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Genetic background in apolipoprotein A-I and cystathionine β-synthase deficiency

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1. ABSTRACT

Double heterozygous mice lacking one allele of *Cbs* and *Apoa1* develop hyperhomocysteinemia and hypoalphalipoproteinemia together with moderate hypertension. To study the influence of the genetic background into this specific phenotype, four groups of male mice were established: control and double heterozygous groups in C57BL/6J and in C57BL/6J x 129 backgrounds, respectively. Nitric oxide levels, systolic blood pressure, plasma lipid parameters, arylesterase activity and aorta histology were analyzed as well as oligonucleotide array hybridization of liver RNA. Results demonstrated that double heterozygous mice in C57BL/6J substrate had a milder phenotype showing lower increase in blood pressure compared to double heterozygous group in hybrid background. The severity of the phenotype in the latter group was associated with lower nitric oxide and arylesterase activity levels, and hyperplasia of the vascular media layer. Hepatic profiling of both genetic substrates showed profound differences in expression of contractile proteins that could explain these pathological findings. In summary, the phenotypic presentation of hypertension is associated with multiple processes from vascular bedside to liver as evidenced by nitric oxide production or paraoxonase levels.

2. INTRODUCTION

Genetic background is the specific collection of allelic gene variants that make individuals to present different and inheritable characters within species. In this sense, inbred mouse strains are widely used to study the effect of different genetic backgrounds on a disease phenotype. For example, changes in cholesterol absorption efficiency among inbred mouse strains have been investigated (1). It has also been observed that mice from the inbred strain C57BLKS/J exhibited increased susceptibility to both diabetes and atherosclerosis compared to C57BL/6J mice (2). In addition to these studies, and in order to study other pathologies, these inbred strains of mice have been backcrossed to gene targeted mice as models of genetic diseases. These new lines are great instruments to determine the effect of genetic backgrounds on the disease phenotype, and to identify those genes whose expressions have changed. This approach has already been used to study the effect of varying genetic backgrounds on hypertension (3), diabetes (4) and obesity (5). These variations may substantially alter the conclusions drawn from experiments, so taking into consideration these observations would be necessary prior to initiate any experimental work.

The use of cystathionine β-synthase (cbs) deficient mouse model of hyperhomocysteinemia and apoA-I deficient mouse model of hypoalphalipoproteinemia have both yielded substantial insight into the pathogenesis of atherosclerosis. Thus, apoA-I deficient mice bred to various knockout or transgenic mice have provided information on the role of HDL apolipoproteins in retarding the development of atherosclerosis as well as in cholesterol reverse transport (2, 6, 7). In the same way, cbs deficient mice have provided valuable data of the atherosclerosis process, endoplasmic reticulum stress, oxidative stress and liver steatosis processes (8-10). In previous studies we were interested in the effect of the combination of moderate hypoalphalipoproteinemia and hyperhomocysteinemia. In order to study this particular situation, double heterozygous mice deficient lacking one allele of both *Cbs* and *Apoa1* genes mice were generated. As we have described in previous works, we concluded that the addition of these two factors produced moderate hypertension due to decrease nitric oxide levels (11).

The current research was designed to determine whether genetic background influences double heterozygous phenotype, in particular, hypertension susceptibility, changes in nitric oxide levels and the extent of endothelium injury. For this purpose, two different strains of double heterozygous mice were used; in one hand we studied mice on the C57BL/6J and in the other on the mixed C57BL/6J and 129 genetic backgrounds.

3. MATERIAL AND METHODS

3.1. Animals

Generation of the cystathionine β-synthase and apolipoprotein A-I double heterozygous with mixed background C57BL/6J x 129 were obtained by breeding heterozygous *cbs* (12) and heterozygous *Apoa1* (13). In order to obtain homogenous background mice were backcrossed for 9 generations to C57BL/6J (Charles River).

Mice were bred in the *Unidad Mixta de Investigación*, Zaragoza. For this study 38 males, aged three months, were housed in sterile filter-top cages in rooms maintained on a 12-h light/12-h dark cycle and had *ad libitum* access to food and water. Body weights and food intake were recorded throughout the experiment. Four groups of study were established: a control (n = 10) and double heterozygous (n = 9) groups in hybrid genetic background (C57BL/6J x 129), and control (n = 10) and double heterozygous (n = 9) groups in homogeneous background (C57BL/6J). All groups were fed on a chow diet Teklad Mouse/Rat Diet no. 2014 from Harlan Teklad (Harlan Iberica, Barcelona Spain). The protocol was approved by Ethical Committee for Animal Research of the University of Zaragoza.

