Genetically based hypertension generated through interaction of mild hypoalphalipoproteinemia and mild hyperhomocysteinemia

Ricardo Carnicer[a], María A. Navarro[a], José M. Arbonés- Mainar[a], Carmen Arnal[b], Joaquín C. Surra[a], Sergio Acín[a], Alfonso Sarría[a], Francisco Blanco-Vaca[c], Nobuyo Maeda[d] and Jesús Osada[a]

Background Hyperhomocysteinemia and hypoalphalipoproteinemia are two well-reported risk factors for cardiovascular disease. The effects of the synergistic combination of these two factors on vascular function need to be investigated.

Methods and results Four groups of male mice were used: a control wild-type group; a group of mice heterozygous for cystathionine β-synthase deficiency; a group of mice heterozygous for apolipoprotein A-I deficiency; and, finally, a group of double heterozygous mice, with both cystathionine β-synthase and apolipoprotein A-I deficiency. To characterize the resulting phenotype, several parameters including plasma apolipoproteins, lipid profiles, homocysteine, blood pressure and aortic protein were analyzed. As expected, our results indicate that double heterozygous mice are a model of mild hypoalphalipoproteinemia and hyperhomocysteinemia. Further, the additive combination of both risk factors resulted in a significant increase in blood pressure compared with control animals (136 ± 8.0 versus 126 ± 7.5 mm Hg, $P < 0.01$) that was not present in single heterozygous mice. The increase in blood pressure was associated with decreased plasma nitric oxide levels, left ventricle hypertrophy and was independent of low-density lipoprotein (LDL) cholesterol, para-oxonase activity and kidney histological changes. Concomitant decreases in levels of apolipoprotein A-IV (APOA-IV) and caveolin-1 content were also found in the double heterozygous group.

Conclusions Our findings suggest an additive adverse effect of hypoalphalipoproteinemia and hyperhomocysteinemia on endothelial function to generate clinical hypertension and cardiac muscle hypertrophy mediated by dysregulation in nitric oxide metabolism. *J Hypertens* 25:1597–1607 © 2007 Lippincott Williams & Wilkins.

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[a]Departamento de Bioquímica y Biología Molecular y Celular, [b]Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, [c]Servicio de Bioquímica, Hospital Santa Creu i Sant Pau, Barcelona, Spain and [d]Department of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, USA

Correspondence to Dr Jesús Osada, Department of Biochemistry and Molecular Biology, Veterinary School, University of Zaragoza, Miguel Servet, 177, E-50013 Zaragoza, Spain
Tel: +34 976 761 644; fax: +34 976 761 612; e-mail: Josada@unizar.es

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Introduction

Homocysteine (Hcy) was first implicated as a risk factor for cardiovascular disease in the late 1960s, when it was noticed that homocystinuric patients had a propensity to thrombosis that resulted in a dramatic increase in mortality from vascular disease [1]. Since then, several epidemiological studies have shown an association between elevated plasma Hcy and vascular disease [2–4]. In the general population, hyperhomocysteinemia has an estimated prevalence of 1 : 70 [5] or even higher [6]. Mild hyperhomocysteinemia, defined as plasma Hcy levels between 15 and 30 mmol/l, is also associated with an increased risk for cardiovascular mortality [7]. Moreover, up to 40% of patients with atherosclerosis exhibit high levels of plasma Hcy [8]. It has been estimated that modest increases in plasma Hcy level (5 μmol/l) may increase two-fold the relative risk for myocardial infarction [2]. Furthermore, different studies have shown that alcohol, obesity, smoking, diabetes and an unhealthy diet contribute to mild hyperhomocysteinemia [9]. Different mechanisms have been proposed to explain why hyperhomocysteinemia increases cardiovascular morbidity and mortality; however, their relative importance is as yet unknown. One of the mechanisms by which hyperhomocysteinemia could induce atherothrombosis is by impairment of endothelial function [10–16] through decreased nitric oxide bioavailability.

Apolipoprotein A-I (APOA-I) is the main protein in high-density lipoprotein (HDL), representing 70% of the total content [17]. The risk of cardiovascular disease from atherosclerosis is inversely proportional to serum levels of HDL and APOA-I [18]. The atheroprotective effect of HDL is generally attributed to its classical function on

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reverse cholesterol transport from peripheral tissues to the liver, but other protective effects of HDL are emerging [19]. Thus, HDL could also exert its atheroprotective effect by mechanisms such as inhibition of oxidized low-density lipoprotein (LDL) signal transduction and the induction of endothelial nitric oxide synthase activity [20–22]. Therefore, a deficiency of HDL due to lower APOA-I may compromise the atheroprotective potential. Mice lacking *Apoa1* did not develop atherosclerosis [23], but the absence of *Apoa1* accelerated atherosclerosis in presence of elevated LDL [24].

Based on the observations described previously, hypoalphalipoproteinemia could alter endothelial function, and this effect might be aggravated in the presence of hyperhomocysteinemia. To evaluate this hypothesis *in vivo*, mice harboring an inactivated copy of *Apoa1* gene were crossed with those lacking one copy of *Cbs* gene, and the vascular phenotype of the resulting double heterozygous animals was studied and characterized.

Material and methods

Animals

Double heterozygous animals (double het), lacking one copy of both cystathionine β-synthase (CBS) and APOA-I, were obtained by breeding mice heterozygous for CBS deficiency (het cbs) [25] and mice heterozygous for APOA-I deficiency (het A-I) [26] and backcrossing for nine generations to C57BL/6J (Charles River) to achieve an homogeneous background.

