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ORIGINAL PAPER

DNA microarray analysis of the antihypertensive effect of milk fermented by *Lactobacillus helveticus* H9 on spontaneously hypertensive rats

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Abstract Although an antihypertensive effect of fermented milk has been demonstrated in many human and animal experiments, the molecular mechanism controlling it is still poorly understood. In this study, we used DNA microarray analysis to determine the molecular mechanism of the antihypertensive effect of fermented milk. The results showed that gene expression in the left ventricle of spontaneously hypertensive rats was regulated when milk fermented by *Lactobacillus helveticus* (*L. helveticus*) H9 was administered. Some hypertension-associated genes were differentially expressed. In-depth analysis showed that these differentially expressed genes were related to NO synthesis, cell proliferation, endothelin binding, and blood clot breakdown. These processes could be responsible for the antihypertensive response and relief of left ventricular hypertrophy observed. It is likely that regulation of the gene expression of endothelial nitric oxide synthase (eNOS), peroxisome proliferator-activated receptor γ (PPARγ), endothelin type A receptor (Ednra), and other hypertension-associated genes play an important role in these processes. In summary, this study provided valuable information that contributes to our understanding of the molecular mechanisms by which antihypertensive effects are provided from milk fermented by *L. helveticus* H9.

Keywords Antihypertensive effect · Fermented milk · Gene expression · Spontaneously hypertensive rats · *Lactobacillus helveticus* H9

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1 Introduction

Hypertension is a critical factor in many cardiovascular diseases including coronary heart disease, stroke, and left ventricular hypertrophy (Neutel et al. 1999; Unger 2002). Patients with hypertension are usually required to take long-term medication in order to control their condition; this long-term medication can have many detrimental side effects. For this reason, scientists have considered the use of functional food to alleviate hypertension; exciting epidemiological studies have shown that the consumption of dairy products was inversely correlated with the risk of hypertension (McCarron et al. 1984). Furthermore, studies also reported that consumption of milk and fermented milk could reduce blood pressure (Buonopane et al. 1992; Green et al. 2000). As the number of these studies grows, it becomes increasingly important to understand the mechanisms for the observed effects; why does the consumption of fermented milk lower blood pressure? It has been proposed that it is the milk-derived peptides in fermented milk products that could be a primary factor conferring the antihypertensive effect. In particular, groups of bioactive peptides with properties such as angiotensin-converting enzyme (ACE) inhibition, opioid-like activities, mineral-binding activity, and antithrombotic activity could be associated with the antihypertensive effect (Minervini et al. 2003). Although many studies have demonstrated that bioactive peptides in fermented milk can reduce blood pressure (Foltz et al. 2007), the molecular mechanisms for this effect are still not clear.

The identity of bioactive peptides present in fermented milk products will depend on the bacterial strain used during fermentation; this is because proteolytic systems are strain-specific. *Lactobacillus helveticus* is a scientifically well-studied homofermentative bacterium that is typically isolated from fermented foods (Beganovic et al. 2013). Many studies have reported that milk fermented by *L. helveticus* has positive antihypertensive activity (Lopez-Fandino et al. 2006; Takano 1998). In our own previous studies, the ACE-inhibitory activity of 259 *L. helveticus* strains was determined in vitro. The results showed that milk fermented by strain H9 had the highest ACE-inhibitory activity, and it was also effective in reducing the extent of left ventricular hypertrophy development (Chen et al. 2014). Left ventricular hypertrophy is the thickening of the myocardium of the left ventricle of the heart. We know that left ventricular hypertrophy is a risk factor for cardiovascular disease. It is strongly related to hypertension and present in a substantial percentage of patients with hypertension (Liebson 2002). The left ventricle mass is an important index in diagnosis and treatment of hypertension. In this current study, the antihypertensive effects of milk fermented by *L. helveticus* H9 were evaluated in a spontaneously hypertensive rat (SHR) model. After long-term administration of fermented milk, changes in gene expression in the left ventricle of these rats were monitored by microarray analysis and validated by real-time quantitative PCR (RT-qPCR). The purpose of this study was to generate novel information at the molecular level that might help us to better understand the mechanisms underlying the antihypertensive effect of fermented milk products.

