THE EFFECT OF ATORVASTATIN LOADED APTAMER CONJUGATED SILICA NANOPARTICLES ON ANTI-INFLAMMATORY SIGNALING PATHWAY IN HUVECs

by

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ABSTRACT

THE EFFECT OF ATORVASTATIN LOADED APTAMER CONJUGATED SILICA NANOPARTICLES ON ANTI-INFLAMMATORY SIGNALING PATHWAY IN HUVECs

In worldwide, cardiovascular diseases (CVD) are first causes of death and affecting millions of humans. Atherosclerosis is a type of chronic inflammatory disease that limits flowing of high oxygen contain blood to organs. Progress of atherosclerosis may result in heart attack and stroke. Drug therapy still is not available to date that directly target and suppress the molecular mechanisms of inflammation. In current clinical practice, only few therapies such as statin and aspirin are effectively diminishing the cardiovascular diseases such as atherosclerosis. However, only 30 to 40 percent of the patients give positive feedback to these treatment strategies. Therefore, it is important to develop the safe delivery of drug administration to the disease region. Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors that prevent production of LDL inside liver. Besides to cholesterol reducing effect of statin, they have various pleiotropic effects in this way they act as vascular protective. This study aim was to evaluate therapeutic efficiency of atorvastatin loaded targeted and controlled release system compared to only atorvastatin via p-Akt/eNOS activation pathway in endothelial cells. For this reason, ICAM-1 specific Aptamer conjugated Atorvastatin encapsulated mesoporous silica nanoparticles (ICAM-1 APT-Atorv MCM41) was developed. HUVEC stimulated six hours with TNF-α for then incubated with Atorvastatin and ICAM-1 APT-Atorv MCM41 for 24 hours. mRNA expression of eNOS and protein levels of eNOS and p-Akt and were observed with RT-PCR and Western blot methods. This study results indicated that targeted delivery of atorvastatin increased eNOS and p-Akt (Ser473) expression level. In conclusion, ICAM-1 Aptamer conjugated atorvastatin encapsulated MCM-41 nanoparticle drug delivery system may provide new candidate approach for preventing atherosclerosis.

ÖZET

APTAMER BAĞLI ATORVASTATIN YÜKLÜ SİLİKA NANOPARTİKÜL SİSTEMİNİN HUVEC HÜCRELERİNDE İNFLAMASYON KARŞITI SİNYAL YOLAĞINA ETKİSİ

Dünya genelinde kardiyovasküler hastalıklar ölüm nedenleri arasında birinci sıradadır ve milyonlarca insanı etkilemektedir. Ateroskleroz, oksijen bakımından zengin kanın organlara akışını sınırlayan kronik enflamatuar hastalıktır. Aterosklerozun ilerlemesi kalp krizi ve/veya inme ile sonuçlanabilir. Enflamasyonun moleküler mekanizmasını doğrudan hedef alan ve baskılayan hiçbir ilaç tedavisi bulunmamaktadır. Mevcut klinik uygulamada, statin ve aspirin gibi sadece birkaç tedavi ateroskleroz gibi kardiyovasküler hastalıkları etkin bir şekilde azaltır. Bununla birlikte, yüzde 30 ila 40'ı bu tedavi stratejilerine olumlu geribildirim veriyor. Bu nedenle, ilaç uygulamasının hastalık bölgesine güvenli bir şekilde verilmesini geliştirmek önemlidir. Statinler karaciğer de LDL üretimini inhibe eden 3-hidroksi-3-metilglutaril-koenzim A (HMG-CoA) redüktaz inhibitörlerdir. Kolesterol düşürücü etiklerine ek olarak, damar koruyucu pleiotropik etkilere sahiptir. Bu çalışmanın amacı, endotel hücrelerinde atorvastatin ilacının en önemli pleitropik etkisini, p-Akt/eNOS aktivasyon yolu, gözlemleyerek atorvastatinin hedefli ve kontrollü salım sistemi yoluyla terapötik etkinliğini değerlendirmektir. Bu nedenle ICAM-1 hedefli aptamer konjuge edilmiş atorvastatin kapsüllenmiş mesoporous silica nanoparçacıkları geliştirildi (ICAM-1 Aptamer-Atorv-MCM41). HUVEC hücreleri altı saat boyunca TNF-α ile ön işleme tabi tutuldu, daha sonra 24 saat boyunca Atorvastatin ve ICAM-1 Aptamer-Atorv-MCM41 ile inkübe edildi. eNOS ve p-Akt (Ser473) mRNA ekspresyonu ve protein seviyeleri RT-PCR ve Western blot yöntemleri ile belirlendi. Sonuçlara göre, atorvastatin ilacının hedefli ve kontrollü salım sistemi ile eNOS ve p-Akt (Ser473) ekspresyonunu artırdığını gösterdi. Sonuç olarak, ICAM-1 aptamer konjuge edilmiş atorvastatin kapsüllenmiş MCM41 hedefli ilaç dağılım sistemi aterosklerozun önlenmesi için yeni aday yaklaşım sağlayabilir.

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LIST OF SYMBOLS/ABBREVIATIONS

°C	Degree centigrade
µg/ml	Microgram per milliliter
µM	Micromolar

Akt	Serine/threonine kinase
BH_4	Tetrahydrobiopretin
BK_{CA}	Calcium-sensitive K channels
CAM	Calcium (Ca^{2+})/Calmodulin
CCL2	C-C motif chemokine ligand 2
CeVD	Cerebrovascular disease
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GTP	Guanyl triphosphate
HAEC	Human aortic endothelial cell
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HRMEC	Human retinal microvascular endothelial cells
HSP90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule1
IHD	Ishcemic heart disease
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
KLF2	Kruppel like factor2

LDL	Low density lipoprotein
LOX-1	Lectin-like ox-LDL receptor-1
MCP-1	Monocyte chemo attarctant protein1
MI	Myocardial infarctiong
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
nNOS	Neuronal bitric oxide synthase
NO	Nitric oxide
ox-LDL	Oxidized-Low density lipoprotein
p-Akt	Phospho-serine/threonine kinase
ROS	Reactive oxygen species
sGS	Soluble guanyl cyclase
SMC	Smooth muscle cells
TNFR-1	TNF receptor1
TNFR-2	TNF receptor2
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cellular adhesion molecule1
VLA-4	Very late antigen4
WHO	World health organization
ZnS_4	Zinc-thiolate cluster

1. INTRODUCTION

1.1. AIM OF THE STUDY

In this study, the aim was to show the pleiotropic effect of Atorvastatin, statin type of drug, on atherosclerosis via a novel targeted and controlled release drug delivery system. For this purpose, ICAM-1 specific Aptamer conjugated Atorvastatin encapsulated mesoporous silica nanoparticles (ICAM-1 APT-Atorv MCM41) were synthesized and its efficacy was tested on TNF-α stimulated human umbilical vein endothelial cells (HUVEC) which mimics the pre-atherosclerotic phase in cell culture. Therapeutic efficacy of targeted and controlled release of Atorvastatin was determined by evaluating the level of eNOS and p-Akt at mRNA and protein level.

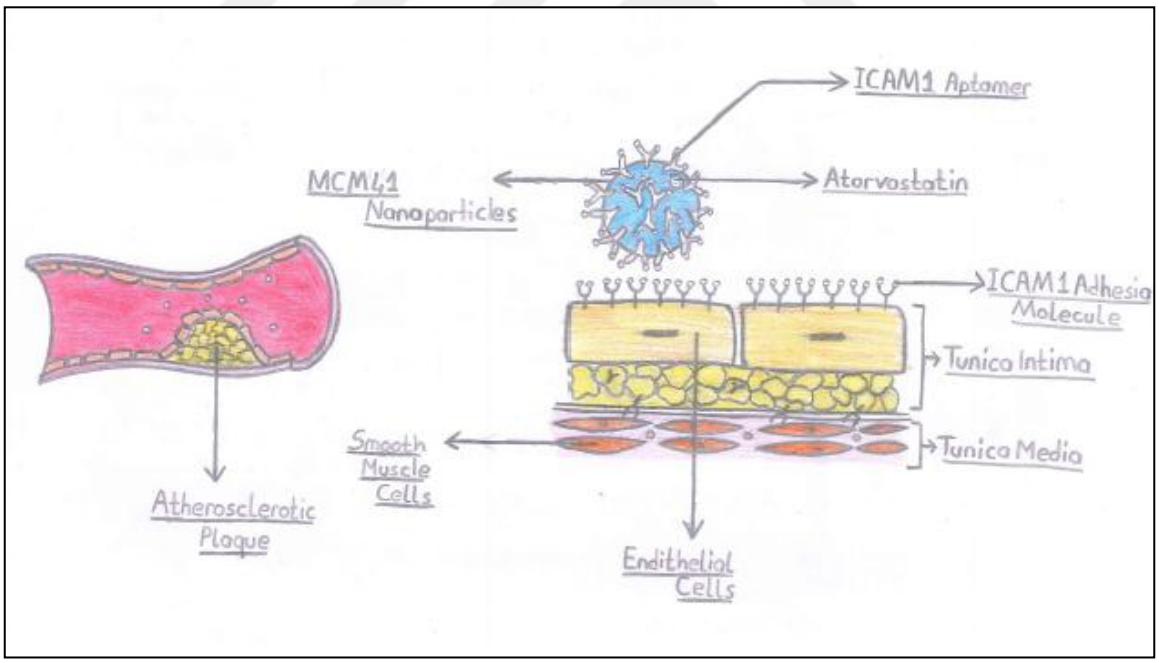


Figure 1.1. ICAM-1 APT-Atorv-MCM41. Schematic representation of targeted Atorvastatin delivery via ICAM-1 APT-Atorv-MCM41 to the inflamed region on artery wall. The release and concentration of Atorvastatin in pre-atherosclerotic region will be controlled by ICAM-1 aptamer gates.

1.2. STRUCTURE OF ARTERY

Circulatory system which is a distribution network of the body is mainly composed of blood vessels. The arteries, capillaries and veins are three different types of blood vessels categorized according to their function and structure. The type of largest of all blood vessels is arteries deliver blood from the heart out to all parts of body. They are also responsible for carrying out foodstuff to all cells and removal of carbon dioxide and biproducts which are secreted by cells. Except pulmonary which carry deoxygenated blood from heart to lungs, most arteries carry oxygen rich blood in system. During heart contractions blood inside ventricles is pumped into the large elastic arteries which branch to smallest arteries which are called arterioles. Blood inside arterioles is feeded into the capillaries which are connected to the tissues and organs. Then capillaries feed blood into the venules, the little vein and subsequently merge into the largest vein consequently it empties into the heart [1-3].

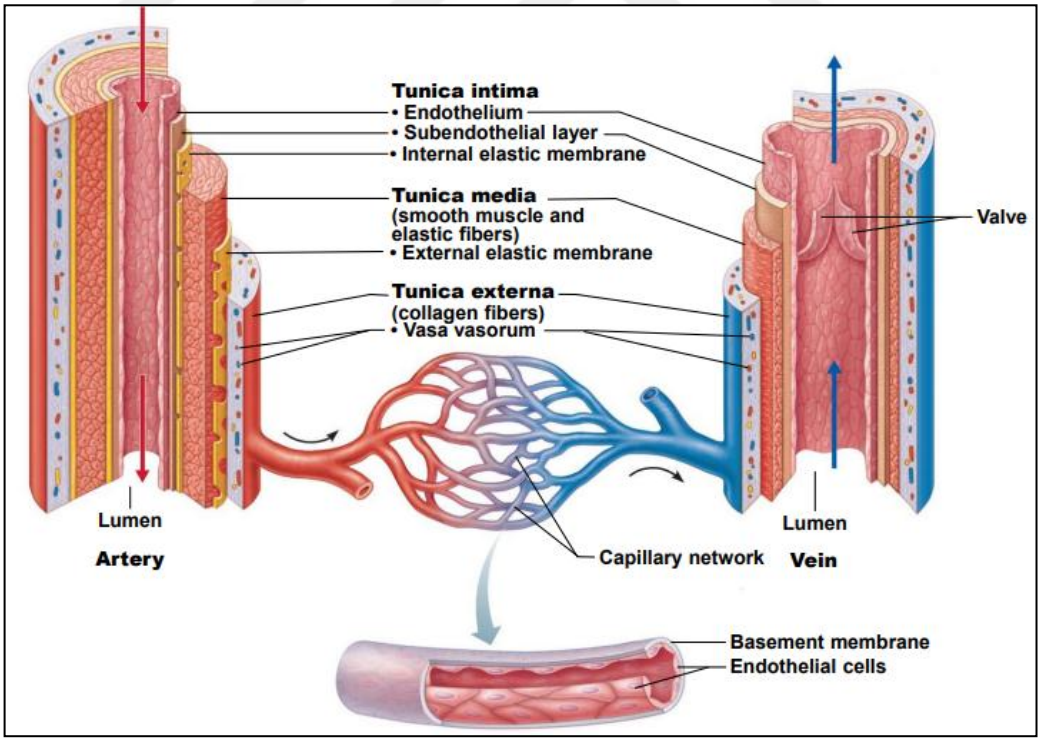


Figure 1.2. Blood vessel layers [4]

Artery wall consists of three functional layers which are tunica intima (or tunica interna), tunica media and tunica externa (or tunica adventitia). The arterial innermost layer is tunica

intima and is lined by smooth muscle tissue epithelium contains endothelial cells. This layer is exposed to hemodynamic force also called shear stress which is employed by flowing blood all the time. Therefore, tunica intima provides a resistance against flowing blood. In this structure, endothelial cells secrets factors that affect the relaxation and/or contraction of smooth muscle cells. These factors secretion depend on the types of shear stress. Specifically, endothelial cells generate nitric oxide (NO) which leads to the relaxation of smooth muscle cells. The arterial wall thickest layer and middle layer is tunica media and consist of both smooth muscle cells and elastic fibers. This layer provides additional support for the vessel and also control the diameter of the artery by smooth muscle constriction. The last part of artery is tunica adventitia, also called as tunica externa is outermost layer of arteries (Figure 1.3). Tunica adventitia, provides mechanical support, comprise of connective tissues including elastic fibers and collagen [1-3].

Arteries play vital roles in cardiovascular system due to their functional properties. Any impairment in normal function or structure of arteries may result in atherosclerosis which may lead to development of severe cardiovascular diseases (CVD) including heart attack and stroke.

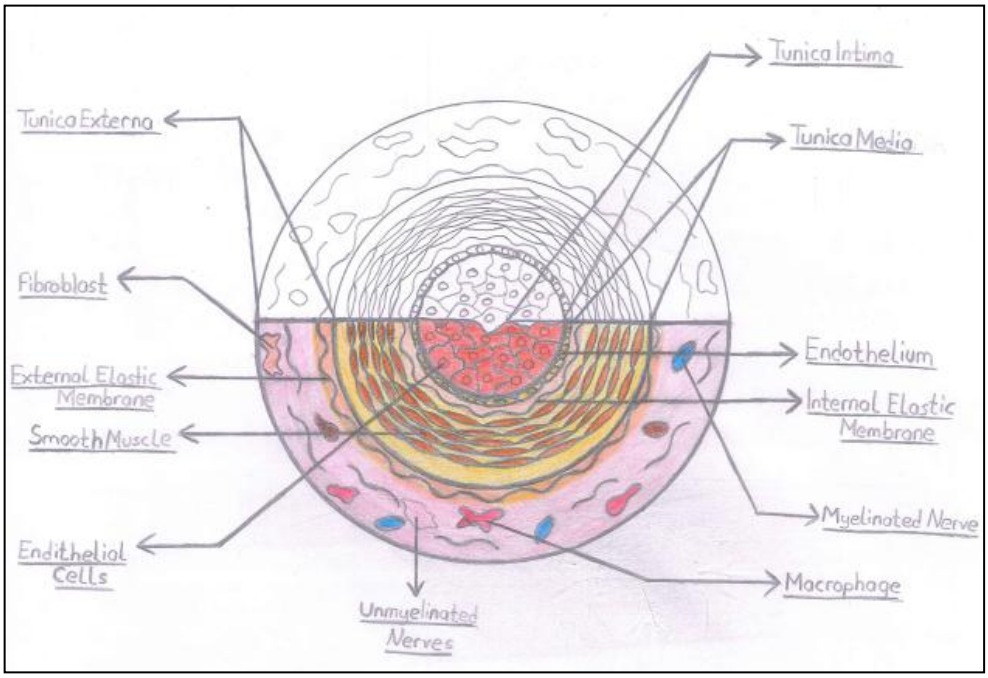


Figure 1.3. Schematic representation of layer of artery

1.3. ATHEROSCLERIS

In worldwide, cardiovascular diseases (CVD) are the first reason of deaths and affecting millions of humans. More than 17.3 million people lost their lives due to cardiovascular diseases in 2013 [5]. Most common CVDs are ischemic heart disease (IHD), or coronary artery disease like heart attack and cerebrovascular disease (CeVD) like stroke. Both IHD and CeVD are in the progress of atherosclerosis which is defined as chronic inflammatory disease. 2013 global statistical analyses reported that IHD was first and CeVD was third cause of all deaths which constitute 84.5 percent of cardiovascular deaths [6]. In 2010, ischemic heart disease and stroke contributed the most reasons of the global deaths [7]. In 2012, 17.5 million deaths occurred because of cardiovascular related diseases in worldwide and it is assumed to raise to 23.3 million by 2030 [8, 9]. According to the world health organization (WHO) reports, an estimate of 17.5 million deaths per annual which composed 31 percent of global deaths occur due to atherosclerotic cardiovascular disease [10]. American Heart Association statistics show that CVD causes nearly 836,546 deaths in U.S and this is account 1 of every 3 deaths. 92.1 million American people live with CVD. In Europe, 1.92 million deaths are reported due to CVD every year [11]. According to TEKHARF studies CVD is leading causes of deaths in Turkey (Table 1.1) [12]. In Turkey, CVD is the most leading reason of mortality with 34 percent and also 2016 WHO reports showed that 407.300 people died due to CVD [13-16]. In addition, it is indicated that death rate from CVD is high and increases each year in Turkey [15]. According to report of Turkish Society of Cardiology, in 2010 IHD was the number one causes of death with 1.8 million and followed by CeVD with 1.2 million in Turkey. In addition, they reported that estimated 3.4 million people were suffered from IHD and CeVD in 2016 and this is expected to increase to 5.4 million by 2035 [17]. In 2016, percent deaths from IHD and CeVD were 40.5 percent and 23.6 percent respectively in Turkey (Table 1.1.). In addition, according to 2017WHO report 81,029 people (108.59 per 100,000 of population) died from coronary heart disease in Turkey [18]. This was equal to about 20.34 percent of total deaths in Turkey in 2017. Recent literature reports that CVD mainly progresses of atherosclerosis is expected to raise even at greater rates in the future [19].

