Life Sciences 237 (2019) 116896

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Physical exercise inhibits atherosclerosis development by regulating the expression of neuropeptide Y in apolipoprotein E-deficient mice

Wei-qiang Wu[a,1], Song Peng[a,1], Xiao-qin Wan[a], Shu Lin[a,b], Lin-yu Li[a,**], Zhi-yuan Song[a,*]

[a] Department of Cardiology, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing, China
[b] School of Medicine, University of Wollongong and Illawarra Health and Medical Research Institute, NSW, 2522, Australia

ARTICLE INFO

Keywords:
Neuropeptide Y
Atherosclerosis
Exercise
Neuropeptide Y receptor
Inflammation

ABSTRACT

Aims: Population-based studies have shown that exercise has anti-atherosclerotic effects, but the mechanisms underlying this cardiac protection are poorly understood. The aim of this study was to investigate if the anti-atherosclerotic effects of exercise are associated with changes in neuropeptide Y (NPY) expression in apolipoprotein E-deficient ($ApoE^{-/-}$) mice.

Main methods: Thirty-one male $ApoE^{-/-}$ mice were randomly divided into regular exercise (5 days/week), occasional exercise (1–2 days/week), and sedentary groups. After 8 weeks, atherosclerotic burden and plaque stability were measured by histological and morphological analysis. Quantitative real-time PCR and immunohistochemistry were used to measure the expression of NPY and its receptors in the aorta.

Key findings: Eight weeks of occasional exercise was equally effective as regular exercise at preventing atherosclerotic plaque formation and enhancing atherosclerotic plaque stability. This was shown by increased plaque collagen and smooth muscle cell content and decreased plaque lipid and macrophage content. The expression of NPY and its receptors in the vasculature was decreased in the regular exercise and occasional exercise groups, and this expression was significantly correlated with the progress of atherosclerosis. Moreover, exercise may reduce the activity of macrophages by down-regulating the expression of NPY Y1 receptors, thereby reducing the release of inflammatory cytokines.

Significance: These results suggest that exercise training can attenuate plaque burden and enhance atherosclerotic plaque stability. The anti-atherosclerotic effect of exercise appears to be, at least in part, dependent on down-regulation of the expression of NPY and its receptors (especially Y1 receptors) in the aorta.

1. Introduction

Existing laboratory and clinical evidence shows that regular physical exercise can reduce the incidence of coronary heart disease [1]. Exercise can reduce obesity, lower blood pressure, increase insulin sensitivity, and reduce inflammation, and is thus able to modify various risk factors of atherosclerotic cardiovascular disease [2]. However, the mechanism by which exercise exerts these cardioprotective effects remains unclear.

Neuropeptide Y (NPY) is a 36-amino-acid residue peptide often released from sympathetic nerve endings together with norepinephrine. NPY is the most abundant neuropeptide in the central and peripheral nervous systems [3]. In addition, NPY has been shown to be synthesized and released in endothelial cells (ECs), immune cells, and megakaryocytes/platelets [4]. To date, five NPY receptors (Y1–Y5) have been identified in mammals, all of which are G protein-coupled receptors [5]. NPY receptors are widely distributed in central and peripheral tissues, playing an indispensable role in appetite regulation and vasoconstriction [6].

In the cardiovascular system, many studies have identified that NPY and its receptors (especially Y1R, Y2R, and Y5R) are involved in the formation of atherosclerotic plaques [4,7,8]. In the periphery, NPY induces long-lasting vasoconstriction via the Y1R [9]. NPY can also stimulate vascular smooth muscle cell (VSMC) proliferation and hypertrophy via the Y1R [10]. In addition, NPY is also an important angiogenic factor, and can stimulate EC migration, proliferation, and differentiation into capillary-like tubes via the Y2R [11]. Furthermore, the Y5R is also involved in EC and VSMC proliferation [12]. Previous

* Corresponding author. No.30 Gaotanyan, Shapingba, Chongqing, 400038, China.
** Corresponding author. No.30 Gaotanyan, Shapingba, Chongqing, 400038, China.
E-mail addresses: 763064610@qq.com (L.-y. Li), zysong2010@126.com (Z.-y. Song).
[1] Authors contribute equally to this article.

https://doi.org/10.1016/j.lfs.2019.116896
Received 29 May 2019; Received in revised form 14 September 2019; Accepted 20 September 2019
Available online 09 October 2019
0024-3205/

studies have identified that SMC hyperplasia and angiogenesis were associated with a greater risk of plaque development [4,13]. In addition, NPY can mediate immunity and inflammatory responses via the Y1/Y2R. The binding of NPY to these receptors influences the migration and adhesion of macrophages, and induces macrophages to release various inflammatory cytokines such as interleukin (IL)-4, IL-12, tumor necrosis factor-alpha (TNF-α), and nitric oxide [14]. Furthermore, Li et al. identified that NPY and its receptors were highly up-regulated in human and murine atherosclerotic plaques compared to normal vascular tissue [4].

Exercise training can reduce sympathetic activation of the cardiovascular system, which is known to be the major source of circulating NPY [11,15]. In addition, many studies have shown that exercise can alter the expression of NPY in peripheral regions. However, there currently exists no consensus on the effects of exercise on the expression of NPY in peripheral regions [16,17]. Therefore, there exists a possible clinical association between NPY and the anti-atherosclerotic effect of physical exercise.

In the present study, we hypothesized that the effects of exercise on atherosclerosis are at least partially due to alterations in the expression of NPY and its receptors in the aorta. In addition, we explored the different effects of regular exercise and occasional exercise on atherosclerosis development.

2. Materials and methods

2.1. Experimental animals

A total of 31 male ApoE$^{-/-}$ mice on a C57BL/6 background (10 weeks old, 20–22 g) were purchased from the Animal Center of Beijing University (Beijing, China). Animals were fed a normal chow diet in the first week to facilitate adaptation to the environment, and were then fed a high fat chow diet (1.25% cholesterol and 20% fat) for the next 11 weeks. Mice were kept in standard cages at 22 °C with a 12 h light-dark cycle and were allowed unlimited access to food and water. All animal experimental protocols were approved by the Third Military Medical University (Army Medical University) Animal Care Committee and were in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication number 8023).

2.2. Exercise protocol

After 3 weeks on a high fat diet, 31 male ApoE$^{-/-}$ mice were randomly divided into regular exercise (n = 11), occasional exercise (n = 10), and sedentary groups (n = 10). Mice assigned to the regular exercise group were run on a motorized rodent treadmill (ZH-PT, Anhui, China) 5 days a week. The mice were progressively familiarized with running during the first week, beginning with a 30-min training period at a speed of 10 m/min on day 1 and ending with a 50-min training period at a speed of 14 m/min on day 5. Each day, the duration and speed of the exercise were increased by 5 min and 1 m/min, respectively [18]. During weeks 2–8, 5 days a week, the regular exercise group were subjected to a 60-min training period at a speed of 15 m/min with 2-min rest intervals every 15 min. The exercise speed and duration of the occasional exercise group were the same as the regular exercise group, but these animals were only exercised 1–2 days a week (the number of days was determined by a random number table). Mice assigned to the sedentary group were confined to their cages throughout the study.