3.2. PCR Genotyping

Tail DNA was prepared and subjected to PCR reaction. 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 pb band from the wild-type gene and a 400 pb band from the knockout gene. In the case of *Apoa1*, 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 results in a 150 pb band from the wild-type gene and a 250 pb band from the knockout gene. PCR amplification for both genes was carried out as (11).

3.3. Plasma determinations

At the end of the experimental period and after overnight fast, animals were sacrificed by suffocation in CO_2 and 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 was determined in a similar manner after phosphotungstic acid- $MnCl_2$ (Roche, Barcelona, Spain) precipitation of apo B containing particles (14). Plasma homocysteine concentrations were assayed with a time resolved immunofluorimetric assay (IMX, Abbot, Madrid). NO levels were measured according to Misko *et al.* (15). Paraoxonase was assayed as previously described (16) and results were expressed as μmol phenyl acetate hydrolyzed $min^{-1}L^{-1}$ (IU L^{-1}).

3.4. Blood pressure

The systemic blood pressure was monitored using a non invasive tail-cuff method (Le 5002 storage pressure meter, LEICA, Barcelona, Spain). It was followed the protocol previously described in (11).

3.5. Histological analysis

The heart and the arterial tree were perfused with phosphate-buffered saline under physiological pressure, after which the hearts and aortas were dissected out, cleaned, and stored in neutral formaldehyde. The aortic base of the hearts and the aortas were included in paraffin, and sections (4 μm) were stained with haematoxylin and eosin. Images were captured and digitized using a Nikon microscope equipped with a Canon digital camera. Morphometric analyses were performed using Scion Image software (Scion Corporation, Frederick, Maryland, USA).

3.6. RNA isolation, Affymetrix oligonucleotide array hybridization, and data analysis

At the moment of sacrifice, livers were obtained and quickly frozen in liquid nitrogen. RNA was prepared from each liver using Trigent reagent MRC (Cincinatti, OH, USA) following the manufacturer's instructions. Equal aliquots of total RNA from each of mouse per group were pooled and purified using RNeasy (Quiagen, Barcelona, Spain). 8 μg of total liver RNA were used for biotin labeling. Hybridization, washing, scanning, and analysis of the Affymetrix GeneChip Murine Genome MOE430A array (Affymetrix, Santa Clara, CA) were carried out according to standard Affymetrix protocols. Fluorimetric

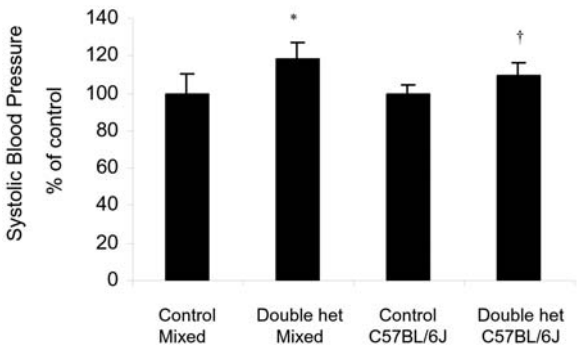

Figure 1. Changes in systolic blood pressure. Data are expressed on percentage of control (mean ± SD) of all groups. Statistical analyses are based on one-way ANOVA. * p<0.05 vs. control hybrid; † p<0.05 vs. control C57BL/6J.

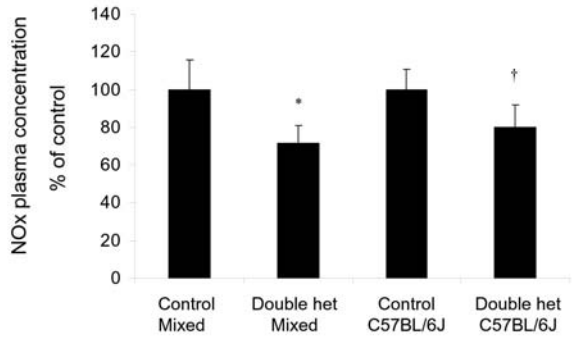

Figure 2. Plasma NOx concentration changes. Data are expressed on percentage of control (mean ± SD). Statistical analyses according to the Mann-Whitney-U test. * p<0.05 vs. control hybrid; † p<0.05 vs. control C57BL/6J.