Table 1.1.Death distribution according to the 2015-2016 statistics in Turkey [12]

	2015		2016	
	Number	%	Number	%
Diseases of the circulatory system	159,194	100.0	162,876	100.0
Ischemic heart diseases	64,692	40.6	65,897	40.5
Cerebrovascular diseases	38,648	24,3	38,395	23.6
Other heart diseases	32,411	20.4	36,317	22.3
Hypertensive diseases	15,446	9.7	14,369	8.8
Other	7997	5.0	7898	4.8

Atherosclerosis is a type of chronic inflammatory disease that development of plaque is occurred inside vessel wall and therefore arteries become narrowed and toughened which limits flowing of highly oxygen contain blood to organs. In Greek "athero" means gruel which is accumulation of lipid and "scleros" meaning hardening which means that artery wall gets thicker and thus elasticity of artery is damaged [20].

Atherosclerotic plaque forms when endothelial cell layer exposed to types of systemic risk factors such as diabetes, smoking high blood pressure, hyperlipidemia (abnormally high level of lipid in blood), genetic inclination, and unhealthy diet. Additionally, exposing of endothelial cells to hemodynamic force involving shear stress such as low and oscillating shear stress which is generated by flowing blood is a local type risk factor and has essential role in the development of atherosclerosis [21-24]. When endothelial cells are injured by systemic risk factors and/or local risk factors, endothelial cells barrier weakens that causes to the permeability of low density lipoprotein (LDL) [25, 26]. After infiltration of LDL to the intima region of artery oxidation of LDL is carried out by reactive oxygen species (ROS) [27, 28]. Oxidized-LDL (ox-LDL) give rise to the releasing of monocyte chemo attractant protein (MCP-1) by stimulated endothelial cells which signals monocytes to attract the dysfunctional endothelial region [29, 30]. ox-LDL also triggers pro-inflammatory genes expression and increasing endothelial cells permeability [31]. At this

point, monocyte transmigrates into the intima and start to eat and digest ox-LDL. This process initiates inflammation and defined as pre-atherosclerotic phase. Following, ox-LDL leads to monocyte differentiation into macrophages which releases the tumor necrosis factor-α (TNF-α) which induces adhesion molecules expression such as intercellular adhesion molecules-1 (ICAM-1), vascular cellular adhesion molecules-1 (VCAM-1), selectins (E-selectin and P-selectin) by endothelial cells and also flux of leukocytes into inflamed region [32-35]. These adhesion molecules permit to adherence of more monocytes to the adhesion molecules on endothelial cells and migration to the tunica intima region [36]. Macrophages laden with ox-LDL changes into foam cells which accumulate and form plaque [30]. Smooth muscle cells (SMCs) proliferate due to cytokines secreted by foam cells. Then, SMCs move from tunica media to the surface of the plaque results in formation of fibrous cap, covered by collagen and SMCs (Figure 1.4.) [24, 37]. The plaque size increases resulting in thickened and hardened of the arterial wall. Over time rupturing of unstable fibrous plaque might triggers formation of thrombosis (clot) by activate platelets on the endothelial surface [26]. At the end, formation of blood clot blocks the flowing of blood and limited blood supplies available in the surrounding area. Thus, neighboring tissue deaths and consequently might result in heart attack [31].

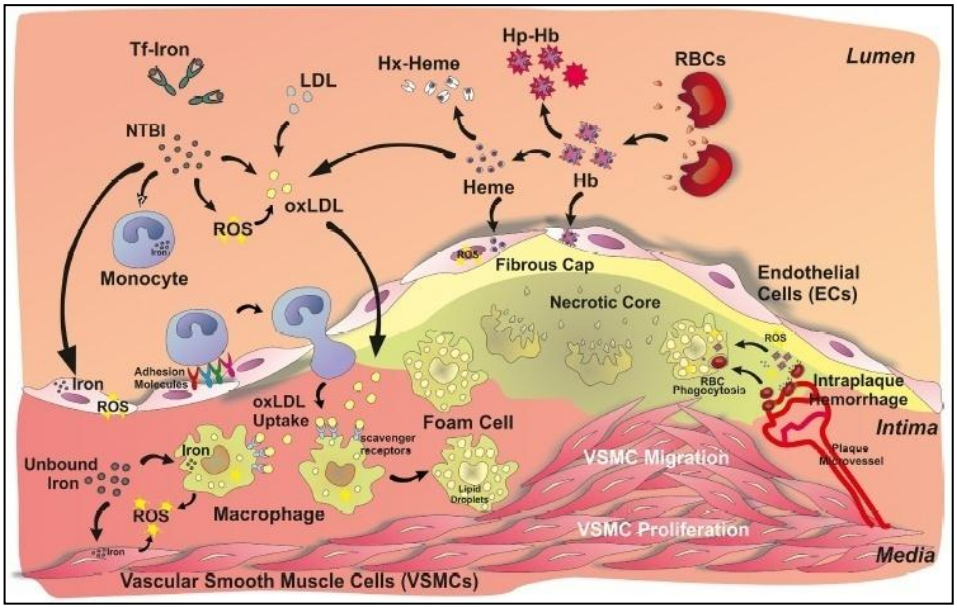


Figure 1.4. The atherosclerotic process mediated by inflammation [38]

1.3.1. Risk Factors of Inflammation

Atherosclerosis is also characterized as chronic inflammation [39]. There are many significant risk factors at molecular level that take part in the initiation of inflammation such as variations in shear stress, stimulation of adhesion molecules, accumulation of immune cells, increased level of LDL and cytokines like TNF-α.

1.3.1.1. Variations of Shear Stress and Adhesion Molecules Expression

Endothelial cells all the time expose to hemodynamic forces which is also known as shear stress caused by flowing blood inside artery. Endothelial cells convert mechanical stimuli due to blood shear stress into biological signals to control artery function [40-43]. As a result, protein and gene expression level and endothelial cells structure change. In static conditions endothelial cells shapes are cobblestone-like however in laminar flow conditions elongation and align of actins filaments, build up cytoskeleton structure of endothelial cells, in the direction of flow is happened [42, 44, 45]. However, endothelial cells that expose to oscillatory shear stress are randomly oriented and cells are polygonal [46]. Disturbed flows like oscillating and low shear stress are shown as pro-inflammatory type of shear stress and they are mostly found in the branches and bifurcations of artery [40, 47].

In vivo, normally endothelial cells are adapted to laminar shear stress at the range of 5-20 dyne/cm^2 [48]. Laminar shear stress which is relatively high is anti-inflammatory in other words it is protective flow type against atherosclerosis. The previous studies reported that laminar shear stress is protective against atherosclerosis by stimulating the expression of specific genes including eNOS [49]. eNOS release nitric oxide (NO) inhibits inflammation and protects endothelial barrier [50]. However, eNOS expression is inhibited by disturbed flow [49]. Inhibition of eNOS is hallmark of endothelial dysfunction and this condition is known as the beginning step of atherosclerosis. Guo et al. observed that laminar shear stress type leaded to enhance eNOS expression and decreased endothelin-1 (ET-1) which has significant role in atherosclerosis development [49]. Dong et al. determined that low shear stress caused to stimulate autophagy and apoptosis involve in the atherosclerotic development [51]. Nam et al. studied partial ligation artery in mouse model to characterize

low and oscillatory shear stress and in this study result endothelial dysfunction, rapid induction of atherosclerosis and pro-atherogenic genes expression were observed [52]. Wang et al. demonstrated that expression of endothelial protective protein kruppel like factor-2 (KLF2) was down-regulated by oscillating shear stress (0.5 ± 4 dyne/cm^2) [53]. Leukocytes are able to attach inflamed endothelial cell under shear stress range of 1-6 dyne/cm^2 [54].

The disturbed shear stress, occurred by flowing blood, has important role in the initial step of atherosclerotic lesion. Types of adhesion molecules such as ICAM-1, VCAM-1 are up-regulated at the sites where atherosclerotic plaques develop [55]. Exposing of endothelial cells to unstable shear stress like low or oscillating result in endothelial dysfunction by stimulating ICAM-1, VCAM-1 and E-selectin. ICAM-1 is expressed on endothelial cells. ICAM-1, VCAM-1 and E-selectin which are shown as markers of atherosclerosis are expressed via nuclear factor kappa B (NF-κB) dependent pathway in result of disturbed shear stress (Figure 1.5) [56]. In addition, disturbed shear stress induces up-regulation of MCP-1 and TNF-α, pro-inflammatory cytokines [57]. The disturbed flow following adhesion molecules expression play significant roles and it is adequate in the initiating of inflammation process, leukocyte-endothelial cell interaction. Ishibazawa et al. observed pro-inflammatory genes expression, ICAM-1 and VACM-1 in human retinal microvascular endothelial cells (HRMECs) at low shear stress (1.5 dyne/cm^2) [58]. Chappell et al. observed enhancement in the level of ICAM-1 and VCAM-1 molecules expression at the prolonged oscillating shear stress ($\pm$ 5 dyne/cm^2) [59]. All these studies showed that adhesion molecules might be a good target for drug delivery systems for the prevention of early atherogenic lesions.

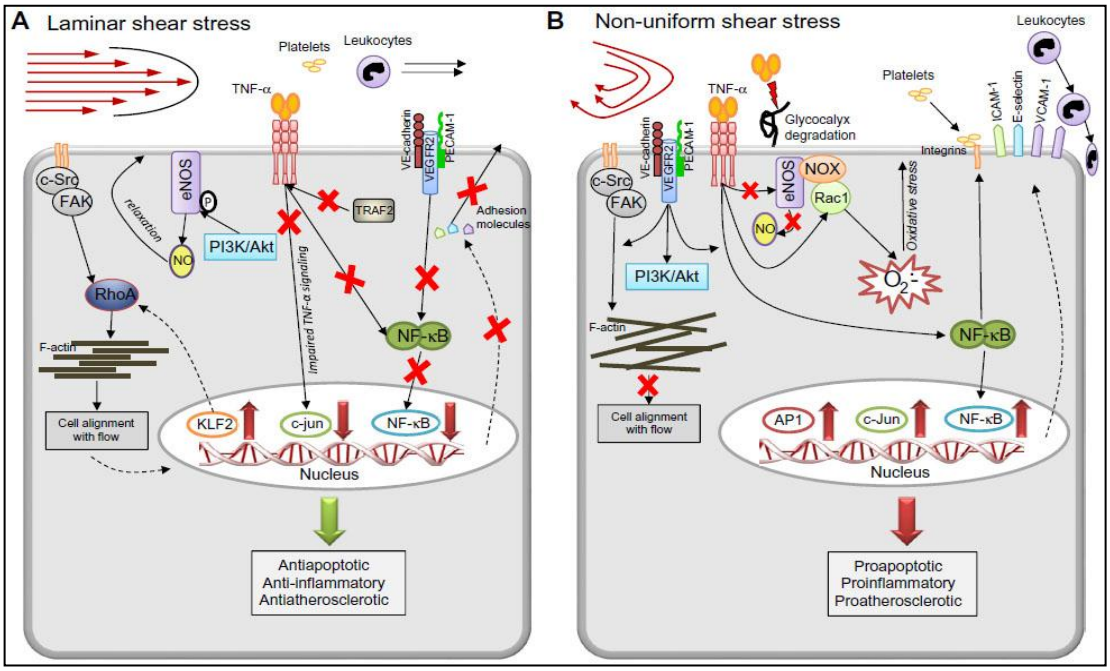


Figure 1.5. Shear stress induced changes of pro-inflammation and anti-inflammation signal pathway [60]

1.3.1.2. Low Density Lipoprotein

LDL which is also known as type of bad cholesterol is another risk factor in early process of atherosclerosis. Damages in endothelial barrier lead to migration of LDL [61]. Cholesterols are fat molecules and because of their water insoluble properties they are carried in blood with combination of certain proteins. These protein and fat combination are called lipoproteins and produced in liver. LDL is one of the lipoproteins which transport cholesterol from liver to cells whereas HDL (high density lipoprotein) that is also called as good cholesterol transport cholesterol back to the liver for ejecting from body. For this reason, if the level of LDL is over than the level of unmanageable amount, LDL constitutes a risk factor for artery where LDL accumulates under intima (Figure 1.8) [62]. After accumulation of LDLs under the artery wall they are oxidized by reactive oxygen species. Uptake of ox-LDLs by monocyte-derived macrophages is described as the early phase of atherosclerosis [63]. Ox-LDL is a cytokine stimulate monocytes differentiate to macrophages, induces the generation of macrophage colony-stimulating factor (M-CSF) by endothelial cells [64]. Ox-LDL ingested macrophages accumulate and form foam cells.

This aggregation leads to formation of the atherosclerotic plague [65]. Additionally, ox-LDL plays important role in increasing expression of MCP-1 receptor which is monocyte receptor. MCP-1 expression is done by endothelial cells and monocytes. MCP-1 stimulates monocytes to migrate and attach damaged endothelial region (Figure 1.8) [66]. Moreover, Zhang et al. determined that ox-LDL not only reduce the proliferation of endothelial cells but also conduce to ROS overproduction [67]. Studies reported that oxidation of LDL in intima leads to adhesion molecules expression and ROS production. Cominacini et al. reported that intracellular ROS production in aortic endothelial cells was increased by ox-LDL [68]. ROS stimulate apoptotic related genes expression such as Bcl-2 which roles as anti-apoptotic and Bax which roles as pro-apoptotic. Ahsan et al. showed that apoptosis increased in HUVEC treated with ox-LDL and also ROS production was observed higher in ox-LDL injured HUVEC [69]. Khan et al. determined that ox-LDL enhanced ICAM-1 expression in HAEC (human aortic endothelial cells) which was activated by TNF-α [70]. Di et al. observed highly VCAM-1 and E-selectin expression in ox-LDL injured HUVEC whereas level of eNOS reduced [29].

Su et al. also demonstrated increment in ICAM-1, VCAM-1 and E-selectin expression in HAEC treated with ox-LDL [71]. ox-LDL damages relaxation of endothelial cells by reducing the NO synthase [72]. ox-LDL causes the increasing of asymmetric dimethylarginine (ADMA) which has role to inhibit L-arginine binding to eNOS resulting in O_2^- production increase and NO decrease [73]. LOX-1 is main receptor of ox-LDL and responsible for LDL uptake by endothelial cells and has a role in induction of ROS production [27, 74, 75]. The expression of LOX-1 is also occurred in macrophages and smooth muscle cells [76].

All these previous studies have implicated that ox-LDL has priority role in atherosclerosis development mechanism. These pro-inflammation features of LDL show that reducing accumulation of LDL in artery wall might be a possible therapeutic candidate for preventing atherosclerosis.

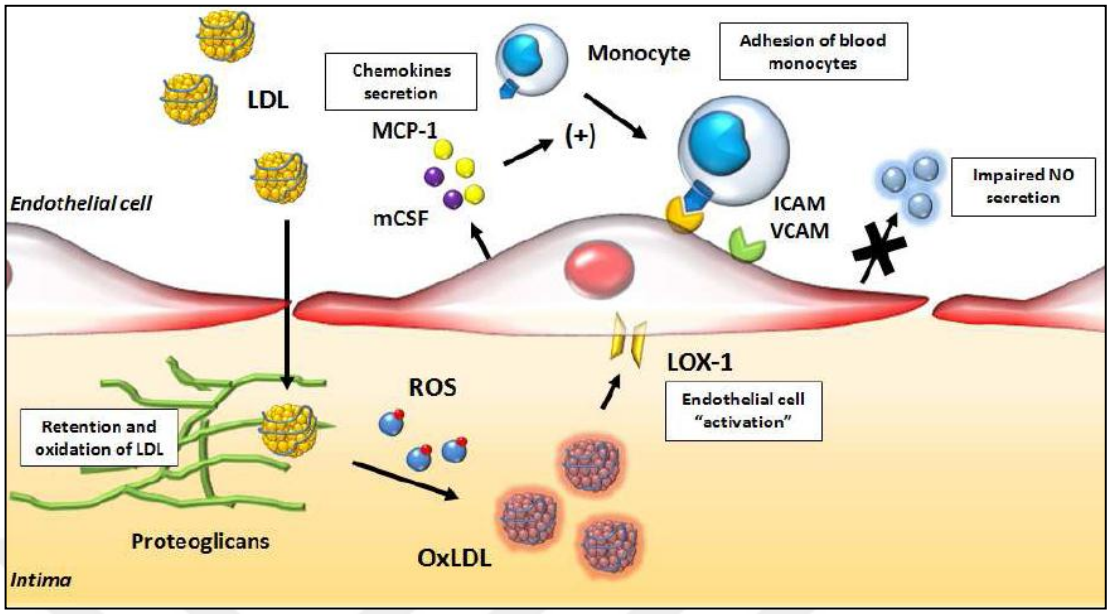


Figure 1.6. The roles of ox-LDL in the of atherosclerotic plaque development [77]

1.3.1.3. Monocytes

Monocytes play crucial roles in the initial step and progression of atherosclerosis. Monocytes are part of innate immunity that produced by hematopoietic stem cells which are in bone marrow and they have short half live (1-2 days) in circulation [78]. Accumulation of monocytes in damaged endothelial area is a risk factor in initial step of atherosclerosis process against inflammatory signal. This signal leads to monocytes migration to inflamed region and by attaching over-expressed adhesion molecules on endothelial cells they transmigrate into the intima region where then monocytes are transformed into macrophages. Macrophages phagocytose ox-LDL via lectin-like ox-LDL receptor-1 (LOX-1). After this process inflammatory macrophages form foam cells and then initiate formation of plaque [24, 79, 80]. Bekkering et al. determined that even low ox-LDL concentration leads to induction of pro-inflammatory phenotype of monocytes which speed up formation of atherosclerosis [81].

