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INHIBITION OF NUCLEAR FACTOR-κB ATTENUATES ATHEROSCLEROSIS IN apoE/LDLR - DOUBLE KNOCKOUT MICE

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Nuclear factor - κB (NF-κB) is a good therapeutic target for cardiovascular disease and numerous efforts are being made to develop safe NF-κB inhibitors. Nowadays many authors address NF-κB as a major therapeutic target in atherosclerosis, especially for preventive measures, in the light of two main hypothesis of atherosclerosis: oxidation and inflammation. We hypothesized that ammonium pyrrolidinedithioocarbamate (PDTC) - a well-known inhibitor of NF-κB could inhibit the development of atherosclerosis in this experimental model. We used apoE/LDLR - DKO mouse model, which is considered as a one of the best models to study the anti-atherosclerotic effect of drugs. In this model PDTC inhibited atherogenesis, measured both by "en face" method (25,15±2,9% vs. 15,63±0,6%) and "cross-section" method (565867±39764 μm^2 vs. 291695±30384 μm^2). Moreover, PDTC did not change the profile of cholesterol and triglycerides in blood. To our knowledge, this is the first report that shows the effect of PDTC on atherogenesis in gene-targeted apoE/LDLR - double knockout mice.

Key words: *atherosclerosis, apoE/LDLR - knockout mice, PDTC*

INTRODUCTION

Since 1992 the mouse has become an excellent model for experimental atherosclerosis research (1-3). In 1992 the first line of gene targeted animal models, namely apolipoprotein E (apoE) - knockout mice was developed (4, 5). The creation of apoE - knockout mice has changed the face of atherosclerotic research (6). It helped in creation the newest theory of atherosclerosis - as an

inflammatory disease. Gene targeting in embryonic stem cells was also used to create LDL receptor (LDLR) - knockout mice (7). More recently, apoE and LDLR - double knockout (apoE/LDLR - DKO) mice have been created, representing a new mouse model that develop severe hyperlipidaemia and atherosclerosis (8). It has been reported that the progression of atherosclerosis is usually more marked in apoE/LDLR - DKO mice than in mice deficient for apoE alone (9). Thus, apoE/LDLR - DKO mouse is considered as a one of the best models to study the anti-atherosclerotic effect of several substances.

We hypothesized that pyrrolidine dithiocarbamate (PDTC) - a well-known inhibitor of nuclear factor - κB (NF-κB) could inhibit the development of atherosclerosis in this experimental model.

MATERIALS AND METHODS

Animals and treatment

Female apoE/LDLR - DKO mice on the mixed C57BL/6J x 129/SvJ background were obtained from Taconic (Bomholt, Denmark). Mice were maintained on 12-h dark / 12-h light cycles in air-conditioned rooms (22.5±0.5°C, 50±5% humidity) and access to diet and water *ad libitum*. At the age of 8 weeks mice were put on Western diet (consisting of 21% fat by weight, 0.15% cholesterol by weight and no cholic acid) made by ssniff, Germany, for 4 months. Experimental group received the same diet, mixed with ammonium pyrrolidinedithioocarbamate - PDTC (Sigma-Aldrich, USA) at a dose of 6 mg/*per* day/*per* mouse. All animal procedures were approved by the Jagiellonian University Ethical Committee on Animal Experiments.

Procedures

At the age of 6 months mice were sacrificed under anesthesia and 1000 UI of fraxiparine (Sanofi-Synthelabo, France) was injected into the peritoneum. The blood was collected from the right ventricle. Plasma was separated by centrifugation at 1000×g at 4°C for 10 min and stored in -80° C. Then, right atrium was incised and the heart was perfused by PBS through the apex of the left ventricle at a constant pressure of 100 mm Hg. Next, the heart and the whole aorta were dissected.

Plasma lipids

Total cholesterol and triglycerides were assayed using commercially available kits (Roche Molecular Biochemical, USA).

Quantitation of atherosclerosis.

The heart and ascending aorta were embedded in OCT compound (CellPath, UK) and snap-frozen. Ten micrometer-thick cryosections were cut from the aortic root using a standardized protocol (10, 11). Serial sections were cut from the proximal 1 mm of the aortic root. Eight sections were collected at 100-μm intervals starting at a 100-μm distance from the appearance of the aortic valves. Sections were thaw-mounted on poly-L-lysine coated slides and air dried. After fixation in 4% paraformaldehyde (pH=7), sections were stained with Meyer's hematoxylin and oil red O (Sigma-Aldrich, USA) (12). Oil red O-stained sections were examined under Olympus BX50 (Olympus,

Tokyo, Japan) microscope and used for quantitative evaluation. Images of the aorta were recorded using Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024x768 pixels. Total area of the lesion was measured semiautomatically in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each animal a mean lesion area was calculated from eight sections, reflecting the cross-section area covered by atherosclerosis (12). The aorta from arch to bifurcation was fixed in 4% formaldehyde, opened longitudinally, pinned onto black wax plates and stained with Sudan IV (Sigma-Aldrich, St. Louis, MO, USA). Aortic lesion area and total aortic area were calculated using LSM Image Browser software.

