Atherosclerosis 237 (2014) 725–733

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Ribose-cysteine increases glutathione-based antioxidant status and reduces LDL in human lipoprotein(a) mice

Tanjina Kader [a], Carolyn M. Porteous [a], Michael J.A. Williams [b], Steven P. Gieseg [c], Sally P.A. McCormick [a], *

[a] Department of Biochemistry, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand
[b] Department of Medicine, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand
[c] School of Biological Sciences, University of Canterbury, Christchurch, New Zealand

ARTICLE INFO

Article history:
Received 6 June 2014
Received in revised form
30 October 2014
Accepted 31 October 2014
Available online 1 November 2014

Keywords:
CVD
GSH
GP*x*
Ribose-cysteine
Oxidised lipids
LDL
apoB

ABSTRACT

Objective: D-ribose-L-cysteine (ribose-cysteine) is a cysteine analogue designed to increase the synthesis of glutathione (GSH). GSH is a cofactor for glutathione peroxidase (GP*x*), the redox enzyme that catalyses the reduction of lipid peroxides. A low GP*x* activity and increased oxidised lipids are associated with the development of cardiovascular disease (CVD). Here we aimed to investigate the effect of ribose-cysteine supplementation on GSH, GP*x*, lipid oxidation products and plasma lipids *in vivo*. **Methods**: Human lipoprotein(a) [Lp(a)] transgenic mice were treated with 4 mg/day ribose-cysteine (0.16 g/kg body weight) for 8 weeks. Livers and blood were harvested from treated and untreated controls ($n = 9$ per group) and GSH concentrations, GP*x* activity, thiobarbituric acid reactive substances (TBARS), 8-isoprostanes and plasma lipid concentrations were measured. **Results**: Ribose-cysteine increased GSH concentrations in the liver and plasma ($P < 0.05$). GP*x* activity was increased in both liver (1.7 fold, $P < 0.01$) and erythrocytes (3.5 fold, $P < 0.05$). TBARS concentrations in the liver, plasma and aortae were significantly reduced with ribose-cysteine ($P < 0.01$, $P < 0.0005$ and $P < 0.01$, respectively) as were the concentrations of 8-isoprostanes in the liver and aortae ($P < 0.0005$, $P < 0.01$, respectively). Ribose-cysteine treated mice showed significant decreases in LDL, Lp(a) and apoB concentrations ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively), an effect which was associated with upregulation of the LDL receptor (LDLR).**Conclusions**: As ribose-cysteine lowers LDL, Lp(a) and oxidised lipid concentrations, it might be an ideal intervention to increase protection against the development of atherosclerosis.

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1. Introduction

Atherosclerosis is the disease process underlying the development of cardiovascular disease (CVD) [1]. It involves the deposition of plasma lipoproteins, namely low density lipoprotein (LDL) and lipoprotein(a) [Lp(a)] in the arterial intima where the lipid components of both are subsequently oxidised to stimulate an inflammatory response. This promotes the formation of foam cells and the progressive development of lipid-filled plaques that are susceptible to rupture and thrombotic events [1]. Elevated LDL and Lp(a) levels are important independent risk factors for the development of CVD [2,3]. An increased oxidised phospholipid content of both lipoproteins is also associated with increased CVD risk [4,5].

As oxidised lipids are a major player in the development of atherosclerosis, endogenous systems that reduce their levels may be important in the prevention of CVD. In mammalian cells, the antioxidant enzyme, glutathione peroxidase (GP*x*) (E.C. 1.11.1.9) constitutes the principal defence system protecting against the effects of oxidised lipids by catalysing their reduction to lipid alcohols [6]. The activity of GP*x* is dependent on the availability of its cofactors, including glutathione (GSH), an endogenous tripeptide involved in many redox reactions protecting against oxidative stress [6]. Depletion of GSH is associated with increased levels of lipid peroxides in the liver [7]. GP*x* deficient mice display an increased level of oxidised LDL and foam cell formation in arteries [8]. Furthermore, a reduced GP*x* activity is associated with an

Abbreviations: CVD, cardiovascular disease; Lp(a), lipoprotein(a); LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides; apoB, apolipoproteinB-100; GSH, glutathione; GP*x*, glutathione peroxidase; TBARS, thiobarbituric acid reactive substances.

* Corresponding author. Department of Biochemistry, 710 Cumberland Street, University of Otago, Dunedin 9016, New Zealand.
E-mail address: sally.mccormick@otago.ac.nz (S.P.A. McCormick).

http://dx.doi.org/10.1016/j.atherosclerosis.2014.10.101
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increased risk of CVD in humans [9,10]. The promotion of GSH synthesis should therefore increase GP*x* activity and reduce the levels of oxidised lipids and associated CVD risk.

The liver has a high content of GSH and is the major organ involved in its synthesis. GSH is synthesised from its precursor amino acids (L-glycine, L-glutamine and L-cysteine) with L-cysteine being the rate limiting amino acid [11]. A readily available supply of L-cysteine for GSH synthesis is crucial for protection against oxidative stress [12]. Free L-cysteine, however, exhibits toxicity [13] so compound forms of L-cysteine that allow for a regulated release into cells to drive GSH biosynthesis without toxic side effects are desirable. Prodrugs of L-cysteine, such as N-acetylcysteine (NAC), have proven to be effective at increasing GSH levels when given at high doses in humans (around 1.0 g/kg body weight) [14,15]. However, side effects have been reported by patients treated with NAC suggesting that NAC releases L-cysteine at a level that can be toxic [16].

