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Methylation of adrenergic β1 receptor is a potential epigenetic mechanism controlling antihypertensive response to metoprolol

Qixia Jiang[1,2], Hong Yuan[1,*], Xiaowei Xing [1], Jingjing Liu[1], Zhijun Huang[1] and Xia Du[1]

[1]Center of Clinical Pharmacology, Third Xiangya Hospital, Central South University, Changsha 410013, China

[2]Department of Cardiology, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai 200003, China

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Although metoprolol is used to treat hypertension, clinical responses are variable and unpredictable. Evidence suggests that adrenergic β1 receptor (*ADRB1*, designated *Adrb1* in rodents) gene polymorphisms influence the level of blood pressure response to this drug therapy, but their presence can not predict the response of the individual patient. The question exists whether epigenetic modifications, such as DNA methylation could cause changes in the gene's expression that are a determining factor in metoprolol's efficacy. The aim of this study was to verify whether DNA methylation could change the expression of the *ADRB1* gene, and epigenetic modification could explain why individuals with identical *ADRB1* gene polymorphisms have different antihypertensive responses to metoprolol. H9c2 rat myocardial cells *in vitro* were randomly divided into 5-aza-2'-deoxycytidine (decitabine)-treated (0.5 to 10.0 μM) and control groups. For the *in vivo* experiments, 45 spontaneously hypertensive rats (SHRs) were divided into metoprolol-treated and control groups, and after a 4-week intervention myocardia were harvested. Genomic methylation-sensitive PCR was used to assess the methylation status of the *Adrb1* promoter after DNA extraction from H9c2 cells and SHR myocardia. Real-time fluorescent quantitative RT-PCR was used to determine levels of *Adrb1* mRNA. In H9c2 cells, the least degree of methylation was observed in the 5.0 μM decitabine treated group. Prolonged exposure of cells to 5.0 μM decitabine resulted in downregulating methylation of the *Adrb1* promoter. Increased levels of *Adrb1* mRNA of the 5.0 μM group demonstrated that this concentration resulted in the highest expression. Accordingly, DNA methylation resulted in the downregulation of *Adrb1* transcription. *In vivo*, the lower level of methylation of the *Adrb1* promoter from SHR myocardial samples demonstrated a better antihypertensive effect by metoprolol. The expression of *Adrb1* mRNA in the effective group of SHRs was significantly upregulated. In conclusion, as shown in both H9c2 cells and SHRs, downregulated methylation of the *Adrb1* promoter is likely to improve the antihypertensive efficacy of metoprolol.

Keywords: Hypertension, Metoprolol, β1-Adrenergic receptor, DNA methylation, Gene

Hypertension is one of the most common cardiovascular diseases in the world with a prevalence of 27% worldwide[1]. It is also a major risk factor for stroke and end-stage renal disease. According to the 2002 China National Nutrition and Health Survey, there are 160 million patients with hypertension, but only 6.16% of the population have their blood pressure adequately controlled[2]. One reason for this low disease control rate is the variable and unpredictable response of individuals to antihypertensive medication. Clinicians sometimes need to consider the individual hypertensive patient's response in order to find an appropriate drug treatment. Pharmacogenetic and pharmacogenomic approaches seek to find genetic predictors of drug response but the discovery of clinically relevant genes that influence responses to antihypertensive drugs remains elusive. To date, more than 40 studies have been conducted to investigate associations between genetic polymorphisms and the response to antihypertensive drugs[3,4].

The adrenergic β1 receptor (ADRB1) mediates many of the effects of endogenous catecholamines that regulate key cardiovascular dynamics[5-8]. It has been shown recently that the *ADRB1* gene has two common functional polymorphisms (Ser49Gly and Arg389Gly), which affect the blood pressure response

*Author for correspondence
Address: No.138 Tongzipo Road, Changsha 410013, PR China.
Tel/Fax: +86-731-88618319
E-mail: yuanhong01@vip.sina.com; dr.liuq@yahoo.com.cn

Abbreviations: ADRB1 or *Adrb1*, adrenergic β1 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gs, stimulus G protein; MSP, methylation-sensitive PCR; decitabine, 5-aza-2'-deoxycytidine; SHR, spontaneously hypertensive rats.

to metoprolol monotherapy in a Chinese population with hypertension[9]. Enhancing the coupling of stimulus G protein (Gs) with downstream signal transduction, a 49 Ser → Gly mutation is considered to have no significant correlation with the incidence and treatment of hypertension[10,11]. On the other hand, the mutation Arg389 to Gly389 can directly affect the antihypertensive effect of metoprolol. During treatment with metoprolol, the blood pressure of hypertensive patients with Arg389Arg declines almost 3-times more than those carrying the heterozygous Gly389Arg. This illustrates that *ADRB1* gene polymorphisms can affect the blood pressure response to metoprolol[12,13]. But while *ADRB1* gene polymorphisms are the main cause for differences in individual drug reaction, the same genotype does not result in the same antihypertensive effect in all patients: those with genotypes sensitive to metoprolol do not always achieve the expected antihypertensive effect.

