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Effects of exercise training and RhoA/ROCK inhibition on plaque in ApoE$^{-/-}$ mice☆

Aya Matsumoto [a,*], Helga D. Manthey [b], Susan A. Marsh [c], Robert G. Fassett [a,d,e], Judy B. de Haan [f], Barbara E. Rolfe [g], Jeff S. Coombes [a]

[a] School of Human Movement Studies, The University of Queensland, Brisbane, Queensland, Australia
[b] Rudolf-Virchow-Zentrum, University of Würzburg, Würzburg, Germany
[c] Program in Nutrition and Exercise Physiology, Washington State University, Spokane, WA, USA
[d] Royal Brisbane and Women's Hospital, Brisbane, Queensland, Australia
[e] School of Medicine, The University of Queensland, Brisbane, Queensland, Australia
[f] Oxidative Stress Laboratory, Diabetic Complications Division, Baker IDI Heart and Diabetes Institutes, Melbourne, Australia
[g] Centre for Research in Vascular Biology, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Queensland, Australia

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ABSTRACT

Background: The molecular mechanisms of exercise-induced cardioprotection are poorly understood. We recently reported that exercise training down-regulated gene expression of the Ras homolog gene family member A (RhoA). RhoA and its first effectors, the Rho-kinases (ROCK), have already been implicated in the pathogenesis of cardiovascular disease. The aim of this study was to compare the effects of a RhoA/ROCK inhibitor (fasudil) and exercise in the Apolipoprotein E knockout (ApoE$^{-/-}$) mouse model of atherosclerosis.
Methods: Four groups of 14 week old ApoE$^{-/-}$ mice were randomised as follows (n = 12/group): i) sedentary controls (Cont); ii) fasudil (Fas) treatment (100 mg/kg bodyweight/day) for 8 weeks; iii) exercise intervention (Ex:free access to running wheel for 8 weeks) and iv) exercise intervention and fasudil treatment (ExFas) for 8 weeks.
Results: Phosphorylation of myosin light chain was significantly reduced in the brachiocephalic artery of all treatment groups compared with sedentary controls, implying an inhibitory effect of exercise and fasudil on the RhoA/ROCK pathway. Furthermore, atherosclerotic lesions were significantly smaller in all treatment and intervention groups compared with the control group (Fas: 34.7%, Ex: 48.3%, ExFas: 40.9% less than Control). The intima: media ratio was reduced by both exercise intervention and fasudil treatment alone or in combination (Fas: 23.6%, Ex: 35.5%, ExFas: 43.9% less than Control). Exercise alone and fasudil treatment alone also showed similar effects on plaque composition, increasing both smooth muscle cell and macrophage density.
Conclusion: These results suggest that the protective effects of exercise on atherogenesis are similar to the inhibitory effects on the RhoA/ROCK signalling pathway.

1. Introduction

Exercise is well established as an effective primary and secondary intervention for atherosclerotic cardiovascular disease (CVD). Exercise improves traditional CVD risk factors such as blood pressure, blood lipids, obesity and insulin sensitivity [1]. It is also known to improve vascular function [2] and reduce inflammation [3]. Despite the positive effects on cardiovascular-associated risk factors, the mechanisms by which exercise exerts these cardioprotective effects are still uncertain [4]. Identification of these mechanisms may have important implications for the development of therapeutic targets.

We recently reported that 14 weeks of treadmill exercise down-regulated gene expression of the Ras homolog gene family member A (RhoA) in the endothelial cells of rats [5]. RhoA is a small G-protein and its first effectors, the Rho-kinases (ROCKs) have already been implicated in the pathogenesis of atherosclerotic CVD [6]. ROCKs have been shown to be up-regulated in inflammatory atherosclerotic lesions [7] and administration of a RhoA/ROCK inhibitor successfully decreased atherosclerotic plaque size [8]. However, the clear role of exercise in RhoA/ROCK pathway has not been established. We therefore hypothesised that the effects of exercise on atherosclerotic plaques are due, at least in part, to the inhibition of RhoA/ROCK signalling, and that RhoA/ROCK inhibition will be similar to the effects of exercise on plaque morphology. Hence the aim of the current study was to compare the effects of the ROCK inhibitor (fasudil) and exercise in the Apolipoprotein E knockout (ApoE$^{-/-}$) atherosclerotic mouse model.

