Dimethylarginine dimethylaminohydrolase-1 contributes to exercise-induced cardiac angiogenesis in mice

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SUMMARY Dimethylarginine dimethylaminohydrolase-1 (DDAH1) maintains nitric oxide (NO) bioavailability by degrading asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of nitric oxide synthase (NOS). It has been well established that DDAH1 and exercise play crucial roles in promoting cardiac angiogenesis under pathological conditions. However, the role of DDAH1 in exercise-induced cardiac angiogenesis remains unclear. In this study, we focused on the change in DDAH1 in response to moderate exercise and the underlying mechanism of exercise-induced cardiac angiogenesis. Eight-week-old male DDAH1 global knockout (KO) mice and DDAH1 flox/flox mice (wild-type) were randomly divided into sedentary groups (control) and swimming groups (exercise). After eight weeks of swimming at five days per week, all the mice were anesthetized and sacrificed. Histological examination and Western blot analysis were performed. There were low levels of myocardial capillaries in DDAH1 KO mice under control and exercise conditions. Notably, exercise elevated DDAH1 protein expression, as observed by Western blot analysis. The common cardiac angiogenesis biomarkers vascular endothelial growth factor (VEGF) and Caveolin-1 were increased during exercise. A significant difference in VEGF was observed between the DDAH1 KO and wild-type groups. Similarly, increased Caveolin-1 expression was abrogated in DDAH1 KO mice. Furthermore, we tested the R-Ras/AKT/GSK3β signaling pathway to study the underlying molecular mechanism. DDAH1 may regulate the R-Ras/AKT/GSK3β pathway due to distinct protein changes in this pathway in the DDAH1 KO and wild-type groups. Our findings suggest that DDAH1 plays an important role in exercise-induced cardiac angiogenesis by regulating the R-Ras/AKT/GSK3β signaling pathway.

Keywords swim, cardiac capillary density, eNOS, VEGF, Caveolin-1, R-Ras

1. Introduction

Angiogenesis is the process of generating new blood vessels from existing ones. Homeostasis of angiogenesis plays an important role in human cardiac health. In animal experiments, myocardial ischemia (1), diabetic heart disease (2) and myocardial aging (3) are all characterized by poor angiogenesis. Inadequate angiogenesis has been viewed as one of the key reasons for the occurrence of these diseases. The cardioprotective effects of regular exercise are an efficient therapeutic strategy to preserve the balance of angiogenesis, including improving cardiac angiogenesis in myocardial infarction (4,5), heart transplantation (6), diabetes (7,8) and aging (9,10). It is well recognized that exercise protects cardiac structure and function via elevating capillary density under pathological conditions (5,11). Exercise-induced angiogenesis plays a vital role in maintaining the homeostasis of angiogenesis in the myocardium.

Dimethylarginine dimethylaminohydrolase-1 (DDAH1) degrades asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of nitric oxide synthase (NOS). Previous studies have demonstrated that DDAH1 is involved in the occurrence and development of multiple diseases, such as vascular disease (12), heart failure (13) and myocardial ischemia (14,15). Moreover, Zhang found direct evidence that DDAH1 expression is upregulated with a decline in ADMA and may take part in a protective effect against myocardial ischemia via promoting angiogenesis (15). DDAH1 and DDAH2 are two isoforms of DDAH. DDAH1 plays a leading role in degrading ADMA. The protein level of DDAH2 is not changed in DDAH1 global KO mice (16) and endothelial DDAH1 gene deletion mice (17). However, these mice show a lack of DDAH activity (increased ADMA level, and blood
pressure and decreased NO generation). Interestingly, the vital role of DDAH1 in regulating cardiac angiogenesis under pathological conditions may be attributed to the distribution of DDAH1. In the myocardium, DDAH1 is mainly distributed in the sarcolemma of cardiomyocytes and the endothelium of cardiac microvessels (18), which provides a basis for the role of DDAH1 in regulating myocardial angiogenesis. However, the role of DDAH1 in exercise-induced angiogenesis in the myocardium remains unclear.

