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Inducible Nitric Oxide Synthase inhibits macrophage migration, a potential explanation for iNOS's proatherosclerotic action

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Chapter I

I. Introduction

Atherosclerosis is known as one of the leading reasons of death in western countries[1]. Atherosclerosis is defined by the chronic accumulation of lipids and fibrous matters in the vessel wall which may lead to progressive stenosis and reduction of blood flow. The initial step, triggering many of the events leading to plaque progression, is considered oxidation of low density lipoprotein (ox-LDL) in the subendothelial space[2].

Nitric oxide synthases (NOS) are the enzymes responsible for generation of nitric oxide (NO), which is one of the smallest known bioactive products of mammalian cells[3] and mediates several biological effects[4]. There are three distinct isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS)[5]. The expression of inducible nitric oxide synthase (iNOS) in early and advanced atherosclerotic human and murine plaques modulates cellular and molecular mechanisms that initiate and propagate atherosclerosis[4,6].

I.1. Atherosclerosis

Atherosclerosis is a growing health problem leading to a significant increase in mortality and morbidity worldwide. The disease is associated with lipid deposition in the arterial vessel wall. The rupture of atherosclerotic plaques may cause death by forming a sudden occlusive thrombus or embolic events leading to ischemia of the heart or the brain. Therefore, the basic mechanism causing atherosclerosis are a major research focus.

Atherosclerosis is considered a chronic inflammatory disease. Fibroinflammatory lipid plaques are accepted as the characteristic atherosclerotic lesions. It is believed that lesion progression in atherosclerosis is following a certain cascade of events. Firstly, the subendothelial deposition of atherogenic lipoproteins facilitate the activation of endothelial cells including adhesion molecule expression and inflammation, which is characterized by the release of some signaling factors

including chemokines[7.8]. Then monocytes are attracted to spots of activated endothelial cells and transmigrate into the intimal space where these monocytes differentiate into macrophages. These macrophages take up excess lipids and transform into foam cells. Finally, foam cells die and liberate their contents, hence attracting more macrophages to the growing plaque (Fig. 1).

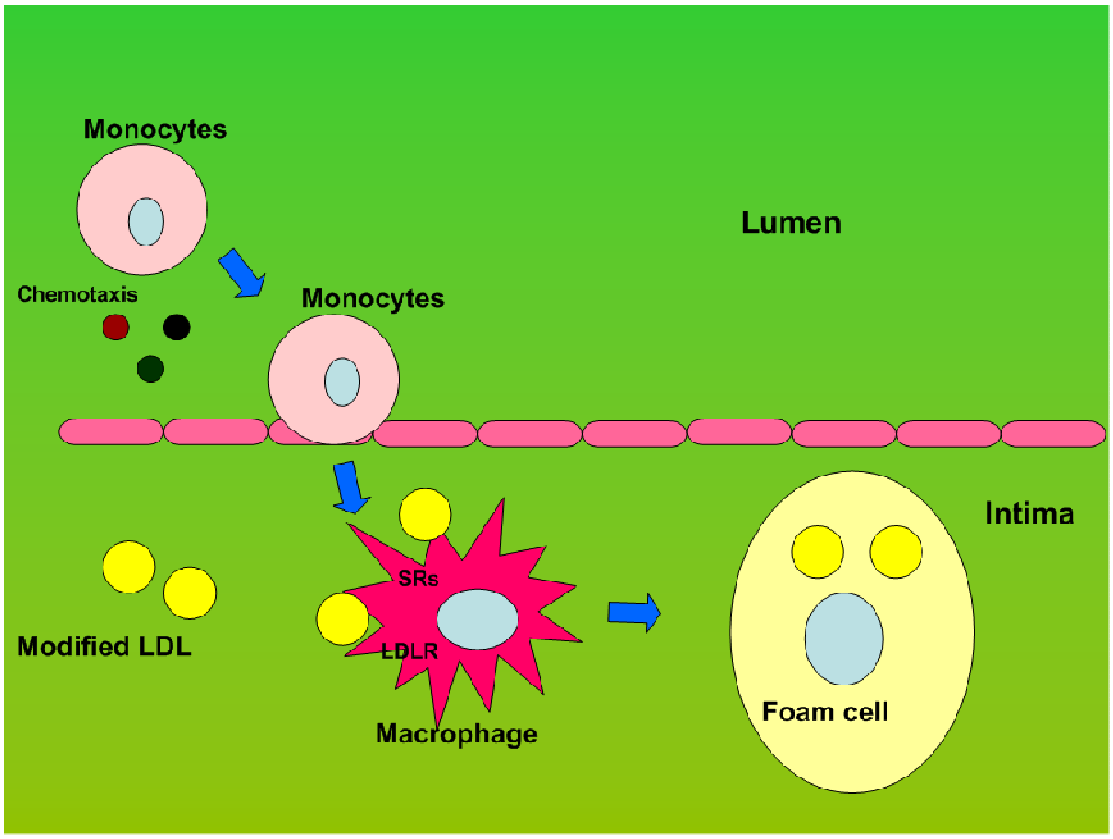

Fig.1 Monocytes play a critical role in all stages of atherosclerosis. Following transmigration into the subendothelial space monocytes transform into macrophages. Subsequently monocyte-derived macrophages upregulate surface expression of scavenger receptors (SRs), which increases their ability to take up modified LDL particles leading to foam cell formation.

In the early stages of atherosclerosis, serum lipids, especially oxidized lipoproteins, are deposited underneath endothelial cells at arterial branch points/vessel bifurcations, in part due to turbulent blood flow in these segments. After endothelial cells are activated, the expression of adhesion molecules on the endothelial cells will increase. Mediated by the chemokine receptors on their surface, monocytes are recruited to

atherosclerotic lesions, adhere to the endothelial cells through the interactions with adhesion molecules, and finally migrate into the subendothelial space. An et al has shown that with genetic deletion or functional blockade of adhesion molecules, such as ICAM-1, VCAM-1, CD18/β2 integrin, and P-selectin, the development of atherosclerosis can be slowed down[9]. Combadière et al showed that the number of circulating monocytes correlates positively with the atherosclerotic plaque size in mice, and that functional alterations in monocyte/macrophages affects the development of atherosclerosis[10].

In the later stages of atherosclerosis, when lesions are already established macrophages continue to engulf extracellular oxidatively modified lipids by receptor-mediated phagocytosis and pinocytosis. Zhou et al demonstrated that the LDL receptor (LDLR) plays a key role in the process of modified lipoprotein internalization by macrophages[11]. However, macrophage-derived foam cells are found in atherosclerotic lesions even in patients with familial hypercholesterolemia despite genetically impaired LDLR. Ashraf et al found a new class of receptors (scavenger receptors, SRs) that bind to modified LDLs using acetylated LDL (acLDL) as a model ligand[12]. Among these SRs, the type A scavenger receptor (SRA) and CD36, a member of the type B family, have been extensively studied [13]. It is well known that ApoE-deficient mice, which are widely used as an atherosclerosis model spontaneously develop atherosclerotic lesions throughout the arterial tree [14,15]. Interestingly, Podrez et al showed that mice with CD36 and ApoE double deficiency have reduced atherosclerotic lesions and greatly reduced uptake of oxLDL by macrophages compared with their littermate controls[16]. Unlike the regulation of LDLR expression, the expression of SRs is not downregulated by increased intracellular levels of cholesterol. As a result, SR-mediated uptake of oxLDL leads to the continuous absorption of lipoproteins and the accumulation of excess lipids by macrophages[17]. The excess lipids are stored in lipid droplets in the cytoplasm, creating a foamy appearance of the so called “foam cell” under the microscope and are named as foam cells, a hallmark of atherosclerosis. Upon the uptake by macrophages, lipoproteins are transported to the late endosome/lysosome, where

cholesterol esters (CEs) are hydrolyzed into free cholesterol (FC) and fatty acids (FAs)[18]. Because of the cytotoxicity of FC, the hydrolysis of CEs and the reesterification of FC are very important in protecting macrophages and foam cells. Similar to other cell types, FAs in macrophages bind to fatty acid-binding proteins which are responsible for their intracellular function. Makowski et al have demonstrated that, in macrophages, the absence of aP2, a fatty acid-binding protein protect ApoE-knockout mice from atherosclerosis development[19]. Ghosh et al recognized neutral cholesterol ester hydrolase (nCEH) as the rate-limiting enzyme in the hydrolysis of CEs in macrophages[20]. In nCEH transgenic mice crossed into an LDLR-null background the western diet induced atherosclerosis was significantly reduced because of the enhancement of cholesterol efflux and reversed cholesterol transport (RCT), which also resulted in increased cholesterol elimination as bile acids in the feces[21]. These results demonstrate that macrophage-specific over expression of nCEH is antiatherogenic and that this protein is potentially a novel target for the treatment of atherosclerosis.

In conclusion, macrophage foam cells play a key role in all stages of atherosclerosis, from the earliest lesions to complex plaques, and are a critical determinant of plaque related complications. During the development of atherosclerotic plaques, monocytes migrate into the arterial intima where they differentiate into macrophages and become lipid-overloaded by uptake of excess lipoproteins. At the same time, cytotoxicity is caused by the sustained increase of intracellular lipid concentrations. With the accumulation of lipids in the macrophages, massive lipid droplets are formed leading to the formation of foam cells. Better understanding of the mechanisms responsible for plaques formation will be important in order to find a cure for atherosclerosis, one of the major causes of death in western countries.

I.2. Oxidative stress (OS)

Oxidative stress (OS) is a condition that occurs when the steady balance between prooxidants and antioxidants is lost, which will lead to damage to the organism.

Prooxidants are defined as free radicals, atoms, or clusters of atoms with a single unpaired electron[22]. Initially, oxidative stress has been described as an imbalance between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS), termed the redox system. These reactive species were first thought to be “only” harmful to cells, but recent data suggests that the redox system involving ROS is also involved in cell signaling i.e. kinase cascade activation, ion transport, regulation of mitogen-activated protein (MAP), calcium mobilization, and apoptosis[23]. The generation of oxidative stress was considered to be associated with alterations in ROS, RNS, and nitric oxide (NO) production, whereby bioavailable NO is decreased and ROS and RNS production are increased[24]. The signaling pathways of the redox system are induced by inflammatory responses. Simultaneously, mitochondrial metabolism generates high concentrations of free radicals. At the same time ROS and RNS could also react with other substrates such as superoxide anion, hydroxyl radical, and peroxynitrite. It was demonstrated that some physiological defense signaling pathways can be offset by the production of ROS and RNS. Under conditions of excessive production of ROS and RNS will damage fatty acids, proteins, and DNA[25].

Recently, a research hotspot focusses on mechanism of production of oxidative stress and the corresponding antioxidant signaling pathways, helping us to understand how the internal environment is preserved from changes in the external environment. ROS are a family of molecules including superoxide (O_2-), hydroxyl (HO), hydrogen peroxide (H_2O_2), nitric oxide (NO), and its derivatives like fatty acid peroxyl radical (R-COO), peroxynitrite (ONOO-), and hypochlorous acid(HOCl)[26]. Via free radicals, the production of one kind of ROS will also lead to the production of several other kinds. The balance between the generation of ROS and NO composes the Redox system. The reaction of O_2- with NO also regulates the production of O_2-. If the production of ROS exceeds the endogenous antioxidant defenses, it will lead to the damage of biological compounds, such as DNA, protein, carbohydrates, and lipids. This situation was often referred as oxidative stress (Fig.2).

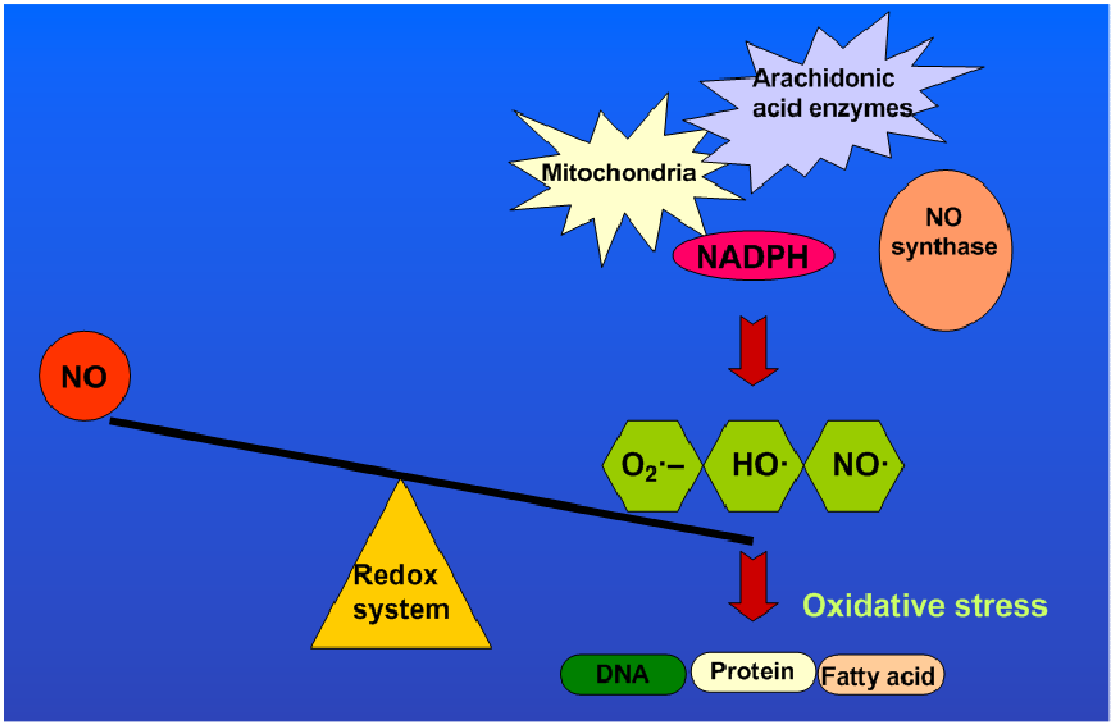

Fig.2 ROS including O_2-, HO, and NO can promote oxidative stress, which could cause damage to biological compounds. The ROS system is counterbalanced by the NO system. Excessive production of free radicals causes oxidative damage.

There is a plurality of latent enzymatic sources of ROS, including the mitochondrial respiratory cycle, heme, arachidonic acid enzymes, cytochrome p450s, xanthine oxidase, NO synthase.

I.3. Reactive oxygen species and vascular disease

Reactive oxygen species (ROS) are associated with the development of most cardiovascular diseases. Vessel injury increases cytokines expression, which in turn up regulates the production of ROS[27]. In contract, low levels of ROS participate in preserving normal vascular function. However, excessive production of ROS leads to monocyte adhesion, platelet aggregation, vascular smooth muscle cell (VSMC) proliferation, migration and apoptosis. Vascular injury is usually initiated by endothelial dysfunction and expression of adhesion molecules induced by cytokines such as tumor necrosis factor-α (TNF-α) following ROS exposure. Then increased

adhesion molecule expression attracts monocytes which transmigrate into the subendothelial space, transform into macrophages and produce high amounts of ROS, which may cause vascular leakage. ROS is also thought to participate in the VSMC proliferation following vascular injury. In that respect, low doses of H_2O_2 mediate most growth related signaling[28]. The intracellular production of H_2O_2 is thought to be important, as ROS producing enzymes are found both in caveolae and focal adhesions[29]. Moreover, high levels of ROS may cause apoptosis of vascular cells following both inflammatory and mechanical injury.

