universität wien

DISSERTATION

Titel der Dissertation

Influence of oxidatively modified LDL on the transcription factor HIF-1α and the scavenger receptor SR-BI in THP-1 macrophages

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1.3 **List of abbreviations**

ARNT	Aryl hydrocarbon receptor nuclear translocator
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
BPB	Brompheol blue
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CD36	Cluster of differentiation 36
cGMP	cyclic guanosin monophosphate
CHO	Chinese hamster ovarian
CVD	Cardiovascular disease
DAPI	4',6-Diamidino-2-phenylindol
DETA-NO	Diethylene triamine-nitric oxide
DEPC	Diethylpyrocarbonate
DHA	Dehydroascorbic acid
DMNQ	2,3-dimethoxy-1-naphthoquinone
DNPH	2,4-dinitrophenylhydrazine
DPI	Dipenyleniodonium
DTPA	Diethylene triamine pentaacetic acid
DTT	Dithiothreitol
ECGS	Endothelial cell growth supplement
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FAD	Flavin adenindinucleotid
FAF-BSA	Fatty acid free bovine serum albumin
FCS	Fetal calf serum
FGF2	Fibroblast growth factor 2

FMD	flow-mediated dilution
FMN	Flavin mononucleotid
GAPDH	Glycerinaldehyd-3-phosphat-dehydrogenase
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GSH	Glutathione
GSNO	S-nitrosoglutathione
HIF	Hypoxia inducible factor
HDL	High density lipoprotein
hLPDS	Human lipid deficient serum
HPLC	High pressure liquid chromatography
HRE	Hypoxia responsive element
HUVEC	Human umbilical vein endothelial cells
LDL	Low density lipoprotein
LOOH	Lipidhydroperoxides
M-CSF	Macrophage colony stimulating factor
MOPS	3-morpholinopropanesulfonic acid
MPA	meta-phosphoric acid
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NAC	N-acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
nLDL	Native LDL
NO	Nitric oxide
NOS	Nitric oxide synthase
ODD	Oxygen-dependent degradation domain
oxLDL	Oxidatively modified LDL
PAF-AH	Platelet-activating factor acetylhydrolase
PBS	Phosphate buffered saline
PHD	Prolylhydroxylase domain
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PON	Paraoxonase
PPAR-γ	Peroxisome proliferator-activated receptor-γ
pVHL	van Hippel-Lindau tumor suppressor protein
RCT	Reverse cholesterol transport
REM	Relative electrophoretic mobility
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Insitute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecylsulfate-Polyacrylamide-Gel-Electrophoresis
SR-A	Scavenger receptor A
SR-BI	Scavenger receptor class B type I
SVCT	Sodium-dependent vitamin C transporter system
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBS	Tris buffered saline
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein

2 Introduction

2.1 Atherosclerosis, a progressive disease of the large arteries

Cardiovascular disease (CVD) is among the major causes of morbidity and mortality in westernized societies [WITZTUM and STEINBERG 2001]. Atherosclerosis is a progressive disease characterized by lipid accumulations and fibrous elements in the large arteries followed by endothelial damages and resulting in clinical complications like stroke or myocardial infarction.

It is commonly accepted that beside genetic predisposition it is caused by enviromental factors like high-fat diet, smoking, high low density lipoprotein (LDL), low high density lipoprotein (HDL) and low antioxidant levels [LUSIS 2000; GLASS and WITZTUM 2001].

Beside the traditional “lipid hypothesis”, which is reviewed elsewhere [STEINBERG 2004; 2005b; a; 2006b; a], focusing on the role of modified lipoproteins, there is growing evidence, that inflammation is also involved in the pathogenesis of atherosclerosis [PACKARD and LIBBY 2008].

2.2 The pathogenesis of atherosclerosis

When the endothelium is inflamed due to high-fat diet, smoking or other factors, leukocytes, normally not adhering to the endothelium, express cell adhesion molecules (CAMs). These cell adhesion molecules are responsible for attachement of leukocytes, in particular monocytes. Among them, Selectin mediates rolling and interaction of leukocytes with inflamed endothelial cells. Chemokines stimulate the cells to migrate into the intima of the endothelium (Fig. 1A).

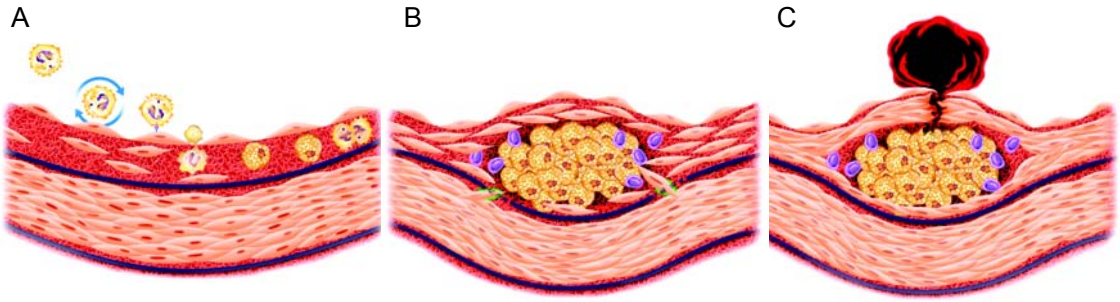


Figure 1: Initiation (A), progression (B) and thrombotic complication (C) of atherosclerosis. Modified from [PACKARD and LIBBY 2008].

Within the intima the macrophage colony stimulating factor (M-CSF) initiates the differentiation of monocytes to macrophages, which is accompanied by the expression of scavenger receptors such as the membrane glycoprotein CD36 or SR-A. These receptors enable internalisation of modified lipoproteins rapidly leading to the formation of the so called foam cells, which are characteristic for early atherosclerotic lesions. The expression of scavenger receptors is mainly regulated by peroxisome proliferator-activated receptor-γ (PPAR-γ), M-CSF and cytokines (TNF-α, IFN-γ) [STEINBERG et al. 1989; BERLINER et al. 1995; HUH et al. 1996; LUSIS 2000; GLASS and WITZTUM 2001; KUNJATHOOR et al. 2002; BOBRYSHEV 2006; LIBBY 2006; PACKARD and LIBBY 2008; SILVERSTEIN and FEBBRAIO 2009].

The oxidative modification of the lipid as well as of the apolipoprotein B (apoB) fraction of native LDL is responsible for its excessive uptake and the subsequent formation of foam cells and early fatty streaks. Oxidative modification is mainly accomplished by reactive oxygen species (ROS), which are provided either by activated neutrophils, endothelial cells or macrophages. LDL can also be modified by enzymes like myeloperoxidase, nitric oxide synthase, sphingomyelinase, secretory phospholipase and 15-lipoxygenase (15-LO) [LUSIS 2000; GLASS and WITZTUM 2001; MALLE et al. 2006].
The heme enzyme myeloperoxidase (MPO), the expression of which has been described to be elevated in human atherosclerotic plaques [DAUGHERTY et al. 1994; MALLE et al. 2000], utilizes H_2O_2 to oxidize halides like Cl^-. HOCl, one of the major reaction products of the MPO/H_2O_2-Cl^--system in human plasma [MALLE et

al. 2006], has been found to modify LDL, and HOCl-modified LDL has also been found in atherosclerotic lesions supporting the possible role of the system *in vivo* [HAZEN and HEINECKE 1997].

Leukocytes and also endothelial cells secrete cytokines which stimulate smooth muscle cells to proliferate and migrate into the intima. The early lesions containing foam cells, called fatty streaks, are not clinically significant, but they are precursors of more advancend atherosclerotic lesions, which are composed of a so called fibrous cap containing smooth muscle cells and extracellular matrix enclosing the lipid rich “necrotic core” (Fig. 1B).
Due to the expression of inflammatory mediators, collagen synthesis is blocked with a concomitant increase of collagenase expression. The resulting reduction of collagen material within the fibrous cap can lead to plaque rupture which might cause acute coronary syndroms (Fig. 1C).

Thus, receptor-mediated endocytosis of oxidized LDL appears to plays a key role in cholesterol accumulation and subsequent foam cell and atherosclerotic plaque formation in the large arteries [STEINBERG et al. 1989; BERLINER et al. 1995; LUSIS 2000; GLASS and WITZTUM 2001; LIBBY 2006; PACKARD and LIBBY 2008].

2.3 **The role of the transcription factor HIF-1α in atherosclerosis**

The hypoxia inducible factor-1 (HIF-1) is a heterodimeric transcription factor. Its transcriptional activity increases as cellular O_2 concentration is decreased.

HIF-1 is composed of an α- and a β-subunit. The ß-subunit, also called ARNT, is constitutively expressed in many cell types, whereas HIF-1α is undetectable under normal oxygen conditions due to rapid proteasomal degradation, but is stabilized under hypoxic conditions (below 5% O_2). After translocation to the nucleus and heterodimerization, HIF1 binds to promotor-specific hypoxia responsive element (HRE) sites of target genes.

Under normoxic conditions HIF-1α is hydroxylated by prolyl-hydroxylase domains (PHD) within a central oxygen-dependent degradation domain on two prolin residues (Pro^{402}, Pro^{564}). HIF-1α prolyl-hydroxylation enables the binding of the van Hippel-Lindau tumor suppressor protein (pVHL), which is recognized by the E3 ubiquitin-protein ligase complex that is responsible for ubiquitination and subsequent proteasomal degradation of HIF-1α (Fig. 2) [SALCEDA and CARO 1997; MAXWELL et al. 1999; IVAN et al. 2001; SCHOFIELD and RATCLIFFE 2005; SEMENZA 2007].

As PHD requires O_2 and α-ketoglutarate as substrates, its activity is reduced under hypoxic conditions due to lack of substrate.

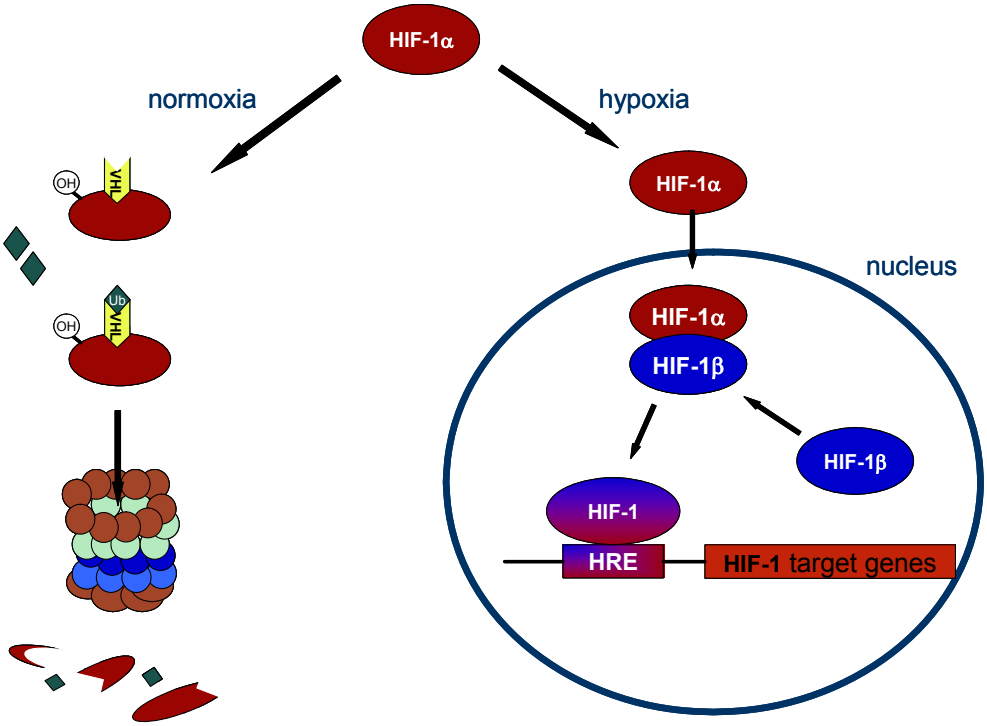


Figure 2: The HIF-1 pathway.

The many target genes that are transactivated by HIF-1 include those encoding erythropoietin, glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (VEGF). Thereby HIF-1 action contributes to increased angiogenesis, enhanced glycolysis, and other properties that promote adaption of the organism to an environment with low oxygen concentrations [SEMENZA 1999; SEMENZA 2000a; SEMENZA 2000b].

Thus, HIF-1α regulates classical hypoxia responsive genes like VEGF, which is important in the pathogenesis of atherosclerosis due to its influence in neovascularization [RIVARD et al. 2000; SCHMITZ and LANGMANN 2005].
Zones of hypoxia also seem to occur within atherosclerotic plaques [BJORNHEDEN et al. 1999]. Recently, it was shown that HIF-1α is present in atherosclerotic lesions and associated with an atheromatous inflammatory plaque phenotype [VINK et al. 2007].

It was suggested that ROS, due to mitochondrial ROS production at complex III, provide a signal for HIF-1 induction under hypoxia [CHANDEL et al. 2000] and moreover, ROS seem to stabilize HIF-1α under normoxic conditions [RICHARD et al. 2000]. In addition, HIF-1α expression is upregulated in activated macrophages [VINK et al. 2007].
In 2003 Shatrov et al. described the stabilization of HIF-1α after oxLDL treatment via redox-dependent mechanisms [SHATROV et al. 2003] and an induction of macrophage VEGF in response to oxLDL was observed in other studies [RAMOS et al. 1998; INOUE et al. 2001; SALOMONSSON et al. 2002]. Vink et al. demonstrated a positive association between HIF-1α staining and VEGF protein in atherosclerotic plaques [VINK et al. 2007].
Hence, the expression of VEGF after oxLDL-treatment and in hypoxic plaques is supposed to be regulated by HIF-1α.
It has also been suggested that HIF-1α is involved in foam cell formation, because it was shown that RNA silencing of HIF-1α in the human monocytic cell line U937 inhibits the oxLDL-induced macrophage foam cell formation [JIANG et al. 2007].

2.4 **HDL and its atheroprotective function**

Circulating plasma high density lipoprotein (HDL) levels are inversely proportional to the risk of atherosclerosis ["Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report" 2002]. The protective effect of HDL during atherosclerosis is widely accepted and supposed to

be founded mainly on its role in reverse cholesterol transport (RCT) by which HDL removes excess cholesterol from peripheral tissues and cells, including those in the artery wall and lipid-laden macrophages (Fig. 3), and delivers it back to the liver for biliary secretion [NOFER et al. 2002; RADER 2003; VILES-GONZALEZ et al. 2003; RADER et al. 2008; RADER and DAUGHERTY 2008]. Reverse cholesterol transport was described first in 1973 by Glomset and Norum [GLOMSET 1973].

However, HDL shows further antiatherosclerotic capacities by inhibiting the expression of adhesion molecules in endothelial cells (Fig. 3) and by preventing the recruitment of blood monocytes into the artery wall [BARTER et al. 2004].
Apart from that, HDL possesses antioxidative and cytoprotective properties [NEGRE-SALVAYRE et al. 2006] and may also function as a scavenger of superoxide anions [CHANDER and KAPOOR 1990]. HDL was shown to reduce the acute infiltration of neutrophils and inhibit the generation of ROS in inflamed, nonatherosclerotic arteries *in vivo* [NICHOLLS et al. 2005].
HDL is enriched with several antioxidant proteins such as paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH) which help to prevent LDL oxidation (Fig. 3) [PARTHASARATHY et al. 1990; WATSON et al. 1995a; WATSON et al. 1995b].
Besides these functions, HDL protects the arterial vasculature by mediating beneficial effects on the endothelium and by inhibiting coagulation. HDL enhances nitric oxide (NO)-dependent relaxation of the endothelium in the large arteries due to HDL-dependent stimulation of eNOS through SR-BI binding and has multiple antithrombotic actions involving attenuation of thrombin generation as well as endothelial and platelet activation [SHAUL and MINEO 2004; MINEO et al. 2006; VAN LEUVEN et al. 2008].

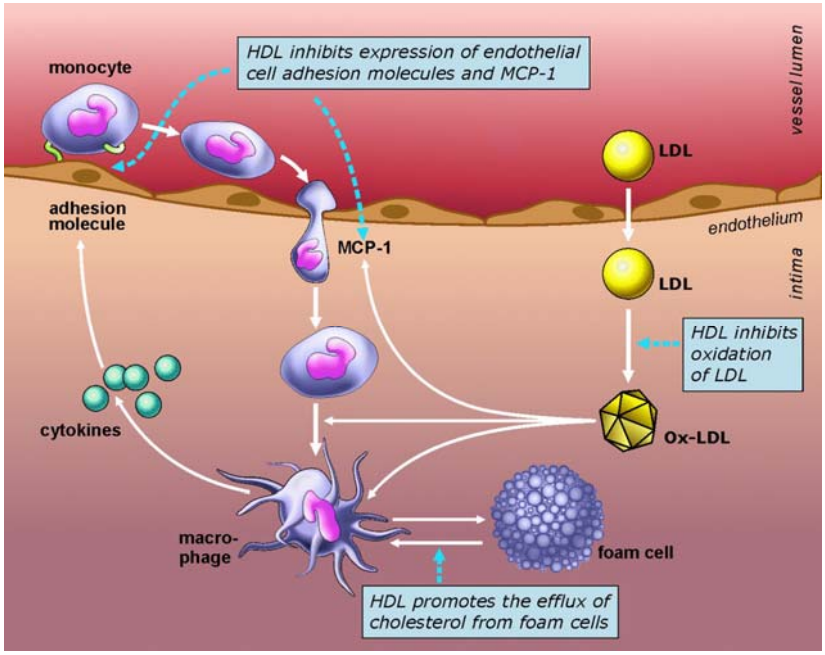


Figure 3: Atheroprotective functions of HDL [VAN LEUVEN et al. 2008].

The selective uptake of cholesteryl esters from HDL in liver and adrenals is mediated by the cell surface “scavenger receptor, class B, type I” (SR-BI) [ACTON et al. 1996; STANGL et al. 1999]. SR-BI is a glycoprotein with a large extracellular domain, anchored in the plasma membrane both at the N and the C termini with short cytoplasmic end parts [RIGOTTI et al. 2003]. Besides its function as an HDL receptor [ACTON et al. 1996] SR-BI is also known to facilitate uptake of modified lipoproteins [ACTON et al. 1994].

During monocyte-to-macrophage differentiation the expression of SR-BI increases [HIRANO et al. 1999]. Macrophage SR-BI expression can be increased by PPAR-γ [CHINETTI et al. 2000]. Whereas Hirano et al. demonstrated that SR-BI mRNA and protein expression is increased by oxLDL [HIRANO et al. 1999], it was also shown that SR-BI decreases after oxLDL treatment in fully differentiated macrophages [HAN et al. 2001].

Hepatic SR-BI expression was shown to be an important positive regulator of the rate of integrated macrophage-to-feces RCT, so the rate of macrophage RCT is not only a function of HDL steady-state plasma concentrations [ZHANG et al. 2005].

The transporter protein ATP-binding cassette transporter A1 (ABCA1) promotes cholesterol efflux of cholesterol from cells to HDL precursors. ATP-binding cassette transporter G1 (ABCG1) transports cholesterol to mature HDL [WANG et al. 2004; JESSUP et al. 2006]. Thus, both transporter proteins contribute to the efflux of cholesterol from macrophages and to general cholesterol transport *in vivo*, whereas SR-BI facilitates only a small amount of macrophage cholesterol efflux [WANG et al. 2007]. The differentiation-dependent induction of the ABCA1 gene in monocytes could also be related to the activation of the HIF-1 pathway [SCHMITZ and LANGMANN 2005].

2.5 **Nitric oxide and atherosclerosis**

Nitric oxide (NO), a soluble gas delivered mainly by the endothelium, mediates important effects regarding blood pressure and vascular tone and can thereby protect vessel walls.

A key process in the early pathogenesis of hypercholesterolemia-induced vascular disease and atherosclerosis is diminished bioavailability of this endothelium-derived signaling molecule.

NO is generated from conversion of L-arginine to L-citrulline (Fig. 4) by a NADPH-dependent nitric oxide synthase (NOS) that requires tetrahydrobiopterin (BH_4), FAD and FMN as cofactors and is regulated by Ca^{++}/Calmodulin (CAL) [NAPOLI and IGNARRO 2001].

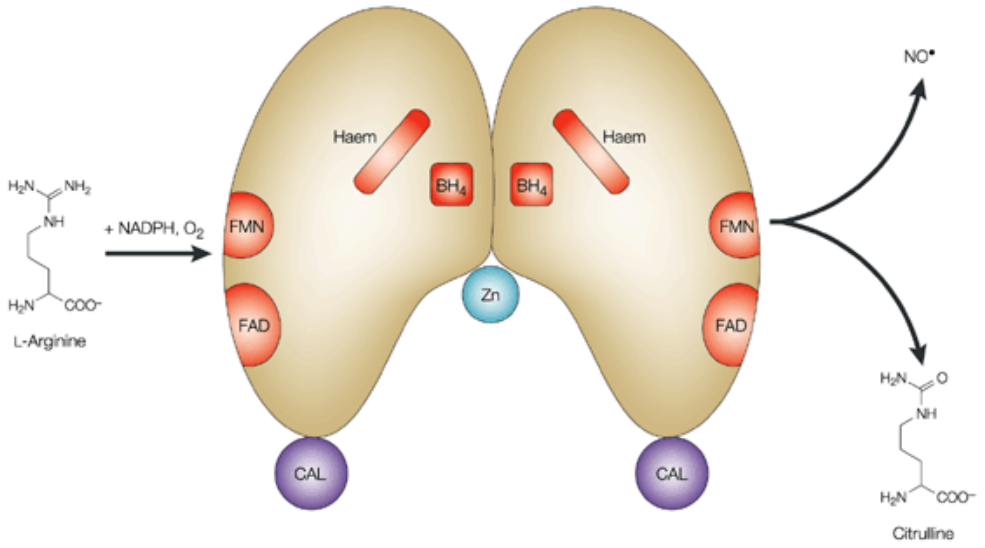


Figure 4: Enzymatic generation of NO. Modified from [VALLANCE and LEIPER 2002].