The aim of this study was to focus on changes in DDAH1 in response to moderate exercise and to investigate the underlying mechanism of exercise-induced cardiac angiogenesis in mice. Our data indicated a pivotal role of DDAH1 in contributing to exercise-induced cardiac angiogenesis.

2. Materials and Methods

2.1. Experimental animals

DDAH1 KO mice (16) and DDAH1^flx/flx^ mice (16) were generated in the lab of the Cardiovascular Division (University of Minnesota Medical School, Minneapolis, USA). The mice were kept in the animal experimental center (SPF level) at Shanghai University of Sport. Twenty-six eight-week-old mice were randomly divided into four groups according to their genotypes and exercise states: the DDAH1 KO sedentary group (KO SED, n = 7), the DDAH1^flx/flx^ sedentary group (WT SED, n = 6), the DDAH1 KO swimming group (KO EXE, n = 7), and the DDAH1^flx/flx^ swimming group (WT EXE, n = 6). All mice were freely fed normal rodent chow and water during the experiments. The mice received a 12 h: 12 h alternating dark and light cycle in well-controlled settings with constant temperature (22-26°C) and humidity (55-60%). All experimental animals conformed to the laboratory rules. The studies were approved by the Institutional Animal Care and Use Committee and the Ethics Committee for Science Research at Shanghai University of Sport.

2.2. Exercise protocol

Mice from the KO EXE group and the WT EXE group were trained to swim in two respective tanks (45 cm × 35 cm × 25 cm), in which the water temperature ranged from 32 to 33°C. The mice were familiarized with the water tank during the first week. The exercise protocol began with a 30 min/day swimming session and increased gradually to 90 min/day for 7 consecutive days. Subsequently, the mice were maintained for seven weeks of swimming (90 min daily, 5 days per week) without any workload increase. Mice in the KO SED and WT SED groups were kept sedentary throughout the study (Figure 1).

2.3. Sample processing

All mice were anesthetized and sacrificed after eight weeks. A portion of the myocardium was fixed in 4% paraformaldehyde and placed in a 4°C refrigerator for histological observation. Another part of the myocardium was placed in liquid nitrogen and then transferred to a -80°C freezer for Western blotting.

2.4. Isolectin B4 and DAPI staining

The cardiac tissue samples were embedded in paraffin and cut at a thickness of 4 μm. After dewaxing in water, repairing antigens and blocking, the sections were labeled with isoelectin B4 (10 μg/mL, DL-1207, Vector laboratories, USA) and DAPI (1 μg/mL, D1306, Thermo Fisher Scientific, Waltham, MA, USA) for angiogenesis observation. The procedures were carried out in accordance with the manufacturer's instructions. Sections (6 sections per group, 5 fields per section) were observed with fluorescence microscopy (Olympus, Japan) and laser scanning confocal microscopy (Zeiss, Germany).

Figure 1. Exercise protocol. Mice from the KO EXE group and the WT EXE group were trained to swim in two tanks. They were trained to swim for the first week (30-90 min/day for 7 consecutive days) and then went into formal exercises for seven weeks (90 min/day, 5 day/week).
2.5. Western blot analysis and antibodies

After the lysis of cardiac tissue homogenates, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was utilized, and the remaining steps were the same as those of conventional Western blotting procedures. The primary antibodies used for Western blotting were as follows: rabbit anti-DDAH1 (1:500, Abcin, abs133884), rabbit anti-phospho-eNOS Ser1177 (1:1000, Abcam, ab215717), rabbit anti-eNOS (1:1000, Abcam, ab199956), rabbit anti-vascular endothelial growth factor (VEGF) (1:500, Abcin, abs131208), rabbit anti-Caveolin-1 (1:1000, Cell Signaling, 3267), rabbit anti-Ras (1:1000, Abcin, ab191791), rabbit anti-AKT (1:1000, Cell Signaling, 4691), rabbit anti-phospho-AKT Ser473 (1:1000, Cell Signaling, 4060), rabbit anti-GSK3β (1:1000, Cell Signaling, 9315), rabbit anti-phospho-GSK3β Ser9 (1:1000, Cell Signaling, 9323) and rabbit anti-GAPDH (1:1000, Cell Signaling, 5174).