In humans, monocytes are classified into three subsets according to the surface markers expression the like cluster of differentiation 14 (CD14) which is lipopolysaccharide (LPS) receptor antigen and CD16 which is IgG Fcγ receptor. $CD14^{++}CD16^{-}$ monocyte is the classical monocyte and it is classified according to high expression level of CD14 and no

expression of CD16 surface receptor. $CD14^{+}CD16^{++}$ monocyte is non-classical monocyte and it is classified according to low expression level of CD14 and high level of CD16 receptor (Figure 1.6). $CD14^{++}CD16^{+}$ is intermediate monocyte and CD14 expression is high whereas expression of CD16 is low. By depending on cell adhesion molecules and chemokine receptors these three types of monocytes have been reported to behave substantially different. 80-95 per cent of total circulating monocytes are consisted by classical monocytes ($CD14^{++}$ $CD16^{-}$) which release interleukin-10 (IL-10) against to LDL and express high levels of MCP-1 receptor which is also called as C-C motif chemokine ligand 2 (CCL2). Increased level of MCP-1 during inflammatory processes and due to high level of receptor of this protein, classical monocyte is the main monocyte subtype that involves in atheromatous plaque. Intermediate monocyte, $CD14^{++}CD16^{+}$, release interleukin-*1β* (IL-1*β*) and TNF-*α* in reaction to ox-LDL [9, 78].

β2 integrins like LFA-1 and Mac-1 which are also known as respectively CD11a/CD18 and CD11b/CD18 are expressed by monocytes. These integrins bind to ICAM-1. In addition, α4β2integrin which is also called as very late antigen (VLA-4) bind to VCAM-1 (Figure 1.6) [30].

Chemokines such as MCP-1 is produced by damaged endothelial cells and that MCP-1 reacts with its receptor CCL2 that highly expressed on monocyte and leads to recruitment of monocytes to inflamed area. Therefore blocking these chemokines to prevent monocytes migration to damaged endothelial cells might be another way for repressing of atherosclerotic plaque development [82].

In atherosclerotic plaque, the strongest chemokine sources are monocytes-derived macrophages. Macrophages show up-regulation in expression level of CCL2 (CCR2 in mouse) which encode MCP-1 that signals monocytes to recruit activated endothelial cells [36]. There are two phenotype of macrophages. M1 is referred pro-inflammatory macrophages and have significant role in progressing plaque additionally. M1 macrophages leads to induction in pro-inflammatory cytokines expression level like TNF-α, interleukin-6 (IL-6) [83]. However, M2 is regarded anti-inflammatory macrophages and is enriched in regressing plaque [30]. In contrast to M1, M2 macrophages have roles in tissue healing and renewal (repair) [83].

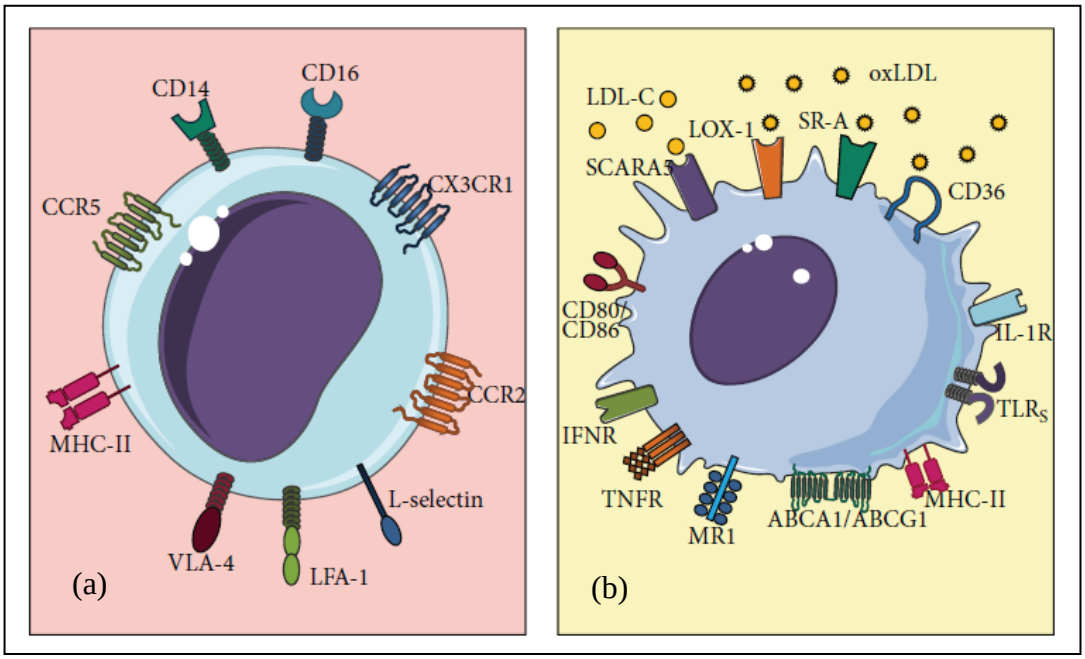


Figure 1.7. (a) Monocyte and (b) Macrophage characteristics [9]

Recruitment of monocytes is main reason to promote developing of inflammation processes by express cytokines and chemokines. Monocytes attach ICAM-1 and VCAM-1 on inflamed endothelial cells via VLA-4 [80, 84]. Monocyte migration to inflamed region is carried out by chemokines which are released by activated endothelial cells and react receptors on monocytes [82].

1.3.1.4. TNF-alpha

TNF-α, is produced 27kDa molecular mass as homotrimer, is cell signaling protein that mainly produced by macrophages and comprise in inflammation. TNF-α has two receptors on cells which are TNF receptor type 1 (TNF1) is a 55kDa and TNF receptor type 2 (TNFR2) is 75kDa. Expression of TNFR1 is present in most of tissues that both membrane-bound and soluble trimeric form of TNF-α can fully active this receptor.

However, expression of TNFR2 is occurred in immune cells and its can be act by membrane-bound form of TNF-α. TNFR1 mediate not only pro-inflammatory but also programmed cell death pathways. It is not well characterized but TNF2 has role in tissue repair and angiogenesis [85]. TNF-α act highly as a pro-inflammatory cytokine and plays

role in the induction of apoptosis and necrosis via TNFR1 [86]. TNF-α is regarded as one of the most significant regulative factors for the generation of cytokines that promote progress of inflammation [87]. Multiple endothelial genes like ICAM-1, VCAM-1 and E-selectin involvement in the development of atherosclerotic plaque are induced by TNF-α via NF-ƙB is suggested as pro-atherosclerotic risk factor (Figure 1.7) [33, 88]. MCP-1 and IL-6 chemokines are increased by TNF-α. Salkowski et al. observed that lipopolysaccharide induced iNOS mRNA expression was regulated and induced by TNFR1 signaling pathway in macrophages [89]. Additionally, TNF-α causes oxidative stress by stimulating production of ROS [90].

TNF-α has an important role in increasing raid of leukocytes to inflammation side of artery wall [35]. Beside to the attraction of leukocyte to inflamed tissue, TNF-α up-regulates cells surface adhesion molecules expression. For instance, ICAM-1, VCAM-1 and E-selectin are stimulated by TNF-α while it is also inducing NF-ƙB [91-93]. In this context, Yan et al. observed that TNF-α significantly increased expression level of VCAM-1compared to control group [94]. Zhao et al. demonstrated that TNF-α up-regulated expression of pro-inflammatory ICAM-1 adhesion molecule [95]. Several studies showed that TNF-α increased ICAM-1 expression by binding TNFR1 on cell surface and activating NF-ƙB (Figure 1.7) [96]. Han et al. observed that monocytes adhesion and ICAM-1, VCAM-1 and E-selectin expression were increased in HUVECs treated with TNF-α [97]. Li et al. was also showed that ICAM-1 and also VCAM-1 adhesion molecules were increased in TNF-α induced HUVECs [98].

All these studies are suggested that TNF-α is another important risk factor in the initiation of atherosclerosis.

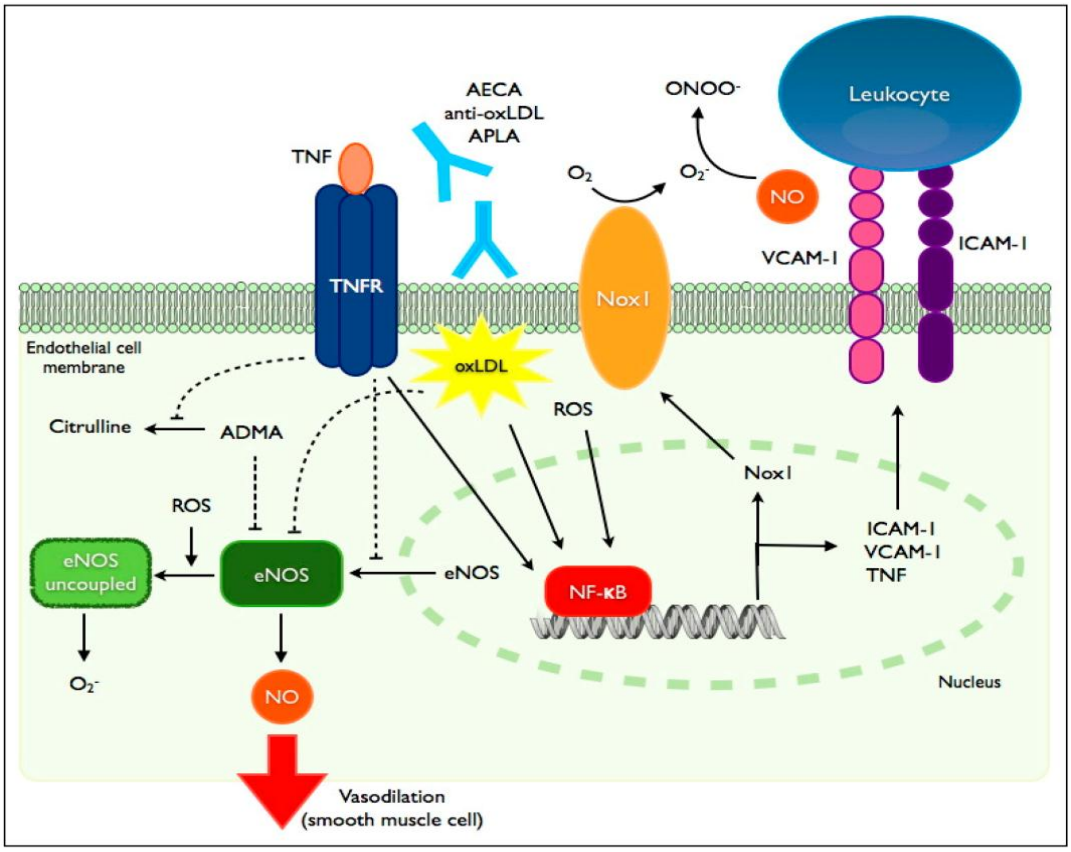


Figure 1.8. Pro-inflammation activation pathway of TNF-α via TNFR1 in damaged endothelial cells [99].

1.4. ENDOTHELIAL NITRIC OXIDE SYNTASE

Endothelial nitric oxide synthase (eNOS), a homodimer, is one of the three isoforms of NOS III enzyme and it is the most important regulator of vascular homeostasis [100]. The other isoforms are inducible nitric oxide synthase (iNOS) which promote inflammation and neuronal nitric oxide synthase (nNOS) which is expressed by neurons in brain [101, 102]. eNOS is consisted by two identical monomers that associate to take form 133kDa eNOS dimer [103]. The promoter region of eNOS comprises binding sites for transcriptional elements which are Sp1 and GATA. KLF2 has been demonstrated to induce eNOS expression [104]. Additionally, there are many transcriptional factors binding sites and some of those are, activation protein-1 and 2 (AP-1, AP-2) and NF-ƙB [105].

The main role of eNOS is to produce nitric oxide (NO) from L-arginine [106, 107]. NO is one of the most significant molecules that maintain the vascular tone. In addition, it has

many important functions such as preventing adhesion of leukocyte to endothelial cells, decreasing of smooth muscle cell proliferation, regulating smooth muscle cells relaxation, reducing monocytes stickiness, reducing releasing of free radicals and inhibiting platelet aggregation [105, 108].

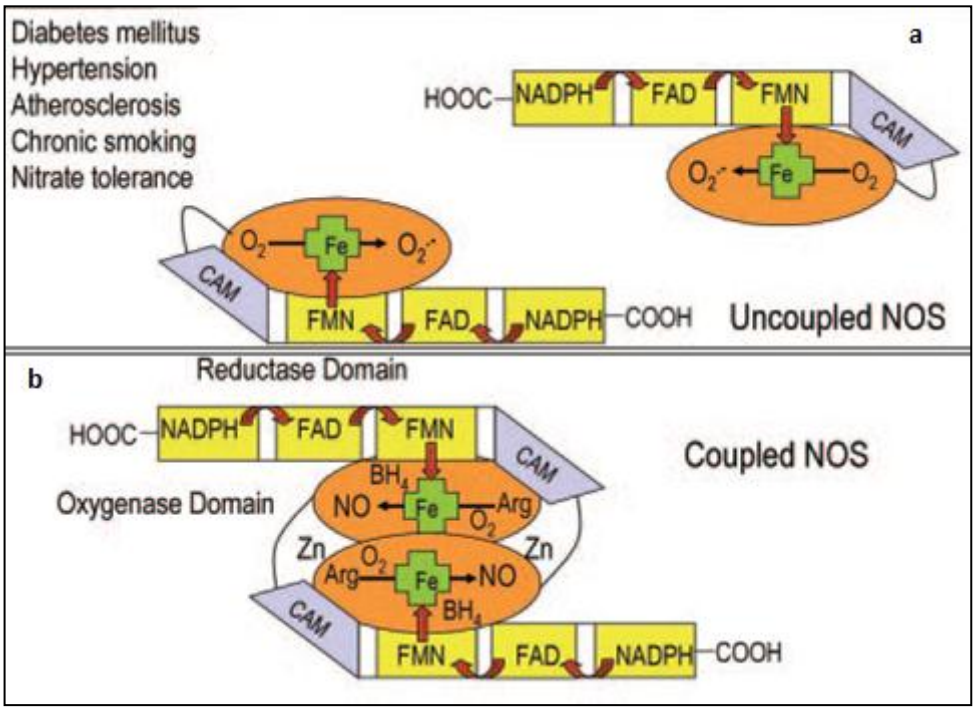


Figure 1.9. The illustration of coupled and uncoupled NOS [109]

eNOS is inactive in monomeric form, in other words when it exerts its uncoupled form (Fig.1.9 a), however it is only active as a homodimer which means when it exerts its coupled form as represented in Fig 1.9 b [110, 111]. Zinc-thiolate cluster (ZnS_4) is formed by zinc ions between cysteine groups of two monomers. Therefore, zinc ions are essential for stability and dimerization of the coupled form. Also, tetrahydrobiopretin (BH_4) and heme is necessary for dimerization. eNOS contains two domains which are C-terminal reductase domain comprised of nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) binding domains and also N-terminal oxygenase domain comprised ofBH_4 cofactor, substrate L-arginine, heme iron, and zinc binding domains [109, 112, 113]. In addition, eNOS hold Calcium (Ca^{2+})/Calmodulin (CAM) binding domain (Figure 1.10). During the production of NO,NADPH is oxidized to NAD^+ and thus electron flows respectively to the flavin FAD,

and then to FMN of the one monomer reductase domain iron heme center and BH_4 of the oxygenase domain of another monomer where L-arginine/O_2 is converted to NO and L-citrulline [114]. CAM controls and increases transfer of electron (Figure 1.10). In monomeric form of eNOS, electron is transferred to the heme center on same eNOS monomer resulting production of superoxide [115].

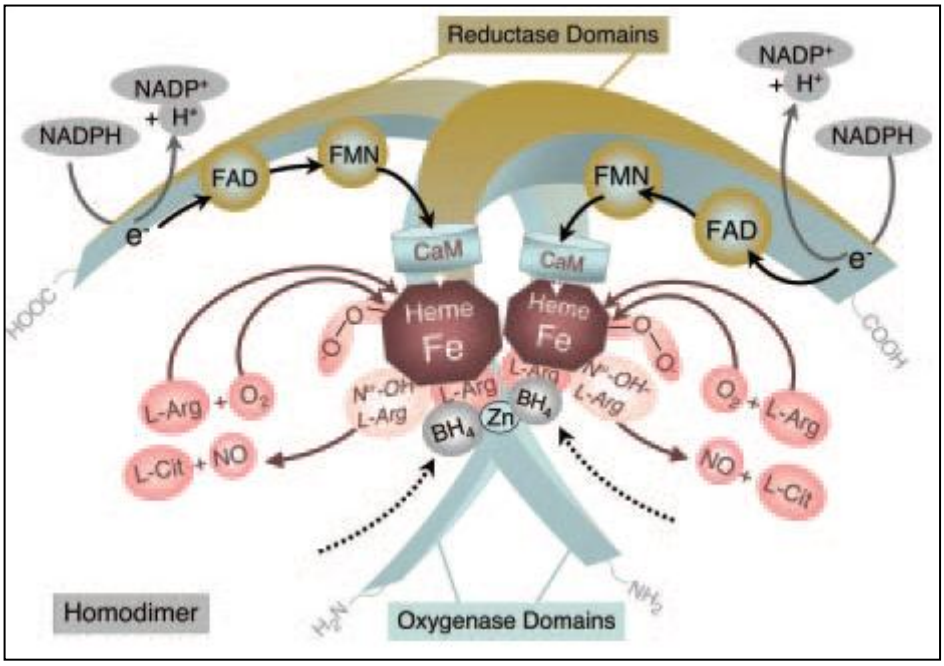


Figure 1.10. NO production via Ca^{2+}/Calmodulin [114]

eNOS activation is not only depends on co-factors but also based on post-translational modification. In humans, eNOS phosphorylation in the amino acid region of Serine 1177 (Ser1177) trigger the activation of eNOS resulting increase in the production of NO while eNOS phosphorylation at Threonine 495 (Thr495) amino acid region inhibits NO production [111, 116, 117]. In this context, eNOS phosphorylation is carried out by serine/threonine kinase (Akt) which is stimulated by VEGF and shear stress [118-123].