2.3. Tissue preparation and collection

At the end of the exercise protocol, the mice were weighed. They were then anesthetized with 1% sodium pentobarbital and blood from the left ventricle of the heart was collected. Plasma was obtained by centrifugation at 3000 rpm for 20 min, and was divided into two portions. One portion was used to measure lipid concentrations (plasma total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured by enzymatic assays), and the other was stored at −80 °C for enzyme-linked immunosorbent assay (ELISA) analysis of NPY. The aorta (n = 5 per group) and heart (n = 8 per group) were dissected and fixed in 4% paraformaldehyde overnight. The whole aorta was stained with Oil Red O (Solarbio, China), and the aortic root was subsequently embedded in optimum cutting temperature compound for cryosectioning. Following this, 10 μm-thick frozen sections of the aortic sinus were collected and stored at −80 °C. The other whole aorta were stored in RNAlater™ Stabilization Solution (Invitrogen, USA) for mRNA measurement.

2.4. Histology and immunohistochemistry

The en face aorta was stained with Oil Red O and the percentage lesion area was measured using Image J software. The atherosclerotic areas, lipid deposition, and collagen content of the aortic root cross-section were identified by hematoxylin and eosin staining (Solarbio, China), Oil Red O staining, and Masson's trichome staining (Solarbio, China), respectively. The mean atherosclerotic area of the aortic root was calculated from eight different mice, using ten serial cryosections from each mouse. The lipid deposition and collagen content were expressed as the ratio of the positively-stained area to the total plaque area of the aortic sinus.

To measure the SMC, macrophage, NPY, and Y1R content of atherosclerotic plaques, sections were respectively incubated with rabbit anti-α smooth muscle actin (1:200, α-SMA, Abcam, UK), rat anti-monocyte + macrophage (1:150, MOMA-2, Abcam, UK), rabbit anti-NPY (1:1500, Abcam, UK), and rabbit anti-NPY1R (1:300, Bioss, China) primary antibodies. The sections were then incubated with appropriate secondary antibodies. The macrophage, SMC, NPY, and Y1R contents of each lesion were evaluated by expressing the positively-stained area as a percentage of the total area of the lesion.

2.5. Quantitative real-time PCR

In order to measure the mRNA expression of NPY, NPY receptors, and inflammatory cytokines in the aorta, total RNA was extracted from the whole aorta using TRNzol Reagent (Tiangen, China). RNA purity and concentration were measured using a spectrophotometer. Reverse transcription and cDNA synthesis were performed using FastKing gDNA Dispelling RT SuperMix (Tiangen, China) according to the manufacturer's instructions. The mRNA expression of NPY, NPY1R, NPY2R, NPY5R, dipeptidyl peptidase IV (DPPIV), and various cytokines was quantified by quantitative real-time PCR (qRT-PCR) with SuperReal PreMix Plus (SYBR Green) (Tiangen, China), and β-actin was employed as a housekeeping gene. The sequences of the forward and reverse primers are shown in Table 1. The relative gene expression was calculated using the $2^{-\triangle\triangle CT}$ method.

3. Statistical analysis

Data are expressed as mean ± standard error of the mean and statistical analysis was conducted using SPSS 23.0 (SPSS, Inc., Chicago, IL, USA). For normally distributed data with equal variances, one-way ANOVA and Tukey's post-hoc test were used to analyze differences between groups. If data were not normally distributed or the group variances were unequal, the Kruskal-Wallis test was used to assess differences between groups. For all statistical analyses, a P value < 0.05 was considered statistically significant.

Table 1
qRT-PCR Forward/Reverse(F/R) primers sequences.

Primers	Sequences 5′-3′
NPY	F: 5′- GTGGACTGACCCTCGCTCTA -3′
	R: 5′- TCAGTGTCTCAGGGCTGGAT-3′
NPY1R	F: 5′- CAGTAAGTACAGGTCCAGTGAG -3′
	R: 5′- GTCGAACACAGTGTTGAAGATG -3′
NPY2R	F: 5′- CGGTACAAGTGTCCACAATAAC-3′
	R: 5′- CAATGATCAGGAAGCTGATTCG-3′
NPY5R	F: 5′- TTTCCAGTCTGGGAGGACTATA-3′
	R: 5′- GATTGCGCTTTTTCATAACAGC -3′
DPPIV	F: 5′- CCAATTCCAGAAGACAACCTTG-3′
	R: 5′- CATCTGCCGTTCCATGAATAAG -3′
ICAM-1	F: 5′-CTGAAAGATGAGCTCGAGAGTG-3′
	R: 5′-AAACGAATACACGGTGATGGTA-3′
VCAM-1	F: 5′-GACATTTACCCAGTTTACAGGC-3′
	R: 5′-TGACGGGAGTAAAGGTTACTTC-3′
TNF-α	F: 5′-ATGTCTCAGCCTCTTCTCATTC-3′
	R: 5′-GCTTGTCACTCGAATTTTGAGA-3′
IL-4	F: 5′- TACCAGGAGCCATATCCACGGATG-3′
	R: 5′- TGTGGTGTTCTTCGTTGCTGTGAG -3′
IL-12	F: 5′- TGAGAAGTATTCAGTGTCCTGC -3′
	R: 5′- CTGTGAGTTCTTCAAAGGCTTC -3′
IFN-γ	F: 5′- CTTGAAAGACAATCAGGCCATC-3′
	R: 5′- CTTGGCAATACTCATGAATGCA-3′
β-Actin	F: 5′- GTGCTATGTTGCTCTAGACTTCG -3′
	R: 5′- ATGCCACAGGATTCCATACC -3′

NPY, neuropeptide Y; NPY1R, neuropeptide Y1 receptor; NPY2R, neuropeptide Y2 receptor; NPY5R, neuropeptide Y5 receptor; DPPIV, dipeptidyl peptidase IV; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; TNF-α, tumor necrosis factor alpha; IL-4, interleukin 4; IL-12, interleukin 12; IFN-γ, interferon gamma; β-Actin, beta-actin.

4. Result

4.1. Exercise had no effect on the body weight or serum lipid profiles of $ApoE^{-/-}$ mice

As shown in Table 2, after 8 weeks of exercise, the serum lipid profiles (TC, TG, HDL-C, and LDL-C) of the three groups were similar. In addition, no significant differences were noted between the exercise and sedentary groups with respect to body weight or serum glucose concentration.

4.2. Exercise attenuated atherosclerotic plaque growth in $ApoE^{-/-}$ mice

As shown in Fig. 1, the cross-sectional plaque area of the aortic sinus was significantly reduced in the two exercise groups compared to the sedentary group (Fig. 1A and B). Moreover, the Oil Red O-stained en face aorta showed a significant decrease in plaque burden in the regular and occasional exercise groups compared to the sedentary group (Fig. 1C and D). However, there was no significant difference in plaque burden between the regular exercise group and the occasional exercise group.

4.3. Exercise enhanced atherosclerotic plaque stability in $ApoE^{-/-}$ mice

The intraplaque lipid content was significantly lower in the two exercise groups than in the sedentary group (Fig. 2A). Furthermore, the plaque collagen content was significantly increased in the mice that received regular exercise compared to the sedentary mice (Fig. 2B). Immunohistochemical analysis showed that the plaque SMC content (α-SMA-positive area) was significantly higher in the two exercise groups than in the sedentary group. In contrast, the plaque macrophage content (MOMA-2-positive area) was significantly lower in the regular and occasional exercise groups (Fig. 2C and D). These data suggest that exercise increases plaque SMC and collagen content and decreases plaque lipid and macrophage content, enhancing the stability of atherosclerotic plaques.