2.6. Statistical analysis

All values are expressed as the mean ± standard deviation and significance was regarded as \( p < 0.05 \). SPSS (19.0 version) was used to analyze the experimental data. Two groups of data were analyzed by unpaired \( t \)-test. Data between four groups were analyzed by two-way ANOVA and post hoc comparisons with the Fisher LSD test.

3. Results

3.1. Effect of exercise on the heart weight/body weight ratio and the left ventricular weight/body weight ratio in WT and DDAH1 KO mice

The heart weight/body weight ratio and the left ventricular weight/body weight ratio had no significant changes in DDAH1 KO and wild-type mice (\( p > 0.05 \)) (Figure 2A and 2B). The results suggested that eight weeks of swimming couldn’t induce cardiac hypertrophy in WT and DDAH1 KO mice.

3.2. Role of DDAH1 in exercise-induced myocardial capillary density

At a basal level, the WT SED group manifested higher cardiac microvessel density than that in the KO SED group (\( p < 0.05 \)). After eight weeks of swimming, compared with the angiogenesis levels in the WT SED and KO SED groups, exercise enhanced cardiac angiogenesis in both WT EXE (\( p < 0.01 \)) and KO EXE (\( p < 0.01 \)) groups. Interestingly, capillary density in the KO EXE group was blunted compared with that in the WT EXE group (\( p < 0.01 \)) (Figure 3A and 3B), indicating that DDAH1 exerted a key effect on exercise-induced cardiac angiogenesis.

3.3. Upregulation of DDAH1, p-eNOS/eNOS, VEGF, and Caveolin-1 protein expression following exercise

According to the histological results, DDAH1 played a crucial role in exercise-induced angiogenesis, but the exercise-induced change in DDAH1 was unknown. Western blot results showed that eight weeks of swimming elevated DDAH1 protein expression in the WT EXE group compared with that of the WT SED group (\( p < 0.01 \)) (Figure 4A and 4B). Similarly, p-eNOS/eNOS was also increased in the WT EXE (\( p < 0.01 \)) and KO EXE (\( p < 0.01 \)) groups (Figure 4A and 4B). Under sedentary and exercise conditions, p-eNOS/eNOS showed a significant difference in the KO EXE group compared with that of the WT EXE group (\( p < 0.01 \)). Moreover, we examined changes in cardiac angiogenesis-related indicators, such as VEGF and Caveolin-1. Eight weeks of swimming increased VEGF and Caveolin-1 expression (\( p < 0.01 \)) (Figure 4A and 4B). The data demonstrated that exercise enhanced cardiac capillary density in mice. The effect of exercise on VEGF and Caveolin-1 was obvious. Exercise for eight weeks upregulated VEGF and Caveolin-1. In addition, the expression of VEGF and Caveolin-1 protein in the KO EXE and KO SED groups was lower.
than those in the WT EXE ($p < 0.01$) and WT SED ($p < 0.01$) groups (Figure 4A and 4B), regardless of exercise or sedentary state.

3.4. Role of DDAH1 in regulating the R-Ras/AKT/GSK3β pathway following exercise-induced cardiac angiogenesis

To further determine the potential molecular mechanism by which DDAH1 affects exercise-induced angiogenesis, we examined the changes in the R-Ras/AKT/GSK3β signaling pathway. R-Ras, p-AKT/AKT and p-GSK3β/GSK3β protein expression was changed under basal conditions in sedentary mice, which showed a novel pathway by which DDAH1 affects myocardial capillary density ($p < 0.05$) (Figure 5A and 5B). After eight weeks of swimming, compared with the expression levels in the WT SED and KO SED groups, R-Ras, p-AKT/AKT and p-GSK3β/GSK3β were expressed at high levels in the WT EXE ($p < 0.01$) and KO EXE ($p < 0.05$) groups (Figure 5A and 5B). Furthermore, the elevation in the protein expression of R-Ras, p-AKT/AKT and p-GSK3β/GSK3β was abrogated in the KO EXE group compared with that of the WT EXE group ($p < 0.01$) (Figure 5A)
and 5B). Corroborating these changes in the R-Ras/AKT/GSK3β signaling pathway, our data suggest that DDAH1 regulates the R-Ras/AKT/GSK3β signaling pathway and plays a crucial role in cardiac angiogenesis.

