lanes 1 and 3, control group; lanes 2 and 4, folate-free group.
the activity of plasma antioxidant enzymes in patients who displayed elevated plasma total Hcy concentrations as a result of inherited defects of Hcy metabolism. Their results suggested that elevated plasma Hcy represents a status of increased oxidative stress, which triggers an adaptive response by increasing the activity of antioxidant enzymes in the circulation. The current study shows, for the first time, that an increase in oxidative stress in kidney induced by HHcy secondary to a complete dietary folate restriction caused a marked increase in the activities of both SOD and GPx. This suggests that a mechanism of homeostatic adaptation might come into play, in which a process of SOD and GPx up-regulation, i.e. enhanced enzymatic protein expression, could represent a protective antioxidant mechanism (Wilcken et al. 2000). It has been recently shown (Sindhu et al. 2005) that the oxidative stress induced in aortic tissue by hypertension is accompanied by an up-regulated increase in the expression of a number of antioxidant enzymes, such as NADPH oxidase, catalase, Cu/Zn-SOD and Mn-SOD, although these authors did not find changes in GPx. They postulated that up-regulation of antioxidant enzymes may be a compensatory response in the face of oxidative stress.

In conclusion, the present study provides evidence that HHcy produces a renal oxidative stress mediated by lipid peroxidation, but not by protein oxidation. The increase of MDA in the kidney is parallel to a rise in antioxidant enzyme activity that probably attenuates kidney damage by reducing the expression of LOX-1 and MCP-1, two main mediators of endothelial dysfunction. Antioxidant therapy might be a convenient therapeutic approach to prevent kidney damage in HHcy subjects.

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