Statistical analysis

Results are expressed as mean±SEM. The nonparametric Mann-Whitney U test was used for analysis of the data. $P<0.05$ was considered as statistically significant.

RESULTS

The aortas differed between control (n=10) and PDTC-treated (n=10) groups (*Fig. 1*) Measured by "en face" method, percentage of total aortic area occupied by Sudan IV-stained changes was in control group 25,15±2,9% whereas in PDTC-treated group 15,63±0,6% ($p<0.05$) (*Fig. 2*). Cross-section of aortic roots revealed also the difference in lesions area (*Fig. 3*). Counting by 8 consecutive sections the mean area±SEM, occupied by ORO-stained lesions was 565867±39764 μm^2 in

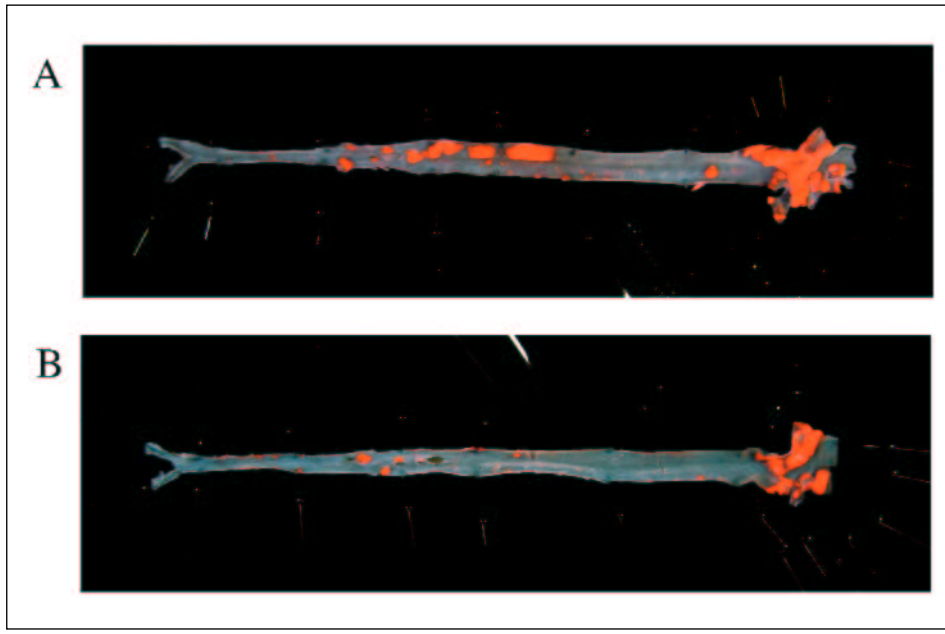

Fig. 1. Sudan IV-stained "en face" preparations of aortas from control (A) and PDTC-treated (B) apoE/LDLR-double knockout mice.

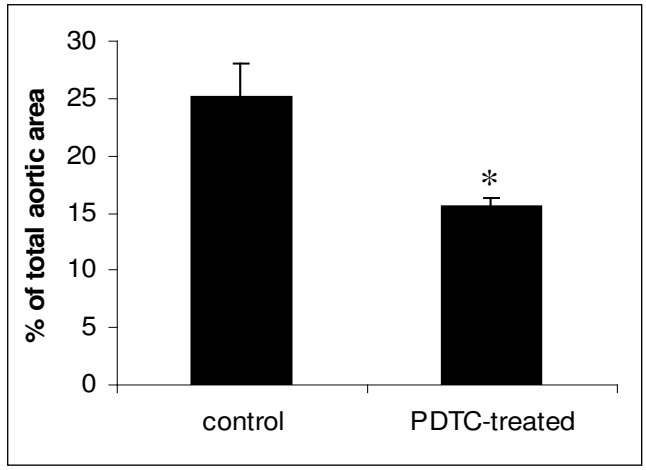

Fig. 2. Lipid lesion area in the entire aorta (percentage of total aortic surface area) in control and PDTC-treated apoE/LDLR-double knockout mice (n=10 per group). Results presented as mean±SEM. *p<0.05