Mice were bred in the Unidad Mixta de Investigación, Zaragoza. For this study 39 males, aged 3 months were housed in sterile filter-top cages in rooms maintained on a 12-h light/12-h dark cycle and had free access to food and water. Body weights and food intake were recorded throughout the experiment. Four groups of study were established: a control group ($n=10$); a heterozygous *Apoa1* group – mice lacking one copy of the *Apoa1* gene ($n=10$); a heterozygous *Cbs* group ($n=10$); and double heterozygous mice, lacking one copy each of *Cbs* and *Apoa1* genes ($n=9$). All groups were fed on a chow diet Teklad Mouse/Rat Diet no. 2014 from Harlan Teklad (Harlan Ibérica, Barcelona, Spain). To avoid the potential confounding effects of variation between batches of chow, a single batch was reserved and used to feed experimental groups throughout the experiment. The experimental protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza.

Polymerase chain reaction genotyping

Tail DNA was prepared and subjected to polymerase chain reaction (PCR). Three oligonucleotides were used in PCR amplification to detect both the endogenous and altered *Cbs* genes simultaneously. The sense primer was *cbs1* (5′-GAA GTG GAG CTA TCA GAG CA-3′). Downstream primers were *neo* (5′-GAG GTC GAC GGT ATC GAT A-3′) and *cbs2* (5′-CGG ATG ACC TGC ATT CAT CT-3′) specific for the endogenous and altered *Cbs* genes, respectively. PCR amplification resulted in a 500 bp band from the wild-type gene and a 400 bp band from the knockout gene. PCR amplification was carried out as follows: 94°C 1 min; 94°C 20 s, 59°C 30 s, 72°C 30 s for 33 cycles followed by a 5-min elongation period at 72°C.

In the case of *Apoa*1 genes, three primers *aI-2* (5′-GGA AGC ATT GGC TAG AAT GG-3′), *aI-1* (5′-AGT GCT GCT ACC TGC CTT CG-3′) and *neo2* (5′-CCG ACT GCA TCT GCG TGT-3′) were used in PCR amplification to detect both the endogenous and altered *Apoa1* genes simultaneously. PCR amplification resulted in a 150 bp band from the wild-type gene and a 250 bp band from the knockout gene. PCR amplification was carried out as follows: 94°C 1 min; 94°C 20 s, 59°C 30 s, 72°C 30 s for 28 cycles, followed by a 5-min elongation period at 72°C.

Plasma determinations

At the end of the experimental period and after an overnight fast, animals were knocked out in a chamber saturated with CO_2 until loss of reflexes and then killed by exsanguination once the blood was drawn from their hearts. Total plasma cholesterol and triglyceride concentrations were measured using commercial kits from Sigma Chemical Co. (Madrid, Spain). HDL cholesterol (HDLc) was determined in a similar manner after phosphotungstic acid-$MnCl_2$ (Roche Diagnostics, Barcelona, Spain) precipitation of particles containing apolipoprotein B [27]. Apolipoprotein concentrations were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described [28], using a rabbit anti-mouse APOA-I and anti-APOA-II (Biodesign, Saco, Maine, USA) and a goat anti-mouse APOA-IV (Santa Cruz Biotechnology, Santa Cruz, California, USA).

Plasma homocysteine concentrations were assayed with a time-resolved immunofluometric assay (IMx; Abbott Diagnostic, Madrid, Spain). Measurement of plasma nitrate/nitrite (NOx) levels was achieved according to Misko *et al.* [29]. Basically, 1 : 10 diluted plasma was centrifuged for 30 min at 13 000 *g* with Millipore YM-10 filters and 100 μl of filtered solution was incubated with reaction buffer containing NADPH and nitrate reductase. The resulting nitrite was quantified by incubation with 10 μl diaminonaphthalene for 10 min and determination of fluorescence (excitation at 360 nm, emission at 430 nm). Para-oxonase was assayed as described previously [30] and results were expressed as μmol phenylacetate hydrolyzed/min per liter (IU/l).

To analyze plasma lipoprotein profiles, 100 μl of pooled plasma samples from each group were subjected to fast protein liquid chromatography (FPLC) gel filtration

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using a Superose 6B column (Amersham Pharmacia, Barcelona, Spain), as described previously [31]. Fractions of 0.5 ml were collected and their total cholesterol, apolipoprotein content and arylesterase activity assayed as described above.

Blood pressure

The systemic blood pressure (BP) was monitored in conscious animals using a non-invasive tail-cuff method and following the protocol described previously [32]. Briefly, mice were kept in a restraining unit mounted on a surface maintained at 32°C. The systolic blood pressure was measured as the pressure of a latex cuff (LE 5002 storage pressure meter, LETICA, Barcelona, Spain, calibrated with a manometer for correcting changes in barometric pressure) fixed around the tail of the animals after 10 preliminary unrecorded measurements. The computer determined heart rate before cuff inflation, and only after 70 measurements showing a stable and normal value was the procedure started. Training sessions for 10 days were also necessary for the mice to become accustomed to the tail-cuff procedure. Sessions of recorded measurements were made by a single investigator (R.C.) from 1200 to 1600 h daily, for 3 weeks. For each session, eight animals (two from each genotype) were tested. The order of the starting genotype, as well as the group of mice selected for each genotype, changed among days. Fifteen measurements per animal were taken in each session. Results represent an average of five sessions, so that a total of 60–75 measurements were used for the determination of the BP of each mouse, and six mice per genotype. For inclusion of each set of measurements for an individual mouse, we required that the computer successfully identify a BP in at least nine of the 15 trials within the set. With these precautions, together with control of genotype, sex and the same batch of chow, reproducibility expressed as coefficient of variation between days was 5%, an improvement compared to the previous description of the method [32] and showing the relevance of using highly inbred mice in well-controlled environmental conditions.