Three isoforms of the enzyme nitric oxide synthase (NOS) have been described, the endothelial NOS (eNOS), which is found in vascular endothelial cells, the inducible NOS (iNOS), which is inducible in a wide range of cells and the neuronal NOS (nNOS), which is predominating in neuronal tissue [ALDERTON et al. 2001].
If oxygen availability and therefore NOS activity is diminished, nitrite can be reduced to NO, which is well known as an alternative to the classical L-arginine-NOS pathway [LUNDBERG et al. 2008].

NO stimulates the soluble guanylyl cyclase resulting in a higher production of cyclic guanosin monophosphate (cGMP), which activates GMP-dependent kinases leading to a decrease in intracellular calcium and therefore to endothel relaxation. NO also displays antiinflammatory actions by blocking the synthesis and expression of cytokines and cell adhesion molecules in the vessel wall [ANDERSON et al. 1995; CANNON 1998].

During atherosclerosis, a reduced concentration or acitivity of L-arginine, tetrahydrobiopterin, iNOS or eNOS and an impaired NO release and diffussion from the damaged endothelium to smooth muscle cells was detected. NO degradation can also be increased due to direct reaction with oxLDL [NAPOLI and IGNARRO 2001].

Oxidative stress within the vasculature leads to NO scavenging and oxidation of tetrahydrobiopterin, whereby the latter can be prevented by the action of ascorbic acid [HELLER et al. 2001]. Superoxide anions and free radicals are also able to oxidize NO to metabolites like peroxynitrite, which cannot activate guanylyl cylase and thus inhibit vascular relaxation [CANNON 1998].

HDL has been reported to activate the endothelial NO-synthase (eNOS) in endothelial cells which leads to a higher NO release within the endothelium [YUHANNA et al. 2001; GONG et al. 2003].

It was shown that NO stabilizes HIF-1α under normoxia, but destabilizes it under hypoxic conditions [BRÜNE and ZHOU 2003; KÖHL et al. 2006; BRÜNE and ZHOU 2007]. NO also attenuates oxLDL-induced HIF-1α accumulation under normoxic conditions [SHATROV et al. 2003]. The underlying mechanisms are still not clarified in detail.

2.6 **Vitamin C and atherosclerosis**

Vitamin C, a well known water-soluble antioxidant in human blood plasma [FREI et al. 1989], exists in two chemically distinct forms, the reduced ascorbate ion form and the oxidized non-ionic form dehydroascorbic acid (DHA). These two chemical forms are transported across the cell membrane of human cells by two different pathways that show absolute specificity for one or the other vitamin form. For ascorbate, it is a high affinity, low capacity sodium-dependent vitamin C transporter system (SVCTs) (Fig. 5A) [DARUWALA et al. 1999; TSUKAGUCHI et al. 1999; WANG et al. 2000] while for DHA there is a low affinity, high capacity sodium-independent system (Fig. 5B), which includes several members of the facilitative glucose transporter family (GLUTs) [VERA et al. 1993; GOLDENBERG and SCHWEINZER 1994].

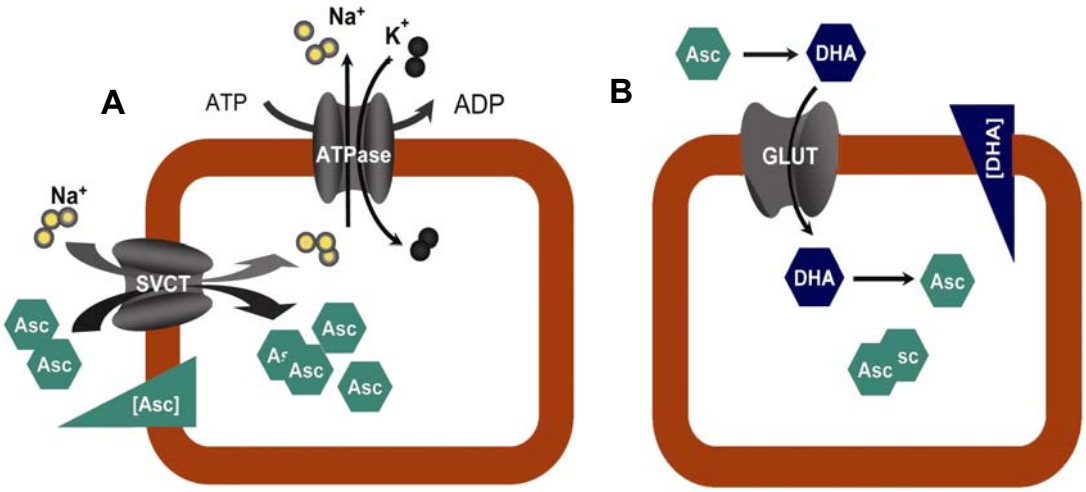


Figure 5: Cellular transport of ascorbate and DHA. Modified from [LI and SCHELLHORN 2007].

Twelve glucose transporter isoforms have been molecularly characterized, and there is evidence that the isoforms GLUT1, GLUT3 and GLUT4 are efficient DHA transporters. Within the cell DHA is reduced back to ascorbate immediately [RUMSEY et al. 1997; RUMSEY et al. 2000].

Vitamin C was shown to play an important role during hypoxia. The dioxygenases, which are responsible for proline hydroxylation during normoxic HIF-1α degradation, contain a non-heme iron group, which requires ascorbate for optimal activity. It was shown that the *in vitro* activity of hydroxylases is enhanced by ascorbate addition [SCHOFIELD and RATCLIFFE 2004] and ascorbate has been reported to decrease hypoxia-induced HIF-1α protein levels in human primary and cancer cell lines [KNOWLES et al. 2003; VISSERS et al. 2007].

Low plasma ascorbate apparently correlates with a higher incidence of cardiovascular disease and mortality [ENSTROM et al. 1992; NYYSSONEN et al. 1997].
Vitamin C was shown to stabilize tetrahydrobiopterin, a cofactor of eNOS. Because of its antioxidative properties it is able to recycle oxidized tetrahydrobiopterin back to the reduced pterin and therefore prevents endothelial dysfunction resulting from NO deprivation [HUANG et al. 2000; HELLER et al. 2001].

On the other hand, it was also shown that vitamin C infusions significantly enhanced flow-mediated dilution (FMD) in patients with metabolic syndrome [CANGEMI et al. 2007]. However, the role of vitamin C in cardiovascular disease is still seen as controversial [MCCALL and FREI 1999].

So far, no data are available of the impact of ascorbate on oxLDL-induced HIF-1α stabilization in macrophages and their role during atherosclerosis.

3 Aim

Foam cell formation, which is characterized by the excessive uptake of oxidatively modified low density lipoprotein (LDL) by macrophages, is one of the initial steps in the pathogenesis of atherosclerosis. Since the transcription factor hypoxia inducible factor (HIF-1) [BJORNHEDEN et al. 1999; SCHMITZ and LANGMANN 2005; JIANG et al. 2007; VINK et al. 2007] and the scavenger receptor class B type I (SR-BI) seem to play a crucial role in this process, we aimed to investigate the expression of these two factors on both mRNA and protein level in atherosclerosis like conditions in the presence and absence of putative antagonists.

HIF-1α protein is known to be degraded under normoxia due to proteasomal action, whereas it is stabilized under hypoxia or in the presence of oxidatively modified LDL [SHATROV et al. 2003; SEMENZA 2007].
Expression of SR-BI, a scavenger receptor for lipoproteins and a designated HDL-receptor [ACTON et al. 1994; ACTON et al. 1996; STANGL et al. 1999], which is involved in reverse cholesterol transport [ZHANG et al. 2005], was shown to be downregulated in the presence of oxidatively modified LDL [HAN et al. 2001].
High density lipoprotein (HDL) was shown to be atheroprotective due to its influence on reverse cholesterol transport, its antioxidative potential and its impact on endothelial function [VILES-GONZALEZ et al. 2003; VAN LEUVEN et al. 2008]. Thus, one of our first aims was to investigate the influence of HDL on the expression of HIF-1α and SR-BI protein, foam cell formation and cholesterol efflux in oxLDL treated THP-1 macrophages.
The antioxidant vitamin C and the gasotransmitter nitric oxide (NO) are known to inhibit hypoxia-induced HIF-1α protein accumulation [BRÜNE and ZHOU 2003; KNOWLES et al. 2003; SHATROV et al. 2003; VISSERS et al. 2007]. Besides this, NO is able to improve endothelial function [NAPOLI and IGNARRO 2001]. Therefore, it was obvious to examine their role on oxLDL-induced HIF-1α stabilization. Additionally, we determined their influence on SR-BI protein expression, foam cell formation and cholesterol efflux.

Currently, little it is known about the interaction of HIF-1α with HDL, NO and vitamin C. There is also little and controversial data available about the role of SR-BI in macrophages during oxLDL-induced pathogenesis. Thus, we aimed to get further insights in oxLDL-induced pathogenesis and foam cell formation.

4 Methods

Used chemicals were from Sigma-Aldrich, Merck or Roth unless noted otherwise.

4.1 **Cell Culture**

4.1.1 THP-1 cells

The human monocytic cell line THP-1 (ATCC TIB-202) was used for the majority of the experiments. This human leukemic cell line was derived from the blood of a one-year old boy with acute monocytic leukemia [TSUCHIYA et al. 1980]. Treatment of this cell line with phorbol esters results in adherence of the cell to the well surface and differentiation into mature cells with functions of macrophages (Fig. 6), which mimic native monocyte-derived macrophages in several aspects. So THP-1 monocytes were differentiated towards macrophages with PMA (phorbol 12-myristate 13-acetate) for 72 hours [TSUCHIYA et al. 1982; AUWERX 1991].

Monocytic THP-1 cells were cultured in suspension in cell culture flasks (Greiner Bio-one) and incubated at 37°C under humified 5 % CO_2 athmosphere (CO_2-incubator C150, Binder, Tuttlingen, Germany) in RPMI 1640 (Roswell Park Memorial Insitute) medium (Biochrom AG, Berlin, Germany), supplemented with 10 % FCS (fetal calf serum, GIBCO), 2 mM glutamin (PAA, Austria), 0.1 mg/ml streptomycin and 100 U/ml penicillin (PAA, Austria).
After two or three days the cells were fed with fresh medium to obtain a density of $0.5*10^6$ cells/ml.
The cell number and viability were measured with CASY® Cell Counter + Analyser System Model TT (Schärfe Systeme GmbH, Reuttlingen, Germany).
For the experiments 3 ml, 1.2 ml or 120 µl of cell suspension with a density of $0.5*10^6$ cells/ml were seeded in 6- (9.6 cm^2 growth area), 12- (3.9 cm^2 growth area) or 96-well plates (0.35 cm^2 growth area) (TPP, Switzerland), respectively. THP-1 cells were differentiated towards adherent macrophages with PMA (160 nM) for 72 hours.

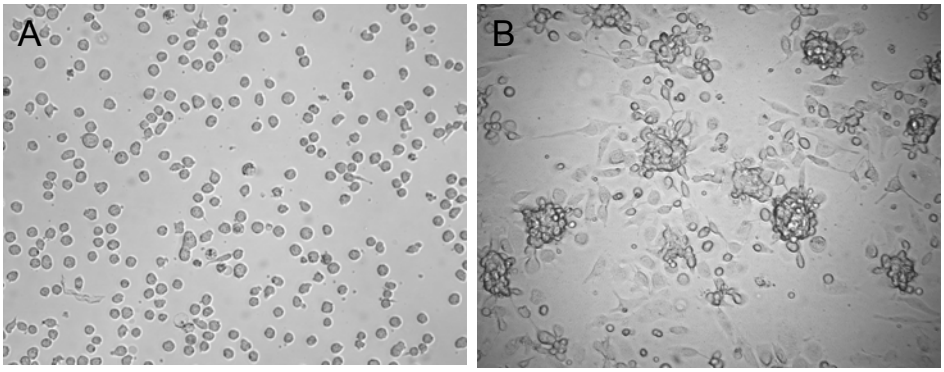


Figure 6: Phaco-microscopy images of THP-1 cells.

THP-1 monocytes (A) were differentiated towards macrophages (B) with 160 nM PMA for 72 h.

4.1.2 HUVEC (human umbilical vein endothelial cells)

HUVEC cells (Fig. 7), isolated from newborn umbilical cords, were kindly provided from Prof. Kapiotis (Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna). These cells were grown in Medium 199 (GIBCO^{TM}), supplemented with 20 % FCS (GIBCO), 2 mM glutamin (PAA), 0.1 mg/ml streptomycin, 100 U/ml penicillin (PAA), 50 ng/ml gentamycin (PAA), 2.5 mU/ml heparin and 0.2 % ECGS (endothelial cell growth supplement) in fibronectin (10 µg/ml) coated 6-well plates and incubated at 37°C under 5 % CO_2.

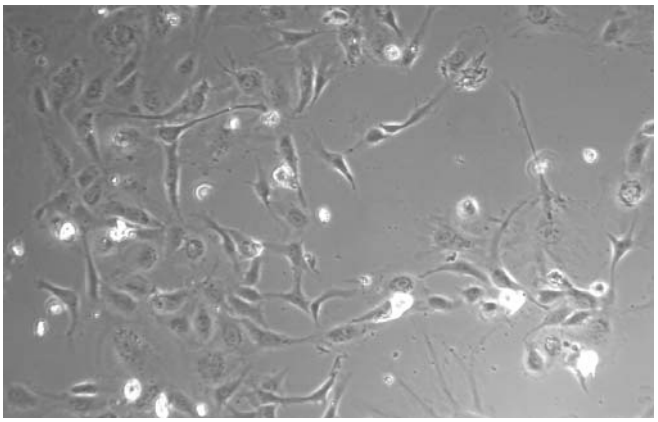


Figure 7: Phaco-microscopy image of HUVEC
(human umbilical vein endothelial cells).

4.1.3 Cell culture experiments under hypoxic conditions

Some incubations were performed under hypoxic conditions. Therefore, cells were placed in a hypoxic chamber (Billups-Rothenberg, MC-101), which was flushed with a specific gas (1 % O_2, 5 % CO_2, 94 % N_2) at a flow rate of 20 l/min for four minutes. The sealed chamber with the cells was placed into an incubator with 37°C during the incubation.

4.2 **Lipoprotein Preparation**

Lipoproteins were isolated using sequential flotation ultracentrifugation.

Reagents and solutions:
0.18 M EDTA (ethylene diamine tetra-acetic acid) pH 8
potassium bromide (KBr)
dialysis buffer (0.9 % NaCl, 0.1 % EDTA, pH 7.4)

Procedure:
Blood samples of healthy fasting female volunteers were collected and EDTA (2 ml of 0.18 M EDTA/50 ml) was added to avoid coagulation. Afterwards, the samples were centrifugated twice at 600xg for 20 minutes (4°C) (Beckmann GPR Centrifuge) and plasma (upper phase) was collected.
Separation of the lipoproteins from plasma was accomplished through different density adjustments [SCHUMAKER and PUPPIONE 1986]. First, the density of the plasma was adjusted to 1.019 g/ml (VLDL) with KBr. The required amount of potassium bromide (KBr) was calculated using following formula:

$$g\ KBr = \frac{\text{sample volume (ml)} * (\text{density}_{final} - \text{density}_{initial})}{1 - (0.312 * \text{density}_{final})}$$

Plasma with adjusted density was centrifugated for 20 hours at 51,000 rpm (200,000xg) and 4°C (Beckmann Ultracentrifuge, Optima XL-70, rotor 55.2 Ti). After centrifugation, the top phase, containing VLDL, was removed by slicing the

centrifugation tube and collecting VLDL with a syringe. Afterwards the density of the infranatant was adjusted to 1.063 g/ml and after over night centrifugation (51,000 rpm, 20 h, 4°C) LDL was collected from the top phase. HDL, obtained by adjusting a density of 1.22 g/ml and two (to ensure complete albumin removal) subsequent centrifugation steps (51,000 rpm, 20 h, 4°C). LDL and HDL were dialysed (Spectra/Por®4) 3 times against 5 l of dialysis buffer for a total of about 24 hours to remove excess KBr.
Protein concentration of the lipoprotein fractions was measured using the Bradford assay (cpt. 4.4.1).

4.2.1 Analysis of the lipoprotein fraction by SDS-PAGE (Sodiumdodecylsulfate-Polyacrylamide-Gel-Electrophoresis)

Principle:
HDL and LDL fractions were analysed routinely for their purity and their apolipoprotein content using SDS-PAGE. Pure HDL fractions show a band at about 28 kDa, corresponding to apoAI. Pure LDL fractions show a band at about 500 kD, indicating the main component of the LDL particle, apoB100 (Fig. 8).

Reagents and solutions:
Diethyl ether
Electrophoresis buffer (25 mM Tris base, 200 mM glycine, 1 % SDS)
4x Sample buffer (500 µl Glycerol, 240 µl 0.5 M Tris-HCl pH 6.8,
210 µl aqua dest., 8 % SDS)
Coomassie stock solution (0.2 %):
0.4 g Coomassie blue dissolved in 80 ml aqua dest. and 120 ml methanol
Coomassie working solution (0.1 %):
50 ml stock solution (filtered) mixed with 10 ml acetic acid and 40 ml aqua dest.
Destain solution (20% methanol, 10% acetic acid, 70% aqua dest.)
1.5 mm polyacrylamide SDS gel (8% and 10%, with 4% stacking gel each, table 1)

	8%	10%	4%
H_20 bidest.	3.2 ml	2.7 ml	2.1 ml
30% Acrylamid/Bis Solution	2.1 ml	2.7 ml	0.5 ml
3 M Tris-HCl, pH 8,45; 0,3% SDS	2.7 ml	2.7 ml	1.3 ml
10% APS	60 µl	60 µl	30 µl
TEMED	6 µl	6 µl	3 µl

Table 1: Composition of a SDS-PAGE gel (1.5 mm).

Procedure:

For gel preparation see cpt. 4.11.2.

First the lipid fraction of the lipoproteins was removed using diethyl ether extraction. Therefore, 50 µg of lipoprotein was filled up to 100 µl with aqua dest. and vortexed with 100 µl diethyl ether. Then the solution was centrifuged for 2 minutes at 10,000 rpm (Eppendorf Centrifuge 5417R) and the upper lipid phase was discarded. The protein fractions of the lipoproteins were applied to SDS gel electrophoresis after mixing 10 - 30 µl of protein solution with 8 µl of sample buffer and 2 µl of 2-mercaptoethanol. Then, 0.9 % NaCl was added to provide a total volume of 40 µl each. A protein ladder was also filled into one gel slot (PageRuler® Prestained Protein Ladder, Fermentas). Electrophoresis was performed at 180 Volt for one hour. Afterwards, the gels were stained with Coomassie blue for half an hour. After destaining the gels with destain solution, the gels were documented with a CCD camera in the ChemiImager™ 4400 (Biozym) for detection.

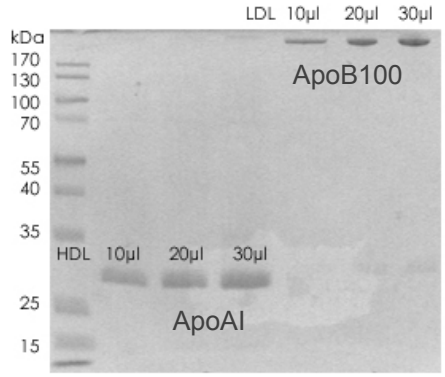


Figure 8: Coomassie staining of LDL- (ApoB100, 500 kDa) and HDL-fractions (ApoAI, 28 kDa), separated by SDS gel electrophoresis (10 % acrylamide).

4.3 **LDL modification**

4.3.1 LDL modification with copper ions

As LDL was stored in 0.9 % NaCl with 0.1% EDTA, EDTA was removed from LDL using a NICK-column (Sephadex G-50, Amersham Biosiences), to avoid complex-building of Cu^{2+} with EDTA, which would prevent $CuSO_4$ from oxidizing LDL particles,. Afterwards, LDL was incubated at a concentration of 0.5 mg/ml with 10 µM $CuSO_4$ for 4 hours at 37°C. Then 50 µM EDTA was added to stop the reaction of $CuSO_4$ with LDL. TBARS (Thiobarbituric acid reactive substances, cpt 4.6) content of LDL was measured routinely to analyse the extent of lipid oxidation.

4.3.2 LDL modification with NaOCl

To oxidize the protein fraction of LDL particles, 1 mg of LDL (2 µM) was incubated with 1 mM NaOCl (molar ratio LDL/NaOCl 1:500) for 1 hour at 37°C. DTPA (Diethylene triamine pentaacetic acid) was added at a concentration of 100 µM to avoid lipid oxidation. The reaction was stopped by adding the same volume of a 0.1 M phosphate buffer (pH 7.5).

4.4 **Protein determination**

4.4.1 Bradford assay

Principle:

The Bradford assay [BRADFORD 1976] is based on the principle that the absorbance maximum of an acidic solution of Coomassie Brilliant Blue G-250 (Bio-Rad® Protein Assay) shifts from 465 to 595 nm when it binds to proteins.

Procedure:

For protein determination cell lysates for Western Blot experiments were diluted 1:200 and cell lysates from oil red o experiments 1:20 with aqua dest. to a final volume of 400 µl. After addition of 100 µl BioRad-reagent the protein concentration of the samples was measured spectrophotometrically at 595 nm. Bovine serum albumin (BSA) was used for calibration.

