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Leukotriene Synthesis Inhibitors Modulate Atherosclerosis Progression In Hypercholestrolemic Rabbits.

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ABSTRACT

This study was undertaken to evaluate the effect of zileuton on the progression of atherosclerosis. A total of 28 local domestic rabbits were used in this study and were randomized into to four groups. Group I (normal control) received standard chow diet, other three groups received atherogenic diet (2% cholesterol) and treated as follows: Group II (atherogenic control) received no treatment, Group III (vehicle control) received ethanol 10 % as solvent, Group IV received zileuton 150 mg\kg daily. Blood samples were collected at the end of experiment (8 weeks) for measurement of total cholesterol (TC), serum triglycerides (TG), HDL-C, plasma high sensitive C-reactive protein (hsCRP), plasma malondialdehyde (MDA) and plasma reduced glutathione (GSH). Immunohistochemical analysis (VCAM-1, MCP-1, IL17 and TNF-α,) and histopathologic assessment of aortic atherosclerotic changes were also performed. Compared to NC, levels of lipid profile, atherogenic index, hsCRP, and MDA are increased while GSH were decreased in animals on atherogenic diet ($p< 0.05$). Immunohistochemical analysis showed that aortic expression of VCAM-1, MCP-1, IL17 and TNF-α were significantly increased in AC group compared to NC group ($p<0.001$). Histopathologic finding showed that animals on atherogenic diet have significant atherosclerotic lesion compared to NC group. Compared to AC group zileuton don't have significant effect on lipid profile. Zileuton causes statistically significant reduction in hsCRP and MDA ($p<0.05$). Zileuton treatment causes significantly increase the level of GSH. Zileuton treatment significantly reduced aortic expression of VCAM-1, MCP-1, IL17 and TNF-α ($p<0.005$). Histopathologic examination of aortic arch showed that zileuton significantly reduced atherosclerotic lesion ($p<0.005$). It thus can be concluded that zileuton reduces lipid peroxidation, systemic inflammation and aortic expression of inflammatory markers used in this study and hence reduce the progression of atherosclerosis.

Keywords: atherosclerosis, zileuton, oxidative stress.

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INTRODUCTION

Atherogenesis is now viewed as the outcome of hypercholesterolemia in combination with inflammation of the vessel wall; an intertwined sequence of events leads to fatty streaks, which may develop to atherosclerosis (1). Cysteinyl leukotrienes (Cys-LTs) are potent inflammatory lipid mediators derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism. Many studies revealed the presence of Cys -LTs in atherosclerotic lesions playing a key role as signaling molecules in atherosclerosis (2). Lipoxygenases can in principle contribute to the pathophysiology of atherosclerosis in two ways: by LDL oxidation and by biosynthesis of proinflammatory leukotrienes (1). Leukotriene B4 (LTB4) promotes atherosclerosis by chemo-attracting monocytes, by providing an amplification loop of monocyte chemotaxis via CCL2 production, and by converting monocytes to foam cells by enhanced expression of CD36 and fatty acid accumulation (3). Furthermore, LTB4 enhances this inflammatory response by several mechanisms, one of which is the induction of pro-inflammatory cytokine release from BLT-expressing leucocytes. These include IL-6, MCP-1 and TNFα, all of which have been linked to pro-atherosclerotic functions (4). Furthermore, BLT receptor antagonism reduces intimal hyperplasia after vascular injury in rats (5). Although healthy human arteries may not express receptors for LTB4, an endothelial BLT1 receptor expression is induced in atherosclerotic lesions (6). Although the understanding of the underlying pathology of atherosclerosis has improved in recent years, the disease is still the main cause of death globally. Current evidence has implicated the role of inflammation in atherogenesis and plaque destabilization. Thus, inflammatory cytokines may attenuate interstitial collagen synthesis, increase matrix degradation, and promote apoptosis in several atheroma-associated cell types, and all these cellular events may enhance plaque vulnerability. Several cell types found within the lesion (i.e., monocyte/macrophages, T cells, mast cells, platelets) contribute to this immune-mediated plaque destabilization (7). Hypercholesterolemia enhances the response to vasoconstrictor agonists and attenuates endothelium-dependent relaxation in isolated vessels and in vivo. Endothelial derived nitric oxide (EDNO) is now recognized to inhibit several pathologic processes that are critical to the development of atherosclerosis. These include monocyte adherence and chemotaxis, platelet adherence and aggregation, and vascular smooth muscle proliferation (8). Morphologic studies have established that, once adherent to the endothelial cell, leukocytes enter the intima by diapedesis between endothelial cells at their junctions. Investigators have defined families of chemoattractant cytokines (chemokines) capable of recruiting leukocytes into the arterial intima. For example, monocyte chemoattractant protein-1 (MCP-1), overexpressed in human and experimental atheroma, can recruit the mononuclear phagocytes that characteristically accumulate in the nascent atheroma. IL-8 may have a similar role as a leukocyte chemoattractant during atherogenesis (9). Atheroma overexpresses other chemokines that may contribute to lymphocyte recruitment, including a trio of CXC chemokines induced by interferon-γ (IFN-γ) (10). Chemoattraction of mast cells found in atheroma may depend on eotaxin, a CC chemokine also overexpressed in these lesions (11). Within the intima, monocytes mature into macrophages under the influence of macrophage colony stimulating factor, which is overexpressed in the inflamed intima (12).

