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Hydralazine-induced promoter demethylation enhances sarcoplasmic reticulum Ca^{2+}-ATPase and calcium homeostasis in cardiac myocytes

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Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) plays an essential role in Ca^{2+} homeostasis and cardiac functions. The promoter region of SERCA2a has a high content of CpG islands; thus, epigenetic modification by inhibiting methylation can enhance SERCA2a expression in cardiomyocytes. Hydralazine, a drug frequently used in heart failure, is a potential DNA methylation inhibitor. We evaluated whether hydralazine can modulate Ca^{2+} handling through an increase in SERCA2a expression via regulating methylation. We used indo-1 fluorescence, real-time RT-PCR, immunoblotting, and methylation-specific PCR to investigate intracellular Ca^{2+}, the expressions of RNA and protein, and methylation of SERCA2a in HL-1 cardiomyocytes with and without (control) the administration of hydralazine (1, 10, and 30 μM) for 72 h. Hydralazine (10 and 30 μM) increased the intracellular Ca^{2+} transients and SR Ca^{2+} contents. Hydralazine (10 and 30 μM) decreased methylation in the SERCA2a promoter region and increased the RNA and protein expressions of SERCA2a. Additionally, hydralazine (10 and 30 μM) decreased the expression of DNA methyltransferase 1. Moreover, treatment with hydralazine in isoproterenol-induced heart failure rats decreased the promoter methylation of SERCA2a and increased SERCA2a RNA expression. In conclusion, hydralazine-induced promoter demethylation may improve cardiac function through increasing SERCA2a and modulating calcium homeostasis in cardiomyocytes.
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Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) plays an essential role in Ca^{2+} homeostasis and regulates cardiac functions. SERCA2a is critical in uptaking $[Ca^{2+}]_i$ in cardiomyocytes to maintain SR Ca^{2+} contents and excitation–contraction coupling. A reduction in the expression of SERCA2a was widely documented in both systolic and diastolic heart failure.[1–3] In contrast, overexpression of SERCA2a was shown to improve cardiac function and attenuate the occurrence of ventricular tachyarrhythmias.[4–6] Those findings indicate that enhanced SERCA2a expression is a potential method for treating cardiac dysfunction and arrhythmias. However, the mechanisms underlying the regulation of SERCA2a are not fully elucidated. Additionally, effective pharmacological treatments to enhance SERCA2a expression are not available. Recently, epigenetic modifications through inhibiting methylation were found to enhance SERCA2a expression and calcium dynamics in cardiomyocytes.[7,8] The proximal promoter region of SERCA2a contains CpG islands, which suggests that demethylation of these sites can reduce transcriptional repression. Therefore, promoter methylation plays an important role in SERCA2a regulation, and demethylation may be a novel treatment for cardiac dysfunction.

Hydralazine, an antihypertensive medication was shown to be effective in treating heart failure, especially in combination with nitrate.[9,10] However, the beneficial mechanisms of hydralazine on heart failure are not fully elucidated. The combined use of nitrate and hydralazine can improve

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hemodynamics through reducing the cardiac afterload and preload caused by venous and arterial vasodilation in patients with heart failure.[9,10] Studies also showed that combined hydralazine and nitrate therapy can decrease nitrate tolerance by modulating nitric oxide bioavailability through its antioxidant ability.[11,12] However, it is not clear whether hydralazine has direct effects on cardiomyocytes.

Hydralazine possesses a DNA-demethylating property through inhibiting mitogen-activated protein kinase , thereby decreasing DNA methyltransferase (DNMT)1 levels and activity.[13–16] As demethylation is a potential strategy for treating heart failure, hydralazine may directly enhance cardiac SERCA2a and improve cardiac function through its inhibitory effects on methylation. The purpose of this study was to investigate the effects and mechanisms of hydralazine on methylation and SERCA2a expression in HL-1 cells and heart failure *in vivo*.

MATERIALS AND METHODS

Cell Culture

HL-1 cells derived from mouse atrial cardiac muscle cells[17] (kindly provided by Dr. Claycomb, Louisiana State University Medical Center, New Orleans, LA, USA) were cultured in a humidified atmosphere of 5% CO_2 at 37 °C in Claycomb medium (JRH Biosciences, Lenexa, KS, USA). Cells treated with hydralazine or vehicle for 72 h were harvested for further study.

