High Dietary Taurine Reduces Apoptosis and Atherosclerosis in the Left Main Coronary Artery

Association With Reduced CCAAT/Enhancer Binding Protein Homologous Protein and Total Plasma Homocysteine but not Lipidemia

Anthony Zulli, Eza Lau, Bagus P.P. Wijaya, Xin Jin, Komang Sutarga, Grace D. Schwartz, Jonathon Learmont, Peter J. Wookey, Angelo Zinellu, Ciriaco Carru, David L. Hare

Abstract—We sought to determine whether taurine could specifically protect against coronary artery disease during an atherogenic diet and whether taurine affects the lipid profile, metabolites of methionine, and endothelial atherogenic systems. Rabbits were fed one of the following diets for 4 weeks: (1) control diet; (2) 0.5% cholesterol +1.0% methionine; or (3) 0.5% cholesterol +1.0% methionine +2.5% taurine. Endothelial function was examined, and the left main coronary artery atherosclerosis was quantified by stereology and semiquantitative immunohistochemistry to determine the endothelial expression of proteins related to the NO, renin-angiotensin, endoplasmic reticulum, and oxidative stress systems, as well as apoptosis. Taurine normalized hyperhomocysteinemia (P<0.05) and significantly reduced hypermethioninemia (P<0.05) but not lipidemia. The intima: media ratio was reduced by 28% (P=0.034), and atherosclerosis was reduced by 64% (P=0.012) and endothelial cell apoptosis by 30% (P<0.01). Endothelial cell CCAAT/enhancer binding protein homologous protein was normalized (P<0.05). Taurine failed to improve hyperlipidemia, endothelial function, or endothelial proteins related to the NO, renin-angiotensin, and oxidative stress systems. Taurine reduces left main coronary artery wall pathology associated with decreased plasma total homocysteine, methionine, apoptosis, and normalization of CCAAT/enhancer binding protein homologous protein. These results elucidate the antiapoptotic and antiatherogenic properties of taurine, possibly via normalization of endoplasmic reticulum stress. (Hypertension. 2009;53:1017-1022.)

Key Words: cholesterol ■ homocysteine ■ methionine ■ atherosclerosis ■ plaque ■ CHOP

Cardiovascular disease deaths have decreased in some developed countries but increased in low- to middle-income countries.1 Coronary heart disease remains the most common cause of death throughout the world and is predicted to remain so in higher-income countries and will become so in lower-income countries by the year 2030.2

The prevention and treatment of atherosclerotic cardiovascular disease have used many interventional modalities. One of the most successful has been the use of statin therapy, which decreases plasma low-density lipoprotein (LDL) cholesterol, has modest effects on raising plasma high-density lipoprotein (HDL) cholesterol, and has pleiotropic effects, of which the clinical importance remains uncertain.3 Although statin therapy has a potent effect on reducing cardiovascular events, all of the randomized clinical trials still indicate a significant residual risk of events in the statin intervention arm of the studies. It is currently unclear whether this is purely from some patients not reaching low enough LDL levels or that additional therapeutic modalities are required.

Populations with higher fish intake have lower cardiovascular death rates than populations with high meat intake.4 Taurine is found in high concentrations in fish, the major human source of taurine.5 Increased taurine intake is inversely related to the incidence of coronary heart disease6 and has been associated with reduced insulin resistance,7 whereas taurine deficiency has been associated with increased obesity.8

Methionine, which is metabolized to homocysteine, is coinjected with cholesterol in meat. Increased levels of plasma total homocysteine (tHcy) have consistently been associated with increased atherosclerotic burden in animal models9,10 and also clinical cardiovascular events. However, simplistic approaches to reduce plasma tHcy by small amounts using B vitamins and folic acid have a possible beneficial role for the reduction of stroke,11–13 but this is not the case for myocardial causes of clinical events.14 Because taurine might affect methionine absorption,14 we postulated...
Figure 1. Blood lipids. A-TC, Total plasma cholesterol significantly increased in both experimental groups. B-Trig, Total plasma triglyceride also rose during the dietary regimen. C-LDL, Total plasma LDL (as determined by the Friedewald formula) significantly increased in both experimental groups. D-HDL, Total plasma HDL significantly increased in both experimental groups after the second week. E-LDL/HDL, Total plasma LDL/HDL ratio significantly increased in both experimental groups after the first week and continued to rise until sacrifice. F-Endothelial function, Endothelial dysfunction was present in the MC group, and taurine failed to restore this effect to normal (P<0.05). G-Methionine, Plasma methionine significantly increased in MC; however, the addition of taurine to the diet significantly reduced the plasma methionine level. H-Homocysteine, tHcy significantly increased only in the MC group after the first week and normalized at the fourth week. I, Plasma taurine was only increased in MCT at the third week (P<0.001). **P<0.01; *P<0.01; †P<0.001.

that a diet high in taurine, while impairing the development of atherosclerosis, might also reduce dietary-induced hyperhomocysteinemia.