2 Materials and methods

2.1 Bacterial strain and preparation of fermented milk

Lactobacillus helveticus H9 was obtained from the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, China. The strain was originally isolated from kurut in Tibet, China (Airidengcaicike et al. 2010) and preserved as a freeze-dried powder at the Lactic Acid Bacteria Collection Centre of the Inner Mongolia Agricultural University. Fermented milk was prepared following the methods of Chen et al. (2014). The strain was activated by two rounds of overnight passage at 37 °C in 11% (*w*/*w*) sterile reconstituted skimmed milk containing 2% glucose and 1.2% yeast extract before being used as a starter culture. The reconstituted skimmed milk was prepared by sterilizing 11% (*w*/*w*) skimmed milk powder (NZMP Ltd., Wellington, New Zealand) at 95 °C for 10 min. Fermented milk was prepared by inoculating the prepared starter culture into the sterile reconstituted skimmed milk at a concentration of 5×10^6 cfu·g^{-1} and allowing fermentation to proceed at 37 °C until a pH of 4.5 was achieved.

2.2 Animal experiment

A total of 30 seven-week-old male SHRs with a systolic blood pressure (SBP) of 142± 18 mmHg and initially weighing 131±8 g were housed in individual stainless steel cages at 20 °C. SHRs were provided with water ad libitum and normal diet throughout the experiment (week 1 to 8). After 1-week adaptation, the rats were randomly divided into two groups (control and H9, 15 rats in each group). The diet of SHRs in the control group was supplemented with physiological saline (15 mL·kg^{-1} body weight) from week 2–8, whereas the diets of those in the H9 group were supplemented with fermented milk (15 mL·kg^{-1} body weight) by intragastric administration.

SBP and diastolic blood pressure (DBP) of the SHRs were measured weekly using a tail cuff blood pressure analyzer. Three consecutive measurements were obtained, and the arithmetic means were recorded. After measurement of blood pressure, the body weight was recorded. At the end of the experiment (week 8), animals were sacrificed by cervical dislocation. The left ventricles were collected, weighed, and stored at −70 °C for further analysis. Left ventricular mass index (LVMI) was calculated as:

$$\mathrm{LVMI} = \frac{\text{Left ventricular mass}}{\text{Body weight}}$$

2.3 Microarray hybridization

Three left ventricle samples in each group were randomly selected for RNA isolation and microarray hybridization. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy Mini kit (Qiagen, Hamburg, Germany). The concentrations of RNA in samples were determined by standard spectrophotometer measurements. RNA purity was checked by agarose gel electrophoresis and ultraviolet spectrophotometer assay. Five to ten micrograms of total RNA from each sample was reverse transcribed using Affymetrix One-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA, USA). cDNAs were isolated as Affymetrix

GeneChip Sample Cleanup Module and used as templates for cRNA synthesis using the GeneChip IVT Labeling Kit (Qiagen, Hamburg, Germany). cRNA fragments were broken down to 35–200-nt long sections and then incubated at 94 °C for 35 mins. The cRNAs were fragmented, labeled, and applied to the Affymetrix Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA, USA), which contained more than 30,000 rat gene probes. Hybridization was carried out in a mixture containing probe array controls, BSA (Invitrogen, Carlsbad, CA, USA) and herring sperm DNA (Promega, Madison, WI, USA) for 16 h at 45 °C. After washing and staining, the arrays were scanned using an Affymetrix GeneArray Scanner, and the fluorescence signal converted to quantify gene expression values using Affymetrix GeneChip Operating Software as described by Affymetrix GeneChip standard procedures.

2.4 Real-time quantitative PCR

RT-qPCR was carried out as described by Di Caro et al. (2005). Eight genes were selected as candidates: Ppia, Actb, and Hk1 (housekeeping genes) and eNOS, Ednra, PPARγ, Nppa, and Cdkn1a (hypertension-associated genes). Primers were designed using Primer 5 (Table 1). The relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an internal control to normalize the amount of RNA added to the reverse transcription reaction. The RNA samples used in RT-qPCR experiments were identical to those used in microarray experiments, each sample was analyzed in triplicate.