2. Materials and methods

2.1. Animals

Fourteen-week old female ApoE$^{-/-}$ mice (on a C57/BL6J background) were obtained from the Animal Resources Centre (Canningvale, WA, Australia). Animals (n = 12/group)

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* Corresponding author at: School of Human Movement Studies, The University of Queensland, Brisbane QLD 4072, Australia.
E-mail address: aya.matsumoto@uqconnect.edu.au (A. Matsumoto).

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were randomly assigned to either a i) sedentary group (Cont); ii) sedentary and fasudil group (Fas); iii) exercise group (Ex) and iv) exercise and fasudil group (ExFas). The mice were housed individually, maintained on a 12/12 hour light–dark cycle and provided with normal chow and water *ad libitum*. Female mice were used due to availability from the supplier. All experiments were approved by The University of Queensland Animal Ethics Committee.

2.2. Exercise protocol

Animals in the Ex and ExFas groups had 24 hour free access to a running wheel for 8 weeks. The wheels were fitted with a monitor to record the daily running distance for each mouse. Running wheels were removed from the cage 24 h prior to euthanasia in order to avoid the acute effects of exercise.

2.3. Fasudil

Animals in the Fas and ExFas groups received 100 mg/kg body weight/day of fasudil (LC Laboratories, Wobum, MA, USA) in their drinking water for 8 weeks. Water intake was recorded daily to calculate the appropriate drug dose per animal. Fasudil supplementation at this dosage and method of delivery has previously been shown to inhibit ROCK in mice [9].

2.4. Tissue processing and histology

.2.4.1. Preparation of tissue for immunohistochemistry and immunofluorescence

Animals were weighed, then anaesthetised with zoletil (Parnell Laboratories, Alexandria, NSW, Australia; 50 mg/kg) and xylazine (Troy Laboratories Pty Ltd, Smithfield, NSW, Australia; 10 mg/kg) *via* intraperitoneal injection. Once a surgical plane of anaesthesia was reached, a thoracotomy was performed and blood was obtained *via* cardiac puncture into heparinised tubes. Plasma was separated and stored at −80 °C until required. Transcardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) was then performed to achieve whole body perfusion fixation. The brachiocephalic artery was removed with the stump of the right subclavian artery attached to aid orientation. Vessels were post-fixed in 4% PFA for 2 h at 4 °C after which time samples were washed 3 times in PBS. The brachiocephalic artery was immersed in 30% sucrose in PBS for 48 h, embedded in Optimal Cutting Temperature (OCT; Sakura Finetek USA Inc., CA, USA) and snap frozen in liquid nitrogen. Tissue blocks were stored at −80 °C until further use. The brachiocephalic artery was cut into 10 μm transverse sections along its length (starting from the proximal end), mounted onto Superfrost plus slides (Menzel-Glaser, Braunschweig, Germany). Four sections per animal were used for all morphometric and morphologic measurements, unless otherwise indicated.

2.5. Involvement of RhoA/ROCK pathway

The effects of exercise and fasudil on the RhoA/ROCK pathway were determined by staining sections with an antibody raised against the down-stream target of RhoA/ROCK, phospho-myosin light chain 2 (pMLC2). Briefly, sections were quenched with 0.6% H_2O_2/TBS for 20 min and blocked with 0.5% milk/TBS for 30 min. Slides were then incubated with rabbit anti-pMLC2 (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. This was followed by incubation with biotinylated secondary antibody (Vector Laboratory, Burlingame, CA) for 10 min at room temperature. Sections were then incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA). Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrocholoride (Sigma-Aldrich, St Louis, MO, USA). Negative controls consisted of sections where the primary antibody was omitted. The area with positive pMLC staining was expressed as a percentage of total vessel area.

2.6. Plaque morphometry

All analyses were performed by a single observer blinded to group allocation. For each analysis, the location of the brachiocephalic artery was matched across different samples by keeping the distance from the proximal end of the artery constant. Sections were stained with Miller's van Gieson and aldehyde-fuchsin [10]. Images were digitised and captured using a Nikon DP70 camera. Morphometric analysis was performed with an image-analysis program (ImageJ, National Institutes of Health Image, Bethesda, MD, USA). The areas enclosed by the external elastic lamina, internal elastic lamina and lumen were measured, from which neointimal area (area enclosed by the internal elastic lamina minus luminal area), medial area (area enclosed by the external elastic lamina minus internal elastic lamina), neointima to media ratio (intima:media ratio), and the degree of stenosis (neointimal area divided by internal elastic laminar X 100%) were calculated. Sections were also stained with Oil-red-O (Invitrogen, Mount Waverley, VIC, Australia) for determination of lipid area within lesions.