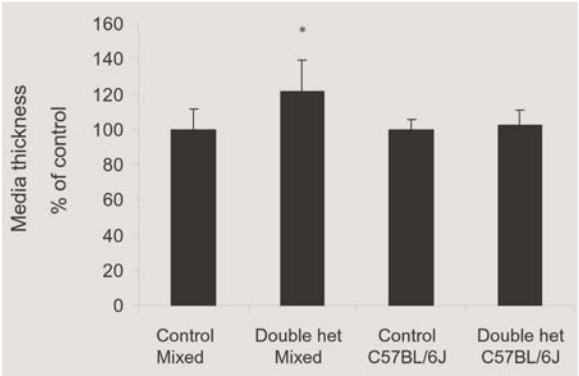

Figure 3. Changes in media thickness in different experimental conditions. Data are expressed on percentage of control (mean ± SD). Statistical analysis according to the one-way ANOVA. * p<0.05 vs. control hybrid.

data were generated by Affymetrix software, and the gene chips were globally scaled to all the probe sets with an identical target intensity value. Data obtained from the microarray hybridizations was processed with Microarray Suite 5.0 (Affymetrix) software. Identification of genes that were activated or repressed by a specific condition was accomplished by comparing the gene expression of the groups at the significant level of P< 0.01. Among those, only differentially regulated genes with a 2-fold cut-off were selected to increase the stringency of identifying candidate genes.

3.7. Statistical analysis

To identify significant differences, one-way analysis of variance (ANOVA) was used when a Kolmogorov and Smirnov test indicated that the variable was normally distributed. Post-hoc tests were performed using Student–Newman–Keuls multiple comparisons test. Differences were considered significant when P<0.05. All statistical analyses were performed using Instat 3.02 for Windows (GraphPad, San Diego, California, USA).

4. RESULTS

4.1. Plasma biochemistry

There was a general decrease in lipid levels in the double heterozygous mice compared to control mice, but it was observed that in mixed genetic background total cholesterol, HDLc and TG decreased to a greater extent than in C57BL/6J (Table 1). Double heterozygous in mixed background showed higher body weights and a concomitant higher liver weight compared with control, whereas in homogeneous background these parameters did not change. Arylesterase activity measured as phenylacetate hydrolysis $min^{-1}L^{-1}$ decreased following the same trend, showing a decrease of 31% in mixed background whereas in homogeneous substrate only a 15%. In contrast to previous results, double heterozygous mice in homogeneous C57BL/6J showed higher increase in homocysteine levels (60%) than those double heterozygous in mixed background (40%).

4.2. Arterial blood pressure

As in our previous results, it was observed that double heterozygous mice showed a moderate hypertension compared to control mice (Figure 1). However, the genetic backgrounds influenced the severity of the phenotype. Thus, the systolic blood pressure increase was higher in the double heterozygous mice in C57BL/6J x 129 substrate compared to homogeneous background (20% vs. 10%).

4.3. Plasma NO levels

Our previous results pointed out to nitric oxide bioavailability as a major cause of moderate hypertension. In the line with this observation, it was observed that double heterozygous with hybrid background had lower nitric oxide levels (30% decrease) according to the more pronounced increase in arterial blood pressure compared to double heterozygous mice in homogeneous genetic background (20% decrease) (Figure 2).

4.4. Histological analyses of aorta

In order to explore the hypertension effects on the endothelium, histological analyses of proximal aorta sections after haematoxylin eosin staining were performed. The results showed no significant changes between groups

Table 1. Plasma parameters in function of genetic background

	C57BL/6J x OLA129		C57BL/6J	
	control (n=10)	double het. (n=9)	control (n=10)	double het. (n=9)
cholesterol	100 ± 18.0	56 ± 23.3*	100 ± 19.5	71 ± 10.3†
HDL-cholesterol	100 ± 35.4	60 ± 28.8*	100 ± 24.9	65 ± 9.5†
triglycerides	100 ± 5.2	64 ± 13.3*	100 ± 20.2	82 ± 13.9†
homocysteine	100 ± 2.0	140 ± 20.0*	100 ± 14.1	161 ± 24.3†
arylesterase activity	100 ± 7.8	69 ± 1.5*	100 ± 13.9	85 ± 8.5†
body weight	100 ± 11.7	112 ± 9.3*	100 ± 19.1	102 ± 12.8
liver weight	100 ± 19.3	107 ± 10.5*	100 ± 19.1	100 ± 13.0

Data are expressed on percentage of control. Results are mean ± SD. Statistical analyses were carried out by the Mann-Whitney-U test. * $p<0.05$ vs. control hybrid; † $p<0.05$ vs. control C57BL/6J.