4. Discussion

The main finding of the current study indicates the essential role of DDAH1 in exercise-induced cardiac angiogenesis. DDAH1 deletion abated myocardial capillary density under sedentary and exercise conditions. At a basal level, DDAH1 global KO mice exhibited increased levels of ADMA in plasma and tissue, decreased NOS, and increased systemic blood pressure (16). ADMA accumulation in DDAH1-/- mice also led to a reduction in angiogenesis and impairment of vascular repairing capacity (12,19). Of note, ADMA accumulation can induce endothelial cell apoptosis. Consequently, it was shown that DDAH1 is pivotal in regulating angiogenesis through degrading ADMA and maintaining NO signaling in a strain of DDAH1 global KO mice. Furthermore, Dowsett confirmed that endothelial deletion of DDAH1 profoundly impaired the angiogenic responses both ex vivo and in vivo (20). In addition, other researchers found that angiogenesis was enhanced in response to overexpression of DDAH1 (21). These findings suggest that DDAH1 increases capillary formation in important organs and blood vessels, including the brain, heart, liver, lung, kidney, skeletal muscle and carotid artery. Under sedentary conditions, DDAH1 KO (16) or DDAH1 silencing (22) reduces the degradation of ADMA, implying that endothelial cell apoptosis increases, while angiogenesis is inhibited. Conversely, overexpression of DDAH1 (22) increases the degradation of ADMA, suggesting that endothelial cell function is protected and angiogenesis is promoted. As the major enzyme for degrading ADMA, DDAH1 can protect against ADMA accumulation induced endothelial cell apoptosis, maintain endothelial function and regulate angiogenesis under sedentary conditions. In the present study, according to our angiogenesis staining results, loss of DDAH1 reduced capillary density in the myocardium under sedentary conditions, which is consistent with previous studies and added robust evidence.

There are two subtypes of DDAH including DDAH1 and DDAH2, which are encoded by distinct genes. Different subcellular distributions and physiological functions of the two isoforms determine that DDAH1 plays a role in cardiac angiogenesis. In terms of subcellular distribution of DDAH, DDAH1 is predominantly distributed in vascular endothelial cells (18) and the heart (23). Based on the physiological functions of DDAH, the leading enzyme degrading ADMA is DDAH1 rather than DDAH2. A large amount of evidence has shown that DDAH1 gene deletion does not change protein expression of DDAH2 (16,17). Unexpectedly, DDAH1 gene deletion increases ADMA levels. To our best knowledge, until now, there is no powerful evidence that DDAH2 plays a role in cardiac angiogenesis. Conversely, a series of strong evidence shows the important effects of DDAH1 on cardiac angiogenesis.

