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Modulation of Vascular Endothelial Growth Factor and Annexin A2 in Response to 4-(Methylnitrosamino)-1-(3-pyridyl)-1-Butanone -Induced Inflammation via Swimming Training

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ABSTRACT

The nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; nicotine derived nitrosamine ketone) is one of the strongest carcinogens in tobacco which is involved in induction of lung cancer by changing the stimulation of vascular endothelial growth factor (VEGF) and annexin A2 expression. The aim of this study was to investigate the changes in resting levels of annexin A2 and VEGF in lung tissues of rats exposed NNK after 12 weeks of aerobic submaximal swimming training.

For this purpose, 46 Wistar rats were randomly divided into five groups consist of training, training + NNK, NNK, saline and control. NNK-induced groups received NNK subcutaneously one day per week at a rate of 12/5 mg per kg body weight and the training groups performed submaximal swimming training for 12 weeks. The levels of VEGF and annexin A2 in lung tissue were measured respectively by ELISA and immunohistochemistry. To analyze the data; ANOVA and Tukey's test were used at a significance level of $p<0.05$.

Findings indicated that 12 weeks submaximal swimming training decreased the levels of VEGF and annexin A2 in lung tissue significantly when compared to NNK group ($p<0.001$). There was no significant correlation between VEGF and annexin A2 levels in all study groups ($p\geq0.05$).

Generally, it could be confirmed that regular submaximal aerobic training plays an important role in inhibition of the effects of lung inflammation induced by NNK via decreased levels of VEGF and annexin A2.

Keywords: Annexin A2; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; Swimming; Vascular endothelial growth factor

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INTRODUCTION

Lung cancer, the leading cause of cancer death worldwide, is a complex disease that develops through

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a series of sequential genetic and epigenetic alterations in oncogenes and tumor suppressor genes, causing subtle alterations in growth control.[1] Generally, about 85–90% of lung cancer cases are attributable to cigarette smoking.[2] Aside from direct carcinogens, inhaled smoke contains other constituents that can stimulate chronic lung inflammatory responses.[3,4] There are more than 20 compounds that are lung carcinogens, such as nicotine derived nitrosamine ketone (NNK; or tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) that systemically induces tumors of the lung and also plays a major role in lung carcinogenesis.[5] As Barta et al reported that chronic extrinsic lung inflammation induced by NNK enhances lung tumorigenesis in mice.[6] The chronic inflammation may promote neoplasia by inducing cell proliferation, resistance to apoptosis, invasiveness, angiogenesis, and secretion of immune suppressive factors.[7]

Proteins that coordinate inflammation and the acute-phase response must mount a rapid, efficient defense against invading microorganisms at local and systemic levels.[8] In this regard, annexin A2 is a 36 kDa peripheral membrane protein that belongs to the annexin family, and localized at the extracellular surface of endothelial cells and various types of tumor cells especially in nonsmall cell lung carcinoma.[9] As, Yao et al have found the increased expression of annexin A2 at both the protein and mRNA levels in lung cancer,[10] particularly in the more aggressive or poorer prognosis phenotype.[11] Luo et al showed that overexpression of annexin A2 is closely associated with proliferation and invasion in non-small cell lung cancer. Analogously, the elevated expression of annexin A2 was also detected in lymph node metastatic tissues of lung cancers.[12]

In addition, annexin A2 also participates in vascular endothelial growth factor (VEGF)-mediated neovascularization.[13] It has been reported that several immunosuppressive cytokines, including VEGF are detected in the tumor microenvironment.[14] Lung's tumor can secrete a large quantity of VEGF and this ability is often associated with a poor prognosis.[15] Wada et al declared that VEGF act as an obstacle against antitumor immunity.[16] Zhang et al revealed that exposure to NNK, stimulates expression of VEGF levels. Therefore, VEGF is increasing by stimulating angiogenesis, inflammation and inflammatory diseases such as cancer.[17]