Zileuton is a potent and selective inhibitor of 5-lipoxygenase activity and thus blocks the formation of all 5-lipoxygenase products. Thus, in addition to inhibiting the formation of the cys-LTs, zileuton also inhibits the formation of LTB4, it is use as anti-asthmatic (13) , it is rapidly absorbed upon oral administration with a mean time to peak plasma concentration (Tmax) of 1.7 hours and a mean peak level (Cmax) of 4.98 µg/mL, the absolute bioavailability is unknown, Systemic exposure (mean AUC) following 600 mg Zileuton administration is 19.2 µg.hr/mL, the apparent volume of distribution (Vd) is approximately 1.2 L/kg, it is 93% bound to plasma proteins. Elimination is predominantly via metabolism with a mean terminal half-life of 2.5 hours. Its activity is primarily due to the parent drug. Zileuton and its N-dehydroxylated metabolite can be oxidatively metabolized by the cytochrome P450 isoenzymes 1A2, 2C9 and 3A4 (CYP1A2, CYP2C9 and CYP3A4) (14). Xian-kun Tu *et al* (2010) found that zileuton decrease cerebral ischemia and infarction size through reduction in inflammatory response(15). Furthermore, zileuton protects against oxidative stress especially against H2O2 induced oxidative stress(16).

MATERIALS AND METHODS.

Animals

A total of 28 local domestic rabbits, weighing (1.5-2) kg, were used in this study. All experiments were conducted in the College of Medicine, Department of Pharmacology, Kufa University, according to the

guidelines for the Care and Use of Laboratory Animals in scientific research. The animals were placed in an animal house, in a group caging system, at controlled temperature (25±2◦C) and ambient humidity. Lights were maintained on a 12-h light/dark cycle. The animals had free access to water *ad libitum*.

Drugs

Zileuton (CORNERSTONE THERAPEUTICS INC. B.N 3083072) was dissolved in ethanol (17), and used in a dose of 150 mg/kg/day (18). This drug was administered once daily to the animal according to body weight by oral route through stomach tube.

Animal model of atherosclerosis

Induction of hyperlipidemia and subsequent development of atherosclerosis were carried out by feeding the rabbits an atherogenic diet (2% cholesterol, BDH Chemicals Ltd Poole England, prod 43011)-enriched diet made by addition of cholesterol powder to chow pellets) for 8 weeks (19).

Experimental Protocol

After 2 weeks of acclimatization period, the animals randomized into 4 groups (of 7 rabbits each): Normal diet control group (NC, group I), high-cholesterol diet group which served as atherogenic control (AC, Group II), high-cholesterol diet group with ethanol 10% as vehicle served as positive control group(PC, Group III) and high-cholesterol diet with zileuton group (Group IV) The NC group was fed normal rabbit chow, whereas the high cholesterol diet groups were fed a 2% high-cholesterol (atherogenic) diet. The duration of treatment was 8 weeks. At the end of the experiment, food was withhold for 16-18 hour and animals were anesthetized by ketamine (HIKMA pharmaceuticals B.N 3310) at 66 mg/kg and xylazine (alfasan B.N 1004111-07) at 6 mg/kg intramuscular (20). The chest was opened by thoractomy, blood sample was collected directly from the heart and aorta was separated before following investigations were performed:

- Lipid profile including total serum cholesterol (TC), low density lipoprotein (LDL), and high density lipoprotein (HDL).
- Immunohisatochemistray for assessment of VCAM-1, TNFα, IL17 and MCP1.
- Oxidation parameter including MDA and GSH.
- Systemic inflammatory marker hsCRP
- Histopathological examination of the aorta for assessment of atherosclerosis.
- All specimens were immediately fixed in 10% formaldehyde solution for subsequent processing.