I.4. Nitric oxide synthases (NOS)

Nitric oxide (NO) is known as a key secondary messenger molecule, which has numerous molecular targets. NO's function is associated with many regulatory signaling pathways such as vascular tone, neurotransmission, regulation of gene transcription, mRNA translation and the production of post-translational modifications of proteins[30,31]. It was suggested that one of the most important functions of NO is its reaction with superoxide anion (O_2-) to form peroxynitrite (ONOO-), which may cause oxidative damage, nitration, and S-nitrosylation of biomolecules including proteins, lipids, and DNA[32]. This nitrosative stress caused by ONOO- has been related with DNA single-strand breaks, followed by poly-ADP-ribose polymerase (PARP) activation[33].

In mammals, NO can be generated by three different isoforms of the enzyme NO synthase (NOS). The isozymes are referred as neuronal nitric oxide synthases (nNOS or NOS I), inducible nitric oxide synthases (iNOS or NOS II), and endothelial nitric oxide synthases (eNOS or NOS III). All of the three isoforms of NOS use L-arginine as the substrate, molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH4) are recognized as the cofactors of all these three isozymes. The NO formed by NOS can react with a number of target enzymes and proteins, such as soluble guanylyl cyclase

which leads to the generation of cyclic GMP[34,35].

Interestingly, all three NOS isozymes have regulatory functions in the cardiovascular system. Neuronal NOS is involved in the central regulation of blood pressure, and nNOS-containing nerves participate in dilating certain vascular beds. In the function of phosphodiesterase 5 inhibitors, the residual nNOS activity is also needed. Inducible NOS is expressed in atherosclerotic plaques and is recognized as an important mediator in septic shock triggered hypotension. Endothelial nitric oxide synthases has the ability to dilate blood vessels, control the blood pressure, and has numerous vasoprotective and anti-atherosclerotic effects.

I.4.1. Neuronal nitric oxide synthases (nNOS)

Neuronal nitric oxide synthases is normally expressed in neuronal tissues, epithelial cells, mesanglial cells, skeletal muscle cells and cardiomyocytes, and involved in a variety of physiological and pathological processes, including neurotransmission, neurotoxicity, skeletal muscle contraction, body fluid homeostasis and cardiac function[36]. Neuronal nitric oxide synthases contains a PDZ domain (PSD-95 discs large/zona occludens-1 homology domain) and can directly react with the PDZ domains of other proteins. These interactions determine the subcellular distribution and the activity of the enzyme[37]. In neurotransmission, nNOS is associated with the rough endoplasmic reticulum and the synaptic membrane, whereas in skeletal muscle, nNOS localizes to the sarcolemma. It has also been shown that nNOS protein is present in the cytosol[38]. Of the three isoforms of NOS, nNOS was the first enzyme which was shown in an “uncoupled” form to produce superoxide instead of nitric oxide[39]. In the presence of L-arginine, nNOS can generate nitric oxide and superoxide, whereas in the absence of L-arginine, nNOS mediates superoxide generation from the oxygenase domain. The proportion of these two radicals depends on the concentration of BH4, which inhibits superoxide production from nNOS in a dose dependent manner. However, independent of BH4, L-arginine may inhibit superoxide production alone, suggesting that BH4 deficiency would not affect the

production of superoxide by nNOS[40]. Besides superoxide, nNOS may also generate hydrogen peroxide (H_2O_2) in the absence of L-arginine, in which BH4 has played a critical role in regulating the generation of superoxide and hydrogen peroxide. The enzymatic activity of nNOS is different compared to the other two NOS isoforms as it has the ability to catalyze the uncoupled reaction. In this way, nNOS produces higher amounts of superoxide than iNOS in the absence of substrate[41]. In both early and advanced atherosclerotic lesions in humans nNOS is detectable in endothelial cells and macrophages, whereas it is absent in normal vessels[42]. Our research suggests that nNOS is expressed in the aorta of apoE ko and apoE/iNOS double knockout (dko) mice[43,44]. As nNOS is induced in various vascular pathologies such as atherosclerosis, vascular injury and hypertension, it not required for normal vessel function. However, as it is constitutively expressed in the nervous system it might normally influence the vascular system indirectly[45]. We have already shown that genetic deletion of nNOS resulted in accelerated atherosclerosis in apoE ko mice, suggesting that nNOS has the ability to protect from atherosclerosis development. We also showed that nNOS improved the survival rate of atherosclerotic mice, as apoE/nNOS dko mice had a 30% increased mortality compared to apoE ko controls. Previously it has been speculated that nNOS localized towards to the lumen of the vessel decreases adhesion of leukocyte and platelet while nNOS expressed in the adventitia may inhibit smooth muscle cell proliferation[46]. Since in the absence of the substrate L-arginine nNOS may be “uncoupled” the role of nNOS derived superoxide and nitric oxide in the formation of atherosclerosis needs further characterisation.

I.4.2. Inducible nitric oxide synthases (iNOS)

Inducible nitric oxide synthases is normally not constitutively expressed, but its expression can be induced by cytokines and other agents in almost all the cell types. With regards to the diseased cardiovascular system, iNOS can be expressed in many nucleated cells such as vascular smooth muscle cells, cardiac myocytes and

leukocytes. Unlike eNOS and nNOS which are usually regulated by intracellular calcium levels, iNOS is independent of intracellular calcium levels since it contains irreversibly bound calmodulin. Therefore, the induction of iNOS will result in generation of massive amounts of nitric oxide[47]. iNOS's expression is normally regulated transcriptionally by cytokines such as tumor necrosis factor-α, interleukin-1b, interleukin-2 and interferon gamma-g, or bacterial lipopolysaccharide stimulation and post transcriptionally by LPS, IFN-γ, transforming growth factor-β (TGR-β). Phosphorylation and the binding to caveolin-1 may also regulate the activity of iNOS, which induces increased degradation of iNOS protein[48,49]. Interestingly, uncoupling of iNOS may also be induced in the presence of high concentrations of L-arginine, which suggests that iNOS is able to generate superoxide when L-arginine is not rate limiting. In contrast to the fact that eNOS and nNOS generate superoxide from their oxygenase domains, iNOS mediates the production of superoxide from its reductase domain. Therefore iNOS was proposed to simultaneously generate nitric oxide from L-arginine bound to its oxygenase domain, while generating superoxide from its reductase domain[50]. The generation of superoxide and nitric oxide results in iNOS mediated peroxynitrite generation, one of the strongest biological oxidants which enhances the anti microbial activity of iNOS[51].

Under normal physiological conditions iNOS's expression has important anti microbial and anti tumor activities since the enzyme produces high cytotoxic concentration of nitric oxide. However, under pathophysiologic conditions, the production of high concentrations of nitric oxide and superoxide may become detrimental. Therefore, the expression of iNOS by macrophages and smooth muscle cells in atherosclerotic lesions has been taken as evidence for its detrimental role in atherosclerosis, due to formation of peroxynitrite[52]. Our research before showed that genetic deletion of iNOS resulted in a significant reduction of lesion formation in apoE ko mice, suggesting a proatherogenic potential of iNOS[6]. iNOS's expression in atherosclerotic lesions has been detected in monocytes/macrophages, vascular smooth muscle cells and lymphocytes. These various cell sources have the ability of producing different amounts of iNOS and hence inducing iNOS's expression in

various compartments of the plaque. Moreover, these cellular sources could induce a specific series of genes co-expressed with iNOS, which may also influence their redox system and result in the formation of peroxynitrite by the production of both nitric oxide and superoxide. Peroxynitrite can oxidize proteins and cause nitrosylation of proteins which may influence protein function.

I.4.3. Endothelial nitric oxide synthase (eNOS)

eNOS is mainly expressed in endothelial cells and myocardial cell. Unlike iNOS, eNOS is constitutively expressed. The activity of eNOS is regulated in multiple steps, including transcription, post-translational modifications, substrate availability, calmodulin, enzymatic cofactors such as FAD, FMN, protein–protein interactions with Hsp-90 and caveolins[53]. Superoxide produced by uncoupled eNOS is believed to result from BH4 deficiency rather than L-arginine deficiency[54]. As eNOS is calcium dependent, activation by calcium could increase the generation of superoxide by eNOS[55].

Endothelium derived nitric oxide has played an important role in the modulation of vascular functions. Nitric oxide generated by eNOS acts as an endothelium derived relaxing factor, and mediates important aspects of vascular functions, including regulation of vascular tone and blood flow, the adhesion and aggregation of platelet and leukocytes, the expression of surface adhesion molecules and the proliferation of vascular smooth muscle cells in response to vascular injury[56].

A deficiency in the bioavailable vascular NO would in turn lead to dysfunction of endothelium and the development of atherosclerosis. Uncoupling of eNOS has been suggested the main reason leading to the reduced NO bioavailability. In many vascular diseases uncoupling of eNOS is found[57,58].

It has been demonstrated that eNOS protects the vessel wall during injury. Kawashima et al proved in a vascular remodeling model, that eNOS expression may decrease intima proliferation, inflammation and the expression of adhesion molecules such as VCAM-1[59]. At the same time genetic deletion of eNOS leads to the

development of arteriosclerosis since our previous research showed that deletion of eNOS results in acceleration of plaque formation in apoE ko mice[60]. Moreover, we found that apoE/eNOS dko mice developed vascular complications like abdominal aortic aneurysms, aortic dissections and distal coronary artery disease as observed in human atherosclerosis[60]. Furthermore, apoE/eNOS dko mice were hypertensive and showed impaired left ventricular function and cardiac hypertrophy, possibly a result of chronic myocardial ischemia, resulting from coronary artery disease[60]. However, in a model of eNOS over expressing apoE ko mice, an increase of atherosclerosis development was observed possibly due to increased uncoupling of the enzyme[61].

I.5. Aims of the investigation

All the three NOS isoforms are expressed in the vascular wall during atherosclerosis development. In the past we generated nNOS/apoE dko, eNOS/apoE dko and iNOS/apoE dko mice to investigate the role of each NOS isoform in atherosclerosis[6,43,60]. Genetic deletion of iNOS resulted in a significant reduction of lesion formation which suggests that iNOS is proatherogenic[6]. However, the detailed mechanism behind iNOS's proatherogenic action is unclear. In the research presented here, we focus on characterisation of the underlying proatherogenic mechanisms of iNOS.

A wealth of evidence suggests that the macrophage-derived foam cell plays a critical role in all steps of atherosclerosis and during the development of plaque related complications. One possible way to stop atherosclerosis could be to change the behavior of these macrophages by changing their ability to migrate into and out of the plaque. As iNOS has a proatheogenic character, we asked ourselves whether the enzyme participates in the regulation of macrophage-derived foam cell migration?

Alleviation of the migratory arrest of macrophage-derived foam cells in the plaque could help these trapped cells to leave the subendothelial space.

Therefore, we set out to test the hypothesis that oxLDL induced inhibition of migration is iNOS-dependent.

Chapter II

II. Materials and Methods

II.1. Materials

II.1.1. Mice

Mice were backcrossed for 10 generations to the C57BL/6J genetic background. iNOS ko and apoE ko mice were obtained from The Jackson Laboratories. Offsprings were crossed and the progenies were genotyped for iNOS and apoE by polymerase chain reaction using a PCR protocol provided by the Jackson Laboratories. iNOS ko and apoE ko animals were crossed to generate apoE/iNOS double knockout mice. Mice were genotyped by PCR using protocols supplied by The Jackson Laboratories. ApoE ko and apoE/iNOS dko animals were weaned at 21 days and fed a western-type diet (42% of total calories from fat; 0.15% cholesterol; Harlan Teklad) for 10 weeks.

II.1.2. Instruments and Accessories

E-scan ESR spectroscope	Bruker BioSpin GmbH
FACScan Calibur	Becton Dickinson Bioscience
Fluorescence Microscope	ZEISS
Mx3000P detector	Stratagene
Spectrophotometer	Pharmacia Biotech
pH meter	InoLab
Incubator	Heraeus
Centrifuge	Eppendorf, Beckman
Water Bath	Thermomix
1.5ml Tubes	Eppendorf
15ml Tubes	Cellstar
75cm^2 culture flasks	Corning, Cell star
6 wells	Nunclon, Corning

25-G needle	BD Bioscience
Trans-wells	Corning

II.1.3. Reagents and antibodies

1400W dihydrochloride (1400W)	Sigma
MCP-1	Sigma
Low-density lipoprotein	Applichem
FITC-Annexin apoptosis kit	BD Bioscience
FITC-phalloidin	Sigma
APC-F4/80	eBioscience
Rat IgG 2a κ iso control apc	eBioscience
siRNA transfection kit	Santa Cruz
FITC-CD36	Abcam
Anti-FAK (phospho Y576+Y577) antibody	Abcam
Secondary antibody to rabbit IgG	Abcam

II.1.4. Cell Culture

RAW 264.7 cells	CLS
RPMI 1640 medium	Gibco
PBS	Gibco
Fetal bovine serum	Gibco
Penicillin/streptomycin	Biochrom
L-glutamine	Biochrom
DMEM	Gibco
DAPI	Vector
Methanol	EMSURE

II.1.5. Real time PCR

TriFast reagent	peqGOLD
iScript cDNA synthesis kit	Bio-Rad
EVA Green PCR Master MIX	Bio-Rad
Chloroform	Sigma
Ethanol	EMSURE

II.1.6. Krebs Hepes Buffer (KHB)

Calcium chloride dihydrate	Sigma
Sodium chloride	Sigma
Magnesium sulphate, heptahydrate	Sigma
Potassium chloride	Sigma
Sodium bicarbonate	Sigma
Potassium dihydrogen phosphate	Sigma
D(+)Glucose	Sigma
Hepes	Sigma

II.1.7. Electron Spin Resonance (ESR)

Iron (II) sulphate heptahydrate	Sigma
DETC	Alexis
CMH	Noxygen
CP	Noxygen
Deferoxamine mesylate salt	Sigma
Pegulated Superoxide Dismutase (PEG-SOD)	Sigma
Apocynin	Sigma
Sodium Hydroxide	Merck
Copper(II) sulfate	Merck

Protein Assay reagent	Bio-Rad

II.2. Methods

II.2.1. Cell Culture

The murine macrophage cell line, RAW 264.7, was cultured and propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine and 1% penicillin/streptomycin in a 75cm^2 culture flasks at 37°C in humidified atmosphere of 5% CO_2 and air.

II.2.2. Bone marrow cells (BMC)s preparation

For BMCs isolation, C57BL/6J mice (6 weeks old) were killed and the lower limbs were removed. BMCs were flushed with Dulbecco's Modified Eagle Medium (DMEM) from the medullary cavities of tibias and femurs using a 25-G needle. Then cells were cultured in DMEM medium supplement with 10% fetal bovine serum , 1% penicillin/streptomycin in a 75cm^2 culture flasks (Corning) and incubated at 37°C in an atmosphere containing 5% CO_2.