As, Recent research determined that the change of multiple key molecules or signal pathways can promote the occurrence and the development of lung cancer via affect lung cancer cell proliferation, migration and invasion. Clinicians and scientists need to find novel strategies for more effective treatments.[18] Researchers suggested Anti-VEGF strategies to treat cancers. Silencing of the annexin A2 inhibited the expression of proangiogenic molecules, including VEGF, leading to the inhibition of neovascularization.[19] Thus, inhibition of VEGF and annexin A2 in tumors seem to be a therapeutic strategy. In this regard, Modified factors such as physical activity can help us to prevent and treat lung cancer by modulating the inflammatory process.[20] However, which kind of exercise could have the best effect as well as the related molecular mechanisms are not yet completely known. Most physiological studies reported that aerobic exercise is a powerful tool against the oxidative stress activation; it also provides a protective mechanism that helps to re-establish cellular homeostasis, decrease the release of pro-inflammatory cytokines, and improve immune responses.[21,22] In this regard, submaximal swimming training is one of the safest training among the types of aerobic exercises as no weight bearing condition in water.[23] Although the possible mechanisms has been suggested, but results about the relationship between physical activity and cancer is inconsistent.[24] Colombo et al reported that 5 weeks of aerobic exercise training on a treadmill, increased the levels of VEGF in lung tissue of rats.[25] While Krenc et al*'* study showed that after a long period of aerobic exercises training, reducing VEGF levels observed.[26]

So, given the importance of prevention of lung cancer, and also the lack of necessary and adequate information regarding the impact of exercise on annexin A2 and VEGF levels in lung's tissue exposed to NNK, the aim of this study was to examine the effects of submaximal swimming training on annexin A2 and VEGF in Wistar rats exposed to NNK.

MATERIALS AND METHODS

Ethical Approval

All procedures in this study were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). This study was approved by HRI Ethics Committee of the Babol University of Medical Sciences (Code No:

MUBABOL.HRI.REC.1395.109).

Materials and Study Design

The experimental design was performed in University of Mazandaran. 46 Wistar rats with age of 6-8 weeks and an average weight of 105.84±27.93 grams were purchased from Pasteur Institute of Iran. After two weeks of becoming familiar with environment and research protocol, Animals were divided randomly into 5 groups consist of training group (n=10), NNK (n=10), training+NNK (n=10), saline (n=10) and control (n=6). Animals in an environment with an average temperature of 22±1.4°C, humidity 55% and the light dark cycle of 12:12 h were kept in cages made of polycarbonate. Keeping animals was carried out accordance with international health institute instructions, as well as the protocol of this study was performed with the declaration of Helsinki on Ethical Principles for Medical Research.[27] Animal were consumed pellets and water freely. NNK is injected subcutaneously 1 times per week at a rate of 12.5 (mg/kg/bw) for 12 weeks and also the saline group received distilled water.[28]

Rats in training groups familiarized with water and swimming in the pool with dimensions of 50×50×100 cm by temperature of 30-32°C for one week (5 days) and 20 minutes each time. The water power was increased 4 lit/min in the first week to 10 lit/min in the 11th, which was fixed for the last week (Table 1).[29]

Measurement of VEGF in Lung Tissue

To eliminate the effect of acute exercise, sampling of the animals was performed 48 hours after the last session of swimming. Then animals were anesthetized by using intraperitoneal injection of ketamine (30-50 mg/kg) and xylazine (3-5 mg/kg). After thoracic surgery, lung tissue was isolated and placed in the microtubes then frizzed in liquid nitrogen at -70°C. To prepare the laboratory analysis, 100 mg of lung tissue with 1 ml of PBS buffer 100 mM homogenized and centrifuged for 15 minutes at 6000 rpm. Then the supernatant obtained was transported to the laboratory for measuring VEGF. VEGF (Shanghai Crystal day Biotech Company; China) were measured and quantified by using ELISA based on the kit instructions. [30]

Method and Evaluation of Immunohistochemistry (IHC)

Immunohistochemistry staining was carried out by a standard streptavidin–peroxidase method. In brief, serial sections (4 μm) from paraffin-embedded blocks were cut and mounted on Poly-L-lysine precoated glass slides and dried. The sections were deparaffinized. Prior to immunostaining, antigen retrieval was carried out. For annexin A2, the slides were heated. Endogenous peroxidase activity was suppressed by hydrogen peroxide in methanol. The sections were incubated with normal goat serum to prevent nonspecific binding. The specific primary antibody, either 1:100 Human/Mouse/Rat annexin A2 Antibody (R&D Systems; USA), was then applied for 2 h at room temperature. Then, the sections were incubated with Rabbit/Mouse antibody (Dako REAL Envision Detection System, Denmark) was used as a secondary antibody (20 min), streptavidin peroxidase as label (20 min) and diaminobenzidine as chromogen. Counterstaining was performed with hematoxylin to enhance nuclear detection. Appropriate positive and negative control slides were stained in parallel. Lung tumors of rat were taken as positive controls for Annexin A2. Primary antibody was omitted in negative controls.[31]

Annexin A2 immunoreactivity was observed both in the cytoplasm and in the membrane. Positive cells were recognized as cells that showed yellowish brown. All counting was done without any knowledge of the diagnosis and results of the other observer's counts. All immunopositive cells were counted in at least 10 high-power fields (×40 objective, ×10 eyepiece) chosen at random.