4.4.2 Lowry assay

Principle:

Because of high SDS-concentrations the protein content of cell lysates from the carbonyl assay (cpt. 4.7) was measured with the Lowry assay [LOWRY et al. 1951]. The first step of this protocol is based on the Biuret reaction, where a blue-violet complex is built between peptide bonds and copper(II)ions under alkalic conditions. In a second step this complex reduces a yellow folin-ciocalteau reagent, the intensity of the resulting blue colour correlates with the protein amount.

Reagents and solutions:

Copper solution:

20 g Na_2CO_3 in 260 ml aqua dest.

0.26 g $CuSO_4$ in 20 ml aqua dest.

0.2 g potassium hydrogen tartrate in 20 ml aqua dest.

After dissolving the 3 solutions were mixed.

2x Lowry reagent

Copper solution:SDS (1%):NaOH (1 M) (3:1:1)

Folin-ciocalteau reagent (0.2 M)

Procedure:

Protein lysates were diluted 1:2.5 with denaturing buffer (cpt. 4.7). 400 µl of protein solution was mixed with 400 µl of 2x Lowry reagent. After 10 minutes of incubation at room temperature 200 µl of Folin reagent was added to the samples during vortexing. After an incubation of 30 minutes the absorbance was measured at a wavelength of 700 nm. BSA was used for calibration.

4.5 **Relative electrophoretic mobility (REM)**

Principle:
Aldehydes, which emerge from Cu^{2+}-induced lipidperoxidation in LDL particles, bind to side-chains of amino acids (Lys, Arg, His) of Apo B100, resulting in an increase of the overall negative charge and therefore in a higher relative electrophoretic mobility (REM). HOCl also converts positively-charged side-chains of His and Lys residues (and possibly Arg, depending on the HOCl excess) to neutral chloramines and/or products, also resulting in an increase of the overall negative charge [MALLE et al. 2006].

Reagents and solutions:
Agarose
TBE buffer (5x, 1 l): 54 g TRIS, 27.5 g borate, 20 ml 0.5 M EDTA (pH 8)
Loading dye (4x): 50 % glycerol, 50 % 0.25 M Tris-HCl, dash of bromphenol blue
GeneRuler^{TM} 100 bp DNA Ladder (Fermentas)
Coomassie working solution (0.1 %)
Destain solution

Procedure:
LDL (native or modified) was mixed with loading dye and subsequently loaded onto an 1 % agarose gel (for preparation, see cpt. 4.15.1 without EtBr). Electrophoresis was carried out for 1 hour in TBE-buffer at 90 Volt. Then the gel was stained with Coomassie blue for half an hour. After destaining the gel with destain solution, it was documented with a CCD camera in the ChemiImager^{TM} 4400 (Biozym). Afterwards, relative electrophoretic mobility (REM) was measured, whereby REM of native LDL (nLDL) was set to 1.

4.6 **TBARS assay**

Principle:

Thiobarbituric acid reactive substances (TBARS) are aldehyde breakdown products of lipid peroxidation, basically from polyunsatured fatty acids. The main component of these products is malondialdehyde (MDA). One molecule of MDA builds a pink chromogen with two molecules of 2-TBA (Fig. 9) at an acidic pH (pH 2-3), which has an absorbance maximum of 534 nm with a molar absorption co-efficient of 156,000 $M^{-1}.cm^{-1}$ [RICE-EVANS 1991].

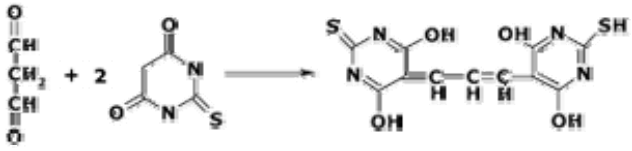


Figure 9: Reaction of malondialdehyde with thiobarbituric acid.
(http://www.nwlifescience.com/index.php?page=shop.product_details&flypage=flypage.tpl&product_id=6&category_id=7&option=com_virtuemart&Itemid=254, 21.08.2009)

Reagents and solutions:

Thiobarbituric acid (1 % in 0.05 M NaOH)

Stored at 4°C

Acetic acid (100 %)

Butylated hydroxytoluene (BHT) (1 mM)

Ethylenediamine tetraacetic acid (EDTA) (1 mM)

Procedure:

Cell supernatants were transferred into an eppendorf tube and 20 µM BHT and EDTA were added to avoid lipid peroxidation during incubation. TBA and acetic acid were added in equal amounts, incubated at 100°C for 45 minutes. After cooling down to room temperature the samples were measured spectrophotometrically at a wavelength of 534 nm (Perkin Elmer UV/VIS Spectrometer Lamda2). The concentration of MDA in the supernatant was calculated from the absorbance with Lambert-Beer's law (extinction coefficient 156,000 $M^{-1}.cm^{-1}$).

4.7 **Measurement of protein carbonyls**

Principle:

Protein carbonyls are products of protein oxidation, which react with 2,4-dinitrophenylhydrazine (DNPH). This reaction leads to the building of a stable, yellowish complex, the 2,4-dinitrophenylhydrazon, which can be measured spectrophotometrically at a wavelength of 355 nm. This method was investigated by Reznick and Packer [REZNICK and PACKER 1994] and modified for quantitation of apoB carbonyl content from Yan et al, to remove interfering lipids [YAN et al. 1995].

Reagents and solutions:

Phosphate buffer (0.1 M , pH 7.5)

Denaturing buffer (0.15 M phosphate buffer, 3 % SDS, pH 6.8)

2,4-Dinitrophenylhydrazine (DNPH) (10 mM in 2 M HCl)

EtOH:ethyl acetate (1:1)

Procedure:

333 µl of native or modified LDL (0.5 mg/ml in 0.1 M PO_4^- buffer) was mixed with 67 µl DNPH or HCl (2 M), respectively, whereas the latter was used as blank value, and incubated for 1 hour at room temperature. Then 200 µl of denaturing buffer was added. After mixing, 600 µl of EtOH and 600 µl of heptan were added and the tubes were vortexed for 1 minute. Then the samples were centrifuged at 14,000 rpm for 2 minutes (4°C, Eppendorf Centrifuge 5417R) and afterwards the precipitated protein from the interface was transferred to a new tube. This protein pellet was washed three times with 1 ml of ethanol:ethyl acetate (1:1) and dissolved in 1 ml denaturing buffer. After overnight incubation at room temperature each DNPH sample was measured spectrophotometrically (Perkin Elmer UV/VIS Spectrometer Lamda2) against the corresponding HCl sample at 355 nm. The concentration of the samples was calculated with Lambert-Beer's law (extinction coefficient 22,000 $M^{-1}.cm^{-1}$) and normalized to the protein concentration of the samples, which was determined using the Lowry assay (cpt. 4.4.2).

4.8 **Oil red o staining**

Principle:

Oil red o, a lipid soluble dye, stains cholesterylester and triglycerides in the cells. After extraction with isopropyl alcohol the absorbance can be monitored spectrophotometrically at 510 nm [RAMIREZ-ZACARIAS et al. 1992].

Reagents and solutions:

Oil red o working solution (2 mg/ml):

87.5 mg oil red o powder was dissolved in 25 ml isopropanol and left at room temperature overnight without stirring. The next day the solution was filtered (589 WH, Schleicher and Schuell), 18.75 ml aqua dest. was added and the solution was left at 4°C overnight. Afterwards the solution was filtered twice and filled into a 50 ml Falcon tube which was sealed with parafilm. The working solution was stored at room temperature and could be reused several weeks.

PBS buffer:

137 mM NaCl

2.7 mM KCl

1.5 mM KH_2PO_4

8.1 mM $Na_2HPO_4.12H_2O$

pH 7.4

Isopropyl alcohol

0.1 M NaOH

Procedure:

THP-1 cells were seeded in 12-well plates at a density of $0.5*10^6$ cells/ml ($6*10^5$ cells/well) and differentiated towards macrophages with 160 nm PMA for three days. After incubation the cells were washed twice with PBS and stained for two hours with oil red o working solution during shaking at the belly dancer. After removing the working solution the cells were washed twice with PBS. Excess PBS was evaporated at room temperature. The lipid dyes in the cells were viewed under

the microscope (Zeiss axiovert 135) and documented by a CCD camera and a digital photograph software (spot camera, Metaview, Fig. 10). In order to determine the extent of lipid accumulations in the cells, isopropanol was added to the stained cells, the extracted dye was removed and the absorbance was monitored spectrophotometrically at 510 nm. The measured absorbance was related to the protein content of the cells. Therefore the cells were lysed after isopropanol extraction with 0.1 M NaOH for one hour at room temperature and protein content was measured with the Bradford assay (cpt. 4.4.1).

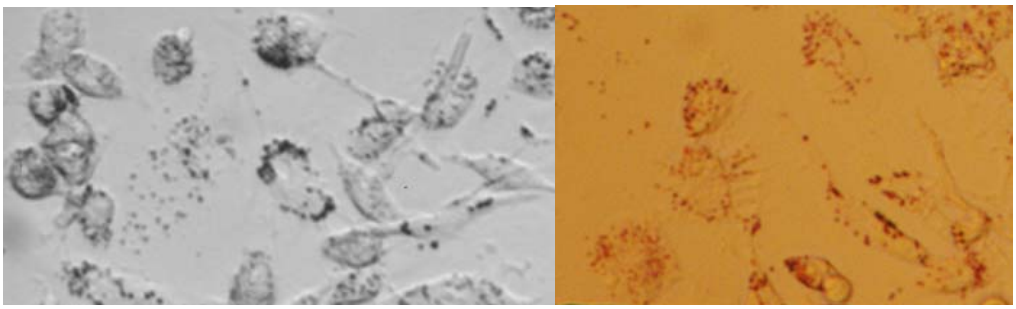


Figure 10: Light microscopy images of THP-1 macrophages after oil red o staining.

4.9 **Neutral red assay**

Principle:

Neutral red assay is a cell viability assay, where the red dye neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is taken up and retained by the lysosomal matrix of living cells (Fig. 11). The amount of dye incorporated in the cells is measured spectrophotometrically at 540 nm. Lower neutral red retention indicates cytotoxic events in the cells [BORENFREUND and PUERNER 1985].

Reagents and solutions:

Neutral red stock solution (0.4% in aqua dest.)

Stored at 4°C

Neutral red working solution

1:80 dilution of the stock solution in RPMI (37°C)

Elution buffer

50 % ethanol

1 % acetic acid

PBS

Procedure:

THP-1 cells were seeded in 96-well plates at a density of $0.5*10^6$ cells/ml ($5*10^4$ cells/well) and differentiated towards macrophages with 160 nm PMA. After 72 hours of differentiation the cells were incubated with the indicated solutions. Afterwards, the cells were washed with 200 µl PBS/well and incubated with neutral red working solution (100 µl/well) for three hours at 37°C and 5 % CO_2. After removing the neutral red solution the cells were washed twice with PBS. 200 µl of elution buffer per well was added for 30 minutes during shaking at the belly dancer to lyse the cell membranes and extract the neutral red dye. Then the absorbance was monitored at 540 nm at a plate reader (Multilabel Counter, Wallac Victor² 1420).

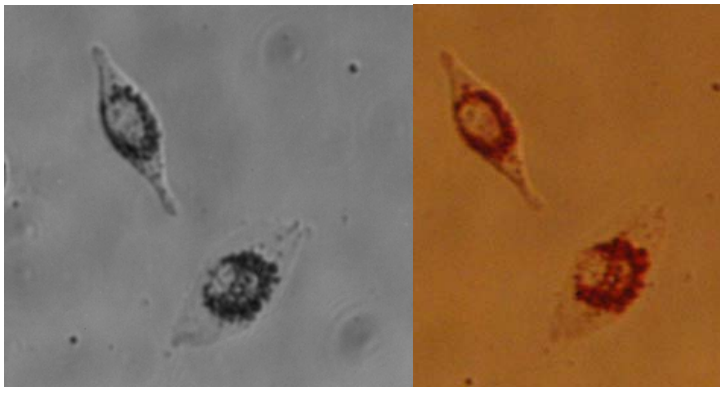


Figure 11: Light microscopy images of THP-1 macrophages, which have incorporated neutral red dye.

4.10 **Measurement of proteasomal activity**

Reagents and solutions:

HEPES-Lysis buffer

- 50 mM HEPES, pH 7.5
- 150 mM NaCl
- 1.5 mM $MgCl_2$
- 1 mM EGTA
- 100 µM NaF
- 10 µM sodium vanadate
- 10 % glycerol
- 1 % Nonidet-P40

s-LLVY-AMC (Succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin) dissolved in DMSO

PBS (ice-cold)

Principle:

s-LLVY-AMC is a synthetic fluoropeptide which acts as substrate for the chymotrypsin-like activity of the proteasome. Due to proteasomal activity AMC is cleaved from this fluoropeptide and can be measured fluorimetrically [GRUNE et al. 1996].

Procedure:

THP-1 monocytes were differentiated towards macrophages with 160 nM PMA for three days in 6-well plates at a density of $0.5*10^6$ cells/ml ($1.5*10^6$ cells/well). Then, the cells were incubated with native or modified lipoproteins for 18 hours at 37°C.

After the incubation the cells were washed twice with ice-cold PBS and then lysed with HEPES lysis buffer. After scraping with a rubber policeman the cell lysates were centrifugated at 14,000 rpm for 10 minutes (4°C, Eppendorf Centrifuge 5417R). The supernatant was transferred to a new eppendorf tube and the protein content was measured using the Bradford assay (cpt. 4.4.1).

The measurement of proteasomal activity was started by addition of s-LLVY-AMC with a final concentration of 20 µM. The samples were transferred to a 96-well plate

and measured with a plate reader (Multilabel Counter, Wallac Victor 1420) at 37°C for 100 minutes at an excitation wavelength of 340 nm and emission wavelength of 480 nm in intervals of 2 minutes.

4.11 **Western Blot**

Principle:
Cell lysates are loaded onto SDS-polyacrylamid gels to separate the proteins. In the presence of the anionic detergens SDS all proteins are negatively charged, the separation in an electric field is mainly due to the molecular weight of the protein. After electrophoretic separation the proteins are blotted from the gel onto a nitrocellulose membran. The proteins of interest are detected immunologically with specific antibodies.

Preparation of Cell Lysates:
Reagents and solutions
PBS-Buffer
Lysis Buffer I:

- 62.5 mM Tris
- 8 M Urea
- 20 mM EDTA
- 20 mM EGTA
- 10% Glycerol
- 1% SDS
- pH 6.8

Lysis Buffer II:

50 mM HEPES, pH 7.5
150 mM NaCl
1.5 mM MgCl
1 mM EGTA
100 µM NaF
10 µM sodium vanadate
10 % glycerol
1 % Nonidet-P40

Protease Inhibitor Cocktail

4x Sample Buffer (1ml):

500 µl glycerol
450 µl buffer (240 µl 0.5 M Tris-HCl pH 6.8, 210 µl aqua dest., 8 % SDS)
a dash of bromphenol blue
50 µl 2-mercaptoethanol (Bio-Rad)
Sample Buffer was always prepared just before use.

THP-1 macrophages were differentiated towards macrophages in 6-well plates at a density of $0.5*10^6$ cells/ml ($1.5*10^6$ cells/well) with 160nM PMA for 72 hours. After incubation the cells were washed twice with cold PBS and solubilized with 75 µl lysis buffer I per well. HUVEC cells were grown until confluency in 6-well plates, after incubation and washing the cells twice they were solubilized with 60 µl lysis buffer II per well. Protease Inhibitor Cocktail was added to lysis buffers (1:100). The cells were scraped with a rubber policeman and transferred into an Eppendorf tube. Afterwards the cell lysates were sonified for about two seconds and incubated on ice for one hour and then centrifugated at 14.000 × g for 10 minutes (4°C) (Eppendorf Centrifuge 5417R). The supernatant was transferred to a new Eppendorf tube. After protein quantification with the Bradford assay (cpt. 4.4.1) the lysates were stored at -20°C.

4.11.1 Sample preparation for electrophoresis

The protein samples were diluted to equal protein amounts with PBS, diluted 4:1 with 4 x sample buffer in a microtube and vortexed. The samples were incubated for 30 minutes at 40°C to denature the proteins. After cooling down the samples to room temperature they were applied to the gel.

4.11.2 SDS-gel electrophoresis

Reagents and solutions:

30% acrylamide/bis solution, 29:1

TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine)

10% sodium dodecyl sulfate (SDS) in aqua dest.

10% ammonium persulfate (APS, Pharmacia Biotech) in aqua dest.

Aliquots of 105 µl were stored at -20°C.

Gel buffer (3 M Tris-HCl, 0.3% SDS, pH 8.45)

Tris/glycine electrophoresis buffer (25 mM Tris base, 200 mM glycine, 1% SDS)

Procedure:

Gel preparation and pouring

	8%	4%
H_2O bidest.	1.6 ml	1.1 ml
30% Acrylamide/Bis Solution	1.1 ml	0.3 ml
3 M Tris-HCl, pH 8.45; 0.3% SDS	1.3 ml	0.7 ml
10% APS	30 µl	20 µl
TEMED	3 µl	2 µl

Table 2: Composition of a SDS-PAGE gel (0.75 mm).

The Mini-PROTEAN®3 Cell (Bio-Rad) was used for electrophoresis.

Prior to use, the glass plates (Bio-Rad) with a spacer thickness of 0.75 mm were cleaned with 70% ethanol and arranged in the gel cassette according to the manufacturer´s instructions.

For the running gel (8%) all components were mixed in a clean tube, 10% APS and TEMED were added immediately before pouring, mixed gently and immediately poured between the glass plates. The gel was overlayed with isopropanol (30%) to prevent the gel from drying out.
After about 30 minutes the running gel was polymerized, isopropanol was removed and the stacking gel (4%) was poured over the polymerized running gel and the comb (10- or 15-well) was inserted.
After additional 30 minutes the gel was fully polymerized and ready to use.

The gel cassettes were put into the electrophoresis chamber according to the manufacturer's instructions. The chamber was filled with Tris/glycine Buffer and after removal of the combs the gel slots were rinsed with Tris/glycine Buffer. Then equal amounts of the samples (5-15 µg protein in 10 µl PBS/15-well slot, 10-30 µg protein in 20 µl PBS/15-well slot) and a protein ladder (PageRuler™ Prestained Protein Ladder, Fermentas) were filled into the slots. Slots without any protein sample were loaded with sample buffer.
Electrophoresis was performed for 80 minutes at 180 Volt in the fridge (4°C).
After the run the gel cassettes were carefully removed and the gels were assembled in the transfer cassettes (cpt. 4.11.3).

4.11.3 Western Transfer Blotting

Reagents and solutions:
Transfer buffer (1x Tris/glycine buffer, 20% methanol)

The buffer was stored at 4°C and reused several times.

Ponceau S (1% Ponceau, 30% trichloroacetic acid, 30% sulfosalicylic acid)
Coomassie stock solution (0.2 %):

0.4 g Coomassie blue dissolved in 80 ml aqua dest. and 120 ml methanol

Coomassie working solution (0.1 %):

50 ml stock solution (filtered) mixed with 10 ml acetic acid and 40 ml aqua dest.

Destain solution (20% methanol, 10% acetic acid, 70% aqua dest.)

Procedure:

For transfer blotting the Mini-Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) was used. The transfer sandwich components were assembled according to the manufacturer's instructions:

white side of the cassette (facing positive electrode)
sponge
Whatman paper
nitrocellulose membrane (Trans-Blot® Transfer Medium, Bio-Rad)
gel
Whatman paper
sponge
black side of the cassette (facing negative electrode)

Sponges, Whatman papers and the nitrocellulose membrane were moisted with transfer buffer prior to assembling. Air bubbles between the gel and the nitrocellulose were removed by rolling with a glass pipet over the Whatman paper. The transfer sandwich was assembled in the blotting apparatus together with an ice block to cool the buffer. The transfer was carried out at 4°C for 45 minutes with 0.4 ampere.

Afterwards the gel was stained with coomassie dye solution and the nitrocellulose with Ponceau S to ensure complete transfer of the proteins.

Thereafter the nitrocellulose membrane was cut according to protein sizes, so that one membrane could be used to detect up to four proteins with different sizes.

The Ponceau S staining was removed by washing with TBS-T (see below) for about five minutes.

4.11.4 Immunodetection

Reagents and solutions:

10x TBS buffer (200 mM Tris base, 1.45 M NaCl, pH 7.4)

Washing buffer TBS-T (1xTBS buffer, 0.1% Tween 20)

Blocking buffer (5% (w/v) milk (non-fat, dry milk; Maresi) or 3% BSA in TBS-T

The blocking buffer was prepared freshly prior to use

Primary antibody:

Primary antibodies were diluted in blocking buffer at the recommended dilution.

anti-ß-Actin (Sigma): mouse IgG2A, 42 kDa, 1:5,000 in 3% BSA in TBS-T

anti-CD36 (Santa Cruz): mouse IgM, 70 kDa, 1:150 in 3% BSA in TBS-T

anti-SR-BI (abcam): rabbit IgG, 82 kDa, 1:3,000 in 5% milk in TBS-T

anti-Calnexin (abcam): rabbit IgG, 90 kDa, 1:10,000 in 5% milk in TBS-T

anti-HIF-1α (BD Transduction Laboratories): mouse IgG1, 120 kDa, 1:300 in 3% BSA in TBS-T

A dash of NaN_3 was added to avoid contamination. Primary antibody solutions were reused several times and stored at 4°C.

Secondary antibody:

Goat anti-mouse IgG-HRP conjugate (Bio-Rad, ß-Actin & HIF-1α), goat anti-rabbit IgG-HRP conjugate (Bio-Rad, SR-BI & Calnexin) as well as rabbit anti-mouse Ig-HRP (DakoCytomation, CD36) were diluted 1:10,000 in blocking buffer.