II.2.3. In vitro migration assay (modified Boyden chamber assay)

Cells were measured in a modified Boyden chamber migration assay using trans-well inserts with a 5µm porous membrane (Corning). Cells were first pretreated with nLDL, oxLDL, 1400W or siRNA. After that, cells were washed twice by PBS 50000 pretreated cells per group were added to 100µl medium and introduced into the upper compartment of the trans-well. Into the lower compartment of the Boyden chamber 600µl medium containing MCP-1 in a concentration of 10ng/ml were added. The chambers were incubated at 37°C in 5% CO_2 for 18 hours. During this time the cells in the upper compartment were allowed to migrate to the lower side of the membrane.

(Fig.3) The membrane was harvested and cells on the lower side of the membrane were fixed with methanol for 8 minutes and then these cells were stained with 1 drop of DAPI. At last, the lower side of the membrane was photographed under a fluorescence microscopy. The number of the cells on each picture was counted by image Pro-Plus software.

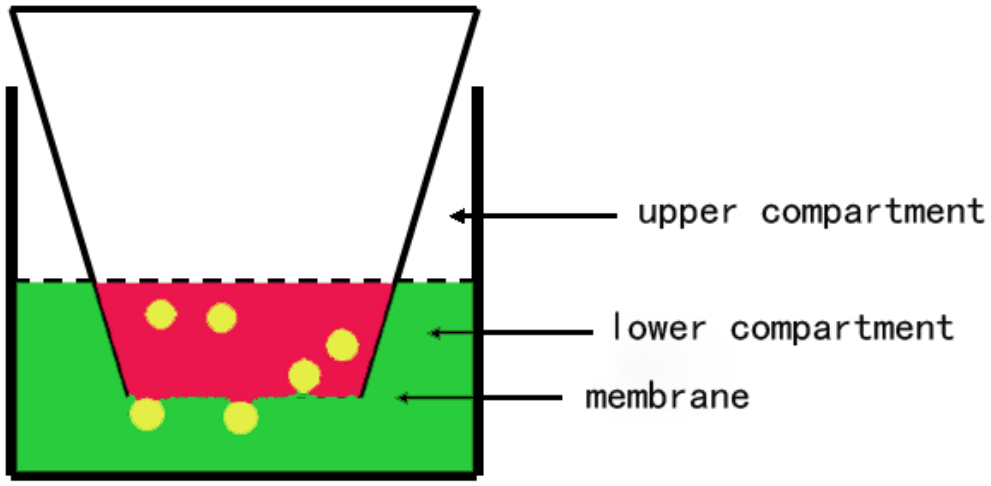

Fig.3 Boyden chamber consists of two compartments, 50,000 pretreated cells in 100µl medium were added to the upper compartment, 600µl medium with MCP-1 in a concentration of 10ng/ml was added to the lower compartment. Cells in the upper wells were allowed to migrate towards the lower side of the membrane.

II.2.4. Flow cytometry assays

To quantify apoptosis in oxLDL and 1400W treated cells, cells were first washed twice by PBS. Then 10^5 cells were suspend with 1×Binding buffer incubated with FITC–annexin V and propidium Iodide (PI) at room temperature for 15 minutes in the dark, and then analyzed.

To measure polymerized actin (F-actin), RAW 264.7 cells were preincubated with nLDL, oxLDL or oxLDL plus 1400W at 37°C in 5% CO_2 for 24 hours. After

incubation, cells were washed twice by PBS, and 10^5 cells were suspended with FACS buffer (PBS with 1% BSA) then fixed with 4% paraformaldehyde for 8 minutes. After washed twice again cells were treated with 0.1% Triton and then stained with FITC-conjugated phalloidin at room temperature for 60 minutes in the dark. Following this the fluorescence intensity was quantified.

To measure phosophorylated focal adhesion kinase (p-FAK), RAW 264.7 cells were preincubated with nLDL, oxLDL or oxLDL plus 1400W at 37°C in 5% CO_2 for 24 hours. After incubation, cells were washed twice with PBS, and 10^5 cells were suspended with FACS buffer (PBS with 1% BSA) then fixed with 4% paraformaldehyde for 8 minutes. After washed twice again cells were treated with 0.1% Triton and then incubated with anti-p-FAK antibody at room temperature for 40 minutes in the dark. Then cells were washed twice again and incubated with Alexa Fluor® 488 conjugated secondary antibody at room temperature for 40 minutes in the dark. After washed twice the fluorescence intensity was quantified.

To measure F4/80 positive bone marrow cells, 10^5 cells were suspend with FACS buffer, then incubated with APC–F4/80 antibody at room temperature for 60 minutes in the dark. After that the fluorescence intensity was quantified.

All flow cytometry were tested using a BD FACScan (Calibur). Data were analyzed by Flowjo 7.6.1.

II.2.5. Real time PCR

Total RNA was extracted from cells using TriFast reagent (peqGOLD). After the medium was removed, 1ml TriFast was directly added to cells in a 6 well chamber. The solution in the 6 wells was passed several times by pipette. For dissociation of the nucleoprotein complexes the samples were kept for 5 minutes at room temperature. After that the solution was transfered into tubes and 0.2 ml chloroform was added. The tubes were vortexed, then kept on ice for 10 minutes. Tubes were centrifugate for 5 minutes, 4°C, at 12,000 g. The mixture then separates into the lower red (phenol-chloroform phase), the interphase and the colorless upper aqueous phase.

RNA is located exclusively in the aqueous phase whereas DNA and proteins move into the interphase and lower phenol phase. Following the transfer of the aqueous phase to a fresh tube RNA was precipitated with 0.5ml of isopropanol per tube. Each samples was incubated on ice for 10 minutes and centrifugated for 10 minutes at 4°C at 12,000g. The RNA pellet forms a gel like precipitate on the bottom and the side of the tube. The supernatant was carefully removed and we then washed RNA pellet twice with 75% ethanol by vortexing. Subsequently the samples were centrifugated for 10 minutes at 4°C at 12,000g. Excess ethanol was removed and the RNA was dried at room temperature. At last RNA was resuspended in 50µl RNase-free water and heated in water bath at 55-60°C. The concentration and the purity of RNA was tested by spectrophotometer (wave length 260/280 nm).

cDNA was synthesized from 1µg of total RNA using iScript cDNA synthesis kit following the instructions of the company. Gene expression was analyzed by quantitative RT-PCR using the EVA Green PCR Master MIX with the Mx3000P detection system and relative quantification software. The expression levels of each gene were determined using the comparative Ct method and normalized to β-actin, as internal control. The primers are listed in Table 1.

Table 1 Primers used for real-time PCR analysis

Gene	Primer sequence	
	Forward	Reverse
iNOS	5′-GTTTCTGGCAGCAGCGGCTC-3′	5′-GCTCCTCGCTCAAGTTCAGC-3′
β-actin	5′-CGTGGGCCGCCCTAGGCACCAGGG-3′	5′-GGGAGGAAGAGGATGCGGCAGTGG-3′
t-FAK	5′-GAGAATCCAGCTTTGGCTGTT-3′	5′-GGCTTCTTGAAGGAACTTCT-3′

II.2.6. Silencing iNOS expression in RAW 264.7 cells employing siRNA

RAW 264.7 cells were seeded in 6-wells for 18h. siRNA was introduced into RAW 264.7 cells by using siRNA transfection reagent following the manufacture instructions.

II.2.7. Electron spin resonance (ESR)

Most usually used methods for measuring the free radicals employ reduction of cytochrome C and chemiluminescence. However because of side reactions of cytochrome C and chemiluminescence, these methods are usually not exact. In contrast, electron spin resonance (ESR), the method which we employed provides specific and direct measurement of free radicals.

The detection by ESR spectroscopy relies on the detection of magnetic properties of unpaired electrons and their molecular environment. Electrons possess a property which was called spin. The unpaired electrons exists in the magnetic field two orientations, parallel and antiparallel. Electrons in the antiparallel state possess higher energy than electrons in the parallel state. The total energy between the two spin states is detected by resonance. Only free radicals which contain unpaired electrons produce an ESR signal. An external magnetic field of a specific strength is applied for the transition to occur such that the difference in energy levels is matched by a microwave frequency.

In biological samples free radicals such like nitric oxide, superoxide and peroxynitrite are found in very low concentrations and are too short lived to be directly detected. This problem can be solved by the addition of exogenous spin traps which can react with free radicals to form secondary ESR detectable radicals in a stable state.

II.2.8.Krebs Hepes Buffer (KHB)

KHB with the following composition, prepared in Milli Q water, was used for the experiments: Sodium chloride 99mM, Potassium chloride 4.69mM, Calcium chloride 2.5mM, Magnesium sulphate 1.20mM, Sodium bicarbonate 25mM, Potassium dihydrogen phosphate 1.03mM, D (+) Glucose 5.6mM, Sodium Hepes 20mM. These reagents were dissolved in 500ml of MilliQ water. The pH was adjusted to 7.4. The pH was checked every day before use and the buffer was filtered using a 0.22µm filter (Millex).

II.2.9. Measurement of NO Production by ESR

NO production of RAW 264.7 was measured in an organ bath using colloid iron (II) diethyldithiocarbamate ($Fe(DETC)_2$) as a spin trap and ESR detection. The method was adapted for detection of baseline NO production in RAW 264.7 cells. Briefly, cells were washed twice by PBS and then suspended at 4°C in chilled Krebs-Hepes Buffer (KHB). A mix of 1000µl of FeSO4 and DETC solutions were bubbled with N_2 at least 20 min, and 1000µl were added to each dish (total amount 4ml). The cells' NO production was trapped with $Fe\text{-}(DETC)_2$ for 1 hour in 37°C KHB. KHB with $Fe(DETC)_2$ was removed from the dish by scraping cell, and 100µl cell suspension were frozen in liquid N_2 and tested. Following ESR measurement to the protein concentration of the samples were quantified with a protein assay reagent (Bio-Rad) and used to normalize the ESR signal intensity. The general settings of ESR were: microwave frequency 9.431GHz, modulation frequency 86 kHz, center field, 3280G; modulation amplitude 6G; field sweep 80G; microwave power 40mW; scan time 120s.

II.2.10. Measurement of reactive oxygen species (ROS) and peroxynitrite by ESR

The generation of ROS by RAW 264.7 cells was measured by ESR detection (e-scan ESR spectrometer). A stock solution of spin probe (10mm, 2.3mg for 1ml KHB) in buffer containing 25μM Defferoxamine and 5μM DETC and a suspension of cells in the concentration of 400,000/ml native or 800,000/ml cultured cells were prepared. Cells were washed twice by PBS and then suspended in KHB containing 25μM DF and 5μM DETC at 4°C. Forty-five μL of cell suspension and 2.5μL of spin probe solution were mixed. ROS production was assessed by pre incubating cells with PEG-SOD (50U/ml) parallel to 1-hydroxy-3-methoxycarbonyl-2,2,5,5--tetramethylpyrrolidine (CMH) for 30min at 37°C. The reaction was stopped by putting the sample on ice.

Uric acid was used as a scavenger for peroxynitrite[62]. Peroxynitrite formation of RAW 264.7 cells was calculated as the difference of ROS production between each group before and following uric acid treatment.

The intensity of the ESR signal was normalized to the sample's protein content. ESR settings: Center field 1.9901g, microwave power 1mW, modulation amplitude 5G, sweep time 10s, field sweep 60G, gain 1×10^3.

II.2.11. Statistical analyses

We performed student'T test and a P-value of less than 0.05 was considered significant. Data are expressed as mean±SD. The analyses were formed using SPSS Statistics 17.0.

Chapter III

III. Results

III.1. Migration assay

III.1.1. Migration of RAW 264.7 cells

RAW 264.7 cells were divided into four groups: 1. unstimulated cells; 2. cells incubated with non-oxidized low density lipoprotein (nLDL); 3. cells incubated with oxidized low density lipoprotein (oxLDL); 4. cells incubated with oxLDL plus 1400W (50μM), an iNOS inhibitor. All groups were incubated at 37°C for 24 hours. Following this, cells were introduced into a modified Boyden chamber in order to quantify cell migration in response to an MCP-1 gradient. Cells which migrated towards the lower side of the membrane were counted using a fluorescence microscope. Four random fields in each filter were examined. Each experiment was performed in five times, and migration was expressed as the mean±S.D. of total cells counted per field.

Migration of oxLDL treated cells was significantly reduced compared to unstimulated cells (228.3±48.9 versus 376.5±28.7, $p<0.05$), while nLDL treated cells showed no difference compared to unstimulated cells (340.9±43.5 versus 376.5±28.7, $p>0.05$). Interestingly, the migratory arrest in response to oxLDL treatment was reversed by coincubation with 1400W (228.3±48.9 versus 358.9±55.1, $p<0.05$) (Fig.4 and 5).

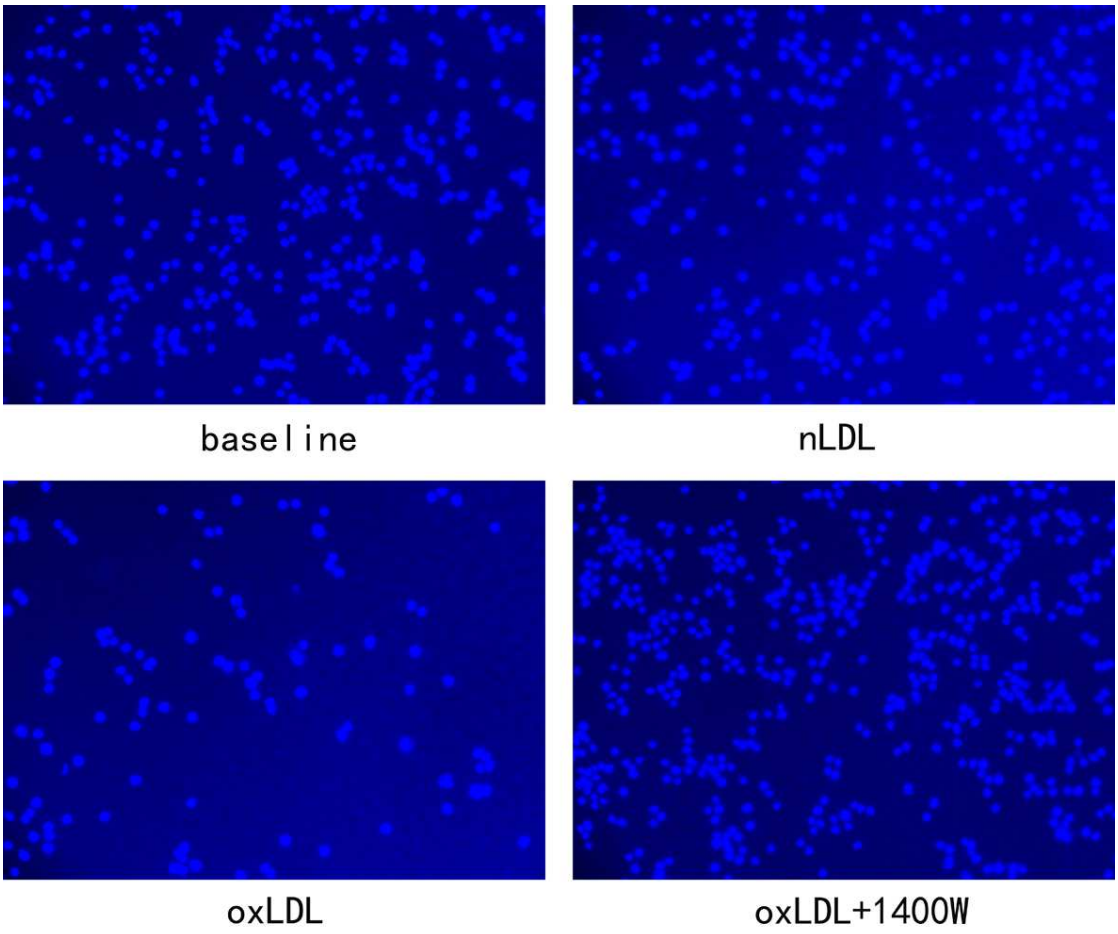

Fig.4 Migrated RAW 264.7 cells on the lower side of the membrane were stained with DAPI and counted under a fluorescence microscope using a ×10 objective. Representative pictures of each treatment group.