In preliminary studies[14], we selected 43 hypertensive patients with the same CYP2D6 and *ADRB1* genotypes, who took metoprolol for 8 weeks (50 mg, twice a day). We found that the systolic and diastolic blood pressure of only 52% and 72% of patients respectively had effectively decreased. In another experiment, treatment of spontaneously hypertensive rats (SHRs) with identical genotypes also demonstrated an inconsistent metoprolol-antihypertensive effect[15].

Epigenetic modifications including DNA methylation do not change coding sequences of genes, but do influence gene expression. DNA hypermethylation is a hallmark of gene silencing, while DNA hypomethylation promotes active transcription. Aberrant DNA methylation has been linked to a number of age-related disorders including cardiovascular diseases, especially the development of atherosclerosis and hypertension[16] and cancer and autoimmune disorders. The hypothesis that DNA methylation regulates *ADRB1* expression is supported by the findings of Yuan and colleagues[17,18]. In these studies, it has been shown that 5-aza-2'-deoxycytidine (decitabine), a typical DNA methyltransferase inhibitor activates the methylated *Adrb1* promoter in rat H9c2 myocardial cells and upregulates the expression of *Adrb1*.

In this study, we investigated the mechanism by which DNA methylation can be associated with the expression of the *ADRB1* gene (designated *Adrb1* in rodents) as well as the variant antihypertensive effects of metoprolol. The correlation of effect with individual epigenetic modification could explain why individuals with the identical *ADRB1* gene polymorphism still have different antihypertensive responses. DNA methylation might be the epigenetic molecular mechanism by which the antihypertensive efficacy of metoprolol could be ensured, potentially leading to *ADRB1* gene-directed therapy in the future.

Materials and Methods

Cell culture and treatment

H9c2 rat heart myocytes were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% fetal bovine serum to 70% confluency, and cultured under either normoxic conditions (5% CO_2, 21% O_2, 74% N_2) in a humidified Napco incubator at 37°C or hypoxic conditions (5% CO_2, 1% O_2, 94% N_2) in an Espec triple gas incubator (Tabai-Espec, Osaka, Japan). Cells grown under either of these oxic conditions at a density of 10^6/mL were then randomly divided into five treatment groups comprising 0.0 (DMSO control), 0.5, 2.0, 5.0, or 10.0 μM decitabine (Sigma). All cells were treated for 72 h.

Animals and treatment with metoprolol

All animal studies were approved by the Animal Ethics Review Board of Central South University (No. 0601). The investigation also conformed to the Guide for the Care and Use of Laboratory Animals published by the U. S. National Institutes of Health. Forty-five male spontaneously hypertensive rats (SHRs) were randomly divided into two groups: 26 SHRs were treated with metoprolol, and 19 with normal saline (control). The rats' tail artery blood pressure was measured, and every week the dose of metoprolol was adjusted according to body weight (see below). Metoprolol tablets were ground and dissolved and the concentration of the suspension adjusted to 2.5 mg/mL in normal saline before oral gavage. Rats in the treatment group were given 10 mL of the metoprolol suspension (i.e., 25 mg metoprolol) per kg body weight, while rats in the control group received 10 mL/kg normal saline, each by oral gavage twice a day at 8-9 AM and 4-5 PM for 4 weeks. After this 4-week intervention, myocardia and blood via the carotid artery were harvested and stored at -80°C until

further analysis. Three litters of rats were studied in each set of analyses.