2.7. Plaque composition

Animals with no plaque formation were excluded from plaque composition measures (see figure legend for numbers). Collagen was stained with Picrosirius red (Sigma, St Louis, MO, USA; 1 mg/ml at pH 2.0) for 1 h [11]. Sections were viewed under an Olympus BX51 polarised light microscope and photos of each section taken under polarised and brightfield light using a Nikon DP70 camera. Polarised and brightfield images were then merged to allow visualisation of collagen within the entire plaque. Each image was split into red and green channels to allow analysis of thick (red) and thin (green) collagen fibers using ImageJ software. Collagen content was calculated as: (mean collagen area/total lesion area) × 100%.

Cell density within lesions was calculated as previously described [12] from sections stained with the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI; 100 ng/ml in PBS; Sigma Aldrich, St Louis, MO, USA).

To measure smooth muscle cell (SMC) and macrophage content, sections were blocked with 1% BSA (Sigma Aldrich, St Louis, MO, USA), 5% goat serum, 0.5% Triton-X 100 (Sigma Aldrich, St Louis, MO, USA), 0.3% fish gelatine (Sigma Aldrich, St Louis, MO, USA) and 0.05% sodium azide (Sigma Aldrich, St Louis, MO, USA) in PBS for SMC staining, and 5% BSA, 0.5% Triton-X 100, 0.3% fish gelatine and 0.05% sodium azide in PBS for macrophage staining. Slides were then incubated with either rabbit anti-α smooth muscle actin (Abcam, Cambridge, UK; 1:100) or rat anti-mouse F4/80 (Abcam; 1:50). Sections were then incubated with the appropriate biotinylated secondary antibody (goat anti-rabbit immunoglobulin (Ig), 1:200 or rabbit anti-rat Ig, 1:200; both from Abcam) before incubation with a streptavidin-conjugated AlexaFluor 555 (Invitrogen; 1:1000). Sections were then mounted in Mowiol solution containing DABCO and DAPI (all from Sigma). Negative controls consisted of sections where the primary antibody was omitted. The number of SMC and macrophages per lesion was calculated and expressed as a percentage of lesion area.

2.8. Total cholesterol and triglycerides

Plasma was analysed for total cholesterol and triglycerides using the Infinity assay kit (Infinity Reagents, Thermo Electron, Noble Park, VIC, Australia), according to the manufacturer's protocol.

2.9. Data analysis

All data are expressed as mean ± SEM, unless otherwise shown. Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc. USA). A 2-way ANOVA followed by Tukey post hoc analysis was used to determine the effects of drug alone (Cont vs Fas), exercise alone (Cont vs Ex), as well as possible interactive effects of the combination treatment (ExFas vs Ex + Fas groups). Statistical significance was accepted as $p<0.05$.

3. Results

3.1. Body weight, running distance and lipid levels

There was no significant difference in the average daily running distance in the Ex and ExFas groups, nor did they significantly affect body weight (Table 1). At the end of the 8-week period, there were no significant differences in plasma triglyceride and plasma cholesterol levels between any of the groups ($p>0.05$).

3.2. Involvement of RhoA/ROCK pathway

Phosphorylation of one of the main down-stream targets of RhoA/ROCK pathway, MLC was decreased in the vessel walls of the Ex, Fas and ExFas groups compared to Cont (Fig. 1, $p<0.05$), implying that both exercise and fasudil inhibited the function of the RhoA/ROCK pathway. Furthermore, the magnitude of inhibitory effect on MLC phosphorylation was similar in all treatment or intervention groups.

3.3. Plaque morphometry

Fig. 2 shows representative sections of brachiocephalic artery from each group and Fig. 3 provides quantitative analysis of atherosclerotic lesions. In all treatment or intervention groups, Oil-Red-O lipid deposition within lesions was less than in the control (untreated) group

Table 1
Assessment of body weight, running distance and lipid levels in the four groups (mean ± SD).

	Control (n = 12)	Exercise (n = 11)	Fasudil (n = 11)	ExFas (n = 11)
Change in body weight over 8 weeks (%)	20.9 ± 5.5	21.1 ± 7.5	19.2 ± 6.8	17.9 ± 8.3
Average daily running distance (km)	–	10.4 ± 1.8	–	10.3 ± 3.3
Plasma triglycerides (mmol/L)	2.0 ± 0.5	2.1 ± 0.8	2.6 ± 0.5	2.3 ± 0.6
Plasma cholesterol (mmol/L)	9.8 ± 2.2	10.7 ± 2.2	11.1 ± 1.0	10.7 ± 1.4