D-ribose-L-cysteine (ribose-cysteine) is a cysteine analogue that has been developed as a prodrug to support GSH synthesis [17]. NMR studies have shown that the release of L-cysteine from ribose-cysteine is much slower than that for NAC and is controlled by the rate of intracellular GSH synthesis and L-cysteine uptake by the liver [18]. Ribose-cysteine administered in a single dose to mice (2.0 g/kg body weight) increased GSH levels without producing toxicity in numerous organs, including the heart, muscle tissue, liver, kidney and lungs [19]. The longer-term effect of ribose-cysteine on GSH, GP*x* activity and oxidised lipids *in vivo* or its effects on plasma lipids has not been previously investigated.

The current study aimed to investigate the effect of ribose-cysteine supplementation on GSH levels, GP*x* activity and oxidised lipids in the liver and circulation as well as its effects on plasma lipids in a transgenic mouse model with elevated LDL levels and Lp(a).

2. Materials and methods

2.1. Mice

Human Lp(a) transgenic mice were used for the study as they have a humanised lipid profile with an elevated LDL and the presence of Lp(a) [20]. Human Lp(a) transgenic mice were generated by breeding mice expressing human apolipoprotein(a) [apo(a)] from a cDNA construct driven by a transferrin promoter [21] to mice expressing human apolipoproteinB-100 (apoB) from an 80-kb P1 genomic clone containing the human apoB gene and native promoter [20]. Both lines were on a C57BL/6 background. All mice were fed a chow-diet (Ruakura 86 Sharpes, Carterton, New Zealand) and housed in a specific pathogen free (SPF) animal facility with a 12 h light/dark cycle at 22 °C. Animals had free access to water and food and daily water intake and weekly body weights were recorded. Ethical approval for this study was granted by the Otago University Animal Ethics Committee. Nine human Lp(a) mice were treated with 1 mg/mL ribose-cysteine in the drinking water for 8 weeks giving an average dose of 4 mg/day/mouse (0.16 g/kg body weight) based on the volume of water consumed. Nine human Lp(a) mice without ribose-cysteine in their drinking water were used as untreated controls. Mice were aged between 12 and 20 weeks and there were three male and six females in each group. Ribose-cysteine was prepared by Chemica Inc. (Los Angeles, CA) and provided by Max International, LLC, Salt Lake City, UT.

2.2. Tissue and blood collection

After 8 weeks of ribose-cysteine supplementation, mice were sacrificed and livers and aortae removed and perfused with phosphate-buffered saline (PBS) to remove blood cells. Whole blood was collected via cardiac puncture into containers with EDTA as anticoagulant and plasma and erythrocytes were isolated. Samples were used fresh or stored at −80 °C until use.

2.3. GSH measurement

The GSH content of liver and plasma samples were measured according to Cotgreave et al. [22] after derivatisation with the thiol-binding dye, monobromobimane (MBB) (Sigma Chemical Co, St Louis, MO). Fresh liver tissue (50–80 mg) was homogenised in PBS and following centrifugation the supernatant was derivatised with MBB. For plasma, 200 μL of fresh plasma diluted 2 fold in PBS was derivatised. Following precipitation of protein from samples with TCA, the derivatised samples were kept at −80 °C until analysis. The GSH-MBB adducts were separated along with derivatised GSH standards (5 μM and 10 μM) (Sigma) by High Performance Liquid Chromatography (HPLC) on a Phenosphere-NEXT 5U reverse phase C_{18} column (100 × 4.6 mm, Phenomenex, Torrance, CA) using the Shimadzu 10 A HPLC system. A mix of 90% solvent A (0.25% acetic acid) to 10% solvent B (100% acetonitrile) was used for the separation with a flow rate of 1.5 mL/min. The fluorescence of the elutent was monitored using a Shimadzu 10xls Spectrofluorometer with excitation at 394 nm and emission at 480 nm. Peak areas were integrated using the Shimadzu LC Solution Software Package (version 1.22 SP1, 2002–2006).

2.4. GPx activity

The activity of GP*x* in liver tissue and erythrocytes was measured by spectrophotometric assay using a commercial kit (RS504, Randox Laboratories, Crumlin, UK). Samples were prepared according to the manufacturer's instructions. Liver tissue (15–20 mg) was homogenised in 1 mL of homogenisation buffer (250 mM mannitol, 70 mM sucrose and 1 mM EDTA adjusted with Tris to pH 7.4). Following centrifugation, the supernatants were sonicated at 300 W for 30 s, in three 10 s intervals then recentrifuged at 20,000 g for 10 min at 4 °C before being used in the assay. Erythrocytes from whole blood were prepared for the GP*x* assay according to the method of McCord and Fridovich [23]. Erythrocytes were washed with 5 mL of 0.15 M NaCl and then lysed by addition of 2 mL of ice-cold distilled water. Cell membranes and haemoglobin were removed by extraction with 1 mL of cold ethanol and 0.6 mL of cold chloroform and the supernatant used for the GP*x* assay. The GP*x* activity was expressed as units per mg of protein.

2.5. Oxidised lipid analysis

Aldehydes in plasma were quantified against a malondialdehyde (MDA) standard (Sigma) using the fluorometric thiobarbituric acid reactive substances (TBARS) assay [24]. TBARS were measured in lipid extracts from the liver and aortae using a protocol from Bartels et al. [25] to extract the lipid component from frozen mouse liver and aortae. Lipids were extracted from homogenised liver and aortae with chloroform/methanol and evaporated under nitrogen gas. Lipids were resuspended in isopropanol containing 1% Triton X-100 and used in the TBARS assay. The TBARS assay was also performed on an Lp(a)-enriched fraction isolated from plasma by Fast Protein Liquid Chromatography (FPLC). Total 8-isoprostanes (free and esterified) were also measured in the liver and in pooled aortae using an 8-isoprostane kit (Cayman Chemical, Ann Arbor, MI) with samples prepared according to the manufacturer's protocol.