Evaluation of therapeutic efficacy of metoprolol

According to the Guidelines For New Drug Research Before Clinical Trials (China), an effective antihypertensive treatment either causes a decrease in blood pressure of more than 20 mm Hg or brings blood pressure to near normal[19]; treatment otherwise is regarded as ineffective. In this experiment, assessment of the hypotensive effect of metoprolol was based on the difference between the systolic blood pressure initially (baseline) and after 4 weeks of treatment. Baseline systolic blood pressures of the prospective metoprolol (n = 26) and saline (n = 10) SHR groups were 183.1 ± 9.0 and 183.0 ± 8.3 mm Hg, respectively. After 4 weeks of treatment, systolic blood pressure in the metoprolol (n = 26) and saline (n = 10) treated groups was 163.0 ± 7.0 and 190.8 ± 8.5 mm Hg, respectively. Effective decline of blood pressure in response to metoprolol was observed in 15 of 26 animals used.

DNA isolation and bisulfite modification

Total DNA was extracted from H9c2 cells and SHR myocardial tissue (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis, MN, USA). DNA (1 μg) in a volume of 50 μL was denatured by NaOH (final concentration, 200 mM) for 10 min at 37°C. For samples of nanogram quantities of human DNA, 1 μg of salmon sperm DNA (Sigma) was added as a carrier before modification. Both 30 μL of 10 mM hydroquinone (Sigma) and 550 μL of 3 M sodium bisulfite (Sigma) at pH 5, freshly prepared, were added to the denatured DNA. Samples were mixed and incubated under mineral oil at 50°C for 16 h. Modified DNA was purified by using Wizard DNA purification resin (Promega) according to the manufacturer's instructions and eluted into 60 μL of water. Modification was completed by treatment with NaOH (final concentration, 300 mM) for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at -20°C.

Sequencing of Adrb1 gene promotor and DNA bisulfite modification

The DNA products were sequenced in both directions using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit in a ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The primer sequence used for the *Adrb1* promotor was: forward, 5'-GGTTTTTGGGGTGTTTTTTAG-3' and rear 5'-CAACAATCCCATAACCAAATC-3'. The sequence used for the DNA bisulfite modification primer was: forward, 5'-GTTTCGTTGTTGTT TTTAGTTAG-3' and rear 5'-CCAAAAATAAA CCCATACC-3'. Performance of this analysis was conducted by the Shanghai National Engineering Center. Sequences were edited, analyzed and compared using Vector NTI software. All of the sequences obtained were compared to the rat sequences available on PubMed.gov (http://www.ncbi.nlm.nih.gov/pubmed/).

Design of primers for methylation PCRs

MethPrimer based on Primer3 is a program for designing PCR primers for methylation mapping. It first takes a DNA sequence as its input and searches the sequence for potential CpG islands. Primers are then picked around the predicted CpG islands or around regions specified by users. In this study, two pairs of primers were needed, one of them specific for modified and methylated DNA (the M pair), and the other for modified and unmethylated DNA (the U pair). To visually display the results for CpG island prediction and primer selection, the Perl GD module (http://stein.cshl.org/WWW/software/GD) was used to generate a portable network graphic (PNG) image for each input sequence. An image map was also generated and embedded into HTML code using a Perl script for each image to display text explanations as tool tips for each element in the image. Results of primer selection were delivered through a web browser in text and in graphic view (Table 1).

PCR amplification

Primer pairs (Table 1) were purchased from Life Technologies. The PCR mixture contained l0× PCR buffer (each at 2.5 μL), dNTPs (2.5 mM each), primers (300 ng each, per reaction) and bisulfite-modified DNA (≈50 ng) or unmodified DNA (50-100 ng) in a

Table 1—Primer results for methylation-specific PCR (MSP)

Primers		Primer sequence	Size (bp)
Methylation primer	Upper primer	5'-TTTCGAATTTTGTAAT TTGTCGTC-3'	200
	Lower primer	5'-AAAAATAAACCCATA CCCGC-3'	
Unmethylation primer	Upper primer	5'-TTTTGAATTTTGTAAT TTGTTGTTG-3'	200
	Lower primer	5'-CCAAAAATAAACCCA TACCCAC-3'	

final volume of 50 μL. PCR specific for unmodified DNA also included 5% dimethyl sulfoxide (DMSO). Reactions were hot-started at 95°C for 5 min before the addition of 1.25 units of Taq polymerase (Gibco-BRL). Amplification was carried out in a Hybaid OmniGene temperature cycler for 40 cycles (5 min at 95°C, 30 s at 94°C, and 30 s at 72°C), followed by a final 10 min extension at 72°C. Controls without DNA were performed for each set of PCRs. Each PCR product (10 μL) was directly loaded on to nondenaturing 6-8% polyacrylamide gels, stained with ethidium bromide and directly visualized under UV illumination.

