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Platelet responses to pharmacological and physiological interventions in middle-aged men with different habitual physical activity levels

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Short title: Training status and platelet function

Abstract

The current guidelines following an acute coronary syndrome recommend dual anti-platelet therapy (aspirin plus a $P2Y_{12}$ antagonist) alongside lifestyle modifications, including more regular physical activity. It is currently unknown if regular exercise affects the pharmacology of dual anti-platelet therapy.

Aim

To explore how exercise-induced improvements in vascular and platelet function affect the efficacy of dual anti-platelet therapy, in a cross-sectional study of men with different physical activity level (training status).

Methods

42 healthy, normal-weight, middle-aged men were divided into 3 groups; untrained, moderately- and well-trained. Their platelet reactivity (agonist-induced %aggregation) was investigated in platelet rich plasma at rest and after inhibition with aspirin and ticagrelor and/or prostacyclin and nitric oxide added to the blood *in vitro*, and after physiological tests of vascular function; passive movement of the leg, flow-mediated dilation and one-leg knee-extensor exercise. Vascular function of the femoral artery (changes in arterial blood flow) was assessed by ultrasound doppler.

Results

Platelets from the well-trained subjects had lower basal reactivity, a higher sensitivity to the anti-aggregatory effects of prostacyclin and were more potently inhibited by dual anti-platelet therapy compared to the untrained subjects. The moderately- and well-trained subjects had a superior vascular function compared to untrained subjects and their platelets were more inhibited by the passive movement, flow-mediated dilation and one-leg knee extensor exercise.

Conclusion

A habitually active lifestyle leads to an increased platelet sensitivity to pharmacological and physiological platelet inhibitors. We suggest that physical activity habits (training status) should be considered when personalising and optimizing anti-thrombotic treatment strategies.

Keywords: Aspirin, Nitric Oxide, Physical Activity, Platelet Aggregation Inhibitors, Prostacyclin

Introduction

The current guidelines after an acute coronary syndrome (ACS) include dual-antiplatelet therapy (DAPT) for at least 12 months, alongside recommendations of more regular aerobic physical activity [1]. DAPT consists of aspirin, inhibiting platelet thromboxane A_2 (TXA_2) production and a $P2Y_{12}$ receptor antagonist, inhibiting the platelet adenosine 5'-diphosphate (ADP) receptor ($P2Y_{12}$) [2]. Some patients have high on-treatment platelet reactivity despite receiving DAPT and this is associated with a higher risk of recurrent thrombotic events [3]. Hence, more optimized treatment strategies are warranted to prevent the high mortality and morbidity of ACS.

We have previously suggested that the efficacy of $P2Y_{12}$ inhibitors may vary markedly in individuals dependent on their vascular function since $P2Y_{12}$ inhibitors dramatically increase the anti-platelet effects of both prostacyclin and nitric oxide [4-6]. Potentiating the inhibitory actions of these endothelial derived mediators may be a central mechanism through which $P2Y_{12}$ inhibitors exert their anti-thrombotic protection *in vivo*.

Since regular physical activity improves endothelial function [7, 8] and enhances platelet sensitivity to prostacyclin [9], the efficacy of anti-platelet drugs, in particular $P2Y_{12}$ inhibitors, may also be affected by an individual's habitual physical activity level.

Insight into how regular physical activity influences anti-platelet drug efficacy is important for assessment of whether the habitual physical activity level of an individual should be taken into account when prescribing anti-platelet drugs.

We hypothesized that regular physical activity is associated with a high level of vascular endothelial function in parallel with a lower basal platelet reactivity and higher platelet sensitivity to DAPT and prostacyclin.

To test this hypothesis, we investigated platelet and vascular function in healthy middle-aged men with different training statuses and tested their platelet responses to pharmacological inhibition with DAPT and their sensitivity to the endogenous physiological platelet inhibitors prostacyclin and nitric oxide.

Results

Subject characteristics

42 male middle-aged healthy subjects with a mean age of 52±1 years participated in the study. They were divided up into 3 groups; untrained (n=13), moderately-trained (n=15) and well-trained (n=14), based on their self-reported IPAQ score and the results of their VO_{2max} test, and there was no difference in age between the groups (Table 1). The untrained subjects had not participated in regular physical activity for the past 9±2 years whereas the moderately-trained and well-trained subjects had had an active lifestyle for the past 28±2 and 26±2 years respectively. Correspondingly, the moderately-trained and well-trained subjects reported in the IPAQ that they participated in more hours of moderate and intense exercise per week (Table 1), and their cardiorespiratory fitness level (peak VO_2 max at exhaustion), was higher than the untrained subjects (Table 1). The well-trained subjects scored higher on all these parameters than the moderately-trained subjects. Resting heart rate was lower in the well-trained subjects compared to the untrained subjects (Table 1). The moderately-trained and well-trained subjects had a larger femoral artery diameter compared to the untrained subjects (Table 1). There was no difference in glycated haemoglobin (haemoglobin A1c) or fasting glucose levels between the groups, but the fasting insulin levels and the Homeostatic Model Assessment of Insulin Resistance ($HOMA_{IR}$) were lower in the moderately and well-trained subjects (Table 1).

The international normalized ratio (INR) prothrombin time test was longer in the moderately and well-trained subject and these two groups also had lower levels of Factor II, VII and X compared to the untrained group (Table 1).

Basal platelet reactivity

When platelet rich plasma obtained from blood drawn at rest was stimulated with the platelet agonists collagen or epinephrine, the aggregation curves from the platelets from the well-trained subjects were lower than the curves from the untrained and moderately-trained subjects, indicating that platelets from the well-trained subjects required more agonist stimulation to aggregate meaning that indicating that they had lower basal platelet reactivity compared to the untrained and moderately-trained subjects (Fig. 1). Basal platelet reactivity to Arachidonic Acid (AA), ADP, thrombin receptor activating peptide (TRAP6), the TXA_2 mimetic U46619 was however not different between the groups (Supplementary material, Fig. S1).

Platelet sensitivity to pharmacological inhibition by DAPT

Platelet aggregation was potently inhibited by DAPT (1μM ticagrelor and 100μM aspirin), (Fig. 2 and Fig. S2). Collagen, epinephrine and U46619-induced platelet aggregation was more potently inhibited by DAPT in the well-trained subjects compared to the untrained and moderately trained subjects (Fig. 2a-e). The inhibitory effect by aspirin (100μM) was similar amongst the subjects (Fig. S3), whilst ticagrelor on its own inhibited AA-induced platelet aggregation potently in the moderately and well-trained subjects compared to the untrained subjects where there was ~20% residual aggregation not inhibited by ticagrelor (Fig. 2f and Fig. S4a).