Histological analysis

Hearts and kidneys were cleaned and stored in neutral formaldehyde. They were embedded in paraffin wax. Serial 4 μm sections were stained with hematoxylin and eosin. For cross-sectional analyses of hearts, three sections taken per mouse ($n = 9–10$), at the location of the papillary muscles, were captured, digitized using a Nikon microscope equipped with a Canon digital camera and evaluated for myocardial cross-sectional area of the left ventricle [33,34]. Average data for each mouse was normalized to the body weight. Morphometric analyses were performed using Scion Image software (Scion Corporation, Frederick, Maryland, USA). Kidneys from all mice (three sections per mouse) were evaluated for the presence of histological features associated with hypertension [35] such as glomerular (thickening of basement membrane and mesangial expansion), vascular (arteriosclerosis) and tubular (hyaline droplets or interstitial fibrosis) lesions.

Western-blot analysis

Aorta homogenates were prepared to analyze protein expression. Briefly, aortic segments were dissected and stored immediately in liquid nitrogen. The frozen segments were pulverized and resuspended in lysis buffer [50 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.1 mmol/l EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 0.5% Nonidet P-40, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF)] and proteinase inhibitors (Roche Diagnostics). Ninety micrograms of total protein were loaded onto a 10% SDS–polyacrylamide gel to determine endothelial nitric oxide synthase (eNOS), and 20 μg onto a 12% gel to analyze caveolin-1, scavenger receptor type BI (SR-BI) and actin expression. Gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Madrid, Spain). The presence of eNOS protein was detected using a rabbit polyclonal antibody against mouse eNOS (BD Transduction Laboratories, Madrid, Spain), followed by detection with a secondary antibody anti-rabbit IgG peroxidase conjugate (GE Healthcare, Madrid, Spain) and chemiluminescence (GE Healthcare). To avoid cross-reactivity with neuronal nitric oxide synthase (nNOS) and loss of signal, the gel section between 130 and 140 kDa was cut and processed for this antibody. Equal loadings were confirmed by using an anti-β-actin antibody obtained from Sigma. Membranes were exposed to enhanced chemiluminescence (ECL) film (GE Healthcare) and films were analyzed using a laser LKB 2202 densitometer. SR-BI and caveolin-1 bands were detected with mouse polyclonal antibodies (Abcam, Cambridge, UK and BD Biosciences, Madrid, Spain) and a rabbit polyclonal antibody, respectively, followed by incubation with an alkaline phosphatase-conjugated anti-IgG secondary antibody (Sigma) as described previously [28].

Statistical analysis

To identify significant differences, one-way analysis of variance (ANOVA) was used when a Kolmogorov–Smirnov test indicated that the variable was normally distributed. After using a Bartlett's test to assess the homogeneity of variances, post-hoc tests were performed using Student–Newman–Keuls multiple comparisons test. When the variables did not exhibit a Gaussian distribution, groups were compared using a Kruskal–Wallis test, and differences between pairs were tested using a Mann–Whitney U-test. To test the strength of the correlations between variables, the Pearson correlation test was used. Unless otherwise stated, results are expressed as mean ± SEM, and differences were considered significant when $P < 0.05$. All statistical analyses were performed using Instat 3.02 for Windows (GraphPad, San Diego, California, USA).

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Table 1 Plasma parameters in function of genotype

	Control ($n = 10$)	het A-I ($n = 10$)	het cbs ($n = 10$)	double het ($n = 9$)
Cholesterol (mmol/l)	2.9 ± 0.50	$1.8 \pm 0.11^{*,\dagger}$	2.7 ± 0.64	$1.8 \pm 0.22^{*,\dagger}$
HDLc (mmol/l)	2.6 ± 0.43	$1.6 \pm 0.11^{*,\dagger}$	2.3 ± 0.55	$1.5 \pm 0.19^{*,\dagger}$
TG (mmol/l)	1.4 ± 0.42	1.0 ± 0.56	1.5 ± 0.51	$0.8 \pm 0.18^{*,\dagger}$
Hcy (μmol/l)	4.2 ± 1.03	4.6 ± 1.07	$11.6 \pm 1.84^{\S,\#}$	$10.6 \pm 2.25^{\S,\#}$
APOA-I (AU)	1.41 ± 0.25	$1.00 \pm 0.09^{\S,\P}$	1.41 ± 0.23	$0.81 \pm 0.15^{\S,\P}$
APOA-II (AU)	1.46 ± 0.30	$1.19 \pm 0.27^{*,\dagger}$	1.45 ± 0.34	$1.05 \pm 0.26^{*,\dagger}$
APOA-IV (AU)	1.18 ± 0.14	1.18 ± 0.23	1.17 ± 0.26	$0.78 \pm 0.19^{*,\ddagger,\dagger}$

APOA-I, apolipoprotein A-I; APOA-II, apolipoprotein A-II; APOA-IV, apolipoprotein A-IV; double het, double heterozygous mice, with both cystathionine β-synthase and apolipoprotein A-I deficiency; Hcy, homocysteine; HDLc, high-density lipoprotein cholesterol; het A-I, mice heterozygous for apolipoprotein A-I deficiency; het cbs, mice heterozygous for cystathionine β-synthase deficiency; TG, triglycerides. Results are mean ± SD. Statistical analyses were carried out using one-way ANOVA and Student–Newman–Keuls multiple comparisons test as post-hoc test. $^{*}P < 0.05$ versus control; $^{\dagger}P < 0.05$ versus het cbs; $^{\ddagger}P < 0.05$ versus het A-I; $^{\S}P < 0.001$ versus control; $^{\#}P < 0.001$ versus het A-I; $^{\P}P < 0.001$ versus het cbs.