Procedure:

First the proteins on the membrane were incubated with blocking buffer for one hour at room temperature with shaking at the belly dancer to block nonspecific sites. Blocking was accomplished with 5% milk in TBS-T, except for detecting CD36, which was blocked with 3% BSA in TBS-T.

After blocking, the membrane was washed twice with washing buffer and incubated with primary antibody overnight at 4°C on a belly dancer.

The next day the antibody was removed and stored in the fridge until the next use.

The blots were washed three times with TBS-T for ten minutes. Afterwards they were incubated with secondary antibody for one hour at room temperature with shaking at the belly dancer. Afterwards the antibody solution was discarded and the membranes were washed again three times.
Finally, the nitrocelluloses were incubated for 5 minutes with ChemiGlow™ West (Alpha Innotech Corporation, CA USA). First, the ChemiGlow™ West working solution was prepared prior to use by mixing the same volume of Luminol/Enhancer Solution and the Stable Peroxidase Solution. Afterwards, the blots were wrapped in plastic wrap and positioned under a CCD camera in the ChemiImager™ 4400 (Biozym) for detection. Quantification was performed using the AlphaEase FC software (Alpha Innotech Corporation, CA USA).

4.12 **Immunocytochemistry**

Reagents and solutions:
10 x TBS^{++} (150 mM NaCl, 20 mM Tris, 2 mM $CaCl_2$, pH 7.4)
Formaldehyde solution (4%):

- 0.8 g paraformaldehyde
- 18 ml aqua bidest
- 50 µl 10 M NaOH
- 2 ml of 10 x TBS^{++}
- pH 7.4
- The solution was stored at 4°C for a maximum of two weeks.

0.2% TritonX-100 dissolved in TBS^{++}
1% glycine dissolved in TBS^{++}
1st antibody dilution in 1% BSA-TBS^{++}

- (SR-BI 1:1000, abcam; CD36 1:100, santa cruz)

2nd antibody dilution in 1% BSA-TBS^{++}

- (SR-BI: TRITC-conjugated mouse antibody 1:100, CD36: TRITC-conjugated mouse antibody 1:100; maximum of absorption: 510nm, emission: 620nm; Dako Cytomation)

4',6-Diamidino-2-phenylindol (DAPI, 100 µg/ml PBS)

Procedure:

THP-1 cells were grown on glass coverslips. Therefore sterile glass coverslips were put in each well of a 12-well plate prior to cell seeding. THP-1 monocytes were differentiated towards macrophages with 160 nM PMA for 72 hours at a density of $0.5*10^6$ cells/ml ($2.5*10^5$ cells/well) to allow growing on glass coverslips at about 50 % confluency with or without subsequent incubation of the cells for 18 hours.

THP-1 monocytes, which are maintained in suspension, were spun onto glass coverslips (120,000 cells per glass coverslip) using the cytospin (Cytospin 2, Shandon) at 350 U for 10 minutes.

Prior to the fluorescent staining procedure the cells were washed three times for 5 minutes with ice cold TBS^{++} and subsequently fixed on the glass coverslips by incubation with paraformaldehyde solution for 20 minutes at room temperatur. Afterwards, the cells were washed again 3 times for 5 minutes. To allow diffusion of reactants to stain HIF-1α protein, which is found intracellularly, cells were permeabilized with TritonX-100 for about 2 minutes. Thereafter cells were washed again. Prior to antibody incubation, free aldehyde-groups were blocked with glycin for 10 minutes, followed by washing three times.

Then THP-1 cells were incubated with primary antibodies for 1 hour at room temperature while shaking. To avoid evaporation, the 12-well plate was wrapped with wet tissues during the incubation and shaken at the belly dancer. The primary antibody was then removed and the cells were washed three times for 5 minutes with ice cold TBS^{++}.

The secondary antibody, which was labeled with TRITC, was added to the cells for 45 minutes with shaking at the belly dancer at room temperature in the dark. Afterwards the cells were washed four times to remove excess antibody.

Finally, cell nuclei were stained with DAPI at a concentration of 1 µg/ml PBS. After 5 minutes of incubation at room temperature, cells were washed again three times.

Then the coverslips were dried in the dark and mounted in glycerol on microscope slides. The coverslips were sealed and fixed at the border to the microscope slides with clear nail polish.

The fluorescent cell preparations were observed under a fluorescence microscope (Zeiss axiovert 135) and photographed and documented by a digital photograph software (spot camera, Metaview).

4.13 **RNA isolation**

Principle:
TriFast™ is a complete ready to use reagent for the isolation of total RNA, DNA and proteins from cells and tissues. TriFast™ includes phenol and guanidinium thiocyanate in a monophasic solution. After addition of chloroform and a centrifugation step the homogenate separates into three phases. The aqueous phase containes the RNA, the interphase the DNA and the organic phase the protein.

Reagents and solutions:
peqGOLD TriFast™ (peqlab)
RNase-free water (0.01% (v/v) of diethylpyrocarbonate (DEPC) in deionized water)
RNAse free plasticware (soaked in RNase-free water overnight and autoclaved)
Chloroform
Isopropanol
75% ethanol in DEPC treated aqua dest.

Procedure:
After two washes with PBS the cells were homogenized in the well (9.6 cm²) by addition of 1 ml TriFast™ per well and repeatedly drawing up and squeezing out the lysate with a pipette. Then the samples were transferred to a 2 ml tube.
The samples were kept for about 10 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. After addition of 200 µl of chloroform they were shaken vigorously for 30 seconds and again kept for 10 minutes at room temperature.
The phases of the samples were separated by centrifugation for 5 minutes at 12,000xg (4°C, Eppendorf Centrifuge 5417R). The mixture separates into the red

phenol-chloroform phase, the interphase and the colourless upper aqueous phase. RNA remains exclusively in the aqueous phase.
The aqueous phase containing the RNA was transferred to a fresh tube containing 500 µl isopropanol to precipitate the RNA. After two hours (-20°C) and centrifugation at 12,000xg for 10 minutes (4°C), the RNA pellet formed a gel-like precipitate on the bottom and the side of the tube.
The supernatant was removed carefully and the RNA pellet was washed twice with ethanol (75%) by vortexing and subsequent centrifugation for 10 minutes at 12,000xg (4°C).
The supernatant was removed carefully and the RNA pellet was air-dryed and resuspended in DEPC treated water by passing through a pipette tip several times and incubating for 10 minutes at 55°C.

4.14 **Reverse transcription reaction**

Reagents and solutions were from Fermentas.
12 µl of the template RNA was mixed with 1 µl (0.2 µg) Random Hexamer Primer, incubated for 5 minutes at 70°C and chilled on ice. Afterwards 8.5 µl mastermix of the remaining substances (Tab.3) was added and the samples were incubated at 37°C for one hour and subsequently at 70°C for 10 minutes to degrade the Reverse Transcriptase and therefore terminate the reaction.

	volume	*final concentration*
Total RNA	*12 µl*	
Random Hexamer Primer (0.2 µg/µl)	*1 µl*	*0.2 µg*
5 x Reaction buffer	4 µl	1 x
dNTP Mix (10 mM)	2 µl	1 mM
Ribonuclease Inhibitor (40 U/µl)	0.5 µl	1 U
M-MuLV Reverse Transcriptase (200 U/µl)	0.5 µl	5 U

Table 3: Pipetting scheme of Reverse transcription sample preparation.

4.15 **RT-PCR**

Reagents and solutions (Fermentas) :
Primer sequences of gene-specific PCR-Primer (VBC Genomics) were designed using PRIMER 3 software available online.

For the PCR-reaction the cDNA was mixed on the basis of the following mastermix (final volume of 50 µl, Tab.4):

	volume	*final concentration*
aqua dest (steril)	31 µl	
Reaction buffer (10x)	5 µl	1x
$MgCl_2$ (25mM)	5 µl	2.5 mM
dNTPs (10 mM)	4 µl	0.8 mM
sense/antisense primer (100 µM each)	1.5 µl	3 µM each
Taq-Polymerase (1U/µl)	1 µl	0.02 U/µl
cDNA	1 µl	

Table 4: Pipetting scheme of RT-PCR master mix preparation.

PCR reaction was carried out in a thermocycler (MWG Biotech) according to the following temperature program (Tab.5). Annealing temperature (AT) and number of cycles were chosen dependent on the gene of interest (Tab.6 and 7).

initial denaturation	94°C, 3 minutes	
Denaturation	94°C, 40 seconds	25-35 cycles
Annealing	AT, 1 minute	
Elongation	72°C, 1 minute	
final elongation	72°C, 5 minutes	
Store at	4°C	

Table 5: RT-PCR temperature cyles.

Gene of interest	*GenBank-Number*	*Annealing-temperature*	*Product Size*	*Number of Cycles*
GAPDH	NM_002006	62°C	185 bp	25
ß-Actin	NM_001101	59°C	579 bp	25
HIF-1α	NM_001530	59°C	453 bp	28
SR-BI	NM_05505	60°C	462 bp	35
CD36	NM_001001547	60°C	428 bp	30
VEGF	NM_001025366	60°C	490 bp	30

Table 6: Description of primers used for RT-PCR.

Gene of interest		*Primer sequences*		
ß-Actin	a	5’	Agagctacgagctgcctga	3’
	b	5’	Caccttcaccgttccagtt	3’
HIF-1α	a	5’	Agatactcaaagtcggacagcc	3’
	b	5’	Tttctgctgccttgtataggag	3’
VEGF	a	5’	Aaggaggagggcagaatcat	3’
	b	5’	Aaatgctttctccgctctga	3’
SR-BI	a	5’	Acctcgtggacaagtggaac	3’
	b	5’	Cagagcagttcatggggatt	3’
CD36	a	5’	Atgtaacccaggacgctgag	3’
	b	5’	Gtcgcagtgactttcccaat	3’
GAPDH	a	5’	Ggagccaaaagggtcatcatctc	3’
	b	5’	Gtcatgagtccttccacgatacc	3’

Table 7: Sequences of primers used for RT-PCR.

4.15.1 PCR-gel electrophoresis

Reagents and solutions:
Agarose
Ethidiumbromide (0.5 ng/ml)
TBE buffer (5x, 1 l): 54 g TRIS, 27.5 g borate, 20 ml 0.5 M EDTA (pH 8)
6x DNA loading dye (Fermentas)
GeneRuler^{TM} 100 bp DNA Ladder (Fermentas)

PCR-products were visualised using agarose gel electrophoresis. After dissolving 40 g of agarose in 40 ml TBE buffer (1 % agarose gel) and boiling in the microwave the solution was allowed to cool down to about 60°C. Then ethidium bromide (EtBr) was added to the gel solution with a final EtBr concentration of 0.2 µg/ml. After mixing the gel solution it was casted into the gel bed, a comb (8 or 15 well) was inserted and the gel was allowed to jell at room temparature. Then the gel was assembled into an electrophoresis chamber, which was filled with TBE buffer.
After mixing the PCR samples with loading dye they were filled into the gel slots. To check the size of the PCR products a DNA ladder was also filled into one gel slot. After electrophoresis at 100 Volt for about 25 minutes the DNA was visualized using a UV transilluminator (Hoefer scientific instruments) and photographed. Density of the bands was measured and quantified using AlphaEase FC software.

4.16 **Northern Blot**

Basic principle:
After isolating, RNA is separated by gel electrophoresis, blotted onto a membrane and detected with radiolabeled olignucleotide probes.

Preparation of the probes:

Reagents and solutions:

LB-agar-plates:

200 ml LB-agar (37 g/l) was prepared, autoclaved, spiked with 100 µg/ml ampicillin and poured into 60 mm dishes.

HIF-1α probe was purchased form LGC Standards (ATCC-10658590, I.M.A.G.E. Clone ID: 3842146, pOT7-vector; LGC Standards).

SR-BI probe was kindly provided by Monty Krieger (murine SR-BI full length cDNA cloned into TOPO vector; Invitrogen).

Procedure:

2 µl of the plasmids were used for transformation with 25 µl One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) according to the manufacturer's manual. Afterwards, the cells were spreaded onto LB-Agar plates containing ampicillin and incubated at 37°C over night.

On the next day 4 ml LB-medium containing 100 µg/ml ampicillin were inoculated with a single colony from the agar plate and allowed to proliferate over night at 37°C during shaking.

The next day the GenElute™ Plasmid Miniprep Kit (Sigma) was used to generate small-scale plasmid DNA preparations according to the manufacturer's manual and the minipreps were screened for a positive clone. To generate large-scale plasmid DNA preparations a HiSpeed® Plasmid Midi Kit (Qiagen) was used according to the manufacturer's manual.

EcoRI was used to digest SR-BI, and both EcoRI and XhoI for HIF-1α. The digests were loaded onto an agarose gel, the corresponding bands were cut into small pieces and eluated from the gel using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Afterwards the probes were concentrated using a SpeedVac concentrator (SVC 100H, Savant). To determine the concentration of the probes they were loaded onto an agarose gel together with a MassRuler DNA LadderMix (Fermentas).

SR-BI-probe (440 bp)

GCTCGGAGAGCGACTACATCGTCATGCCCAACATCCTGGTCTTGGGTGCGGCGGTGATGATGG
AGAATAAGCCCATGACCCTGAAGCTCATCATGACCTTGGCATTCACCACCCTCGGCGAACGTGC
CTTCATGAACCGCACTGTGGGTGAGATCATGTGGGGCTACAAGGACCCCCTTGTGAATCTCATC
AACAAGTACTTTCCAGGCATGTTCCCCTTCAAGGACAAGTTCGGATTATTTGCTGAGCTCAACAA
CTCCGACTCTGGGCTCTTCACGGTGTTCACGGGGGTCCAGAACATCAGCAGGATCCACCTCGT
GGACAAGTGGAACGGGCTGAGCAAGGTTGACTTCTGGCATTCCGATCAGTGCAACATGATCAAT
GGAACTTCTGGGCAAATGTGGCCGCCCTTCATGACTCCTGAGTCCTCGCTGGAGTTC

HIF-1 α probe (604 bp)

AATTCTCAACCACAGTGCATTGTATGTGTGAATTACGTTGTGAGTGGTATTATTCAGCACGACTT
GATTTTCTCCCTTCAACAAACAGAATGTGTCCTTAAACCGGTTGAATCTTCAGATATGAAAATGAC
TCAGCTATTCACCAAAGTTGAATCAGAAGATACAAGTAGCCTCTTTGACAAACTTAAGAAGGAAC
CTGATGCTTTAACTTTGCTGGCCCCAGCCGCTGGAGACACAATCATATCTTTAGATTTTGGCAGC
AACGACACAGAAACTGATGACCAGCAACTTGAGGAAGTACCATTATATAATGATGTAATGCTCCC
CTCACCCAACGAAAAATTACAGAATATAAATTTGGCAATGTCTCCATTACCCACCGCTGAAACGC
CAAAGCCACTTCGAAGTAGTGCTGACCCTGCACTCAATCAAGAAGTTGCATTAAAATTAGAACCA
AATCCAGAGTCACTGGAACTTTCTTTTACCATGCCCCAGATTCAGGATCAGACACCTAGTCCTTC
CGATGGAAGCACTAGACAAAGTTCACCTGAGCCTAATAGTCCCAGTGAATATTGTTTTTATGTGG
ATAGTGATATGGTCAATG

4.16.1 RNA isolation

For Northern Blot analyses THP-1 cells were differentiated towards macrophages with 160 nM PMA for 3 days in petri dishes (8 cm diameter) at a density of $0.5*10^6$ cells/ml ($10*10^6$ cells/dish). RNA was isolated using Rneasy Mini Kit (Qiagen) according to the manufacturer's manual. The concentration of RNA was determined by measuring the absorbance at 260 nm spectrophotometrically (Ultraspec 2100 pro, Amersham Pharmacia Biotech). Then the RNA was precipitated over night with sodium acetate (3 M, pH 5, 1/10 of the RNA volume) and 2-fold RNA volume of ethanol at -20°C. The next day the RNA precipitate was centrifuged for 10 minutes at high speed (14.000 rpm, 4°C, Eppendorf Centrifuge 5417R), washed with EtOH (70 %) and then resuspended in DEPC-H_2O (cpt. 4.13) at a final concentration of 1 µg/µl.

4.16.2 Gel electrophoresis

Reagents and solutions:

20x MOPS (3-morpholinopropanesulfonic acid, pH 8)

83.6 g MOPS

13.6 g NaOAc

40 ml EDTA (0.5 M, pH 8)

3.2 g NaOH

filled up to 1 l with DEPC-H_2O, steril filtrated (0.2 µM) and stored in the dark at 4°C

Denaturant:

620 µl formamide

220 µl formaldehyde (37 %)

62 µl 20x MOPS

Sucrose/BPB:

5 g sucrose

10 mg bromphenol blue

856 mg EDTA

filled up to 10 ml with DEPC-H_2O

Loading dye:

100 µl sucrose/BPB

33 µl ethidium bromide

Agarose gel:

3 g of RNAse free agarose was dissolved in 280 ml DEPC-H_2O in the microwave. After cooling down to about 60°C 15 ml of 20x MOPS and 5.5 ml of formaldehyde (37 %) were added. Then the gel was casted to the gel bed, a 15-well comb was inserted, and the gel was allowed to jell at room temperature.

Procedure:

After soaking the electrophoresis equipment over night in SDS (5 % in DEPC-H_2O) it was washed with DEPC-H_2O. After preparing the gel the samples were prepared as follows: 9 µl RNA (1 µg/µl)

27 µl denaturant

4 µl loading dye

After incubating the samples for 15 minutes at 65 °C they were cooled down on ice and filled into the gel slots. GeneRuler™ Plus (Fermentas) was mixed with ethidium bromide and also filled into one slot.

Electrophoresis was accomplished on ice in MOPS buffer at 100 Volt at the first hour, 120 Volt at the second and 130 Volt at the third hour.

Then the gel was viewed and documented in a UV chamber (UVP DigiDoc-H® Digital Imaging workstation) with a digital camera (Olympus C-4000). 18s and 28s bands were marked with a needle in the gel.

4.16.3 Northern blotting

Reagents and solutions:

20x SSC: 175.3 g NaCl

88.2 g tri-sodium citrate

filled up to 1 l with DEPC-H_2O, pH 7.0

Procedure:

The gel was cut to size and washed three times in 10x SSC for ten minutes, the nylon membrane (Hybond-N, amersham pharmacia biotech) was cut to the same size as the gel and soaked in 10x SSC.

Northern Blot sandwich was assembled as follows:

A glass plate was fixed over a 6x SSC reservoir. Two pieces of Whatman paper were placed on the glass plate crosswise, large enough to reach the SSC reservoir. Then the gel, top-side down, and afterwards the membrane were placed onto the sandwich. Air bubbles between paper, gel and membrane were removed by rolling a glass pipet across. A nylon foil dam was assembled around the border of the gel

and the membrane to avoid drying out. Then a Whatman paper of the same size as the gel and the membrane was placed onto the membrane. After adding a thick layer of paper towels and a big glass plate on top and weighing down the sandwich the transfer was allowed to happen over night.
The next day the 18s and 28s bands were marked with a pencil through the hole in the gel and then the sandwich was broken down. After drying the membrane the RNA, transferred to the membrane, was crosslinked at 1200 Joule (uvilink UV crosslinker, CL-E508, UVITEC Ltd). The membrane, wrapped into plastic wrap, was stored at 4°C until hybridization.

4.16.4 Hybridization

Reagents and solutions:

50x Denhardt's:

1 % Ficoll
1 % polyvinylpyrrolidon
1 % BSA (fraction V)
sterilfiltrated aliquots (1.6 ml) were stored at -20°C

ssssDNA (Sigma, 10 mg/ml):

After stirring the solution at room temperature for 3 hours it was pulled through a 17-gauge-needle 12 times, boiled for 10 minutes and stored in 0.8 ml aliquots at -20°C.

20x SSPE:

3 M NaCl
0.2 M NaH_2PO_4
0.02 M EDTA
in DEPC-H_2O, pH 7.4

10 % SDS:

dissolved in DEPC-H_2O, pH 7.2

10x TE buffer pH 7.6:

100 mM TRIS-HCl
10 mM EDTA

Final concentration	Reagent	Total 40 ml
50 %	Formamide	20 ml
5x	20x SSPE	10 ml
2x	50x Denhardt's	1.6 ml
0.1 %	10 % SDS	0.4 ml
200 µg/ml	ssssDNA (10 mg/ml)	0.8 ml
10 %	Dextran sulphate	4 g dissolved in 7.2 ml DEPC-H_2O

Table 8: Pipetting scheme of hybridization.

Each blot was prehybridized for at least 2 hours in 40 ml hybridization buffer at 42°C.

During prehybridization the HIF-1α and SR-BI probes were prepared as follows:
150 ng of the oligonucleotide probe was filled up to 45 µl with TE buffer and incubated at 95°C for 5 minutes and on ice for additional 5 minutes. Then it was transferred to the labelling reaction tube (Random Prime Labeling Kit, Amersham Biosciences), mixed by pipetting up and down and spiked with 5 µl of [^{32}P]dCTP (Hartmann Analytic). After vortexing the probe was incubated at 37°C for 10 minutes. Then 5 µl of EDTA (0.2 M) was added and nucleotide removal (QIAquick® Nucleotide Removal Kit, Qiagen) was carried out according to the manufacturer's protocol. The probe was eluted in 100 µl, stored at 4°C and shortly before adding to the blot it was incubated at 95°C for 5 minutes. After cooling down the radioactivity was measured in a ß-counter (2000 CA TRI-CARB® Liquid Scintillation Analyzer, PACKARD). The probe should have about 1 to 1.5 µCu and was added to the hybridization buffer. Hybridization was performed at 42 °C over night.