Platelet prostacyclin sensitivity and response to DAPT in the presence of prostacyclin

Platelets from the well-trained subjects were more sensitive to the inhibitory effects of prostacyclin compared to the two other groups (Fig. 3). The concentration of prostacyclin required to inhibit 10μM TRAP6-induced platelet aggregation by 50% (IC_{50}), was lower in the well-trained subjects (mean, 95% confidence interval) (15nM, 12-19) compared to the untrained subjects (32nM, 28-37). Adding epinephrine (10μM) in combination with TRAP6 (10μM), diminished the inhibitory potency of prostacyclin in all groups (Figure 3d). In

combination with DAPT, platelet sensitivity to prostacyclin was strongly enhanced in all subjects, but most potently so in the well-trained subjects: (IC_{50} 2nM, 2-3) versus (4nM, 4-5) in the untrained subjects (Fig. 3).

Platelet sensitivity to nitric oxide and response to DAPT in the presence of nitric oxide

There was a concentration-dependent inhibitory effect on platelet aggregation by the DEA/NONOate which was further potentiated by DAPT, but there was no difference in responses between the different groups (Fig. S5).

Platelet and vascular responses to 3 physiological tests:

1. *Passive leg movement*

Femoral arterial blood flow was higher in the well-trained subjects at rest and 3 minutes of passive leg movement led to a higher increase in femoral arterial blood flow in the moderately and well-trained subjects compared to the untrained subjects (Fig. 4a). There was a moderate positive correlation (r=0.41) between the increase in femoral arterial blood flow following the passive movement and the subjects' VO_{2max} (Fig. 4b). There was right-hand shift in the ADP-induced platelet aggregation curves (indicating inhibition of platelet reactivity) following the passive movement, that became increasingly separated and shifted to the right with increasing training status (Fig. 4c+d+e).

2. *Flow-mediated dilation*

There was a significant increase in femoral arterial diameter in all the groups after the FMD and the largest increase was seen in the well-trained group. (Fig.5a). There was a positive correlation (r=0.55) between the FMD response (% difference between the diameter of the femoral artery at rest and the peak diameter of the femoral artery after the FMD) and the passive movement response (Fig.5b). The FMD test did not affect collagen-induced platelet reactivity in the untrained subjects (Fig. 5c), but there was an inhibitory effect (separation of the curves) in the moderately-trained subjects (Fig.5d) and a clear inhibitory effect on platelet

reactivity in the well-trained subjects where there was a reduction in maximum platelet aggregation to collagen after the FMD (Fig.5e). A similar pattern was seen for ADP and TRAP-6-induced aggregation (Fig.S6). Plasma levels of the prostacyclin breakdown product (6-keto $PGF_{1\alpha}$) did not differ between the groups at baseline and was not altered by the FMD (Table 1).

3. One-leg knee extensor exercise

Femoral arterial blood flow increased to a similar level in the three groups (~3000ml/min) following the set load (12W) (low to moderate intensity) one-leg knee extension exercise (Fig.6a). Epinephrine-induced platelet aggregation was unaffected in the untrained subjects (Fig.6b), but was progressively inhibited in the moderately-trained (Fig.6c) and the well-trained subjects (Fig.6d). There was a higher increase in femoral arterial blood flow following the relative load (40% of max.) in the moderately and well-trained subjects compared to the untrained subjects (Fig.7a), reflected by their higher workload (17±2 W in the untrained vs. 23±2 W in the moderately-trained vs. 32±1 W in the well-trained subjects). The 40% max. workload increased platelet reactivity to epinephrine in the untrained subjects, shifting the concentration-response curve to the left (Fig.7b), whereas platelet reactivity in the other two groups was not affected (Fig.7c+d). Plasma levels of 6-keto $PGF_{1\alpha}$ did not change after the 40% exercise compared to baseline (Table 1). WBC count, MPV and % of large platelets (P-LCR) increased in the blood samples from all subjects after both the 12W and 40% one-leg knee exercise (S7+S8). Platelet count, however, only increased in the untrained and moderately trained subjects after the exercise (S7b+S8b).

Discussion

The main findings of the current study were: (1) Basal platelet reactivity to collagen and epinephrine was lower in well-trained subjects compared to anthropometrically matched, moderately-trained or untrained middle-aged men. (2) Platelets from the well-trained subjects were more potently inhibited by DAPT *ex vivo*. (3) Platelet sensitivity to the anti-aggregatory

effects of prostacyclin was also higher in the well-trained subjects. (4) Active one-leg knee extension exercise, passive movement and FMD had a greater inhibitory effect on platelets from the moderately-trained and well-trained subjects compared to the untrained subjects.

The subjects included in the three study groups were selected to be healthy, non-smokers, of similar age and their body composition (BMI and waist circumference), cholesterol levels and fasting levels of plasma insulin/glucose were below the WHO definition of the levels considered as cardiovascular risk factors [10]. Thus, with regard to cardiovascular risk factors, the three groups differed significantly from each other only in terms of habitual physical activity level.

To our knowledge, this is the first study to investigate basal platelet reactivity in healthy middle-aged men with different training status. The lower basal platelet reactivity observed in the well-trained subjects is in line with results from previous cross-sectional studies performed in younger male [11] and female subjects [12]. Using the same optimul platelet aggregation assay as in the current study, we have previously reported that a 3-month exercise intervention lowered basal platelet reactivity to collagen in premenopausal women of a similar age to the men in the current study [9]. Taken together, these studies show that habitual physical activity modulates platelet function at rest, lowering the reactivity threshold of platelets.

In agreement with our hypothesis, platelets from the well-trained subjects were more potently inhibited by DAPT compared to the untrained subjects. Platelets from moderately-trained subjects were also more strongly inhibited by the $P2Y_{12}$ inhibitor ticagrelor compared to the untrained subjects. In order to mimic the physiological environment where these drugs normally act, where the endogenous platelet regulator prostacyclin is continuously released from the vascular endothelium, we added low (nM) concentrations of prostacyclin to the assay and found that the inhibitory potency of DAPT was further enhanced, mostly so in the well-trained subjects. We have previously suggested that potentiating the inhibitory actions of prostacyclin may be a central mechanism through which $P2Y_{12}$ inhibitors exert their anti-

thrombotic protection in vivo [4, 5]. The results from the current study suggests that the habitual physical activity levels of individuals may need to be considered in the optimization of their anti-thrombotic treatment strategies. Accordingly, ACS patients with a history of a physically active lifestyle may have a different baseline platelet reactivity and DAPT efficacy compared to sedentary patients and may therefore need less medication, for instance mono-therapy with a $P2Y_{12}$ antagonist [13]. Conversely, stronger anti-platelet medication regimes may be warranted in patients with a sedentary lifestyle. These findings may also serve as a motivator to sedentary patients highlighting previously unknown benefits to regular physical activity- increasing the efficacy of their DAPT medication and potentially the level of anti-thrombotic protection.

We found that platelets from the well-trained subjects were more sensitive to the anti-aggregatory effects of prostacyclin but not to nitric oxide. This finding is in agreement with our previous results from a longitudinal intervention study in middle-aged women showing increased platelet sensitivity to arterially infused prostacyclin after training, without a change in the sensitivity to nitric oxide [9]. Combined these data suggest that exercise training seems to particularly affect the platelet prostacyclin signalling pathway.