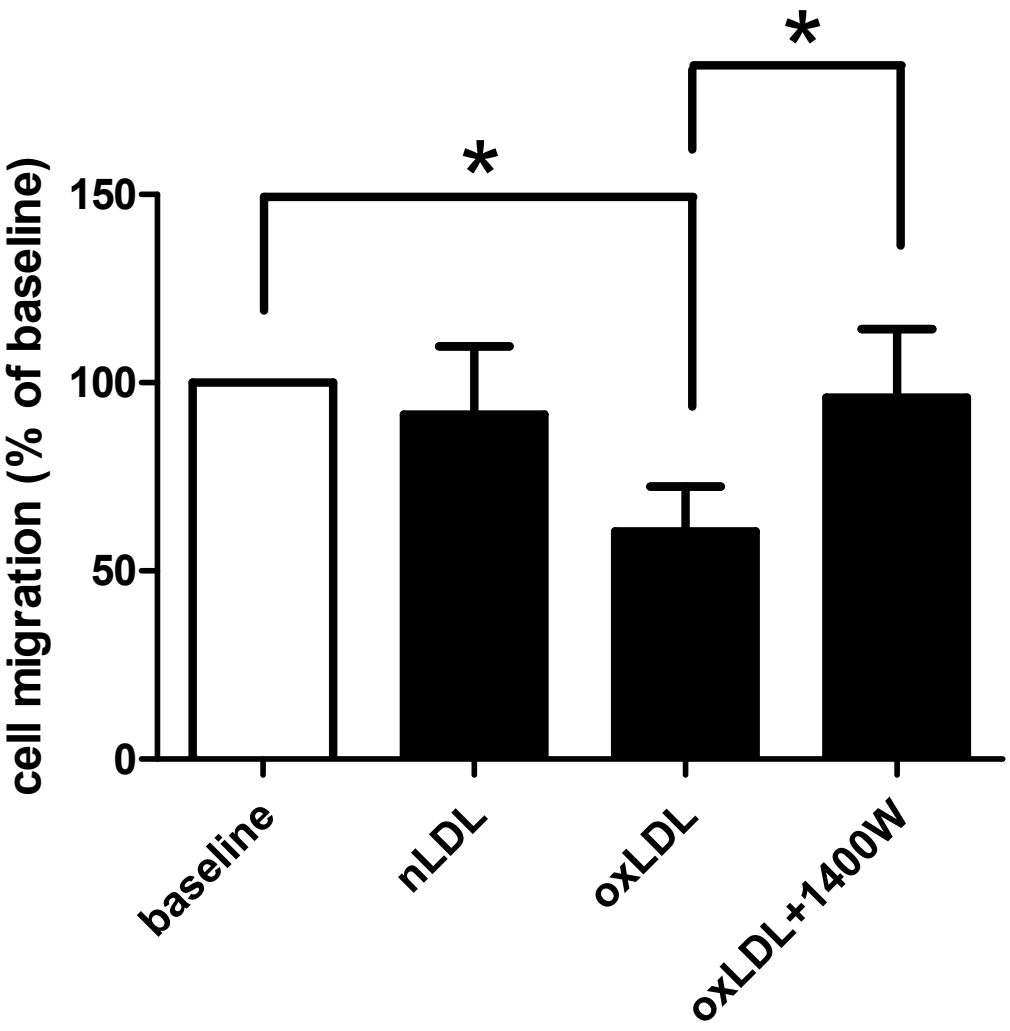

Fig.5 Migrated cells of each goup. Significance was determined by T-test. (*, P<0.05, n=5)

To detect the effect of NO on cell migration we used SIN-1, an NO donor to stimulate RAW 264.7 cells and measured cell migration employing the migration assay as described above.

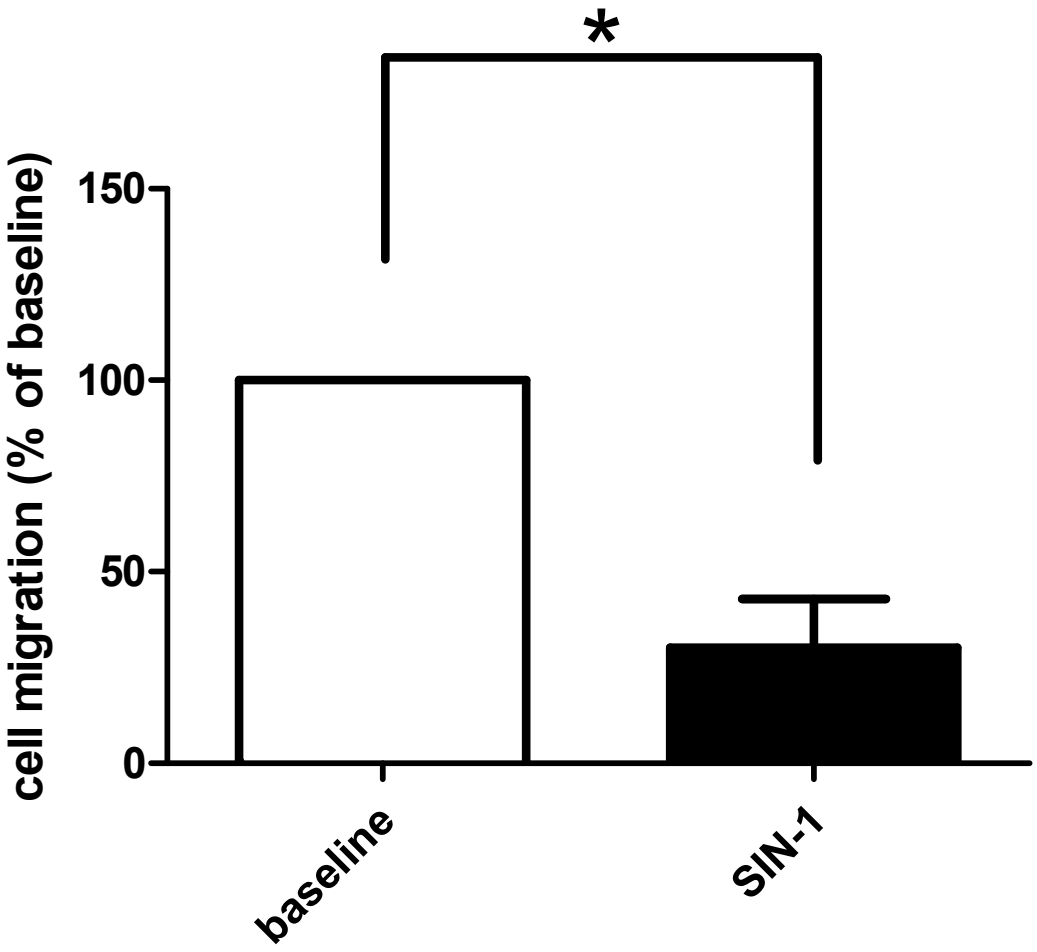

Fig.6 RAW 264.7 cells were pretreated with SIN-1(0.3mM). Migrated cells quantified as described above.(*, P<0.05, n=4)

As shown in Fig.6, when cells were stimulated with SIN-1, the migration was significantly inhibited. We then used Trolox, a strong antioxidant to test whether an antioxidant known to reduce the production of lipid peroxides could reverse the inhibition of cell migration caused by oxLDL. As shown in Fig.7, the inhibition induced by oxLDL was reversed by Trolox.

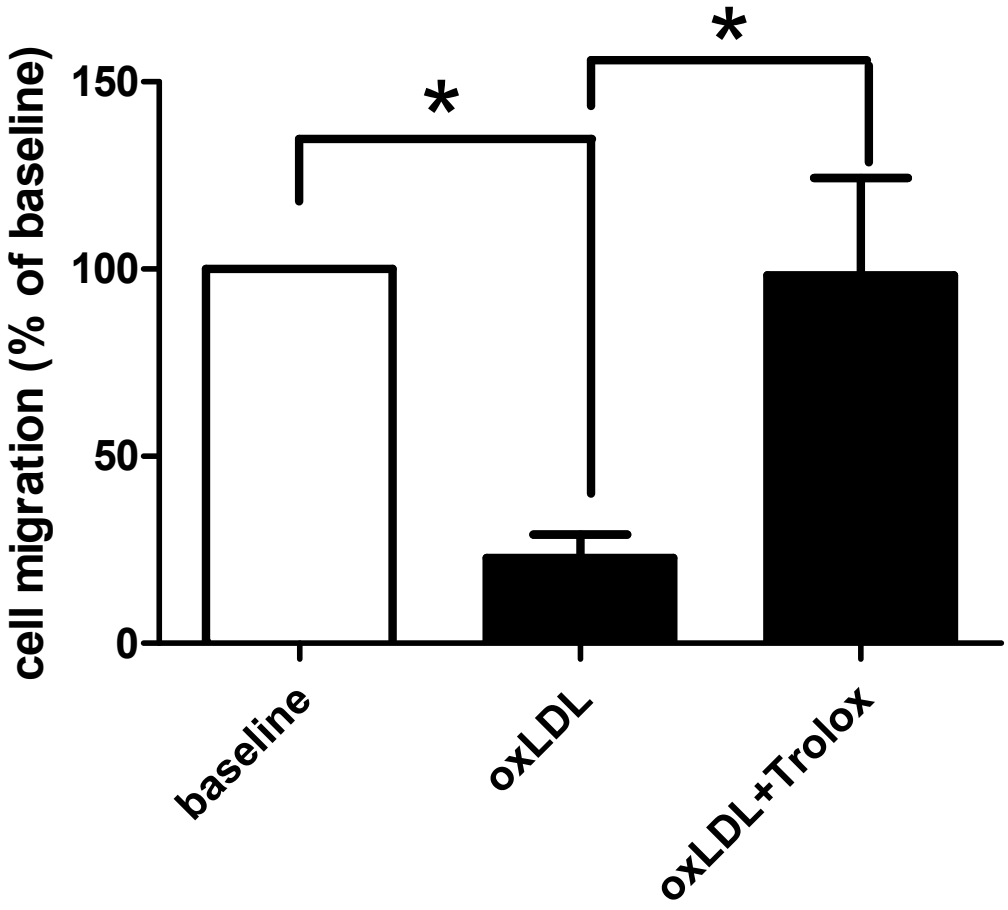

Fig.7 RAW 264.7 cells were pretreated with oxLDL or pretreated with oxLDL plus Trolox (0.2mM). Migrated cells quantified as described above. (*, $P<0.05$, n=6)

III.1.2. Apoptosis

To exclude the possibility that the inhibitory effect of oxLDL was due to induction of raw cell apoptosis, we analyzed Annexin V and propidium lodide staining of the raw 264.7 cells by flow cytometry.

In the flow cytometry scatter plot (Fig.8), the cells in the lower left quadrant (FITC negative, PI negative) were defined as live cells, in the lower right quadrant (FITC positive, PI negative), cells were defined as apoptotic cells and in the upper right quadrant (FITC positive, PI positive), cells were defined as dead cells.

All the measurement were repeated for five times for each group. There were no significant differences between the percentage of live cells, apoptotic cells and dead

cells in each group: live cells, baseline (80.7±3.67), nLDL (78.8±2.65), oxLDL (79.5±1.97), oxLDL+1400W (79.2±2.19); apoptotic cells, baseline (0.60±0.48), nLDL (0.73±0.29), oxLDL (0.534±0.32), oxLDL+1400W(0.58±0.26); dead cells, baseline (10±6.63), nLDL (14.7±2.99), oxLDL(13.3±2.54), oxLDL+1400W (13.7±1.64).