Results

Plasma biochemistry

Total cholesterol, HDLc, triglycerides, Hcy and apolipoprotein levels are shown in Table 1. Double het and het A-I groups had significantly lower levels of total cholesterol (1.8 ± 0.22 and 1.8 ± 0.11 versus 2.9 ± 0.50 mmol/l in the control group) as well as HDLc (1.5 ± 0.19 and 1.6 ± 0.11 versus 2.6 ± 0.43 mmol/l in the control group). However, only the double deficiency decreased triglyceride levels (0.8 ± 0.18 versus 1.4 ± 0.42 mmol/l for the control group). Both the double heterozygous and the single heterozygous CBS groups showed increased Hcy levels (10.6 ± 2.25 and 11.6 ± 1.84 μmol/l, respectively, versus 4.2 ± 1.03 μmol/l for the control group) in accordance with the *Cbs* deficiency. As expected, double heterozygotes showed significantly decreased plasma levels of APOA-I, that were accompanied by statistically significant decreases in APOA-II and APOA-IV (Table 1). In contrast, the significant decrease in APOA-I observed in the het A-I group was accompanied by a decrease in APOA-II, while APOA-IV levels remained unchanged.

Lipoprotein analysis by FPLC

To further characterize apolipoprotein behavior, lipoproteins were separated by FPLC and the obtained fractions were analyzed. Most plasma cholesterol (80%) was present in HDL particles (Fig. 1a). There was a markedly significant decrease in HDLc in the double heterozygote compared with control group animals, confirming the data obtained previously. As shown in Fig. 1b, APOA-I was only associated with HDL fractions and its levels were in accordance with the plasma determinations (Table 1). No change in the distribution of this apolipoprotein was observed among animal groups, and consequently its distribution appeared to be genotype independent. Arylesterase activity of HDL particles was also assayed (Fig. 1c). The arylesterase activity was linked to the presence of HDL particles and decreased in the het cbs, het A-I and double het groups compared with the control group (Fig. 1c).

Arterial blood pressure and histological analyses

To evaluate the effect of each single genetic deficiency, as well as their interaction upon vascular function, blood pressure measurements were performed on the caudal artery (Fig. 2).

Systolic blood pressure was significantly increased, with respect to control animals, solely in double heterozygous mice (136 ± 8.0 versus 126 ± 7.5 mmHg, $P < 0.01$). These results demonstrate a contribution of both factors – hyperhomocysteinemia and hypoalphalipoproteinemia – to the elevation of systolic blood pressure.

Histological analyses of hearts unveiled that the double heterozygous mice presented increased left ventricular cross-sectional area in a concentric way (Fig. 3b, c) compared to the control group when normalized to body weight (Fig. 3a, c). However, kidney histological examination did not show morphological alterations in any of the groups (Fig. 3d, e).

Plasma nitrate/nitrite (NOx) levels

To evaluate whether the increased blood pressure in double heterozygotes could be mediated by alterations in eNOS function, plasma NOx levels were determined. As shown in Fig. 4, plasma NOx levels in double heterozygous mice were decreased with respect to the control group. No differences were observed in the single heterozygotes compared to the control group, suggesting again the interaction of both deficiencies in the induction of decreased NOx levels.

Western analysis of eNOS, caveolin-1 and SR-BI

Since the double heterozygote mice suffered mild hypertension in a setting of decreased NO plasma levels, we wanted to determine if a decrease in aortic endothelial NO synthase (eNOS) content or function could underlie these effects. Western blotting of aorta homogenates indicated that eNOS protein diminished in all three genetic deficient groups when compared to the control group (Fig. 5a). In contrast, a tendency to increased SR-BI protein levels was observed in all deficient groups, even though this increase only reached significant levels in the het cbs experimental groups (Fig. 5b). Expression of caveolin-1 (Fig. 5c), a major protein involved in the regulation of eNOS activity, showed a significant

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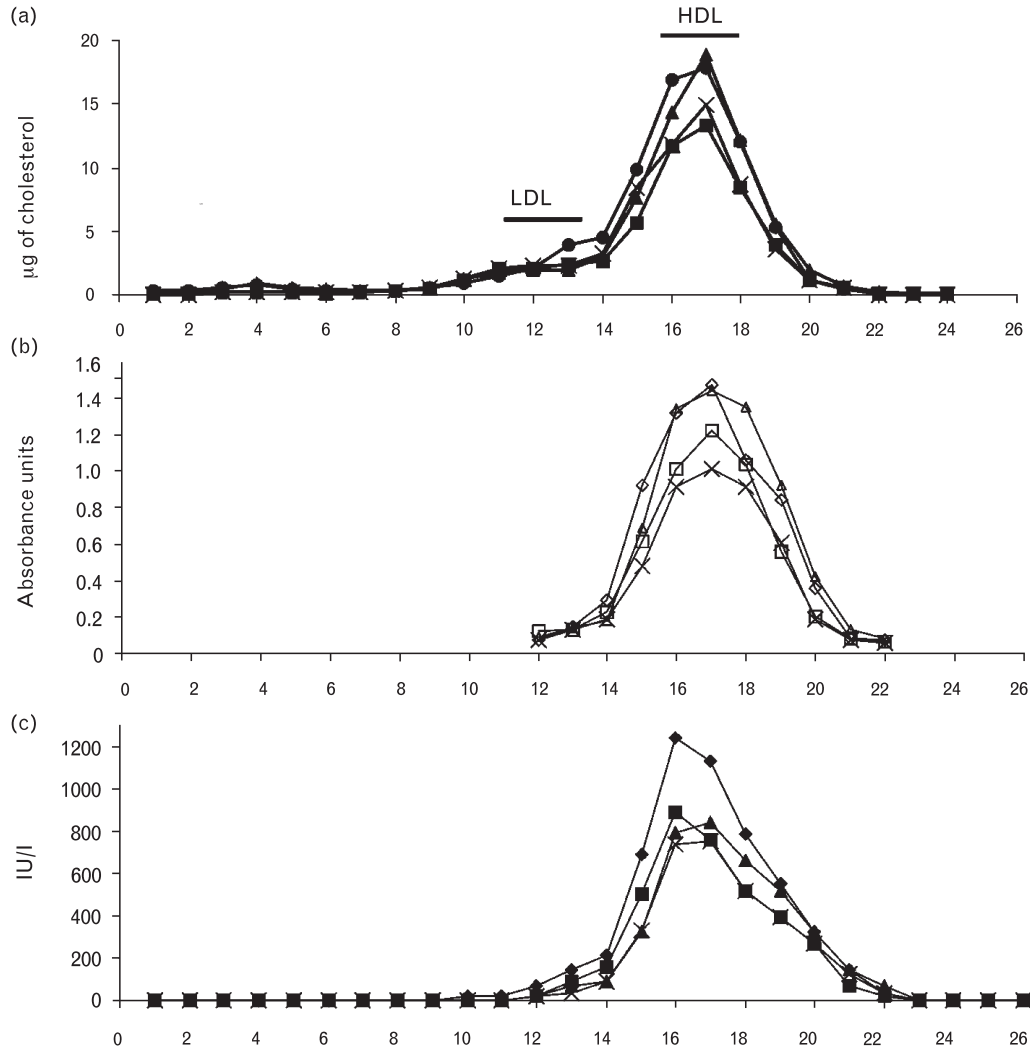
Fig. 1