RNA isolation and quantitation

Total RNA was extracted from H9c2 cells and SHR myocardial tissue (Puregene RNA Isolation Kit, Gentra Systems, Minneapolis, MN, USA), quantitated, and reverse-transcribed. The reverse transcription (RT) reaction was performed in 20 μL with SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) using random hexamer oligonucleotides (Amersham Pharmacia, Piscataway, NJ). Real-time PCR was performed using 5 μL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 3 μL of 20 pM-specific primers, 1 μL cDNA, and water to a final volume of 10 μL.

Quantitative real-time RT-PCR (LightCycler 2.0, Roche) was performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the house-keeping gene was 5'-TGCTACAACGACCCCAAGTG-3' and 5'-AGAAGGAGACGACGGACGAG-3'. The amplification program consisted of initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95°C for 5 s, annealing at 55°C for 20 s and extension at 72°C for 30 s. After amplification, extension was repeated for 30 s. The threshold cycle (Ct) value for each gene of interest was measured for each RT sample. The Ct value for GAPDH was used as an endogenous reference for normalization. Real-time RT-PCR assays were done in duplicate or triplicate, with each set of assays repeated 2-4 times.

Statistical analysis

Biomedical variables obtained from this study were expressed as mean ± standard error (SE) or median value ± quartile range (M ± QR). Data were analyzed using SPSS® 11.0 software (SPSS, Chicago, IL, USA). Data comparisons were made using Student's *t*-test or the chi-square (χ^2) test. *P*-values <0.05 for both Student's *t*-test and the χ^2 test were considered statistically significant.

Results

Decitabine activates Adrb1 expression in H9c2 myocardial cells

The *Adrb1* promoter was expressed in H9c2 myocardial cells. Cells were treated with 0.0 (control), 0.5, 2.0, 5.0 and 10.0 μM concentrations of decitabine. Methylation-specific PCR was performed to detect the methylation status of the *Adrb1* promoter in the cells. Electrophoresis was used to resolve both the methylated (Fig. 1) and unmethylated (Fig. 2) methylation-sensitive PCR products of the *Adrb1* promoter in all decitabine-treated and control groups. It was found that the *Adrb1* promoter in cells of all concentration groups was partly methylated. However, the least degree of methylation was observed in the 5.0 μM group, as demonstrated by its being the darkest band in the test for methylated primers (Fig. 1) and the lightest for unmethylated (Fig. 2). Prolonged exposure of the cells to the 5.0 μM concentration of decitabine caused the downregulation of methylation of the *Adrb1* promoter. We also explored the possibility whether the downregulated methylation status of the *Adrb1*

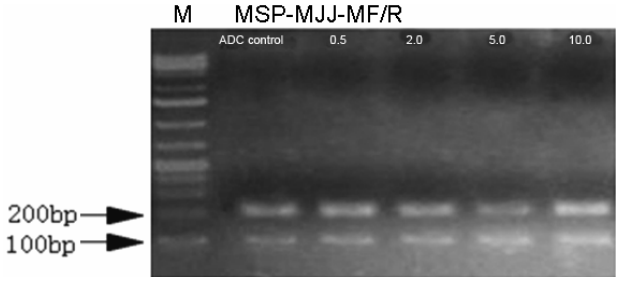

Fig. 1—Electrophoresis results of the methylated products of methylation-sensitive PCR (MSP) for the *Adrb1* promoter of the control and decitabine groups in H9c2 myocardial cells ["ADC Control" rightward to "10.0" represents control treated with DMSO and decitabine treatment at 0.5 μM, 2.0 μM, 5.0 μM and 10.0 μM, respectively. M: molecular marker/DNA fragments separated by size]

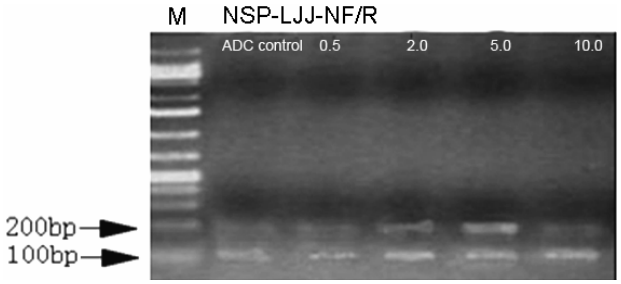

Fig. 2—Electrophoresis results for the unmethylated products of MSP of the *Adrb1* promoter of the control and decitabine groups in H9c2 myocardial cells [See the legend of Fig. 1 for abbreviation and drug treatment]

promoter contributed to reactivation of gene expression. The expression of *Adrb1* was tested by fluorescent quantitative RT-PCR. GAPDH was used as the internal control and the differential expression of samples and GAPDH was observed by the $2^{-\Delta\ \Delta Ct}$ method. The expression of the *Adrb1* mRNA of the 5.0 μM group exceeded that of the other concentration groups (Fig. 3).