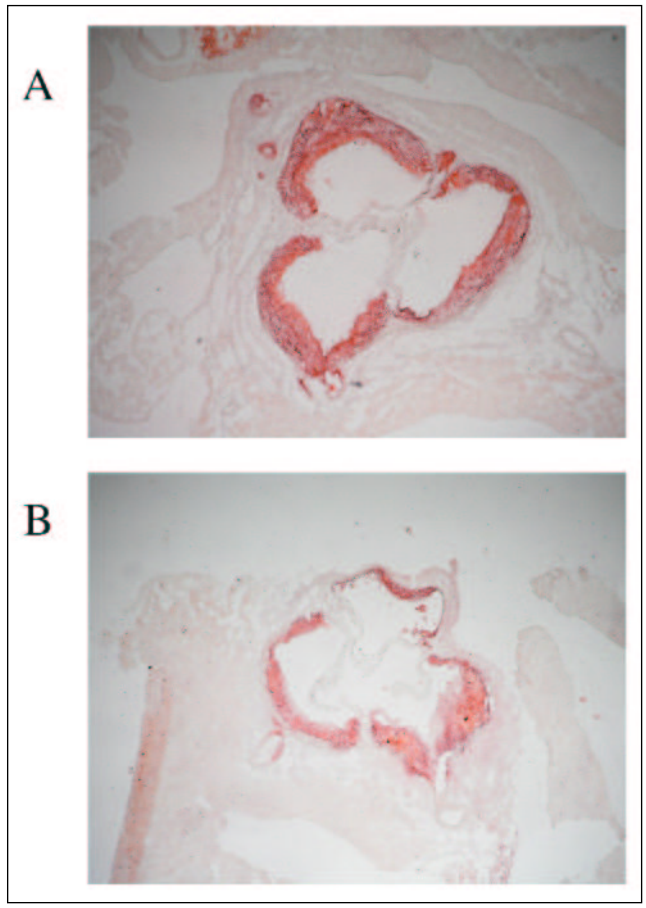

Fig. 3. Representative micrographs showing oil red O - stained lesions in control (A) and PDTC-treated (B) apoE/LDLR-double knockout mice (magnification × 40).

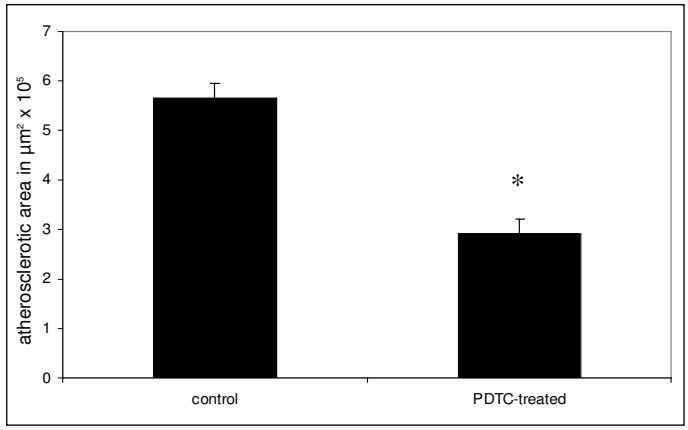

Fig. 4. Lesion size in the aortic root ($\mu m^2 \times 10^5$) stained by oil-red O in control and PDTC-treated apoE/LDLR-double knockout mice (n=10 per group). Results presented as mean±SEM. *p<0.05

Table 1. Cholesterol (TCH) and triglycerides (TG) levels in control and PDTC-treated groups, presented as mean±SEM. NS: non-significant difference between groups.

group	TCH (mmol/l)	TG (mmol/l)
control (n=10)	26.8±1.3	2.01±0.1
PDTC treated (n=10)	26.1±1.1 (NS)	1.8±0.2 (NS)

control group versus 291695±30384 μm^2 in PDTC-treated group ($p<0.05$) (*Fig. 4*). PDTC did not change the profile of cholesterol and triglycerides in blood (*Table 1*).

DISCUSSION

Nuclear factor - κB (NF-κB) is a good therapeutic target for cardiovascular disease and numerous efforts are being made to develop safe NF-κB inhibitors (13-15). Nowadays many authors address NF-κB as a major therapeutic target in atherosclerosis, especially for preventive measures, in the light of two main hypothesis of atherosclerosis: oxidation and inflammation (16-21). It was shown that reduction of NF-κB activity by simvastatin leads to decrease of atherosclerosis (22).

Dithiocarbamates were first synthesized in the mid-1800s, and since then have found numerous applications in the chemical, agricultural, and pharmaceutical industries (23). Their utility in a wide variety of settings can be attributed to their metal binding and antioxidant properties. Dithiocarbamates are well known for their antioxidant properties and effects on cellular transcriptional events. Pyrrolidine dithiocarbamate (PDTC) is widely used as an inhibitor of nuclear factor kappa B and this, or related compounds may have therapeutic potential in inhibiting atherosclerosis. However, the precise molecular mechanisms through which PDTC could elicit antioxidant or cell signaling effects in a cellular setting remain unclear (24).

A critical event in the pathogenesis of atherosclerosis is the infiltration of inflammatory cells into the vessel wall (25). Vascular cell adhesion molecule-1 (VCAM-1) plays a pivotal role in this process by mediating leukocyte binding to endothelial cells. PDTC markedly represses cytokine-induced VCAM-1 gene expression in cultured human endothelial cells (23). PDTC is also a potent inducer of the stress-inducible gene, heme oxygenase-1 (HO-1). Recently, it has been shown that HO-1 induction prevents oxidant-stressed endothelial upregulation of adhesion molecules and the development of transplant atherosclerosis in normal mice (26).

In experimental gene-targeted mice fed by atherogenic Western diet, lesion formation is greatly accelerated and lesion size is increased. Moreover, advanced lesions develop at a significantly earlier age (27). That is why we have chosen this diet to feed our mice.

To our knowledge, this is the first report that shows the effect of PDTC on atherogenesis in gene-targeted apoE/LDLR - double knockout mice.

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