Separation of plasma lipoproteins by fast protein liquid chromatography (FPLC). (a) Cholesterol distribution; (b) apolipoprotein A-I; and (c) arylesterase activity of para-oxonase. Control (diamond), mice heterozygous for cystathionine β-synthase deficiency (triangle), mice heterozygous for apolipoprotein A-I deficiency (square) and double heterozygous mice (cross).

decrease of this protein in both hyperhomocysteinemic animal groups and increased levels in heterozygous APOA-I animals when compared to control mice.

Discussion

It is well established that a high plasma concentration of Hcy or low HDLc levels are independent risk factors for the development of atherosclerosis and its thrombotic complications. However, little is known about the interactions of both risk factors. In this paper we investigated the results and the mechanisms resulting from the interaction of genetically induced hypoalphalipoproteinemia and hyperhomocysteinemia, in the absence of any other pro-atherosclerotic factor. For this purpose, we generated and used heterozygous mice lacking both *Apoa1* and *Cbs* genes. These double heterozygotes developed moderate hyperhomocysteinemia (10.6 versus 4.2 µmol/l) and hypoalphalipoproteinemia (1.5 versus 2.6 µmol/l) with respect to control animals.

The major finding of this study is that double heterozygotes can be used as a mild hypertension model as they presented higher blood pressure levels compared to control animals (136 ± 8.0 versus 126 ± 7.5 mmHg). It has been reported previously that heterozygous CBS deficient mice presented endothelial dysfunction [36,37], but in our experiments we could not detect any blood pressure changes that might be related to the reported endothelial dysfunction of these animals. In this regard, endothelial dysfunction with mild hyperhomocysteinemia has been demonstrated in a large number of animal models and humans [38], but this

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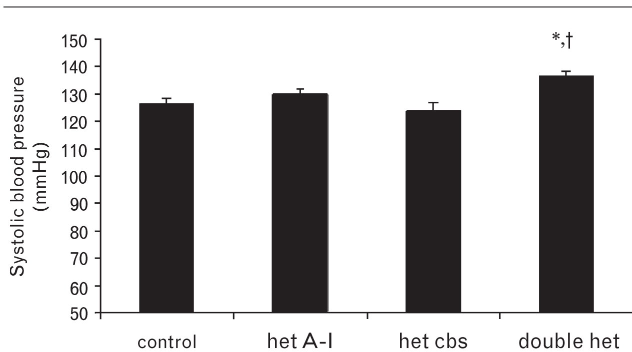
Fig. 2



Systolic blood pressure in mmHg (mean ± SEM) of all experimental groups. Statistical analyses were carried out using one-way ANOVA and Student–Newman–Keuls multiple comparisons test as post-hoc test. $^{*}P < 0.01$ versus control; $^{\dagger}P < 0.01$ versus het cbs. double het, double heterozygous mice, with both cystathionine β-synthase and apolipoprotein A-I deficiency; het A-I, mice heterozygous for apolipoprotein A-I deficiency; het cbs, mice heterozygous for cystathionine β-synthase deficiency.

impairment, likely due to increased oxidative stress and impaired nitric oxide bioactivity, was not translated into higher blood pressure levels [39]. Our results suggest that mild homocysteinemia requires an additional factor to be translated into clinical hypertension, and this is the decrease in the APOA-I-containing particles of HDL. Some HDL deficiencies are characterized by the development of endothelial burden. For example, heterozygous carriers of *abca1* gene mutations present endothelial dysfunction due to impairments in NO bioavailability [40]. In this case, an increase in HDL results in the complete recovery of the endothelial vasomotor response. This observation indicates that HDL directly exerts beneficial effects on the vasculature. In this respect, it is worth noting that recent studies have shown that HDL appears to protect the endothelium directly through SR-BI-dependent eNOS activation [20,21]. However, no changes in blood pressure have been found associated with this situation. In line with these observations, we have observed hypertension only in double heterozygous animals, suggesting that hypertension development requires the contribution of both hypoalphalipoproteinemia and hyperhomocysteinemia. This hypertension state in the double heterozygous mice may be responsible for the concentric left ventricular hypertrophy (LVH), as shown by the heart histological analysis, and by a positive, although non-significant, association between both parameters ($r = 0.28$). In this experimental setting, homocysteine concentration may play an important role due to its high association with LVH ($r = 0.467$, $P < 0.02$) corroborating previous data of a role of this agent on heart pathology independent of hypertension [41,42]. Furthermore, a combination of homocysteine and apolipoprotein A-IV levels, in our model, was able to explain 41% ($P < 0.01$) of the variance of LVH. These results point to a new role of these plasma compounds in cardiac pathology and the emergence of new biomarkers to predict the outcome of LVH. Interestingly, kidneys in this group showed no histological damage, in agreement with previous results where initial hypertension was not associated with renal dysfunction [43].