It is well established that exercise promotes cardiac angiogenesis in animals and human studies (6,24). The increase in myocardial capillaries is an important manifestation of exercise benefits to the heart. Exercise-induced cardiac angiogenesis in both WT and KO mice showed that our designed swim protocol was effective. In this exercise protocol (90 min/day, 8 weeks, without any workload increase), the intensity for the mice was moderate. Evangelista (25) demonstrated that different swim training volumes (min), body weight loads (%), frequencies (sessions/day) and durations (weeks) played essential roles in triggering cardiac hypertrophy in mice. As evidences showed in the heart weight/body weight ratio and the left ventricular weight/body weight ratio, our protocol did not meet the minimum requirement of exercise training-induced cardiac hypertrophy caused by swimming. There may be a threshold in the model of swim training induced cardiac hypertrophy. Thus, in this study, exercise-induced cardiac angiogenesis did not belong to myocardial remodeling caused by high-intensity exercise training-induced cardiac hypertrophy. Although moderate and high intensity exercise (4) can both increase myocardial capillaries, they should be essentially different, which may facilitate the future study of their respective mechanisms.
After eight weeks of swimming, the protein expression of DDAH1 in wild-type mice was increased compared with that of the control group. The results of this study demonstrate a direct role of DDAH1 in exercise-induced capillary formation. Combined with the decrease in angiogenesis in KO mice under exercise conditions, DDAH1 deficiency indeed reduced exercise-induced myocardial capillary density. Thus, to the best of our knowledge, these findings provide the first evidence that exercise elevates the protein expression of DDAH1. The protein expression of DDAH1 was upregulated during exercise. It may be hypothesized that DDAH1 directly increases in the endothelium of cardiac microvessels, which is the basis by which DDAH1 participates in exercise-induced cardiac angiogenesis. In fact, DDAH1 is involved in the regulation of myocardial capillary formation and has a protective effect on the heart. Other researchers had similar results in heart diseases, such as heart failure (13) and myocardial infarction (14), for DDAH1 protecting myocardium. In the present study, we have found that exercise elevated the expression of DDAH1 and increased angiogenesis, suggesting that exercise could inhibit ADMA to promote angiogenesis by increasing the expression level of DDAH1. The result is consistent with the findings that regular aerobic exercise can reduce ADMA levels in human experiments (26,27). Therefore, studying the regulatory angiogenesis mechanism of DDAH1 is essential.

Our finding that exercise increased myocardial eNOS expression is well established in many studies (28,29). DDAH1, which directly interacts with eNOS in cardiac tissues (13), degrades ADMA to preserve eNOS activity and NO. It has been noted that exercise elevates both DDAH1 and p-eNOS/eNOS in the myocardium. Thus, the changes in DDAH1 and p-eNOS/eNOS are consistent. In addition, p-eNOS/eNOS in the DDAH1 KO mice was blunted compared with that in wild-type mice under sedentary or exercise conditions. Thus, DDAH1 plays an important part in cardiac angiogenesis by regulating NO signaling. This is consistent with previous research (22). However, the question of whether there is synergism between exercise and DDAH1 needs further study.

It is already known that DDAH1 takes part in the regulation of exercise-induced angiogenesis, but how DDAH1 regulates angiogenesis still needs more research regarding related indicators and pathways of angiogenesis. VEGF and Caveolin-1 are crucial to the vascular system. A recent report confirmed that exercise improves angiogenesis through the Caveolin-1/VEGF pathway in the ischemic penumbra (30). However, the evidence above was determined in the brain and not in the heart. Other research demonstrated that the Caveolin-1/VEGF signaling pathway is an important target in angiogenesis in acute myocardial infarction (31). However, the purpose of the study was drug treatment rather than exercise intervention. In this study, the results are not different. Exercise induced the expression of VEGF and Caveolin-1, which demonstrated that exercise directly increases myocardial capillary density. Our finding that VEGF and Caveolin-1 are affected by the deletion of DDAH1 showed that DDAH1 may play an important role in modulating myocardial angiogenesis induced by exercise through VEGF and Caveolin-1. VEGF is a leading regulator of angiogenesis. A large amount of evidence has demonstrated that the VEGF signaling pathway could directly promote blood vessel formation (32,33). Thus, the results indicated that DDAH1 contributes to exercise-induced cardiac angiogenesis. Different from the widespread use of VEGF in angiogenesis, Caveolin-1 begins to show new features. Chen demonstrated the primary colocalization of DDAH1 and Caveolin-1 in the endothelium of cardiac microvessels (18). In fact, Caveolin-1 is mainly expressed in endothelial cells (34). It is conceivable that similar localization of DDAH1 and Caveolin-1 substantiates the same function in regulating cardiac angiogenesis. Caveolin-1 plays an important role in controlling the biological process of angiogenesis by binding to eNOS. VEGF and Caveolin-1 are key indexes of cardiac angiogenesis. Since DDAH1 regulates cardiac angiogenesis rather than DDAH2, DDAH1 has a dominant effect on the expressions of VEGF and Caveolin-1. In this paper, DDAH1 played an important role in exercise-induced cardiac angiogenesis. The expressions of VEGF and Caveolin-1, which are key indexes of cardiac angiogenesis, changed significantly, suggesting that DDAH1 has an important effect on the expressions of VEGF and Caveolin-1. There is no strong evidence that DDAH2 has an effect on the expression of VEGF and Caveolin-1. To our best knowledge, DDAH1 has a dominant effect on the expression of VEGF and Caveolin-1 in the myocardium. Based on the explanations above, our data provide new direct evidence for the impact of DDAH1 on VEGF and Caveolin-1 in exercise-induced cardiac angiogenesis.