Biochemical Procedures

Serum lipid profile, including total cholesterol and TG, were determined by enzymatic methods using an automatic analyzer (Abbott, Alcyon 300, USA). Plasma GSH levels was determined using methods of Beutler (21) .Plasma MDA level was determined by using competitive inhibition enzyme immunoassay technique ((cusabio; Catalog No.CSB-E13712Rb). While Determination of hsCRP was done by using rabbit high-sensitive CRP ELISA kit supplied by (KAMIYA BIOMEDICAL COMPANY; Cat. No. KT-097) the measurement was carried out according to the manufacturer's instructions.

Histological examination of the aorta

For histological evaluation of atherosclerosis, the specimens were processed in usual manner, and embedded in paraffin and cut into 5 µm thick sections. The tissue sections were stained with hematoxylin and eosin. The assessment of atherosclerotic changes was performed according to the American Heart Association classification of atherosclerosis; Type I and Type II lesions (early lesions), Type III lesions (intermediate lesions or preatheroma), Type IV lesions (atheroma), Type V lesions (fibro-atheroma or advance lesion) and Type VI (complicated lesion) (22).

Immunohistochemistry

Immunohistochemistry was performed with polyclonal goat antibodies, raised against rabbit VCAM-1, TNFα, IL17 and MCP-1 Staining procedure was carried out according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc). The stain intensity was scored to 0: Indicated no staining, 1: Weak, 2: Moderate, 3: Strong, 4: Very strong stain intensity (23) (Figure1).

Statistical analysis

Statistical analyses were performed using SPSS 12.0 version. Data were expressed as mean ± SEM. Paired t-test was used to compare the mean values within each group at different time. Analysis of Variance (ANOVA) was used for the multiple comparison among all groups. The histopathological grading was assessed by Mann-Whitney test. In all tests, P< 0.05 was considered to be statistically significant.

RESULTS

Effect of high cholesterol diet

Compared to NC group, rabbits fed on cholesterol-enriched diet showed significant changes in serum lipid profile, oxidation and inflammatory markers. Serum levels of TC, TG and LDL-C as well as plasma level of MDA and hs-CRP were significantly ($p<0.001$) 7 increased. In addition plasma levels of GSH were significantly ($p<0.001$) lower in rabbits fed on cholesterol-enriched diet in comparison to animals on normal diet.

Effects of zileuton treatment

Compared to atherogenic control, treating hyperlipidemic rabbits with zileuton resulted in significantly ($p<0.001$) lower levels of plasma hs-CRP and MDA with significantly ($p<0.001$) higher levels of plasma GSH levels. However zileuton treatment caused no significant ($p>0.05$) alteration in the serum lipids.

Immunohistochemistry

The result of immunohistochemical analysis for rabbit's aortic arch of VCAM-1, MCP-1, IL17 and TNF-alpha were significantly different between all the 4 study groups. The median intensity of these markers was highest in AC group (very strong for all markers) and lowest in NC group (normal for all markers). There is no statistically significant difference in median intensity of these markers between PC and AC control groups on atherogenic diet. Zileuton treated group was associated with a moderate median stain intensity that is significantly lower than the atherogenic control.

Histopathological findings

The atherosclerotic lesions of aortic arch were graded as normal, initial, intermediated, advance and complicated lesions (figure2). The median was highest in atherogenic control (advance) and vehicle control (PC) whereas lowest in the normal diet control (no abnormality). Zileuton treated group was associated with a median aortic change (initial) that is significantly lower than the atherogenic control.

Table (1): Change in serum lipid profile in the normal control (NC), atherogenic control (AC), vehicle control (PC) and montelukast treated groups.

Groups				*Parameters*
Zileuton treated	PC	AC	NC	
1170.7±39.96^{N}	1116.4±42.9	1121.4±41.6*	45.1±0.94	TC (mg/dl)
358.7±42.77^{N}	357±35.18	357.7±41.3*	56±1.13	TG(mg/dl)
22.7±1.17^{N}	22.1±0.77	23±1.35^{N}	18.1±0.99	HDL(mg/dl)
1076.3±41.91^{N}	1022.9±38.7	1026.9±37.61*	15.8±1.71	LDL(mg/dl)
71.7±8.55^{N}	71.4±7.04	71.5±8.27*	11.2±0.23	VLDL (mg/dl)

Results are expressed as mean ± SEM.
*$p < 0.05$, as compare to NC group, ^{N} not significant as compare to PC group

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Table (2): Change in mean plasma levels of hs-CRP, MDA and GSH among the four study groups.