Although clinical trials of simple, oral antioxidant therapies, eg, vitamin E or combinations of vitamins C, E, and β-carotene, have focused on the absorption of the oxygen radical,15,16 the hypochlorite anion has not as yet been targeted. The hypochlorite anion (OCl⁻/H⁺) is a powerful oxidant that is able to oxidize both HDL and LDL into hypochlorite-modified atherogenic forms (hypochlorous LDL17 and hypochlorous HDL18). Hypochlorous LDL particles are recognized by the scavenger receptor class B type I, which also impairs reverse cholesterol transport from cells.19 Taurine removes the oxidant hypochlorite and, thus, might impair the hypochlorite modification of LDL to hypochlorous LDL. Furthermore, taurine has several potentially beneficial cardiovascular effects, which involve regulating the NO system and endothelial function,20–23 the renin-angiotensin system (RAS),24–27 the oxidative stress system, and apoptosis,28–38 as well as the endoplasmic reticulum (ER) stress system.39,40

Thus, we hypothesized that reduction of circulating HOCl by taurine41 during an atherogenic diet aimed at increasing both tHcy and LDL19,10 would impair the formation of plasma hypochlorous LDL and endothelial cell apoptosis and that high dietary taurine would be associated with beneficial changes in the NO system, renin-angiotensin system, oxidative stress system, and ER stress system in the endothelial layer of the left main coronary artery.

Methods

Male New Zealand white rabbits at 3 months of age were randomized into 3 groups and fed one of the following diets for 4 weeks: (1) control (n=5); (2) a normal rabbit chow diet supplemented with 0.5% cholesterol+1.0% methionine+5.0% peanut oil (n=5; MC); or (3) a normal rabbit chow diet supplemented with 0.5% cholesterol+1.0% methionine+2.5% taurine+5.0% peanut oil (n=5; MCT). The animals were housed in individual cages and maintained at a constant temperature of ~21°C. Food and water were supplied ad libitum. The experiments were carried out according to the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The animals were then euthanized by an overdose IV injection of ketamine and xylazine via the main ear vein, as described previously in our laboratory.9,10 The aorta and heart were then excised. The aorta was cleaned of connective tissue and fat and used for isometric tension studies, and an apical section of the heart, which included the left main coronary artery, was cut and immersed in freshly prepared 4% paraformaldehyde solution in 1×PBS (pH 7.4).

For detailed methods relating to isometric tension studies, left main coronary artery analysis, apoptosis detection by single-strand DNA (ssDNA), methionine, taurine, plasma thiols, lipids, and homocysteine, please see the online data supplement at http://hyper.ahajournals.org.
Data Analysis
Comparisons between MC and MCT were performed by Student t test for single comparisons. A 1-way ANOVA followed by Newman-Keuls posthoc test was performed for comparison among the control, MC, and MCT groups. A 2-way ANOVA followed by Bonferroni posthoc test was performed for comparison of weekly effects. In all of the cases, P<0.05 was accepted as significant. All of the data are expressed as mean±SEM.

Results
Blood total cholesterol (TC) in both MC and MCT significantly increased after the first week of the diet and continued to increase until week 4 (Figure 1A, TC). There was no significant difference in TC between the MC and MCT groups. As well, plasma triglycerides also increased over the dietary period in both the MC and MCT groups, but again without difference between groups (Figure 1B, Trig). When LDL was calculated using the Friedewald formula, LDL remained higher throughout the dietary protocol in the MCT group, and this result was significant at the third week (P<0.05; Figure 1C, LDL). However, plasma HDL did remain lower throughout the dietary protocol in the MCT group, and this decrease was significant at the 2-week point (P<0.05; Figure ID, HDL). The overall worse lipid profile is illustrated by the TC:HDL (second and third weeks; P<0.01; figure not shown) and LDL:HDL (second and third weeks; P<0.05; Figure 1E, LDL/HDL) ratios. After 4 weeks of dietary manipulation, endothelial dysfunction was evident in the MC group (P<0.05), but taurine did not improve endothelial function in the abdominal aorta, which remained significantly impaired compared with the control (P<0.05; Figure 1F). Taurine markedly inhibited the increase in plasma methionine (Figure 1G) and completely inhibited the increase in the tHcy level observed in the MC group (P<0.05; Figure 1H). Plasma taurine was only increased in the third week in MCT versus MC (Figure 1I). Interestingly, there was no significant change in plasma methionine or tHcy at the end of the regimen.