4.4. NPY was involved in the anti-atherosclerotic effect of exercise

In order to determine if atherosclerosis is associated with elevated circulating levels of NPY, plasma NPY level was measured by ELISA. The regular exercise and occasional exercise groups exhibited similar plasma NPY levels. Furthermore, the sedentary group exhibited a significantly higher plasma NPY level (Fig. 3C). Interestingly, we also found that plasma NPY level was significantly correlated with the percentage en face lesion area of the entire aorta (Fig. 3D). Similarly, immunohistochemical and qRT-PCR results showed that the regular exercise and occasional exercise groups exhibited significantly decreased NPY expression in atherosclerotic plaques, which was consistent with the attenuation of atherosclerotic plaque growth by exercise in ApoE −/− mice (Fig. 3).

We further investigated whether NPY receptors were involved in the exercise-induced regulation of atherosclerotic plaques. PCR results showed that the mRNA expression of NPY1R, NPY2R, NPY5R, and DPPIV in the aorta was significantly down-regulated in the two exercise groups compared to the sedentary group (Fig. 3E and F). The regular exercise group exhibited significantly lower aortic NPY and NPY5R mRNA expression compared to the occasional exercise group. The mRNA expression of NPY1R, NPY2R, and DPPIV was also lower in the regular exercise group than in the occasional exercise group, although these differences were not statistically significant (Fig. 3E and F).

4.5. Exercise reduced Y1R expression, inhibiting macrophage activation and inflammatory cytokine expression in atherosclerotic plaques

Macrophages play a key role in atherosclerosis by accumulating cholesterol and producing inflammatory mediators and cytokines [19]. Y1R-deficient mice exhibited a reduced inflammatory response, suggesting the involvement of Y1Rs in inflammation [20]. Immunohistochemistry confirmed that the expression of Y1Rs in the exercise group was significantly lower than that in the sedentary group, especially in plaque areas with high macrophage expression, suggesting that Y1Rs may be involved in regulating the activity of macrophages in atherosclerotic plaques (Fig. 4A and B). Y1Rs have been shown to be involved in the release of inflammatory cytokines by macrophages [14]. Therefore, we investigated whether the expression of inflammatory cytokines in the aorta was affected by 8 weeks of exercise. As expected, the expression of inflammatory cytokines (intercellular adhesion molecule 1, TNF-α, IL-4, and IL-12) was significantly lower in the regular exercise group and occasional exercise group than in the sedentary

Table 2
Body weight, serum lipid profiles and glucose concentration.

Groups	BW (g)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	Glucose (mmol/L)
Regular	27.76 ± 0.59	5.94 ± 0.50	0.21 ± 0.02	0.62 ± 0.03	4.96 ± 0.42	3.77 ± 0.20
Occasional	28.50 ± 0.65	6.00 ± 0.64	0.26 ± 0.04	0.54 ± 0.04	4.94 ± 0.48	3.06 ± 0.31
Sedentary	29.10 ± 0.15	7.14 ± 0.29	0.24 ± 0.02	0.56 ± 0.02	5.80 ± 0.18	3.72 ± 0.17
P	ns	ns	ns	ns	ns	ns

BW: body weight; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; ns: not significant.

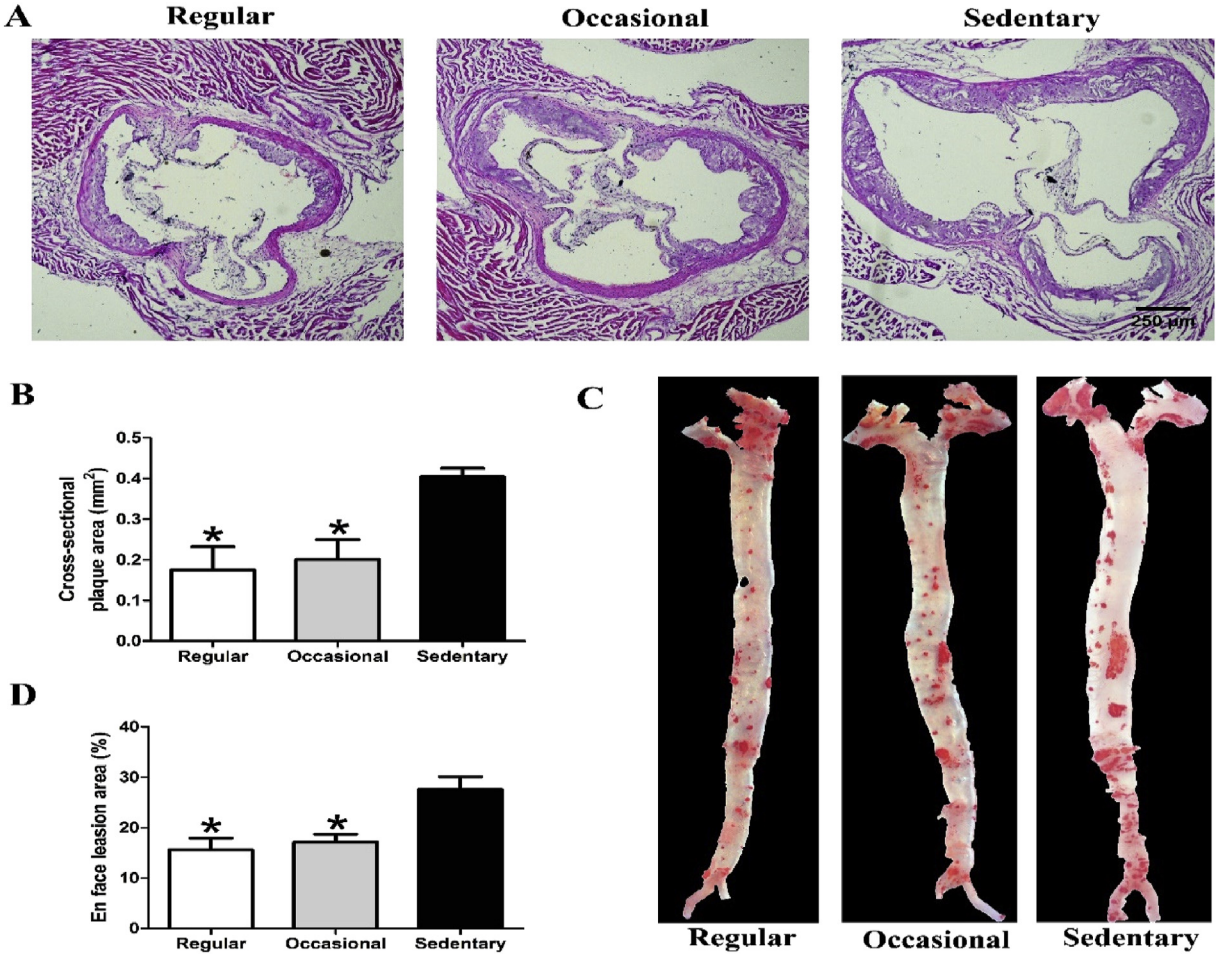

Fig. 1. Exercise attenuated atherosclerotic plaque growth in ApoE −/− mice. (A) Representative cross-sectional images of the aortic root plaques by H&E staining. (B) Quantitative of cross-sectional plaque area in aortic roots. (C) Representative images of Oil Red O staining of en face aorta. (D) Quantitative analysis of en face aorta lesions area of the entire aorta. $^{*}P < 0.05$ vs. Sedentary group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

group (Fig. 4C–E). These data suggest that exercise reduces the expression of Y1Rs, thus inhibiting the activation of macrophages and the expression of inflammatory cytokines in plaques.