Animals and Experimental Design

Heart failure was induced by a subcutaneous injection of 100 mg isoproterenol/kg (Sigma, St. Louis, MO, USA) in male Wistar rats (weighing 240 ~ 270 g) as described previously.[18] One week after isoproterenol administration, hydralazine (10 mg/kg) or the vehicle was administered intraperitoneally (i.p.) once daily for 7 days. Then, the rats were anesthetized with an i.p. injection of sodium pentobarbital (40 mg/kg, Sigma). A midline thoracotomy was then performed, and the heart was removed from each rat for further analysis.

Methylation Analysis

The effect of hydralazine on the methylation status of the SERCA2a gene promoter was investigated by methylation-specific PCR (MSP) and direct sequencing after bisulfite conversion. Briefly, genomic DNA (1 μg) isolated from HL-1 cells or left ventricle of Wistar rats was modified with sodium bisulfite that converts only unmethylated cytosines to uracil according to the procedures of the EZ DNA Methylation-Gold™ Kit (Zymo, Orange, CA, USA). The modified DNA was amplified by a set of primers (U and M) for MSP. One primer (U) anneals unmethylated DNA, while the second primer (M) anneals methylated DNA that has undergone chemical modification. The PCR products were separated in 1.8% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination. Bisulfate modified DNA was amplified and ligated into pGEM-T vector (Promega, Madison, WI, USA) for direct sequencing.

RNA Isolation and Real-Time Reverse Transcription (RT)-PCR

Total RNA isolated from HL-1 cells and rat hearts was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Messenger RNA (mRNA) expression of SERCA2a was analyzed by a quantitative PCR with the ABI PRISM7300 system (Applied Biosystems, Foster City, CA, USA) using SYBER Green (Applied Biosystems). Relative changes in transcript levels of SERCA2a were estimated from the threshold cycle (Ct) value and normalized to the respective Ct value of GAPDH determined in corresponding samples and subsequently to control cells.

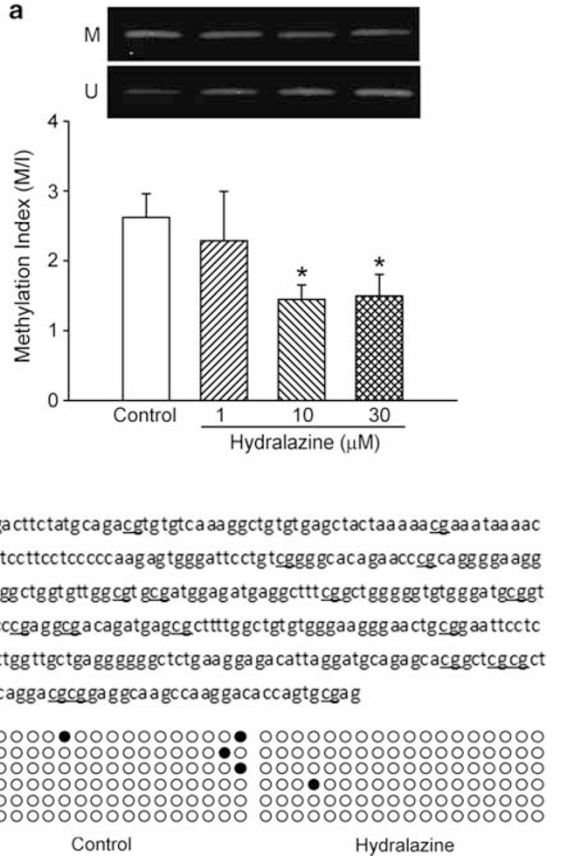

Figure 1 Effects of hydralazine on SERCA2a methylation. (**a**) Upper panel represents agarose gels of methylation-specific PCR products for promoter methylation of SERCA2a from control and hydralazine-treated HL-1 cells. Lower panel shows the methylation index obtained from the densitometric intensity of methylated bands divided by unmethylated bands in control and hydralazine-treated cells ($n = 5$). * $P < 0.05$ *vs* control cells. (**b**) Direct bisulfate sequencing in control and hydralazine-treated cells. Upper panel shows CpG island of SERCA 2 promoter positioned from −816 to −479 bp (18 CG sites) relative to transcriptional start site. Lower panel shows bisulfite sequencing of CpG island in the SERCA2 promoter from control and hydralazine (30 μM)-treated HL-1 cells. Six clones were picked and sequenced, respectively. Symbols: ○, ummethylated cytosines; ●, methylated cytosines.