2.6. Lipid and apolipoprotein analysis

Total concentrations of plasma cholesterol and triglycerides (TG) were measured using enzymatic reagents from Roche Diagnostics (Mannheim, Germany). High density lipoprotein (HDL) cholesterol concentrations were measured after apoB precipitation using the method described by Purcell-Huynh et al. [26]. Total LDL cholesterol concentrations were calculated using the Friedewald equation [27]. The concentrations of Lp(a) in plasma and apo(a) in liver homogenates were measured using an apo(a)-specific enzyme-linked immunosorbent assays (ELISA) as previously described [28]. The concentrations of apoB in plasma and liver homogenates were measured using the same ELISA but with an anti-human apoB polyclonal capture antibody (Roche) and an anti-human apoB monoclonal detection antibody, IDI-hrp [29]. Western blot analysis of Lp(a) in plasma was performed under non-reducing conditions using an anti-human apo(a) monoclonal detection antibody, a5-hrp [28]. Lipoprotein fractions were isolated by separation of plasma samples by FPLC on a Superose 6HR 10/30 column from GE Healthcare Bio-Sciences (Uppsala, Sweden). Separated fractions were measured for cholesterol concentration by enzymatic assay (Roche) and subjected to western blot analysis using the monoclonal antibodies a5-hrp [28] and IDI-hrp [29] to identify Lp(a) and LDL-containing fractions, respectively.

2.7. Quantitative RT-PCR analysis

The level of mRNA transcript for apoB and the low-density lipoprotein receptor (LDLR) was measured by quantitative RT-PCR with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β_2-microglobulin (B2M) as reference genes. Total RNA was extracted from liver tissue using the Quick-RNA Miniprep Kit (Zymo, Irvine, CA) according to the manufacturer's protocol with genomic DNA digested with DNase. 500 ng of RNA was reversed transcribed to cDNA using a Prime Script ™ RT reagent kit (Perfect Real Time) (Takara Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer's protocol. Quantitative PCR was performed on an Applied Biosystem 7900HT sequence detection system (Life Technologies, Carlsbad, CA) using the SYBR Green I detection system. The cDNA was amplified with the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Gene expression was determined in triplicates for all samples and the mean value was normalized to the mean value of GAPDH and B2M.

2.8. Western blot analysis of LDLR

Liver homogenates (60 μg) were separated by SDS PAGE on 7.5% polyacrylamide gels under reducing conditions and subject to western blot analysis using an anti-LDL receptor antibody (Abcam, Cambridge, MA) and an anti-actin antibody (Sigma). Blots were

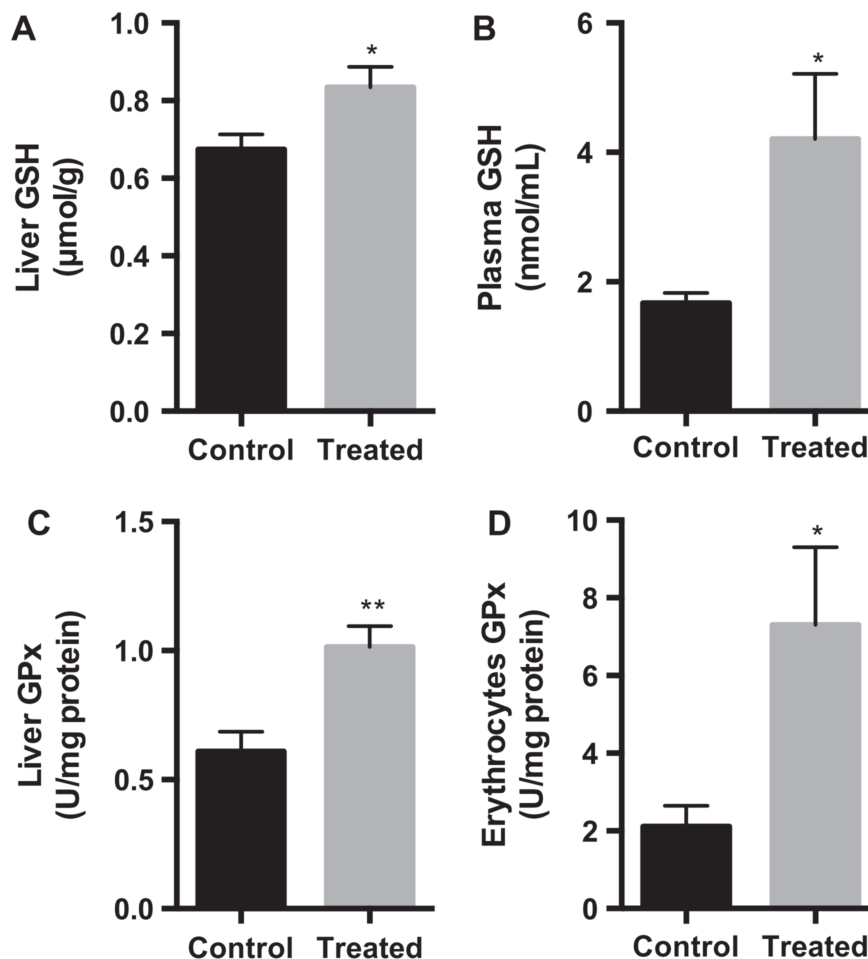

Fig. 1. Ribose-cysteine increases GSH levels and GP*x* activity in human transgenic Lp(a) mice. Human transgenic Lp(a) mice were treated with 4 mg/day ribose-cysteine in their drinking water for 8 weeks alongside human transgenic Lp(a) control mice given normal drinking water ($n = 9$ per group). Tissue GSH content was measured by HPLC and GP*x* activity was measured by spectrophotometric assay. (A) Liver GSH ($n = 9$), (B) Plasma GSH ($n = 9$), (C) Liver GP*x* ($n = 9$), (D) Erythrocytes GP*x* ($n = 5$). $^*P < 0.05$, $^{**}P < 0.01$.

washed and then incubated with a goat anti-rabbit IgG-hrp antibody (Thermo Scientific, Waltham, MA). Membranes were developed using enhanced chemiluminescence (ECL) on a LAS-3000 Imager (Fujifilm, Tokyo, Japan). Protein quantification was performed by Image Quant TL Software (Amersham Biosciences, Piscataway, NJ) with protein normalised against actin.