Table 1 Genes and primers used in real-time quantitative PCR

Gene	Probeset ID	Description	Primer sequence (5′ to 3′)
GAPDH	AFFX_Rat_GAPDH_3_at	Glyceraldehyde-3-phosphate dehydrogenase	Forward-GAGTCTACTGGCGTCTTCA
			Reverse-TGTCATATTTCTCGTGGTTC
Ppia	1398850_at	Peptidylprolyl isomerase A	Forward-CCACCGTGTTCTTCGACAT
			Reverse-TAGCCAAATCCTTTCTCCC
Actb	1398836_s_at	Actin, beta	Forward-CGTTGACATCCGTAAAGACC
			Reverse-GGAAGGTGGACAGTGAGGC
Hk1	1386929_at	Hexokinase 1	Forward-GAATGTGGAGATGGTGGAG
			Reverse-TTTGTTTCCCAGAGTTTAGAG
eNOS	1371166_at	Nitric oxide synthase 3	Forward-CAATCTTCGTTCAGCCATCA
			Reverse-GCAGACAGCCACATCCTCA
PPARγ	1369179_a_at	Peroxisome proliferator-activated receptor gamma	Forward-AATGCCACAGGCCGAGAA
			Reverse-GCTTTGGTCAGCGGGAAG
Ednra	1369511_at	Endothelin receptor type A	Forward-TTCTCAACCTTCCCAATG
			Reverse-AGGGCAGACTAAGACTATCA
Nppa	1367564_at	Natriuretic peptide A	Forward-TTCTCAACCTTCCCAATG
			Reverse-AGGGCAGACTAAGACTATCA
Cdkn1a	1388674_at	Cyclin-dependent kinase inhibitor 1A	Forward-GACGGTAGGCTCCTTAGTGC
			Reverse-TCTAGGCTGTGACTGCTTCA

2.5 Data analysis

Statistically significant differences in gene expression between the treatment and the control were determined using Student's *t* test. Upregulated genes were identified when the gene expression value in the H9 group was more than onefold relative to that in the control group at $P<0.05$. Meanwhile, downregulated genes were identified when the gene expression value in the H9 group was less than onefold relative to that in the control group at $P<0.05$. Differentially expressed genes were grouped using the DAVID database (Dennis et al. 2003). Biochemical parameters were presented as means±standard deviation and compared using one-way analysis of variance with SPSS 11.5 software.

3 Results

The SBP between weeks 5 and 7 and the DBP between weeks 6 and 7 were significantly lower in the H9 group (diet supplemented with milk fermented by *L. helveticus* H9) compared with the control group (diet supplemented with physiological saline) ($P<0.05$) (Figure S1 and Figure S2, respectively in the supplementary material). Specifically, in weeks 5, 6, and 7, SBP decreased by 10, 11, and 11 mmHg, respectively, and DBP decreased by 10, 11, and 10 mmHg, respectively.

The body weights of SHRs in the H9 group seemed higher than those in the control group during weeks 1 to 6, but this difference only became significant ($P<0.05$) in week 7 (Figure S3 in the supplementary material). This may indicate that the SHRs in H9 group grew slightly faster than those in the control group after long-term intake of fermented milk. LVMI was also significantly smaller in the H9 group (3.06±0.08 $mg \cdot g^{-1}$) compared with the control group (3.19±0.12 $mg \cdot g^{-1}$) ($P<0.05$).

A microarray analysis was used to compare gene expression levels in the H9 group with the control group, and statistically significant up- and downregulations were observed for 573 and 604 genes, respectively (Table S1 in supplementary material). Up- and downregulated genes were analyzed and grouped by their biological functions using the DAVID database. The 573 upregulated genes were mainly associated with transcription, cell death, apoptosis, macromolecule biosynthesis and metabolism, nitrogen compound metabolism, and RNA metabolism (Table S2 in supplementary material); whereas the 604 downregulated genes were mainly related to protein transport and localization, organization of mitochondrion and membranes, and protein and nuclear transport (Table S3 in supplementary material).

In-depth analysis showed that some hypertension-associated genes were differentially expressed in the H9 and control groups (Table 2). We found that eight hypertension-associated genes were upregulated in the H9 group compared with the control: PPARγ, eNOS, Nfkb1, Slc4a7, Hmox1, Cdkn1a, Plat, and Thbd. There were three genes associated with hypertension that were downregulated in the H9 group compared with the control: Ednra, Nppa, and Alas1.

RT-qPCR of eight selected genes (Ppia, Actb, snd Hk1 [housekeeping genes] and eNOS, Ednra, PPARγ, Nppa, and Cdkn1a [hypertension-associated genes]) showed good correlation with the microarray results and exhibited a positive correlation ($r=0.89$, $P<0.01$) (Fig. 1) between the two methods. This result demonstrates

Table 2 Differentially expressed genes associated with hypertension

Gene	Fold change	*P* value	Protein encoded	Function associated with hypertension
eNOS	1.16	<0.01	Endothelial nitric oxide synthase	NO synthesis
Nfkb1	1.21	0.012	Nuclear factor NF-kappa-B p105 subunit	NO synthesis
Slc4a7	1.22	0.041	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	NO synthesis
Hmox1	5.29	< 0.01	Heme oxygenase (decycling) 1	NO synthesis, cell proliferation
Alas1	0.47	0.025	Delta-aminolevulinate synthase 1	NO synthesis
PPARγ	1.17	0.047	Peroxisome proliferator-activated receptor γ	NO synthesis, cell proliferation
Cdkn1a	3.27	< 0.01	Cyclin-dependent kinase inhibitor 1A	Cell proliferation
Ednra	0.71	0.018	Endothelin type A receptor	Endothelin binding
Plat	1.49	0.044	Tissue plasminogen activator	Blood clot breakdown
Thbd	1.49	0.017	Thrombomodulin	Blood clot breakdown
Nppa	0.46	0.026	Natriuretic peptide A	Vasodilatation

reproducibility of the microarray experiment and confirmed DNA microarray studies are a reliable way to study the expression of a large number of genes.