Immunoblot Analysis

Equal amounts of total proteins from HL-1 cells were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis. Blots were probed with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against DNMT1 and SERCA2a and secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were detected with an enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA) and analyzed by AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). Targeted bands were normalized to cardiac α-sarcomeric actin (Sigma) to confirm equal protein loading.

SERCA2a Activity

The ATPase activity of the cardiac SR Ca^{2+} pump was measured using an enzyme-coupled assay.[19] SR vesicles were prepared from HL-1 cells as described by Münch *et al.*[20] SR protein (25 μg) was incubated in 250 μl buffer containing 21 mM MOPS, 100 mM KCl, 3 mM $MgCl_2$, 0.06 mM EGTA, 4.9 mM NaN_3, 1 mM glycerophosphate, 1 mM phosphoenopyruvate, 0.1 mM NADH, 8.4 units pyruvate kinase, and 12 units lactate dehydrogenase. The reactions received 1 mM of ATP at 30 °C (basal activity). SERCA2a activity was measured as the change in absorption at 340 nm divided by the extinction coefficient of NADH. The consumption of NADH is considered to be equivalent to the hydrolysis of ATP by SERCA2a with or without Ca^{2+}.

Measurement of Intracellular Calcium ($[Ca^{2+}]_i$)

Spontaneous $[Ca^{2+}]_i$ transients in HL-1 cells were monitored using the fluorometric ratio technique (indo-1 fluorescence) as described previously.[21] In brief, cells were loaded for 30 min with 10 μM of indo-1/AM (Sigma) followed by perfusion of cells with Tyrode's solution at 35 ± 1 °C for at least 20 min to allow intracellular deesterification of indo-1. Cells were excited with UV light at 360 nm, and the ratio of the emitted fluorescence values at 410 and 485 nm ($R_{410/485}$) was used as an index of $[Ca^{2+}]_i$. The SR Ca^{2+} content was estimated by rapidly adding 20 mM of caffeine, and the total SR Ca^{2+} content was measured from the peak amplitude of caffeine-induced $[Ca^{2+}]_i$ transients.

Statistical Analysis

All quantitative data are expressed as the mean ± s.e.m. One-way repeated analysis of variance (ANOVA) with *post-hoc* Fisher's least significant difference was used to compare the effect of different drug concentrations. A P value of <0.05 was considered statistically significant.

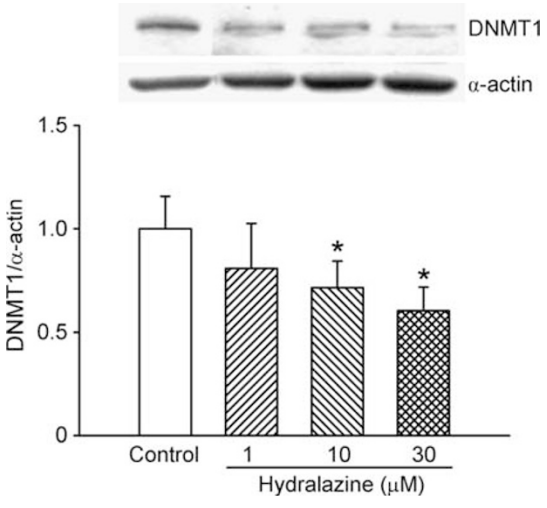

Figure 2 Effects of hydralazine on DNMT1 protein expression determined by immunoblot analysis. An example and average data ($n = 10$) of hydralazine's effect on DNMT1 protein expression determined by an immunoblot analysis. The expression of DNMT1 was normalized to α-sarcomeric actin as the internal control. *$P < 0.05$ *vs* control cells.