Therefore, Akt plays important role in activation of eNOS to produce NO. Heat shock protein-90 (Hsp90) is other effector which regulates eNOS activity [116, 124].

Ca^{+2}/CAM dependent manner is another activation way for eNOS [125]. Functional eNOS is found as bounding to caveolin-1 which is a plasma membrane protein. The Caveolin-1 attached to CAM binding sites as a result inhibits transfer of electron from NADPH at the

reductase domain to heme center of oxygenase domain. Ca^{+2} helps the binding of CAM protein to the CAM binding region of eNOS which causes the disruption of eNOS/Caveolin-1 complex in the plasma membrane [126]. Therefore, the flow of electron from reductase region to oxygenase region is initiated which results in the up-regulation of this enzyme.

The activation of eNOS is regulated by certain factors such as acetylcholine, histamine or bradykinin which take part in the increased intracellular level of Ca^{+2} by endoplasmic reticulum (ER). In this manner, shear stress represent the most significant factor in the regulation of eNOS enzyme expression [127].

NO that is produced by eNOS functions as a most protective molecule in cardiovascular diseases. Blocking the synthesis of NO has been shown to lead serious patho-physiological conditions such as atherosclerosis and hypertension [128]. For example, Kuhlencordt et al. showed that inadequacy in eNOS expression in ApoE-KO mice caused the acceleration of atherosclerosis with high lesion area in aortic valve [100, 129]. The most significant vasculature function of NO in the is relaxation of smooth muscle cells regulated through NO/cGMP signaling pathway [130]. Produced NO is diffused into smooth muscle cells where it bounds to and thus activates soluble guanylyl cyclase (sGC). Active sGC converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) that activates calcium-sensitive K channels (BK_{CA}) while inhibiting Ca^{2+} channels concluded in the relaxation of SMCs (Figure 1.11.) [130-132].

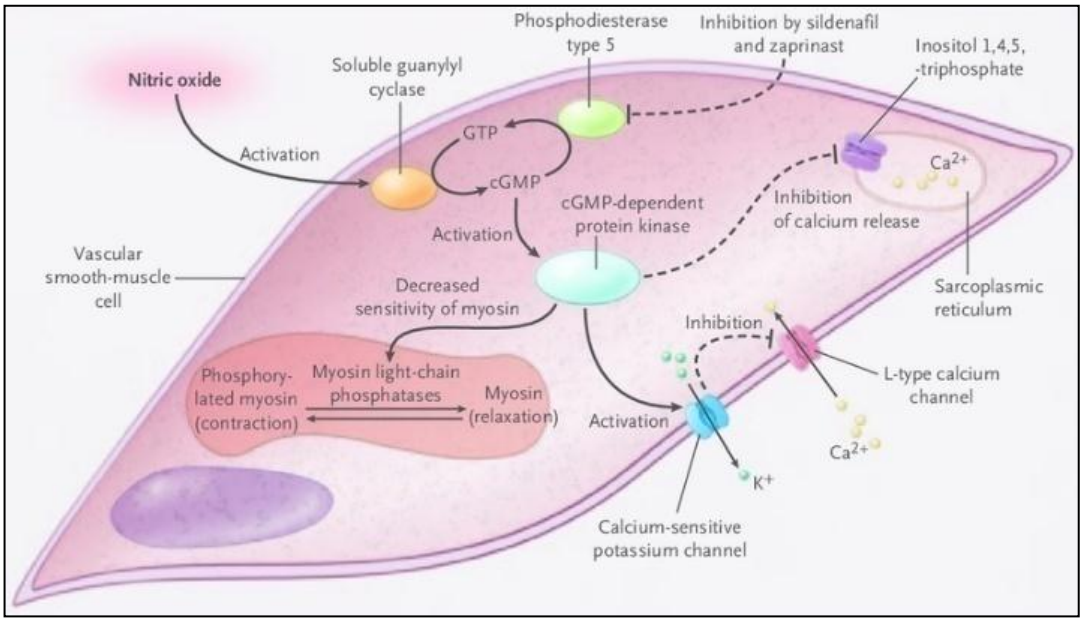


Figure 1.11. Smooth muscle cell relaxation processes [130]. NO activates sGC and BK_{CA} are activated while inhibiting Ca^{2+} channels. Thus, resulting smooth muscle relaxation and control of artery tonus.

NO exerts anti-inflammatory effects on vascular maintenance, prevents endothelial dysfunction and predominantly declines permeability of endothelial cells. Kosonen et al demonstrated that neutrophil adhesion and E-selectin expression were inhibited due to NO-releasing compound treatments in lipo-polysaccharide stimulated HUVECs [133]. It has been shown that NO diminished the level of leukocyte C11/CD18 receptors and prevented the binding of leukocytes to ICAM-1, VCAM-1 and E-selectin [127, 134]. In addition, NO inhibits the production of free radicals which take part in oxidation of LDL in intima region of artery during endothelial dysfunction [135].

Impairment in the availability of NO triggers the development of the atherosclerosis which can contribute to severe clinical events including myocardial infarction (MI) and stroke [101]. Reduced level of NO has been demonstrated to cause endothelial dysfunction that is also known an early step for atherosclerosis in the human arterial wall [126]. Therefore, the maintenance of the availability of NO by activating eNOS in endothelial cells could be the primary therapeutic approach for preventing atherosclerosis and by this way restrain acute clinical symptoms.

1.5. STATINS

Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors. These hypolipidemic drugs are used to treat hyperlipidemia which is abnormal increase of lipids such as cholesterol in blood [10]. Statins are known as the most beneficial drugs to reduce LDL level in blood by inhibiting cholesterol production pathway in liver (Figure 1.12) [136]. HMG-CoA reductase enzymes are working by catalyzing HMG-CoA to mevalonate which is important substrate for cholesterol production as shown in figure 1.12 [137]. LDLs are found in atherosclerotic lesions and they are involved in atherosclerosis related cardiovascular diseases [138].

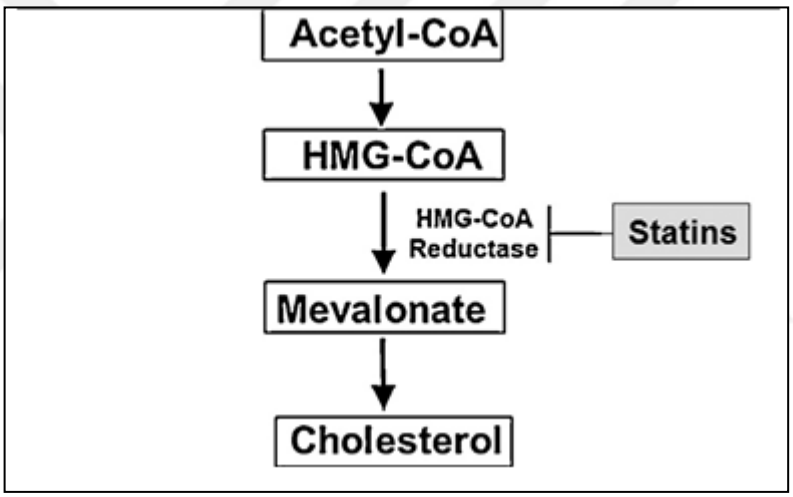


Figure 1.12. Reaction of statin in cholesterol production [137]

In addition, statins have various pleiotropic effects, in this way they protect vasculature [139]. Beneficial effects of statins are including decreased inflammation, protection of endothelial barrier function, decreasing expression of adhesion molecules on vascular wall, increased plaque stability. In addition, platelet aggregation, apoptosis and also thrombus formation decreasing are helpful effects of statins as represented in Figure 1.13 [140]. To conclude, statins have been studied as different therapeutic strategy to avoid development of atherosclerosis resulting in heart attack.

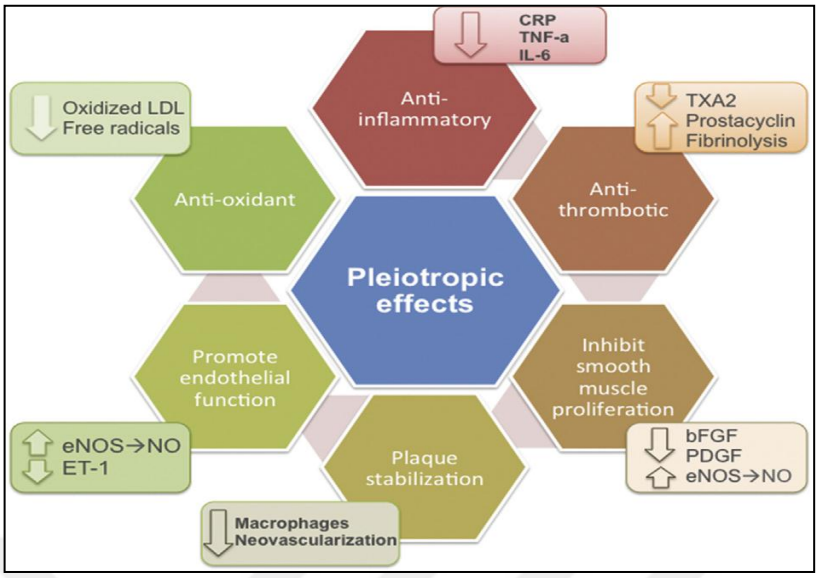


Figure 1.13. Pleiotropic effects of statin drug [140]

Randomized clinical trials indicate that decreasing LDL level results in reduction of cardiovascular disease risks [126]. High level of cholesterol in blood is main reason of CVD development and consequently deaths. However, statins have capacity to reduce significantly the deaths risk. According to 2013 American College of Cardiology/American Heart Association Guidelines, recent randomized clinical trials showed that 18 out of 1000 people were treated with statins for five years without any CVD events [141]. Clinical studies evidenced that statins might stimulate activation of anti-inflammatory pathways because of its action in inhibition of HMG-CoA reductase enzyme [137]. Due to LDL level reduction ranges 30 to 63 percent statin drugs are characterized primary powerful drugs for prevention of cardiovascular disease (6-10).

1.5.1. Types of Statins

There are different statins types such as atorvastatin, rosuvastatin simvastatin, pitavastatin, pravastatin and fluvastatin. According to their different characteristics such as chemical properties, derivation, solubility and metabolic pathway their bioavailability and half-life is variable (Figure 1.15). Beside their differences they all have been shown to be beneficial in cardiovascular diseases in literature.

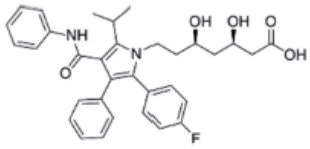
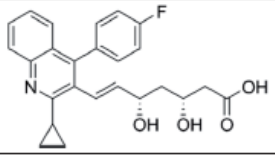
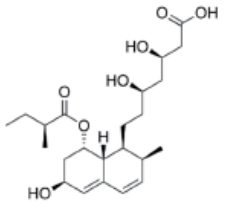
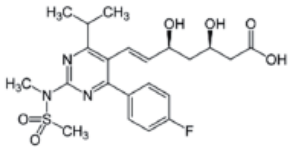
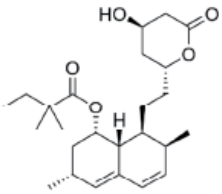
Name	Chemical structure	Derivation	Solubility	Metabolic pathway	Bio-availability	Half-life (hours)
Atorvastatin		Synthetic	lipophilic	CYP450 3A4	14%	14
Pitavastatin		Synthetic	lipophilic	Biliary, CYP2C9/2C8	50%	12
Pravastatin		Fermentation derived	hydrophilic	Non CYP	17%	77
Rosuvastatin		Synthetic	hydrophilic	CYP2C9, CYP2C19	20%	19
Simvastatin		Synthetic derivative of a fermentation derived product	lipophilic	CYP450 3A4	<5%	1.9

Figure 1.14. Pharmacological and chemical properties of different statin types [142]

Simvastatin, pravastatin and lovastatin are obtained from fungal by fermentation. Atorvastatin, fluvastatin and cerivastatin are synthesized. After 31 cerivastatin treated patients' deaths this drug was withdrew by Bayer on 2008. Circulation concentrations of statins are low such as 12 percent Atorvastatin, 20-30 percent fluvastatin, 5 percent simvastatin and lovastatin. Active acid form compounds are dispended in form of statin drugs like atorvastatin, fluvastatin and pravastatin. However, simvastatin and lovastatin are inactive form thus they are required to enzymatically activated [143-145].

Long-Term intervention with Pravastatin in ischemic disease study group indicated that 40 mg/day pravastatin therapy reduced the mortality of coronary heart disease in patients who

suffered from myocardial infarction previously [146]. Rosenson et al. demonstrated 10-40 mg rosuvastatin treatment reduced the level of LDL 52-63 percent compared to HDL and the level of triglycerides by 28 percent in hypercholesterolemia patients [147]. Jones et al investigated the effect of 10, 20, 40 and 80 mg of rosuvastatin, atorvastatin, simvastatin or pravastatin treatment on 2431 hypercholesterolemia patients for six weeks. As a result they showed that rosuvastatin and atorvastatin treatment decreased the level of LDL cholesterol by 82 to 89 percent and 60 to 85 percent respectively [148]. Another study showed that 10 mg atorvastatin was more effective to reduce LDL level in hypercholesterolemia suffered patients compared to 10 mg simvastatin, 10 and 20 mg pravastatin, 20 and 40 mg fluvastatin and lovastatin [149]. Hoeke et al. showed that atorvastatin enhanced activity of brown adipose tissue, plays role in atherosclerosis and hypercholesterolemia with its anti-atherosclerotic and lipid lowering effect, in APOE*3-Leiden mice [150]. Brown et al. also compared different types of statins like atorvastatin, simvastatin, fluvastatin and lovastatin in role of LDL cholesterol reduction level in patients who suffer from atherosclerosis. The indicated results showed that the target LDL cholesterol concentration was achieved importantly with atorvastatin usage at the starting dose of 10 mg when compared to other statins [151]. Li et al. studied effect of E-selectin targeted atorvastatin calcium with curcumin, polyphenol that has antioxidant and anti-inflammation effect, loaded of liposome delivery system in reduction of lipids and adhesion molecules in vitro (HAECs) and in vivo (ApoE$^{-/-}$mice model). The results of study showed that the inflamed cells targeted drug delivery system reduced ICAM-1 and E-selectin expression in vitro and decreased atherosclerotic lesions in vivo [152]. All these studies confirmed that both atorvastatin and rosuvastatin drugs are more effective than another existing statin in LDL reduction. Moreover, Sever et al. have applied 10 mg atorvastatin in hypertensive women and men by following up to 5 years and they utilized a reduction in the main cardiovascular events [153]. Nohria et al. studied with 35 atherosclerotic patients who treated with 10 mg/day and 80 mg/day atorvastatin doses and the results showed that atorvastatin reduces pro-atherogenic Rho/Rho kinase (ROCK) pathway [154].

Therapeutic effect of statins has been indicated not only on LDL levels but also in different anti-inflammatory pathways occur in arteries and cardiovascular pathologies. For example, statin drugs are reported to increase NO bioavailability, decrease inflammation stimulated expression levels of adhesion molecules, cytokine, having antioxidant effects and prevent

endothelial dysfunction [155, 156]. Statins have been documented in eNOS expression and activity. In this context, Igarashi et al. reported that atorvastatin and pitavastatin were induced sphingosine-1 phosphate ($S1P_1$) receptor that binds to G-protein coupled $S1P_1$ receptor and activate eNOS in vascular endothelial cells. They concluded that pleiotropic effect of statin may be vascular protective through increasing bioavailability of NO [156]. Ota et al. demonstrated that PI3K/Akt pathway was activated and eNOS expression was increased with atorvastatin, pravastatin and pitavastatin treated HUVEC [157]. In another study, Loboda et al. determined that atorvastatin increased eNOS expression in microvascular endothelial cells [158]. Chen et al demonstrated that Atorvastatin increased proliferation of ox-LDL pretreated HUVEC also increased eNOS mRNA and protein expression while decreasing HSP22 level, is pro-inflammatory heat shock protein type, in $ApoE^{-/-}$ mice and in HUVEC [159]. Chang et al showed that atorvastatin protected proliferation mechanism of HUVEC by inhibiting Angiotension II (AngII), plays role in endothelial dysfunction [160].

Downar et al. showed that mRNA expression of VCAM-1 and ICAM-1 were decreased and NF-ƙB was inhibited in simvastatin treated HUVEC which is stimulated by TNF-α [161].

BAO et al. observed that ROS accumulation and apoptosis of endothelial cells might be inhibited by using atorvastatin [162]. Studies demonstrated that apoptosis of endothelial cells in terms of ROS is vital in endothelial dysfunction. It is recognized that people suffer from diabetic mellitus tend to have cardiovascular mortality. Ekstrand et al. observed that five days treatment of atorvastatin reduced ROS level in atherosclerotic lesion of high fat died fed mice model [163]. According to recent studies, glycation and oxidative stress has a significant role in cardiovascular disease development. Feng et al. investigated atorvastatin effect through receptor for advanced glycation end products (RAGE) and MCP-1 which is downstream target gene of RAGE. RAGE is cell surface receptor of immunoglobulin super-family and has role in inflammation. Obtained results demonstrated that atorvastatin decreased RAGE and MCP-1expression in HUVEC and in rats fed with high fat diet [164].

Minami et al. was determined that pleiotropic effect of statin which was up-regulated effect GTP-binding protein dissociation stimulator (SmgGDS), regulator of small GTP- binding proteins (Rho, Rac, Ras), by degrading Rac1 whose role is ROS production through β1-

integrin/Akt1 pathway [155]. Recent studies showed that of pro-inflammatory cytokines expression are controlled by statin. Rezaie-Majd et al. demonstrated that MCP-1, IL-6 and IL-8 were decreased in simvastatin treated hypercholesterolemia patients [165].