Apoprotic endothelial cells, as detected by ssDNA, were present in the left main coronary artery of the control group (arrows, Figure 2A). However, the MC group showed a 17% increase in endothelial ssDNA staining (P<0.05; Figure 2B). The addition of taurine significantly reduced this staining to −13% below control levels (P value not significant versus control; P<0.01 versus MC; Figure 2C). Plaque cells also showed positive ssDNA cells in both MC (Figure 2D) and MCT (Figure 2E) groups, and apoptosis is quantified in Figure 2F. Despite the worse lipid profile, wall pathology (intima:media ratio) was also decreased by the addition of taurine to the diet. Intimal thickening, as determined by a neointima devoid of macrophages, decreased by 28% in the taurine-treated group compared with the MC group (Figure 2D and 2E and quantified in Figure 2G; P<0.05). As well, atheroma in the left main coronary artery was also reduced by 64% (Figure 2D and 2E and quantified in Figure 2G). ER stress, as measured by endothelial CCAAT/enhancer binding protein homologous protein (CHOP), increased by 74% in MC (P<0.05), and this was normalized by taurine treatment (Figure 2H).

For analysis of plasma cysteinylglucose, glutathionine, and cysteine please see Figure S1. For analysis of NO synthase (NOS) proteins (total eNOS, peNOS–S1177, peNOS–T495, and caveolin–1), for RAS (angiotensin-converting enzyme 2, angiotensin II type 2 receptor, angiotensin-converting enzyme, and angiotensin II type 1 receptor), and for oxidative stress system (heat shock protein 70, heme-oxygenase-1, myeloperoxidase, nitrotyrosine, inducible NOS), please see Figure S1. For Western blot analysis of hypochlorous LDL, plaque hypochlorous LDL, correlation
between plasma homocysteine, and hypochlorous acid LDL, please see Figure S2.

Discussion

This is the first comprehensive study examining the effect of high dietary taurine supplementation on the left main coronary artery. The major findings of this investigation are as follows: (1) taurine supplementation inhibited the development of hyperhomocysteinemia and hypermethioninemia and temporal effects of diet on plasma tHcy and methionine levels; (2) taurine supplementation inhibited endothelial cell apoptosis possibly by reduction in ER stress; (3) taurine supplementation reduced left main coronary artery atherosclerosis; and (4) taurine supplementation did not significantly affect the endothelial level of proteins associated with the NOS, RAS, or oxidative stress systems.

The reduction in tHcy by dietary taurine presented in this study was not attributed to increased metabolism of homocysteine to cysteine or other sulfur-containing amino acids, nor the reduced formation of homocysteine from methionine. Indeed, we observed that high dietary taurine significantly impaired the increase in plasma methionine compared with the untreated group, indicating that other possible routes of methionine metabolism are upregulated by taurine or that taurine can impair the absorption of methionine. Indeed, this latter hypothesis is supported by a recent study in cultured CaCo-2 cells, whereby methionine transport across the apical membrane of Caco-2 cells was affected by extracellular pH and taurine. Thus, it appears that taurine can impair the absorption of methionine and, thus, provide a novel way to reduce plasma tHcy. These results might have implications in nutrition. As the popularity of processed fast foods high in methionine is increasing and has been linked to increased tHcy, the addition of taurine to the diet might help stem the increase in tHcy and, thus, reduce cardiovascular disease risk. Further research to determine whether these results hold true in humans is warranted.

Furthermore, impaired methionine transport across the intestinal epithelia because of other factors could be causing the temporal effect on tHcy and methionine after the first dietary week. Indeed, we first eluded to this temporal effect in a similar study in rabbits on a 3-month dietary protocol. It is unclear why this phenomenon occurs; however, it is possible that both gut Na+ dependent and Na+-independent mechanisms are involved. As well, these results suggest that if these effects hold true in humans, plasma methionine or tHcy might not be a reflection of dietary methionine intake.

In the study presented here, taurine inhibited apoptotic coronary endothelial cells even on a background of a worse lipid profile. Apoptosis could be inhibited by a reduction in ER stress, as measured by a normalization of CHOP protein. In vitro research suggests that homocysteine causes ER stress, and this stimulates CHOP mRNA in human umbilical vein endothelial cells and apoptosis in cultured endothelial cells. Our study confirms this theory, because CHOP protein was significantly increased in the atherogenic group, which also had higher plasma tHcy levels, and, thus, a reduction in tHcy would impair apoptosis.

Furthermore, novel insights into the mechanisms involved in homocysteine-induced cellular damage include homocysteinylation of proteins. Both HDL and the intracellular atheroprotective enzyme metallothionein can become dysfunctional via homocysteinylation. For example, Barbato et al found that homocysteinylation of metallothionein impairs its zinc binding function, thus impairing its superoxide scavenging properties and possibly amplifying oxidative stress in endothelial cells. Thus, targeting a reduction in both tHcy and cellular homocysteine to reduce protein homocysteinylation could be a novel avenue for the treatment of homocysteine-induced vascular damage.