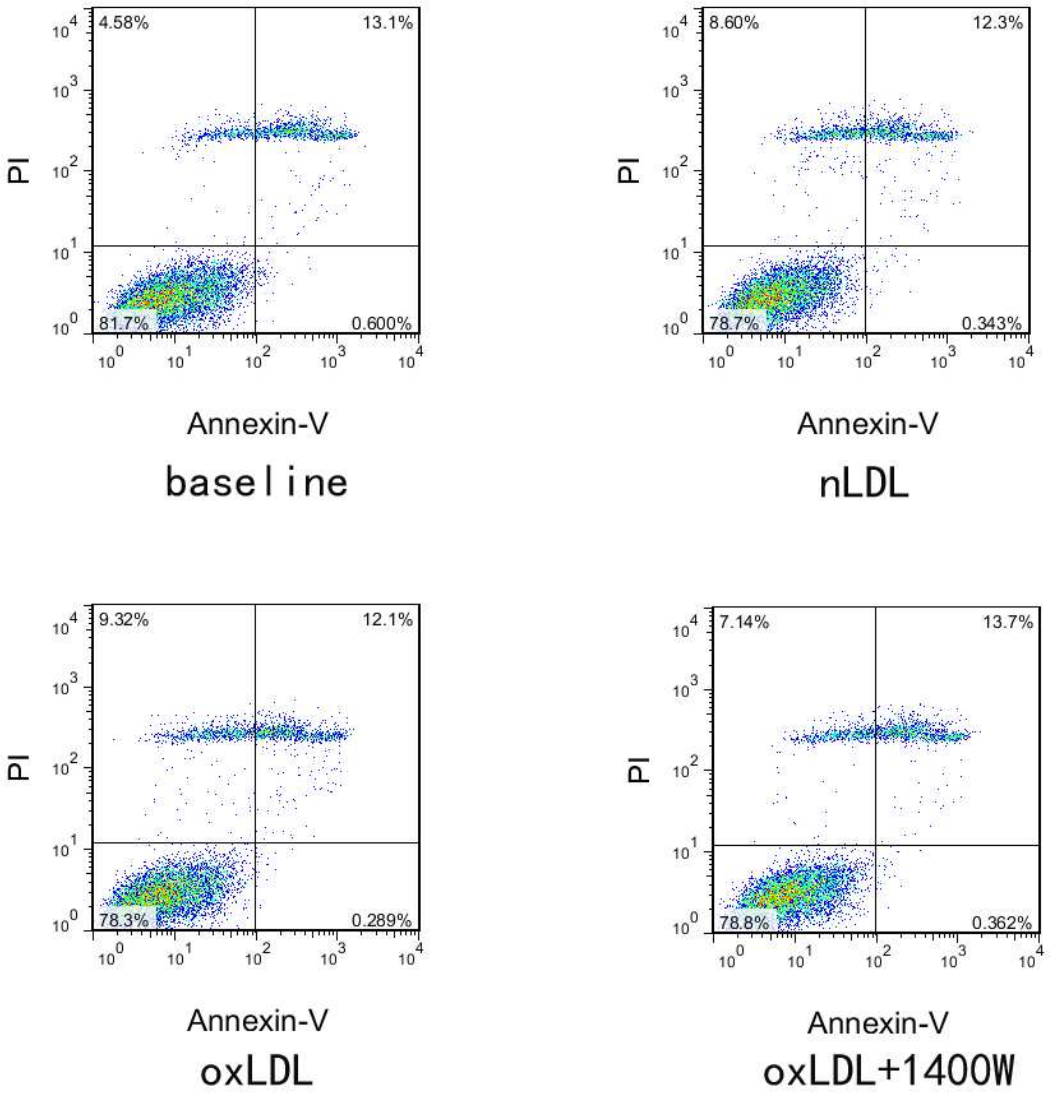

Fig.8 RAW 264.7 cells were stained with annexin V and PI and subjected to analysis by flow cytometry. (n=5)

III.1.3. Migration of siRNA treated RAW 264.7 cells

To confirm the importance of iNOS in oxLDL induced inhibition of macrophage migration, we treated RAW 264.7 cells with small inhibitory RNA (siRNA) to silence iNOS gene expression and quantified cell migration in the same way as described before. These experiments revealed that the inhibition of migration by oxLDL was totally reversible by the treatment of the cells with iNOS-siRNA (Fig.9).

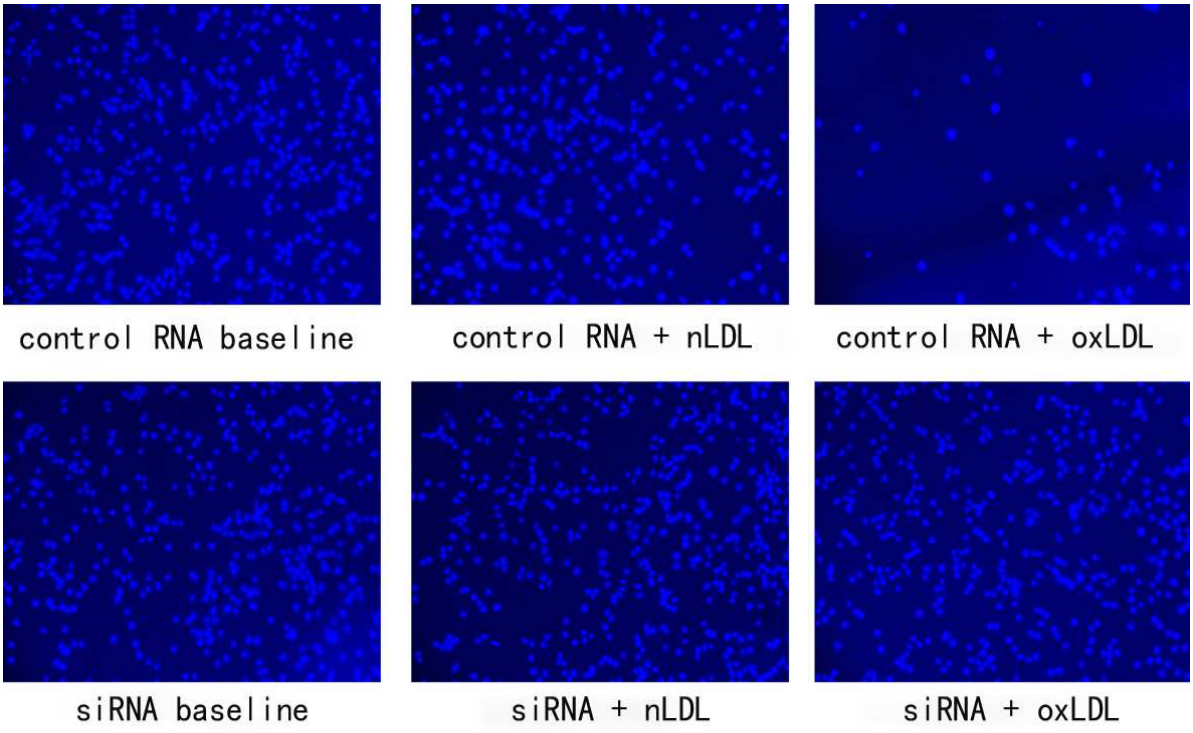

Fig.9 RAW 264.7 cells pretreated with control RNA or siRNA were exposed to nLDL and oxLDL and migrated cells quantified as described above.

In the control RNA (nonsense RNA/no inhibition of iNOS expression) treated groups oxLDL ("goup 3") significantly reduced migration compared with "group 1" cells (188.7±58.1 versus 390.8±42.3, $p<0.05$) or nLDL treated "group 2" cells (385.8±31.9, $p<0.05$). In contrast, siRNA treatment abrogated the migratory arrest by oxLDL such that no significant difference in migration was seen between "group 1" (397.6±11.5), nLDL "group 2" (390.2±52.3) compared to oxLDL "group 3" (395.6±23.5) cells ($p>0.05$). (Fig.10)

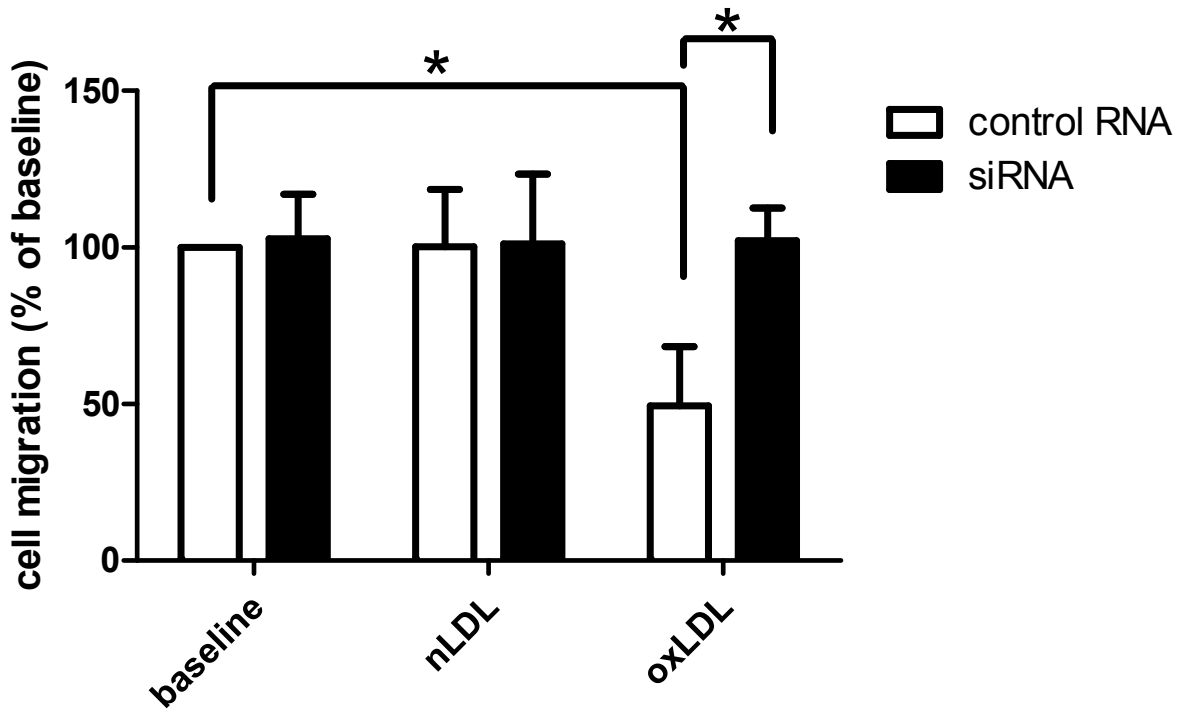

Fig.10 Migrated cells of each goup expressed as percent of baseline migration. (*, P<0.05, n=5)

III.1.4. Bone marrow derived macrophage migration

Here we used bone marrow derived macrophages of apoE/iNOS double knockout and apoE knockout mice to test differences in cell migration in a model of chronic iNOS deletion. (Fig.11)

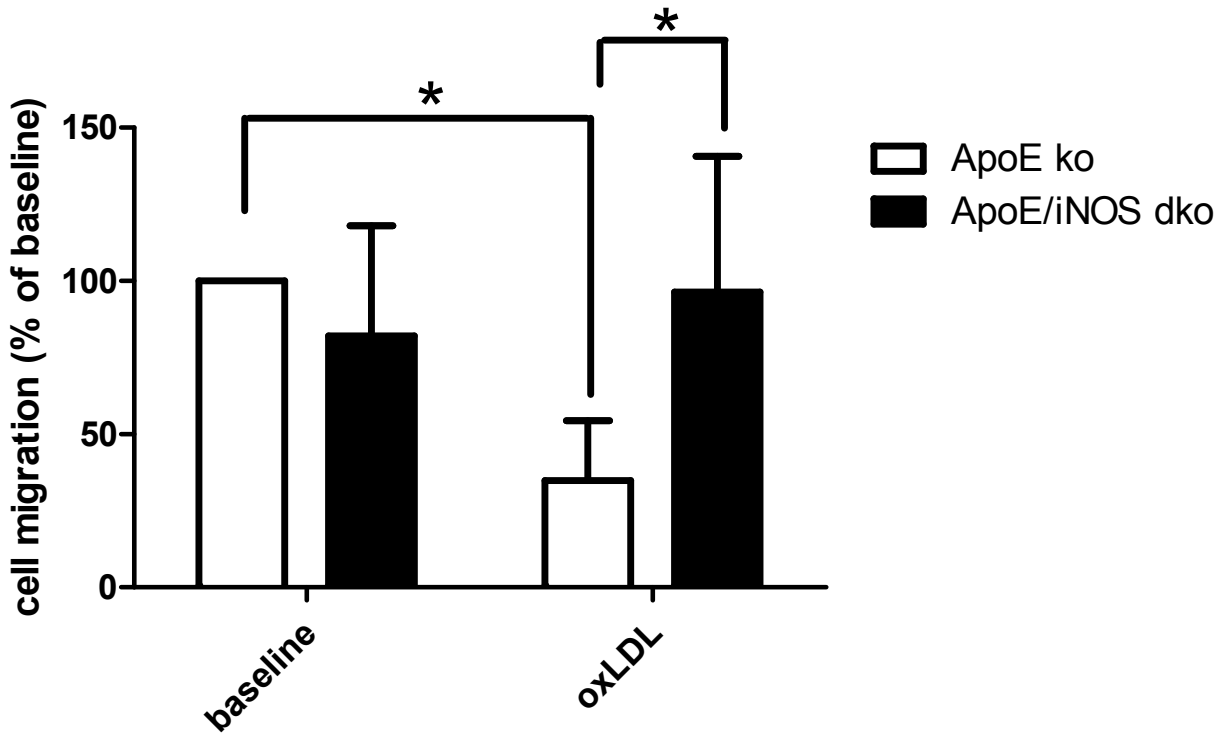

Fig.11 Bone marrow derived macrophages of apoE ko mice or apoE/iNOS dko mice were exposed to oxLDL, introduced into a Boydem chamber and migrated cells were quantified as described above. (*, $P<0.05$, n=5)

To verify that bone marrow derived cells were indeed macrophages, we stained the cells for F4/80, a marker for macrophages and analyzed them by flow cytometry. (Fig.12)

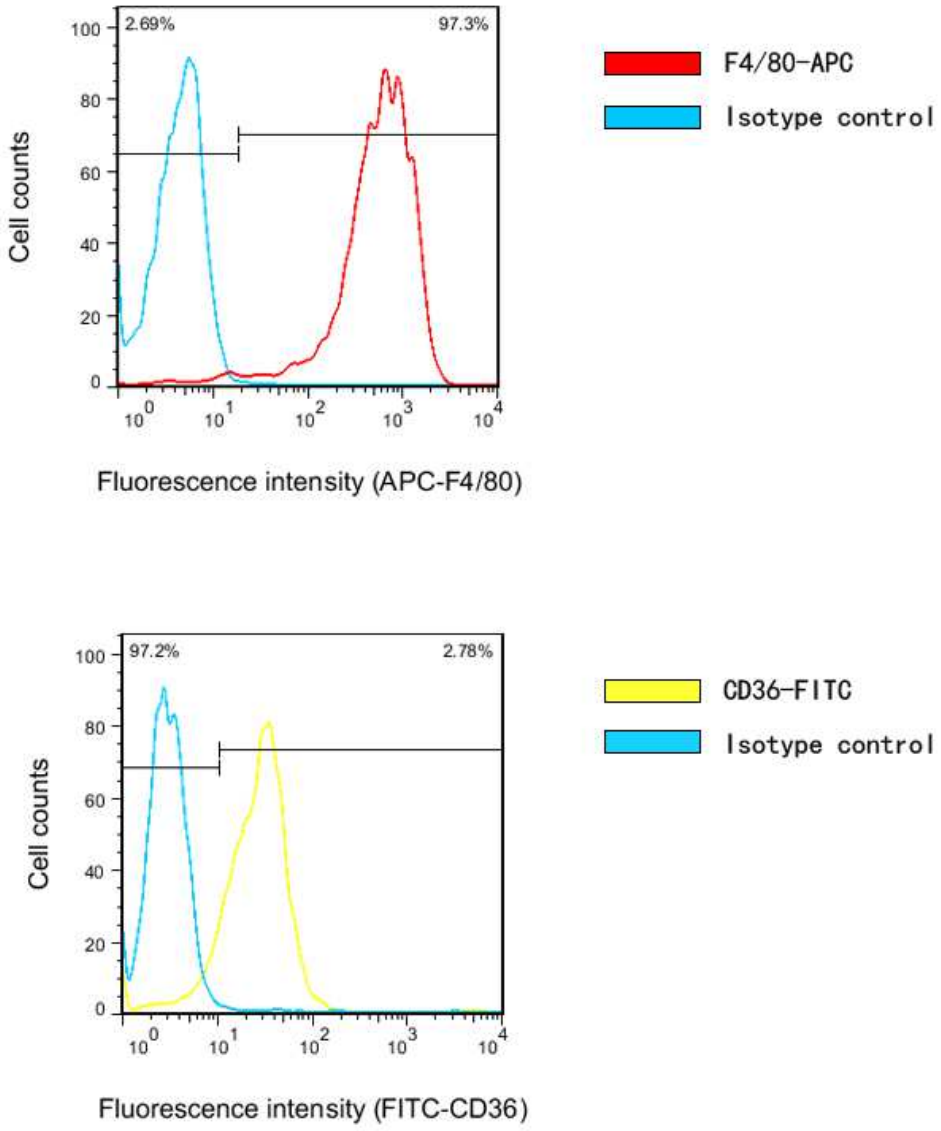

Fig.12 Bone marrow derived macrophages were stained with fluorochrome labeled anti-F4/80 and anti-CD36 antibodies. Fluorescence intensity was analyzed by flow cytometry.