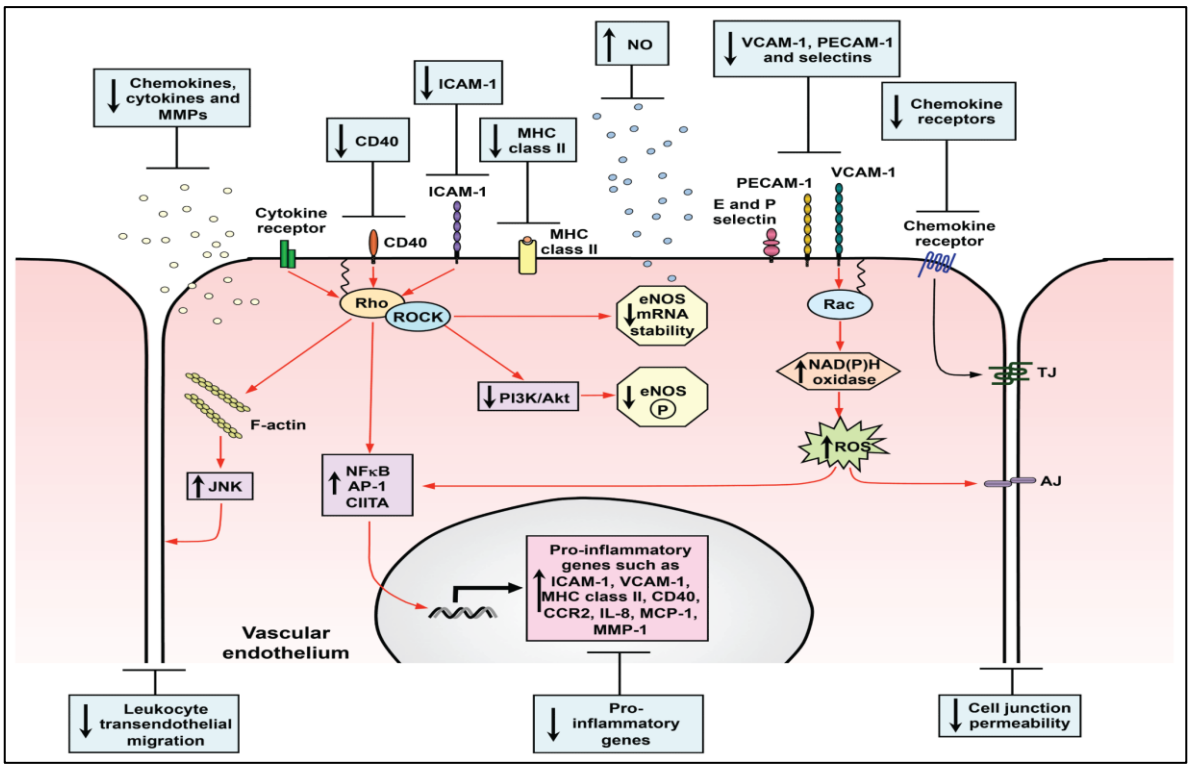


Figure 1.15. Anti-atherosclerotic action of statin in vascular endothelium [166]

Apart from therapeutically effect of statins, there are serious side effects of these drugs like myopathy (muscle weakness), liver abnormalities, diabetes, peripheral neuropathy (damages of nerve that carry message to and from brain) and insomnia (inability to sleep) [141, 167]. Some studies showed that adverse effects are caused because of increasing concentration of statins in blood circulation [168]. It was suggested that simvastatin dosage should not be more than 40 mg/day because it was stated that 80 mg/day resulted in high rates of myopathy. Food and Drug Administration (FDA) advise to avoid 80 mg dosage for simvastatin [169]. Some studies have shown in patients with hypercholesterolemia that atorvastatin and rosuvastatin are dose-dependently more efficient by decreasing level of triglycerides than other types of statins depending on dose rises [148, 170, 171]. The most prevalent musculoskeletal adverse effects that caused by statins therapy are muscle weakness, cramps aches and creatinine kinase (CK) elevations [172]. CK, type of enzyme,

muscle cells are damaged releases therefore clinical trials observed that releasing of this enzyme depends on statins and their usage doses [167].

The effective therapy by using statin in order to eliminate statin-related muscle symptoms during treatment of patients is still required. Most patients are unable to tolerate high statin dose therefore they are under risk [138]. For this reason, in order to reduce drug dosage, side effects and increase therapeutic efficacy, development of disease area specific targeted drug delivery systems become more an issue. Targeted delivery approach may be beneficial in order to reduce side effects of statins which are indicated in studies describing the therapeutic potential of the drug.

1.6. APTAMERS

Aptamers are short RNA and single-stranded DNA oligonucleotides [173]. Aptamer has been named from Latin as *"Aptos"* which means -to fit- and from Greek word *"meros"* which means -region part- [174]. In the early of 1990, aptamer technology was invented by Ellington et al. with various studies on targeted dye molecules using RNA aptamer. These studies helped scientists to discover specific aptamer to target specifically intercellular molecules that are inaccessible by antibodies [174, 175]. Thus aptamers have some advantages compared to antibodies [176]. Antibody molecular weight ranges from 150 kDa to 1000 kDa, however weight of aptamer ranges from 15 to 50 kDa. Aptamers are smaller than antibodies. Their smaller size makes aptamer easily penetrate between tissue or cells and also make them more attractive for targeting drug delivery preventions [177]. Also, production of aptamer is easier than antibody. In other words, aptamer can be synthesized in a short time. On the contrary, cost of antibodies production is high and need to produce in living animal which is more difficult and expensive. In addition, aptamers have higher binding affinity and highly specific to their target. Other advantages of aptamers are that they are not identified by immune system in body because they are nucleic acid [178, 179]. These features of aptamer could make them attractive for therapeutic prevention and diagnosis of cardiovascular diseases. High affinity aptamers are selected with a method which is called Systematic Evolution of Ligand by Exponential Enrichment (SELEX) [180, 181]. This SELEX method which needs about 10-15 cycles isolates highly target specific aptamer from whole aptamers library [182, 183]. There are

four steps for SELEX. Firstly, nucleic acid with random sequence is synthesized. Secondly, these random sequences are incubated with target molecules to bind. Third, unbounded sequences are removed by washing and eluting. Finally, the isolated DNA/RNA sequence is amplified by using polymerase chain reaction (PCR) [184]. In clinical, Macugen® (or pegaptinib) which is vascular endothelial growth factor (VEGF) antagonist is the most successful RNA aptamer. This aptamer is applied for wet age-related macular degeneration disease [185]. Pathophysiology of endothelial cells represent therapeutically target for studying targeted drug interventions [186]. Aptamer can function as cell signaling pathway suppresser by binding to target ligand which leads to prevent of cytokines to bind their receptor [182].

Pro-inflammatory protein expression on inflamed endothelial cell make them attractive target molecule to develop targeted drug delivery system. Therefore, these features of aptamers make them attractive for therapeutic prevention and diagnosis of atherosclerosis especially at early stages which the plaque do not protrude into the lumen and it is called *"silent plaque"* [187]. This silent plaque can lead to severe clinical symptoms.

In this context, Ji et al. studied thrombospondin-1 (TSP-1) specifically detects DNA aptamer in atherosclerotic cell model using HAEC via cell-SELEX method. TSP-1 protein expression activates the adhesion molecules expression, proliferation of smooth muscle cell and also leads to monocytes to attach to endothelium in the initially stages of atherosclerosis [188]. Damare et al. developed RNA aptamer that was specific to plasminogen activator inhibitor 1 (PAI-1), which is an important risk factor for types of cardiovascular diseases development, in order prevent interaction of PAI-1 with tissue-type plasminogen (tPA) and urokinase-type plasminogen (uPA) activators. The results showed that PAI-1 RNA aptamer inhibited binding of PAI-1 with tPA [189]. Ojima et al. developed advanced glycation end products (AGE) aptamer and studied in balloon injured rat carotid arteries whether this aptamer inhibits neointimal hyperplasia. Interaction of AGE with its receptor RAGE causes the production of oxidative stress and leads to inflammatory reactions in this way including in the process that accelerated atherosclerosis [190]. Site specific delivery of drug is important for minimizing delivery to normal cell thereby reducing site effects. Mann et al. developed E-selectin specific aptamer and observations showed that this aptamer detected E-selectin in human microvascular endothelial cells (HMVEC) with high affinity [191]. Highly target specific and affinity

properties of aptamer make them suitable for delivering of less-site specific therapeutic peptide. For that reason, Romanelli et al. developed aptamer peptide chimera (Gint4.T-MP) to deliver of MP peptide which mimic $Ca_v\beta 2$, has role in cardiac contractility, to cardiac cells to treat cardiac diseases. Gint4.T was specific to platelet-derived growth factor receptor-β (PDGFR-β) whose expression is observed in cardiomyocytes [192].

C-reactive protein (CRP) is an another significant biomarker and the concentration ranges three mg/ml to 10 mg/ml of CRP constitutes high risk factor for cardiovascular diseases [193]. There are different techniques to detect CRP level such as immunofluorescence, immunochemiluminescence, ELISA and radioimmunodiffusion. Main limitation of these methods includes equipment cost, sophisticated experimental procedure and low detection limit. Therefore, Jarchzewska et al. discovered as sensor based on electrochemical DNA-aptamer to observe CRP as an alternative analyzer of this protein [194]. Kao et al. found CRP-specific ssDNA aptamer with high specificity and sensitivity using field effect transistor (FET) equipment to observe CRP in an integrated micro fluidic system. Results showed with concentration from 0,625 mg/ml to 10 mg/ml aptamer-FET assay is capable of identify CRP which could be potential therapy for early diagnosis of CVDs [193]. Yu et al. improved highly specific and selective DNA aptamer to detect CRP with SELEX [183].

These literature studies show that aptamers are practicable alternative to antibody and they look great potential to be applied as therapeutic and targeting agent [195]. For nanoparticle-based drug delivery system aptamers are promising much hope.

1.7. NANOPARTICLE BASED TARGETED RELEASE

According to World Health Organization approximately 31 percent of deaths around the world base upon to the atherosclerosis and that's why this is crucial for prevention of the development of this disease which result in heart attack [196, 197]. In order to prevent atherosclerosis varied therapeutics have been carried out, tested as clinically and researched in laboratories. Most of studies showed that delivered drugs as orally lead to non-specific taken of drugs by healthy cells. Thus, non-specific distribution can be resulted in severe side effects particularly during long-term treatment. In some cases, higher doses of statin concluded to be efficient in hyperlipidemia treatment however it leads to adverse effects like myopathy that is weakness of muscle during long time treatment. In addition,

lower dosage of statin drugs could not be effective in the treatment of atherosclerosis. Nanoparticle based targeted drug delivery remains great potential to develop therapeutic delivery of drug to disease site for atherosclerosis [198]. Nanoparticles based targeted drug delivery has been studied over years in order to obtain therapeutic effects while reducing side effects. The currently drugs that are applied for atherosclerosis have not proper function to directly inhibit inflammation mechanism resulting in atherosclerosis [199]. Therefore, there is still a demand for more potential and safe therapy that directly target inflammation mechanism [200]. In this way, nanoparticle technology promises an important approach to overcome these issues. Nanoparticle based drug delivery overcomes some drawbacks including poor availability, fast metabolism, water insoluble and reverse effects of drugs. Additional risk factors in atherosclerosis show that there are various targeting opportunities for using nanoparticles for diagnosis and treatment of atherosclerosis. During circulation of drugs along blood, endothelium is excessive to drugs however without targeting the drugs could be taken by healthy cells. Therefore, nanoparticles loaded with drugs can be conjugated with determinants which are specific for endothelial cells. As targeting agent receptors, peptides and inflammatory proteins have been utilized for atherosclerosis targeted therapy (Figure 1.16) [199].

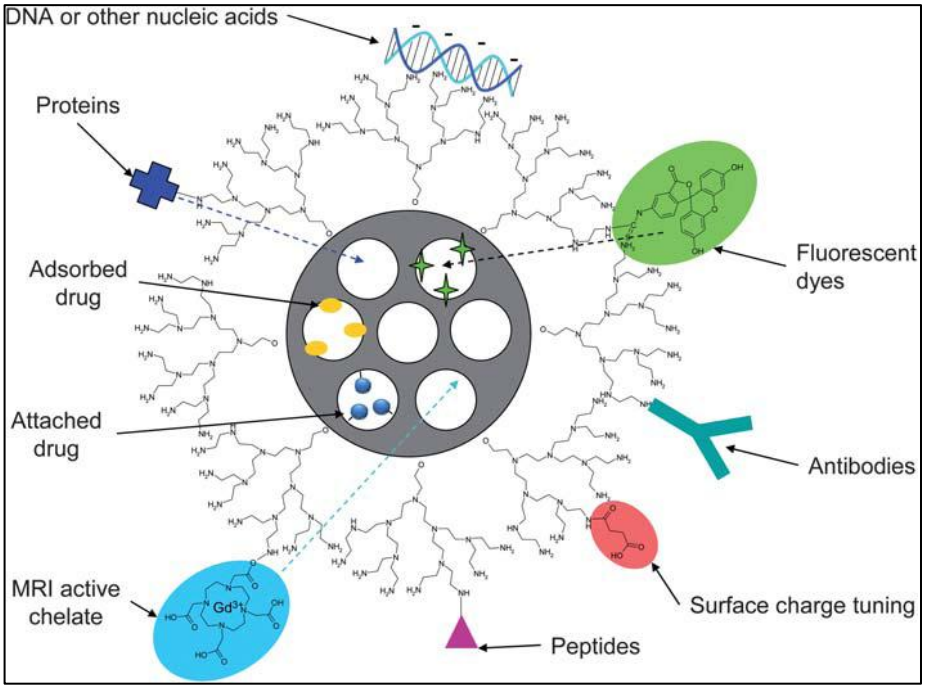


Figure 1.16. An example of MSN modified nanoparticle [201]

Nanoparticles ranges from 1 to 100 nm in size [202]. There are different types of nanoparticles according to their chemical properties such as liposome, mesoporous silica nanoparticles, HDL and dendrimers.

Liposomes are composed of phospholipid bilayers and they are studied targeted drug delivery system [203]. Scott et al. developed P-selectin, is protein expressed on endothelial cells during inflammatory stimuli, conjugated VEGF containing liposome to improve cardiac function using MI rat model [204]. Homem de Bittencourt et al. synthesized VACM-1 antibody conjugated cyclopentenone prostaglandin, anti-inflammatory agent, loaded liposome to target atherosclerotic area in ldl receptor knockout mice [205]. Valk et al. studied prednisolone loaded liposomal nanoparticle (LN-PLP) in $LDLr^{-/-}$ mice and the results of this study demonstrated that recruitment of monocytes were induced by LN-PNP [206]. Atherosclerosis targeted nanotechnology is still challenged.

In the early of 2000s, mesoporous materials have been introduced for drug delivery and in recent years, mesoporous silica nanoparticle (MSNs) has been shown to encourage hope in applying cell-specific drug delivery [201]. Due to the uniform nano-pore size and surface chemistry, MSNs are manageable to protect loaded drug for controlled release as well as suitable to minimize side effects that is done by drug [201, 207]. MSNs are also applicable for hydrophobic drugs and can prevent drugs from degradation [208]. Nanoparticles have various advantages, for example they can be modified including particle size, shape, bioavailability and surface charge which give nanoparticles site specific characteristics [209]. The nano-scale pores of MSNs provide drugs to efficiently disperse [210]. In the present study, for encapsulating Atorvastatin Drug Mobile crystalline material (MCM-41) mesoporous silica nanoparticles were applied. This nanoparticles made of silicon dioxide (SiO_2) [211]. Fernandez et al. studied MCM-41 nanoparticles that loaded with caspase-1 inhibitor VX-765 (S2-P) and attached with poly-lysine as a molecular target in THP-1 cells and in inflamed mice model. The results showed that MCM-41 provide potential advantage to treat inflammatory diseases [208]. Geng et al. studied hollow mesoporous silica nanoparticles (HMS) to show these nanoparticles have high drug encapsulating capacity and importance in the regulation of release drug [210].

In current clinical practice, only few therapies such as statin and aspirin are effectively diminishing the cardiovascular diseases such as atherosclerosis. However, only 30 to 40 percent of the patients give positive feedback to these treatment strategies [212]. In

addition, statin drugs require long administration time for treatment which causes strong side effect. Therefore, it is important to develop the safe delivery of drug administration to the disease region.

In this current study, we investigated the effect of non-targeted delivery of drug which contained only Atorvastatin and targeted delivery of drug system which was ICAM-1 Apt-Atorv-MCM41 on TNF-α stimulated HUVEC. Therefore, we evaluated in vitro therapeutic potential of Apt-Atorv-MCM41 delivery system on HUVEC by checking p-Akt/eNOS activation at RNA expression and protein level.

2. MATERIALS

2.1. INSTRUMENTS

- Cell Culture Hood (Esco, Class II BSC)
- Cell Culture Incubator (Esco, CCL-050B-8)
- Light Microscope (American Optical Corporation, 1810)
- Centrifuge (Microfuge16, BECKMAN COULTER)
- PCR Machine (MULTIGENE)
- Nanodrop 2000 (ThermoFisher)
- UltraFreezer (Haier-86 UltraFreezer, DW86L338)
- Fridge (Arçelik, No-Frost)
- -20 Freezer (Panasonic, MDF-U731M)
- Spectrophotometer (ThermoFisher Scientific, VARIOSKAN LUX)
- Elisa Plate Reader (ThermoLab systems, Multiskan Ascent)
- Centrifuge (SIGMA, 3-18K)
- Vortex
- Visualization (ChemiDoc^{TM}XRS+System, 1708265)
- Real Time PCR Detection System (BIORAD, CFX96^{TM} Touch)
- Vertigal Gel Electrophoresis Apparatus JY-ZY5 Western Blotting System (Junyi, China)

2.2. EQUIPMENTS

- Cell Culture Flasks T75, T25 (TPP, 90075-25)
- Micropipette tips 1000µl, 200µl, 10µl (Axygen, 301-03-051)
- Serological Pipette 25ml, 10ml, 5ml (SPL, 91005,10,25)
- Microcentrifuge tubes 2ml, 1.5ml (CAPP, 5101550C, 52000C)
- Cryotube 2ml (Isolab, I.091.11.102.100)
- Falcon Tubes 50ml, 15ml (Axygen, SCT15ML25S)

- Falcon Tube 5ml (SSI BIO, 1410-09)
- 24-Well Plate (Corning, 3526)
- PCR Tubes (Thermo Fisher, AB0620)
- Hemocytometer (Sigma, Z359629)
- Micropipette 1000µl, 200µl, 20µl, 2µl (Finn Pipette, 4642010,50,80,90)
- PCR Plate and Seal (BIO-RAD, 2239441)
- Western Blot Apparatus (ThermoFisher Scientific)
- Filter Paper (ThermoFisher, 88600)

2.3. CHEMICALS

- Atorvastatin calcium salt (Sigma, PZ0001-25MG)
- DMEM 1X (Gibco, 11965118)
- FBS (Gibco, 105000064)
- PBS (Gibco, 13190094)
- Pen/Strep (Gibco, 15140122)
- Trypsin (Gibco, 25200056)
- DMSO (Sigma, D8418)
- SuperSignal™ West Femto Maximum Sensitivity Subtrate (ThermoFisher, 34096)
- TNFα (Invitrogen GIBCO, PHC3011)
- RIPA Lysis Buffer (Santa Cruz Biotechnology, I1815)
- SureCast™ 40% (w/v) Acrylamide (ThermoFisher Scientific, 793535-17)
- SureCast™ Resolving Buffer (ThermoFisher Scientific, 1799334)
- SureCast™ Stacking Buffer (ThermoFisher Scientific, 1794649)
- Dodecyl sulfate sodium salt (SDS, Merck, 8.22050.1000)
- eNOS(49G3) Rabbit mAb (Cell Signaling, 9586)
- Phospho-Akt (Ser473) Antibody (Cell Signaling, 9271)
- Anti-Rabbit IgG, HRP-linked Antibody (Cell Signaling, 7074)
- Page Ruler Prestained Protein Ladder (ThermoFisher, 00590517)
- Trizma base (Sigma Aldrich, T1503-1KG)
- Bovine serum albumin (BSA, Sigma Aldrich, 05470)

2.4. KIT

- Aurum™ Total RNA Mini Kit (BIO-RAD, 732-6820)
- iScriot™ cDNA Synthesis Kit (BIO-RAD, 170-8891)
- iTaq™ Universal SYBR Green Supermix (BIO-RAD, 172-5121)3

3. METHODS

3.1. APTAMER AND NANOPARTICLE CONJUGATION AND CHARACTERIZATION

Characterization of silica nanoparticles MCM-41 and drug delivery system of Apt-NP were carried out by applying TEM.