The decreased intimal thickening and reduced atherosclerosis in the left main coronary artery of this model during taurine treatment could be attributed to the impaired increase in tHcy. Although clinical trials involving the reduction of tHcy by vitamin supplementation have failed to significantly reduce myocardial events, our studies in rabbits and others in mice show that hyperhomocysteinemia on a hyperlipidemic background does enhance the development of atherosclerotic plaque burden in animal models. The human studies only managed small reductions (eg, 2.4 µmol/L) in plasma tHcy, using an intervention that would reduce tHcy by increasing methionine, which might not be the most advantageous way of reducing tHcy. In addition, it is possible that the role of hyperhomocysteinemia might be more important in the earlier development of atherosclerotic plaque rather than in reducing events in patients with existing plaque.

It is unclear whether increased triglyceride can directly induce apoptosis or is affected by dietary taurine. In this study, we showed that plasma triglyceride is not affected by dietary taurine and that the prevention of endothelial apoptosis can occur regardless of the triglyceride level. This finding is supported by in vitro experiments, whereby Nyblom et al showed reduced β-cell apoptosis, although the triglyceride level did not change. Taken together, these results suggest that triglyceride might not be an important determinant of cellular apoptosis, at least in endothelial or β cells.

Taurine supplementation did not significantly affect the endothelial level of proteins associated with the NOS, RAS, or oxidative stress systems. For this discussion, please see the data supplement.

In conclusion, we show that the addition of 2.5% taurine to an atherogenic diet reduces left main coronary artery wall pathology on a background of a worse lipid profile. As well, taurine also significantly reduces endothelial ER stress, hyperhomocysteinemia, and hypermethioninemia and impairs left main coronary artery endothelial cell apoptosis without detectable effects on the NOS, RAS, or oxidative stress systems.

Perspectives

Atherogenesis is clearly related to factors other than purely the lipid profile. It is possible that dietary taurine might be used independently to not only impair coronary artery disease but also to reduce the burden of hyperhomocysteinemia caused by excess dietary intake of processed foods high in methionine. As well, therapeutic intervention aimed at reduc-
tion in ER stress could be a novel avenue for drug development for the further prevention of cardiovascular disease.

Acknowledgment
We acknowledge the expert technical support of Angela Kourakis, who aided in the processing of the left main coronary artery for paraffin and immunohistochemistry.

Sources of Funding
This work was supported by the National Health Medical Research Council and National Heart Foundation of Australia.

Disclosures
None.

References
31. Ootsuka U, Yilmaz E, Ootsuka L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun C, Hotamisligil GS. Chemical chaperones reduce ER stress and...


High Dietary Taurine Reduces Apoptosis and Atherosclerosis in the Left Main Coronary Artery: Association With Reduced CCAAT/Enhancer Binding Protein Homologous Protein and Total Plasma Homocysteine but not Lipidemia

Anthony Zulli, Eza Lau, Bagus P.P. Wijaya, Xin Jin, Komang Sutarga, Grace D. Schwartz, Jonathon Learmont, Peter J. Wookey, Angelo Zinellu, Ciriacio Caru and David L. Hare

Hypertension. 2009;53:1017-1022; originally published online April 27, 2009;
doi: 10.1161/HYPERTENSIONAHA.109.129924

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/53/6/1017

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2009/04/20/HYPERTENSIONAHA.109.129924.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

HIGH DIETARY TAURINE REDUCES APOPTOSIS AND ATHEROSCLEROSIS IN THE LEFT MAIN CORONARY ARTERY: ASSOCIATION WITH REDUCED CHOP AND TOTAL PLASMA HOMOCYSTEINE BUT NOT LIPIDEMIA.

Anthony Zulli\textsuperscript{a,b,*}, Eza Lau\textsuperscript{a}, Bagus PP Wijaya\textsuperscript{a}, Xin Jin\textsuperscript{a}, Komang Sutarga\textsuperscript{a}, Grace D Schwartz\textsuperscript{a}, Jonathon Learmont\textsuperscript{a}, Peter J Wookey\textsuperscript{a,b}, Angelo Zinello\textsuperscript{c}, Ciriaco Carru\textsuperscript{c}, David L Hare\textsuperscript{a,b}

Departments of Cardiology\textsuperscript{a}, and Medicine\textsuperscript{b}, University of Melbourne, Austin Health, Australia
Department of Biomedical Sciences\textsuperscript{c} - University of Sassari Viale S.Pietro 43/b - 07100 Sassari- Italy