Under baseline conditions apoE/iNOS double knockout macrophages migrated to the same extent as apoE knockout macrophages. In contrast, in the presence of oxLDL, apoE knockout macrophage migration was significantly inhibited, while apoE/iNOS double knockout macrophage migration was not inhibited. This finding in this chronic iNOS deletion model further supported the hypothesis that oxLDL-induced inhibition of macrophage migration in vitro is partially dependent on iNOS.

III.2. OxLDL regulates expression of iNOS in RAW 264.7 cells

To test whether oxLDL increases the iNOS expression in RAW 264.7 cells we used quantitative RT-PCR and quantified iNOS mRNA levels. RAW 264.7 cells were divided into five groups: Unstimulated cells, cells treated with nLDL, cells treated with oxLDL, cells treated with oxLDL plus 1400W (50μM) and cells treated with LPS (1μg/ml) as a positive control. The iNOS mRNA expression of oxLDL treated cells was significantly higher compared to unstimulated cells and nLDL treated cells ($p<0.05$) (Fig.13).

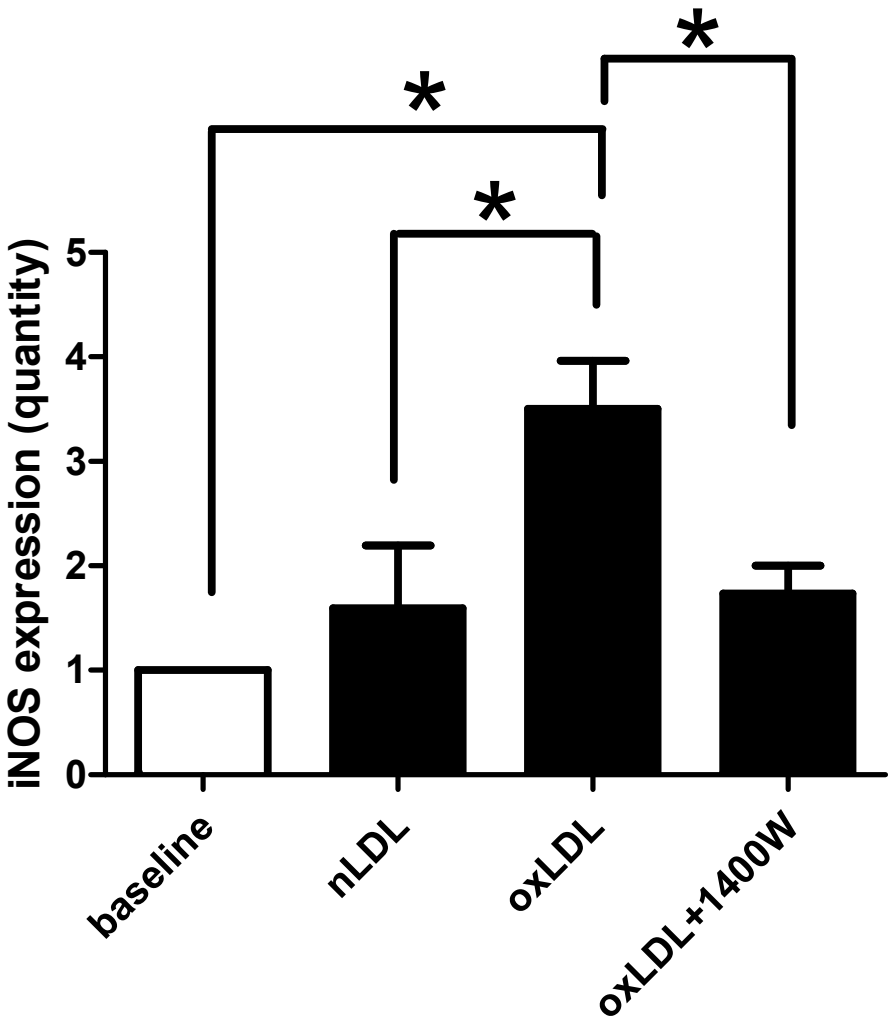

Fig.13 The iNOS expression of RAW 264.7 cells of each group was tested by Real time PCR

using the comparative CT method. (*, P<0.05, n= 3)

III.3. iNOS inhibition with 1400W was associated with a reduction in NO and peroxynitrite formation and an increase in superoxide generation

Pharmacologic iNOS inhibition with 1400W was associated with a significant reduction in NO and peroxynitrite formation and a significant increase in superoxide generation. (Fig.14, 15, 16)

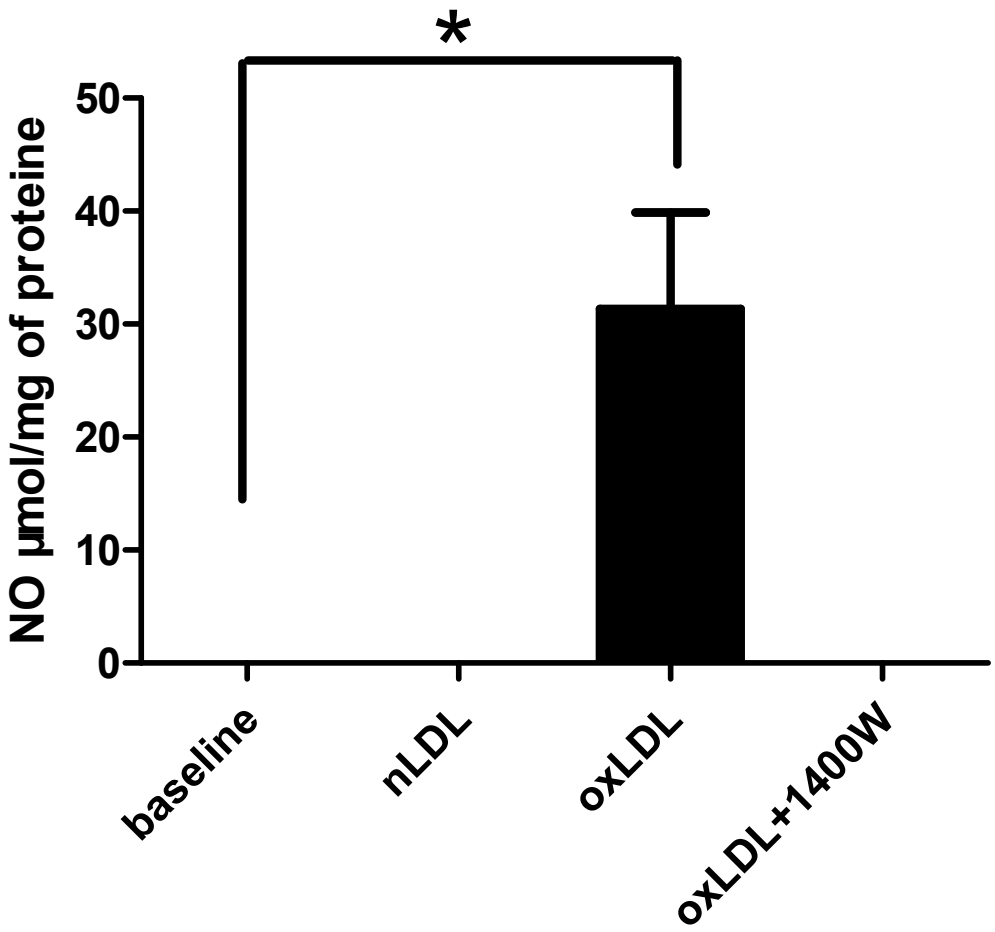

Fig.14 The intracellular NO production was measured by ESR. (*, P<0.05, n=5)

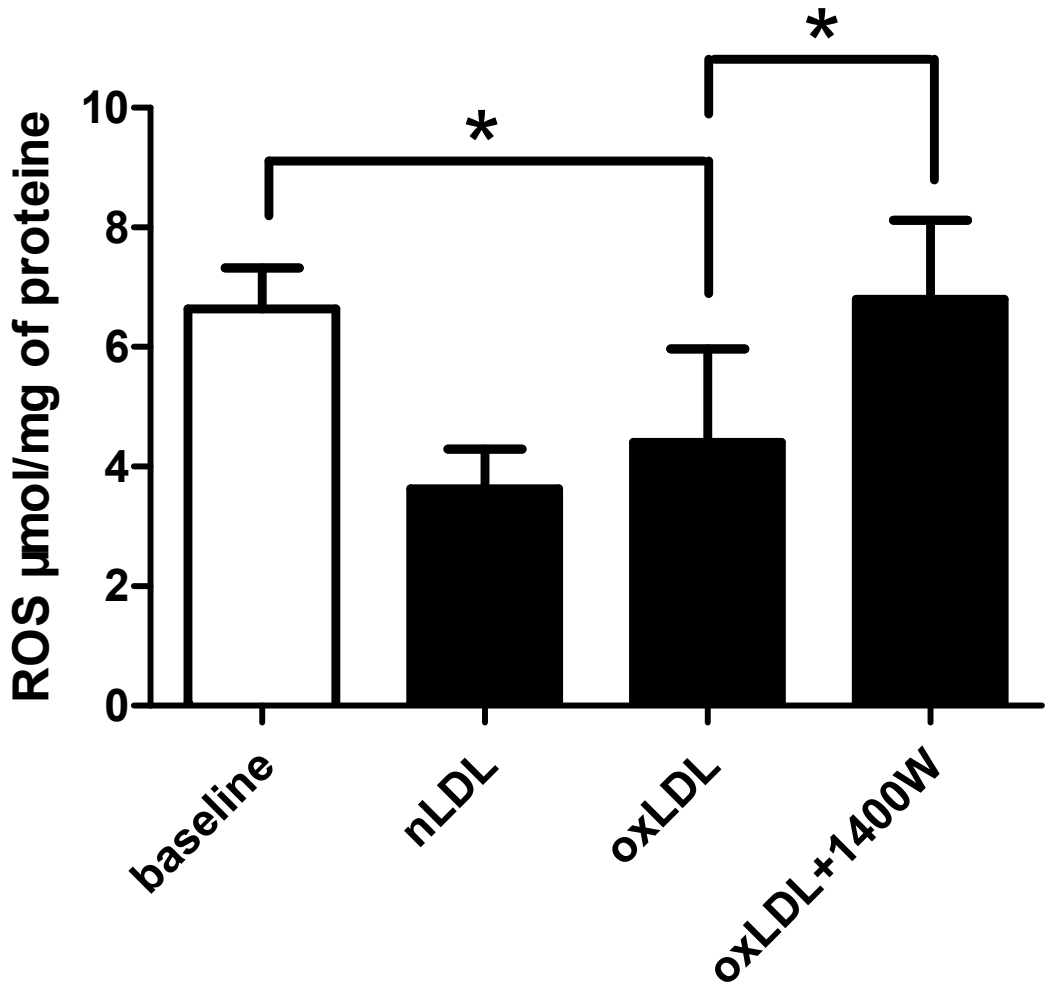

Fig.15 The intracellular ROS was measured by ESR. (*, P<0.05, n=5)

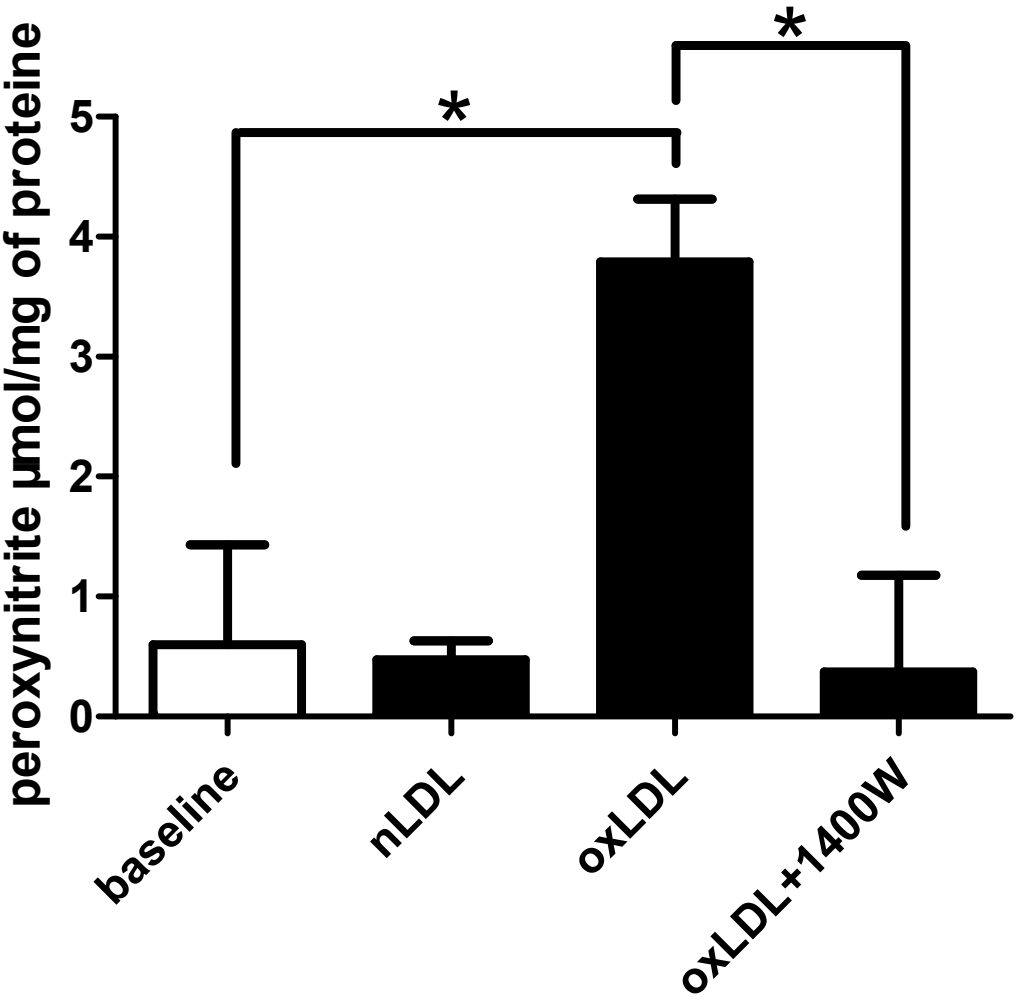

Fig.16 The intracellular peroxynitrite was measured by ESR. (*, P<0.05, n=6)

The NO and peroxynitrite production was significantly increased in oxLDL treated cells. Moreover, this increase could be abrogated by pharmacologic iNOS inhibition with 1400W. ROS production was significantly decreased by oxLDL treatment, which could also be reversed by 1400W.