5. Discussion

To the best of our knowledge, this is the first study to directly compare the anti-atherosclerotic effects of regular and occasional exercise in ApoE$^{-/-}$ mice. Our results also show for the first time that the beneficial effects of physical exercise may be mediated, at least in part, by alterations in the NPY-related pathway in this mouse model. Exercise reduces the activity of macrophages through the NPY Y1R pathway, which could potentially underlie the anti-atherosclerotic effects of exercise.

It is well known that physical exercise can prevent and treat human atherosclerotic cardiovascular disease [21]. Compelling evidence has identified that physical exercise improves lipid metabolism, insulin resistance, and glucose intolerance, and reduces inflammation, ultimately preventing and reducing many established atherosclerotic risk factors [21,22]. In addition, Pascal et al. also demonstrated that long-term exercise stabilized atherosclerotic plaques in ApoE$^{-/-}$ mice [23]. However, this anti-atherosclerotic effect was observed only in response to regular exercise [23,24]. Our data provide the first evidence that 8 weeks of occasional exercise was equally effective as regular exercise at preventing atherosclerotic plaque formation and enhancing atherosclerotic plaque stability. This was shown by increased plaque collagen and SMC content and decreased plaque lipid and macrophage content. This may indicate that there is no significant dose-response curve between medium-term exercise and atherosclerosis under high-fat diet conditions. Atherosclerosis is a complex multifactorial chronic disease, and diet and exercise have a close relationship in the prevention of atherosclerosis. Previous studies have found that a high fat diet significantly increased serum TC, TG, LDL, and very low-density lipoprotein, major risk factors of atherosclerosis [25,26]. These effects contrast with the anti-atherosclerotic effects of exercise. Cesar et al. reported that exercise did not reduce plaque accumulation in ApoE$^{-/-}$ mice fed a high fat diet compared to sedentary mice [25]. The anti-atherosclerotic effects of regular exercise may therefore be significantly decreased by a high fat diet which elevates the levels of atherogenic lipids. Clearly, further studies are needed to address this point.

The precise molecular mechanisms underlying the anti-atherosclerotic effect of exercise are largely unknown. Previous studies have suggested that NPY may play a critical role in the effects of exercise on energy balance and myocardial ischemia [27,28]. In the present study, we investigated if NPY is involved in the mechanism underlying the anti-atherosclerotic effect of exercise. Exercise can reduce sympathetic modulation of the cardiovascular system [15]. Additionally, the major source of circulating NPY in vivo is the sympathetic nerves [11]. Furthermore, many animal and human studies have suggested that NPY and its receptors are involved in mechanisms related to the development of atherosclerosis [4,7]. Shah et al. identified that NPY gene polymorphisms increased the risk of early-onset atherosclerosis due to

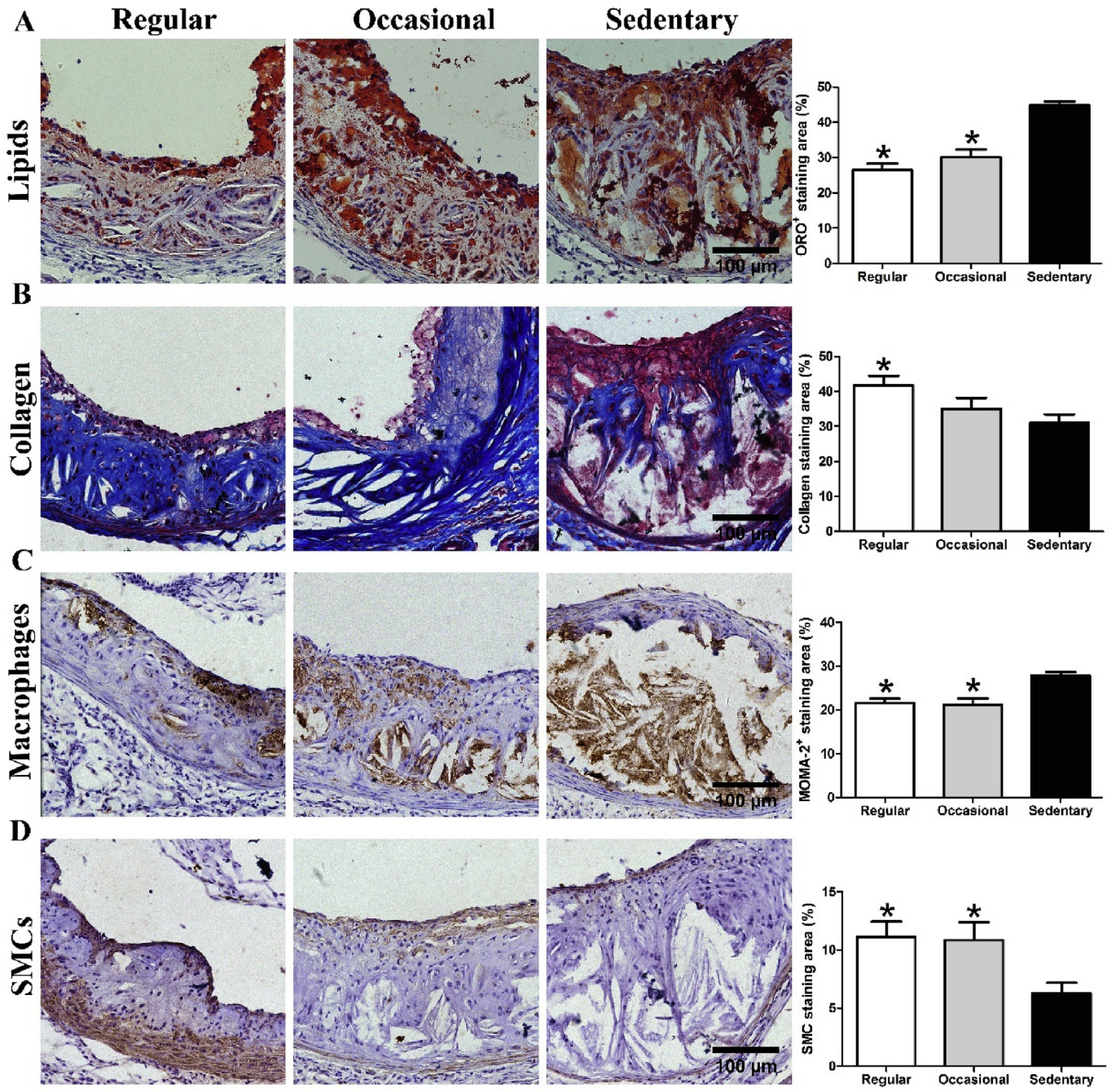

Fig. 2. Exercise enhanced atherosclerotic plaque stability in ApoE−/− mice. Representative image and quantification of the plaque composition of lipid (A), collagen (B), macrophages (C), smooth muscle cells (D). *$P < 0.05$ vs. sedentary group.