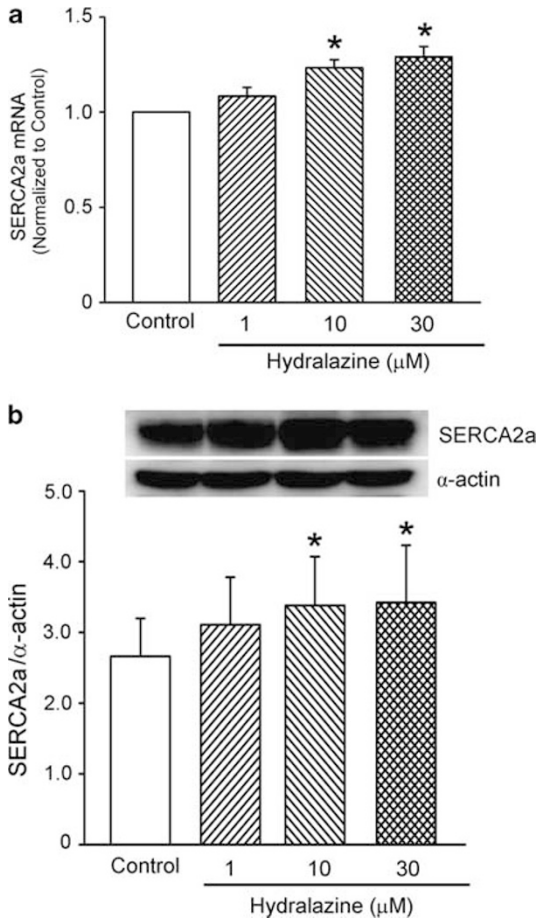

Figure 3 Effect of hydralazine on SERCA2a levels in HL-1 cells. (**a**) Expression of SERCA2a increased with hydralazine treatment ($n = 8$) as determined by a quantitative real-time PCR. The expression of SERCA2a was normalized to GAPDH as an internal control, and then normalized to the value of control cells. (**b**) SERCA2a protein level increased with hydralazine treatment ($n = 6$) as determined by an immunoblot analysis. The expression of SERCA2a was normalized to α-sarcomeric actin as the internal control. *$P < 0.05$ *vs* control cells.

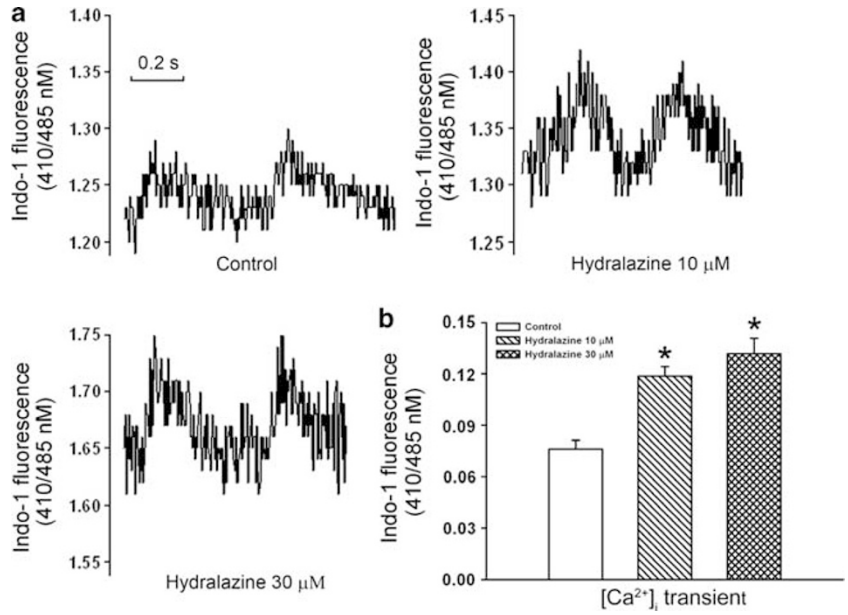

Figure 4 Effects of hydralazine on $[Ca^{2+}]_i$ transients in HL-1 cells. (**a**, **b**) Tracings and average data of $[Ca^{2+}]_i$ transients in control cells ($n = 12$) and different concentrations of hydralazine-treated cells ($n = 12$ for each concentration). $^*P < 0.05$ *vs* the control.