Groups				
Zileuton treated	PC	AC	NC	**Parameters**
0.798±0.0164**	0.52±0.013	0.568±0.024*	1.102±0.0258	Plasma GSH (mmol/L)
0.229±0.0117**	0.51±0.0142	0.51±0.0145*	0.133±0.005	Plasma MDA(µmol/L)
62.3±1.69**	135.9±2.09	135.3±1.4*	32.9±0.88	Plasma hsCRP (µg/L)

*p < 0.05, as compare to NC group, **p < 0.05, as compare to AC group.

Table (3): The difference in median tissue (VCAM-1, MCP-1, IL17 and TNF alpha) immunostain intensity among the four study groups.

Groups				
Zileuton treated	Pc	AC	NC	**Markers**
Moderate**	Very strong *	Very strong *	Negative	VCAM-1
Moderate **	Very strong*	Very strong*	Negative	MCP-1
Moderate **	Very strong*	Very strong*	Negative	IL17
Moderate **	Very strong*	Very strong*	Negative	TNFα

*p < 0.05, as compare to NC group, **p < 0.05, as compare to PC group.

Table 4:The difference in median atherosclerotic lesion Phase between the 4 study groups. P (Kruskal-Wallis<0.001)(

	Normal diet control group (NC)		Atherogenic diet control group (AC)		Ethanol solvent+atherogenic diet positive control		Zileuton treatment	
	N	%	N	%	N	%	N	%
Negative lesion	7	100	0	0	0	0	0	0
I	0	0	0	0	0	0	2	28.6
II	0	0	0	0	0	0	3	42.9
III	0	0	2	28.6	2	28.6	2	28.6
IV	0	0	3	42.9	3	42.9	0	0
V	0	0	1	14.3	1	14.3	0	0
VI	0	0	1	14.3	1	14.3	0	0
Total	7	100	7	100	7	100	7	100
Median	Negative		IV		IV		III	
Mean rank	4		27.93		27.93		14.71	
P (Mann-Whitney) for difference in median between 2 groups: AC group X NC group=0.001 Positive control (PC) X AC group=1[NS] Zileuton X Positive control (PC)=0.003 Zileuton X NC group=0.001								

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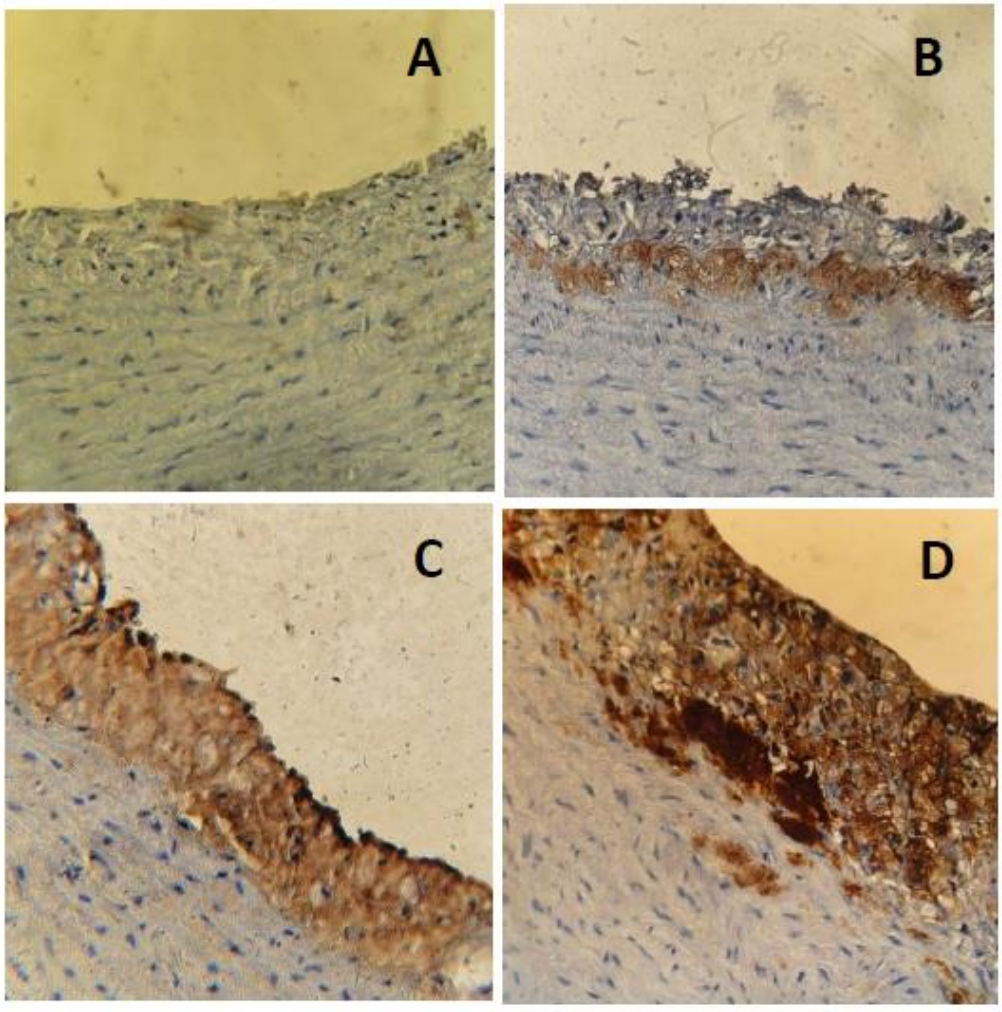

