Effect of Cigarette Smoke on Rhoa/Rho-Kinase Signalization Pathway in Lung

Akciğerde Rhoa/Rho-Kinaz Sinyalizasyon Yolu Üzerine Sigara İçmenin Etkisi

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ÖZET

Anahtar kelimeler: Sigara dumanı, RhoA, rho-kinaz, akciğer, fareler.

ABSTRACT
Cigarette smoke exposure has side effects on lungs, cardiovascular systems and some tissue and organs. Rho/rho-kinase signalization pathway has important role bronchial smooth muscle contractions and cancer development of lung fibroblast. Molecular mechanism of some side effects due to cigarette smoke exposure has not yet been clearly identified. In the present study we aimed to research effect of cigarette smoke exposure on rho/rho-kinase signalization pathway in lungs. For this propose mice (male age: 8 weeks) separated into two groups as control and smoke exposed. The cigarette smoke application continued 7 days in a week during two months. At the end of two months, Mice were sacrificed by cervical dislocation and their lungs were isolated. Then rhoA and rho-kinase enzymes expression and rho-kinase enzyme activity have been determined. Cigarette smoke exposure caused unregulated rhoA and rho-kinase enzyme expression and elevated rho-kinase enzyme activity. As a result cigarette smoke exposure elevated the activity of rho/rho-kinase signalization pathway.

Key words: Cigarette smoke, rhoA, rho-kinase, lung, mice.
INTRODUCTION
Smoking is one of the most important health problems in our day. Cigarette smoke exposure affects many organs in the body negatively primarily such as the lungs and cardiovascular system and disrupt and leads to damages and function failures in the relevant organs. Smoking is a great risk in cardiovascular diseases. Epidemiological studies have revealed the fact that smoking in addition to causing death due to thrombosis, atherosclerosis development, myocardial infarction, vascular graft failure and coronary artery diseases also is one of the major causes of hypertension and endothelial dysfunction (1, 2, 3, 4).

Smoking reduces the density of combined nitrite, nitrate and antioxidants in plasma (5). Cigarette smoke exposure reduces NO production by affecting L-arginine-NO synthase pathway in endothelial cells. It has been reported in studies that it also increases oxidative stress. In addition, it reduces L-arginine transport and nitric oxide synthase expression and activity (6). Long-term cigarette smoke exposure irreversibly disrupts the mouse carotid artery structure and reduces the elasticity thereof (7). Smoking increases the risk of chronic obstructive pulmonary disease. It leads to destruction of the lung parenchyma cells due to reduction of Vitamin A and development of emphysema, a disease caused due to reduced elasticity thereof (8). Smoking also causes the increase of free radicals thereby increasing oxidative stress. The increasing oxidative stress causes the inactivation of NO and contributes to endothelial dysfunction as well as reduction of blood flow in normal coronary arteries (9).

Nicotine incubation reduces the relaxation response given to acetylcholine after spasm is created in coronary artery by norepinephrine and additionally application of nicotine to cells in carotid artery cell culture reduces eNOS expression (10).

Rho proteins are members of the Rho subfamily of the Ras superfamily of monomeric GTPases. Effector regions of RhoA, RhoB and RhoC have the same amino acid sequences and cellular functions of these GTPase proteins are similar. A lot of described functions of Rho is based on the studies conducted with RhoA (11, 12). RhoA is the most commonly available and the most studied Rho protein sub-type in the body (11). RhoA protein is activated by many receptors and activates the Rho-kinase enzyme (13, 14).

Rho kinase activated by RhoA is also named as ROCKα or ROCK2 (also known as ROCK1). Rho is an isoform of kinase (11, 15). ROCK1 and ROCK2 genes in humans are in 18th chromosome (18q11.1) and 2nd chromosome (2p24) respectively (16). Rho-kinase enzyme has been reported to play a role in Ca 2+ sensitivity of vascular smooth muscle cells (6). It has been reported that Rock2 is expressed in brain and heart more while ROCK1 is expressed in lung, liver, spleen, kidney, and testis more. Presence of Rho-kinase enzyme in almost every tissue has been revealed (11, 15, 17, 18, 19).

Phosphorylation degree of the light chain Myosin (MLC), a substrate of Rho-kinase in contraction degree of vascular smooth muscle cells induced by agonist is the determinant of the degree of contraction force. Amount of MLC phosphorylation is based on the balance
between Ca 2+/calmodulin-dependent myosin light chain kinase (MLCK) and myosin phosphatase (MYPT) (20).

Smoking mostly affects the lungs and leads to emphysema. It also increases the proliferation of smooth muscle cells. It has been reported that nicotine, which is the most important component of cigarette smoke, contracts smooth muscle cells of airways. Furthermore, superoxide radicals within the smoke are also known to cause contraction in smooth muscle cells through increase of the intracellular calcium levels. As such, we aimed to investigate the effect of cigarette smoke exposure on cell proliferation in the lungs and RhoA/Rho-kinase signaling pathway which plays a role in contraction of smooth muscle cells in our study.

MATERIAL AND METHOD

8 week old balb/c albino male mice that are obtained from the Experimental Animal Center in Çukurova University, in Adana are used in the study. This study was approved by the Animal Care Committee and Ethics Committee of Çukurova University. Mice were divided into two groups as control and smoke exposure.