The 28s probe was prepared as follows:

Reagent	Total 50 µl
Oligonucleotide 28S 10 pmol (VBC Genomics)	1 µl
T4 PNK Buffer 10 x (usb corporation)	5 µl
[γ-^{32}ATP] (Hartmann Analytic)	5 µl
DEPC-H_2O	37 µl
T4 Polynucleotide Kinase (3U/µl; usb corporation)	2 µl

Table 9: Pipetting scheme of 5'-end labeling of the 28S probe.

28S probe solution was vortexed, spun down and incubated at 37°C for 30 minutes. The reaction was stopped by adding 1 µl of EDTA (0.5 M, pH 8) and the solution was cleaned using the Nucleotide Removal Kit (QIAquick® Nucleotide Removal Kit, Qiagen) according to the manufacturer's protocol. Then it was incubated at 95°C for 5 minutes, cooled down and added to the hybridization buffer.

4.16.5 Detection

The next day the blots were washed for half an hour with 2x SSC at 42°C, followed by another washing step with SSC/0.1% SDS. The blots were then wrapped in plastic wrap, exposed to a phosphor screen (Storage Phosphor Screen GP, Kodak) in a cassette for 0.5 - 2 hours and then documented (Molecular Imager® FX, Bio-Rad). Density of the bands was measured using AlphaEase FC software and SR-BI and HIF-1α bands were normalized to 28S band density. Phosphor screens were erased at a Screen Eraser (Screen Eraser-K, Bio-Rad).

4.17 **Cholesterol efflux measurements**

Reagents and solutions:
^{3}H-cholesterol (Perkin Elmer)
Fatty acid free BSA (FAF-BSA, PAA)
PBS

THP-1 cells were differentiated towards macrophages with 160 nM PMA for 72 hours in 12-well plates at a density $0.5*10^{6}$ cells/ml ($6*10^{5}$ cells/well). After 48 hours of differentiation ^{3}H-cholesterol (2 µCi) was added to the cells overnight. Then, DETA-NO (300 µM) and/or HDL (150 µg/ml) was added for 8 hours. 72 hours after addition of PMA the cells were washed with warm, sterile PBS and incubated 16 hours overnight with oxLDL (100 µg/ml) and/or HDL and DETA-NO in FCS-free RPMI medium with 5% hlpds (human lipoprotein deficient serum).
The next day the supernatant of the cells was removed and the cells were equilibrated twice for 20 minutes at 37 °C with RPMI/FAF-BSA (2 mg/ml). Then the cells were incubated for 6 hours at 37°C with HDL (10 µg/ml) in RPMI/FAF-BSA (2 mg/ml). After this treatment cell supernatant was collected and centrifuged (3000 rpm, 10 min, eppendorf centrifuge 5403). The counts per minute (cpm) within the supernatant were subsequently measured in a ß-counter (2000 CA, TRI-CARB® Liquid Scintillation Analyzer, Packard).
After removal of cell supernatant and two washing steps with ice-cold PBS the cells were lysed with 0.1 M NaOH. The cell lysates were divided into two parts, whereas one was utilized for scintillation counting and the other for protein determination (Bradford assay, cpt. 4.4.1).
After protein adjustment of the measured counts cholesterol efflux in THP-1 cells was calculated as follows:

$$\% \text{ efflux} = \frac{\text{cpm (supernatant)} \times 100}{\text{cpm (total)}}$$

4.18 Quantification of Vitamin C and cholesterol by high performance liquid chromatography (HPLC)

4.18.1 Determination of Vitamin C

Reagents and solutions:

Ascorbate

Ascorbate oxidase

TBSG:

- 140 mM NaCl
- 20 mM Tris
- 5mM glucose
- pH 7.4

meta-phosphoric acid (MPA, 10%, 5%)

mobile phase:

- 100 mM Na_2HPO_4
- 2.5 mM Na_2EDTA
- 0.2 mM n-dodecyltrimethylammoniumchloride

0.125 M Tris

10 mM dithiothreitol (DTT) in Tris (0.5 M)

Principle:

After chromatographic separation vitamin C is measured by electrochemical detection using its oxidizability for detection. This detection method is very specific and sensitive, but only ascorbate can be detected, as dehydroascorbate (DHA) is not electrochemically active. Therefore, DHA has to be reduced back to ascorbate with 1,4-dithiothreitol (DTT) before detection.

Procedure:

THP-1 cells were differentiated towards macrophages with 160 nM PMA in 6-well-plates for three days (cpt. 4.11). Cells were washed with warm TBSG and then incubated at 37°C in RPMI (without FCS) with either ascorbate (100 µM) alone, or ascorbate (100 µM) and ascorbate oxidase (1 Unit/well) together to generate dehydroascorbate (DHA), for different time points.

For measuring extracellular ascorbate or DHA respectively, 300 µl of the cell supernatant was used. Then the cells were washed with ice-cold TBSG to stop the incorporation of vitamin C.

4.18.1.1 Measurement of extracellular ascorbate:

300 µl of cell supernatant was mixed with 300 µl MPA (10%). 250 µl of this mixture was pipetteted into an HPLC vial. Then 250 µl of MPA (5%) and 500 µl of TRIS (0.125 M) were added.

4.18.1.2 Measurement of extracellular dehydroascorbate (DHA):

250 µl of the supernatant/MPA 10% (see above) was incubated with 250 µl MPA (5%) and 250 µl 10 mM DTT (in 0.5 M TRIS) for 20 minutes at room temperature in a HPLC vial, to reduce DHA back to ascorbate. Then 250 µl of H_2SO_4 (0.2 M) was added to stop the reaction.

4.18.1.3 Measurement of intracellular ascorbate:

Within the cells DHA is reduced back to ascorbate, so only ascorbate content of THP-1 macrophages was measured.

After washing the cells with cold TBSG, 300 µl of MPA (5%) was added to the wells. Then the cells were scraped with a rubber policeman and the mixture was transferred to an Eppendorf tube. Then the remaining cells in the well were scraped again with 300 µl of MPA (5%). The cell suspension was sonified to solubilize the cells and 500 µl of this cell suspension was mixed with TRIS (0.125 M) in a HPLC vial.

The other part of the cell suspension was used to perform a Bradford assay (cpt. 4.4.1) to determine cell protein content of the samples.

4.18.1.4 HPLC-System:

After vortexing the vials were put into the HPLC autosampler (AS-1550-10, Jasco), which was cooled to 4°C. For the measurement 20 µl of the sample was injected, transported to the columns through a pump (PU-1580, Jasco) and the flow was adjusted to 1 ml/min. The separation occured according to Lykkesfeld et al. through a reverse-phase column (Spherisorb ODS-2 3µm, 125 x 4.6 mm) with a preponed precolumn (Hypersil C18 ODS) [LYKKESFELDT et al. 1995].
The signals were generated by an electrochemical, amperometric detector (BAS, LC-4C) with a glass/carbon-electrode (CC-5E), which was directed against an Ag/AgCl-reference electrode of 600 mV. The amperage was setted to 50 nA.
The measuring time of each sample was 7 minutes.
A standard curve was generated to quantify ascorbate concentrations.
The HPLC system was flushed after every set of measurements with 50% methanol.

4.18.2 Analysis of cellular cholesterol content

Reagents and solutions:
PBS
PBS^+: 0.2% BSA dissolved in PBS buffer
hexane:isopropanol (3:2)
ergosterol stock (1 mg/ml Fluka)
0.1 M NaOH
ethanolic KOH (EtOH 96%:KOH 50%, 47:3)
EtOH 96%:chloroform (8:2)
Mobile phase (acetonitril:isopropanol, 8:2)

Principle:
After lipid extraction of the cells cholesteryl esters were hydrolyzed with KOH (3%) to measure total cholesterol content. The saponified samples were dried and then taken up in ethanol:chloroform (8:2) and applied to HPLC analysis using a dual absorbance detector at a wavelength of 210 nm.

Procedure:

THP-1 cells were differentiated towards macrophages with 160 nM PMA for 3 days in 60 mm dishes at a density of $0.5*10^6$ cells/ml ($4.5*10^6$ cells/dish). Then the cells were treated with native or modified LDL (100 µg/ml) for 18 hours at 37°C. After the incubation period, the cells were washed twice with PBS^+ and twice with PBS. Then 3 ml hexane:isopropanol (3:2) was added to the cells for 30 minutes to extract the lipids. To quantitate intracellular cholesterol, an internal standard of ergosterol (12 µg/dish, dissolved in hexane:isopropanol 3:2) was added. The lipid extracts were then transferred to glass tubes and the cells were washed with 3 ml hexane:isopropanol (3:2), which was also transferred to the glass tube. The cells were lysed with 0.1 M NaOH and protein content was measured using the Bradford assay (cpt. 4.4.1).

The hexane:isopropanol solution was divided, after drying the extracted lipids under a N_2 athmosphere one part was stored at 4°C until HPLC measurements for assessment of free cholesterol, the other part was saponified with ethanolic KOH to measure total cholesterol.

Therefore, the extracted lipids were taken up in 5 ml ethanolic KOH and saponified at 70°C for one hour. The samples were mixed every ten minutes during saponification.

Then the tubes were cooled down to room temperature, 5 ml of aqua dest. was added and the lipids were extracted with 10 ml hexane:isopropanol (3:2). After vortexing for 30 seconds the upper organic phase was collected. The lower phase was extracted a second time with 10 ml hexane:isopropanol and then the upper phase was added to the tubes of the first extraction, dried under N_2, and taken up in 1 ml ethanol:chloroform (8:2). The samples were filtrated through a syringe filter (sterile Acrodisc, 0.45 µM, Gelman Sciences) into HPLC screw neck vials (Waters) to get rid of solid components and applied to HPLC analysis.

HPLC analysis was performed on a separation module (Waters 2695, Waters, Vienna, Austria) using a Nova-Pack C18 4 µm column (3.9 x 150 mm) and a Dual Absorbance detector (Waters 2487, Waters, Vienna, Austria) set at a wavelength of 210 nm. Acetonitril:isopropanol (8:2) was used as running solution at a constant flow of 1 ml/min. Every sample was run for 30 minutes.

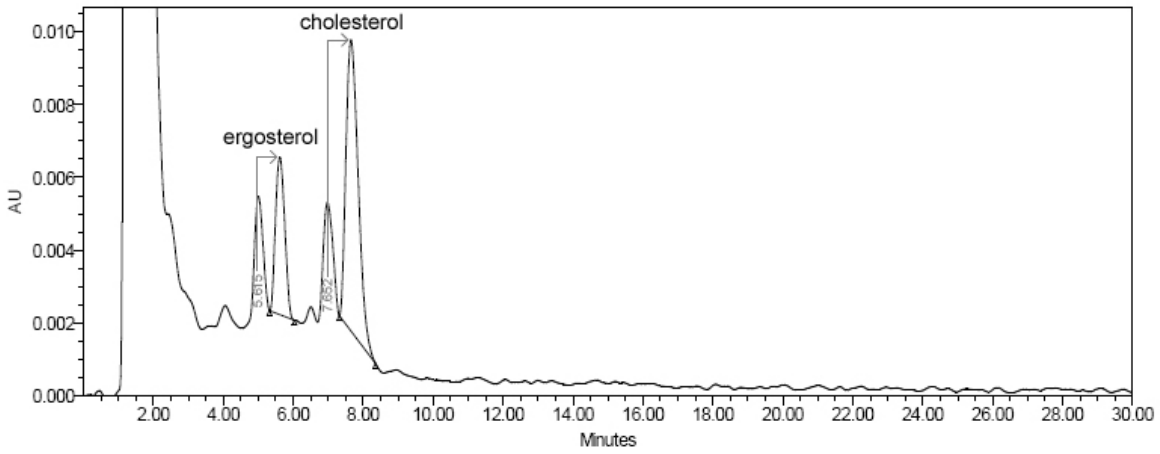


Figure 12: Representative HPLC-Peaks of ergosterol and cholesterol.

Ergosterol was detected at an elution time of about 5.6 and cholesterol after 7.6 minutes.

For quantification areas under both peaks were measured and total cholesterol content of the cells was calculated relating the cholesterol peak area to the ergosterol peak area and to protein content. At same mass (µg), the ratio of peak area ergosterol/cholesterol = 1.7. Cholesteryl ester content was calculated as total cholesterol minus free cholesterol.

The HPLC column was flushed with aqua dest for 1 hour to remove excess acetonitril and with 50% methanol for several hours at a column temperature of 50°C to remove the residual lipids from the column.

4.19 **Statistics**

The results are presented as mean values (MEAN) of 3 to 6 experiments ± standard deviation (SD). Specific effects were evaluated by one-way analysis of variance (ANOVA) with *post hoc* testing using Newman-Keuls´ test. Values of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were considered as statistically significant.

5 Results

5.1 Expression of CD36 and SR-BI protein during differentiation of THP-1 cells

THP-1 monocytes were differentiated towards macrophages with 160 nM phorbol 12-myristate 13-acetate (PMA) for up to 72 hours. After cell lysis the protein expression levels of the macrophage scavenger receptors SR-BI and CD36 were assessed by Western blot analysis.

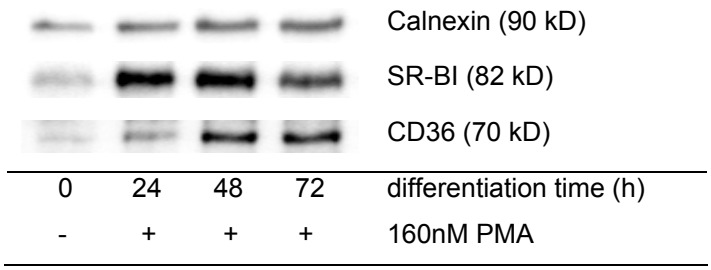


Figure 13: SR-BI and CD36 protein expression during differentiation towards THP-1 macrophages. THP-1 cells were incubated with 160 nm PMA for up to 72 h at 37°C. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed for CD36 and SR-BI protein. Calnexin was used as loading control.

SR-BI and CD36 protein were hardly detectable in native THP-1 cells. SR-BI protein was expressed after 24 hours of phorbol ester differentiation in THP-1 cells (Fig. 13) and showed the highest expression after 48 hours. 72 hours after addition of phorbol ester SR-BI protein expression remained at relatively high levels. After 24 hours CD36 protein was hardly detectable. After 48 and 72 hours PMA differentiated THP-1 cells clearly expressed CD36 protein. For the following experiments, THP-1 cells were differentiated for 72 hours prior to the experiments.
An immunocytochemical approach was used to confirm these results. Therefore, THP-1 cells were differentiated towards macrophages with 160 nM PMA for 72 hours on sterile glass plates. THP-1 monocytes were spun onto glass plates. Nuclei were stained with DAPI. CD36 protein was immunodetected by a monoclonal mouse antibody, SR-BI protein by a polyclonal rabbit antibody, followed by TRITC conjugated secondary antibodies for detection under the microscope.

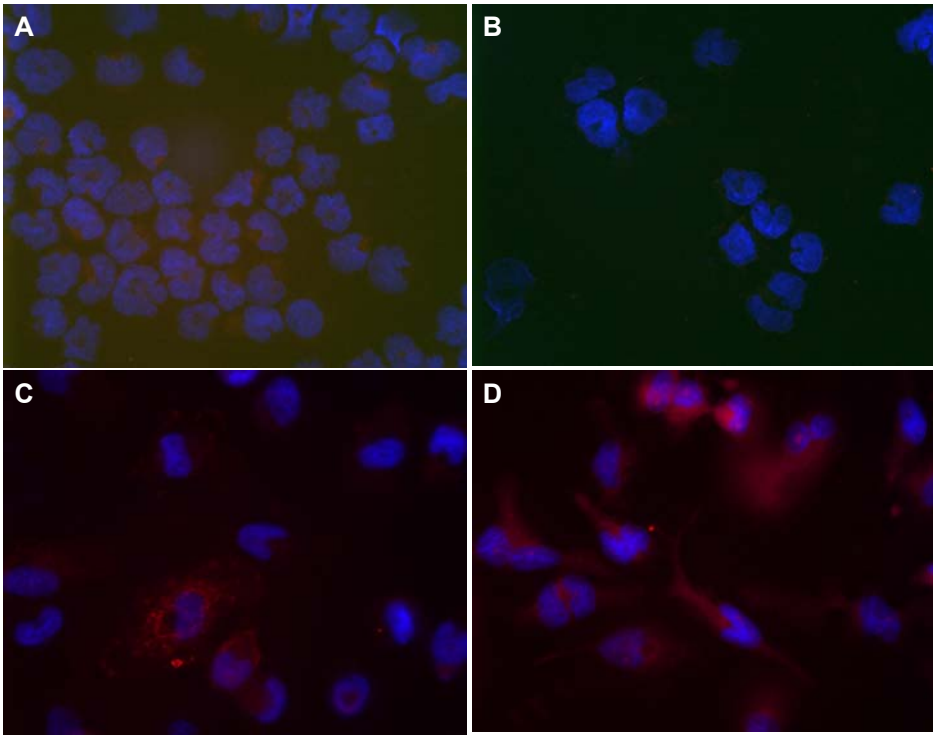


Figure 14: Immunofluorescent staining of CD36 and SR-BI protein in THP-1 cells.
Immunostaining of CD36 protein in undifferentiated THP-1 monocytes (A) and THP-1 macrophages (C). Immunostaining of SR-BI protein in undifferentiated THP-1 monocytes (B) and THP-1 macrophages (D). THP-1 monocytes were spun onto glass plates using cytospin (A,B), THP-1 macrophages were grown and differentiated with 160 nM PMA for 72 h on sterile glass plates (C,D).

CD36 (Fig. 14A) and SR-BI (Fig. 14B) protein were hardly detectable in THP-1 monocytes. In THP-1 cells, which were differentiated towards macrophages for 3 days, both CD36 (Fig. 14C) and SR-BI protein (Fig. 14D) could be detected predominantly on the cell surface.

5.2 **Modification of low density lipoproteins**

LDL was isolated from healthy young women by sequential flotation ultracentrifugation. To investigate different kinds of LDL modification we measured relative electrophoretic mobility and protein carbonyl accumulation of the lipoprotein particles. TBARS accumulated within Cu^{2+}-oxidized, but not in HOCl-modified LDL (data not shown).

5.2.1 Relative electrophoretic mobility of modified lipoproteins

HOCl mainly oxidizes the protein fraction of the particle and fragments it. $CuSO_4$ oxidizes both the protein and the lipid part of the LDL particle. Oxidation of apolipoprotein B100 leads to an overall loss of positive charge resulting in an enhanced electrophoretic mobility in agarose gels compared to native LDL [MALLE et al. 2006].

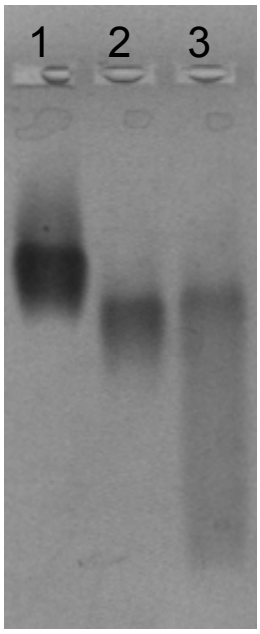


Figure 15: Relative electrophoretic mobility of modified LDL.
Native (1), Cu^{2+}-oxidized (2) and HOCl-modified (3) LDL were loaded onto a 1.5% agarose gel. After 1.5 h of native electrophoresis the gel was stained with Coomassie blue and photodocumented.

Cu^{2+}-oxidized LDL showed a relative electrophoretic mobility of 1.24 and HOCl-modified LDL of 1.17 compared to native LDL, whose relative electrophoretic mobility was set to 1. Due to a reaction of HOCl with reactive side-chains of the protein moiety of the LDL particle [MALLE et al. 2006], HOCl-modified LDL showed some smaller protein fragments.

5.2.2 Accumulation of protein carbonyls in the LDL particle after treatment with oxidizing agents

To measure the extent of protein modification within the protein moiety of the LDL particle after treatment with copper ions or HOCl, carbonyls were measured. Protein carbonyls are representative products of protein oxidation and can be measured spectrophotometrically after reaction with 2,4-dinitrophenylhydrazine at 355 nm.

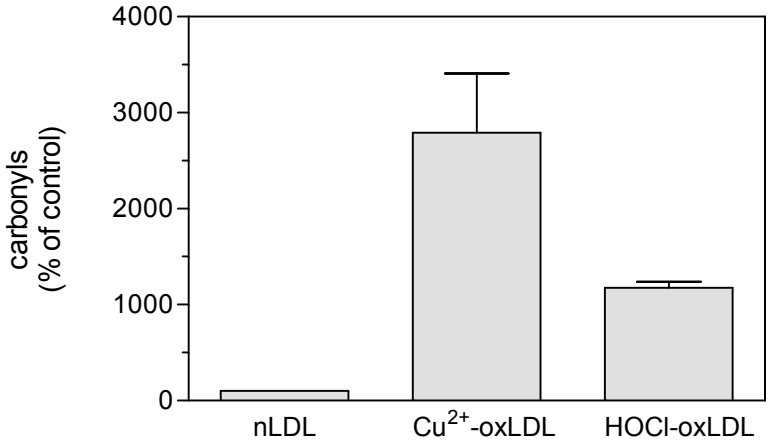


Figure 16: Protein carbonyls in the LDL particle after treatment with modified LDL.
LDL was incubated with or without Cu^{2+} (10 µM, 4 h, 37°C) or HOCl (1 mM, 1 h, 37°C), and protein carbonyls within the LDL particle were measured spectrophotometrically at 355 nm after reaction with 2,4-dinitrophenylhydrazine, (mean ± SD, n=2).

After incubation of LDL with $CuSO_4$ (10 µM) for 4h at 37°C a massive, 25-fold increase in protein oxidation products was found compared to the control. Treatment of LDL with NaOCl (1 mM) for 1h at 37°C led to a 10-fold increase in protein carbonyls.