III.4. The inhibition of migration was associated with actin polymerization

As cell spreading requires actin polymerization to form lamellipodia[63], the inhibition of migration is usually associated with actin polymerization, a marker for

cell spreading and migratory arrest. We therefore examined the effect of oxLDL on actin polymerization in macrophages. RAW 264.7 cells were divided into four gourps: unstimulated cells, treated with nLDL, oxLDL or oxLDL plus 1400W (50μM) and then stained with fluorescein-conjugated phalloidin to detect polymerized actin. Each experiment was performed five times, and fluorescence intensity was detected by flow cytometry. (Fig.17).

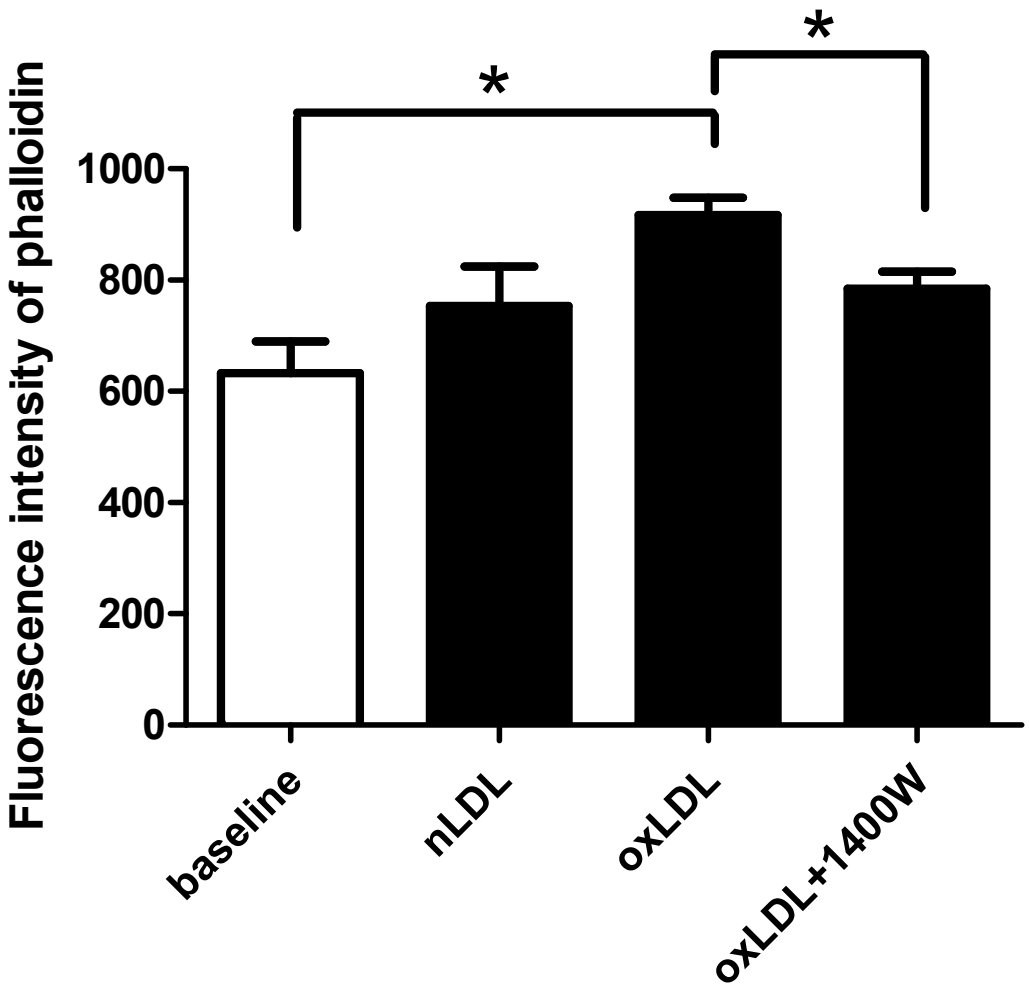

Fig.17 RAW 264.7 cells were stained with FITC-phalloidin and subjected to analysis by flow cytometry. (*, $P<0.05$, n=5)

Flow cytometric analysis showed that the amount of polymerized actin did not differ between baseline and nLDL treatment. However, oxLDL treatment increased phalloidin staining, an effect which was abrogated by pharmacologic iNOS inhibition

with 1400W.

III.5. Inhibition of migration was associated with increased phosphorylation of focal adhesion kinase

First, we used real time PCR to see whether the RNA content of total focal adhesion kinase (t-FAK) differed between the groups. As shown in Fig.18, there is no difference of t-FAK expression in each group.

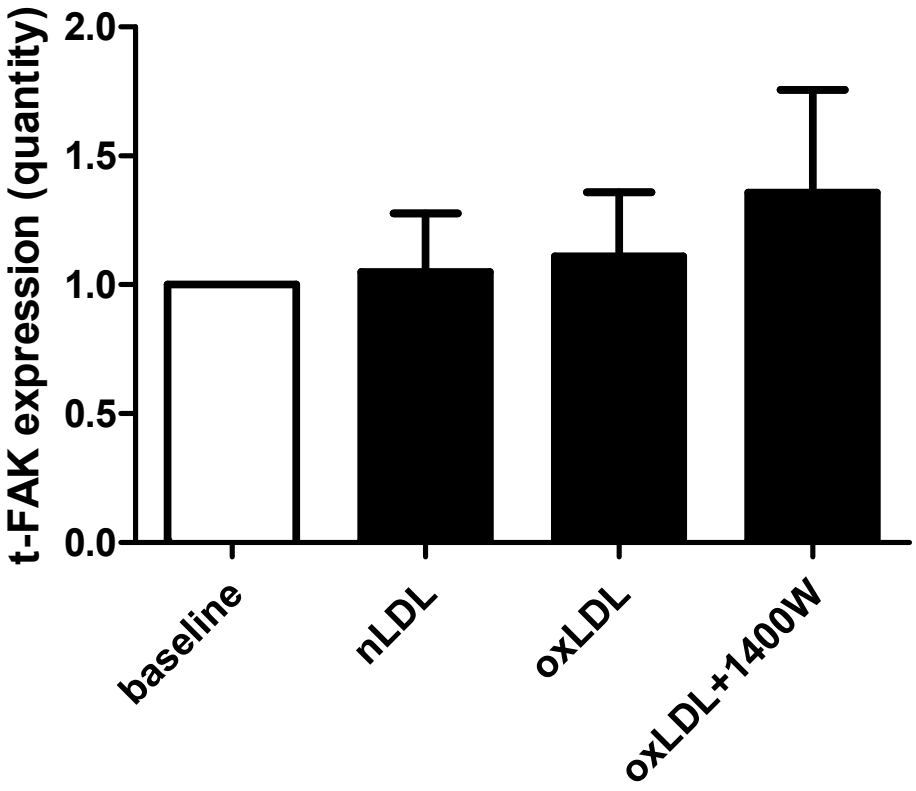

Fig.18 The t-FAK expression of RAW 264.7 cells of each group was test by Real time PCR using comparative CT method. (*, $P<0.05$, n=3)

Then we used FACS to analyze the expression of phosphorylated focal adhesion kinase (p-FAK). As shown in Fig.19, expression of p-FAK was significantly increased in the oxLDL treated group, an effect that was completely reversed by 1400W.

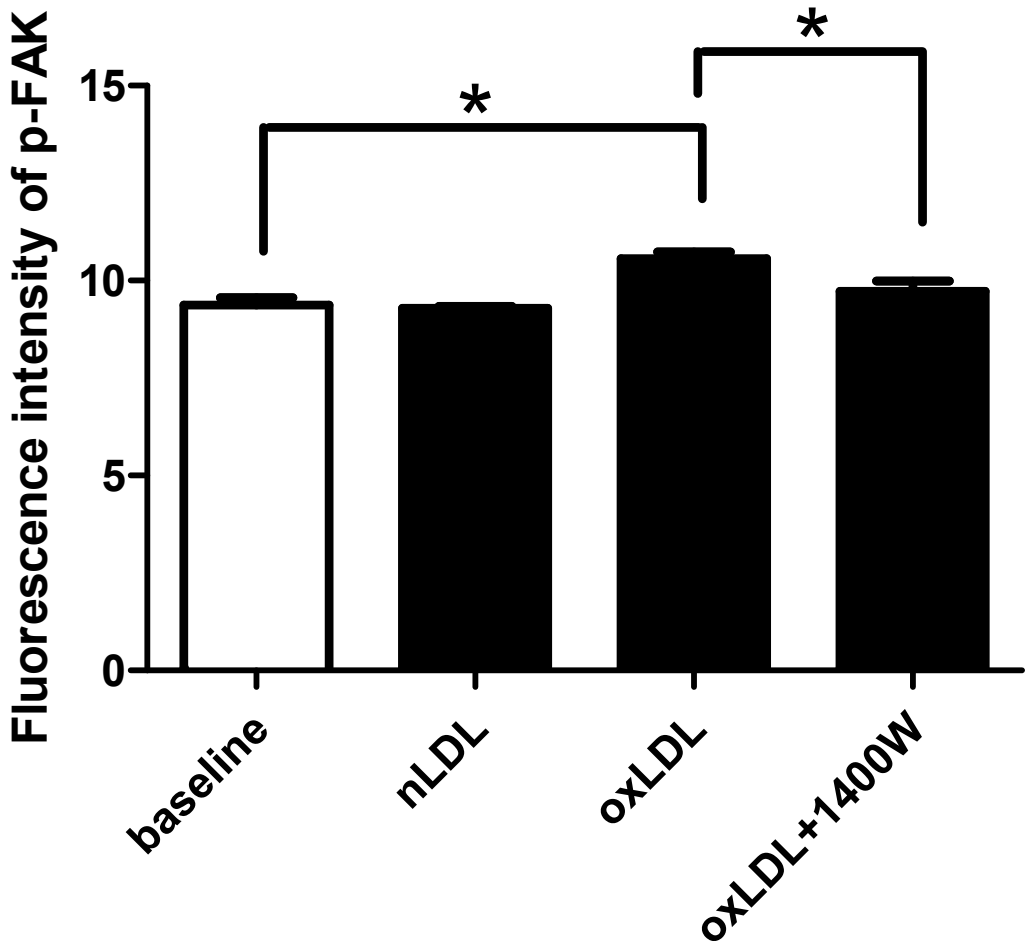

Fig.19 RAW 264.7 cells were stained with p-FAK and subjected to analysis by flow cytometry. (*, $P<0.05$, n=3)

Chapter IV

IV. Discussion

With substantial advances in our understanding of the molecular and cellular biology of atherosclerosis progression and regression, macrophage derived foam cells have been seen as key player in all stages of atherosclerosis, from the earliest discernable lesions to complex plaques, and are a key determinant of related cardiovascular complication.[64].

Previous research regarding atherosclerotic plaque progression and regression were focused on the development of atherosclerotic lesions and the important role of macrophages in plaque development and macrophage emigration to regional lymph nodes in lesion regression[64–68]. The formation of macrophage-derived foam cells is central to atherosclerotic plaque development[7]. The molecular mechanism of foam cell formation is generally believed to consist of the following steps: first the endothelium is activated following the accumulation of modified lipoproteins in the subendothelial space; monocytes are attracted by chemoattractants and migrate into the intima; monocytes differentiate into macrophages and take up modified lipoproteins; excess lipids accumulate in macrophages forming lipid-laden foam cells, foam cells die and release their contents, thus attracting more macrophages[69]. It is known that oxidized low density lipoproteins (oxLDL) inhibit macrophage migration and trap cholesterol laden foam cells in arteriosclerotic plaques. But the mechanisms involved in macrophage-derived foam cell trapping in the inflamed atherosclerotic neointima are incompletely understood, yet represent a novel target for potential therapeutic interventions[70]. Therefore, if we can inhibit the signals which cause the migratory arrest and reverse the ability of macrophage-derived foam cells to migrate again, it may be possible to reverse the development of plaques and potentially atherosclerosis.

As we know, unlike the other two isoforms of NOS, eNOS and nNOS which are constitutively expressed, iNOS is expressed only when induced by external stimuli. iNOS is not found in healthy vessels, however, in response to stimulation with inflammatory cytokines or low PH (PH7.0) in the microenvironment of inflammatory

lesions, iNOS is expressed by macrophages and vascular smooth muscle cells[71,72]. Aside from its transcriptional regulation, multiple post-translational modifications of iNOS have been identified that may allow complex regulations of the catalytic activity of iNOS[48,73-76]. iNOS protein also produces high amounts of nitric oxide (NO) which is highly reactive with other free radicals. NO reacts with superoxide (O_2-) to form peroxynitrite (ONOO-), which in turn leads to protein nitration, DNA damage and PARP activation. Peroxynitrite reacts with other molecules to form a variety of oxgen- and nitrogen free radicals i.e. Nitrogen dioxide (·NO2), peroxynitrous acid (·ONOOH) and hydroxyl radical (·OH)[77,78]. While eNOS and nNOS generate superoxide from their oxygenase domains, iNOS may catalyse the production of superoxide from its reductase domain. Therefore, iNOS was proposed to simultaneously generate nitric oxide from L-arginine bound to its oxygenase domain, while generating superoxide from its reductase domain[50].

Under physiological conditions, iNOS is unlikely to have a functional role in the cardiovascular system due to its low expression level. However, under pathologic conditions iNOS expression is thought to have important anti-microbial and anti-tumor activities since it is able to generate high cytotoxic concentration of nitric oxide. A large number of reports are available which provide evidence for the expression of iNOS under pathophysiological conditions, both in humans as well as in animal models. Recent research revealed that iNOS had both positive and negative effects on atherosclerosis. As described before, vascular expression of iNOS could protect from developing transplant arteriosclerosis and vein graft arteriosclerosis by inhibiting vascular smooth muscle cell proliferation and neointimal hyperplasia[79,80,81]. On the other hand iNOS deletion could act as a negative regulator of leukocyte trafficking in the microcirculation, decrease smooth muscle cell proliferation and prevent neointima formation following balloon angioplasty, protect aortic allografts from developing allograft arteriosclerosis, improve cardiac reserve following myocardial infarction, and partially protect against acute cardiac mechanical dysfunction mediated by pro-inflammatory cytokines[82].

The seemingly opposing effects of iNOS under different pathological conditions may

be due to the differences in the cellular compartment of iNOS expression. However, iNOS expression relevant to atherosclerosis development was detected in vascular smooth muscle cells, mononuclear cells and lymphocytes. These various cellular sources are capable of generating different amounts of iNOS and subsequently target iNOS expression to various compartments of the plaque. The expression of iNOS by macrophages and smooth muscle cells in atherosclerotic lesions has been taken as evidence for its detrimental role in atherosclerosis, due to the formation of peroxynitrite[52].