3.2. TEM ANALYSIS

MCM-41 type silica nanoparticle crystalline and compositional properties were characterized by applying TEM analysis. Fabrication of nanoparticles was done using ultrasonication of powder silica nanoparticles inside ethanol for five minutes. Later suspension was allowed to get dry applying on standard holey carbon TEM grid. TEM analysis was performed in Middle East Technical University Central Laboratories, 200kV in TEM mode.

3.3. CELL CULTURE

HUVECs (ATCC) were grown in DMEM supplied 10 percent FBS and one percent Pen/Strep. HUVECs seeded to T75 flasks were incubated at 37°C in five percent CO_2 humidified incubator. Medium of cells was changed every two or three days. During reached up of cells to 70 or 80 percent they were sub-cultured. For sub-culturing, medium inside flask was taken off and cells were washed using PBS. To remove cells from surface one ml of trypsin was added on cells and after softly shaker cells were again incubated at 37°C incubator for three or four minutes. Then cells were poured with fresh medium. Cells were counted by using Hemocytometer. After cells number was determined, the appropriate number of suspended cells was added on centrifuge tubes and tubes were centrifuged at 1500 rpm for five minutes. Pellet was re-suspended and cells were used as required. For freezing, one million cells that were re-suspended with one ml of total freezing medium with five percent DMSO was transferred into cryotubes. Before

transferred cells into ultra-freezer, cryotubes were placed in cell freezing chamber for overnight. For the experiment of this study passage numbers for cell were used from 11 to 15.

3.4. CYTOTOXIC EFFECT OF MCM-41 SILICA NANOPARTICLE ON HUVEC

Drug delivery system targeting ICAM-1 with its aptamer consisted of silica nanoparticle MCM-41 and loaded with Atorvastatin. In order to determine MCM-41 type silica nanoparticle cytotoxic effect, HUVECs were incubated with different concentrations of MCM-41 silica nanoparticles. 5×10^3 cells/well HUVEC were seeded to 96-well plate after that, cells were incubated at 37ºC for overnight. Then HUVEC were incubated with 25, 50, 75, 100, 200 and 500 µg/ml concentrations of silica nanoparticles MCM-41 contained DMEM and control group which included only 0.5 percent DMSO for 24 hours. Then silica nanoparticles contained medium was taken off and washing of cells were done using PBS. Total 100 µl DMEM which contained 10 µl cell proliferation reagent of WST-1 was put on cells and then cells were placed inside incubator for one hour at 37ºC. The absorbance of medium was calculated by applying Elisa microplate reader.

3.5. EVALUATING THE ATTACHMENT OF ICAM-1 APTAMER-MCM41-FLUORESCEIN IN HUVEC

The in vitro attachments of ICAM-1-Aptamer-MCM41-Fluorescein (APT-NP-FI) to HUVEC cells were determined. 3×10^4 cells/well HUVEC was seeded to 96-well plate and placed at 37ºC for overnight. Then, cells were stimulated with TNF-α for six hours and there was a control group that was not stimulated with TNF-α. Afterwards, HUVECs were placed with 500 nM of APT-NP-FI for four hours. The images were taken with fluorescence microscopy.

3.6. EVALUATING ICAM-1 INDUCING DOSE OF TNF-ALPHA

ICAM-1 up-regulating concentration of TNF-α was determined. 6×10^5 cells/well HUVEC were seeded to 6-well plate after that they were incubated at 37ºC for overnight. HUVECs

were incubated with 0.1, 1, 10 and 20 ng/ml TNF-α for four hours. Then cells were trypsinized after centrifuge pellet was freeze at -20ºC for RNA isolation.

3.7. DRUG TREATMENT OF HUVECS

Confluent cells were passage and seeded into 24-well plate. HUVECs were stimulated with 20 ng TNF-α for six hours. After that, HUVECs were treated with only Atorvastatin and ICAM1 Aptamer conjugated Atorvastatin loaded MCM-41 (ICAM1-Apt-Atorv-MSNP) which was dissolved in DMSO, and culture cell media at concentrations 1nM, 2nM, 1μM, 2μM and 5μM. Experimental groups were as shown in Table 3.1.

Table 3.1. Experimental groups that were treated with drug

Group 1	HUVEC
Group 2	HUVEC + TNFα
Group 3	HUVEC + TNFα + Atorvastatin
Group 4	HUVEC + TNFα + ICAM1 Aptamer conjugated Atorvastatin loaded MCM-41

3.8. REAL-TIME PCR

3.8.1. RNA Isolation

Total RNA isolation was carried out using BIO-RAD Aurum^{TM} Total RNA Mini Kit. Briefly, 350 μl lysis solutions were added into HUVECs and pipet up and down. 350 μl of 70 percent ethanol added on lysis solution. Whole homogenized lysates were put into RNA binding column which was placed into 2 ml capless tube. The centrifugation of tubes was done at 12.000 g for 35 seconds. The bottom solution was disposed of and the column was replaced into same wash tube. 700 μl RNA Low Stringency wash solution was put into the

RNA binding column and centrifugation was done at 12.000 g for 35 seconds. Solutions in bottom of tubes were discarded and the columns were replaced into the same wash tube. Five µl DNase I and 75 µl DNase dilution solution were mixed and 80 µl was used for one sample then incubation of samples was done at room temperature for 15 minutes. 700 µl RNA high stringency wash solution was added on RNA binding column and centrifuged at 12.000 g for 35 seconds. Bottom solutions were removed, and the columns were replaced with same wash tube. 700 µl of RNA low stringency wash solution put on column and centrifuged at 12.000 g for one minute 30 seconds. The bottom solution was discarded and again centrifugation of the tubes was done at 12.000 g for two minutes. RNA binding column was taken and put into 1.5 ml micro centrifuge tube and 40 µl elution solution added onto column and samples incubation was done at room temperature for one minute and then centrifuged at 12.000 g for two minutes. Finally, the column was removed, and RNA contained tubes stored at -20°C.The concentration of RNA samples was measured using Nanodrop spectrophotometer by taking one µl from samples.

3.8.2. cDNA Synthesis and Real Time Polymerase Chain Reaction

cDNA synthesis was performed out using 1000 ng total RNA. 1000 ng RNA and master mix containing random primer, reaction mix, and enzyme mix were mixed followed by addition of nuclease free water in an eppendorf tube. Samples were placed in the cDNA synthesis machine in following conditions: 25°C for five minutes, 42°C for 120 minutes, 85°C for five minutes.

Following that, real time polymerase chain reaction was applied. A master mix was prepared which for 1x containing 10 µl SYBR Green, two µl cDNA, six µl nuclease free water, one µl reverse and forward primer. Two µl cDNA was mixed with 16 µl master mix that prepared for eNOS primer forward 5-'GCACAGTTACCAGCTAGCCA'-3, reverse 5-'AGGATTGTCGCCTTCACTCG-'3, ICAM-1 and βeta-actin in RT-PCR plate. Samples were placed in the RT-PCR machine and program was arranged in the conditions as shown in Table 3.2.

Table 3.2. RT-PCR machine was set up according to these values

Step	Time/cycles	Temperature
Initial activation step	3 min.	95 ºC
Denaturation	30 sec.	95 ºC
Annealing Extension	40 sec. 40 sec.	60-72 ºC
Number of cycles	39 X	
End of PCR cycling	Indefinite	4 ºC

3.9. WESTERN BLOTTING

3.9.1. Cell Lysis

The protein lysate was harvested from the cultured cells in RIPA lysis buffer supplemented with PMSF, Na-Orthovanadate and Protease inhibitor. The medium inside 24-well plate was removed. After that, washing of cells were done with cold PBS one time. Then 100 µl lysis of buffer was added onto cells and cell scraper was used to remove cells from surface. Final lysate was pull together into 1.5 ml eppendorf tubes and after cells break up with injector, they were placed on ice for 30 minutes. Finally, centrifugation of cells was done at 18000 rpm at 4°C for 10 minutes and supernatant was discarded, and pellet was stored at -20°C.

3.9.2. Detergent Compatible Protein Assay

Serial dilution was performed from a standardized 20 mg/ml BSA solution which was diluted 1:1 in RIPA lysis buffer for obtaining the final concentrations; 0 (RIPA), 0.125, 0.25, 0.50, 0.75, 1, 1.5 and 2 mg/ml. 200 µl Reagent B and 25 µl Mix Reagent (Reagent A and Reagent S) was added to 5 µl of both standards and samples in 96 well plate as two replicates. Protein content was calculated by measuring absorbance at 750 nm using Spectrophotometer and comparing the protein concentration of the known standards to the samples using Microsoft Office Excel.

3.9.3. SDS PAGE

Western Blotting was performed using four percent Stacking Gel and eight percent of Resolving Gel with BOLT Running Buffer. Total protein concentrations used in the experiments were 40 µg. LDS Sample Buffer (4x) diluted to 1x and the Reducing agent (10x) diluted to 1x were added to samples. Then sample mixtures were denaturant at 70°C for 10 minutes. After denaturation, the BOLT running buffer (20x) was prepared with dH_2O as final concentration 1x. The apparatus was set up and gel was placed. 200 ml of BOLT running buffer was added to the inner tank and it was checked in case of leakage. The fill up of outer chamber with 600 ml running buffer was done. Four µl of protein ladder was taken and it was loaded into first well and then samples were loaded one by one. The gel was run at 60V until dye reaches to resolving gel then voltage was raised to 120V for about two hours. Protein was blotted to a PVDF membrane by using wet transfer system at 25V, 35mA for overnight. The membrane blocking was done with using 10 ml five percent BSA in TBS-T at room temperature by shaking for one. After that the membrane incubation was done overnight at 4°C by using primary antibodies of eNOS (1:500), p-Akt (1:2000) diluted in 10 ml five percent BSA in TBS-T and β-actin (1:2000) diluted in 10 ml five percent milk powder in TBS-T on shaker. Then the membrane was washed with TBS-T with three times for five minutes. After that, incubation of the membrane was done with Anti-Rabbit secondary antibodies of eNOS (1:5000), p-Akt (1:5000) diluted in 10 ml five percent BSA in TBS-T and Goat-Anti-Rabbit β-actin (1:10000) diluted in 10 ml five percent milk powder in TBS-T at room temperature for one

hour by placing on shaker. After that the membrane was rinsed in TBS for ECL application. 500 µl each of ECL and Enhancer were mixed and added on top of membrane drop by drop then incubate membrane for one minute. Finally, the membrane was scanned by using BIO-RAD Molecular Imager.

3.10. STATISTICAL ANALYSIS

GraphPad Prism 7.00 was used to perform statistical analysis of the data. Experiment results were shown as mean ± SD. For cytotoxicity assay one-way Anova test was performed. For TNF-α dose determination one-way Anova test and t-test were done in order to evaluate differences between different concentrations. RT-PCR and Western Blot results were analyzed using one-way Anova test and t-test to provide differences between specific groups where it is appropriate. 'n value' represents the repeated experiments number. P value is smaller than 0.05 ($p<0.05$) that is statistically significant.

4. RESULTS

The aim of this study was to evaluate therapeutic efficiency of atorvastatin loaded targeted and controlled release system compared to only atorvastatin via p-Akt/eNOS activation pathway in endothelial cells.

4.1. APT-NP DRUG DELIVERY SYSTEM CHARACTERIZATION

Mesoporous silica nanoparticles MCM41 characterization was carried out applying different methods. At first, silica nanoparticles hexagonal image was shown using TEM [213].

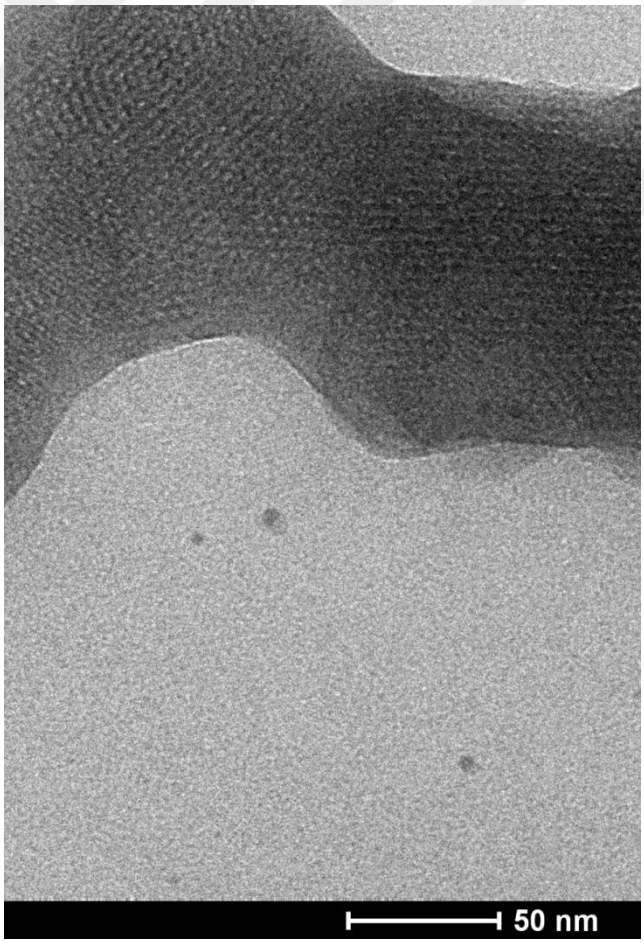


Figure 4.1. Mesoporous silica nanoparticles MCM41 TEM images was shown [213].

4.1.1. MCM-41 Silica Nanoparticles Cytotoxic Effect on HUVEC

To determine cytotoxicity of silica nanoparticles on HUVEC cells, WST-1 assay was carried out. HUVECs were incubated with different silica nanoparticle concentrations (ranges from 25 to 500 μg/ml) for 24 hours. The experiments were carried out as triplicates and results were normalized to the groups that HUVEC were not exposed to silica nanoparticles. As shown in Figure 4.1, we determined that there were no significant differences between the groups that were incubated with different doses of silica nanoparticles compared to 0.5 percent DMSO treated control group. These results showed that silica nanoparticles do not have cytotoxic effect on HUVEC cells including increasing concentrations up to 500 μg/ml.

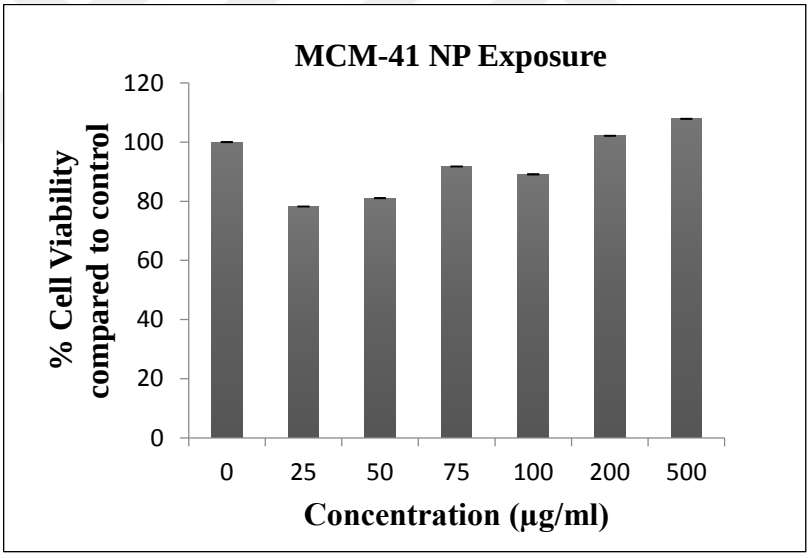


Figure 4.2. Effect of MCM-41 nanoparticles on cell viability of HUVEC compared to control group treated with 0.5 percent DMSO (0 μg/ml). HUVEC treated with different concentrations (25-500 μg/ml) MCM-41 nanoparticles. Data was represented by mean±standard deviation. One-way Anova was used to compare groups (n=3).