METHODS SUPPLEMENT

\textit{Isometric tension in the abdominal aorta}
Abdominal aortae were dissected into 3 x 3mm rings and sequentially mounted between two metal hooks in organ baths attached to force displacement transducers (Grass FT03) coupled to a data acquisition system (MacLab). The baths will be filled with Krebs solution and kept at a constant temperature of 37 °C and continuously bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2}. After 1 hour, vessels were gently stretched to a resting tension of 2.5 g. After 30 minutes, the vessels were gently re-stretched to a resting tension of 2.5 g. After the vessels reached plateau tension, maximum constriction was determined by a high potassium krebs solution (KPSS, 124 mM K\textsuperscript{+}). After plateau (6 minutes), vessels were rinsed with Krebs solution. After 45 minutes, the vessel rings were subjected to a phenylephrine concentration curve (10\textsuperscript{-8} – 10\textsuperscript{-5} M, half log units). After the final concentration of phenylephrine was added and the constriction reached plateau, the rings were subjected to an acetylcholine dose response curve (10\textsuperscript{-8} – 10\textsuperscript{-6} M, half log units).

\textit{Left main coronary artery analysis}
The left main coronary artery was excised from the heart and processed for paraffin. Groups were staggered into 2 batches and the LMCA from each rabbit from each batch were mounted in the same paraffin block. Sections were cut at 5 micron until the bifurcation. Approximately 30 sections were studied. Sections were randomly selected, dewaxed, rehydrated and placed in 10 mM TrisCl, pH 7.4. Sections were then preincubated with 1% goat serum in 10 mM TrisCl (pH7.4) for twenty minutes before incubating with the primary antibody diluted in 1% goat serum in 10 mM TrisCl (pH7.4). Mouse monoclonal IgG, ACE2 (Cat# ALX 804-715, Alexis Biochemicals, diluted 1:150), AT2R (Cat# MAB3659, R&D Systems, Minneapolis, diluted 1:150), AT1R (Cat# sc-57036, Santa Cruz Biotechnology, diluted 1:50), ACE (Cat# MAB3502, Millipore, diluted 1:50), eNOS, (Cat#610296, BD Australia, diluted 1:100), phosphor-eNOS 1177 (Cat#612392, BD Australia, diluted 1:100), phosphor-eNOS 495 (Cat#612706, BD Australia, diluted 1:100), caveolin-1 (Cat# 036000, Zymed Laboratories, diluted 1:50), HSP70 (Cat#MAB3516, Millipore, diluted 1:50), nitrotyrosine (Cat#MAB5404, Millipore, diluted 1:100), HO-1 (Cat#ab13248, Abcam, diluted 1:100), myeloperoxidase (Cat#sc-59600, Santa Cruz Biotechnology, diluted 1:50), CHOP (Cat#MA1-250, ABR, USA) were incubated overnight and immunohistochemistry was performed as previously described \textsuperscript{1,3}. Antigenic sites were developed with DAB, counterstained with hematoxylin, dehydrated and mounted with DPX mounting media.
**Apoptosis detection by ssDNA**

Apoptotic endothelial cells were detected by a monoclonal antibody to single stranded DNA (ssDNA). Sections were cut from paraffin blocks and attached to superfrost plus slides. Slides were placed in oven at 60°C for 1 hour, deparaffinised and hydrated. Slides were then incubated with saponin (0.1mg/ml in PBS) at room temperature for 20 minutes. Sections were washed in 1xPBS and incubated with Proteinase K (20ug/ml in 1xPBS) for 20 minutes at room temperature. Slides were then washed in three changes of 1xPBS and subsequently transferred into a coplin jar containing 50ml of 50% formamide (v/v dH₂O) preheated in water bath to 56°C and incubated for 20 minutes. Importantly, the temperature of formamide solution inside the jar was kept at 56°C. The slides were transferred into a container of ice-cold 1xPBS for 5 min. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 5 minutes and then rinsed in dH₂O and then treated with 3% nonfat dry milk + 1% goat serum diluted in 1xPBS for 15 minutes to block non-specific antibody binding. Sections were then rinsed in 1xPBS and incubated with primary antibody to ssDNA (Cat# MAB3034, 1:100 dilution in 1%goat serum in 1xPBS) and left overnight. Sections were rinsed in 1xPBS for 5 minutes, and then incubated with the ‘Envision’ molecule (Cat# K4001, Dakocytomation) for 1 hour at room temperature. Slides were rinsed again in 1xPBS for 5 minutes, incubated with DAB chromagen for 1 minute, rinsed in dH₂O for 1 min, counterstained with haematoyxlin for 1 minute, rinsed in dH₂O, blued in Scotts tapwater, dehydrated and then mounted in DPX media mount.