The number of annexin A2 positive cell was given as a percentage for each case. Extent of immunostaining (based on the percentage of positive cells) was scored as 0 (0 to 5%), 1 point (6% to 24%), 2 points (25% to 49%), 3 points (50% to 74%), and 4 points (75% to 100%). Staining intensity was graded as 0 (negative), 1 point (weak), 2 points (moderate), and 3 points (strong). The immunohistochemical staining for annexin A2 was assessed according to the immunoreactive score (IRS) value by multiplying the individual scores of extent by intensity. Expression of annexin A2 within stromal cells was recorded by the percentage of positive cells.[31]

Statistical Analysis

In order to determine the normal distribution of data and homogeneity of variance we used Shapiro-Wilk test and Leven's test respectively. Then, to analyze the

Table 1. The effect of swimming submaximal training on vascular endothelial growth factor in wistar rats exposed to tobacco-derived nitrosamine ketone

Week	1	2	3	4	5	6	7	8	9	10	11	12
Time (min)	25	30	35	40	45	50	55	60	60	60	60	60
Power(lit/min)	4	4	5	5	6	6	7	7	8	9	10	10

data; ANOVA and Tukey's test were used at a significance level of $p<0.05$ to investigate changes of VEGF and annexin A2 levels. Mean and standard deviation were used to report the values of measured variable. All statistical analyses were performed using the SPSS version 24.0(IBM Corp., Armonk, N.Y., USA).

RESULTS

Our findings did not show significant changes in body weight of different research groups. The results of ANOVA test showed that the levels of VEGF in lung tissue of research groups had a significant difference ($p<0.001$) and result of post-hoc test showed that VEGF levels in lung tissue of NNK group increased significantly when compared to the saline group (34.07%) ($p<0.001$).

In addition, findings noted that 12 weeks submaximal swimming training decreased the levels of VEGF in lung tissue significantly when compared to control (20.64%) and NNK (27.34%) groups (Respectively: $p=0.03$, $p<0.001$), While these differences were not significant when compared to the saline and training+NNK groups (respectively: $p=0.99$, $p=1.00$).

Moreover, it was shown that intervention training with induction of NNK resulted in significant decreased in VEFG levels of lung tissue when compared to NNK group (26.55%) ($p<0.001$), While this difference was not significant when compared to the saline group ($p=1.00$) (Figure 1).

Annexin A2 Expression in Lung Tissue

The results of ANOVA test showed that the levels of annexin A2 in lung tissue of research groups had a significant difference ($p<0.001$) and result of post-hoc test showed that annexin A2 levels in lung tissue of NNK group increased significantly when compared to the saline group (144%) ($p<0.001$).

Also, findings demonstrated that 12 weeks submaximal swimming training decreased the levels of annexin A2 in lung tissue significantly when compared to control (75%) and NNK (90%) training + NNK groups (60%) (Respectively: $p\leq0.001$, $p\leq0.001$, $p=0.01$).

Furthermore, it was shown that intervention training with induction of NNK resulted in significant decreased in annexin A2 levels of lung tissue when compared to NNK (77%), saline groups (44%) ($p<0.001$) (Figure 2).

There is weak expression of annexin A2 in control (0.08 HPF), training+NNK (0.05 HPF), saline (0.09 HPF) and NNK groups (0.22 HPF), While we saw negative expression of annexin A2 in training group (0.02 HPF) (Figure 3). Furthermore, histopathologic studies showed that the structure of the parenchymal segment of the lung tissue is completely normal in the control and saline groups. In the NNK group, the accumulation of atypical cells with inflammatory cells was observed in lung tissue, and it can be stated that a small area of the tumor was formed. Also, in the training group, most of the lung parenchymal structure was maintained. In the NNK+training group, mild acute inflammation was observed and parenchymal structures have been well-preserved, in addition alveolar and emphysema reduction occurred in lung tissue.

Furthermore, Pearson correlation analysis used for determining the relationship between VEGF and annexin A2 levels. There was no significant correlation between VEGF levels and annexin A2 in all study groups.