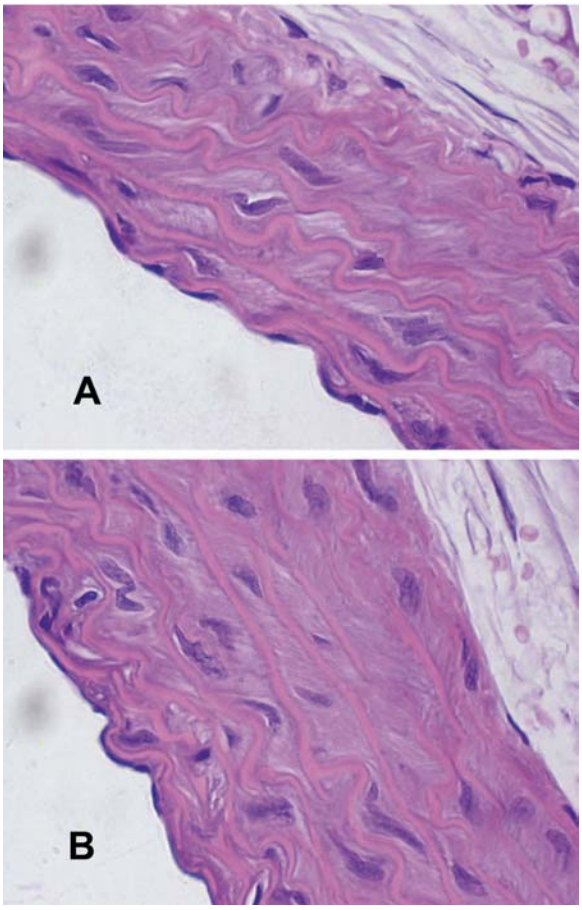

Figure 4. Representative micrographs from aorta of mice on C57BL/6J x 129 genetic background. Aorta sections (4 μm) were stained with haematoxylin and eosin A) control 40x and B) double heterozygote 40x.

in the C57BL/6J genetic background (Figure 3). However, it was found a significant increase in the media thickness in double heterozygous hybrid group compare to its control (Figures 3 and 4). This increase appeared to be caused by media hypertrophy rather than by an increase in the number of cells, as the number of nucleus per section did not change in both groups (data not shown).

4.5. Affymetrix oligonucleotide array hybridization

To explore the genetic differences between both backgrounds, liver RNA was isolated from animals aged 9 days. RNA hybridization in the Affymetrix GeneChip Murine Genome MOE430A array displayed differently expression of 1331 genes. Among these, 717 were over expressed and 614 were repressed. Those genes that showed significant changes (Signal log ratio > 2.0) were selected and listed in Table 2. Hybrid mice showed increase expression in different processes such as muscle contraction (troponin C; myosin, heavy polypeptide 6 alpha) and cytoskeleton structure (myosin, light polypeptide 2; actin, alpha). In the other hand other genes implicated in different processes were downregulated, as well as various genes with unknown function.

5. DISCUSSION

Our results showed significant changes between both genetic backgrounds in relationship with the severity of the genotype present in double heterozygous lacking one allele of *Cbs* and *Apoa1* mice. Thus, hybrid substrate exhibited higher changes between double heterozygous and control groups in most parameters. Likewise, hybrid double heterozygous mice showed more pronounced lowering levels of nitric oxide (30%) vs. control than that homogeneous double heterozygote (20%) vs. control. This decreased nitric oxide level was translated into further increased systolic blood pressure levels in double heterozygous hybrid mice than in double heterozygous mice in homogeneous background (18% vs. 10%, respectively).