**Wall pathology**

Wall pathology was quantified by image analysis software (MCID Elite 6.0). Briefly, digital images of the LMCA (4 images, top, bottom, left and right) were obtained using a Leica DC480. The area of the intima and media was obtained by digital trace. The areas of plaques were determined separately. The intima:media ratio or plaque:media ratio was then obtained. To determine the intensity and proportional area of the endothelium that was immunostained, each trace was repeated three times, each time re-selecting the hue, saturation and intensity to obtain the most accurate representation of colour. The ribbon tool (MCID software) was selected, and the endothelial layer was traced, including other binding cells. The data from all four images were averaged to obtain one result. This was done for three traces. All three traces were then averaged to obtain one result from each LMCA. This value was then used as n=1. All experimental groups were normalised to a percentage increase over control.

**Western blot analysis**

Plasma samples (0.5µL) were added to 9.5µL sample buffer, heated to 100°C for 5 minutes, immediately placed on ice, and loaded into PAGE gels (5% stacking, 8% resolving). Initial voltage was 100V until samples entered resolving gel, and then 180V for 2 hours. Proteins were transferred onto PVDF membrane using a semidyry transfer cell (Bio rad) using standard transfer buffer. Membranes were washed with 25 ml TBS for 1 min at room temperature and non-specific binding was blocked by incubating membrane with 5% skim milk for 45 min. Then, membranes were washed 5 times for 3 minutes each with 50 ml TBS-Tween, incubated with HOCl-LDL antibody (3µL of Cat#MAB 3232, in 10 ml) for 1 hour. Serum HOCl-LDL levels were expressed as HOCl-LDL/LDL ratios. For comparison between MC and MCT, these ratios were normalized to MCT increases equivalent to 0%. Membranes were washed 5 times for 3 minutes each with 50 ml TBS-Tween and then incubated with ‘Envision’ molecule (25µL of Cat# K4003, in 10mL, Dakocytomation). Membranes were then washed 5 times for 3 min each with 50 ml TBS-Tween, and exposed to ECL. Then, membranes were stripped with Stripping Buffer (Pierce, Cat #46430), and then washed with 25 ml TBS for 1 min at room temperature and non-specific binding was blocked by incubating membrane with 5% skim milk for 45 min. Then, membranes were washed 5 times...
for 3 minutes each with 50 ml TBS-Tween, incubated with a monoclonal antibody to ApoB (3µL of Cat# MAB4124, R&D systems, in 10 ml) for 1hour. Membranes were wash 5 times for 3 minutes each with 50 ml TBS-Tween and then incubated with goat anti mouse IgG conjugated to peroxidise (1:20,000 dilution, Sigma Cat# AO168). Membranes were then washed 5 times for 3 min each with 50 ml TBS-Tween, and exposed to ECL and detected on a Fuji Film LAS3000 and western blot bands were then analysed using FujiFilm MultiGauge Software.

Amino acid profile

Methionine was measured by capillary electrophoresis (CE) UV detection as previously described with some modifications 6. In brief, 400 µL of obtained plasma was filtered in Vivaspin 500 microconcentrators by centrifugation at 3000g for 20min to remove proteins. Filtered samples were directly injected into CE. An MDQ capillary electrophoresis system equipped with a diode array detector was used (Beckman Instruments, Fullerton, CA, USA). Analysis was performed in an uncoated fused-silica capillary (75 µm I.D. and 60.2 cm length), injecting 39 nL of sample. Separation was carried out in a 125 mmol/L Tris buffer titrated with 1mol/L phosphoric acid to pH 2.3, 15 ºC, and 15 kV.

Taurine was measured by capillary electrophoresis laser induced fluorescence detection as previously described7. Briefly, 50 µL of plasma was mixed with 50 µL of internal standard homocysteic acid (200 µmol/L) and 100 µL of trichloroacetic acid (10%) was then added to precipitate the proteins. After centrifugation at 3,000g for 5 min, 10 µL of clear supernatant was mixed with 90 µL of 100 mmol/L Na2HPO4 of pH 9.5 and 11 µL of 15 mmol/L FITC (fluorescein isothiocyanate). After 20 min incubation time at 100°C, the samples were diluted 100-fold and injected in CE. Analysis of taurine was performed by a CE system (P/ACE 5510) equipped with a laser-induced fluorescence (LIF) detector (Beckman, Palo Alto, CA, USA). Analysis was performed in an uncoated fused silica capillary, 75 µm I.D. and 47 cm length, injecting 18 nL of sample. Separation was carried out in a 20 mmol/L tribasic sodium phosphate buffer, pH 11.8, 23°C at normal polarity 22 kV.