elevated plasma NPY levels [8]. Li et al. found that NPY and its receptors were highly up-regulated in human and murine atherosclerotic plaques compared to normal vascular tissue, especially in the SMCs, ECs, and macrophages/foam cells within these plaques [4]. In addition, Lagraauw et al. identified that NPY expression was increased up to 2-fold in unstable human plaques compared to stable plaques. In ApoE$^{-/-}$ mice, overexpression of NPY in the carotid artery significantly increased atherosclerotic plaque size compared to controls [7]. Previous work from our group has demonstrated that NPY can mediate VSMC proliferation via the Y1R [29]. The Y1R also mediates NPY-induced vasoconstriction which contributes to vascular tone by enhancing the action of norepinephrine and epinephrine [30]. Moreover, Han et al. identified that NPY was responsible for approximately 30% of sympathetic nerve-mediated vasoconstriction in the mesenteric vascular bed [31]. NPY also substantially stimulates neointima formation via the Y1R [32]. In addition, NPY is a potent angiogenic factor, and activates multiple steps of angiogenesis via the Y2R [6]. Zukowska-Grojec et al. reported the first evidence that NPY promotes vessel sprouting and adhesion, migration, and capillary tube formation by ECs [11]. Additionally, recent studies have demonstrated that Y2R knockout mice exhibit significantly diminished NPY-induced angiogenesis compared to wild type mice [33,34]. Furthermore, the Y5R plays an additional, though lesser, role in promoting neointimal hyperplasia and angiogenesis [4]. DPPIV is an enzyme which converts NPY1-36 to NPY3-36. As a result, NPY is unable to bind to the Y1R but retains affinity for the Y2R and Y5R, further promoting the angiogenic

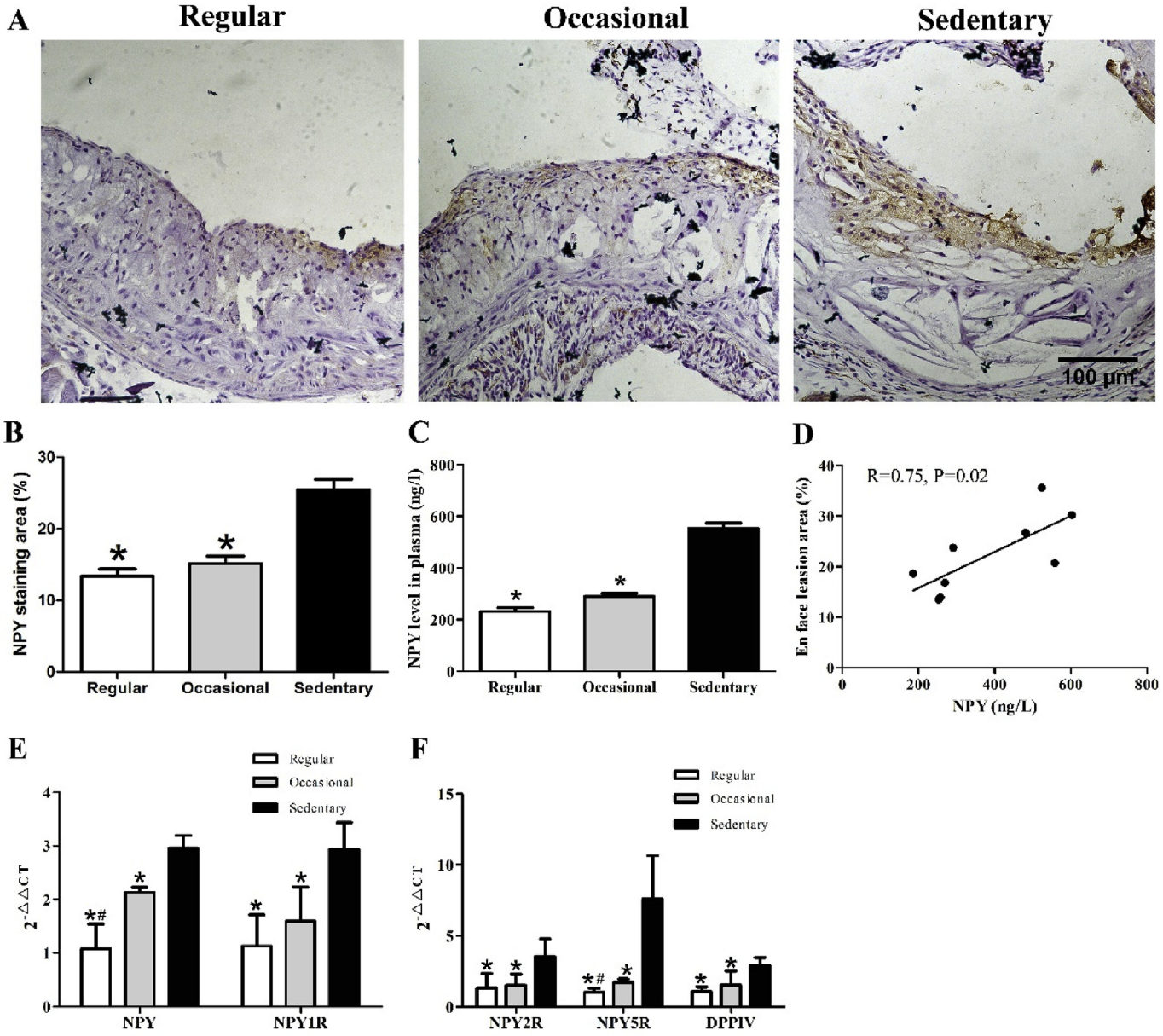

Fig. 3. Exercise regulated NPY and its receptors expression in ApoE−/− mice. (A) Representative immunohistochemical staining of NPY in atherosclerotic lesions with different groups. (B) Charts showing percentage of NPY in atherosclerotic lesions with different groups. (C) Plasma NPY expressions were measured by ELISA. (D) The correlation of plasma NPY and the percentage of en face lesion area. (E–F) The mRNA expression of NPY, NPY1R, NPY2R, NPY5R and DPPIV in aorta were measured by qRT-PCR. *$P < 0.05$ vs Sedentary group; #$P < 0.05$ vs occasional exercise group.

response [35,36]. It has been shown that a hypervascularized plaque is more likely to develop an intraplaque hemorrhage, which promotes further plaque growth and aggravates plaque instability [13]. Our results showed that exercise could significantly reduce plasma NPY level. We also demonstrated that the expression of NPY, NPY1R, NPY2R, NPY5R, and DPPIV in atherosclerotic plaques was lower in trained mice than in sedentary mice, which was consistent with the decrease in plasma NPY. Furthermore, we identified a significant positive correlation between plasma NPY level and the percentage en face lesion area of the entire aorta. Taken together, these results strongly suggest a potential role of NPY in the anti-atherosclerotic effect of exercise.