Gene targeting and transgenic technology give us the opportunity to explore the role of the proteins in biological processes. These techniques have produced a large variety of animal models that reproduce several pathologies, but also, they have been paralleled by reports describing mouse strain dependent phenotypic changes (17, 18). Genetic heterogeneity among strains used to generate transgenic and knockout mice, make us to take into consideration the possibility of unpredictable phenotypic effects caused by variation in the genetic background. It is recommended to describe these changes among the strains prior to develop an experimental design. A variety of processes observed in these models are influenced by genetic background, among these accounted the atherosclerosis and diabetes pathologies, renal development and cholesterol absorption (1, 2, 19, 20). As example, aortas from C3H/HeJ and C57Bl/6 mice were studied in response to atherogenic stimuli, being the latter strain the one with higher propensity to develop inflammation (21). In line with these previous observations, a model of hypertension, the cystathionine β-synthase and apolipoprotein-I double heterozygous mice, was studied in two different genetic backgrounds. Initially, mice were in

Table 2. Fold changes of hepatic genes activated or repressed depending on genetic backgrounds

Biological process	GenBank	Affymetrix ID	Name	Gene Symbol	hybrid au	C57BL/6J au	Signal Log Ratio
signal transmission	NM_023879	1421144_at	retinitis pigmentosa GTPase regulator protein	Rpgrip1	22.8	1585.8	5.5
progesterone metabolism	NM_134066	1419136_at	aldo-keto reductase family 1, member C18	Akr1c18	63	737.9	3.6
---	AK004806	1422731_at	LIM domains containing 1	Limd1	52.7	299.2	2.7
amino acid phosphorylation	NM_024182	1460670_at	RIO kinase 3	Riok3	30.4	211.2	2.4
acute-phase response	NM_011316	1419319_at	serum amyloid A 4	Saa4	33.5	193.6	2.3
---	AI182092	1434906_at	EST	0610005C13Rik	10.5	862.6	2.1
---	AU046270	1438758_at	EST	---	58.7	1099.3	4
Biological process	GenBank	Affymetrix ID	Name	Gene Symbol	hybrid	C57BL/6J	Signal Log Ratio
cytoskeleton organization	NM_010861	1448394_at	myosin, light polypeptide 2,	Myl2	520.9	4.1	-6.3
acute-phase response	BE628912	1451054_at	orosomucoid 1	Orm1	835.9	12	-6
cytoskeleton organization	NM_009608	1415927_at	actin, alpha	Actc1	200.3	4.3	-5.4
---	BF234005	1423866_at	proteinase inhibitor, A, 3K	Serpina3k	8465.6	236.3	-5.2
oxygen transport	BC025172	1451203_at	myoglobin	Mb	151.9	4.2	-4.5
cellular morphogenesis	NM_007598	1417461_at	CAP, adenylate cyclase-associated protein	Cap1	489.3	34.3	-3.9
muscle contraction	NM_009393	1418370_at	troponin C, cardiac/slow skeletal	Tnnc1	156.9	10.7	-3.5
immune response	NM_010259	1420549_at	guanylate nucleotide binding protein 1	Gbp1	73.7	5.6	-3.5
muscle contraction	BB481540	1448826_at	myosin, heavy polypeptide 6 alpha	Myh6	95.9	8.2	-3.4
transport	NM_010174	1416023_at	fatty acid binding protein 3	Fabp3	141.8	13.7	-3.3
---	AV006463	1434484_at	RIKEN cDNA 1100001G20 gene	1100001G20Rik	1104.3	155	-3
transport	NM_021370	1420451_at	amiloride-sensitive cation channel 5	Accn5	121.3	28.6	-2.4
carbohydrate metabolism	NM_009669	1416055_at	amylase 2, pancreatic	Amy2	636.6	130	-2.2
---	BG862223	1455869_at	---	---	592.2	163.7	-2.2
---	NM_133217	1421221_at	beta-carotene 9', 10'-dioxygenase 2	Bcdo2	76	20	-2.2

Data are expressed as arbitrary units of absorbance

hybrid genetic background, achieved from C57BL/6J and 129 founders. This substrate was obtained from the genetic technique used to generate the *Cbs* knock out mice derived from two different backgrounds to overcome the poor reproductive performance of inbred strains (22). Through breeding for 9 generations the double mutation *Cbs x Apoa1* has been transferred to the C57BL/6J strain, used frequently in atherosclerosis, blood pressure and cancer research (23-25). The availability of animal models in both substrates and its phenotypic characterization provides further support to the influence of genetic background on the severity of hypertension.

