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Molecular basis of vascular damage caused by cigarette smoke exposure and a new approach to the treatment: Alpha-linolenic acid

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ABSTRACT

Exposure to cigarette smoke (CS) causes vessel damage and mechanism of this damage has not yet been clearly identified. Therefore, in this study we aimed to investigate whether vessel damage due to the CS exposure will be prevented by the alpha-linolenic acid (ALA) or not which has anti-inflammatory effect in mice. For this reason, mice were grouped as controls (with and without CS) and ALA (with and without CS). The CS application continued 5 days a week for two months. At the end of two months, the mice were killed by cervical dislocation and their blood and thoracic aortas were isolated. ALA Treatment increased acetylcholine relaxations. CS decreased acetylcholine relaxation. CS with ALA treatment increased acetylcholine relaxations versus just CS treatment. CS caused rising in cyclooxigenase-2 and phospholipase A2 levels. This rise is inhibited with ALA treatment. CS decreased eNOS levels. But this result was not statistically significant. Furthermore, according to electron microscopic study CS damaged both smooth muscle and endothelium. While ALA treatment prevented smooth muscle damage it didn't prevent endothelial damage. Using cigarette and CS exposure is a risk factor for cardiovascular disease. Our study showed that this disease.

1. Introduction

Cigarette smoking (CS) is a recognized risk factor for cardiovascular disease and is known to promote the development of atherosclerosis and thrombosis [1,2]. Epidemiological studies have established that there is a high order of correlation between cigarette smoking and cardiovascular disease, especially atherosclerosis. Animal experiments have confirmed the devastating effects of smoking on the endothelium. Endothelial cells seem to play a prominent role in the early development of atherosclerosis [3]. The eNOS is essential in maintaining basal vascular NO production that regulates blood flow, particularly coronary blood flow. Reduction in basal NO release may cause a predisposition to hypertension, thrombosis, vasospasm, and atherosclerosis [3–5]. In vivo, CS and nicotine infusion impair the endothelium-dependent relaxation mediated by NO in human arteries and veins. CS exposure reduces NO production, eNOS activity and eNOS expression in endothelium. Furthermore, CS exposure influences L-arginine transport [6]. CS enhances oxidative stress by increasing free oxygen radicals. Oxidative stress causes NO inactivation and decreasing coroner blood flow by contributing endothelial dysfunction [7]. CS exposure causes atherosclerosis by enhancing cyclooxygenase-2(COX-2), prostaglandin E2 and ICAM-1 which are mediator of inflammation [8].

Nicotine is main content of CS. Some studies show that direct nicotine treatment affects vessels activity. Nicotine treatment reduces acetylcholine relaxations in coroner arteries. Furthermore, it reduces eNOS expression in nicotine treated carotid artery cells [9]. Intragastric high dose of nicotine treatment causes increase of mucosal prostaglandin E2 synthesis. Moreover, nicotine constricts rat basilar artery. The main origin of this contraction is in the endothelium and nicotinic receptors accompanies this contraction. However, inhibitors of phospholipase A2, phospholipase C and cyclooxygenase-2 inhibits this contraction [10,11].

α-linolenic acid (ALA) is found in some plant such as flax seed and fish oil. ALA is known as omega-3 fatty acid and have protective effects on cardiovascular systems [12,13]. ALA is

precursor of Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) [14,15]. Diet of ALA reduces systolic and diastolic blood pressure [16]. ALA is also an antioxidant due to double bonds in its structure. It reduces oxidative stress and this effect accompanies prevention of inflammation [17]. Furthermore, it inhibits LPS induced

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inflammation by inhibiting translocation of NF-kB and MAPK thus this inhibition causes expression of inflammatory factors such as iNOS,COX-2 and TNF-α [18].

In our study we planned to examine molecular mechanism of CS induced inflammation and anti-inflammatory effect of ALA in thoracic aorta.

2. Materials and methods

Approval from the Institutional Animal Research Committee was obtained. 8 weeks old male mice were separated into 4 groups as control, only ALA treated, CS treated and CS + ALA treated. ALA treatment is done orally (gavage, 200 mg/kg everyday) to mice. 12 mice were used in each group. Mice were exposed to the smoke of 20 commercial filtered cigarettes per day (same brand was used during the study), for 5 d/wk for two months. 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 to the suction vacuum pump on the upper of chamber and inhaling the smoke through the chamber the smoke was dispersed throughout the chamber by a ventilator. 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 (Blood samples were obtained 6 hours later after the last cigarette smoke exposure) 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 for 5 d/wk for two months under the same conditions but without using any cigarette, so that only room air was being aspirated into the chamber.