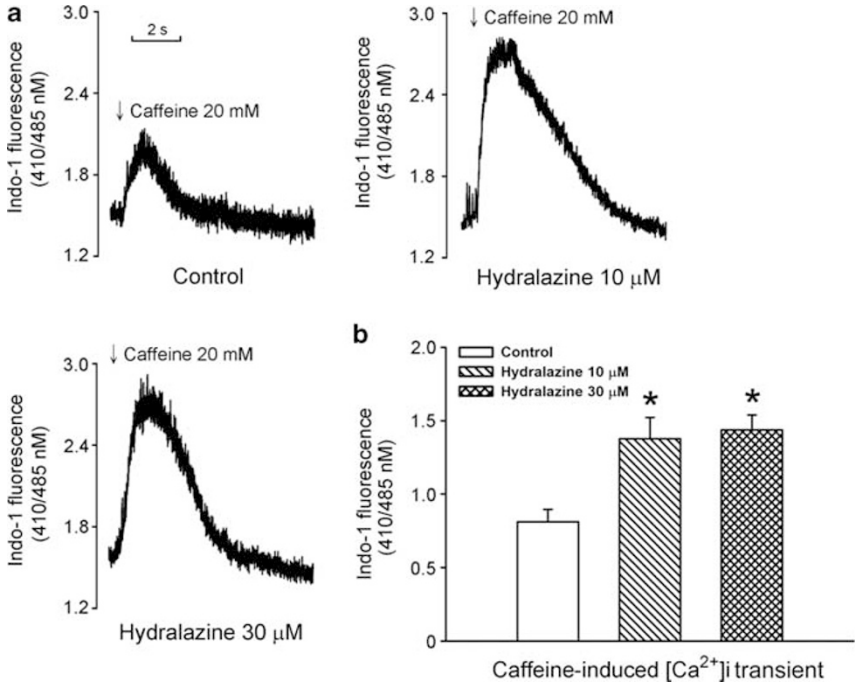

Figure 5 Effects of hydralazine on caffeine-induced Ca^{2+} transients. (**a**, **b**) Tracings and average data of SR Ca^{2+} contents from control cells and different concentrations of hydralazine-treated cells ($n = 10$ for each concentration). $^*P < 0.05$ *vs* the control.

RESULTS

Hydralazine Decreases Methylation of the SERCA2 Promoter

Figure 1 shows the methylation status of the SERCA2 promoter in HL-1 cells with and without different concentrations of hydralazine assayed by the MSP. Compared to the control, hydralazine (10 and 30 μM, respectively) decreased methylation in the SERCA2 promoter by 45 ± 4 and $44 \pm 7\%$ (Figure 1a). Moreover, we performed direct bisulfite sequencing analysis for SERCA2 methylation and found that hydralzine (30 μM) decreases the methylated CpG sites in promoter area of SERCA2 in HL-1 cells (Figure 1b). We further examined whether DNMT1 was involved in the demethylation effects of hydralazine, and found that

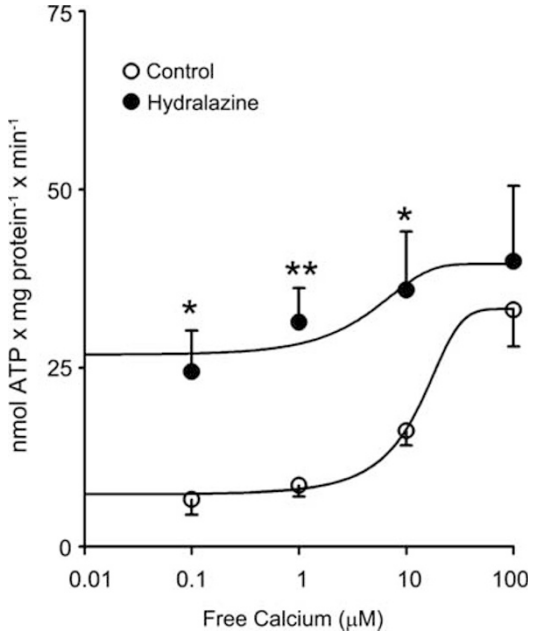

Figure 6 Effects of hydralazine on SERCA2a activity in HL-1 cells. Hydralazine modulates SERCA2a activity with different concentrations of Ca^{2+} ($n=7$). The SERCA2a activity was measured from the decreases of 340 nm absorption. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control at the respective concentration of Ca^{2+}.