2.9. Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad, San Diego, CA). Statistical analysis for the effect of ribose-cysteine on GSH, GP*x* and oxidised lipids were assessed using an unpaired student *t*-test. The correlation between plasma and aortae TBARS was analysed by simple linear regression. Statistical analysis for lipid and apolipoprotein analysis, quantitative RT-PCR analysis and western blot analysis were assessed using an unpaired student *t*-test. All values are expressed as means ± SEM. A difference with $P < 0.05$ was considered as significant.

3. Results

3.1. Effect of ribose-cysteine on GSH and GPx

Daily water consumption was similar between groups (4.07 mL ± 0.24 vs 4.04 mL ± 0.02). Liver weights of treated versus control animals were similar (1.27 *g* ± 0.04 vs 1.31 *g* ± 0.06) and

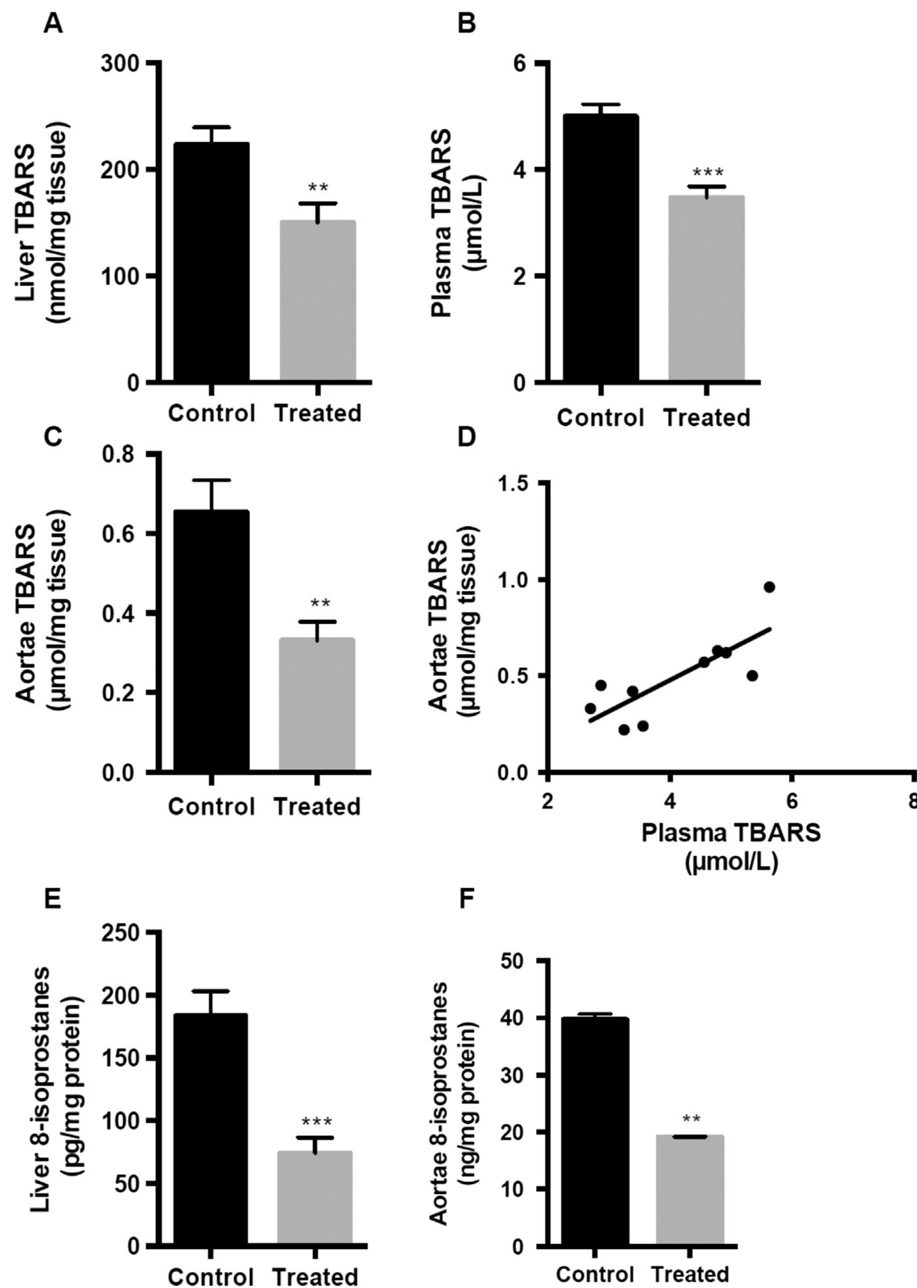

Fig. 2. Ribose-cysteine decreases TBARS and total 8-isoprostanes in human transgenic Lp(a) mice. Concentrations of aldehydes in ribose-cysteine treated and control mice were measured in various tissues by the TBARS assay and total 8-isoprostanes in liver and aortae were measured by an 8-isoprostane EIA kit (A) TBARS content of the liver ($n = 9$), (B) TBARS content of plasma ($n = 9$), (C) TBARS content of aortae ($n = 5$), (D) Correlation between plasma and aortae TBARS ($n = 5$ treated and 5 controls), (E) Total 8-isoprostane in the liver ($n = 9$), (F) Total 8-isoprostane in a sample of 5 pooled arteries analysed in duplicate. $^{**}P < 0.01$, $^{***}P < 0.0005$.