With respect to the pathophysiology of atherosclerosis, inflammation plays a key role in plaque development and destabilization [37]. NPY is a potent pro-inflammatory peptide with the ability to directly modulate the activity of macrophages and induce the release of various inflammatory cytokines [14]. NPY itself has been shown to modulate the recruitment of monocytes by acting as a chemoattractant at a physiological concentration of 10^{-8} to 10^{-10} M [38]. In addition, NPY has also been shown to increase the adherence capacity of peripheral blood monocytes in pathological conditions [39]. In the present study, we found that the exercise-induced decreases in plasma NPY level and atherosclerotic plaque formation were accompanied by a decrease in the macrophage content of atherosclerotic plaques. This suggests that the exercise-induced reduction of plaque macrophage content may be related to a decrease in NPY expression. Of the five receptors, the Y1R plays the greatest role in inflammation [14]. Hassani et al. demonstrated significantly reduced inflammatory responses in Y1R-deficient mice and mice administered a Y1 antagonist [20]. In addition, macrophages from Y1R-deficient mice produced less TNF-α and IL-12 [40]. Zhou et al. also found that NPY promoted TGF-β1 production in Raw264.7 cells via the Y1R [41]. In the present study, we found that exercise reduced the expression of Y1Rs in plaque areas with high

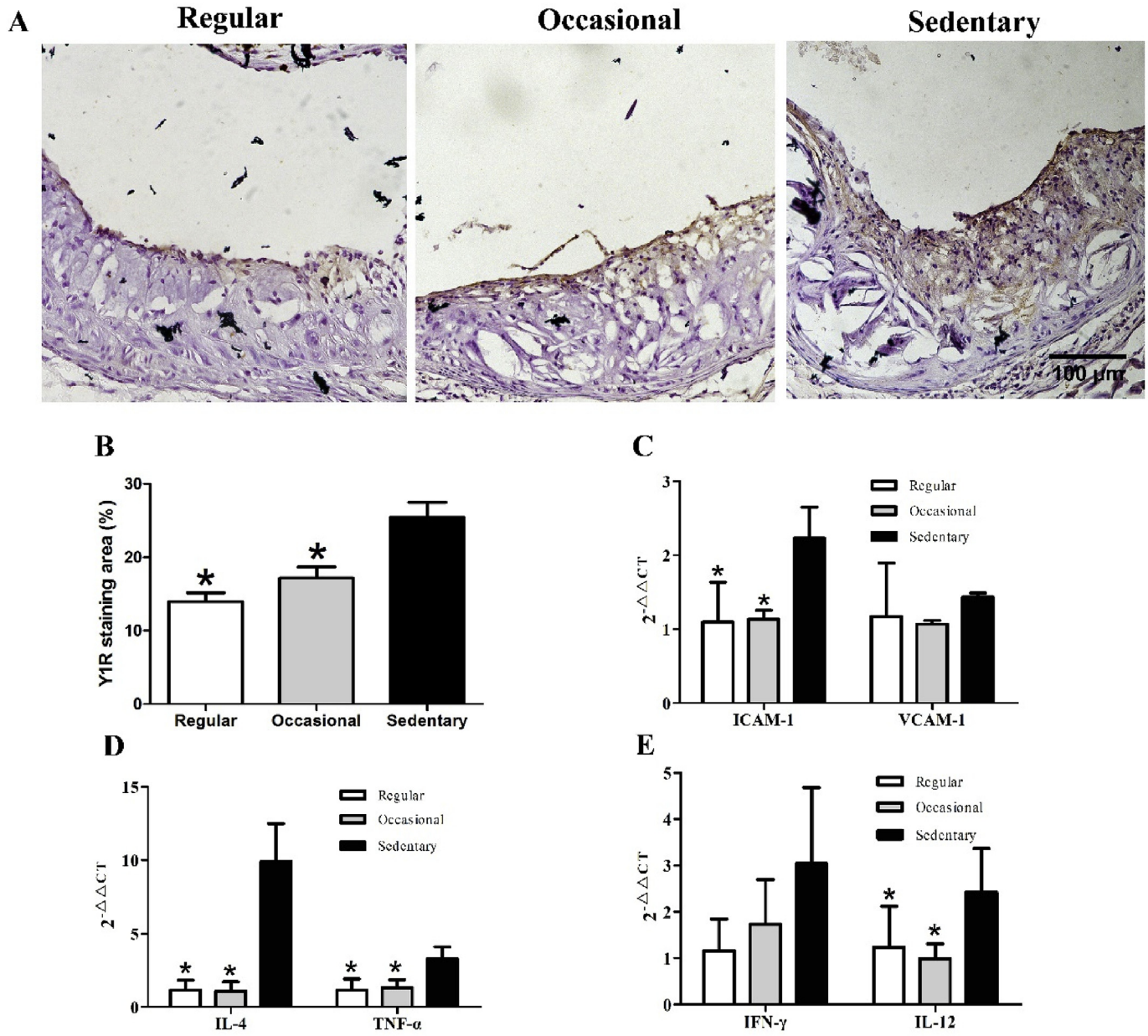

Fig. 4. Effects of exercise on the expression of Y1R and pro-inflammatory cytokines. (A) Representative immunohistochemical staining of Y1R in atherosclerotic lesions with different treatments. (B) Charts showing percentage of Y1R in atherosclerotic lesions with different treatments. (C–E) The mRNA expression of ICAM-1, VCAM-1, IL-4, TNF-α, IFN-γ and IL-12 were measured by qRT-PCR. *$P < 0.05$ vs. sedentary group.

macrophage expression. Furthermore, the expression of inflammatory cytokines in plaques was also significantly reduced after exercise. Taken together, these results suggest that exercise may reduce macrophage migration, adhesion, and activity by down-regulating the expression of NPY and Y1Rs, thereby reducing the release of inflammatory cytokines and retarding the progress of atherosclerosis.

In the present study, we examined the anti-atherosclerotic effect of only medium-term exercise (8 weeks) under high fat diet conditions, so the dose-response curve between exercise and atherosclerosis could not be ascertained. Furthermore, the forced running mouse model entails stress and is considered a non-physiological model. Further studies combining diet and voluntary exercise protocols should be conducted to examine the anti-atherosclerotic effect of physical exercise. Finally, it should be noted that experimental models of atherosclerosis, including ApoE$^{-/-}$ mice, cannot exactly reproduce the pathophysiology of human atherosclerosis, especially plaque instability. As a result, human studies may be required to further confirm our results.

6. Conclusions

In conclusion, we here demonstrate that occasional exercise and regular exercise were equally effective at preventing atherosclerotic plaque formation and enhancing atherosclerotic plaque stability under high fat diet conditions. Exercise training decreased the expression of NPY and its receptors in atherosclerotic plaques, and this was significantly correlated with the progress of atherosclerosis. In addition, exercise may reduce the activity of macrophages through the Y1R, thereby inhibiting the release of pro-inflammatory mediators. The above results identify NPY as an important factor in the regulation of atherosclerosis by exercise.

Funding

This work was supported by National Natural Science Foundation of China (No. 81700694).This work was also supported by Southwest

Hospital Project (No. SWH2018LJ-12).

Author contributions

WQW, SP, SL, LYL, ZYS designed the study methodology. WQW and SP collected the data and analyzed the results. WQW and SL drafted the article. All authors reviewed and revised the work. All authors reviewed the final article and approved it for submission.

Declaration of competing interest

There is no conflict of interest to declare.