To explore the potential mechanism by which DDAH1 regulates exercise-induced myocardial angiogenesis, a new pathway regulating vascular density has been found in recent years. In 2005, Komatsu reported that R-Ras is a pivotal regulator of vascular regeneration in tumors by employing R-Ras-null mice (35). Based on this evidence, Komatsu and colleagues found that the R-Ras/AKT axis induces endothelial lumenogenesis and regulates the potency of regenerating vasculature in vitro and in vivo (36). The integrity of angiogenesis includes proliferation, migration, increased permeability and lumenogenesis of endothelial cells because the vessels are three-dimensional and form a complicated reticular structure. The sequential steps of angiogenesis should recapitulate sprouting, branching, and lumen formation (36). R-Ras, a member of the Ras family of small GTPases, not only increases AKT,
GSK3β phosphorylation and microtubule stabilization in endothelial cells (36) but also compensates for the conventionally insufficient effect of VEGF on angiogenesis. R-Ras activates AKT (37) and GSK3β, which is a known downstream substrate of AKT (38). Thus, we observed exercise-induced changes in the expression of R-Ras, p-AKT, AKT, p-GSK3β and GSK3β in the R-Ras/AKT/GSK3β signaling pathway. Our data demonstrates that exercise increased R-Ras, p-AKT/AKT, and p-GSK3β/GSK3β. It seems that exercise activates the R-Ras/AKT/GSK3β pathway. Exercice promotes angiogenesis in terms of improving the quantity and quality of microvessels. In addition, the expression of R-Ras and the ratio of p-AKT to AKT and p-GSK3β to GSK3β in DDAH1 KO mice was significantly different from those in wild-type mice. These results show that DDAH1 directly regulates this pathway. Consistent with Komatsu's finding, the R-Ras/ AKT/GSK3β pathway indeed plays a crucial role in regulating angiogenesis. Combined with the data of this study, DDAH1 may play an important role in exercise-induced myocardial angiogenesis by regulating the R-Ras/AKT/GSK3β signaling pathway. On the one hand, the current study expands the understanding of increased capillary density by exercise; on the other hand, this study provides a new pathway in the regulation of angiogenesis by DDAH1. The new discovery of the function of the R-Ras/AKT/GSK3β pathway also reveals a novel role of DDAH1, which is of great significance in explaining the regulatory role of DDAH1 on angiogenesis. DDAH1 promotes exercise-induced angiogenesis, which may be exerted partly through the regulation of DDAH1 on the R-Ras/AKT/ GSK3β signaling pathway. DDAH1 may be a potential molecular target to regulate angiogenesis with exercise in future gene therapy studies.

DDAH1 plays a major role in exercise-induced cardiac angiogenesis not DDAH2. In this study, DDAH1 played a key role in exercise-induced cardiac angiogenesis through regulating the R-Ras/AKT/GSK3β signaling pathway. So we found a novel role for DDAH1. Does DDAH2 have this new role? It depends on whether DDAH2 affects exercise-induced cardiac angiogenesis. Until now, there is no powerful evidence that DDAH2 plays a role in cardiac angiogenesis. Thus, DDAH1 has a leading effect on the regulation of the R-Ras/AKT/ GSK3β signaling pathway in the myocardium.

Based on the above analysis, we think that DDAH1 plays a leading role in our experiments. Therefore, we have not studied DDAH2. If there are sufficient conditions in the future, we will also consider further research on DDAH2.

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