there were no obvious signs of hepatoxicity. Liver GSH was significantly increased in ribose-cysteine treated mice compared to controls (0.84 ± 0.05 vs 0.67 ± 0.04 μmol/g tissue, $P < 0.05$, Fig. 1A). Plasma GSH was also significantly increased in treated mice compared to controls (4.21 ± 1.01 vs 1.67 ± 0.15 nmol/mL, $P < 0.05$, Fig. 1B). The GP*x* activity in liver tissue was higher in treated mice compared to controls (1.01 ± 0.08 vs 0.61 ± 0.08 U/mg protein, $P < 0.01$, Fig. 1C). The GP*x* activity in erythrocytes was also significantly higher in treated mice (7.30 ± 1.99 vs 2.11 ± 0.54 U/mg protein, $P < 0.05$, Fig. 1D).

3.2. Effect of ribose-cysteine on oxidised lipids

Treated mice had significantly decreased concentrations of TBARS in the liver compared to controls (149.90 ± 18.28 vs 223.30 ± 16.21 nmol/mg tissue, $P < 0.01$, Fig. 2A). Plasma TBARS concentrations were significantly reduced in treated mice compared to controls (3.47 ± 0.21 vs 4.99 ± 0.23 μmol/L, $P < 0.0005$, Fig. 2B). Aortae TBARS concentrations were also significantly decreased in treated mice compared to controls (0.33 ± 0.05 vs 0.65 ± 0.08 μmol/mg tissue, $P < 0.01$, Fig. 2C). There was a significant positive correlation between plasma TBARS and aortae TBARS using data points from all animals ($n = 10$, $R^2 = 0.62$; $P < 0.01$, Fig. 2D). Separation of the plasma lipoproteins by FPLC and western blot analysis of the fractions for apo(a) and apoB identified an Lp(a)-enriched fraction which was measured for TBARS content (Suppl. Fig. 1A). The Lp(a)-associated TBARS content appeared to be decreased in the ribose-cysteine treated animals, but the decrease did not reach significance ($P < 0.09$) (Suppl. Fig. 1B).

Analysis of total 8-isoprostanes in the liver and aortae showed a significant reduction in both tissues with ribose-cysteine treatment compared to controls (74.37 ± 12.28 vs 183.50 ± 19.58 pg/mg protein, $P < 0.0005$ and 19.15 ± 0.03 vs 39.72 ± 0.98 ng/mg protein, $P < 0.01$, respectively, Fig. 2E, F).

3.3. Effect of ribose-cysteine on plasma lipid levels

There was a significant decrease in total plasma cholesterol concentrations in the ribose-cysteine treated mice compared to control mice (2.25 ± 0.23 vs 2.79 ± 0.08 mmol/L, $P < 0.05$, Fig. 3A). The concentrations of HDL and TG were not significantly different between the two groups (Fig. 3B, C). There was a decrease in plasma Lp(a) concentrations in treated mice compared to controls (28.13 ± 2.56 vs 42.73 ± 3.16 nmol/L, $P < 0.01$, Fig. 3D). Western blot analysis of plasma for apo(a) showed only Lp(a) bands and no free apo(a) in control or treated samples (Suppl. Fig. 2). The LDL cholesterol concentrations were significantly reduced in treated mice compared to controls (0.70 ± 0.17 vs 1.13 ± 0.11 mmol/L, $P < 0.05$, Fig. 3E) as were apoB concentrations (0.71 ± 0.13 vs 1.02 ± 0.05 μmol/L, $P < 0.05$, Fig. 3F).

Separation of the plasma lipoproteins by FPLC showed that the LDL and HDL cholesterol fractions increased from 0 to 8 weeks in both the control (Fig. 4A) and treated mice (Fig. 4B) but the increase in LDL cholesterol (as indicated by the area under the curve) was much less in the ribose-cysteine treated mice (Fig. 4B). A comparison of total cholesterol concentrations before and after treatment showed that before treatment (0 week) both groups had similar concentrations (control 2.19 ± 0.20 vs treated 2.21 ± 0.14 mmol/L, Suppl. Fig. 3A) but by 8 weeks total cholesterol concentrations in control mice had increased (2.79 ± 0.08, $P < 0.05$) whereas treated mice showed no change (2.25 ± 0.23 mmol/L, $P = 0.89$) (Suppl. Fig. 3A). Similarly, LDL concentrations in control mice increased significantly from 0 to 8 weeks 0.65 ± 0.16 vs 1.13 ± 0.11 mmol/L ($P < 0.05$) whereas treated mice showed no increase (Suppl. Fig. 3B). These results were mirrored by the apoB concentrations with control animals showing a significant increase from 0 to 8 weeks 0.63 ± 0.13 vs 1.02 ± 0.05 μmol/L ($P < 0.05$) and treated mice showing no significant increase (0.68 ± 0.11 vs 0.71 ± 0.13 μmol/L, $P = 0.82$, Suppl. Fig. 3C).