Since the vascular endothelium plays a key role in regulating platelet reactivity *in vivo*, we wanted to see if the lower basal reactivity in the well-trained men was associated with a superior vascular function. It is well known that regular physical activity leads to structural and functional improvements both at the macrovascular and the microvascular level [14]. We therefore assessed vascular function by three different (shear stress-induced nitric oxide dependent) physiological tests; passive leg movement, FMD and one-leg knee-extension exercise. We also wanted to see if we could capture any anti-aggregatory effects on the platelets by these physiological interventions, to see if the interactions between the blood vessels and blood platelets differed with training status.

Using the passive movement test [15], we were able to show that vascular function in the moderately-trained and the well-trained subjects was superior compared to the untrained

subjects. We also made the novel observation that the passive movement had a larger inhibitory effect on platelet aggregation in the moderately-trained and well-trained subjects, indicating that they had a higher bioavailability of shear stress-induced endothelial-derived platelet inhibitors (presumably nitric oxide) following this physiological challenge. The subjects that had the largest increase in femoral arterial blood flow following the passive leg movement also had the highest FMD response. We also show for the first time that FMD inhibits platelet aggregation in a training status dependent way, having the strongest inhibitory effect on platelets from well-trained subjects.

Acute exercise has been shown to affect platelet function in an intensity dependent manner where moderate exercise tends to suppress platelet reactivity whereas strenuous exercise has been shown to temporarily increase platelet reactivity [16, 17]. In the current study we used a localized model of exercise involving a single active muscle group in the leg [18]. We selected this model since we wanted to investigate platelet responses to acute exercise in a setting with lower levels of catecholamine release compared to full body exercise [19]. We found that 12W had an inhibitory effect on platelet reactivity in the moderately and well-trained subjects only, despite increasing the MPV and % of large platelets in the blood samples. Large platelets are typically considered to be more reactive to aggregate [20], but in the current study this was not the case in the moderately and well-trained subjects, since their platelet reactivity was lower after exercise, indicating that the exercise triggered an inhibitory milieu strong enough to also inhibit these larger platelets. Increasing the workload to 40% relative load triggered a hyper-aggregation response of the platelets in the untrained subjects only and removed the inhibitory effect of the lower intensity exercise in the trained subjects. Hence, exercise at lower (moderate) intensities may be more favourable when it comes to acute modifications of platelet reactivity. Overall, the hyper-aggregatory response to acute exercise in the untrained subjects highlights the importance of caution when introducing high intensity exercise to sedentary middle-aged male subjects.

Study limitations

A limitation of this cross-sectional study is that the favourable anti-thrombotic function in the well-trained subjects could in part be due to genetic selection or differences in characteristics other than training status between groups. Future studies should follow up on our findings and assess the longitudinal effects of physical activity on platelet function. Since this study population was limited to healthy middle-aged men, further research is needed to determine the effects of training on platelet activity and sensitivity to DAPT in people with other risk factors for cardiovascular diseases and/or in ACS patients. Another limitation with this study was that the DAPT was added to the blood in vitro rather than dosed in vivo, which may yield different results due to differences in pharmacokinetics and acute effects of DAPT in vivo versus in vitro.

The investigators performing the platelet aggregation assay were not blinded to the identity of the blood samples, but the potential for bias was minimal since this is a quantitative test of platelet aggregation. The investigator analysing the FMD files was however blinded to the training status of the individuals since the potential for bias was higher in this analysis.

Conclusions

We have shown that middle-aged men with a physically active lifestyle have platelets that are more easily inhibited by pharmacological platelet antagonists (DAPT) and have a higher sensitivity to the physiological platelet inhibitor prostacyclin, allowing for a more responsive communication between their blood vessels and blood. Conversely it can be argued that healthy and normal weight middle-aged men with a sedentary lifestyle have platelets that require more prostacyclin and DAPT to be inhibited and have a hyperactive response to acute exercise. Overall, this study clearly demonstrates that regular physical activity has a beneficial impact on platelet function and that DAPT is most efficacious at inhibiting platelet aggregation in healthy middle-aged men with a physically active lifestyle. These results indicate that training status may need to be considered when personalising and optimizing anti-thrombotic treatment strategies in patients receiving DAPT. Future studies should

investigate if a longitudinal exercise training intervention in previously sedentary ACS patients can improve the efficacy of their DAPT treatment.

Materials and Methods

The study protocol was approved by the ethics committee of Copenhagen (H-2-2014-112). All subjects were recruited after providing their written informed consent in accordance with the Declaration of Helsinki. The material submitted in this paper conform with Good Publishing Practice in Physiology [21].

Recruitment of subjects

The subjects were recruited via newspaper advertisements where the inclusion criteria were: healthy middle-aged men, 45-60 years old, normal weight (BMI>18.5 and <27 kg/m^2), normotensive (blood pressure <140/90 mmHg) and normal waistline (<94cm). The waistline criteria were strictly adhered to since central obesity have been associated with a reduced sensitivity to prostacyclin and nitric oxide [22]. Exclusion criteria were: smoking (the past 15 years), excessive alcohol intake (>14 units/week), regular medication intake and blood donation <4 months before participating in study. Training status was defined as: untrained (no regular physical activity in the past 2 years or more), moderately-trained (2-3 hours of moderate regular physical activity per week, for the past 15-20 years or more) and well-trained (>4 hours of moderate to intense regular physical activity per week, for the past 15-20 years or more).

Study design

The study consisted of a health screening day and two experimental days: a platelet function testing day and a vascular function testing day.

Health screening day

All participants underwent a health examination, including a 10-point resting electrocardiogram (ECG), to confirm that the subjects were healthy and that the inclusion criteria were met. A blood test was taken to ensure that hematological markers, cholesterol levels and coagulation factors were within normal range (analyzed within 2 hours at Rigshospitalet in Copenhagen, Denmark). The subjects then performed a one-leg knee extension exercise incremental max test to determine the watt (W) representing 40% of their maximum (ahead of the vascular function testing day). Each subject completed the short version of the International Physical Activity Questionnaire (IPAQ) to quantify their self-reported physical activity habits [23]. To objectively characterize fitness level, their pulmonary maximal oxygen uptake (VO_{2max}, Oxycon Pro, Intramedic) was assessed in an incremental bicycle ergometer exercise test until exhaustion (respiratory exchange ratio >1.1).

Experimental days- platelet function testing day and vascular function testing day

The subjects were advised to avoid non-steroidal anti-inflammatory drugs for at least 2 weeks and not to perform exercise within 48 hours of the experimental days (to avoid confounding effects of acute exercise). In preparation for each experimental day the subjects had fasted overnight.