hydralazine (10 and 30 μM) significantly decreased the DNMT1 protein level by 28 ± 8 and 39 ± 8%, respectively, compared to the control (Figure 2).

Hydralazine Increases SERCA2a Expression

As shown in Figure 3a and 3b, hydralazine (10 and 30 μM) upregulated the RNA and protein expressions of SERCA2a measured by both a real-time PCR and an immunoblot assay. Compared to the control, hydralazine (10 and 30 μM) significantly increased SERCA2a mRNA by 21 ± 4 and 26 ± 6%, and increased the SERCA2a protein by 28 ± 7 and 24 ± 5%, respectively. These findings confirmed that hydralazine (10 and 30 μM) can modulate SERCA2a expression through transcriptional regulation.

Hydralazine Increases $[Ca^{2+}]_i$ Transients

We examined whether hydralazine can directly modulate Ca^{2+} homeostasis in HL-1 cells. Using an indo-1 fluorescence assay, we found that hydralazine (10 and 30 μM)-treated cells had respective larger amplitudes of $[Ca^{2+}]_i$ transients than control cells by 60 ± 11 and 75 ± 10% (Figure 4). Additionally, hydralazine (10 and 30 μM)-treated cells had a tendency to enhance Ca^{2+} decay (τ_{Ca}) of $[Ca^{2+}]_i$ transients compared to control cells (34 ± 5 and 30 ± 3 *vs* 44 ± 7 ms, $P>0.05$). As assessed by caffeine-induced $[Ca^{2+}]_i$ transients, hydralazine (10 and 30 μM)-treated cells had larger respective SR Ca^{2+} contents than control cells by 79 ± 19 and 88 ± 19% (Figure 5). Moreover, the SERCA activity increased in hydralazine-treated cells at 0.1, 1, and 10 μM Ca^{2+} compared to the controls (Figure 6).

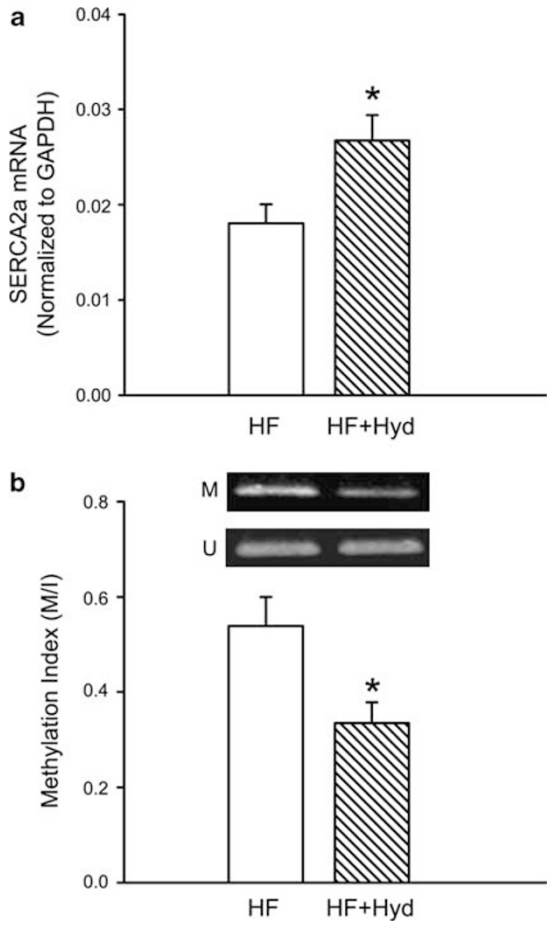

Figure 7 Effects of hydralazine on isoproterenol-induced heart failure. (**a**) Cardiac SERCA2a mRNA expressions from heart failure rats (HF, $n=6$) and hydralazine-treated heart failure rats (HF + Hyd, $n=6$). (**b**) Representative agarose gels and average data of methylation-specific PCR products for promoter methylation of SERCA2a from rat hearts with heart failure ($n=6$) or heart failure rats treated with hydralazine ($n=6$). $^{*}P<0.05$ *vs* heart failure without hydralazine.