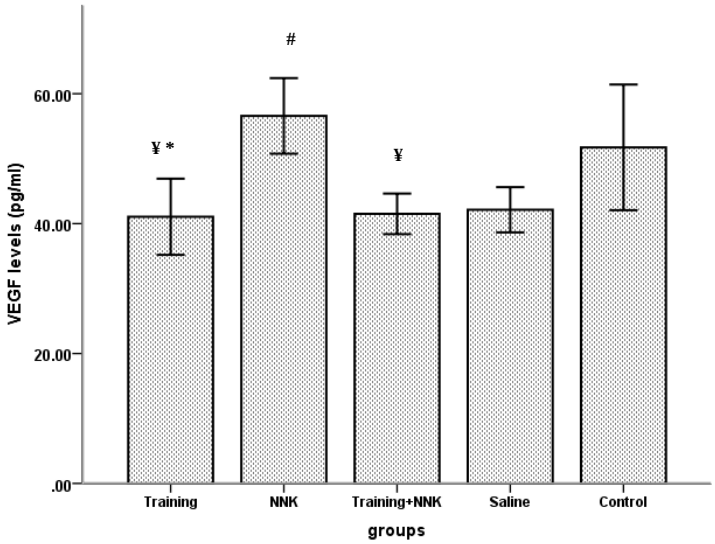

Figure 1. The levels (mean±SEM) of vascular endothelial growth factor (Pg/ml) in lung tissue of Training (n=10), NNK (n=10), training+NNK (n=10), saline (n=10) and control groups (n=6).
Statistical significance for the difference between the data of training and saline *vs* control: * $p<0.001$
Statistical significance for the difference between the data of NNK and control *vs* saline: # $p<0.001$
Statistical significance for the difference between the data of training, training+NNK and saline vs NNK: ¥ $p<0.001$
The statistical comparisons were made using ANOVA and Tukey's pot test at a significance level of $p<0.05$.

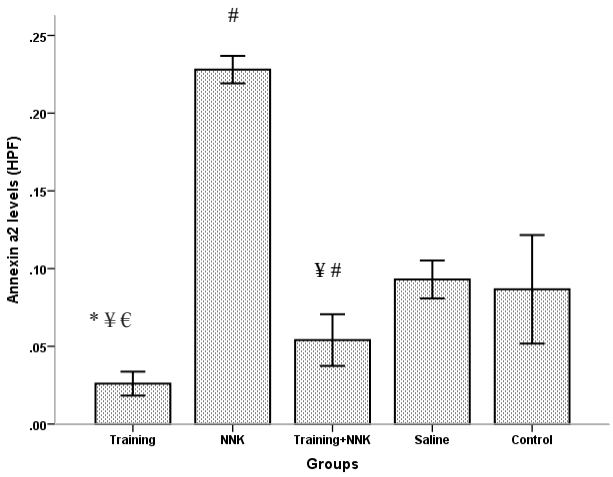

Figure 2. The annexin A2 (mean±SEM) expression (high power field) in lung tissue of Training (n=10), NNK (n=10), training+NNK (n=10), saline (n=10) and control groups (n=6).
Statistical significance for the difference between the data of training and saline vs control: * $p<0.001$
Statistical significance for the difference between the data of NNK and control vs saline: # $p<0.001$
Statistical significance for the difference between the data of training, training+NNK and saline vs NNK: ¥ $p<0.001$
Statistical significance for the difference between the data of NNK and training vs training+NNK: € $p<0.001$
The statistical comparisons were made using ANOVA and Tukey's pot test at a significance level of $p<0.05$.