4.1.2. In Vitro Attachment of Apt-MCM-41 in HUVEC

In order to examine cellular attachment of ICAM-1 targeted aptamer drug delivery system, silica nanoparticle conjugated aptamers with fluorescent attachment (Apt-NP-FI) and fluorescence microscopy has been used. In control group, HUVECs were incubated with Apt-NP-FI using 500 nM concentrations for four hours. Also, another group HUVECs was firstly pre-incubated with TNF-α for six hours and then cells were incubated with Apt-NP-FI using 500 nM concentration for four hours. The images obtained by fluorescence microscopy are shown in Figure 4.2. DAPI, FAM and merged DAPI/FAM are shown below separately. The detected fluorescence signal showed that Apt-NP-FI nanoparticles more attached cells more when pre-incubated with TNF-α. These results evaluated that TNF-α stimulates ICAM-1 adhesion molecules expression and increase attachment of targeted drug delivery system to HUVECs. This result shows the attachment of APT-NP-FI to HUVEC in inflammatory conditions such as TNF-α stimulation.

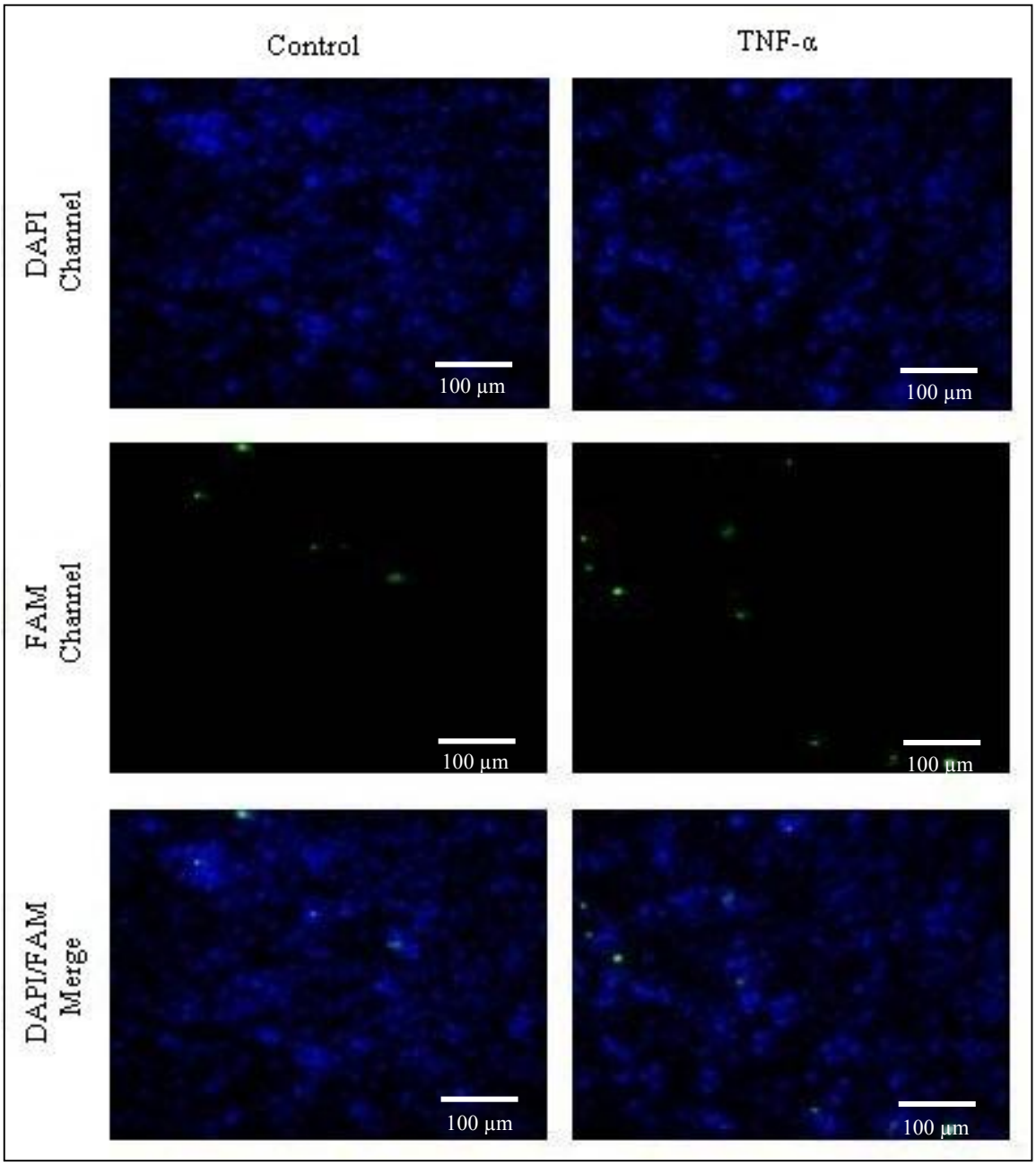


Figure 4.3. In vitro attachment of ICAM-1 Aptamer-MCM41 Nanoparticle-Fluorescein (Apt-NP-FI) in HUVEC. Cells were incubated with 500 nM Apt-Np-FI for four hours. (Blue: DAPI; Green: Apt-NP-FI)

4.2. TNF-α INDUCES ICAM-1 mRNA EXPRESSION OF IN HUVEC

We evaluated whether TNF-α stimulate inflammatory response in HUVEC. Therefore, HUVEC cells were stimulated with different TNF-α concentrations such as control (only 0.5 percent DMSO), 0.1, 1, 10 and 20 ng/ml for 4 hours. As shown in Figure 4.2 we observed that TNF-α increased ICAM-1 mRNA level in dose-dependently. 10 and 20 ng/ml was significantly up-regulated ICAM-1 mRNA expression when comparing to control group. With this experiment, TNF-α pro-inflammatory effect has been shown in HUVEC.

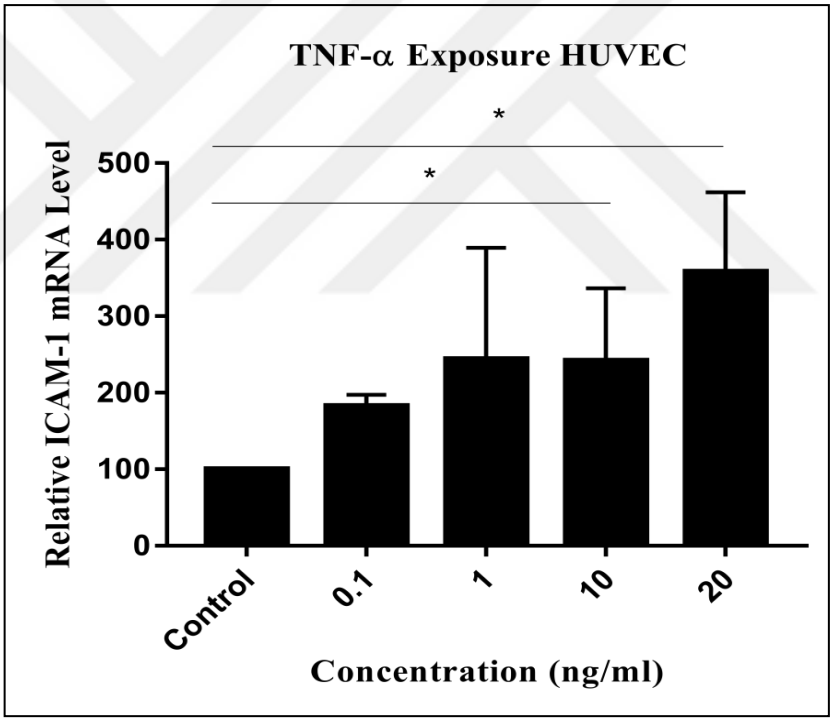


Figure 4.4. Effect of TNF-α on ICAM-1 mRNA expression level compared to control group treated with 0.5% DMSO. HUVEC were pre-treated with different concentrations of TNF-α for four hours. Data was represented by mean±standard deviation. One-way Anova was used to compare groups (n=3). * represents significant differences ($p<0.05$).

4.3. EVALUATING THE EFFECT OF NON-TARGETED AND TARGETED DELIVERY OF ATORVASTATIN IN mRNA EXPRESSION OF eNOS IN PRE-TREATED HUVECS

To examine the effect of Atorv and ICAM-1-APT-Atorv-MCM41 on eNOS mRNA expression, we firstly activated HUVEC by inducing with 20 ng TNF-α for 6 h. Then HUVEC cells were pre-treated with different concentrations (1nM, 2nM, 2μM and 5μM) of Atorv drug and ICAM-1-APT-Atorv-MCM41 targeted drug delivery system for 24 hours. We measure mRNA level using RT-PCR. The results were normalized to β-actin. We determined that TNF-α tend to reduce eNOS level compared to control group. In addition,

Atorv and ICAM-1-APT-Atorv-MCM41 targeted drug delivery system showed tendency in increasing of eNOS mRNA level dose dependently compared to TNF-α stimulated group. Also, we observed that pretreatment of HUVEC with ICAM-1-APT-Atorv-MCM41 had tendency to more increase mRNA expression of eNOS dose dependently compared to Atorv pretreated groups as shown in Figure 4.3. The results of this study show that although there is not significantly increases more increased in eNOS mRNA level was observed in HUVEC group pretreated with Atorv targeted and controlled release drug delivery system.

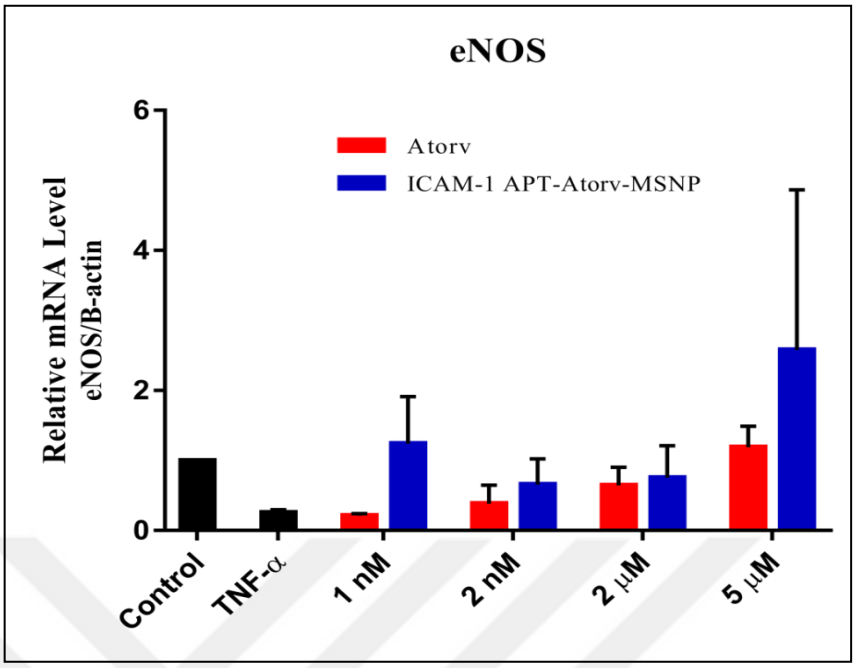


Figure 4.5. Effect of targeted and non-targeted delivery of Atorvastatin on eNOS mRNA expression in TNF-α pre-treated HUVEC. HUVEC were treated with different concentrations of Atorv drug and ICAM-1-APT-Atorv-MCM41 targeted drug delivery system. Data was represented by mean±standard deviation. T-test was used to compare groups (n=2/4).

4.4. THE EFFECT OF TARGETED AND NON-TARGETED DELIVERY OF ATORVASTATIN ON PROTEIN LEVEL OF eNOS AND p-Akt IN PRE-TREATED HUVECS

Following, effect of both non-targeted and targeted atorvastatin delivery on p-Akt/eNOS pathway was observed to demonstrate therapeutic efficiency of nanoparticle based targeted drug delivery system when compared to non-targeted drug delivery. Here, in vitro targeted and non-targeted delivery of Atorv was evaluated in HUVEC activated by 6 hours TNF-α. The effect of Atorv and ICAM-1 APT-Atorv-MCM-41 on protein expression of eNOS and p-Akt were determined. The results were normalized to βeta-actin. According to results of Western Blot, TNF-α tend to reduced level of total eNOS compared to control group. Also, both only Atorv and ICAM1 APT-Atorv-MCM41 respectively 1.8 and 1.73 fold increased total eNOS protein level when compared to TNF-α treated group (Figure 4.6; Figure 4.7).

In addition, 1 nM Atorv and ICAM-1 APT-Atorv-MCM-41 drug delivery system increased eNOS level respectively 1.3 and 1.2 fold when compared to TNF-α stimulated HUVEC. Also 2 nM Atorv and ICAM-1 APT-Atorv-MCM-41 drug delivery system raised level of eNOS 1.3 and 1.6 fold respectively comparing to TNF-α group. 2 μM and 5 μM concentration of only Atorv increased eNOS protein level 1.1 and 1.19 fold however 2 μM and 5 μM ICAM-1 APT-Atorv-MCM-41 drug delivery system increased total eNOS level 1.2 and 1.69 fold compared to TNF-α group as shown in Figure 4.7. Besides, 2 μM of ICAM-1 APT-Atorv-MCM-41 drug delivery system increased protein level of total eNOS 1.43 fold when compared to 2 μM only Atorv and also 5 μM ICAM-1 APT-Atorv-MCM-41 drug delivery system 1.41 fold increased eNOS protein level when compared to Atorv (Figure 4.7).

In addition, as shown in Figure 4.8, 1 nM and 5 μM concentration of Atorv and ICAM-1 APT-Atorv-MCM-41 drug pre-treatment leaded to increase of p-Akt protein level significantly when compared to only TNF-α stimulated group. Also, 2 μM concentration of ICAM-1 APT-Atorv-MCM-41 significantly increased p-Akt protein expression comparing to TNF-α stimulated group (Figure 4.8). In addition, 2 μM and 5 μM concentration of ICAM-1 APT-Atorv-MCM-41 significantly increased protein level of p-Akt when compared to 2 μM and 5 μM Atorv pre-treated groups as shown in Figure 4.8. Targeted atorvastatin drug delivery system (ICAM-1 APT-Atorv-MCM41) was significantly up-regulated Akt phosphorylation in pre-atherosclerotic HUVEC. These results showed that targeted and controlled atorvastatin drug release system is more effective than only Atorv drug to increase eNOS and p-Akt level.

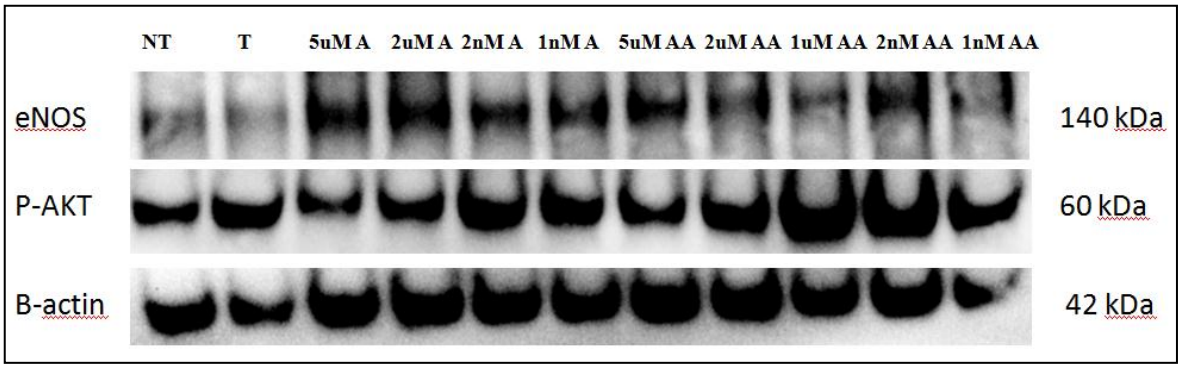


Figure 4.6. Concentration-dependent manner of eNOS and p-Akt protein level. After 6 h TNF-α activation, HUVEC were pre-treated with Atorv and APT-Atorv-MCM-41 for 24 h and Western Blot analysis was carried out using eNOS and p-Akt antibody. (A:Atorv; AA:ICAM-1 APT-Atorv-MCM41)

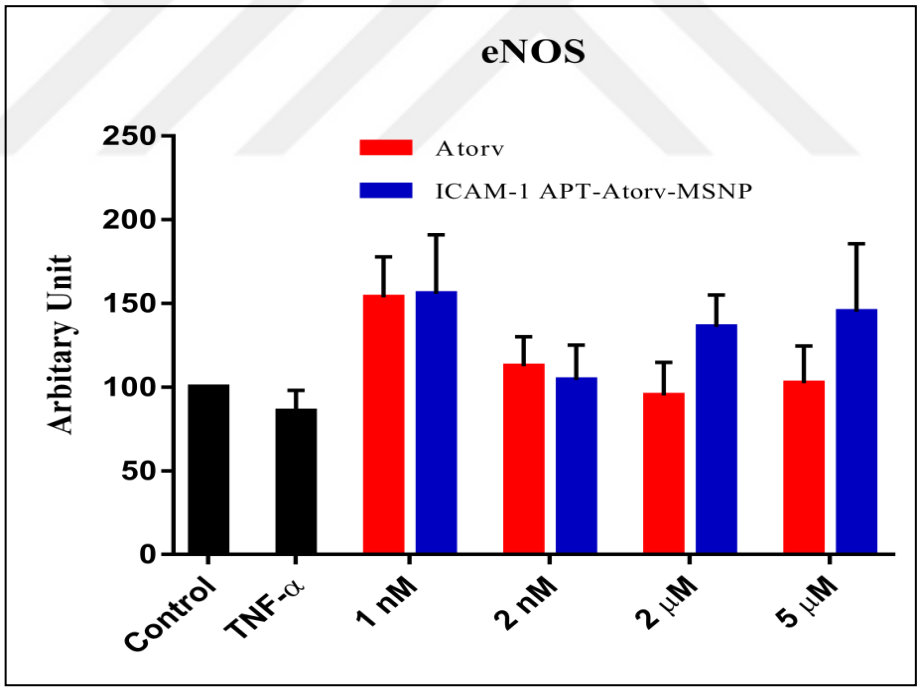


Figure 4.7. Effect of targeted and non-targeted delivery of Atorvastatin on eNOS protein level in 6 h TNF-α pre-treated HUVEC. For statistical analysis, one-way Anova and t-test were performed to compare groups (n=3/4). Data was represented by mean±standard error.