Platelet function testing day

The primary outcomes of this day were: 1) Investigate the anti-aggregatory responses to pharmacological inhibition with DAPT and 2) Examine if platelet prostacyclin and nitric oxide sensitivity differs with training status. To this end, blood was drawn from the antecubital vein (after a 10 minutes rest in a supine position), into 3.2% citrate monovettes (s-monovette, Sarstedt). The blood was immediately analyzed for platelet count, mean platelet volume (MPV), large platelet ratio (P-LCR; >12fL and <30fL), haemoglobin, haematocrit and white blood cells (WBC) using a hematology analyzer (XP-300, Sysmex). Blood samples were then centrifuged (5804 R, Eppendorf) at 180xg for 10 minutes at 20°C, to obtain platelet rich plasma (PRP). Remaining blood was centrifuged further at 15000xg for 2 minutes to obtain

platelet poor plasma (PPP). The PRP was then treated with either; 1) the reversible purinergic $P2Y_{12}$ receptor antagonist ticagrelor (1μM, 15425, Cayman Chemical), or 2) aspirin (acetyl-salicylic acid, 100μM, 5376, Sigma), or 3) DAPT (ticagrelor 1μM and aspirin 100μM), or 4) vehicle (0.5% DMSO and 0.03% ethanol in phosphate buffer saline, PBS) for 30 minutes at room temperature. The PRP was then plated out and treated with vehicle (1mM NaOH), prostacyclin (1-300nM, epoprostenol 2989, R&D systems) or nitric oxide (0.1-100μM, D5431, Sigma) for 1 minute before transferring the PRP to a pre-prepared freeze-dried vacuum-packed 96-well optimul plates [9, 24-26] coated with platelet agonists: 20μM ADP (A2754 Sigma), 10μg/ml collagen (1130630, Takeda), 10μM of the TXA_2 analogue U46619 (16450, Cayman Chemical Company), 10μM thrombin receptor activating peptide (TRAP-6, SFLLRN H2936, Bachem), 10μM epinephrine (E4375, Sigma) or TRAP-6 + epinephrine. Remaining ticagrelor, aspirin, DAPT and vehicle treated PRP was added to freeze-dried 96-well optimul plates precoated with; ADP (0.02–40μM), collagen (0.01-10μg/ml), epinephrine (0.004–40μM), TRAP-6 (0.02–40μM), U46619 (0.02–40μM) or arachidonic acid (AA, 0.06-1mM, 4425, Sigma). The plates were placed on a shaker (BioShake iQ; Q Instruments) at 37°C to mix at 1200rpm for 5 minutes. Absorbance was measured at 595nm (Emax Precision Microplate Reader, Molecular Devices) and %platelet aggregation calculated [24-26]. All assays were completed within 2.5 hours of drawing the blood.

Vascular function testing day

The primary outcomes with this day were: 1) Test if the trained subjects had lower basal reactivity than then untrained subjects. Basal platelet reactivity was defined as agonist-induced platelet aggregation in PRP obtained from blood drawn at baseline (after 30 minutes rest). 2) Evaluate the subject's vascular function and 3) Platelet aggregation responses to physiological tests (to examine the interactions between platelet function and vascular function). An intravenous catheter (Vasofix Safety, 19G; B. Braun) was placed in the antecubital vein, followed by 30 minutes rest in an upright seated position before blood was drawn to test basal platelet reactivity. Blood samples drawn into 3.2% citrate monovette

containers and were immediately centrifuged to obtain PRP which was processed as outlined in experimental day 1 (minus the DAPT/prostacyclin or nitric oxide incubations) and plated out on pre-prepared optimul 96-well plates testing ADP, collagen, epinephrine, TRAP6, and U46619-induced platelet aggregation at full concentration-response curves. The assay took about 25 minutes from blood drawn to plate read. The subjects then commenced the passive movement test [15] by passively relaxing all the muscles in the right leg, whilst the lower part of the right leg was strapped to an ergometer and moved by one of the investigators at 60rpm frequency for 3 minutes. Femoral arterial blood flow was measured at baseline and after the passive movement with ultrasound Doppler (Vivid E9, GE Healthcare) equipped with a linear probe (L5) operating at an imaging frequency of 4/8 MHz and a Doppler frequency of 4.2 MHz. The site of blood velocity measurements in the common femoral artery was distal to the inguinal ligament but above the bifurcation into the superficial and profound femoral branch to avoid turbulence from the bifurcation. All recordings were obtained at the lowest possible insonation angle (always <60 degrees). The sample volume was maximized by choosing the widest section of the vessel and the measurements were made without interference of the vessel walls. A low-velocity filter (velocities <1.8 m s^{-1}) rejected noise caused by turbulence at the vascular wall. Doppler tracings and B-mode images were recorded continuously and Doppler tracings were averaged over 45 seconds. After a 15-minute rest in a supine position, the flow mediated dilation (FMD) test [27] was initiated, by rapidly inflating a cuff (E20, Hokanson) around the subject's thigh, holding the pressure at 240mgHg for 5 minutes, followed by rapid deflation. A recording of superficial femoral arterial blood flow was preformed 30 seconds before cuff deflation and in the following 5 minutes. The response to the FMD test was calculated by comparing the diameter of the femoral artery at rest to the diameter at the peak dilation of the artery following the opening of the cuff. Blood samples for platelet function testing were drawn from the arm at baseline and immediately after opening the cuff. After a 15-minute rest in a seated position, the one-leg knee exercise protocol started with the subject's right leg being strapped into the ergometer and subjects were instructed to actively kick forward and

passively return their leg at a kicking frequency of 60rpm. The workload was set to 12W (representing low to moderate intensity). Blood samples and femoral blood flow measures were obtained at baseline and after 10 minutes. The workload was then increased to 40% of the subjects' personal maximum and blood samples and femoral arterial blood flow measures were captured after 10 minutes. Blood samples for prostacyclin $PGF_{1\alpha}$ was drawn at baseline, after the FMD and after the 40% one-leg exercise into EDTA vacutainers and placed on ice until they were centrifuged at 1000xg 15 minutes, 4˚C, plasma was pipetted off and stored at -80℃ until analysis using an immunoassay kit (EIA; 515211, Cayman Chemical Co).

Statistical analysis

Power calculations of the primary outcome measure (platelet function and vascular function) were made to determine the study size, using the standardized difference method [28] where the α-level was set to 0.05 and the power level to 0.8. The effect size was set to detect a 50% difference in femoral blood flow following the passive movement and a 50% increased platelet sensitivity to the inhibitory effects of prostacyclin, based on previous reports [9, 22]. Data are reported as mean ± standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism5 and groups were compared using one-way or two-way ANOVA as appropriate, with Bonferroni multiple comparison post-test. Differences in platelet count, WBC, MPV, and % large platelets from baseline to one-leg exercise were detected using a paired two-tailed t-test. The Kolmogorov–Smirnov test and the Shapiro–Wilk test of normality confirmed that the data was normally distributed. Final number of replicates are indicated in each figure legend.

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Conflict of Interest

There are no conflicts of interest.