3.4. Effect of ribose-cysteine on liver transcripts and proteins

The level of LDLR mRNA transcript was 3.9 fold higher in ribose-cysteine treated mice compared to controls ($P < 0.0001$, Fig. 5A). Western blot analysis of the LDLR protein (Fig. 5B) and subsequent quantification (Fig. 5C) showed a 2.3 fold higher protein level in treated mice ($P < 0.0005$) compared to controls. There was no difference in the level of apoB transcript or protein in the liver between control and treated mice (Suppl. Fig. 4A, B). There was also no significant difference in the level of apo(a) protein between control and treated mice (Suppl. Fig. 4C).

4. Discussion

GSH is an important cofactor for many redox reactions that protect cells from oxidative stress [12]. L-cysteine is a limiting component for GSH synthesis and multiple cysteine analogues have been developed to enhance its delivery to the liver as a means of increasing GSH synthesis [12]. It is thought that enhancing GSH production will add protection against diseases involving oxidative stress. Here we investigated the effect of the cysteine analogue, ribose-cysteine [17], on GSH, GP*x* and oxidised lipids due to their association with the development of CVD. We show that ribose-cysteine increases GSH and activates the GP*x* system to reduce the level of oxidised lipids. Furthermore, ribose-cysteine has the added benefit of lowering LDL cholesterol and Lp(a) in mice.

Previous *in vivo* studies of ribose-cysteine have been mainly limited to its acute protective effects against drug induced renal and hepatotoxicity in mice [30,31]. These studies showed that i.p administration of ribose-cysteine at high doses (0.1–2 g/kg body weight) increased GSH levels and reduced hepatotoxicity without any side effects [30,31]. One of these studies also used oral supplementation of ribose-cysteine in the diet (1.2 g/kg body weight over 48 h), which also reduced hepatotoxicity [30]. Here we investigated the effect of a longer-term and lower oral dose of ribose-cysteine (0.16 g/kg body weight for 8 weeks) on the GP*x* system and lipid factors involved in the development of CVD. We used a transgenic mouse model that has elevated LDL levels and produces Lp(a) [20] as both lipoproteins are risk factors for developing CVD and are known to accumulate oxidised phospholipids [4,5].

Ribose-cysteine supplementation modestly but significantly increased the concentration of liver GSH and this was associated with a substantial (2.5 fold) increase in plasma GSH (Fig. 1A, B). This suggests that ribose-cysteine is absorbed and L-cysteine released for uptake by the liver presumably by non-enzymatic hydrolysis [18]. Furthermore, the GSH produced seems to be exported back into plasma. GSH is known to be exported from the liver into plasma by carrier-mediated transporter(s) to allow uptake by other tissues to maintain GSH homoeostasis [11,12]. The increase in liver and plasma GSH was associated with significant increases in the activity of GP*x* in the liver and erythrocytes (1.7 and 3.5 fold, respectively) (Fig. 1C, D). Our results are in keeping with a previous study showing that garlic-derived cysteine containing compounds increase liver and plasma GP*x* activity in mice [32] and a recent study in humans which has shown that NAC supplementation enhances plasma GP*x* activity and reduces MDA concentrations [33].

The increase in GP*x* activity in both liver and erythrocytes promoted by ribose-cysteine would be expected to be associated with a reduction in lipid peroxidation products in the liver and in