DNA bisulfite modification sequences validate the methylated modification position of the Adrb1 promoter

Comparison of the *Adrb1* promotor sequence before and after the bisulfite treatment showed that the cytosine of no-CG in the original sequence changed to thymine because of the none-methylation modification. The cytosine of CG did not change. This result verified that methylation occured in the *Adrb1* promotor (Fig. 4).

Lower methylation activates the responses of metoprolol in SHR

We chose the SHR developed by Okamato[20] as the research model because SHRs carry the same adrenergic β1 receptor gene polymorphisms as humans. Of 26 SHRs given metoprolol, 15 responded with a lowering of blood pressure with a drug effective rate of 57.7%. Therefore, metoprolol treatment was shown to be capable of producing an antihypertensive response. Bands of methylated primers from SHR myocardial samples were amplified by PCR at 200 bp for rats in both the effective and ineffective groups and bands of unmethylated primers from those of the effective group only. Accordingly, it was found that the *Adrb1* promoter isolated from myocardia in the ineffective group were partly unmethylated. Most importantly, the least degree of methylation of the *Adrb1* promoter from SHR myocardia demonstrated a better antihypertensive effect by the drug metoprolol (Fig. 5).

We explored the possibility whether the downregulated methylation status of the *Adrb1* promoter contributed to reactivation of gene expression. The expression of *Adrb1* was tested by fluorescent quantitative RT-PCR. GAPDH was used as the internal control and the differential expression of samples and GAPDH was observed by the $2^{-\Delta\Delta\ Ct}$ method. The expression of the *Adrb1* mRNA in the effective group was significantly upregulated (P<0.05) (Fig. 6).

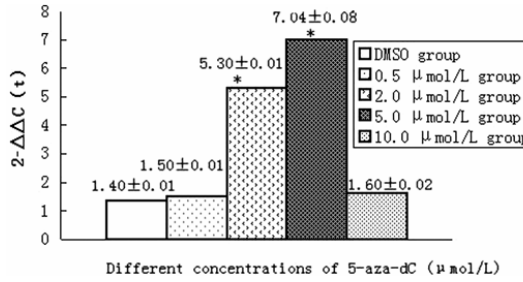

Fig. 3—Expression of *Adrb1* mRNA in H9c2 myocardial cells [There was a significant difference between control (DMSO) and decitabine-treated groups at concentrations of 2.0 and 5.0 μM; P<0.05]

Fig. 4—Analysis of DNA sequencing results of the *Adrb1* promoter in H9c2 cells treated by sodium sulfite [Lane 1: the sequence before the sodium sulfite treatment; Lane 2: the sequence after the sodium sulfite treatment. The red C represents cytosine of no-CG, which was changed to thymine after the bisulfite treatment, because of the none-methylation modification. The underlined CG represents cytosine of the none-methylation modification, which kept cytosine after the bisulfite treatment]

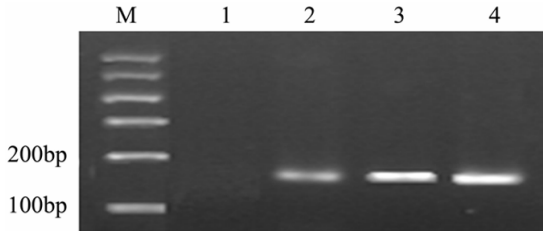

Fig. 5—Electrophoresis results of the unmethylated and methylated products of MSP of the *Adrb1* promoter of SHR myocardial samples in both the effective and ineffective groups [Lane 1: unmethylated product of SHR myocardial samples in the ineffective group; 2: methylated product of SHR myocardial samples in the ineffective group; 3: unmethylated product of SHR myocardial samples in the effective group; and 4: methylated product of SHR myocardial samples in the effective group; M: molecular marker/DNA fragments separated by size]