Cigarette Smoke Exposure
8 weeks old male mice were exposed to the smoke of 20 commercial filtered cigarettes per day, during 8 weeks. The smoke exposure was accomplished by enclosing the animals in a chamber 100 cm long, 60 cm wide, and 80 cm high. The animals were exposed to the smoke by lighting two cigarettes which are mounted the suction vacuum pump upper of chamber and inhaling the smoke through the chamber the smoke was dispersed throughout the chamber by a ventilotior. Two cigarettes were lit and "smoked" over a period of 10 min and followed by a period of 20 min without cigarette smoking. The cycle was repeated until a total of 20 cigarettes were "smoked" over a period of about 6 h. To confirm that this system led to significant smoke inhalation, we obtained blood measurement of cotinine level by ELISA in another group of animals exposed to cigarette smoke under identical conditions. As control group for the effects of cigarette smoke exposure, we also studied control group placed in a similar chamber for a similar period of time during 8 weeks under the same conditions but without using any cigarette, so that only room air was being aspirated into the chamber. Mice were sacrificed by cervical dislocation and lungs were been isolated and frozen to be used in ELISA experiments.

Quantitative Analysis

Tissue Homogenization
3 ml/gram RIPA (Radio-immunoprecipitation Assay) buffer, 30 μl PMSF (phenylmethanesulfonyl fluoride), 30 μl sodyum vanadate, 30 μl protease inhibitor were applied on frozen tissue samples that were stored in Eppendorf tubes then homogenates were obtained by using ultrasonication on those tubes on ice. Homogenates were then centrifuged at 10.000 RPM for 10 minutes and supernatants were taken and pellets were discarded.

Protein Quantification
Bradford method was used to quantify the protein in homogenized tissues. By using Bovine serum albumin (1μg/ml), 1, 2, 3, 5, 7, 8, 10 (μg/ml) standarts were prepared. 10 μl was
taken from every sample and completed to 100 μl by adding distilled water. 1 ml Bradford solution was added to standards and samples, vortexed and absorbances at 595 nanometer were measured manually. Protein quantification (μg/μl) was done according to the standart curve drawn in Prism software.

ELISA (Enzyme Linked Immunosorbent Assay) Test
ELISA test was used to examine the expression and of RhoA (CUSABIO, Inc), rho-kinase II (CUSABIO, Inc) and the activity of rho-kinase (Cell Biolabs, Inc).

Statistic Analyzes
For the comparison of parameters from control and smoke exposed group unpaired Student’s t test was used. Data is presented as means ± SEM. Statistical analysis of differences with p < 0.05 was taken as the indicator of significance.

RESULTS

ELISA RhoA Protein Quantification
Cronical smoke treatment increased expression of rhoA (fig 1).

ELISA Rho-kinase II Enzyme Quantification
Cronical smoke treatment increased expression of rho-kinase II (fig 2).

Figure 2. Effect of cronical smoke treatment on rho-kinase II expression. The data are presented as mean ± SEM. Differences between parameters of control and smoke treated group were analyzed by applying unpaired Student’s t test. *difference between control and smoke treated group is significant with p < 0.05

ELISA Rho-kinase Activity Quantification
Cronical smoke treatment elevated activity of rho-kinase (fig 3).

Figure 3. Effect of cronical smoke treatment on rho-kinase activity. The data are presented as mean ± SEM. Differences between parameters of control and smoke treated group were analyzed by applying unpaired Student’s t test. *difference between control and smoke treated group is significant with p < 0.05.
DISCUSSION
We investigated what kind of an effect chronic cigarette smoke exposure would have on RhoA/Rho-kinase signaling pathway which is one of the intracellular communication mechanisms of lungs and which has an important role in cell proliferation and development in our study. Numerous revealed functions of Rho proteins are based on studies conducted with RhoA (10). RhoA is the most commonly available and the most studied Rho protein sub-type in the body (11, 12). RhoA carries out a variety of functions as a result of the stimulation of G-protein-linked receptors by some agonists (14). Rho-kinase enzyme is activated by the RhoA protein (7, 8, 9). Activated Rho-kinase is involved in many activities within the cell. It has been observed in our results that cigarette smoke exposure leads to upregulation of RhoA protein in the lungs. In an in vitro research it was revealed that RhoA is upregulated in airway smooth muscle cells of rats exposed to cigarette smoke (21). Cigarette smoke was given by inhalation in our study. RhoA protein also was upregulated in our study as it was in the cited study. The increase in the activity of RhoA protein may be caused by superoxide radicals in cigarette smoke. Superoxide radicals were reported to increase the activity of RhoA protein in another study (22). This situation also supports our findings. RhoA-kinase which is another enzyme we examined in our study is a sub-effector of RhoA protein. The results we obtained have shown that cigarette smoke exposure causes an increase in expression and activity of rho-kinase enzyme. In the studies conducted it has been reported that super oxide radicals increase the activity of rho-kinase (23). There are many active substances in cigarette smoke other than the superoxide radicals and these substances may have additionally activated RhoA/Rho-kinase signaling pathway in addition to superoxide radicals. Consequently activation of RhoA/Rho-kinase signaling pathway may disturb the physiology of the lung by causing bronchospasm. Furthermore, studies have shown that increase of activation in this pathway leads to onset of apoptosis known as the death of cells (24, 25). Activation of the RhoA/Rho - kinase signaling pathway has been reported to increase the proliferation and migration of cancer cells in the studies conducted to that effect (26, 27). Cigarette smoke is one of the biggest causes of lung cancer. Besides it also prepares the ground for COPD which is as dangerous as cancer. It has been shown in studies that RhoA/Rho-kinase signaling pathway is activated in the lungs of COPD patients (28). In conclusion, our study has revealed the fact that cigarette smoke exposure activates RhoA/Rho-kinase signaling pathway. Furthermore, it was shown that activation of this pathway by cigarette smoke may cause spasms thereby forming the basis for development of COPD as well as helping the development of lung cancer.

REFERENCES