(Figure 1): Immunohistochemical staining for, IL 17 expression in aortas arch from cholesterol-fed rabbits (x40). **A**: negative, **B**: weak stain intensity, **C**: moderate stain intensity, **D**: strong stain intensity, **E**: very strong stain intensity.

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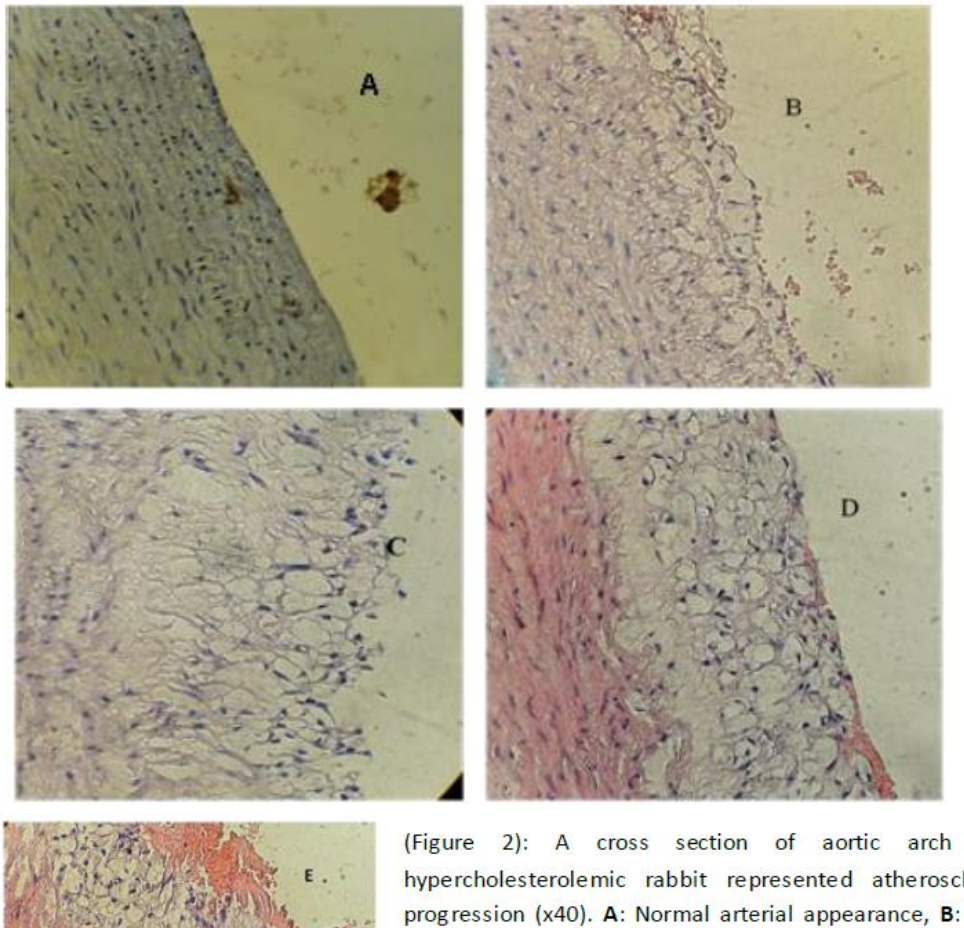

(Figure 2): A cross section of aortic arch from hypercholesterolemic rabbit represented atherosclerosis progression (x40). **A**: Normal arterial appearance, **B**: Initial atherosclerotic lesion characterized by lipid laden macrophage (foam cells), **C**: Intermediate atherosclerotic lesion characterized by extracellular lipid pool. **D:** Advance atherosclerotic lesion characterized by core of extracellular lipid and. **E**: Complicated atherosclerotic lesion characterized by haemorrhagic thrombus.