After the period of smoke exposure, the mice were sacrificed by cervical dislocation. Thereafter the thoracic cage was opened and thoracic aorta isolated. Connective tissue was removed and then the aorta was cut into 2–3 mm ring segments in the Petri dish containing cold Physiological Salt Solution (PSS). Special care was performed to not damage endothelium during the isolation of the rings. Some of tissues saved to use in ELISA and Electron Microscopic Study.

2.1. Organ bath experimental protocol

The ring was then mounted horizontally between two fine stainless steel rods. The lower rod was connected to the base of the organ bath, while the upper rod was attached to the isometric force transducer (COMMAT, Ankara, Türkiye) which was coupled to the Biopac computerized recorder (Biopac systems Inc., CA, USA). This was used in recording the force displacement by the tissue. The rings were superfused in 20 ml double jacketed organ bath with PSS at 37 °C and gassed with 95% O_2: 5% CO_2 mixture. The pH of the PSS was usually between 7.35–7.40, and all baths used simultaneously had a parallel connection to the source of PSS. The PSS consisted of (mM): NaCl 118, KCl 4.8, CaCl2 2.5, KH2PO4 1.2, NaHCO3 24, glucose 11, MgSO4 1.2, Na2EDTA 0.01.

After mounting, a passive tension of 0.5 g was applied to each ring. The mounted ring was then allowed to equilibrate in the PSS for 90 min, after this period for relaxation response to acetylcholine, aortic rings were precontracted with 5×10^{-6}M phenylephrine and after the contraction had reached a plateau, a doses of acetylcholine (Ach) (5×10^{-6}M) were added to the organ bath.

2.2. Immunochemical protocol

In immunochemical study we used Enzyme-linked immunosorbent assay (ELISA) technique. We used this technique for quantitative estimation. $cPLA_2$, COX-2 and cotinine ELISA kits were obtained from Calbiotech and eNOS ELISA kit was obtained from Uscn Life Science Inc.

2.3. Electron microscopic study

Tissues for electron microscopic examination were immediately placed in 5% glutaraldehyde buffered at pH 7.4 with Millonig phosphate buffer for 4 h. The tissue pieces were subsequently fixed in 1% osmic acid for 2 h. The tissue samples were then dehydrated in graded ethanol and embedded in araldite and processed for electron microscopy using conventional methods.

2.4. Statistic analyzes

Results were expressed as means ± S.E.M., and *n* refers to the number of animals used for each experiments. Differences in results between tissues were tested by analysis of variance (ANOVA) corrected for multiple comparisons (Bonferroni corrections). P values less than 0.05 were considered to be significant.

3. Results

3.1. Blood cotinine levels of smoke treated mice

Cotinine levels of CS exposured mice were 113 ± 12 ng/ml (n = 25)

3.2. Electron microscopic findings

In only ALA treated group the ultrastructure of the endothelial cells, the internal elastic lamina and the smooth muscle cells of the tunica media appeared normal (Fig. 1).

In control group the fine structures of the vessels were similar to those of only ALA treated group (Fig. 2).

ALA + CS treated group in the tunica intimae, endothelial cells exhibited cytoplasmic vacuoles. Furthermore, the endothelial cells were partially separated from the basal lamina in some areas. The subendothelial layer was irregularly outlined and enlarged (Fig. 3). The smooth muscle cells of the tunica media were appeared normal.

In only CS treated group the ultrastructural changes of arterial wall were more prominent in this group. In the tunica intimae, most of the endothelial cells exhibited an increase in nuclear heterochromatin and cytoplasmic vacuoles with membranous structures. Additionally, the endothelial cells showed protrusions towards the lumen. The subendothelial layer was irregularly organized and exhibited membranous structures and large edematous areas. Although most of the smooth muscle fibers showed normal structures in the tunica media, some of muscle fibers revealed increase in nuclear heterochromatin and cytoplasmic vacuolations (Fig. 4).

4. Organ bath experiments

4.1. Ach relaxant results

ALA treatment increased ach relaxation versus control group. This increase is statistically significant. While only CS exposure inhibited ach relaxations smoke exposure plus ALA treatment reversed these inhibitions (Fig. 5). Mean values of % ach relaxation for control, ALA treated, CS and CS + ALA group are found to be 61,33 (SEM 3,746), 80,25 (SEM 5,508), 36,64 (SEM 3,099), 63,09 (SEM 3,114) respectively

4.2. ELISA phospholipase A2 enzyme quantification

While CS treatment increased phospholipase A2 enzyme expression. ALA treatment decreased CS treatment induced increase of cPLA2

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