Plasma thiols (Cys, GSH, Glu-Cys and Cys-Gly) were measured by capillary electrophoresis laser induced fluorescence detection as previously described 8. In brief, 100 µL of plasma sample were mixed with 10 µL of tri-n-butylphosphine (10%) were mixed, vortexed for 30 s and subsequently incubated at 4°C for 10 min. At the end of incubation 100 µL of 10% trichloroacetic acid were added, vortexed for 10 s and then centrifuged for 10 min at 3,000g. 100 µL of supernatant were mixed with 100 µL of 300 mmol/L Na3PO4 at pH 12.5 and with 25 µL of 5-iodoacetamidofluorescein (4.1 mmol/L), and subsequently incubated at room temperature for 10 min. The mix was diluted 1/100 before injection on CE-LIF. Thiols analysis was carried out on a P/ACE 5510 system. The dimension of the uncoated fusedsilica capillary was 75 µm I.D. and 57 cm length. Analysis was performed applying 14 nL of sample under nitrogen pressure and using 5 mmol/L sodium phosphate/ 4 mmol/L boric acid as electrolyte solution with 75 mmol/L N-methyl-D-glucamine at pH 11. The separating conditions (28 kV, 70 µA, normal polarity, 40°C ) were reached in 30 s and held at a constant voltage for 5 min.
RESULTS SUPPLEMENT

Analysis of plasma cysteinylglycine (Figure S1– A), glutathionine (Figure S1 – B) and cysteine (Figure S1 – C) showed no changes between groups.

Analysis of the expression of endothelial proteins of the NOS system showed a trend towards increased total eNOS and peNOS-S1177 in the MC group, however the addition of taurine to the diet increased endothelial caveolin-1 protein by 29% vs control (p=0.059) and endothelial peNOS-T495 by 34% (p=0.095). This was not associated with a concurrent increase in eNOS or peNOS-S1177.

Analysis of the expression of endothelial proteins of the oxidative, nitrative and endoplasmic reticulum stress system shows that the MC group exhibited elevated oxidative stress, as detected by an increase in HSP 70 by 33% vs control (p<0.05), and taurine treatment further increased endothelial HSP70 (p<0.02). Interestingly, endothelial heme-oxygenase-1 (HO-1), myeloperoxidase (MPO), nitrotyrosine (NT), and iNOS appeared to decrease in the MC group and the addition of taurine only resulted in a trend towards increased levels above control (p=ns). Analysis of the expression of endothelial proteins of the RAS system in the MC group showed a significant increase of ACE2 by 19% (p<0.01) and ACE by 11% (p=0.01) vs control. In addition, endothelial AT1R appeared to increase but this did not reach significance. The addition of taurine to the MC diet appeared to reduce the expression of endothelial ACE2, AT2R, ACE and AT1R, but this was not significant from MC. There was no significant change in endothelial AT2R.

Analysis of serum hypochlorous LDL/LDL ratio by western blot (Figure S2-A) showed a trend to increase hypochlorous LDL in the MC group, but this normalized at week 4 (Figure S2-C). Plaque hypochlorous LDL appeared to be increased in the MCT group, but this failed to reach significance (Figure S2-B). As taurine in the MCT group completely inhibited the increase in tHcy observed in the MC group, a very strong trend towards a positive correlation (r²=0.6986) was observed (p=0.0401, Figure S2-D) between plasma tHcy and the plasma hypochlorous LDL/LDL ratio in the MC group (normalized to MCT).
DISCUSSION SUPPLEMENT

The failure of the following relationships to reach significance could possibly be a type 2 error due to an insufficient number of animals studied in these exploratory studies and have thus not been included in main text

Taurine resulted in a significant reduction in plasma tHcy, this being strongly associated with reduction in serum hypochlorous LDL. Thus, it is possible that a potentially atherogenic property of homocysteine might include the formation of hypochlorous LDL, although independent yet still closely correlated, effects of taurine could be implicated. Certainly one can say that this current study suggests a role for high dietary taurine in the prevention of dietary induced hyperhomocysteinemia. It is possible that this could be a better method of lowering tHcy than the methods used in previous clinical studies, although this is obviously speculative at this stage.

Moreover, the increase in endothelial HSP70 in both the atherogenic group and the taurine treated group indicates a clear role for HSP70 in coronary artery atherosclerosis. HSP70 is a cytoprotective protein and acts as a molecular chaperone to restore normal protein function. The further increase in HSP70 observed by taurine treatment could be preventing the observed endothelial cell apoptosis in this model, as HSP70 is a regulator of apoptosis. Furthermore, HSP70 has been shown to be an indicator for endothelial cell proliferation, indicating that the endothelial cell layer in the taurine treated group might be proliferating, and this theory is also supported by the lack of apoptotic endothelial cells in this group.

As oxidised LDL is a potent inducer of endothelial cell apoptosis, it is possible that taurine might inhibit apoptosis by impairing the formation of oxidized LDL. Potentially taurine could impair oxidised LDL by absorbing the hypochlorite anion (HOCl/OCl) produced by myeloperoxidase in macrophages, and thus reduce the formation of hypochlorous LDL in serum. As circulating plasma hypochlorous LDL originates from mild oxidation in the arterial wall by myeloperoxidase, we studied both plaque and serum hypochlorous LDL. We found that there was a trend towards reduced circulating hypochlorous LDL by the addition of taurine to the atherogenic diet over the four week period, compared to the atherogenic diet alone. This trend towards lower plasma hypochlorous LDL was associated with a trend towards increased plaque hypochlorous LDL in the taurine treated groups. If these trends are real, it could be possible that retaining hypochlorous LDL in plaque might result in less release of hypochlorous LDL into the circulation.