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Table 1. Subject characteristics

Subject characteristics	Untrained n=13	Moderately-trained n=15	Well-trained n=14
Age (years)	52.5 ± 1.5	52.0 ± 1.4	50.1 ± 1.5
Weight (kg)	75 ± 2	76 ± 2	77 ± 1
Body mass index (BMI, kg/m^2)	24.5 ± 0.5	23.1 ± 0.6	23.3 ± 0.5
Waist circumference (cm)	87.2 ± 1.1	82.5 ± 1.5 *	83.3 ± 1.2*
SBP/DBP (mm Hg)	128/78 ± 3/1	124/76 ± 2/1	122/76 ± 3/2
Femoral artery diameter (mm)	94 ± 2	106 ± 1*	111 ± 3 * #
IPAQ total hours of exercise per week	1.4 ± 0.8	7.2 ± 1.3 *	12.5 ± 1.0 * #
IPAQ hours of mod. exercise per week	1.2 ± 0.8	4.4 ± 1.4	5.9 ± 1.6 *
IPAQ hours of intense exercise per week	0.1 ± 0.1	2.7 ± 0.5 *	6.6 ± 0.9 * #
Heart rate at rest (beats/min)	60 ± 2	54 ± 2	51 ± 1 *
One-leg knee exercise max test (W)	43.4 ± 3.6	58.5 ± 3.9 *	82.5 ± 3.6 * #
Peak workload at exhaustion, bike test (W)	248 ± 9	332 ± 10 *	404 ± 8 * #
VO_2 max. (ml min^{-1})	2626 ±122	3544 ± 117 *	4580 ±106 * #
VO_2 max. (ml min^{-1} kg^{-1})	34.5 ± 1.2	46.6 ± 1.0 *	60.0 ± 1.6 * #
Total cholesterol (mg dl^{-1})	216 ± 10	186 ± 7 *	189 ± 8 *
HDL cholesterol (mg dl^{-1})	55 ±4	62 ± 4	65 ± 3
LDL cholesterol (mg dl^{-1})	137 ± 9	109 ± 6 *	113 ± 8
Triglycerides (mmol L^{-1})	143 ± 27	98 ± 10	103 ± 17
Haemoglobin A1c (%)	5.4 ± 0.05	5.3 ± 0.07	5.3 ± 0.06
Fasting glucose (mg dl $^{-1}$)	91 ± 2	91 ± 2	87 ± 2
Fasting insulin (pmol L^{-1})	70 ± 11	41 ± 4 *	23 ± 1 * #
$HOMA_{IR}$	2.3 ± 0.4	1.3 ± 0.1 *	0.7 ± 0.04 * #
Coagulation factors (INR)	1.03 ± 0.02	1.11 ± 0.02 *	1.11 ± 0.02 *
Factor II VII X (units L^{-1})	0.98 ± 0.04	0.83 ± 0.03 *	0.80 ± 0.04 *
Haematocrit (% L L^{-1})	47 ± 1	45 ± 1	46 ± 1
Haemoglobin (mmol L^{-1})	8.0 ± 0.1	7.8 ± 0.2	7.9 ± 0.1
6-keto $PGF_{1\alpha}$ (pg ml^{-1}) Baseline	54 ± 4	51 ± 3	62 ± 5
6-keto $PGF_{1\alpha}$ (pg ml^{-1}) FMD	61 ± 5	51 ± 3	65 ± 6
6-keto $PGF_{1\alpha}$ (pg ml^{-1}) 40% max. exercise	54 ± 4	47 ± 3	53 ± 5

Table 1. SBP/DBP mm Hg: Systolic blood pressure/ diastolic blood pressure. IPAQ: International Physical Activity Questioner. W: Watt $HOMA_{IR}$: homeostasis model assessment of insulin resistance. INR: International normalized ratio. WBC: white blood cell count. Data are mean ± SEM. P<0.05 * compared to the untrained group, # compared to the moderately trained group and ¤ compared to baseline.

Figure legends

Figure 1. Basal platelet reactivity (agonist-induced % platelet aggregation) to 6 concentrations of the platelet agonists (a) collagen and (b) epinephrine. Platelet rich plasma from venous blood drawn at rest from the antecubital vein of untrained (n=13), moderately-trained (n=15) and well-trained (n=14) middle-aged men. ~~ϕ~~ *P<0.05*: the platelet aggregation curve from the well-trained subjects was lower than the curves from the untrained and moderately-trained subjects (indicating lower basal platelet reactivity). ~~overall difference between the curves between the groups.~~

Figure 2. The anti-aggregatory effects of dual anti-platelet therapy (DAPT) in platelet rich plasma (PRP) from untrained (n=13), moderately-trained (n=15) and well-trained (n=13-14) subjects was pre-incubated for 30min with DAPT consisting of the $P2Y_{12}$ inhibitor ticagrelor (1μM) and the cyclooxygenase inhibitor aspirin (100μM) or vehicle (0.5% DMSO + 0.03%EtOH in PBS) before inducing platelet aggregation by (a) Collagen (full concentration response curve), (b) 10μg/ml Collagen, (c) Epinephrine (full concentration response curve), (d) 10μM Epinephrine or (e) the thromboxane A_2 mimetic U46619 and (f) % platelet aggregation in response to 0.6mM Arachidonic acid (AA) in PRP treated only with ticagrelor. * *P*<0.05 well-trained or moderately-trained compared to untrained subjects and # well-trained compared to moderately-trained subjects.

Figure 3. Platelet sensitivity to the anti-aggregatory effects of prostacyclin, with or without dual anti-platelet therapy (DAPT). Platelet rich plasma (PRP) from untrained (n=13), moderately (mod.) trained (n=15) and well-trained (n=14) middle-aged men was pre-incubated with vehicle (0.5% DMSO + 0.03%EtOH in PBS) or DAPT (1μM ticagrelor and 100μM aspirin) for 30min, followed by 1min prostacyclin, before the PRP was added to 96-well plates pre-coated with the platelet agonists (a) 20μM Adenosine diphosphate (ADP) (b) 10μM the TXA_2 mimetic U46619 (c) 10μM thrombin receptor activating peptide 6 (TRAP-6) or (d) 10μM TRAP-6 + 10μM epinephrine to induce platelet aggregation. *P*<0.05 well-trained compared to (*) untrained and (#) moderately-trained subjects.

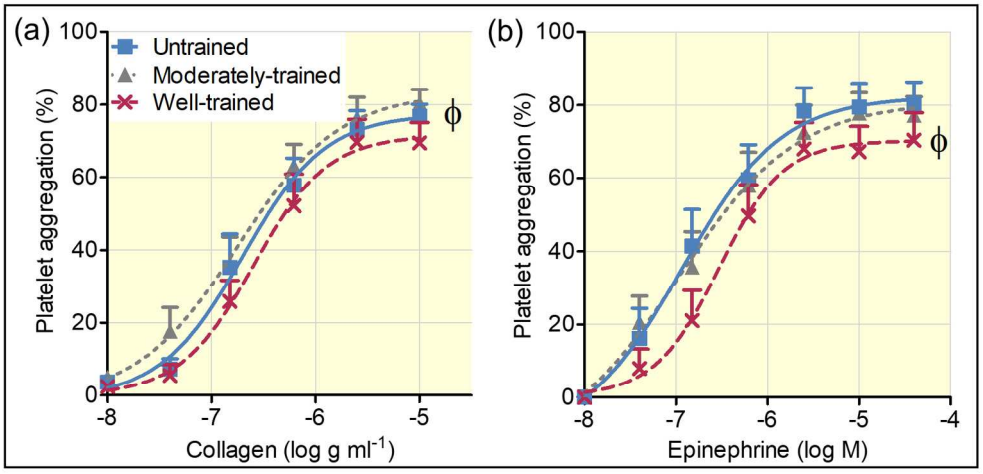
Figure 4. (a) Femoral arterial blood flow at rest and after 3 min of passive one-legged knee-extension movement in untrained (n=12), moderately-trained (n=15) and well-trained (n=14) middle-aged men.