There are scant data describing the impact of this combination of factors upon blood pressure. In the Third National Health and Nutrition Examination Survey, Lim and Cassano [44] reported that Hcy was inversely associated with HDLc; however, HDLc was neither an effect modifier nor confounder of the Hcy–blood pressure association. These observations could be explained by taking into account that HDL is a heterogeneous lipoprotein group, where different subclasses may have potentially different functions. In their study, Lim and Cassano [44] analyzed the effects of HDLc without addressing the existence of different HDL subclasses. In our model, however, we observe that increased blood pressure is associated with a decrease in APOA-I-enriched HDL particles. In what could be a related observation, a previous study revealed that women with pre-eclampsia, a hypertensive disorder of pregnancy, showed increased Hcy levels and depressed HDL levels [45]. Recently, an inverse relationship between Hcy and apolipoprotein A-I has been found [46,47]. Our present report, together with the previous results, strongly suggest that future hypertension studies should take into consideration the potential concerted contribution to disease development of hyperhomocysteinemia and hypoalphalipoproteinemia, especially due to apolipoprotein A-I deficiency.

Endothelial burden in our model did not seem to be modified by arylesterase activity of para-oxonase. This enzyme, which is transported in HDL, protects the endothelium by inhibiting LDL lipid peroxidation [48]. Moreover, HDL-associated human serum paraoxonase hydrolyzes thiolactone, thereby minimizing protein *N*-homocysteinylation [49]. As, in our study, we observed a similar decrease in arylesterase activity in the three groups harboring a genetic deficiency, we have no indication that para-oxonase could be responsible for either the decrease in NO levels or hypertension development in the double heterozygous animals. Furthermore, the observed decrease of arylesterase activity in CBS heterozygous mice, in the absence of a parallel decrease in HDL, could represent a down-regulatory mechanism of hyperhomocysteinemia on para-oxonase activity.

Low-density lipoprotein cholesterol (LDLc) has a close relationship with cardiovascular diseases and is considered a major promoter in the early stages of these pathologies, where it accumulates in the subendothelial

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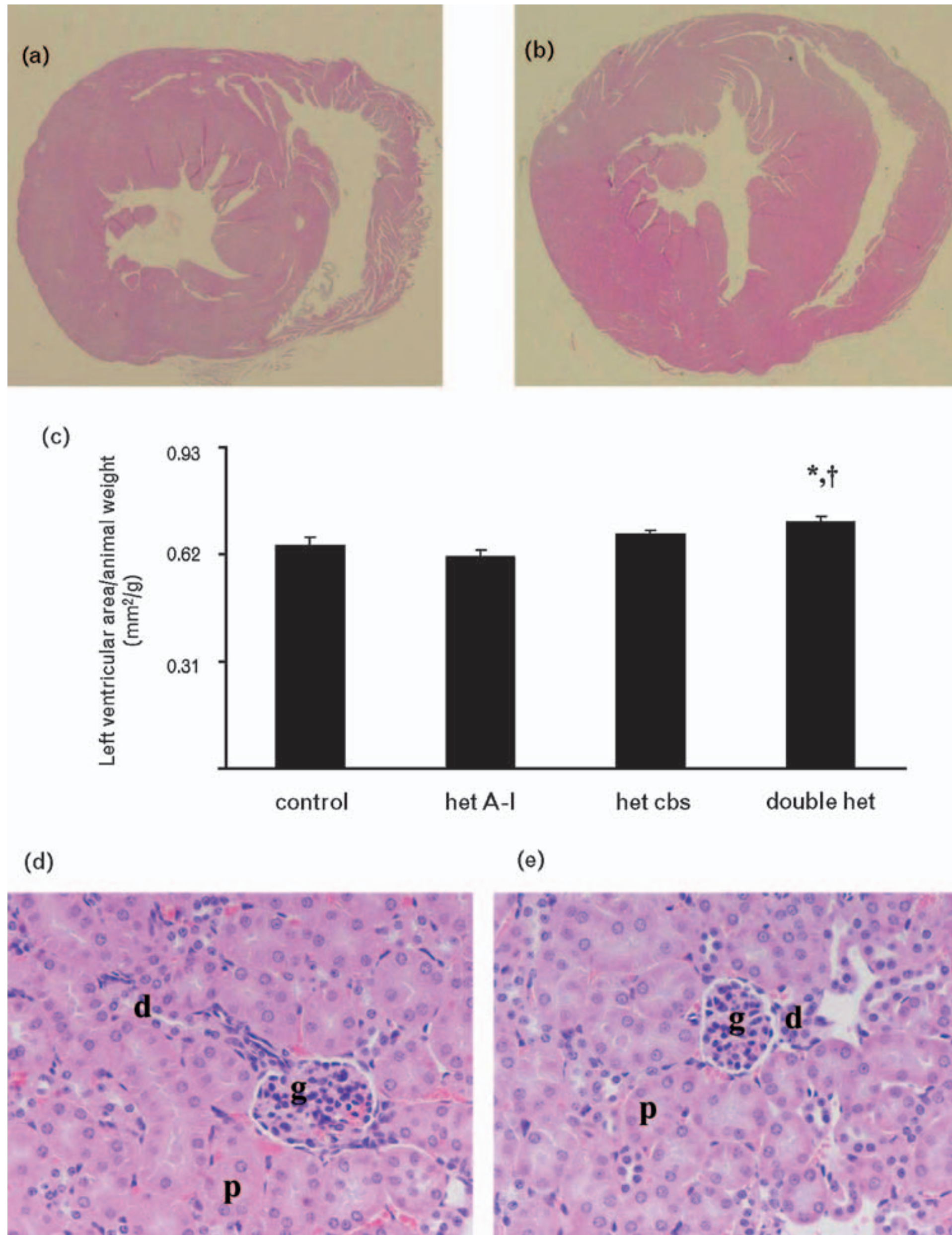
Fig. 3