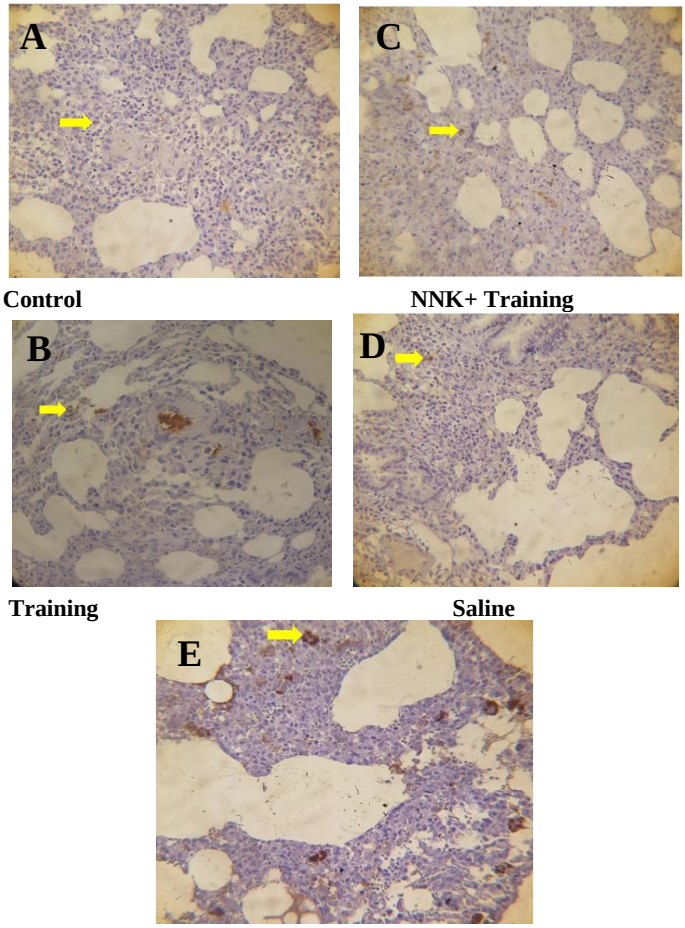

Figure 3. Immunohistochemical expression of annexin A2 in lung tissue parenchyma of the study group to evaluate the effect of swimming submaximal training on vascular endothelial growth factor in wistar rats exposed to tobacco-derived nitrosamine ketone(immunohistochemistry test with 3,3'-Diaminobenzidine (DAB) chromogen, differential chromatography of hematoxylin with a magnification of 400×).
(A); the section from the lung of control group (B); training group (C); NNK+ Training group (D); and Saline group (E); NNK group. Brownish reactions (yellow arrow) in the tissue were indicated the annexin A2 expression. The expression of annexin A2 significantly increased in (E) group compared to other groups.

DISCUSSION

In present study the effect of submaximal swimming training on resting levels of VEGF and annexin A2 in lung tissue of rats exposed to NNK had been examined. The results showed that the VEGF levels in training group were decreased significantly when compared to the control group. In this regards, Krenc et al reported decreased levels of VEGF after a long period of aerobic exercise training.[26] Ribatti et al noted that the sst2-R-mediated antiangiogenic action of somatostatin could either be direct, involving the inhibition of endothelial cell proliferation or indirect, being mediated by the suppression of production of growth factors, including VEGF.[32] Furthermore, Czarkowska et al stated that increased capillary density in response to exercise, resulted insufficient oxygen in the tissues and thus causes the activation of VHL, which is to be disabled by HIF-1. The inactive HIF-1 is also reduced VEGF expression.[33] The results of some studies indicate increased levels of VEGF following physical activity, which contradicts the results of the present study.[34,35] However, Mirdar et al reported that after three weeks of swimming training in pregnant

mice resulted no significant changes in lung VEGF levels.[34] Also, Mirdar and colleagues stated that a swimming training leads to increased levels of VEGF in kidney of neonatal rat.[35] One of the possible reasons for inconsistent findings of this study with the results of other researchers can be noted features and duration of the exercise. As Colombo et al reported an increase in VEGF levels of lung tissue in hypertensive mice following 5 weeks of aerobic exercise training on treadmill.[26] Although in Mirdar's study the nature of training was swimming, but the duration of training was 3 weeks, which seems short-term exercises may justify a lack of changes in VEGF levels.[34] The other one may be related to the characteristics of the participants, as Colombo measured VEGF levels in lung tissue of hypertensive rats.[26]

In other hand, results showed that swimming training decreased the levels of annexin A2 in lung tissue significantly when compared to control group. In this regard, Leggate et al demonstrated significant reductions in annexin A2 as a response to high-intensity intermittent training. Annexin A2 activate macrophages and stimulate cytokines and chemokines including IL-6, TNF-α, and ICAM-1 production in macrophages in vitro.[36] Also, Hord et al reported that the intervention of voluntary wheel running was capable of maintaining at or near control levels of annexin A2 and suggesting protection against cell membrane damage and thus the need for heightened repair.[37] The results of Hussey et al' study is inconsistent with our results. They stated that annexin A2, was increased after the exercise training. A possible reason for inconsistent findings of this study can be noted that this research was conducted on patients with type II diabetes and training protocol was different.[38]