(b) Correlation between the change in femoral arterial blood flow following the passive movement and cardiorespiratory fitness level (VO_2 max, $ml^{-1}min^{-1}kg^{-1}$). Platelet reactivity (% platelet aggregation induced by adenosine diphosphate, ADP) at rest and after the passive movement in platelet rich plasma obtained from (c) untrained (n=10), (d) moderately-trained (n=15) and (e) well-trained (n=14) subjects. (¤) *P*<0.05: compared to baseline and (*) *P*<0.05: compared to the untrained subjects.

Figure 5. Figure 5. (a) Flow Mediated Dilation (FMD) response (change in femoral artery diameter from rest to peak during 5 min deflation of occlusion cuff around the thigh) in untrained (n=7), moderately-trained (n=8) and well-trained (n=9) middle-aged men. (b) Correlation between the femoral arterial response to passive movement (blood flow ml min^{-1}) and response to FMD (% change in femoral arterial diameter). Platelet reactivity (collagen induced % platelet aggregation) at baseline and immediately after opening the 5-min occlusion (FMD test) in platelet rich plasma obtained from (c) untrained (n=12), (d) moderately-trained (n=15) and (e) well-trained (n=12) subjects. (ϕ) P<0.05 curve is lower than baseline. (¤) P<0.05, (¤¤) P<0.005 and (¤¤¤) P<0.0005 compared to resting baseline.

Figure 6. (a) Femoral arterial blood flow at rest and after 10 minutes of acute dynamic one-leg knee-extension exercise at a set workload (12Watt, W). Epinephrine-induced platelet aggregation in platelet rich plasma from (b) untrained (n=12), (c) moderately-trained (n=15) and (d) well-trained (n=12) middle-aged men, before and after the 12W acute one-leg knee extension exercise. (¤) *P*<0.05: compared to baseline and (ϕ) *P*<0.05 curve is lower than baseline. (*) *P*<0.05: compared to untrained subjects.

Figure 7. (a) Femoral arterial blood flow at rest and after 10 minutes of acute dynamic one-leg knee-extension exercise at a relative workload: 40% of the maximum ability (determined in a max test performed on the health screening day), corresponding to 17±2 Watt (W) for the untrained, 23±2 W for the moderately-trained and 32±1 W for the well-trained subjects. Epinephrine-induced platelet aggregation in platelet rich plasma from (b) untrained (n=12), (c) moderately-trained (n=15) and (d) well-trained (n=12) subjects, before and after the 40%. max acute exercise. (¤) *P*<0.05: compared to baseline (*) *P*<0.05 compared to untrained subjects. (ϕ) *P*<0.05 curve is lower than baseline. (*) *P*<0.05: compared to the untrained subjects.



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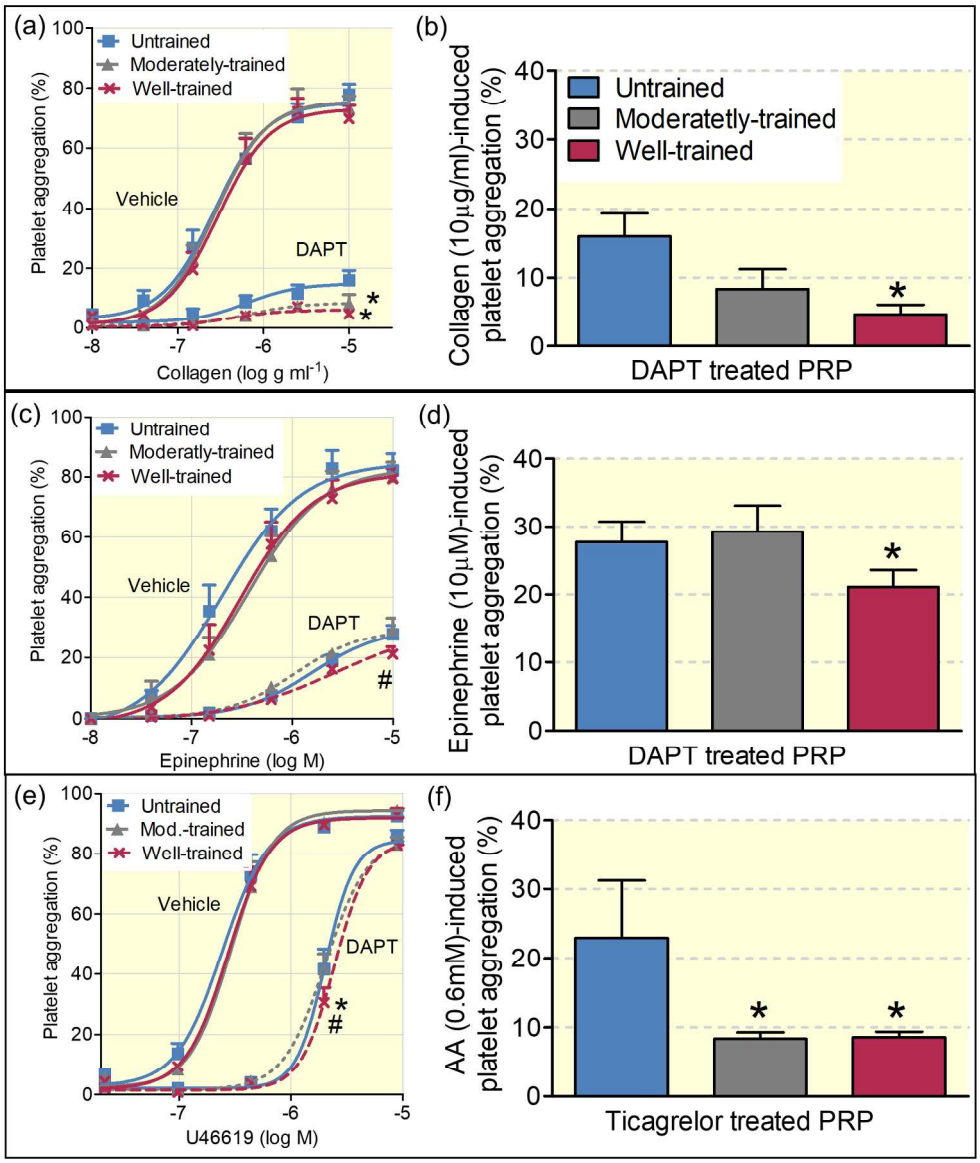