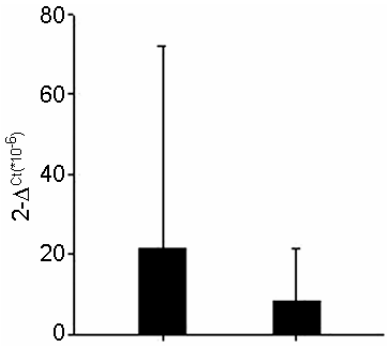

Fig. 6—Expression of *Adrb1* mRNA of the effective (left column) and ineffective group (right) in SHR myocardial samples [There was a significant difference between the two groups; P<0.05]

Discussion

The epigenetic expression of a gene, although resulting in a stably inherited phenotype, is due to chromosomal changes that do not involve alterations in its DNA sequence. DNA methylation, the post-synthetic methylation of cytosine bases at position 5 of the pyrimidine ring by a DNA methyltransferase represents one of the most important kinds of epigenetic modifications. Methylated cytosines are almost exclusively found in CpG pairs; approximately 70% of CpG dinucleotides in human DNA are constitutively methylated, whereas most of the unmethylated CpGs are located in CpG islands. A considerable number of methylated CpGs exist in transposable elements and potentially active transposable elements when methylated cannot initiate transcription[21]. CpG islands are CG-rich sequences located near coding sequences and serve as promoters for their associated genes. Approximately half of mammalian genes have CpG islands[22]. DNA methylation suppresses gene transcription by binding methyl-CpG-binding proteins, such as MECP2 and MBD2. It may also interfere with the binding of some transcription factors[23].

DNA methylation helps stabilize chromatin and hypomethylation can lead to genomic instability by predisposing chromatin to strand breakage and recombination within derepressed repetitive sequences. Promoter CpG hypomethylation may result in gene activation, also contributing to aberrant gene expression. In contrast to hypomethylation, aberrant hypermethylation of CpG islands can cause inappropriate gene silencing and disease states[24].

Research progress in DNA methylation has enlightened its role in hypertension[25]. Recent studies have demonstrated that aberrant modification of DNA methylation can influence the expression of the *HSD11B2* and *ADRB1* genes, resulting in blood pressure elevation[26-28]. Therefore, aberrant modification of DNA methylation might be an important hereditary factor in the pathogenesis of hypertension.

We propose that while gene-directed individualized therapy for hypertension currently emphasizes gene polymorphisms of receptors and metabolic enzymes, it ignores other possibilities such as epigenetic mechanisms. From our study results, it was obvious that expression of the rat *Adrb1* mRNA was significantly different between the metoprolol-effective and ineffective groups. We, therefore, conclude that DNA methylation may regulate the expression of *Adrb1*, which then influences the antihypertensive effect of metoprolol. If this hypothesis is true, significant individual differences in humans in the status of DNA methylation of *ADRB1* could explain why different people with the same *ADRB1* gene polymorphism have different antihypertensive responses.

In our study, the results of *in vitro* experiments using H9c2 rat heart myocytes showed that the *Adrb1* promoter had many methylated positions. The downregulated methylation of the promoter resulted in the upregulated expression of *Adrb1* mRNA. According to the results of the animal model experiment, SHRs who had a better antihypertensive response to metoprolol also showed a lower level of DNA methylation modification and a higher expression of *Adrb1* in their myocardial tissues. The ineffective group had a higher level of DNA methylation modification and lower expression of *Adrb1* in their myocardial tissues. Between the two groups, there were significant differences.

In this study, we used methylation-sensitive PCR, which is the most widely used assay for the detection of hypermethylation in CpG islands[29]. Although methylation-sensitive PCR is useful at multiple CpGs for methylation, it is not adapted to the detection of more subtle variations. It is also not the only available method for the analysis of methylation of a single specific cytosine either within or outside a CpG island[30]. Newer bisulfite sequencing and cassette methylation techniques greatly augment the utility of the older decitabine treatment and DNaseI hypersensitivity analyses[31]. We plan to incorporate these newer techniques into our future studies.

Altogether, our results demonstrate the possibility that in humans DNA methylation regulates the transcription of *ADRB1*. DNA methylation could be the epigenetic molecular mechanism that directs the antihypertensive efficacy of metoprolol. If so, this discovery could lead to *ADRB1* gene-directed therapy. Furthermore, it would be important to investigate whether epigenetic changes of *ADRB1* also take place in the blood vessels.

Acknowledgements

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