5.3 **Effect of modified LDL on THP-1 macrophages**

Next, we investigated the influence of modified LDL on uptake and accumulation of lipids and on cell viability in differentiated THP-1 cells. Therefore, we visualized foam cell formation, measured lipid uptake by oil red o staining, TBARS formation in the cells, cholesterol uptake via HPLC and toxicity of modified LDL on THP-1 macrophages by neutral red assay.

5.3.1 OxLDL-induced foam cell formation of THP-1 macrophages

After differentiation of THP-1 cells towards macrophages with 160 nM PMA for three days, cells were incubated with BSA (2 mg/ml), native LDL (100 µg/ml), Cu^{2+}-oxidized LDL (100 µg/ml) or HOCl-modified LDL (100 µg/ml), respectively, for 18 hours at 37°C. THP-1 cells took up modified LDL excessively through scavenger receptors, predominatly CD36 and SR-A [KUNJATHOOR et al. 2002], developed lipid deposits and thus built foam cells.

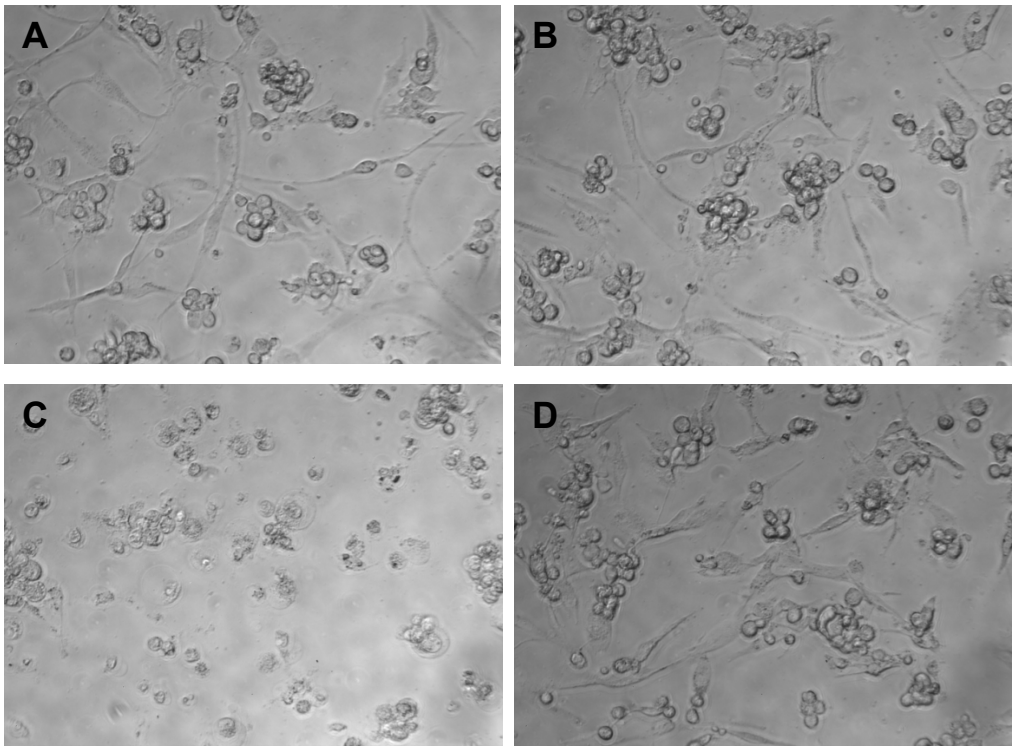


Figure 17: Foam cell formation of THP-1 macrophages.
Images of THP-1 macrophages (A) after incubation with native LDL (100 µg/ml) (B), Cu^{2+}-oxidized LDL (100 µg/ml) (C) or HOCl-modified LDL (100 µg/ml) (D) for 18 h at 37°C, documented by phaco-microscopy.

Micrographs clearly depict that THP-1 macrophages (Fig. 17A) changed their morphology towards foam cells after incubation with Cu^{2+}-oxidized LDL (100 µg/ml, Fig. 17C) due to massive lipid accumulation. After treatment with native (100 µg/ml, Fig. 17B) or HOCl-modified LDL (100 µg/ml, Fig. 17D) cell morphology was comparable to that of untreated THP-1 macrophages (control, Fig. 17A).

To follow foam cell formation in a time-dependent manner, THP-1 monocytes were differentiated towards macrophages and were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) for different time periods (0-20 hours). Every 2 hours a dish was taken and photographed under the microscope, showing representative micrographs after 6, 8 and 18 hours in Fig. 18.

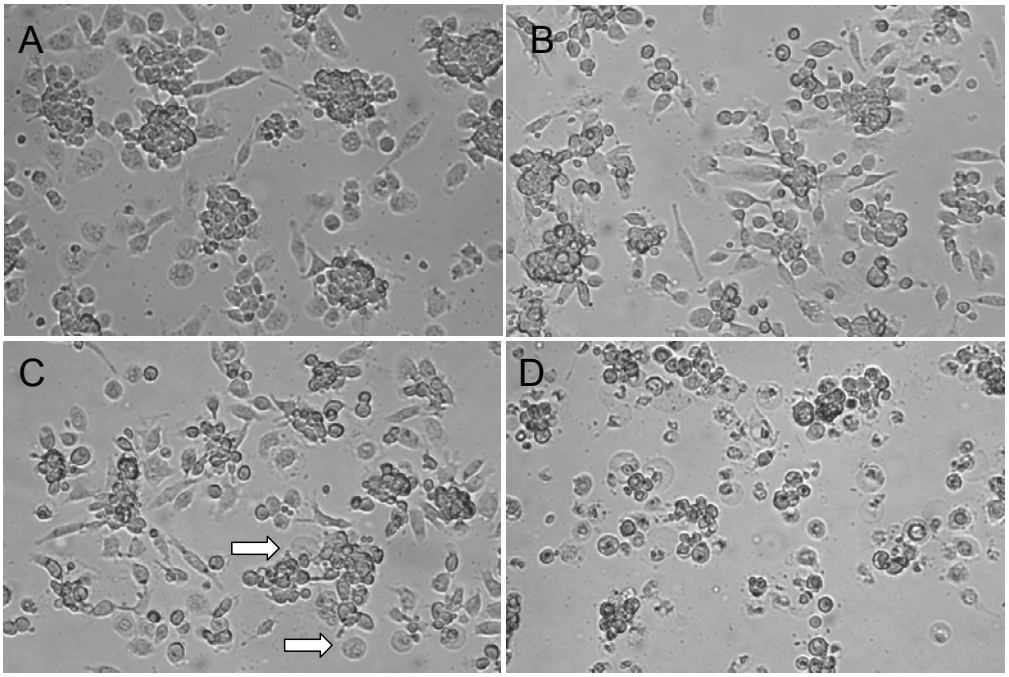


Figure 18: Foam cell formation of THP-1 macrophages at different time points.
Images of THP-1 macrophages (A) after incubation with Cu^{2+}-oxidized LDL (100 µg/ml) for 6 (B), 8 (C) and 18 h (D) at 37°C, documented by phaco-microscopy. Arrows in the picture indicate foam cells.

Incubation of THP-1 macrophages (Fig. 18A) with Cu^{2+}-oxidized LDL (100 µg/ml) led to observable foam cell formation. After 6 hours of incubation (Fig. 18B) no change on cell morphology was visible. The first foam cells were detectable after 8 hours of treatment with Cu^{2+}-oxidized LDL (Fig. 18C). After 18 hours nearly all THP-1 macrophages had converted to visible foam cells (Fig. 18D).

To investigate foam cell formation in the presence of differently modified LDL in native monocytic THP-1 cells, these cells were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) and HOCl-modified LDL (100 µg/ml) for 18 hours at 37°C. Afterwards pictures were taken under the microscope to document cell morphology.

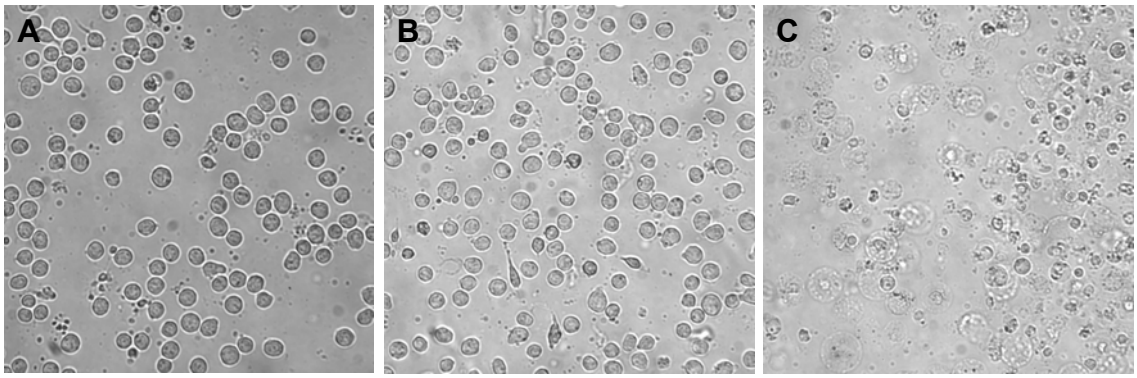


Figure 19: Foam cell formation of THP-1 monocytes.
Images of undifferentiated THP-1 cells (A) and THP-1 monocytes after incubation with HOCl-modified LDL (100 µg/ml) (B) and Cu^{2+}-oxidized LDL (100 µg/ml) (C) for 18 h at 37°C, documented by phaco-microscopy.

In concordance with THP-1 macrophages, THP-1 monocytes (Fig. 19A) built visible foam cells after incubation with Cu^{2+} (100 µg/ml, Fig. 19C) but not with HOCl-modified LDL (100 µg/ml, Fig. 19B). Incubation of undifferentiated THP-1 cells with native LDL (100 µg/ml) did not lead to foam cell formation (data not shown).

5.3.2 Accumulation of lipid deposits after incubation with modified LDL in THP-1 macrophages

Lipids were stained with oil red o, a dye which stains cholesterylesters and triglycerides within the cell. After extraction of the dye with isopropanol the amount of lipids was quantified spectrophotometrically at 510 nm.

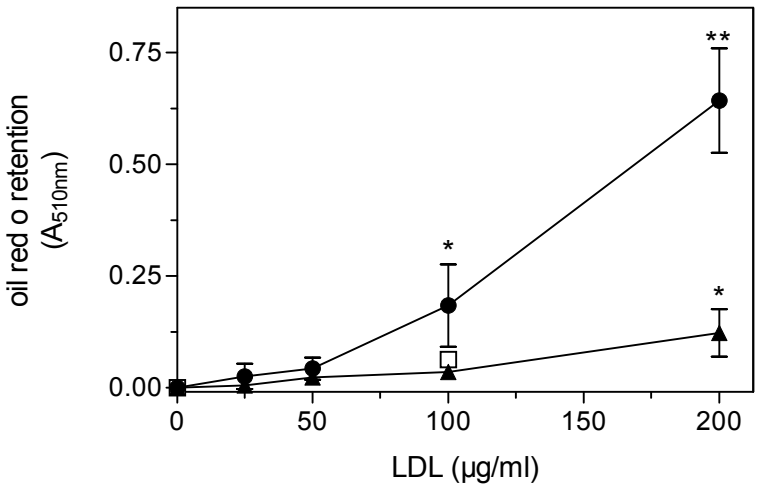


Figure 20: Lipid deposits in THP-1 macrophages after treatment with modified LDL.
THP-1 macrophages were incubated for 18 h with increasing concentrations (12.5 – 200 µg/ml) of native (□), Cu^{2+} (●) or HOCl (▲) modified LDL. Then the cells were stained with oil red o for 2 h. Accumulation of lipid deposits in the cells was monitored spectrophotometrically after extraction of the dye with isopropanol at 510 nm. * $p<0.05$, ** $p< 0.01$ vs. control (without lipoproteins), (mean ± SD, n=4).

Treatment of THP-1 macrophages with 100 ($p<0.05$) or 200 µg/ml ($p<0.01$) Cu^{2+}-oxidized LDL enhanced lipid deposits in the cells dose-dependently compared to the control cells without any lipoprotein treatment. After treatment with HOCl-modified LDL THP-1 macrophages accumulated lipids significantly at a concentration of 200 µg/ml ($p<0.05$). Native LDL had no effect on lipid accumulation in THP-1 macrophages.

5.3.3 TBARS formation in THP-1 macrophages during foam cell formation

Thiobarbituric acid reactive substances are products of lipid peroxidation with malondialdehyde (MDA) as one of the main components. After differentiation of THP-1 cells they were incubated with native LDL (100 µg/ml) or with Cu^{2+}- and HOCl-modified LDL (100 µg/ml). Afterwards, TBARS formation within the cell supernatant was measured spectrophotometrically at 534 nm and quantified as malondialdehyde content (µM) with the molar extinction coefficient of MDA (ε=156,000 $M^{-1}.cm^{-1}$). It has to be considered that also other aldehydes, like the

highly reactive unsaturated aldehyde 4-hydroxynonenal (4-HNE) can react with thiobarbituric acid.

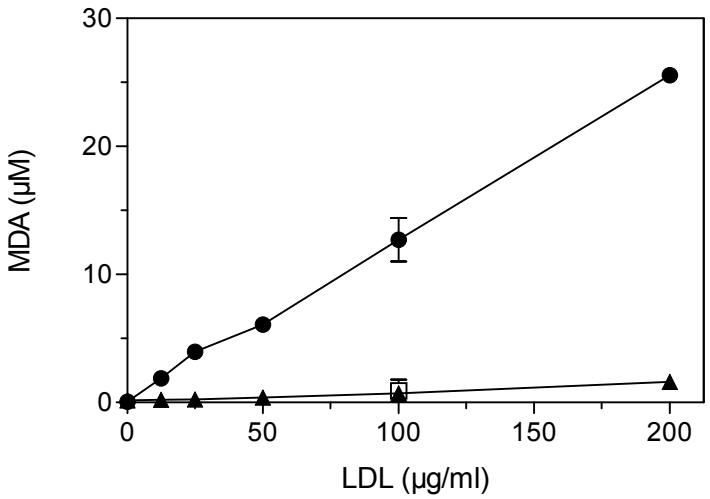


Figure 21: OxLDL-induced TBARS formation in THP-1 macrophages.
THP-1 macrophages were incubated for 18 h with increasing concentrations (12.5 – 200 µg/ml) of native (□), Cu^{2+} (●) or HOCl (▲) modified LDL. Lipidperoxidation of the cells was monitored as the increase of malondialdehyde, measured spectrophotometrically after the reaction with thiobarbituric acid at 534 nm, (mean ± SD, n=3).

Incubation with Cu^{2+}-oxidized LDL for 18 hours in THP-1 macrophages raised malondialdehyde formation dose-dependently, 12.7±1.7 µM of MDA were measured at a concentration of 100 µg/ml and 25.5±0.5 µM at 200 µg/ml. Incubation with different concentrations of HOCl-modified LDL or native LDL had no effect on TBARS formation (Fig. 21).

To investigate time dependent responses of Cu^{2+}-oxidized LDL (100 µg/ml) on TBARS formation in THP-1 macrophages, cells were incubated for up to 20 hours. After every 2 hours cell supernatant was collected for TBARS assay. Native LDL (100 µg/ml) was measured as control after an incubation of THP-1 macrophages for 2 and 20 hours.

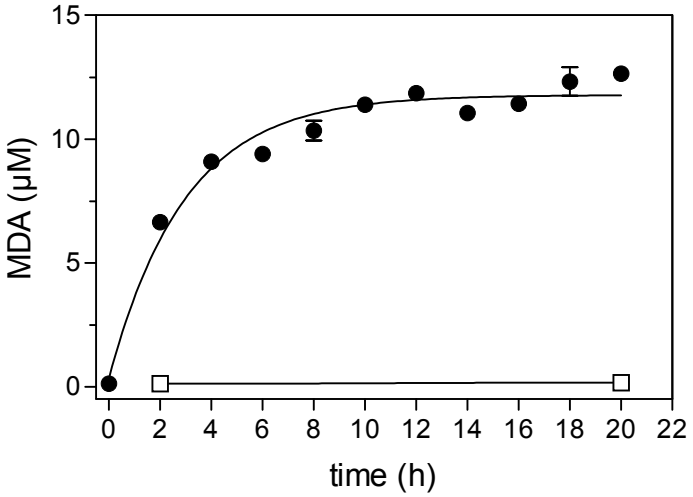


Figure 22: Time course of Cu^{2+}-oxidized LDL mediated TBARS formation of THP-1 macrophages. THP-1 macrophages were incubated with Cu^{2+}-oxidized LDL (100 µg/ml, ●) for up to 20 h and native LDL (100 µg/ml, □) for 2 and 20 h, respectively. Lipidperoxidation was monitored as the increase of malondialdehyde formation (µM), measured spectrophotometrically after the reaction with thiobarbituric acid at 534 nm, (mean ± SD, n=3).

Incubation of THP-1 macrophages with Cu^{2+}-oxidized LDL induced TBARS accumulation time-dependently (Fig. 22). After two hours of incubation 6.7±0.1 µM of malondialdehyde accumulated within the cell supernatant reaching a plateau of about 12 µM after 10 hours with no further increase in TBARS formation at later time points. Incubation of THP-1 macrophages with native LDL over a time period of 20 hours did not trigger TBARS formation.

5.3.4 Effect of native and modified LDL on cholesteryl ester, free and total cholesterol content of THP-1 macrophages

Next, we aimed to quantify the cholesterol content of THP-1 macrophages after incubation with differently modified LDL. Therefore, THP-1 cells were differentiated towards macrophages for 3 days and incubated with either BSA (2 mg/ml, control), native, Cu^{2+} or HOCl-modified LDL (100 µg/ml) for 18 hours. After lipid extraction without (free cholesterol) or with saponification with ethanolic KOH (total cholesterol) cholesterol content of the cells was measured by HPLC/UV detection.

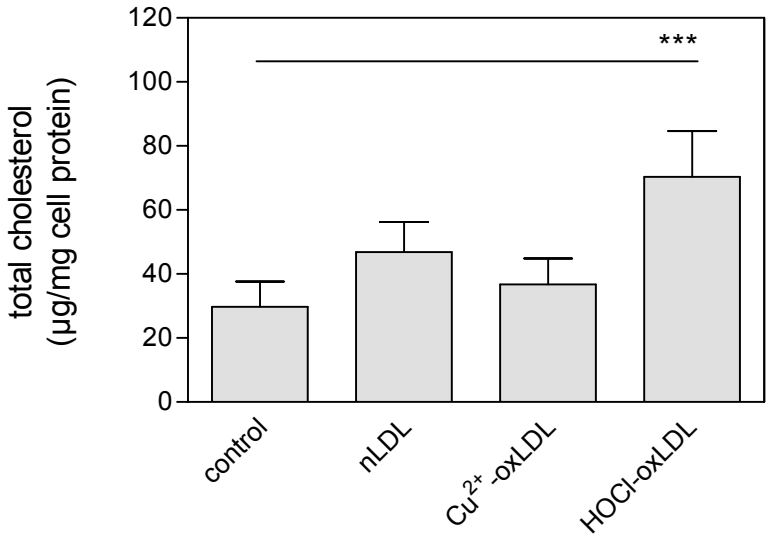


Figure 23: Effect of native and modified LDL on total cholesterol content of THP-1 macrophages. THP-1 macrophages were incubated with BSA (2 mg/ml, control), native, Cu^{2+}- or HOCl-modified LDL (100 µg/ml). After adding ergosterol as internal standard the lipids were extracted with hexane:isopropanol (3:2) and saponified with ethanolic KOH, dried and taken up in EtOH:chloroform (8:2). The resulting samples were analyzed using HPLC-UV. $p<0.001$ vs. control, (mean ± SD, n = 4).

Control THP-1 macrophages (BSA, 2 mg/ml) showed an intracellular cholesterol content of 29.7±7.8 µg/mg cell protein (basal cholesterol level), including free and esterified cholesterol. Incubation with native LDL (100 µg/ml) resulted in a total cholesterol content of the cells of 46.8±9.4 µg/mg cell protein, Cu^{2+}-oxidized LDL (100 µg/ml) in a content of 36.7±8.1 µg/mg cell protein, which is a rather small elevation of total cholesterol content compared to control. Surprisingly, incubation with native LDL showed a higher content than incubation with Cu^{2+}-oxidized LDL. Administration of HOCl-modified LDL (100 µg/ml) to the cells led to an increase to 70.4±14.3 µg/mg cell protein ($p<0.001$) which is more than double as basal levels (Fig. 23).

Cholesteryl ester content was calculated by dividing the lipid extractions, both total and free cholesterol were measured.