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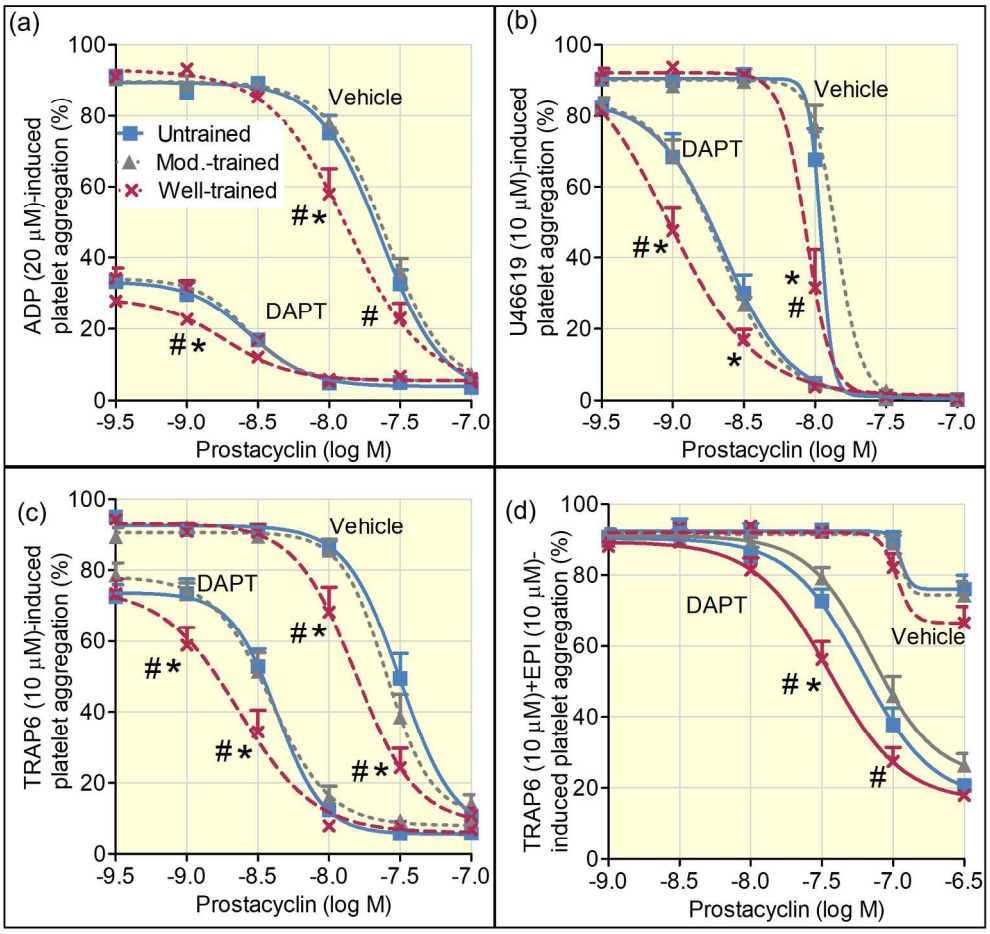

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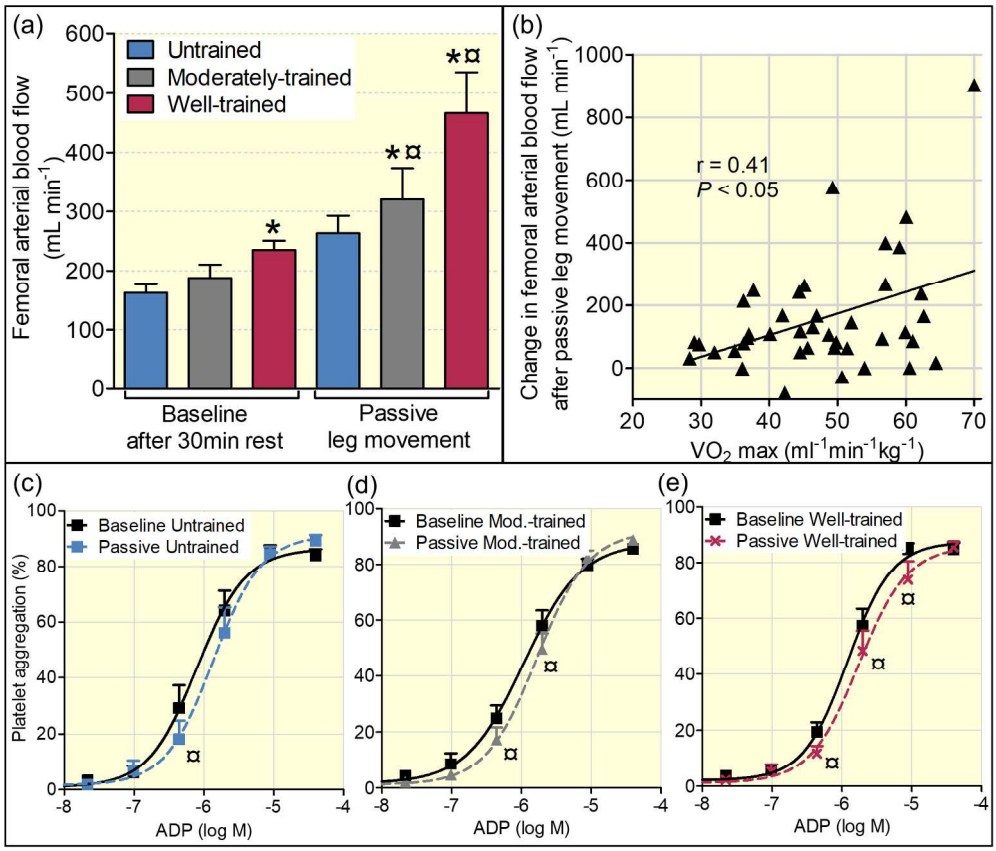