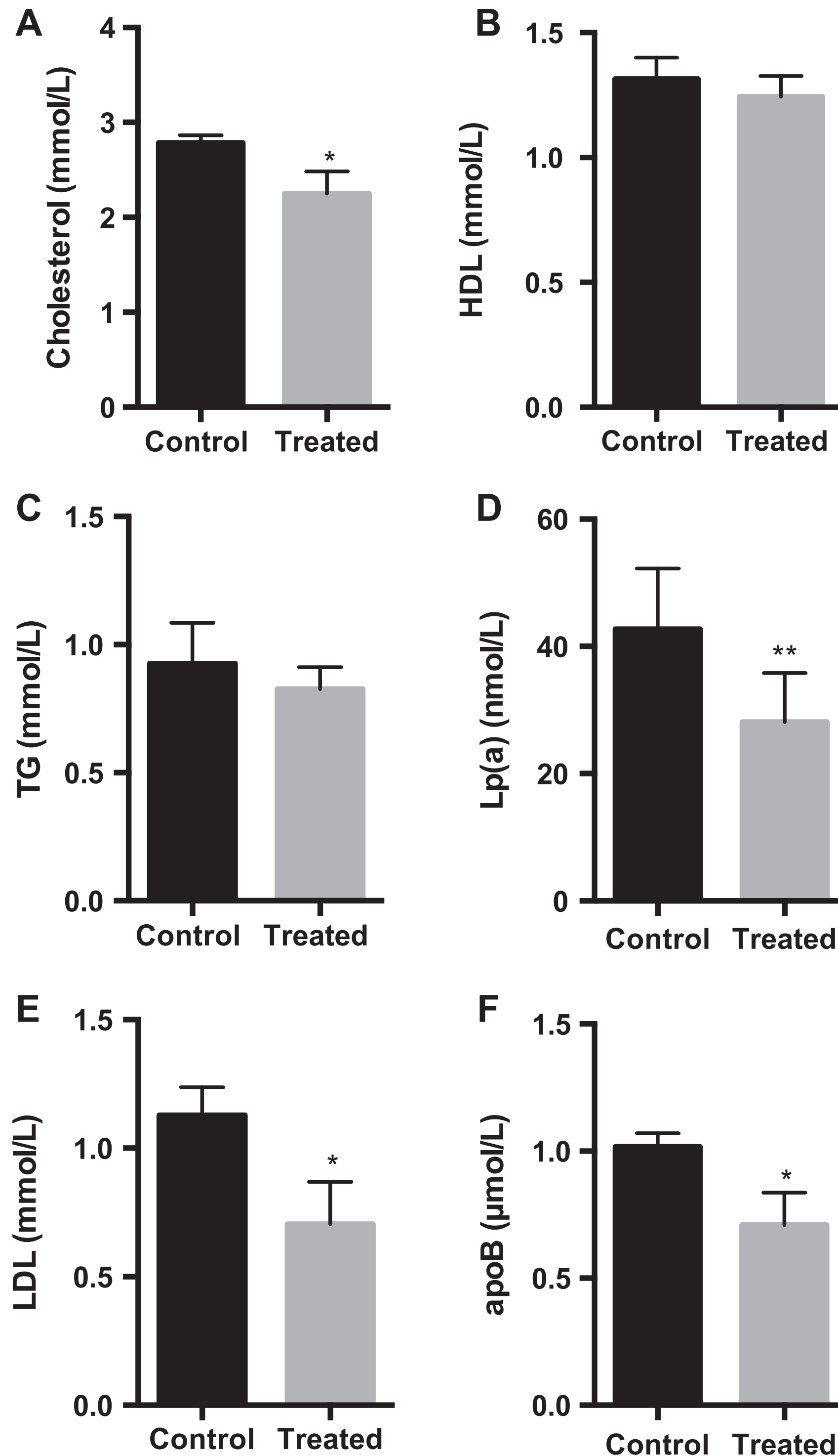

Fig. 3. Ribose-cysteine decreases total plasma cholesterol, LDL, Lp(a) and apoB levels in human transgenic Lp(a) mice. Total plasma cholesterol, HDL cholesterol and TG levels were measured in ribose-cysteine treated and control mice ($n = 9$ per group) by enzymatic assay and Lp(a) and apoB levels were measured by ELISA. Total LDL levels were calculated by the Friedewald equation. (A) Total cholesterol, (B) HDL cholesterol, (C) TG, (D) Lp(a), (E) LDL cholesterol, (F) apoB. $^{*}P < 0.05$, $^{**}P < 0.01$.

plasma. Indeed lipid peroxidation products as measured with the TBARS assay were significantly reduced in the liver, plasma and aortae (Fig. 2A, B, C) of ribose-cysteine treated mice. A positive correlation between TBARS in the artery and plasma TBARS has been reported [34] and elevated plasma TBARS are found in patients with CVD and hyperlipidaemia [35,36]. We also observed a significant positive correlation between TBARS in the plasma and arteries of Lp(a) mice (Fig. 2D), although we caution that this was only based on a small number of samples. An alternative and more stable marker of lipid peroxidation, 8-isoprostanes [37], was also measured and was shown to be reduced in the livers and aortae with ribose-cysteine treatment (Fig. 2E, F). By reducing lipid oxidation, ribose-cysteine may protect against the effect of oxidised lipids in the arterial intima where they promote inflammation and foam cells formation contributing to atherosclerotic plaque formation.

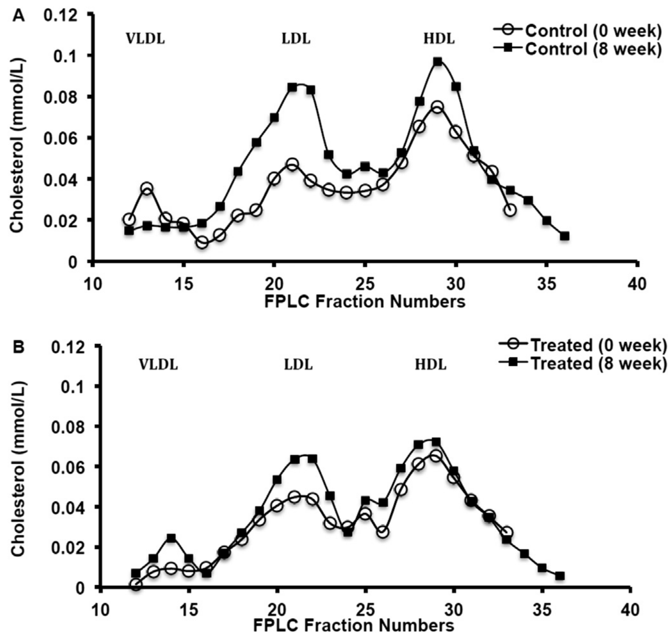

Fig. 4. Plasma lipoprotein analysis by FPLC. Plasma lipoproteins were separated by FPLC and the cholesterol concentration in each fraction measured by enzymatic assay. Each data point represents the average cholesterol concentration per fraction from 9 animals. (A) Plasma lipoproteins in control mice at 0 and 8 weeks, (B) Plasma lipoproteins in ribose-cysteine treated mice at 0 and 8 weeks.

As antioxidant compounds often alter plasma lipid levels [38], we investigated the effect of ribose-cysteine supplementation on plasma lipids. Total plasma cholesterol, LDL cholesterol, Lp(a) and apoB concentrations were all significantly reduced in ribose-cysteine treated mice (Fig. 3A, E, D, F), while concentrations of HDL cholesterol and TG remained unchanged (Fig. 3B, C). The effect of ribose-cysteine on total cholesterol, LDL cholesterol and apoB seemed to be due to its ability to abrogate the increase in LDL cholesterol and apoB levels with age (Fig. 4A, B; Suppl. Fig. 3A, B, 3C). In humans, LDL levels are known to increase with age and this is thought to be due to the decrease over time in LDLR expression [39]. Here we saw an increase in LDL concentrations of 0.47 mmol/L over 8 weeks in control animals but this increase was almost entirely prevented by ribose-cysteine. To establish the mechanism by which LDL levels were being decreased by ribose-cysteine, we analysed the level of apoB and LDLR in the liver. While apoB levels were similar between control and treated animals at both the transcript and protein level (Suppl. Fig. 4A, B), the LDLR transcript and protein levels were both significantly upregulated (Fig. 5A, B, C). This suggests that ribose-cysteine promotes an increased catabolism of LDL via upregulation of the LDLR. It is likely that ribose-cysteine may activate sterol regulatory binding protein 2 (SREBP2), a key transcriptional activator of LDLR known to be upregulated by antioxidants [40].