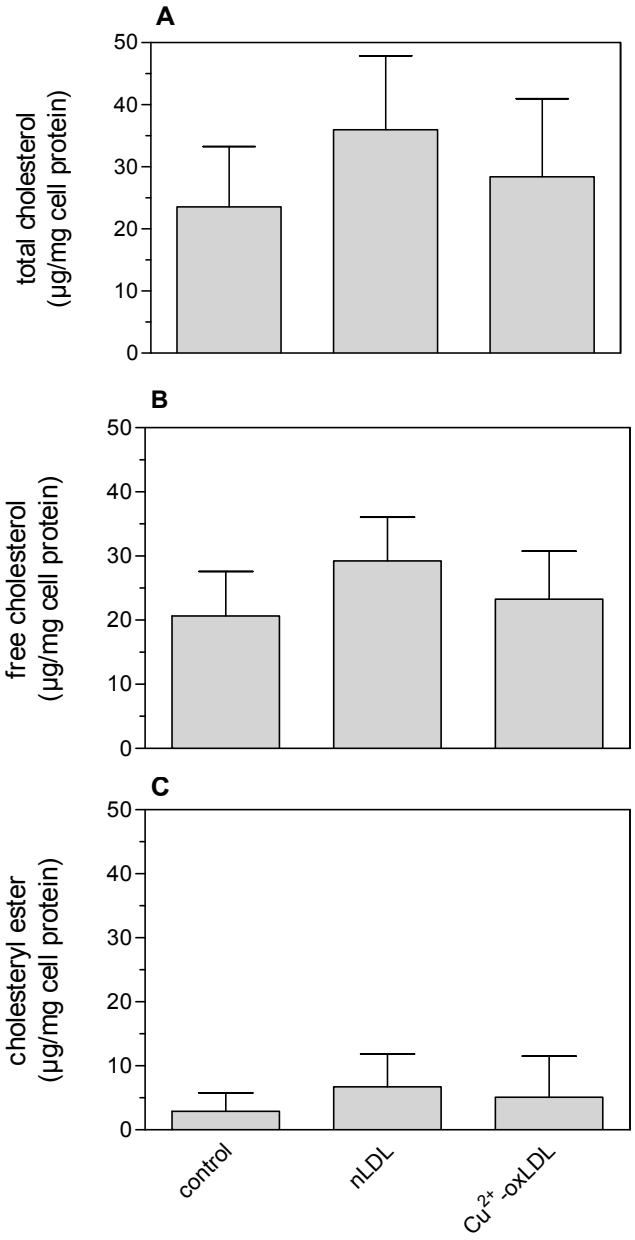


Figure 24: Effect of native and Cu^{2+}-oxidized LDL on cholesteryl esters, free and total cholesterol content of THP-1 macrophages.

THP-1 macrophages were incubated with BSA (2 mg/ml, control), native and Cu^{2+}-oxidized LDL (100 µg/ml). After adding ergosterol as internal standard the lipids were extracted with hexane:isopropanol (3:2) and saponified with ethanolic KOH (total cholesterol) or not (free cholesterol), dried and taken up in EtOH:chloroform (8:2). The resulting samples were analyzed using HPLC-UV. Cholesteryl ester content was calculated as total cholesterol minus free cholesterol, (mean ± SD, n = 3).

23.6±9.7, 36±11.9, 28.4±12.6 µg/mg cell protein were measured for the control, native or Cu^{2+}-oxidized LDL treated THP-1 macrophages, respectively (Fig. 24A). Whereas free cholesterol levels of oxLDL treated cells (23.3±7.5 µg/mg cell protein) were comparable to control cells (20.7±6.9 µg/mg cell protein) it was highest in THP-1 macrophages incubated with native LDL (29.2±6.8 µg/mg cell protein) (Fig. 24B). Cholesteryl ester amount of both native (6.7±5.1 µg/mg cell protein) and Cu^{2+}-oxidized LDL (5.1±6.4 µg/mg cell protein) was higher than in control cells (2.9±2.8 µg/mg cell protein) (Fig. 24C).

5.3.5 Effect of modified LDL on cell viability of THP-1 macrophages

Cell viability of THP-1 macrophages was measured with neutral red assay. The dye neutral red is assimilated and retained by the lysosomal matrix of living cells. Lower neutral red retention indicates cytotoxic events in the cells [BORENFREUND and PUERNER 1985].

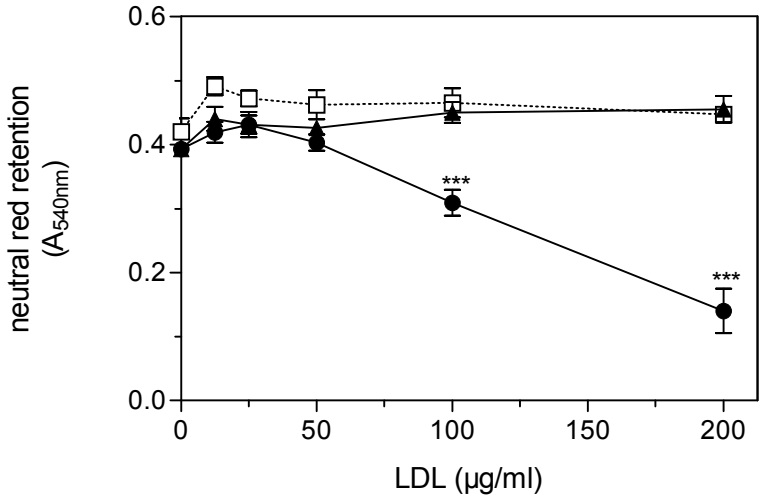


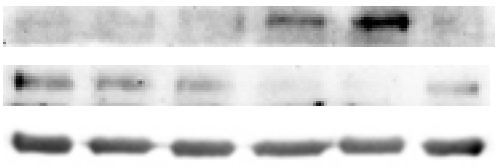
Figure 25: Cell viability of THP-1 macrophages after treatment with modified LDL.
THP-1 macrophages were incubated for 18 h with different concentrations (12.5 – 200 µg/ml) of native (□), Cu^{2+} (●) or HOCl (▲) modified LDL. Then the cells were stained with neutral red solution. Neutral red retention in the cells was monitored after extraction of the dye at 540 nm. *** $p < 0.001$ vs. control (without lipoproteins), (mean ± SD, n=6).

Incubation of THP-1 macrophages with 100 or 200 µg/ml Cu^{2+}-oxidized LDL for 18 hours led to a loss of cell viability ($p<0.001$) of about 25 or 50%, respectively, compared to the control without any lipoprotein treatment. Incubation with native or HOCl-modified LDL had no effect on cell viability in THP-1 macrophages. Therefore, we used 100 µg/ml of modified LDL for the further investigations, where loss of cell viability is not more than 25% (Fig. 25).

After showing that modified LDL increases the uptake and accumulation of lipids and cholesterol and slightly decreases cell viability in differentiated THP-1 cells, our aim was then to investigate the influence of modified LDL on the protein expression of the transcription factor HIF1α and the scavenger receptor SR-BI.

5.3.6 Effect of Cu^{2+}-oxidized LDL on HIF-1α and SR-BI protein expression in THP-1 macrophages

To investigate the effect of native and modified LDL on HIF-1α and SR-BI protein expression, THP-1 cells were incubated with increasing concentrations of Cu^{2+}-oxidized LDL (12.5 – 100 µg/ml) and native LDL (100 µg/ml) for 18 hours and Western blot analyses were performed with whole cell lysates.



HIF-1α (120 kD)
SR-BI (82 kD)
ß-Actin (42 kD)

-	-	-	-	-	100	nLDL (µg/ml)
-	12.5	25	50	100	-	Cu^{2+}-oxLDL (µg/ml)

Figure 26: Concentration dependent expression of HIF-1α and SR-BI protein in THP-1 macrophages after treatment with Cu^{2+}-oxidized LDL.
THP-1 macrophages were incubated with increasing concentrations (12.5 - 100 µg/ml) of Cu^{2+}-oxidized LDL and native LDL (100 µg/ml) for 18 h at 37°C. Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed. ß-Actin was used as loading control.

THP-1 macrophages accumulated HIF-1α protein dose-dependently after incubation with Cu^{2+}-oxidized LDL (12.5 – 100 µg/ml). Native LDL (100 µg/ml) did not show any effect.

SR-BI protein diminished in THP-1 macrophages after treatment with Cu^{2+}-oxidized LDL dose-dependently, while again, native LDL (100 µg/ml) did not affect THP-1 macrophages concerning SR-BI protein expression (Fig. 26).

Immunofluorescence experiments were performed to confirm these results. Therefore, THP-1 cells were differentiated towards macrophages in 12-well plates on glass coverslips. After incubation with native or Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours at 37°C, cells were fixed on the glass coverslips and after incubation with HIF-1 α or SR-BI antibody. Bound antibody was detected under a fluorescence microscope with TRITC or FITC labeled secondary antibody.

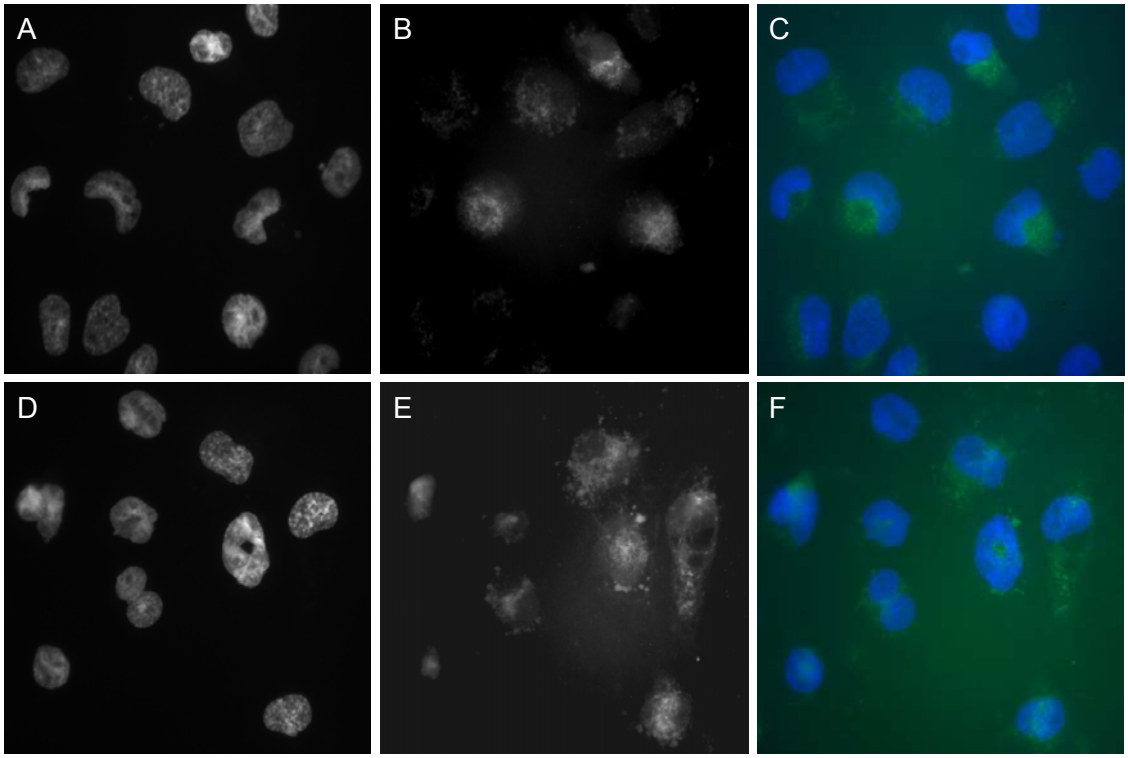


Figure 27: Immunofluorescent staining of HIF-1α protein in THP-1 macrophages.

THP-1 macrophages were grown and differentiated with 160 nm PMA for 72 h at 37°C on glass plates and incubated with 100 µg/ml of native (A, B, C) or Cu^{2+}-(D, E, F) modified LDL. Nuclei were stained with DAPI (A, D). HIF-1α protein (B, E) was immunodetected by a polyclonal mouse antibody and a FITC conjugated mouse secondary antibody. C and F show a merge of DAPI and FITC staining.

Under normal oxygen conditions HIF-1α protein is degraded permanently by the proteasome. After incubation of THP-1 macrophages with native LDL (100 µg/ml) for 18 hours a small amount of this transcription factor was detected within the cells (Fig. 27B). A merge of cell nuclei (Fig. 27A) and HIF-1α protein (Fig. 27B) is shown in Fig. 27C and depicts clearly that HIF-1α protein is distributed within the cytosol whereas in the nuclei there is almost no HIF-1α protein detected (Fig. 27C).
HIF-1α protein clearly accumulated (Fig. 27E) in THP-1 macrophages incubated with Cu^{2+}-oxidized LDL (100 µg/ml), confirming our results of Western blot analysis.

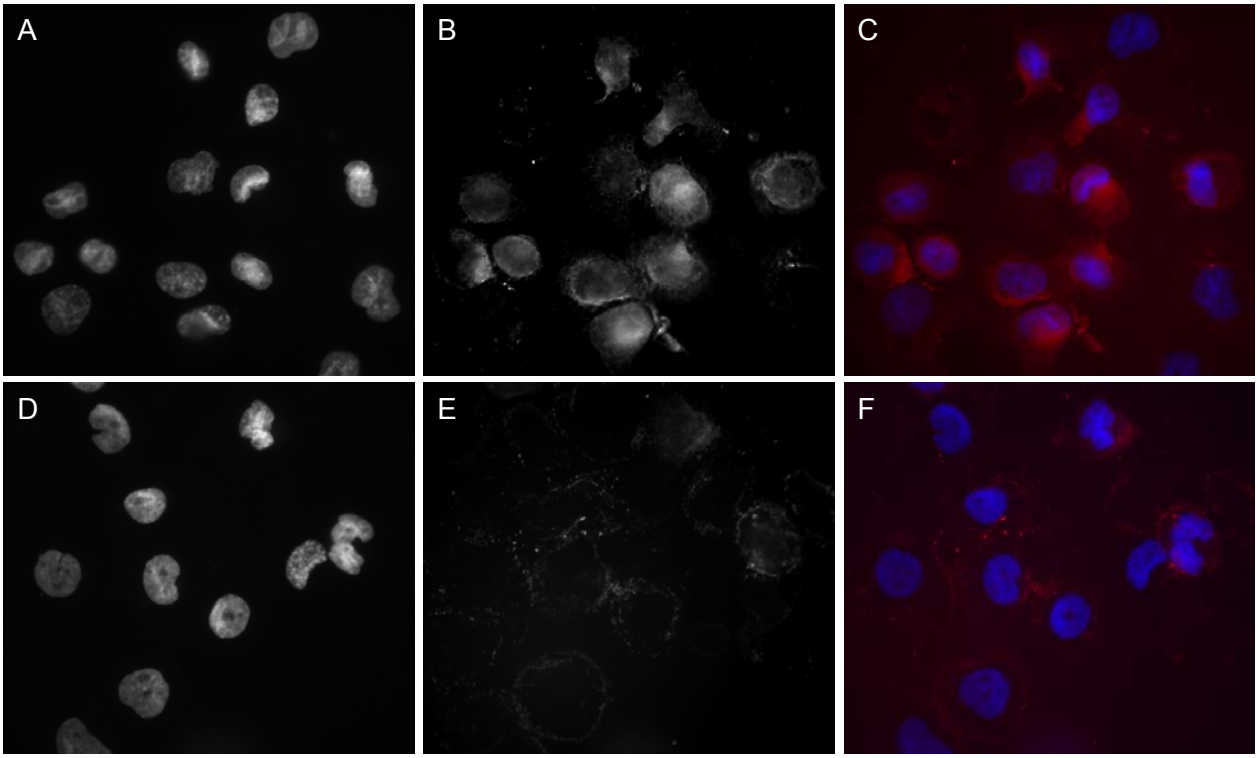


Figure 28: Immunofluorescent staining of SR-BI protein in THP-1 macrophages.
THP-1 macrophages were grown and differentiated with 160 nm PMA for 72 h at 37°C on glass plates and incubated with 100 µg/ml of native (A, B, C) or Cu^{2+} (D, E, F) modified LDL. Nuclei were stained with DAPI (A, D). SR-BI protein (B, E) was immunodetected by a polyclonal rabbit antibody and a TRITC conjugated rabbit secondary antibody. C and F show a merge of DAPI and TRITC staining.

Incubation of THP-1 macrophages with Cu^{2+}-oxidized LDL (100 µg/ml, Fig. 28E) led to a decrease of SR-BI protein compared to treatment with native LDL (100 µg/ml, Fig. 28B), which could also been demonstrated by Western blot. A merge of nuclei and SR-BI protein staining (Fig. 28C,F) shows that SR-BI protein is expressed mainly at the cell membrane.

To investigate time dependend responses of HIF-1α protein accumulation and SR-BI protein attenuation on Cu^{2+}-oxidized LDL (100 µg/ml) in THP-1 macrophages, cells were incubated for up to 20 hours. Every 2 hours pictures of cells were taken (cpt. 5.3.1) and cell supernatant was used for TBARS assay (cpt. 5.3.3). After washing the cells twice with ice-cold PBS whole cells were lysed and Western blot analysis was performed. Native LDL (100 µg/ml) was lysed as control after incubation for 2 and 20 hours. ß-Actin was used as loading control.

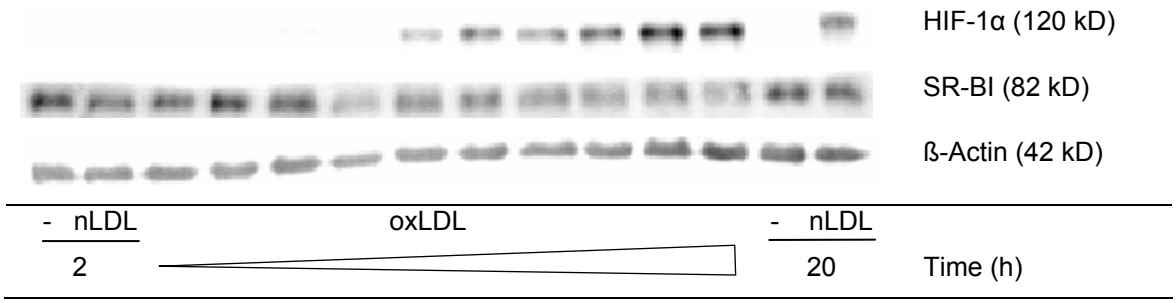


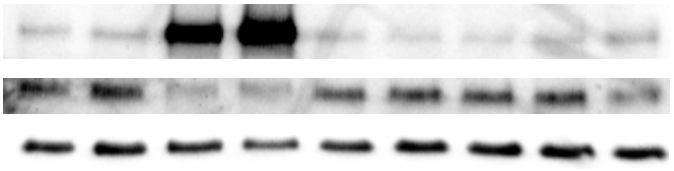
Figure 29: Time response of HIF-1α and SR-BI protein expression in THP-1 macrophages after treatment with Cu^{2+}-oxidized LDL.
THP-1 macrophages were incubated with native (100 µg/ml) and Cu^{2+}-oxidized LDL (100 µg/ml) for 2 to 20 h at 37°C. Every two hours cells were lysed and Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed. ß-Actin was used as loading control.

As mentioned before, treatment with Cu^{2+}-oxidized LDL (100 µg/ml) triggers HIF-1α protein accumulation in THP-1 macrophages, which started after 10 hours of incubation and increased in a time dependent manner. Incubation of THP-1 macrophages with native LDL (100 µg/ml) for 20 hours also increased HIF-1α protein accumulation compared to the incubation with nLDL (2 hours).

SR-BI protein was mitigated in THP-1 macrophages after treatment with Cu^{2+}-oxidized LDL (100 µg/ml) with the highest attenuation after 20 hours. Native LDL (100 µg/ml) had no effect on SR-BI levels (Fig. 29).

5.3.7 Effect of HOCl-modified LDL on HIF1α and SR-BI protein expression in THP-1 macrophages

HIF-1α protein accumulation in THP-1 macrophages, incubated with HOCl-modified LDL (100 µg/ml), where solely the protein fraction is oxidized, was compared to Cu^{2+}-oxidized LDL (100 µg/ml), which possesses modified protein and lipid moities.



-	100	-	-	-	-	-	-	-	nLDL (µg/ml)
-	-	100	200	-	-	-	-	-	Cu^{2+}-oxLDL (µg/ml)
-	-	-	-	12.5	25	50	100	200	HOCl-oxLDL (µg/ml)

Figure 30: Expression of HIF-1α and SR-BI protein in THP-1 macrophages after treatment with Cu^{2+} and HOCl-modified LDL.
THP-1 macrophages were incubated with increasing concentrations (12.5 - 100 µg/ml) of HOCl-modified LDL, native (100 µg/ml) and Cu^{2+}-oxidized LDL (100 - 200 µg/ml) for 18 h at 37°C. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed. ß-Actin was used as loading control.

THP-1 macrophages did not accumulate HIF-1α protein significantly after treatment with HOCl-modified LDL (12.5 – 200 µg/ml). 200 µg/ml of HOCl-modified LDL decreased SR-BI protein in THP-1 macrophages compared to the control without lipoprotein treatment, but there was no effect on SR-BI protein detected after treatment with lower concentrations of HOCl-modified LDL (Fig. 30).

As HOCl-modified LDL does not induce accumulation of HIF-1α protein in THP-1 macrophages, Cu^{2+}-oxidized LDL (100 µg/ml) was used for the further experiments to generate foam cells, and was used as model for HIF-1α protein stabilization. At

this concentration the extent of HIF-1α protein accumulation (Fig. 30) is comparable to a concentration of 200 µg/ml of oxLDL but the loss of cell viability is not that high (cpt. 5.3.5).

5.3.8 Proteasomal activity in THP-1 macrophages after treatment with modified LDL

The normoxic accumulation of HIF-1α protein in THP-1 macrophages after treatment with Cu^{2+}-oxidized LDL raised the question whether oxLDL diminishes proteasomal activity resulting in an inhibition of HIF-1α protein degradation. Hence, proteasomal activity in THP-1 macrophages was measured after treatment with native, HOCl and Cu^{2+}-oxidized LDL (25 – 100 µg/ml) for 18 hours.

After addition of the synthetic fluoropeptide s-LLVY-AMC as substrate to the cell lysates, AMC is cleaved through 20S proteasomal activity and can be measured fluorimetrically.