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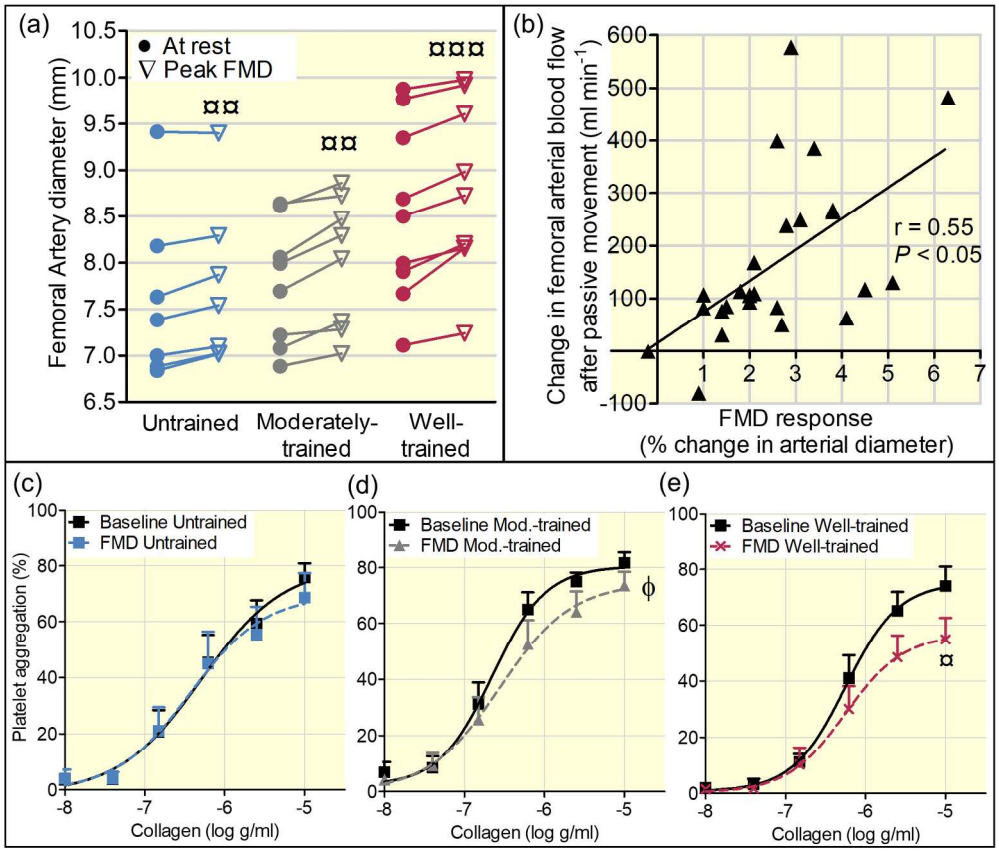

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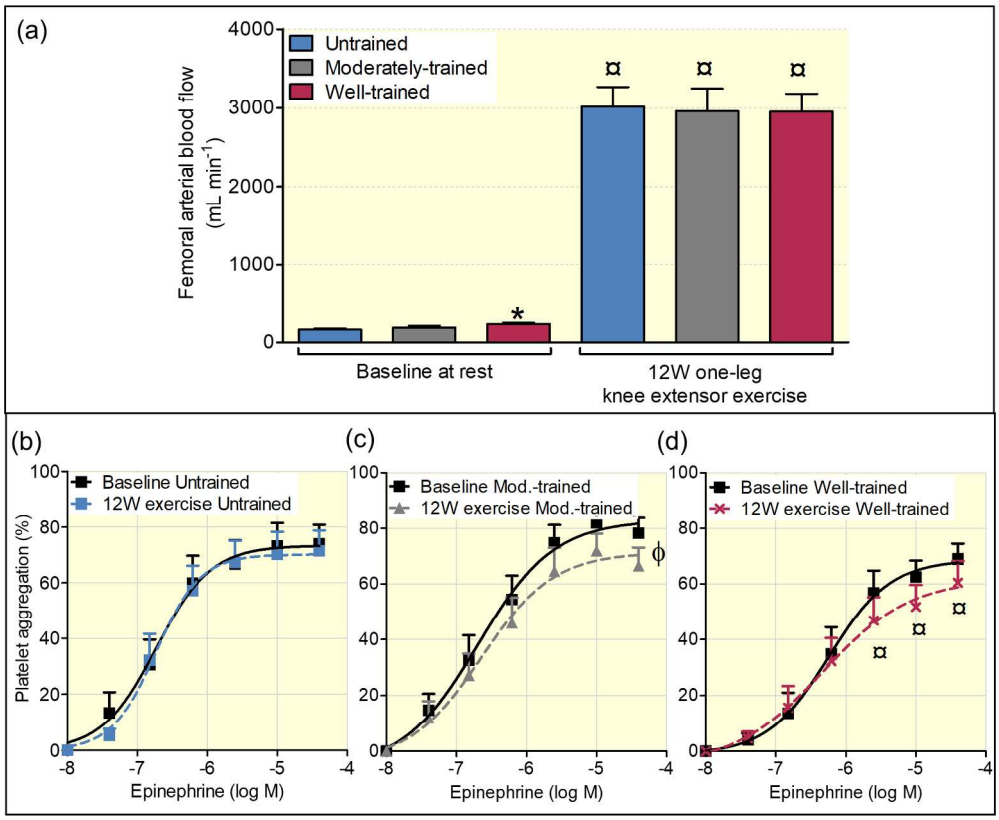

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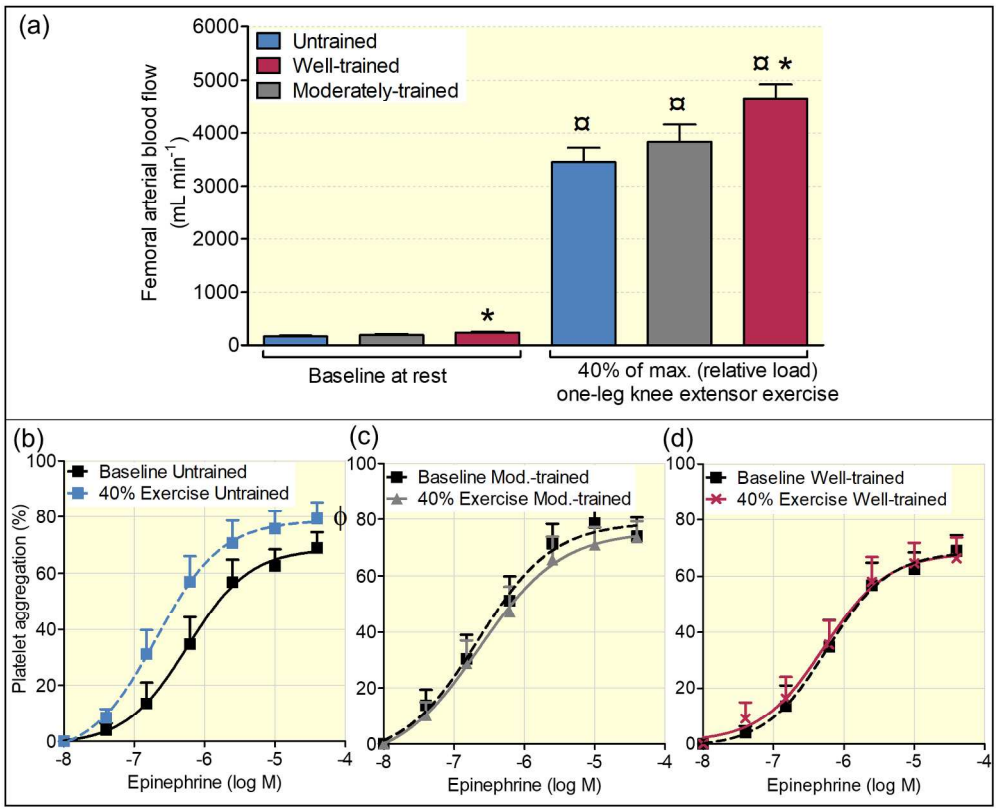

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