References

[1] H.D. Sesso, R.S. Paffenbarger, I. Lee, Physical activity and coronary heart disease in men, Circulation 102 (9) (2000) 975–980, https://doi.org/10.1161/01.CIR.102.9.975.

[2] N.P.E. Kadoglou, F. Iliadis, C.D. Liapis, Exercise and carotid atherosclerosis, EUR J VASC ENDOVASC 35 (3) (2008) 264–272, https://doi.org/10.1016/j.ejvs.2007.08.022.

[3] Z. Zukowska-Grojec, A.C. Vaz, Role of neuropeptide Y (NPY) in cardiovascular responses to stress, Synapse 2 (3) (1988) 293–298, https://doi.org/10.1002/syn.890020319.

[4] L. Li, A.H. Najafi, J.B. Kitlinska, R. Neville, J. Laredo, S.E. Epstein, M.S. Burnett, Z. Zukowska, Of mice and men: neuropeptide Y and its receptors are associated with atherosclerotic lesion burden and vulnerability, J. Cardiovasc. Transl. 4 (3) (2011) 351–362, https://doi.org/10.1007/s12265-011-9271-5.

[5] K. Abe, L. Kuo, Z. Zukowska, Neuropeptide Y is a mediator of chronic vascular and metabolic maladaptations to stress and hypernutrition, Exp. Biol. Med. 235 (10) (2010) 1179–1184, https://doi.org/10.1258/ebm.2010.009136.

[6] Z. Zukowska, Atherosclerosis and angiogenesis: what do nerves have to do with it? Pharmacol. Rep. 57 (Suppl) (2005) 229–234.

[7] H.M. Lagraauw, M.M. Westra, M. Bot, A. Wezel, P.J. van Santbrink, G. Pasterkamp, E.A.L. Biessen, J. Kuiper, I. Bot, Vascular neuropeptide Y contributes to atherosclerotic plaque progression and perivascular mast cell activation, Atherosclerosis 235 (1) (2014) 196–203, https://doi.org/10.1016/j.atherosclerosis.2014.04.025.

[8] S.H. Shah, N.J. Freedman, L. Zhang, D.R. Crosslin, D.H. Stone, C. Haynes, J. Johnson, S. Nelson, L. Wang, J.J. Connelly, M. Muehlbauer, G.S. Ginsburg, D.C. Crossman, C.J. Jones, J. Vance, M.H. Sketch, C.B. Granger, C.B. Newgard, S.G. Gregory, P.J. Goldschmidt-Clermont, W.E. Kraus, E.R. Hauser, Neuropeptide Y gene polymorphisms confer risk of early-onset atherosclerosis, PLoS Genet. 5 (1) (2009) e1000318, , https://doi.org/10.1371/journal.pgen.1000318.

[9] Y. Zhou, W. Shi, H. Luo, R. Yue, Z. Wang, W. Wang, L. Liu, W.E. Wang, H. Wang, C. Zeng, Inhibitory effect of D1-like dopamine receptors on neuropeptide Y-induced proliferation in vascular smooth muscle cells, Hypertens. Res. 38 (12) (2015) 807–812, https://doi.org/10.1038/hr.2015.84.

[10] J. Pons, J. Kitlinska, H. Ji, E.W. Lee, Z. Zukowska, Mitogenic actions of neuropeptide Y in vascular smooth muscle cells: synergetic interactions with the β-adrenergic system, Can. J. Physiol. Pharm. 81 (2) (2003) 177–185, https://doi.org/10.1139/y02-166.

[11] Z. Zukowska-Grojec, E. Karwatowska-Prokopczuk, W. Rose, J. Rone, S. Movafagh, H. Ji, Y. Yeh, W.T. Chen, H.K. Kleinman, E. Grouzmann, D.S. Grant, Neuropeptide Y: a novel angiogenic factor from the sympathetic nerves and endothelium, Circ. Res. 83 (2) (1998) 187–195, https://doi.org/10.1161/01.RES.83.2.187.

[12] L. Li, E.W. Lee, H. Ji, Z. Zukowska, Neuropeptide Y–induced acceleration of post-angioplasty occlusion of rat carotid artery, Arterioscler. Thromb. Vasc. Biol. 23 (7) (2003) 1204–1210, https://doi.org/10.1161/01.ATV.0000071349.30914.25.

[13] R. Virmani, F.D. Kolodgie, A.P. Burke, A.V. Finn, H.K. Gold, T.N. Tulenko, S.P. Wrenn, J. Narula, Atherosclerotic plaque progression and vulnerability to rupture, Arterioscler. Thromb. Vasc. Biol. 25 (10) (2005) 2054–2061, https://doi.org/10.1161/01.ATV.0000178991.71605.18.

[14] B. Chandrasekharan, B.G. Nezami, S. Srinivasan, Emerging neuropeptide targets in inflammation: NPY and VIP, Am. J. Physiol-Gastr L. 304 (11) (2013) G949–G957, https://doi.org/10.1152/ajpgi.00493.2012.

[15] M. Bertagnolli, P.C. Schenkel, C. Campos, C.T. Mostarda, D.E. Casarini, A. Bello-Klein, M.C. Irigoyen, K. Rigatto, Exercise training reduces sympathetic modulation on cardiovascular system and cardiac oxidative stress in spontaneously hypertensive rats, Am. J. Hypertens. 21 (11) (2008) 1188–1193, https://doi.org/10.1038/ajh.2008.270.

[16] S.A. Benite-Ribeiro, D.A. Putt, J.M. Santos, The effect of physical exercise on orexigenic and anorexigenic peptides and its role on long-term feeding control, Med. Hypotheses 93 (2016) 30–33, https://doi.org/10.1016/j.mehy.2016.05.005.

[17] R. Rämson, J. Jürimäe, T. Jürimäe, J. Mäestu, The effect of 4-week training period on plasma neuropeptide Y, leptin and ghrelin responses in male rowers, Eur. J. Appl. Physiol. 112 (5) (2012) 1873–1880, https://doi.org/10.1007/s00421-011-2166-y.

[18] M. Pynn, K. Schäfer, S. Konstantinides, M. Halle, Exercise training reduces neointimal growth and stabilizes vascular lesions developing after injury in apolipoprotein E–deficient mice, Circulation 109 (3) (2004) 386–392, https://doi.org/10.1161/01.CIR.0000109500.03050.7C.

[19] N. Shibata, C.K. Glass, Regulation of macrophage function in inflammation and atherosclerosis: fig. 1, J. Lipid Res. 50 (Supplement) (2009) S277–S281, https://doi.org/10.1194/jlr.R800063-JLR200.

[20] H. Hassani, G. Lucas, B. Rozell, P. Ernfors, Attenuation of acute experimental colitis by preventing NPY Y1 receptor signaling, Am. J. Physiol-Gastr L. 288 (3) (2005) G550–G556, https://doi.org/10.1152/ajpgi.00182.2004.

[21] P.D. Thompson, D. Buchner, I.L. Piña, G.J. Balady, M.A. Williams, B.H. Marcus, K. Berra, S.N. Blair, F. Costa, B. Franklin, G.F. Fletcher, N.F. Gordon, R.R. Pate, B.L. Rodriguez, A.K. Yancey, N.K. Wenger, Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease, Arterioscler. Thromb. Vasc. Biol. 23 (8) (2003), https://doi.org/10.1161/01.ATV.0000089628.63625.D4.