Other cysteine-containing compounds have been shown to lower total cholesterol concentrations in mice [32]. One study showed that NAC was able to prevent the increase in LDL concentrations that occurs with fat feeding in mice through suppression of SREBP and 3-hydroxy-3-methylglutaryl coenzyme A reductase [41]. It was of interest in our study that ribose-cysteine also reduced Lp(a) concentrations as there has previously been one report of high doses of NAC significantly lowering Lp(a) levels [42]. One possibility for the effect of ribose-cysteine on Lp(a) is that the apo(a) transgene, which is under the control of a transferrin promoter, could be affected. Transferrin is a negative acute phase response protein regulated by inflammatory cytokines [43], the levels of which could be altered by the antioxidant effects of ribose-cysteine. However, this possibility seems unlikely given that there was no significant change in apo(a) levels in the livers of ribose-cysteine treated animals (Suppl. Fig. 4C), suggesting that there was no effect on apo(a) transgene expression. The decrease in Lp(a) may be associated with the decrease in LDL via upregulation of LDLR although the role of the LDLR in Lp(a) catabolism remains controversial [44]. Alternatively, ribose-cysteine could have an

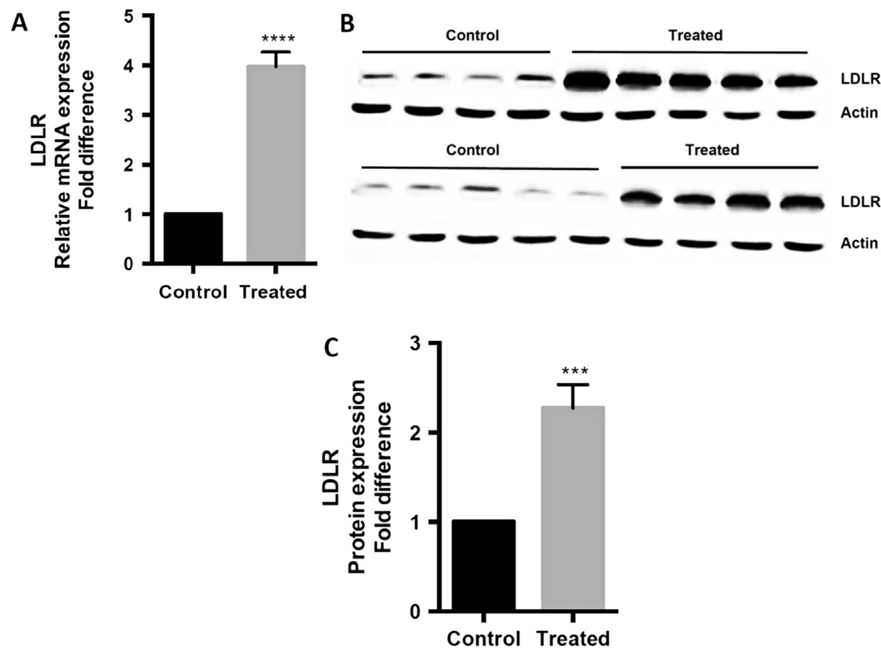

Fig. 5. Ribose-cysteine increases LDLR gene and protein expression. Relative levels of LDLR mRNA were measured by quantitative RT-PCR against GAPDH and B2M. Fold differences in relative mRNA levels were calculated and compared between control and treated mice ($n = 9$ per group). The levels of LDLR protein were analysed by western blotting and quantified after normalisation to actin in both control and treated mice. (A) Fold difference of relative LDLR mRNA, (B) Western blot of LDLR, (C) Fold difference of relative LDLR protein. ****$P < 0.0001$, ***$P < 0.0002$.

independent effect on Lp(a) clearance for which the receptor responsible remains unclear [45]. It is noteworthy that ribose-cysteine only reduced the concentrations of the atherogenic lipoproteins, LDL and Lp(a) and not that of the atheroprotective HDL. This is in contrast with other antioxidants in use to protect against CVD which produce unfavourable lipid profiles such as vitamin E and vitamin C which increase LDL [46] and probucol which reduces LDL but also HDL [47].

In conclusion, ribose-cysteine increases GSH-based antioxidant status in mice and this is associated with a reduction in oxidised lipid content. Furthermore, ribose-cysteine suppresses the level of atherogenic lipoproteins in plasma via upregulation of the LDLR. These properties suggest that ribose-cysteine may be an ideal intervention to increase protection against the development of CVD. Clinical trials with ribose-cysteine would be required to establish any cardioprotective effects in humans. Ribose-cysteine is currently a compound patented specifically for enhancing glutathione levels and is marketed as a dietary supplement.

Conflict of interest

None.

Acknowledgements

This work was supported by a University of Otago Research Grant and an Otago Medical School Summer Scholarship. Ribose-cysteine was kindly provided by Max International, LLC, Salt Lake City, UT. Antibodies for the Lp(a) ELISA (MAb a-6 and a-1) and apo(a) western blot (MAb a-5) were kindly provided by Prof. Santica Marcovina, Department of Medicine, University of Washington.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2014.10.101.

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