It is conceivable that dietary taurine, by impairing oxidation of LDL in plaques, might contribute to plaque stability and thus reduce the likelihood of acute coronary syndromes, histological examination of lethal plaques demonstrating more oxidised LDL and unstable angina having been previously correlated with circulating oxidised LDL. In this regard, further studies into the mechanisms involved in inhibiting the release of hypochlorous LDL from plaque to the circulation are warranted, as this would decrease circulating oxidised LDL and possibly impair endothelial cell apoptosis and thus thrombosis.

Clinical data clearly established a strong association between higher cardiovascular events and a high total cholesterol/HDL ratio, and even more so for an apoB100/apoA1 ratio. The current study highlights the possibility of discordance between a worsening lipid profile and a demonstrable improvement in coronary artery atherosclerosis. This possibility has been suggested in some clinical studies. For example, it is well known that there is no evidence of...
premature coronary artery disease in human carriers of the ApoA1-Milano despite very low HDL levels. Our results clearly show that a high LDL/low HDL lipid profile does not further induce atherosclerosis in the left main coronary artery if induced by high dietary taurine, clearly indicating other factors besides the lipid profile are involved in atherogenesis.

It is suggested that enhancing endothelial function via the restoration of NOS function or reduction in oxidative stress is a pre-requisite for the impairment of atherosclerosis. In contrast, we show that atherosclerosis in the coronary artery is not related to an improvement in endothelial function in the abdominal aorta. To study endothelial function in the left main coronary artery, we investigated eNOS, RAS and oxidative activity in the endothelial layer. We found no evidence to suggest that eNOS activity was increased, as detected by eNOS activity markers phosphorylated eNOS at serine 1177, dephosphorylated eNOS at threonine 495, and the eNOS inhibitor caveolin-1 in the endothelial layer left main coronary artery or that oxidative stress was decreased. However, we did find that ACE, ACE2, AT2R and AT1R were all slightly decreased, but these findings did not reach significance. Whether the activity of these enzymes are changed remain to be elucidated. Indeed, we did find that taurine treatment might decrease eNOS activity in the coronary artery, as suggested by a trend to increase in endothelial caveolin-1 (p=0.059) and phosphorylated eNOS at the threonine site (p=0.095). If these results hold true, taurine could be impairing the dysfunctional eNOS, thus reducing the amount of O$_2^-$.

This view is supported by Ozaki and colleagues, who show that ApoE deficient mice overexpressing eNOS accelerated atherosclerosis. As well, caveolin-1 has been shown to regulate apoptosis in cell lines and vascular smooth muscle cells, raising the possibility that the increase in caveolin-1 might also impair the apoptosis observed in this study.
REFERENCES


ENDOTHELIAL IMMUNOQUANTIFICATION

% of control change

- eNOS
- Cav-1
- PeNOS-S
- PeNOS-T
- HSP70
- HO-1
- MPO
- NT
- iNOS
- ACE2
- AT2R
- ACE
- AT1R

P-values:
- P<0.05
- P<0.02
- P<0.01
- P=0.059
- P=0.095
- P=0.07
S2

A. Week 0, Week 1, Week 2, Week 3, Week 4

MC

MCT

B. Plaque HOCl-LDL

% increase

MC

MCT

C. Serum HOCl-LDL/LDL normalised to MCT

% increase

Week

0 1 2 3 4

D. y = 3.4x + 21

R² = 0.8

p = 0.0401

Hcy (µM)

Normalised serum HOCl-LDL/LDL in MC to MCT
Figure S1
Plasma cysteineglycine (A), glutathione (B) and cysteine (C) were not increased in any diet. Immunohistochemical quantification of factors involved in atherogensis in the LMCA endothelial layer. The addition of taurine to the MC diet failed to significantly affect the NOS system, oxidative stress system or renin-angiotensin system. For all endothelial immunoquantification bar graphs, the first column is from the Con group, the second (white box) is from the MC group, and the third is from the MCT group (black box).

Figure S2
Western blot analysis of serum hypochlorous LDL/LDL (A,C) as well as immunoquantification of plaque hypochlorous LDL (B). Taurine appears to increase plaque hypochlorous LDL (B) and reduce serum hypochlorous LDL/LDL (C). Interestingly, a positive association between plasma tHcy in the MC group and serum hypochlorous LDL was observed (p<0.05).