Differences in circulating lipids among inbred strains of mice have been observed (26, 27). In our model it is important to note that cholesterol is mainly transported in HDL particles. HDLc exhibited a similar behaviour independently of the genetic background what it is in agreement with other reports where it has been well documented that mouse strains exhibit similar HDL composition. In addition to these similarities, it is remarkable that each strain shows its own phenotypic variation of HDL electrophoretic pattern, evidencing size and density heterogeneity between them (26, 28). These changes in structure are related to several genes that finally may determine the genetic component in atherosclerosis (20, 29) and may be more complex than previously suggested by initial studies of recombinant inbred strains (30). In our case a more profound response was found in TG levels between both genetic substrates in accordance with studies reporting different behavior in response to the diets in function of genetic background (31, 32). TG levels have been found to be associated with polymorphism sites in apolipoprotein C-II, C-III and apoA-V as well as lipoprotein lipase in human (33). In the same direction, some reports have identified in inbred mice strains the possible loci where candidate genes for TG regulation may be located (27). Overall, our data indicate that TG is also a parameter that may change dramatically along genetic background.

One of the proteins transported in HDL particles is paraoxonase. It is established that this enzyme exerts beneficial effects on endothelium due to its ability to inhibit

the activity of mildly oxidized low density lipoproteins (LDL) (34). The present study found significant higher decrease in arylesterase activity in hybrid double heterozygous mice compared to its control than in homogeneous C57BL/6J double heterozygous compared to its counterparts. Our data is suggesting an enhanced harmful effect over the endothelium function in hybrid double heterozygous mice, contributing to the negative lower nitric oxide levels described previously. Variations in arylesterase activity among strains have also been reported in other studies. For example, arylesterase activity associated to HDL particles decreased in C57BL/6J strain whereas the opposite effect was observed in C3H/HeJ when both groups were maintained on an atherogenic diet high in fat and cholesterol (35). These results are indicating that a variation in activity of this enzyme is a good candidate to explain severity of phenotype in vascular biology.

The increase in systolic blood pressure was accompanied by hypertrophy of the media in the aorta, but only in the hybrid substrate. No changes in this parameter were observed for the C57BL/6J background double heterozygote. It has been demonstrated that hypertrophy of vascular smooth muscle cells are linked to enhanced blood pressure sensitivity and increased vasoconstriction of arteries as described in angiotensin II receptor deficient mice (36). Furthermore, they suggested the regression of vascular hypertrophy as a potential therapeutic target for the reduction of complications associated with hypertension. Variability in this gene among inbred strains could explain the different morphological phenotype.

It is established the general hypothesis that high levels of homocysteine are toxic to the endothelium (37, 38). Consistent with this idea our C57BL/6J double heterozygous that showed higher increase in homocysteinemia would potentially suffer more harmful effects on vascular function. Our results previously exposed are in opposition to this hypothesis, what might suggest that previous *in vitro* studies were done with concentrations of homocysteine that far exceeded those reached by our animal model, and that this risk factor would be less relevant than the others in order to influence endothelium response.

From the array results, it is clear that these differences observed between both strains are caused by different gene expression patterns. Furthermore, these changes are caused not only by a single gene; they are produce by the interaction of several routes since it appeared various metabolic pathways implicated. Remarkable muscle contraction and cytoskeleton organization are important in smooth muscle cells to control vascular tone (39-41). We suggest that this increased expression in hybrid double heterozygous mice could be in part responsible for the impaired vascular response.

In conclusion, we have demonstrated that the severity of hypertensive phenotype depends on the genetic background. Double heterozygous mice in mixed genetic substrate exhibited higher reductions in nitric oxide and in arylesterase activity that their counterparts in C57BL/6J background. Consequently, they also presented higher blood pressure levels than the double heterozygous in C57BL/6J genetic background.

6. ACKNOWLEDGMENTS

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Abbreviations: apoA-I, apolipoprotein A-I; HDLc, high density lipoprotein cholesterol; cbs, cystathionine β-synthase; NO, nitric oxide; TG, triglycerides; Hcy, homocysteine

Key Words: Hypertension, Nitric Oxide, Homocysteine, High Density Lipoproteins, Apolipoprotein

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