Effects of Hydralazine on Isoproterenol-Induced Heart Failure

Comparison between heart failure with and without hydralazine treatment showed that hydralazine-treated hearts had increased SERCA2a mRNA (Figure 7a). In addition, the promoter methylation of SERCA2 significantly decreased in hydralazine-treated hearts (Figure 7b), which was consistent with the *in vitro* findings in HL-1 cells.

DISCUSSION

In this study, for the first time we demonstrated that hydralazine at the concentrations of 10 and 30 μM has significant direct effects on cardiomyocytes by increasing $[Ca^{2+}]_i$ transients and the SR Ca^{2+} content. As the SR Ca^{2+} content plays a critical role in determining $[Ca^{2+}]_i$ transients, hydralazine-enhanced $[Ca^{2+}]_i$ transients may be

caused by a higher SR Ca^{2+} content. Previous studies also showed that SERCA2a overexpression in transfected cardiomyocytes or in transgenic rat hearts can increase calcium transients and SR calcium contents, and improve cell contractility.[6,22] Moreover, increasing SERCA2a expression can reverse heart failure-induced electrical and structural remodeling as a result of decreased cardiac arrhythmias and improved cardiac function.[23] Therefore, hydralazine-increased SR Ca^{2+} contents may act through an increase in SERCA2a mRNA with enhancing SERCA2a protein and activity. Accordingly, hydralazine seems to be a promising drug for treating heart failure in addition to its hemodynamic effects. Moreover, hydralazine increased SERCA2a mRNA and decreased promoter methylation of SERCA2 in animal models of heart failure, which were consistent with the *in vitro* findings in HL-1 cells. A previous study indicated that enhanced SERCA2-mediated calcium uptake can induce arterial relaxation.[24] As vascular smooth muscles undergo a slowly sustained tonic contraction, sequestration of Ca^{2+} by SERCA2 may result in arterial relaxation and contribute to the antihypertensive effect of hydralazine. We also checked the promoter sequences in other important calcium-handling proteins, but found no CpG islands in the promoter area of sodium–calcium exchanger and ryanodine receptor. Therefore, the effect of hydralazine on calcium regulation was suggested to arise from its effect on SERCA2a.

Previous studies showed that hydralazine contains demethylating and gene-reactivating activities in treating cancers.[25–27] Moreover, hydralazine-induced systemic lupus erythematosus was also proposed for its inhibition of T cell DNA methylation and induction of autoreactivity.[14] Therefore, the demethylation activity of hydralazine is clinically related. In this study, hydralazine also effectively reduced methylation at the concentrations of 10 and 30 μM in cardiomyocytes. Through an MSP analysis, we also found that hydralazine at 10 and 30 μM decreased the DNMT1 level and attenuated the methylation of the SERCA2 promoter in cardiomyocytes with an increase in SERCA2a, which indicated epigenetic regulation by hydralazine. These findings are consistent with previous findings that epigenetic modification by promoter methylation or demethylation plays an important role in regulating SERCA2a transcription.[7]

The data should be interpreted with caution due to the limitations of this study. First, the standard PCR followed by agarose gel-EtBr staining used in this study may not be quantitative. However, direct bisulfate sequencing analysis also suggested a decreased methylation in SERCA2 promoter in hydralazine-treated cells. Second, it is not clear whether the effects of hydralazine on isoproterenol-induced heart failure are similar to those in other models of pathologically induced heart failure. In conclusion, hydralazine decreased DNMT, thus decreasing methylation in the SERCA2a promoter region as a result of the enhanced SERCA2a expression. These findings suggest that inhibition of SERCA2a methylation may be a novel treatment strategy for cardiac dysfunction.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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