[22] A. Niessner, B. Richter, M. Penka, S. Steiner, B. Strasser, S. Ziegler, E. Heeb-Elze, G. Zorn, A. Leitner-Heinschink, C. Niessner, J. Wojta, K. Huber, Endurance training reduces circulating inflammatory markers in persons at risk of coronary events: impact on plaque stabilization? Atherosclerosis 186 (1) (2006) 160–165, https://doi.org/10.1016/j.atherosclerosis.2005.06.047.

[23] M. PELLEGRIN, C. MIGUET-ALFONSI, K. BOUZOURENE, J. AUBERT, V. DECKERT, A. BERTHELOT, L. MAZZOLAI, P. LAURANT, Long-term exercise stabilizes atherosclerotic plaque in ApoE knockout mice, Med. Sci. Sport. Exerc. 41 (12) (2009) 2128–2135, https://doi.org/10.1249/MSS.0b013e3181a8d530.

[24] J. Szostak, C. Miguet-Alfonsi, A. Berthelot, P. Laurant, Training-induced anti-atherosclerotic effects are associated with increased vascular PPARgamma expression in apolipoprotein E-deficient mice, Acta Physiol. 216 (2) (2016) 221–230, https://doi.org/10.1111/apha.12615.

[25] L. Cesar, S.V. Suarez, J. Adi, N. Adi, R. Vazquez-Padron, H. Yu, Q. Ma, P.J. Goldschmidt-Clermont, A. Agatston, P. Kurlansky, K.A. Webster, An essential role for diet in exercise-mediated protection against dyslipidemia, inflammation and atherosclerosis in ApoE⁻/⁻ mice, PLoS One 6 (2) (2011) e17263, , https://doi.org/10.1371/journal.pone.0017263.

[26] A.S. LEON, O.A. SANCHEZ, Response of blood lipids to exercise training alone or combined with dietary intervention, Med. Sci. Sport. Exer 33 (Supplement) (2001) S502–S515, https://doi.org/10.1097/00005768-200106001-00021.

[27] S. Bi, K.A. Scott, J. Hyun, E.E. Ladenheim, T.H. Moran, Running wheel activity prevents hyperphagia and obesity in otsuka long-evans tokushima fatty rats: role of hypothalamic signaling, Endocrinology 146 (4) (2005) 1676–1685, https://doi.org/10.1210/en.2004-1441.

[28] L. Gullestad, B. Jorgensen, T. Bjuro, J. Pernow, J.M. Lundberg, C.D. Dota, C. Hall, S. Simonsen, B. Ablad, Postexercise ischemia is associated with increased neuropeptide Y in patients with coronary artery disease, Circulation 102 (9) (2000) 987–993, https://doi.org/10.1161/01.cir.102.9.987.

[29] Z. Jiang, Y. Zhou, X. Chen, L. Li, S. Liang, S. Lin, M. Shu, Different effects of neuropeptide Y on proliferation of vascular smooth muscle cells via regulation of Geminin, Mol. Cell. Biochem. 433 (1–2) (2017) 205–211, https://doi.org/10.1007/s11010-017-3028-7.

[30] B. Fallgren, P. Arlock, L. Edvinsson, Neuropeptide Y potentiates noradrenaline-evoked vasoconstriction by an intracellular calcium-dependent mechanism, J. Auton. Nerv. Syst. 44 (2–3) (1993) 151–159.

[31] S. Han, C.L. Yang, X. Chen, L. Naes, B.F. Cox, T. Westfall, Direct evidence for the role of neuropeptide Y in sympathetic nerve stimulation-induced vasoconstriction, Am. J. Physiol. 274 (1) (1998) H290–H294, https://doi.org/10.1152/ajpheart.1998.274.1.H290.

[32] L. Li, A. Jönsson-Rylander, K. Abe, Z. Zukowska, Chronic stress induces rapid occlusion of angioplasty-injured rat carotid artery by activating neuropeptide Y and its Y1 receptors, Arterioscler. Thromb. Vasc. Biol. 25 (10) (2005) 2075–2080, https://doi.org/10.1161/01.ATV.0000179601.19888.19.

[33] E.W. Lee, D.S. Grant, S. Movafagh, Z. Zukowska, Impaired angiogenesis in neuropeptide Y (NPY)-Y2 receptor knockout mice, Peptides 24 (1) (2003) 99–106, https://doi.org/10.1016/S0196-9781(02)00281-4.

[34] M. Koulu, S. Movafagh, J. Tuohimaa, U. Jaakkola, J. Kallio, U. Pesonen, Y. Geng, M. Karvonen, E. Vainio Jylhä, M. Pöllönen, K. Kaipio Salmi, H. Seppälä, E. Lee, R. Higgins, Z. Zukowska, Neuropeptide Y and Y2-receptor are involved in development of diabetic retinopathy and retinal neovascularization, Ann. Med. 36 (3) (2009) 232–240, https://doi.org/10.1080/07853890410031236.

[35] L. Li, H.U. Demuth, Z. Zukowska, Dipeptidyl peptidase IV: a molecular switch of vascular actions of neuropeptide Y, Adv. Exp. Med. Biol. 575 (2006) 135–140, https://doi.org/10.1007/0-387-32824-6_14.

[36] E.W. Lee, M. Michalkiewicz, J. Kitlinska, I. Kalezic, H. Switalska, P. Yoo, A. Sangkharat, H. Ji, L. Li, T. Michalkiewicz, M. Ljubisavljevic, H. Johansson, D.S. Grant, Z. Zukowska, Neuropeptide Y induces ischemic angiogenesis and restores function of ischemic skeletal muscles, J. Clin. Invest. 111 (12) (2003) 1853–1862, https://doi.org/10.1172/JCI16929.

[37] P. Libby, P.M. Ridker, G.K. Hansson, Progress and challenges in translating the biology of atherosclerosis, Nature 473 (7347) (2011) 317–325, https://doi.org/10.1038/nature10146.

[38] R.H. Straub, M. Mayer, M. Kreutz, S. Leeb, J. Schölmerich, W. Falk, Neurotransmitters of the sympathetic nerve terminal are powerful chemoattractants for monocytes, J LEUKOCYTE BIOL 67 (4) (2000) 553–558, https://doi.org/10.1002/jlb.67.4.553.

[39] K. Mitić, S. Stanojević, N. Kuštrimović, V. Vujić, M. Dimitrijević, Neuropeptide Y modulates functions of inflammatory cells in the rat: distinct role for Y1, Y2 and Y5 receptors, Peptides 32 (8) (2011) 1626–1633, https://doi.org/10.1016/j.peptides.2011.06.007.

[40] J. Wheway, C.R. Mackay, R.A. Newton, A. Sainsbury, D. Boey, H. Herzog, F. Mackay, A fundamental bimodal role for neuropeptide Y1 receptor in the immune system, J. Exp. Med. 202 (11) (2005) 1527–1538, https://doi.org/10.1084/jem.20051971.

[41] J. Zhou, Z. Xu, C. Jiang, Neuropeptide Y promotes TGF-β1 production in RAW264.7 cells by activating PI3K pathway via Y1 receptor, Neurosci. Bull 24 (3) (2008) 155–159, https://doi.org/10.1007/s12264-008-0130-6.