Moreover, VEGF levels increased significantly in NNK group when compared to the training groups. Consistent with our results, other researchers reported that exposure to NNK contributes to the stimulation of VEGF levels.[17,39] *Zhang* et al found that expression of VEGF, a HIF-1α target gene, significantly increased after cells were treated with NNK, compared with control group.[39] Zhang et al result's showed that NNK significantly promoted VEGF expression,[17] which is regulated by growth factors, hormones, and small molecules including PDGF, bFGF, TGFs and epidermal growth factor. VEGFs and their receptors are expressed in solid tumors of lung carcinoma. These results suggested that NNK promoted tumor growth by inducing the formation of tumor vessels via the beta-adrenoceptor signaling pathway.[17] Studies on NNK-exposed lung epithelial cells constitutively showed DNA damage upon exposure to 50 mg NNK/kg body weight 3 times a week for eight weeks, suggesting the importance of NNK-mediated CIN initiation during lung tumorigenesis.[40]

Furthermore, the findings noted that annexin A2 levels in NNK group increased significantly as compared to the training groups. This results confirms the previous findings by Wang et al that annexin A2 levels is overexpressed in tumors of NSCLC Patients compared with adjacent non-tumor tissues.[41] Wang and Feng Lin reported that Intense annexin A2 immunoreactivity is detected in lung adenocarcinoma and squamous cell carcinoma, compared to noncancerous control.[42] Annexin A2 heterotetramer on the cell surface binds to t-PA and activates plasminogen conversion to plasmin. Plasmin results in activation of MMPs and lead to ECM degradation. Increased annexin A2 expression results in increased plasmin generation and enhances cancer invasion and metastasis.[43]

We also demonstrated that training intervention with induction of NNK resulted in significant decreased in VEGF levels when compared to NNK group. The exercise-induced plasma VEGF decreased is in accordance with previous studies.[44] Amani Shalamzari et al reported significant reduction in level of VEGF after endurance exercise in breast cancer bearing mice. Since the Regular exercise reduces the level of IL-6 in tumor tissues and this cytokine has an effective role in producing VEGF. Thus, this reduction after exercise training indicated the reduction of angiogenesis in tumor by suppressing the production of IL-6.[44] In addition, after training in normoxia the endostatin levels were significantly elevated. Endostatin, is highly expressed in blood vessels and has been shown to be an inhibitor of VEGF-induced angiogenesis.[45]

The results of this study shall provide the first data regarding the effect of training intervention with induction of NNK on annexin A2 levels of lung tissue that showed a significant decreased when compared to NNK group. Possible mechanisms to reduce levels of annexin A2 induced by endurance exercise is binding annexin A2 to angiostatin, a powerful anti-angiogenic molecule that is generated from plasminogen

processing. Angiostatin binding to the lysine binding domain of annexin A2 in endothelial cells results in an anti-angiogenic effect. Moreover, the interaction between annexin A2 and angiostatin resulted in reduced plasmin generation in Lewis lung carcinoma cells. These findings indicated that plasminogen and angiostatin may bind to the same annexin A2 binding site and the anti-angiogenic action of angiostatin is mediated via interactions with annexin A2.[43]

We have demonstrated that there was no significant correlation between VEGF and annexin A2 levels in all groups. The results showed that expression of annexin A2 is regulated by many functional proteins, such as VEGF and VEGFR2. Suppressing the ischemia-induced expression of VEGFR2 increased the annexin A2 mRNA. This suggests that VEGF may play major roles and may be involved in regulating the expression of annexin A2.[13] On one hand, annexin A2 as one of the receptors for plasminogen and tPA is involved in the angiogenic process.[11,13] On the other hand, presumably annexin A2 as mRNA binding protein influences the stability of mRNA expression of VEGF, or is involved in the angiogenic process by VEGF/VEGFR pathway. The reasons for lack of relationship between these two variables in the present study can be the intensity and duration of exercise and exposure to NNK Conditions. The intensity and duration of the exercise can have a direct effect on the VEGF, and an increase in stimulation of this level can change the level of annexin A2. Although, in this study many variables such as species, sex, weight, environmental factors (noise, light, humidity, and temperature), training factors (type, duration and intensity of exercise) and diet were under control, one of the limitations of this study, was that the overnight physical activity of the subjects was not under control and the intake of NNK in subjects was not measured.

VEGF-targeted therapies were developed with the notion that they would inhibit new blood vessel growth and thus starve tumors of necessary oxygen and nutrients. However, VEGF-targeted therapy is complex, and probably involves multiple mechanisms. In addition, Annexin A2 has also been identified as a molecular target for a peptide with tumor-targeting and anti-angiogenic effects in lung cancer cells. Thus, it seems that submaximal swimming training is effective protocol for reducing the levels of annexin A2 and VEGF induced by NNK in rats.

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