4 Discussion

Hypertension is a multifactorial disease and can be regulated by many cellular chemical signals. Among these signals, NO is considered as one of the most important regulators of blood pressure. The physiological actions of NO include the regulation of vascular tone and blood pressure, prevention of platelet aggregation, and inhibition of vascular smooth muscle proliferation (Moncada and Higgs 2006). The gene eNOS is associated with NO synthesis and was upregulated in the H9 group in our study. This result is in accordance with the results reported by Yamaguchi et al. (2009) that showed eNOS was upregulated in SHRs fed with antihypertensive peptides. eNOS catalyzes the synthesis of nitric oxide in blood vessels and is involved in regulating vascular tone and platelet aggregation; an increase in eNOS expression reduced hypertension in fructose-treated rats (Zhao et al. 2009). Unlike the report of Yamaguchi et al. (2009), we did not find differential expression of the gene connexin 40 but did find differential expression of Nfkb1 and Slc4a7, which were also upregulated in H9 group compared with the control. Nfkb1 is a transcription factor and binding of Nfkb1 subunits p50 and p65 to the eNOS promoter can increase eNOS transcription (Davis et al. 2004). Slc4a7 is an electroneutral cotransporter and plays a critical role in regulation of intracellular pH in vascular smooth muscle cells and endothelial cells. It modulates eNOS by regulating the intracellular pH, because eNOS is pH sensitive (Boedtkjer et al. 2011). Hmox1 and Alas1 are rate-limiting enzymes in the catabolism of heme, which is a component of eNOS. Iwasaki et al. (2005) reported Hmox1 and Alas1 were both upregulated in monocrotaline-induced hypertension. In our study, Hmox1 was upregulated with a

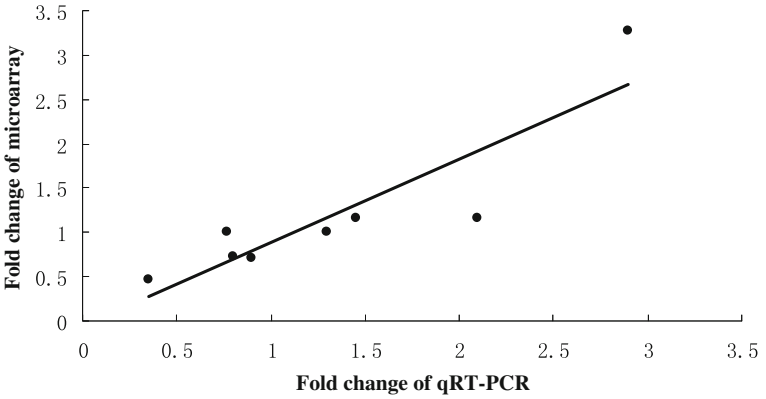

Fig. 1 Validation of gene expression by real-time quantitative PCR. qRT-PCR experiments were performed on eight target genes using the same RNA samples as were used in the microarray experiments. The qRT-PCR data showed good agreement with the microarray result, and there was a positive correlation (r=0.89, P<0.01) between the two methods

high-fold change, while Alas1 was downregulated in the H9 group (Table 2). This result is puzzling and need further study.

In addition to eNOS, activation of endothelial PPARγ can also increase NO bioavailability. Previous studies reported that activation of PPARγ in endothelial cells enhanced NO production, whereas the disruption of PPARγ caused a reduction in NO release (Kleinhenz et al. 2009). The upregulation of eNOS and PPARγ genes after the long-term intake of milk fermented by *L. helveticus* H9 may in turn result in an increase in the activity and bioavailability of NO to heart tissue, which may partially explain the antihypertensive effect of fermented milk. But in the report of Yamaguchi et al. (2009), the expression of PPARγ gene was decreased in the aorta of SHRs after repeated administration of Val-Pro-Pro and Ile-Pro-Pro; this contradiction is puzzling and need more verification.