Effect of atherogenic diet and treatment on aortic atherosclerotic lesion

Severity (phase) As shown in table 4, the median of the atherosclerotic lesion phase was highest in control animals on atherogenic diet with or without Ethanol solvent (Phase-IV) and lowest in control animals on normal diet (normal arterial wall). Atherogenic diet was associated with statistically significant higher median of atherosclerotic lesion phase (median = IV) compared to NC group (median = negative). Among animals on atherogenic diet zileuton treated group showed a statistically significant changes less severe atherosclerotic lesion (phase III) was observed compared to untreated positive control group (median = IV).

DISCUSSION

In comparison to atherogenic control, the present study demonstrated that zileuton treatment had no effect on lipid profile. Further this study revealed that the values for the lipid peroxidation marker (MDA) were significantly reduced by zileuton treatment. GSH levels were significantly higher in zileuton treated animals. These finding suggested that Zileuton markedly reduce oxidative stress. Xian-kun Tu et al. (2010) demonstrated that zileuton reduced MDA content in rats. The selective 5-LOX inhibitor zileuton inhibited the

activation of NF-κB and 12 reduced the expression and activation of iNOS , In addition, NF-κB has been shown to regulate the expression of iNOS and other inflammatory mediators, attenuation of iNOS expression and NO production has been demonstrated to have protective role (16). Concerning hsCRP this study showed that zileuton treatment significantly reduces plasma hsCRP compared to AC group. Jean et al. (2010) showed that treatment with 5-Lipoxygenase inhibitor VIA-2291 (Atreleuton) in patients with recent acute coronary syndrome caused a significant reduction in hs-CRP (24). Other findings of this study revealed that zileuton significantly reduces the expression of aortic inflammatory marker (MCP-1, TNF-α, VCAM-1, and IL17). Regarding to MCP-1, this finding was in agreement with Li Huang et al. (2004), they found that LTB4 strongly induces MCP-1 production in primary human monocytes; this induction is mediated through the BLT1 pathway increasing MCP-1 transcription. Activation of ERK1/2 or JNK MAPK is essential for this induction, the NF-κB activation may be involved in LTB4-increased MCP-1 expression. The LTB4-induced MCP-1 in human Monocytes may play a critical role in the atherogenicity of LTB4 (25). Regarding to TNF-α , this finding was in agreement with Marco et al. (2006) they found that Pharmacological inhibition of leukotrienes by zileuton in an animal model of bleomycin-induced acute lung injury resulted in a marked reduction in TNF-α immunostaining in lungs Zileuton treated group (26). The mechanisms of TNF-α pro-inflammatory activity are likely to involve both direct effects of TNF-α itself on regulation of adhesion molecule expression and induction of other cytokines and growth factors capable of mediating leukocyte chemotaxis and survival (27). Regarding to VCAM-1, Salvatore et al. (2005) revealed that 5-Lipoxygenase modulates colitis through the regulation of adhesion molecule expression and neutrophil migration; they concluded that the upregulation of P-selectin, E-selectin, ICAM-1, and VCAM-1 in the lung was largely attenuated with Zileuton treatment (28). Mehrabian et al. (2002) reported that heterozygotes for the 5-LO gene on the LDLR_/_ background had considerably reduced aortic lesions despite hypercholesterolemia as compared with the advanced lesions of LDLR_/_ mice (29). Dwyer et al. (2004) showed that variant alleles of 5-LO genes were associated with a significant increase of carotid intima thickness (30). Helgadottir et al. (2004) demonstrated a significant association between the gene encoding 5-LO activating protein (FLAP) and myocardial infarction by analysis of single-nucleotide polymorphism haplotype in humans (31). The beneficial effect of zileuton on the progression of atherosclerosis may be due to their 13 favourable effect on oxidative stress, systemic inflammation and aortic inflammatory marker (VCAM-1, MCP-1 IL17 and TNF-α).

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