Histological analysis of heart and kidney. Representative hearts from control (a) and double heterozygous (b) mice. (c) Quantification of left ventricular area normalized to the animal weight (mean ± SEM) for each group. Statistical analysis according to one-way ANOVA and Student–Newman–Keuls multiple comparisons test as post-hoc test. $^{*}P < 0.05$ versus control; $^{\dagger}P < 0.01$ versus het A-I. double het, double heterozygous mice, with both cystathionine β-synthase and apolipoprotein A-I deficiency; het A-I, mice heterozygous for apolipoprotein A-I deficiency; het cbs, mice heterozygous for cystathionine β-synthase deficiency. Representative sections at ×400 from kidneys showing the renal cortex of (d) control and (e) double heterozygous mice. Glomeruli are indicated by g, and distal and proximal tubules are indicated by d and p, respectively.

space. In our model of double deficiency, LDLc potential effects over the endothelium are minimized, given that the majority of cholesterol (80–90%) is HDL-transported. Moreover, double heterozygous mice showed lower cholesterol levels compared to controls and similar to single heterozygotes. We believe these are the reasons why this model does not develop spontaneous atherosclerosis on normal chow or on atherogenic diets (data not shown).

We studied plasma HDL apolipoproteins, the major role of which is thought to be the transport of lipids, although they may also exert diverse protective effects upon the endothelium. Most of APOA-I and APOA-II were

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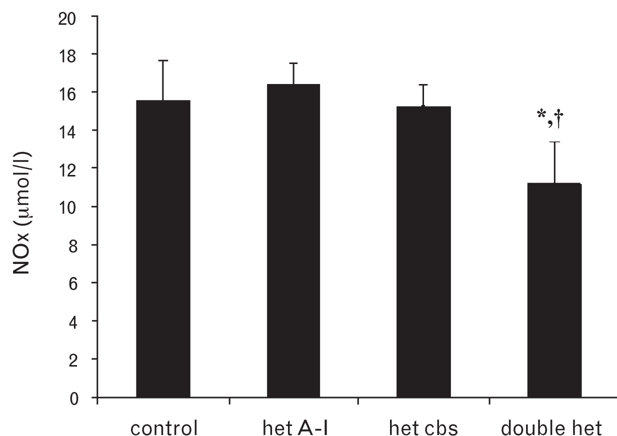
Fig. 4



Plasma nitrate/nitrite (NOx) levels expressed in micromolar concentrations (mean ± SEM) for each group. Statistical analysis according to the Mann–Whitney U-test. $^{*}P < 0.05$ versus control; $^{\dagger}P < 0.05$ versus het A-I. double het, double heterozygous mice, with both cystathionine β-synthase and apolipoprotein A-I deficiency; het A-I, mice heterozygous for apolipoprotein A-I deficiency; het cbs, mice heterozygous for cystathionine β-synthase deficiency.

distributed under the monodisperse peak of HDL cholesterol previously described in mice [50]. As expected, APOA-I diminished in groups with *Apoa1* genetic deficiency, while APOA-II mimicked APOA-I behavior. In fact, an association analysis between both variables showed a strong and significant value ($r = 0.58$, $P < 0.001$). This suggests that APOA-II recruitment into hepatic HDL is, at least in part, dependent on APOA-I. In contrast, APOA-IV did not entirely co-elute with HDL, as described previously by other authors [51], as 50% of the protein was present in the lipoprotein-deficient fractions. Although its biological relevance is presently largely unknown, it should be remarked that a decrease in APOA-IV is the only apolipoprotein-related alteration that co-segregates with the decrease in NOx and blood pressure observed in double heterozygous animals. In this respect, it should be noted that, in this animal model, there is a significant inverse correlation between APOA-IV concentration and blood pressure ($r = -0.5481$, $P = 0.0007$). Although APOA-IV function(s) in the atherosclerotic process is not well characterized, it has been shown that overexpression in mice fed a high cholesterol diet produced significant protection against atherosclerosis [52]. In addition, there is also some evidence suggesting that APOA-IV plays an important role during the inflammatory response. Thus, APOA-IV appears to be an acute-phase protein in mouse [53], and its exogenous administration has been shown to restore the physiological state in a mouse model of experimental colitis [54]. Recently, we have found that APOA-IV-enriched small, dense HDL has a potent anti-atherosclerotic effect, due to a great ability to inactivate LDL peroxides [55]. From these observations, we hypothesize that decreased levels of APOA-IV in the double heterozygous group could impair the anti-inflammatory response and vascular response to oxidative stress.

NO has a key role in the endothelium-dependent responses [56]. A large body of evidence indicates that eNOS regulates arterial pressure [57]. Our findings are in agreement with these observations, since hypertensive double heterozygous mice showed a significant decrease in plasma NOx levels. As is the case for hypertension, our results indicate that both deficiencies need to act in concert to cause major dysfunction, since no change in nitric oxide concentration in either single heterozygous group was observed. To gain further insight into the mechanism underlying decreasing NOx levels, we measured the expression of different proteins implicated in NO homeostasis. The most important enzyme in this system is the eNOS enzyme which catalyzes the conversion of arginine into citrulline, generating one NO molecule. This enzyme is highly regulated through different pathways [58], and is considered to be one of the key factors of vascular health. Our results indicate that aortic eNOS expression was decreased in the three groups harboring genetic deficiencies. It seems that both hypoalphalipoproteinemia and hyperhomocysteinemia, independently, result in decreased expression of eNOS, in agreement with in-vitro studies [59]. However, decreased NOx levels – which could also represent, in part, increased NO usage – is only apparent in animals where both conditions coexist. SR-BI acts as the HDL receptor in the endothelium, allowing further eNOS phosphorylation and activation [60], therefore potentially acting as a protective mechanism by which endothelium might reactivate NO production. Aortic SR-BI expression seems to be rather increased, even though this only reached statistical significance in het cbs mice. It is thus possible that this increase could represent a compensatory mechanism to maintain NO action. Caveolin blocks eNOS activity by decreasing access of its cofactor to the eNOS enzyme [61]. The decrease in aortic caveolin-1 levels manifested by both groups of hyperhomocysteinemic mice is noteworthy because this could represent an additional attempt to further protect the endothelium from the decrease in NO.