Besides modulation of NO, the activation of PPARγ in another pathway can inhibit the proliferation of smooth muscle cells. Previous studies showed that the PPARγ agonist rosiglitazone activated PPARγ which induced the expression of Hmox1, which upregulated the expression of Cdkn1a, which in turn suppressed the proliferation of smooth muscle cells (Li et al. 2010), as Cdkn1a is a regulator of cell cycle progression. In our study, we found PPARγ, Hmox1, and Cdkn1a were all upregulated in the H9 group, indicating that the elements in fermented milk, such as bioactive peptides, could also activate this pathway and inhibit the proliferation of smooth muscle cells in the left ventricle. This might explain the observed relief of left ventricular hypertrophy in SHRs that had been fed on fermented milk.

In addition to NO, endothelin is another regulator of blood pressure. Endothelins can induce vasoconstriction and play a key role in the regulation of vascular tone (Kedzierski and Yanagisawa 2001). Previous studies suggested that endothelins were involved significantly in the regulation of hypertension (Hynynen and Khalil 2006). Upregulation of the endothelin system occurred in human hypertension and experimental animal models of severe hypertension (Schiffrin 2001). The expression of endothelin was often enhanced in intramyocardial coronary arteries in hypertensive rats (Schiffrin 1999). In our study, there was no significant difference in expression of

endothelin between the H9 group and the control group. However, the gene Ednra, which encodes for the protein ETa, was downregulated in the H9 group. ETa is a kind of endothelin receptor. Binding endothelins to ETa will result in vasoconstriction and an increase in blood pressure (Hynynen and Khalil 2006). Previous studies also reported that ETa antagonists could block the endothelin-induced effects and prevent cardiovascular diseases (Schiffrin 1998). It is known that certain bioactive peptides in fermented milk might act as ETa antagonists (Ihara et al. 1992). From the results of our study, it is logical to hypothesize that certain components, e.g., bioactive peptides, present in fermented milk, contribute to downregulation of the expression of ETa and consequently reduce the binding of endothelin to ETa.

As a multifactorial disease, hypertension can also be induced by blood clotting which obstructs the flow of blood through the circulatory system. In our study, the genes Plat and Thbd, which are associated with clot breakdown, were upregulated in the H9 group. Plat is a serine protease on endothelial cells, and it catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown. Because it works on the clotting system, Plat is used in some cases of diseases that feature blood clots. Thbd is an integral membrane protein expressed on the surface of endothelial cells, and it reduces blood coagulation by converting thrombin to an anticoagulant enzyme from a procoagulant enzyme. These two genes' upregulation may indicate that milk fermented by *L. helveticus* H9 can induce the breakdown of blood clots.

Many studies reported the bioactive peptides Val-Pro-Pro and Ile-Pro-Pro in fermented milk were responsible for the antihypertensive effect, and the gene expression profile in the aorta of SHRs was detected after repeated administration of Val-Pro-Pro and Ile-Pro-Pro (Yamaguchi et al. 2009), but we paid more attention on blood pressure and less on the left ventricular hypertrophy. Here, we have shown that some genes associated with hypertension are regulated by provision of milk fermented by *L. helveticus* H9 and that this may indeed be responsible for the observed antihypertensive effect and relief from left ventricular hypertrophy. In accordance with previous study (Yamaguchi et al. 2009), we also found gene eNOS were upregulated in left ventricle. Furthermore, gene Hmox1 and Cdkn1a were also upregulated in SHRs; this may be responsible for the relief from left ventricular hypertrophy. However, our method does not provide more information on the detailed mechanism driving these effects; which components of the fermented milk induce the change in gene expression? Further studies are now required to determine whether it is bioactive peptides or nonpeptidic compounds that exert the effect and how changes are induced at the transcriptional level.

5 Conclusion

Milk fermented by *L. helveticus* H9 has the ability to reduce blood pressure and relieve left ventricular hypertrophy. DNA microarray analysis showed that some hypertension-associated genes were differentially expressed. In-depth analysis showed these differentially expressed genes were associated with NO synthesis, cell proliferation, endothelin binding, and clot breakdown. The findings of this study provide new insights into the mechanism behind the ability of milk fermented with *L. helveticus*

H9 to deliver antihypertensive effects, although the full details are still not entirely clear.

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Conflict of interest Yongfu Chen, Zhi Zhong, Jiangang Xue, Jie Yang, Qing Zhang, Jia Yue, and Heping Zhang declare that they have no conflicts of interest.

Statement of animal rights All applicable institutional and/or national guidelines for the care and use of laboratory animals were followed.

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