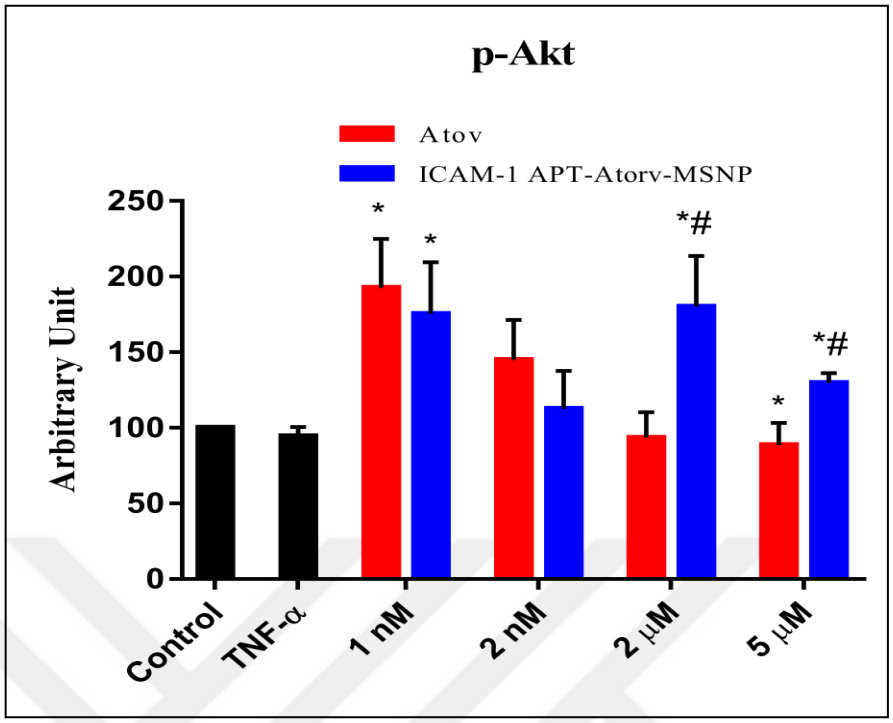


Figure 4.8. Effect of targeted and non-targeted delivery of Atorvastatin on p-Akt protein level in 6 hours TNF-α pre-treated HUVEC. Data was represented by mean±standard error. One-way Anova was used while comparing 3 groups (Control, TNF-α and treatments) and t-test was used to compare two groups (between treatments) for statistical analysis. *represents significant difference compared to TNF-α control group and #represents significant difference compared to treatment group (*$p<0.05$, #$p<0.05$, n=3/8).

5. DISCUSSION

Atherosclerosis is major contributor for developing of CVD such as myocardial infarction and stroke. There are various drug treatments that are applied to prevent heart diseases such as atherosclerosis, hyperlipidemia and hypertension in order to restrain the development of thrombotic complications [200]. Atherosclerosis remains to be the number one cause of death in worldwide, despite of the various researches that is conducted and clinically tested to prevent it [214]. Besides, there is no available drug therapy to date to directly target and suppress the molecular mechanisms of inflammation. For example, the drugs that are currently used for therapeutic prevention of atherosclerosis are consumed orally resulting in non-specific distribution of drugs which are able to cause strong side effects and requires long-term usage [199]. Therefore, there is a need for more efficient and safer treatment strategies to directly target the inflammation area to prevent the process of developing of atherosclerosis [215]. In addition, inflammation site targeted nano-medicines seem very attractive approaches for diagnosis, monitoring, and treatment for all stages of atherosclerosis [216]. Moreover, nanoparticles based drug delivery can overcome few drawbacks such as toxicity, water non-soluble and poor bioavailability of drugs [217]. Nanoparticles are successfully applied both in vivo and in vitro drug delivery, imaging of disease region, anticancer drugs, biological tagging application and research [215].

TEM imaging technique was used to characterize MCM-41 mesoporous silica nanoparticles. Mesoporous silica nanoparticles MCM-41 hexagonal characteristics were revealed using TEM imaging in Figure 4.1 [213].

To determine biological effect of MCM-41 silica nanoparticle on HUVEC, cell viability assay was done. Previously, 25 and 50 μg/ml silica nanoparticles have not shown any toxic effect on endothelial cells however 200 μg/ml concentrations decreased cell viability to 82.3 percent when compared to control group [218]. In the current study, all doses did not show cytotoxic effect on HUVEC in Figure 4.2.

The attachment of Apt-NP-FI targeted system was determined on HUVEC. We have shown that ICAM-1 aptamer conjugated silica nanoparticles have been attached on HUVEC under fluorescence microscopy as shown in Figure 4.3. This result revealed that

this targeted nanoparticle is able to attached ICAM-1 expressed on pre-atherosclerotic endothelial cells model.

After that, we have studied the pro-atherosclerotic effect of TNF-α on HUVEC by determining expression of adhesion protein like ICAM-1. In the previous study it has been shown that ICAM-1 expression was increased in HUVEC treated with TNF-α for four hours [219]. Another study showed that ICAM-1 mRNA expression significantly increased after six hours incubation with TNF-α [220]. Previously, it has been determined that adhesion molecules such as ICAM-1 expression were increased by TNF-α [221, 222]. Similarly, we also determined that TNF-α significantly stimulated expression of ICAM-1 in HUVEC. 10 and 20 ng/ml concentrations of TNF-α significantly was increased expression of ICAM-1 for four hours as shown in Figure 4.4. TNF-α is a critical pro-inflammatory cytokine and in this study TNF-α has been used to stimulate pro-inflammatory condition in endothelial cells to study effect of atorvastatin.

Then the one of the most important pleiotropic effect of atorvastatin, upregulating eNOS gene expression, in endothelial cells was studied. Previously, Dang et al. has shown that atorvastatin had no effect on cell viability of HUVEC treated with different doses (1, 10, 20 and 50 μM) of atorvastatin [223]. eNOS mRNA expression level and protein level were determined respectively with RT-PCR and Western blot in order to evaluate the therapeutic efficiency of ICAM-1 Apt-Atorv-MCM41 drug delivery system when compared to non-targeted delivery of atorvastatin. Both targeted and non-targeted delivery of atorvastatin did not affect significantly mRNA expression and protein level of total eNOS as shown in Figure 4.5, 4.6 and 4.7. However, RT-PCR and Western blot results showed that eNOS level tended to reduce in TNF-α treated HUVEC compared to control group. In addition, although both Atorv and ICAM-1 Apt-Atorv-MCM41 showed tendency to increase level of eNOS however ICAM-1 Apt-Atorv-MCM41 tended to increase more the mRNA and protein level of eNOS comparing to Atorv treated HUVEC. In a study by Loboda et al. 0.1 μM concentration of atorvastatin increased eNOS expression in human microvascular endothelial cells [158]. Previously, pretreatment of HUVEC which treated with ox-LDL with different concentration (0.01, 0.1 and 1 μmol/l) of rosuvastatin leaded to significantly increasing in level of phosphorylation of eNOS (p-eNOS s1177) however total eNOS amount did not changed [224]. In another study, Western blot analysis showed that 0.1 and 1 μM concentration of pitavastatin significantly increased phosphorylation of eNOS and

total eNOS level has not been changed [225]. Another study also showed that 1μM simvastatin enhanced p-eNOS on Ser^{1177} however has not changed total eNOS level in rat mesenteric artery [226]. However, another study evaluated that 1 μmol/L concentration of simvastatin and 10 μmol/L concentration of lovastatin significantly increased total eNOS level in ox-LDL treated human saphenous vein endothelial cells [227]. Next, we studied the efficiency of ICAM-1-Apt-Atorv-MCM41 in the up-regulation of p-Akt (Ser473) protein level which is important protein for eNOS activation. Akt is important protein for expression and activation of eNOS. In the present study, we observed that p-Akt (Ser473) level tended to reduce in TNF-α treated HUVEC compared to control group. In addition, although both Atorv and ICAM-1 Apt-Atorv-MCM41 significantly increased level of p-Akt (Ser473) when compared to TNF-α treated HUVEC group. Also, ICAM-1 Apt-Atorv-MCM41 targeted drug delivery system significantly enhanced protein level of p-Akt (Ser473) comparing to Atorv treated HUVEC (Figure 4.7). Previously, it has been evaluated that 10 and 30 μmol/L atorvastatin increased p-Akt level in HUVEC [155]. Another study showed that atorvastatin, pravastatin and pitavastatin enhanced eNOS expression dose dependently (10, 30 and 100 nmol/L) and 100 nmol/L atorvastatin increased expression of p-Akt at Ser473. The result of this study indicated that statin increased eNOS level via p-Akt pathway [157]. NO which is product of eNOS has significant role in maintaining vascular tone with its anti-inflammatory effects. Therefore, providing NO bioavailability is essential for preventing development of atherosclerosis. Instead of LDL lowering effect statin drugs have different protective effect and eNOS activation pathway is one of these repair effects [228, 229]. However, statin drugs have serious adverse effects such as myopathy, neurological and metabolic damages [167]. For this reason, in this study we tried to develop drug delivery system that safely delivery drug to disease side by this way increasing therapeutic efficiency while reducing side effects. We showed that targeted delivery of atorvastatin showed higher efficiency by increasing level of eNOS and p-Akt in TNF-α treated HUVEC. Previously, it has been shown that Akt signaling pathway has roles in expression and activation of eNOS [224, 230, 231].

There are also different nanoparticles-based studies to prevent development of atherosclerosis and prevent cardiovascular diseases. Macrophages and leukocytes targeting drug loaded nanoparticles are another strategy to keep their pro-inflammatory effects under control to prevent development of atherosclerosis.

Some studies are focused on developed nanoparticles with imaging agents for the detection of atherosclerotic plaque especially in the early stages which is also called "silent plaque" that is difficult to detect [214]. In this manner, Yao et al. studied the detection of connective tissue growth factor (CTGF) which is expressed in atherosclerotic plaque thus imaging atherosclerotic lesion in vivo ApoE-deficient ($apoE^{-/-}$) mice, which are types of atherosclerosis developed mice model, by developing connective tissue growth factor conjugated-polyethylene glycol coated ultra-small super-paramagnetic iron oxides (Anti-CTGF-USPIO) [232]. Muhammed et al. investigated the cardio-protective effect of ethyl acetate fraction of P.emodi loaded gold nanoparticle (Pe.EA40-AuNPs) [233]. In another study, PEG functionalized 5-aminolevulinic acid loaded AuNps (ALA:AuNPs) were utilized as therapeutic drug delivery approach for early diagnosis of atherosclerosis [234]. Zhu et al. took another approach to observe the prevention effect and mechanism of selenium quantum dots (SeQDs) *in vivo* using atherosclerotic *$Apo^{-/-}$ mice* model [235]. Most research are used chemotherapeutic drugs as their anti-inflammatory feature for preventing vascular inflammation [236].

In another study, it is claimed that anti-cancer drugs have anti-atherosclerotic action therefore docetaxel, anti-cancer drug, loaded lipid core nanoparticle as vesicle (LDE-DXT) was fabricated and the study showed that LDE-DXT has anti-atherosclerotic action *in vivo* using cholesterol-fed rabbit [237].

Platelets attachment to inflamed endothelial cells especially after rupturing of vulnerable plaque is important process for development of atherosclerosis resulting in thrombotic complications.

In this manner, Song et al. studied specificity and effective treatment of platelet mimicking drug therapy by developing platelet membrane coated rapamaycin encapsulated nanoparticles (RAP-PNP) by carrying out in $ApoE^{-/-}$ mice model. Spyropoulos-Antonakakis et al. developed polyamidoamine dendrimer conjugated with zinc phthalocyanine (PAMAM/ZnPc) in order to study nano-drug distribution effectiveness and

therapeutic efficiency of PAMAM with ZnPc photosensitizer [238]. Iron oxide nanoparticles have fundamental (intrinsic) imaging properties and can be modified for drug delivery [239]. Poon et al. studied the potential imaging of vulnerable atherosclerotic plaque by developing non-invasive MRI technique by engineered fibrin-binding peptide conjugated metal oxide encapsulated micelles [240]. Furthermore, integrin α4β1 antagonist, which is leukocyte receptor resulting in binding of leukocyte to adhesion molecules on endothelial cells, incorporated liposomal Gd contrast agent, MR imaging agent, was used to image atherosclerotic plague as overall [241]. Li et al. also studied the detection of atherosclerosis by MR imaging system using tenascin-C-targeted-ultra-small super-paramagnetic iron oxide nanoparticles [242]. Another study was focused on the treatment of atherosclerosis by developing curcumin which is spice turmeric extraction loaded linear-dendrimer copolymer nanoparticles [243]. Gomes et al. used anti-cancer chemotherapeutic drug such as paclitaxel in lipid core nanoparticles for restricting high atherosclerotic lesion [236].

There are various targeting methods to use nanoparticles in the diagnosis and treatment of atherosclerosis. During circulation of drugs throughout blood, endothelium is excessively exposed to drugs however without targeting the drugs can be taken up by healthy cells. Therefore, nanoparticles loaded with drugs can be conjugated with determinants which are specific for endothelial cells.

Besides to therapeutic usage, nanoparticles can also be used to modify by conjugating peptides, aptamers, antibodies or another ligands which can target molecules in disease region [244]. Two main cause of the failure of therapeutic drugs in the treatment of atherosclerosis are the short bioavailability and inability selection of targeted tissue. In this manner, target molecule attached drug encapsulated nanoparticles are able to place atherosclerotic region [245].

Combination of targeting markers which are only found in disease site with nanoparticles provides powerful diagnostic strategy for delivering of nanomedicine for atherosclerosis [246]. Different peptides which are expressed on endothelial cells, smooth muscle cells, monocytes and macrophages have been studied and pre-clinically tested for atherosclerosis [247].

Adhesion molecules are frequently used as targeting peptide and have been identified as therapeutic target for managing inflammation. Endothelial cells are stimulated to express ICAM-1, VCAM-1 and E-selectin adhesion molecules on their surfaces during endothelial dysfunction [56]. These adhesion molecules have roles in binding of leukocytes to endothelial cells. Kelly et al. developed VHSPNKK peptide, which was specific to bind VCAM-1 on inflamed endothelial cells, carrying multimodal nanoparticles to images inflamed endothelial cells in vitro by confocal microscope and in vivo atherosclerotic lesion detection in $ApoE^{-/-}$ mice via MRI [248]. Imanparast et al. utilized modified ZD7349 peptide conjugated simvastation loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticle and the effectiveness of this mZD7349-SIM-PLGA-NPs on eNOS phosphorylation (p-eNOS) was evaluated in TNF-α activated HUVEC. ZD7349 peptide is an antogonist for inhibiting the binding of VLA-4 (very late antigen-4), which is expressed on monocyte and has role in recruitment of monocyte by interacting with ICAM-1, to ICAM-1 [196]. Krishna et al. investigated the effect of an ionic nanoliposome application, which consists phospholipid bilayers, to prevent dyslipidemia in low-density lipoprotein knock-out mice ($Ldlr^{-/-}$). It was suggested that nanoliposome and plasma lipoprotein interaction could be a mechanism to promote the purification of LDL from blood due to the quick uptake of nanoliposome by liver which is the excretion organ of LDL. Dyslipidemia is the abundance of lipoprotein in blood therefore it is a main initiative factor in atherosclerosis [249]. Coating surface of nano-carrier with antibodies against ICAM-1 (anti-ICAM-1) could be a target for drug delivery systems however large targeting protein may lead to induction of immune response that can damage the explication of therapeutic effects. In this context, Carmen Garnacho and his colleagues (2011) reported that γ3 nanocarriers (NCs) targeted ICAM-1 with specificity similar to the anti-ICAM-1 NC using short peptide derived from fibrinogen (γ3) which is a natural ligand of ICAM-1. In addition, internalization of γ3 NCs by cells both *in vitro* (HUVEC) and *in vivo* (C57BL/6) was observed while cell junctions remained undisturbed [250]. Aanei et al. utilized VCAM-1 attached MS2 viral capsid nanocarrier to detect atherosclerotic lesion at early stages by applying on ApoE knockout mice [251]. Calin et al. improved VCAM-1 recognizing peptide conjugated Teijin compound 1 which is a human chemokine (C-C motif) receptor 2 (CCR2) antagonist, encapsulated liposome nanocarrier in order to inhibit adhesion of monocytes to inflamed region on endothelium [252]. Due to short half-life and vasculature protective features of NO, it is crucial to supply NO to inflamed region. Therefore, Rink et al. developed NO

delivering high density lipoprotein nanoparticle to supply distribution of NO donor to inflamed region [253]. Robbins et al. studied to improve strong adhesion of polymers one by designing leuko-polymer some which was developed using two adhesion molecules, P-selectin and ICAM-1, attached polymersome and applied on shear stress stimulated inflamed HUVEC in order to establish polymersomes based drug delivery system [254]. It has been demonstrated that ROS have a significant role in proceed of atherosclerosis therefore this could be another way for targeting. Nakhlbanda et al. utilized a different approach to investigate protective effect of Marrubiin, an antioxidant and water-soluble drug, on the generation of ROS by loading this drug inside solid lipid nanoparticles (SLNs) in order to increase not only pharmacokinetics but also bioavailability of drugs for the treatment of atherosclerosis [255]. Combining water-soluble drugs with nanoparticles results in enhanced bioavailability of drugs inside aqueous environment as well as improves therapeutic effect. All these studies show that there are different nanoparticles-based approaches to detect and prevent atherosclerotic plaque.

6. CONCLUSION

In conclusion, disease site specific nanoparticle encapsulating drug delivery has proceeded a significant strategy for prevention of cardiovascular related diseases. In this study, therapeutic efficiency of atorvastatin via ICAM-1 Apt-Atorv-MSNP drug delivery system was studied by comparing with non-targeted delivery of atorvastatin.

Mostly studies using nanotechnology for atherosclerosis focused on detection of atherosclerosis plaque development. However, therapeutic approaches for prevention of atherosclerosis took less attention and there are only a few studies using this kind of perspective. In this thesis a novel targeted drug delivery system, ICAM-1 Apt-Atorv-MSNP drug delivery system is developed to prevent atherosclerosis at the early phase. This novel targeted drug delivery system can increase the efficacy of statin via increasing the dose in the inflammation area on artery and lead to protection for endothelial cells. ICAM-1 aptamer conjugated atorvastatin encapsulating mesoporous silica nanoparticle drug delivery of may provide new therapeutic approach for suppress atherosclerosis in this way preventing clinical symptoms like heart attack or stroke.

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