Our previously research has shown that iNOS increases plaque development and plasma lipid peroxides in atherosclerotic apoE knockout mice[6,44]. Furthermore, our past results have shown that iNOS simultaneously increases NO and O_2- production and nitrosative stress in the atherosclerotic plaques. Therefore, iNOS may directly increase proatherosclerotic oxidative stress[83].

Our results show that iNOS is involved in the process of inhibition of foam cell migration. Here, we present pharmacologic and genetic evidence that oxLDL induced iNOS expression inhibits macrophage-derived foam cell migration. Therefore, strategies which reduce iNOS expression in the plaque potentially reverse migratory arrest of macrophage-derived foam cells and reverse plaque formation. In our modified Boyden chamber test, the migration of macrophages was significant reduced by oxLDL treatment. Using 1400W, a pharmacologic inhibitor, migration was fully restored. Furthermore, iNOS siRNA mediated silencing of iNOS transcription additionally restored migration in oxLDL treated macrophages. Since our observation of iNOS's pro-atherosclerotic action was made in apoE/iNOS dko mice, we isolated bone marrow cells from these mice, and subjected them to migration. In this genetic model of chronic iNOS deletion, lake of iNOS led to a resistance towards oxLDL mediated cell arrest as apoE/iNOS dko macrophages migrated normally following uptake of oxLDL. As we already described above, oxLDL is thought to be one main reason in vivo which promotes endothelia dysfunction and the formation of foam cells. Progress of atherosclerosis is tightly associated with the accumulation of macrophage-derived foam cells induced by oxLDL in plaques. However, the

particular mechanism involved in the regulation of macrophage migration and the signaling pathway involved are still unclear. Here, on the basis of our previous research on iNOS, we have proved that iNOS modulates the migratory ability of macrophage-derived foam cells. iNOS inhibition could be a target to revers deposition of macrophage-derived foam cells in atherosclerosis.

We have also showed that oxLDL-mediated signals led to enhancement of actin polymerization and phosphorylated focal adhesion kinase (p-FAK), which are associated with iNOS expression. As we know, actin-based motility, driven by the assembly of actin filaments may be one important way to direct movements of cells. Actin-based motility describes a variety of cellular processes through which living cells change shape in response to environmental signals, or extend protrusions like lamellipodia and filopodia, or wrap around a particle in a phagocytic cup. Progress in understanding the mechanism by which actin polymerization generates movement resulted from advances in different fields[63]. The cell migration process consists of actin polymerization driven lamellipodia extension, disruption of existing focal contacts, and formation of new focal contacts[84]. Mechanistically, this inhibition was associated with enhancement of focal adhesion kinase (FAK) phosphorylation and actin polymerization[70]. Since the signaling pathway required for actin polymerization includes activation of FAK[85], it has been suggested that the sustained activation of FAK may lead to inhibition of migration[86]. Moreover, the hyperphosphorylation of FAK in cells is associated with the inhibition of migration[87].

Interestingly, when we inhibit iNOS expression, actin polymerization and p-FAK were also significantly reduced. That is to say, the iNOS expression mediated by oxLDL may lead to an increase of actin polymerization and FAK phosphorylation.

For a better understanding of the underlying pathomechanism we performed a detailed analysis of oxidative stress using Electron Spin Resonance (ESR) spectroscopy measurement of reactive oxygen species (ROS), NO and peroxynitrite production. As we described before, oxidative stress caused by ROS, NO and peroxynitrite may lead to vascular injury. Moreover, in the atherosclerosis lesions

oxidative stress is increased compared to healthy vascular tissue, suggesting that oxidative stress is one important mediator or direct factor to causing the trapping of macrophages in the intima. Our studies revealed that oxLDL significantly increases macrophage-derived foam cell production of NO in an iNOS dependent manner. Interestingly, ROS production of the macrophage-derived foam cells following oxLDL treated is significantly reduced compared to unstimulated cells. We conclude that this may be the result of excess NO which reacts with ROS to form peroxynitrite, one of the strongest known oxidants in biological samples. We speculate that increased peroxynitrite formation leads to increased production of lipid peroxides which are strong inducers of protein tyrosine phosphatases (PTPs) by their oxidation[88]. Today, there is a wealth of evidence supporting peroxynitrite mediated lipid peroxide oxidation[89]. With regards to this, we found increased levels of malondialdehyde (MDA) in plasma of apoE/iNOS dko mice[6]. In this way, oxidative inactivation of PTP would result in sustained phosphorylation of FAK and increased actin polymerization. The expression of iNOS signaling will then cause the inhibition of migration.

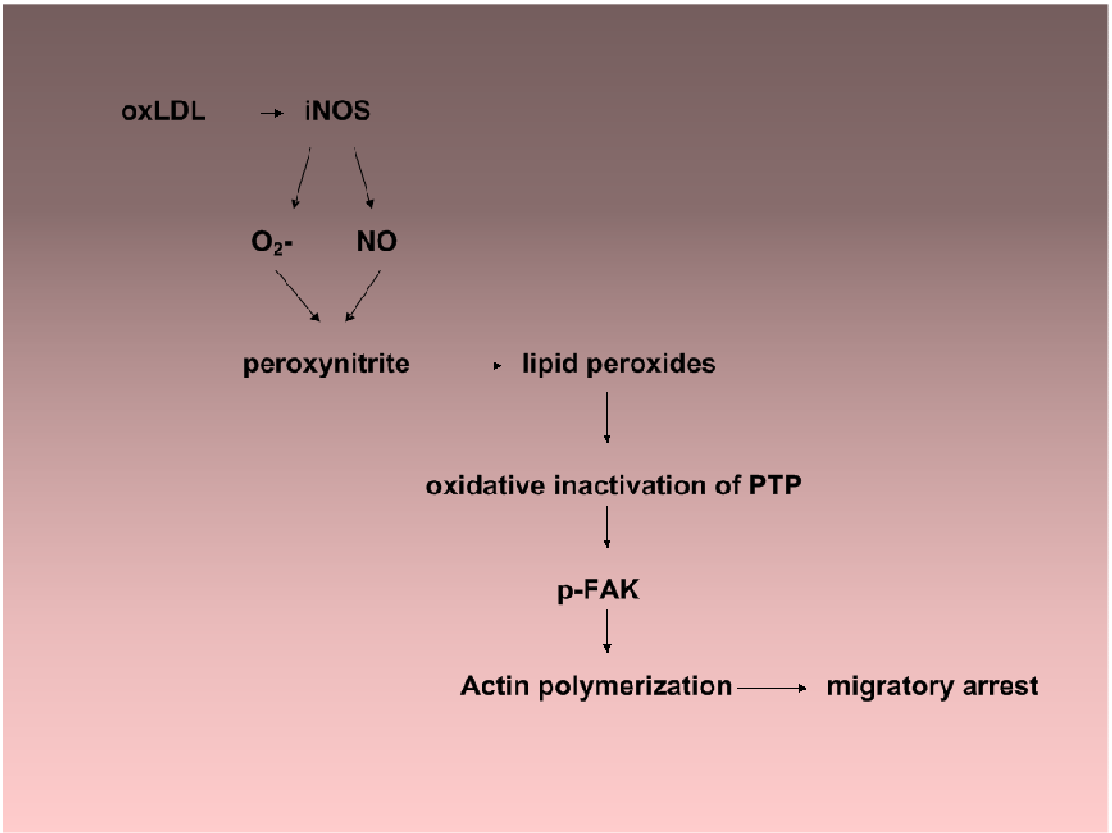

Fig.20 Model depicting mechanisms of macrophage migratory arrest.

Park et al showed that oxLDL-CD36 interactions in macrophages led to NADPH oxidase-mediated generation of intracelluar ROS, which in turn led to oxidative inactivation of SHP-2 and migration[70]. Unpublished data from our lab suggests that THP-1 cells used in the Park publication do not produce iNOS following oxLDL treatment. Furthermore, they did not investigate NO production used in their murine macrophage preparations. In our experiments iNOS induction led to a decrease in superoxide formation, most likely because superoxide was scavenged by NO to form peroxynitrite. Given the above presented references which showed that peroxynitrite is capable of generating lipid peroxides it is interesting that recent data found that lipid peroxides are much more effective in PTP oxidation compared to ROS (nanomolar range for lipid peroxides vs. micromolar range for ROS) [88]. Our results show that Trolox (VitE), an antioxidant known to reduce lipid peroxidation, can reverse the inhibition of migration caused by oxLDL. Thus, we hypothesise that iNOS driven peroxynitrite mediated formation of lipid peroxides may drive a much stronger

signal which can inhibit migration in macrophages.

In conclusion, our data identified a novel iNOS depending pathway modulating macrophage-derived foam cell migration. This finding may explain some of the proatherogenic functions of iNOS in our model and suggests that iNOS might be a target gene to reverse atherosclerosis.

Chapter V

Summary

Macrophage-derived foam cells play a critical role in all stages of atherosclerosis, from the earliest discernable lesions to complex plaques. oxLDL is thought to be a main trigger for endothelial release of pro-inflammatory cytokines, subsequently causing transmigration of the monocytes into the vessel wall. Moreover, formation of macrophage-derived foam cells is mainly induced by oxLDL. Deposition of macrophage-derived foam cells in the lesions is induced by oxLDL uptake, as this uptake causes migratory arrest of the cells. Therefore, reversion of migratory arrest of macrophage-derived foam cells might enable these cells to leave the plaques resulting in reduction of plaque sizes.

Our results show that iNOS participates in the mechanisms of oxLDL induced inhibition of macrophage-derived foam cell migration. Inhibition of iNOS expression completely reversed oxLDL mediated migratory arrest of macrophage-derived foam cells. Inhibition of iNOS was associated with enhanced phosphorylation of focal adhesion kinase (FAK) and subseqent actin polymerization. Furthermore, the p-FAK triggered increase in actin polymerization is dependent on iNOS mediated increased oxidative stress. Our results suggest that iNOS may be an interesting target gene to reverse the process of atherosclerosis.

Zusammenfassung

Die aus den Makrophagen entstehenden Schaumzellen spielen eine wichtige Rolle in allen Stadien der Atherosklerose – von den frühesten erkennbaren Läsionen bis hin zur Entstehung komplexer atherosklerotischer Plaques. Man nimmt an, dass oxLDL einer der Hauptgründe für die Freisetzung von Entzündungsfaktoren von Endothelzellen ist, die Monozyten zu einer Transmigration in die Gefässwand verleiten. Außerdem wird die Entstehung von Schaumzellen aus Makrophagen hauptsächlich durch oxLDL verursacht. Man weiß, dass die Ablagerungen von Schaumzellen die Hauptbestandteile der atherosklerotischen Läsion darstellen, was vermuten lässt, dass der Verlust der Migrationsfähigkeit der Schaumzellen einen wesentlichen Anteil in der Entstehung von Plaques hat. Die Wiedererlangung der Migrationsfähigkeit der Zellen könnte dazu führen, dass Schaumzellen die Gefässwand verlassen können und somit eine Regression der Plaquebildung erreicht werden kann.

Unsere Ergebnisse zeigen, dass iNOS in dem Mechanismus des Migrationsarrestes eingebunden ist. Durch die Hemmung der iNOS-Expression konnte der migrationshemmende Effekt des oxLDLs signifikant reduziert werden. In unseren Experimenten förderte die oxLDL induzierte iNOS-Expression die p-FAK Bildung und Polymerisation von Aktinfilamenten, was den Effekt dieses Gens auf die Migrationshemmung des oxLDLs erklärt.

Die Hemmung der iNOS vermindert oxidativen Stress, was die von uns gezeigte proatherogene Funktion von iNOS erklärt. Unsere Ergebnisse identifizieren iNOS als vielversprechendes Zielgen zur Bekämpfung der Arteriosklerose. Unsere Studien weisen in die Richtung eines bislang unbekannten Makrophagen-Signalwegs, ausgelöst durch oxLDL und gefödert durch Induktion der iNOS Expression. Sie bieten eine Erklärung für den durch oxLDL ausgelösten Migrationsarrest von Makrophagen in atherosklerotischen Läsionen, erklären eine mögliche Rolle von

iNOS als Enzym mit proatherosklerotischer Funktion und zeigen neue Wege der Mobilisation und Emigration von Schaumzellen aus atherosklerotischen Plaques.

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Abbreviation

Apo E:	Apolipoprotein E
BH4:	(6R-)5,6,7,8-tetrahydro-L-biopterin
CEs:	Cholesterol esters
CMH:	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrr
CP:	3-carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
DETC:	Diethyldithiocarbamic acid. Sodium salt. Trihydrate
ESR:	Electron Spin Resonance
FAD:	Flavin adenine dinucleotide
FAs:	Fatty acids
FC:	Free cholesterol
FMN:	Flavin mononucleotide
KHB:	Krebs Hepes Buffer
LDL:	Low density lipoprotein
MAP:	Mitogene-activated protein
MCP-1:	Monocyte Chemotactic Protein 1
NADPH:	Nicotinamide-adenine-dinucleotide phosphate
NO:	Nitric oxide
NOS:	Nitric oxide synthases
nNOS:	neuronal NOS
iNOS:	inducible NOS
eNOS:	endothelial NOS
nCEH:	Neutral cholesterol ester hydrolase
OS:	Oxidative stress
PEG-SOD:	Pegulated Superoxide Dismutase
RCT:	Reverse cholesterol transport
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species

SRs:	Scavenger receptors
TNF-α:	Tumor necosis factor-α
VSMC:	Vascular smooth muscle cell
1400W:	N-(3-aminomethyl) benzyl-acetamidine

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1. The molecular mechanism of Bis Ⅷ selectively eliminate activate T cells in heart transplant rejection.

2. Inducible Nitric Oxide Synthase inhibits macrophage migration, a potential explanation for iNOS's proatherosclerotic action.

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4. Effects of Rivaroxaban on Restenosis in mice with endothelial dysfuntion/eNOS deficiency.

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The molecular mechanism of Astilbin inhibits smooth muscle cell proliferation of transplanted heart arteries. (The national natural science funds)

Lab Skills:

Animal model building：Rat heart tansplantion model；Myocardial infarction model, Gene transfection, Cell culture, Migration assay, RT-PCR, Flow Cytometry, Western blot, SPSS.

Publications：

1. Hua Huang, Jin-ping Zhao, Si-hai Gao. Effection of Bis Ⅷ in Heart tansplantion.(Master's thesis)

2. Hua Huang, Jin-ping Zhao, Si-hai Gao. Granular Cell Tumor of the inferior segment of Esophagus: Report of one Cases and Review of the Literature.Acta Medicinae Universitatis Scientiae et Technologiae Huazhong, 2010,39(5) 729-730.

3. Si-hai Gao, Hua Huang , Jin-he Yuan et,al. Bronch- pulmonary artery plasty in the treatment of centre long cancer (the 11th Cardiac and Thoracic surgery meeting of six provinces in south of China)

4. Hua Huang, Peter J. Kuhlencordt et,al. Inducible Nitric Oxide Synthase inhibits

macrophage migration, a potential explanation for iNOS's proatherosclerotic action. (Oral presentation in the 41st annual conference of the German Angiology)