In conclusion, the double heterozygous CBS/APOA-I deficient mouse that develops moderate hyperhomocysteinemia and hipoalphalipoproteinemia is a potentially good model for the study of hypertension associated with decreased NO levels and left ventricular hypertrophy. The present research fuels the notion that an interaction of decreased APOA-I levels concomitant with mild hyperhomocysteinemia might generate endothelial dysfunction, clinical hypertension and cardiac alterations. Further studies to validate and characterize the molecular mechanisms involved in this

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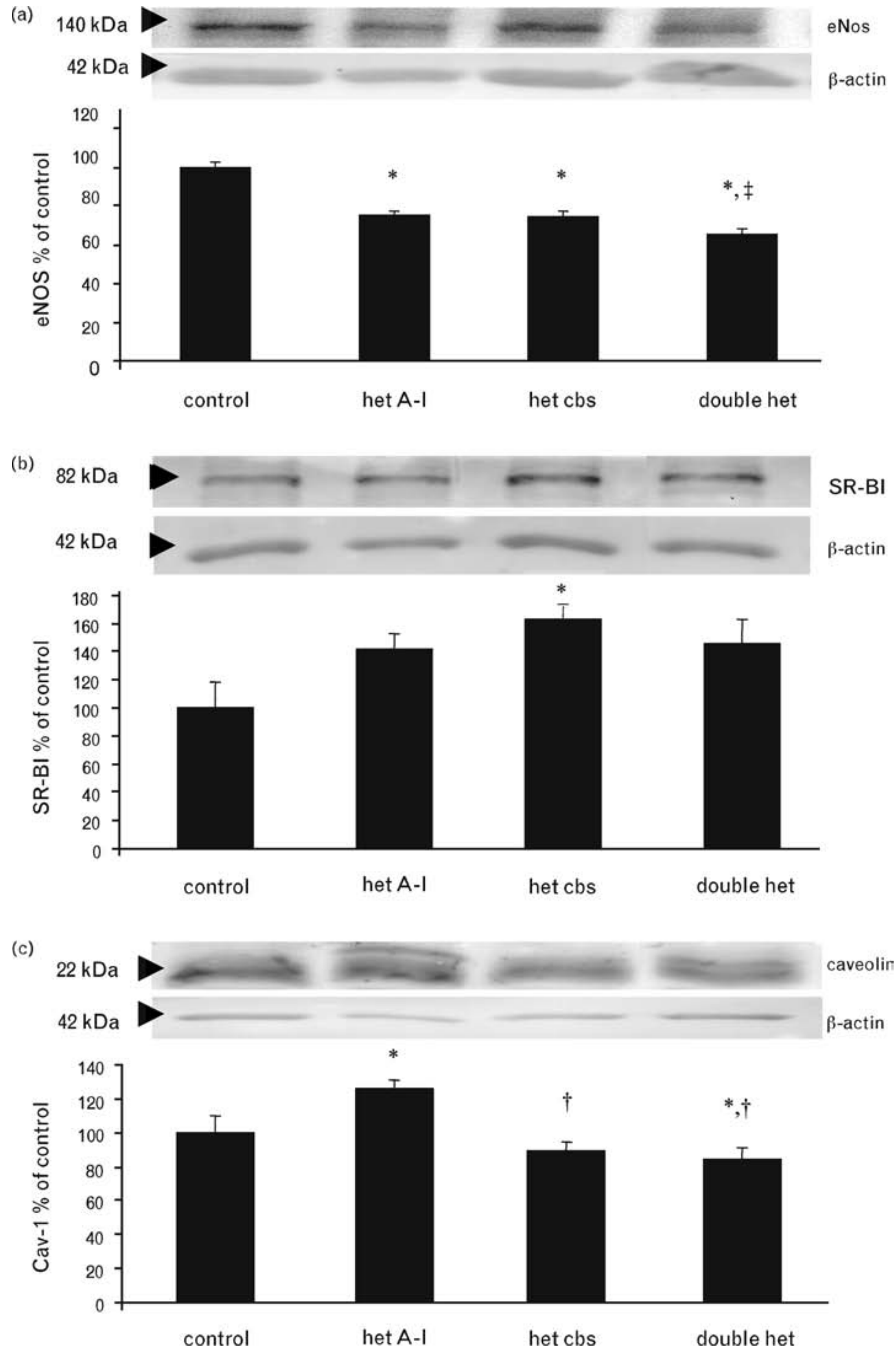
Fig. 5



Effect of mutation on aortic-specific protein expression. The results represent data obtained by densitometric analysis of immunoblotted signals for proteins normalized to those of actin on the same gels. Representative blots and data expressed as percent of control values (mean ± SEM) are given for (a) endothelial nitric oxide synthase (eNOS); (b) scavenger receptor type BI (SR-BI); and (c) caveolin-1 (Cav-1). Two homogenates of pooled aortas of each group ($n = 3$) were prepared and analyzed in duplicate. Statistical analyses were carried out using one-way ANOVA and Student–Newman-Keuls multiple comparisons test as post-hoc test. $^{*}P < 0.05$ versus control; $^{\dagger}P < 0.05$ versus het A-I, $^{\ddagger}P < 0.05$ versus het cbs. double het, double heterozygous mice, with both cystathionine β-synthase and apolipoprotein A-I deficiency; het A-I, mice heterozygous for apolipoprotein A-I deficiency; het cbs, mice heterozygous for cystathionine β-synthase deficiency.

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pathophysiological interaction of cardiovascular risk factors are thus warranted.

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