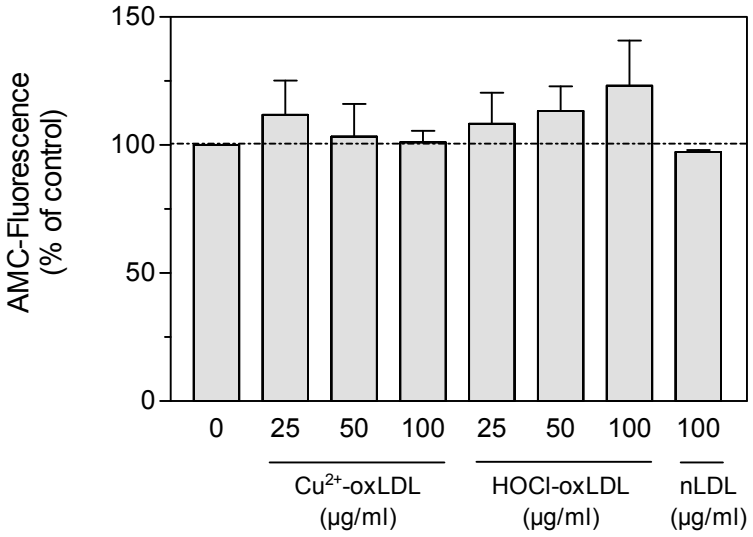


Figure 31: 20S-proteasomal activity of THP-1 macrophage cell lysates after incubation with native, copper- or HOCl-modified LDL.

THP-1 macrophages were incubated with increasing concentrations (25 - 100 µg/ml) of HOCl and Cu^{2+}-oxidized LDL and with native LDL (100 µg/ml) for 18 h at 37°C. After cell lysis proteasomeal activity was measured fluorometrically with the synthetic fluoropeptide s-LLVY-AMC after AMC-release, (mean ± SD, n=2).

Treatment of THP-1 macrophages with up to 100 µg/ml of native, Cu^{2+} or HOCl-modified LDL did not affect proteasomal activity. Thus, oxLDL-induced HIF-1α protein accumulation is not due to diminished 20S proteasomal activity.

5.4 **Effect of vitamin C and HDL on oxLDL-induced modifications in THP-1 macrophages**

Vitamin C is known to attenuate hypoxia-induced HIF-1α protein accumulation in human primary and cancer cell lines [KNOWLES et al. 2003; VISSERS et al. 2007]. The atheroprotective role of HDL is mainly due to its role in reverse cholesterol transport. Therefore we aimed to investigate the role of ascorbate and HDL as putative antagonists in oxLDL treated THP-1 macrophages.
To investigate the effect of vitamin C and HDL on oxLDL-induced events in THP-1 macrophages the cells were preloaded with both ascorbate and the oxidized form, dehydroascorbic acid (DHA).

5.4.1 Transport of vitamin C into THP-1 macrophages

In human cells both chemical forms of vitamin C are transported across the cell membrane with the participation of two different pathways (cpt. 2.6). For this experiment THP-1 macrophages were incubated with either ascorbate or DHA (100 µM) for different time periods (0-30 min). Extra- and intracellular vitamin C content was measured using HPLC with electrochemical detection.

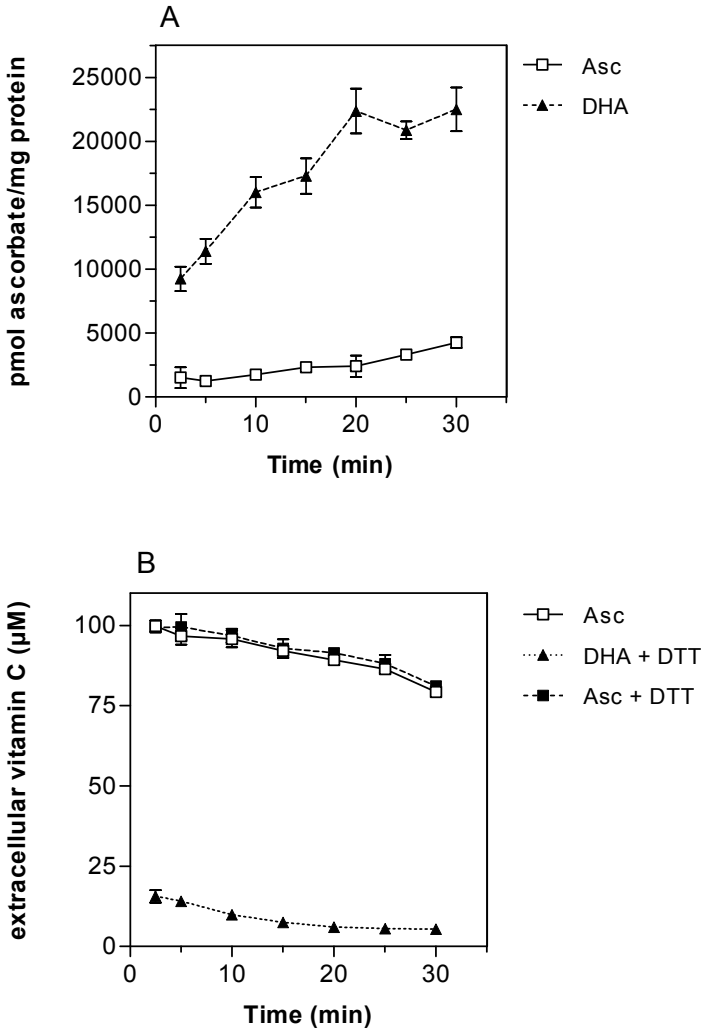


Figure 32: Time course of intra- and extracellular vitamin C in THP-1 macrophages.
After loading of THP-1 macrophages with 100 µM ascorbate or DHA for the indicated periods of time (2.5 – 30 minutes), vitamin C content within the cells (A) and extracellular vitamin C content (B) was measured with HPLC, (mean ± SD, n=3).

Concomitant with a loss of extracellular ascorbate (Fig. 32B, squares) from 100 to 81.1±0.9% after 30 minutes of ascorbate incubation (100µM) THP-1 macrophages accumulated ascorbate intracellularly during this time period (Fig. 32A). After 30 min of incubation THP-1 macrophages showed about seven times higher intracellular ascorbate concentration after DHA preload (100 µM) compared to those loaded with ascorbate (100 µM). Extracellularly dehydroascorbate (which was reduced back to ascorbate with DTT for HPLC measurement) was nearly undetectable (15.6±2%

after 2.5, 5.4±0.1% after 30 minutes). This loss of more than 80% is not due to its uptake (only 1 – 5% of DHA is taken up), it is due to its instability.
Considering a cell water content of 1.6 pl per cell, as measured with Casy (cpt. 4.11), and an intracellular content of 22.5±17.1 nmol ascorbate per mg cell protein after incubation with 100 µM DHA for 30 min, THP-1 macrophages accumulate about 2 mM ascorbate within the cells.
Therefore, a preload of the cells with DHA (100 µM) for 30 minutes was used for the following experiments.

5.4.2 Effect of vitamin C and HDL on oxLDL-induced cell toxicity, lipid deposits and malondialdehyde release in THP-1 macrophages

THP-1 macrophages were incubated for 18 hours at 37°C with Cu^{2+}-oxidized LDL (100 µg/ml) together with or after an HDL (150 µg/ml) pre-incubation for 8 hours with or without a previous DHA load (100 µM, 30 min). Neutral red assay was performed to examine the effect of HDL on the celltoxic events of Cu^{2+}-oxidized LDL. To examine the effect of HDL and vitamin C on the lipid accumulating events of Cu^{2+}-oxidized LDL, cells were stained with oil red o and extracted with isopropanol to measure the amount of intracellular dye spectrophotometrically. TBARS formation was measured in cell supernatant to investigate the effect of HDL and vitamin C on lipid peroxidation of Cu^{2+}-oxidized LDL.

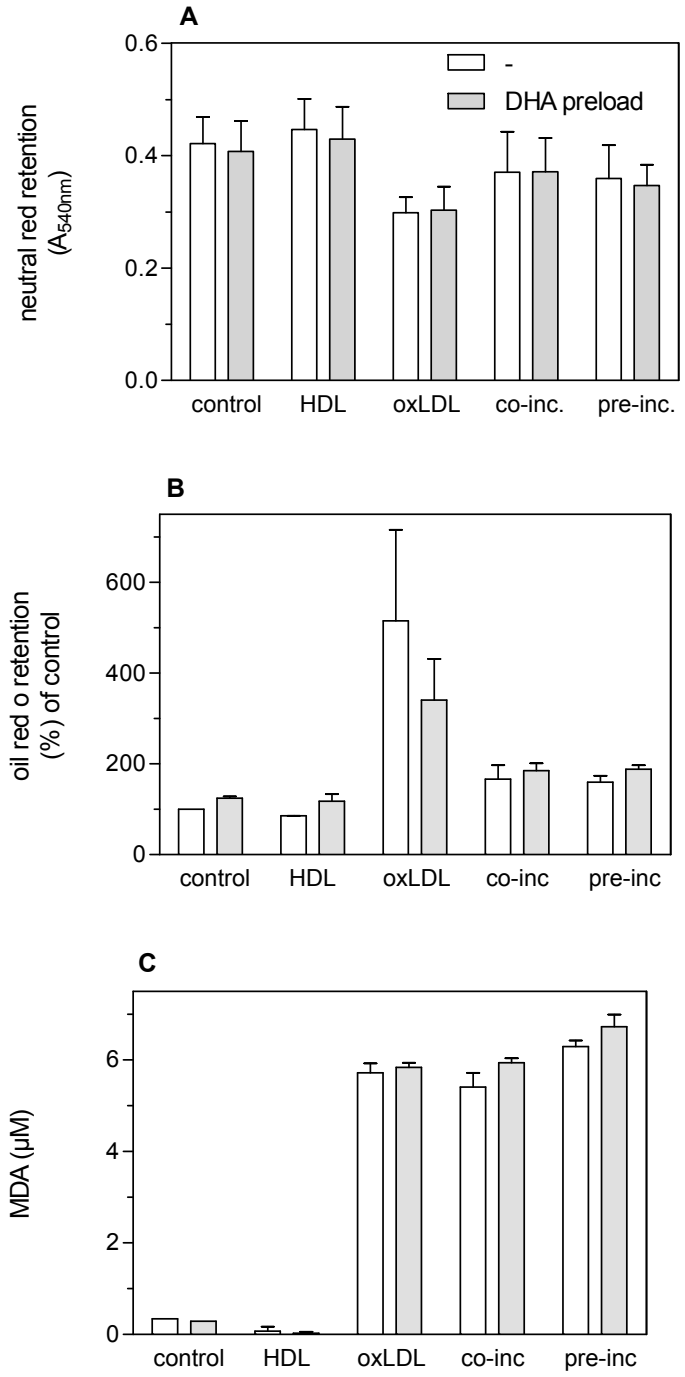


Figure 33: Effect of HDL and DHA on oxLDL-induced changes in cell viability (A), lipid deposits (B) and malondialdehyde release (C) in THP-1 macrophages.

THP-1 macrophages were incubated at 37°C with HDL (150 µg/ml) or Cu^{2+}-oxidized LDL (100 µg/ml) alone, together (co-incubation) or with HDL 8 h before oxLDL was added (pre-incubation) for 18 h, with (grey bars) or without (white bars) preloading of DHA (100 µM) for 30 minutes. Neutral red assay was performed to measure cell viability (A), accumulation of lipids within the cells was monitored with oil red o staining (B) and the amount of MDA release was measured spectrophotometrically within the cell supernatant (C), (mean ± SD, n=2).

As shown before (cpt. 5.3.5), treatment of THP-1 macrophages with Cu^{2+}-oxidized LDL (100 µg/ml) led to a loss of cell viability (about 25%). This effect was reversed in the presence of HDL (150 µg/ml, co-incubation) or when HDL was given 8 hours before (pre-incubation) Cu^{2+}-oxidized LDL was added to the cells, almost reaching levels of control cells. HDL alone and preloading of the cells with DHA (100 µM, 30 minutes) had no effect on cell viability in THP-1 macrophages (Fig. 33A).

OxLDL treatment (100 µg/ml, 18 hours) led to a massive lipid accumulation (515±200%) within THP-1 macrophages compared to cells without lipoprotein treatment, which was set to 100%. Preloading of the cells with DHA (100 µM, 30 min) reduced triglycerides and cholesterylesters in the cells to 340±90%. Treatment of cells with HDL (150 µg/ml), together or 8 hours before oxLDL addition, resulted also in a decrease of lipid deposits in the cells compared to oxLDL treatment alone whereby vitamin C had no further effect (Fig. 33B).

OxLDL (100 µg/ml, 18 hours) dramatically induced TBARS formation (5.7±0.2 µM), measured as malondialdehyde released from THP-1 macrophages. Treatment of the cells with HDL (150 µg/ml) together with or 8 hours before oxLDL was added had no effect on oxLDL-induced TBARS formation. HDL alone did not trigger malondialdehyde formation. Preloading of the cells with DHA (100 µM) for 30 minutes did not affect oxLDL-induced malondialdehyde accumulation (Fig. 33C) either.

Taken together, HDL rescued oxLDL-induced cytotoxicity and lipid accumulation in THP-1 macrophages, vitamin C showed minor effects.

5.4.3 Effect of vitamin C and HDL on HIF-1α and SR-BI protein expression in oxLDL treated THP-1 macrophages

THP-1 macrophages were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours at 37°C together with increasing concentrations of HDL (50 - 150 µg/ml) with

or without a previous preload with DHA (100 µM) for 30 minutes. The effect of DHA and HDL on HIF-1α and SR-BI protein levels in Cu^{2+}-oxidized LDL treated THP-1 macrophages was investigated performing Western blot analyses using whole cell lysates. ß-Actin was used as loading control.

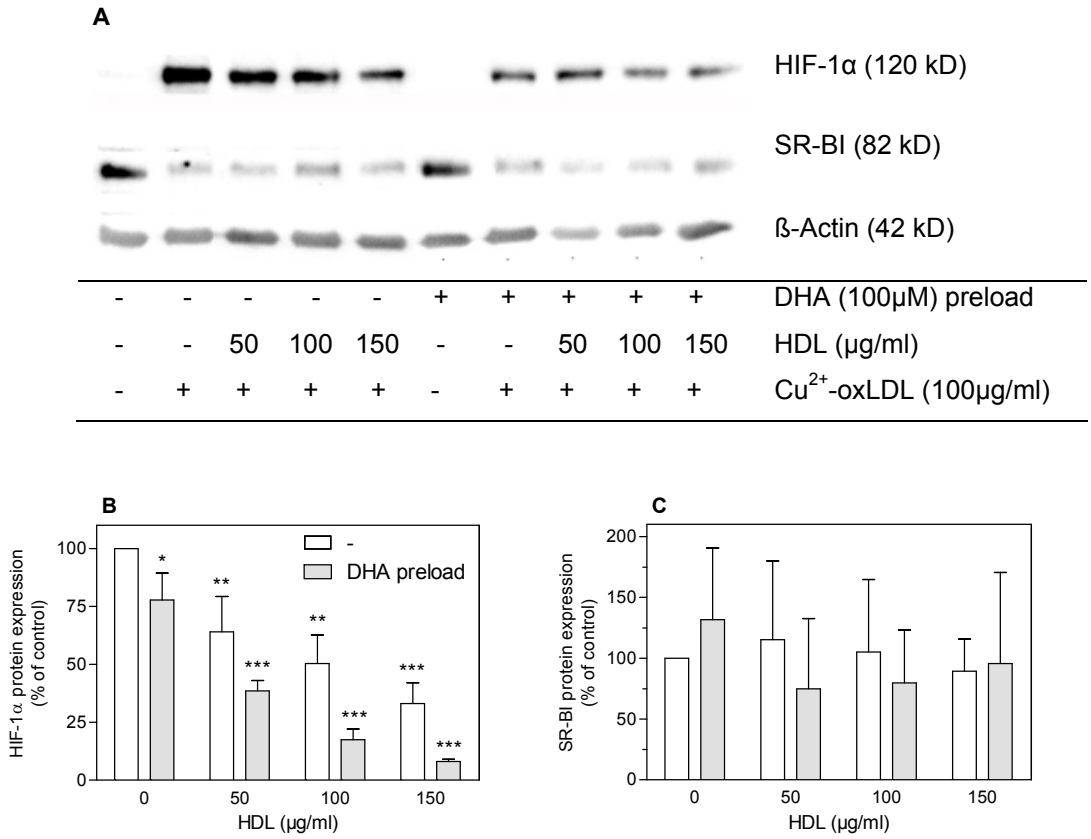


Figure 34: Effect of DHA and HDL on HIF-1α and SR-BI protein levels in oxLDL treated THP-1 macrophages.

Cells were treated with oxLDL (100 µg/ml) alone or together with increasing concentrations of HDL (50 - 150 µg/ml) for 18 h at 37°C with (grey bars) or without (white bars) DHA (100 µM) preloading for 30 minutes. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed (A). ß-Actin was used as loading control.

Graph (B) shows normalized intensity of HIF-1α protein expression in % of control (oxLDL-treatment alone). * $p<0.05$, ** $p< 0.01$, *** $p<0.001$ vs. oxLDL treatment without DHA-preloading (100%), (mean ± SD, n = 3).

Graph (C) shows normalized intensity of SR-BI protein expression in % of control (oxLDL-treatment alone), (mean ± SD, n = 3).

Preloading of the cells with DHA (100 µM) for 30 minutes at 37°C resulted in a decrease of the oxLDL (100 µg/ml) induced accumulation of HIF-1α protein in THP-1 macrophages to about 75% ($p<0.05$). Co-incubation of the cells with HDL impaired the oxLDL-induced HIF-1α protein stabilization in THP-1 macrophages dose-dependently (50 – 150 µg/ml) compared to the control with oxLDL incubation alone ($p<0.01$, $p< 0.001$). This effect was even more pronounced when combined with DHA preloading of the cells (Fig. 34B). HDL alone did not cause HIF-1α protein accumulation in THP-1 macrophages (data not shown).
Concerning oxLDL-induced attenuation of SR-BI protein, neither DHA preload (100 µM, 30 min) nor HDL co-incubation were found to reverse this effect (Fig. 34C).

5.5 **Effect of DETA-NO on oxLDL-induced modifications in THP-1 macrophages**

Recently, Shatrov et al. demonstrated that NO, given by the donor S-nitrosoglutathione (GSNO, 1 mM), is able to attenuate oxLDL-induced HIF-1α protein accumulation in MM6-cells [SHATROV et al. 2003]. Hence, we aimed to investigate the effect of diethylene triamine-nitric oxide (DETA-NO), a synthetic NO donor with a steady state concentration of NO about 1/2000 of the DETA-NO concentration [KÖHL et al. 2006], on oxLDL-induced events in THP-1 macrophages.

5.5.1 Effect of DETA-NO on oxLDL-induced foam cell formation

To examine the effect of DETA-NO on oxLDL-induced foam cell formation in THP-1 macrophages they were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) together with DETA-NO (300 µM). Pictures were taken to record foam cell formation in THP-1 macrophages.

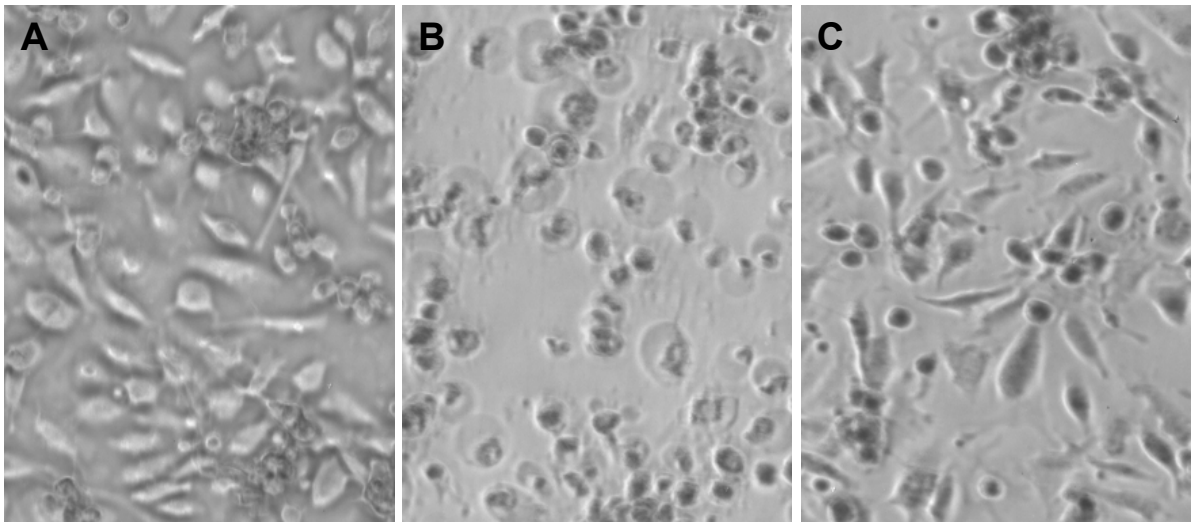


Figure 35: Effect of DETA-NO on oxLDL-induced foam cell formation in THP-1 macrophages.
Image of THP-1 cells, documented by phaco-microscopy, after differentiation towards macrophages with 160 nM PMA for 72 h (A) and THP-1 macrophages after incubation with Cu^{2+}-oxidized LDL (100 µg/ml) (B) and oxLDL together with DETA-NO (300µM) (C) for 18 h at 37°C.

THP-1 macrophages (Fig. 35A), incubated with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours at 37°C, differentiated towards foam cells (Fig. 35B), which was inhibited in the presence of DETA-NO (300µM) (Fig. 35C).

5.5.2 Effect of DETA-NO on oxLDL-induced cell toxicity, lipid deposits and malondialdehyde release in THP-1 macrophages

THP-1 macrophages were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) together with increasing concentrations of DETA-NO (37.5 - 600 µM) for 18 hours at 37°C. Afterwards, neutral red assay was performed to examine the effect of DETA-NO on the celltoxic events of Cu^{2+}-oxidized LDL (A). Oil red o staining was used to examine the effect of DETA-NO on lipid accumulation due to Cu^{2+}-oxidized LDL (B) and MDA release (C) was measured to assess the extent of lipid peroxidation in THP-1 macrophages.

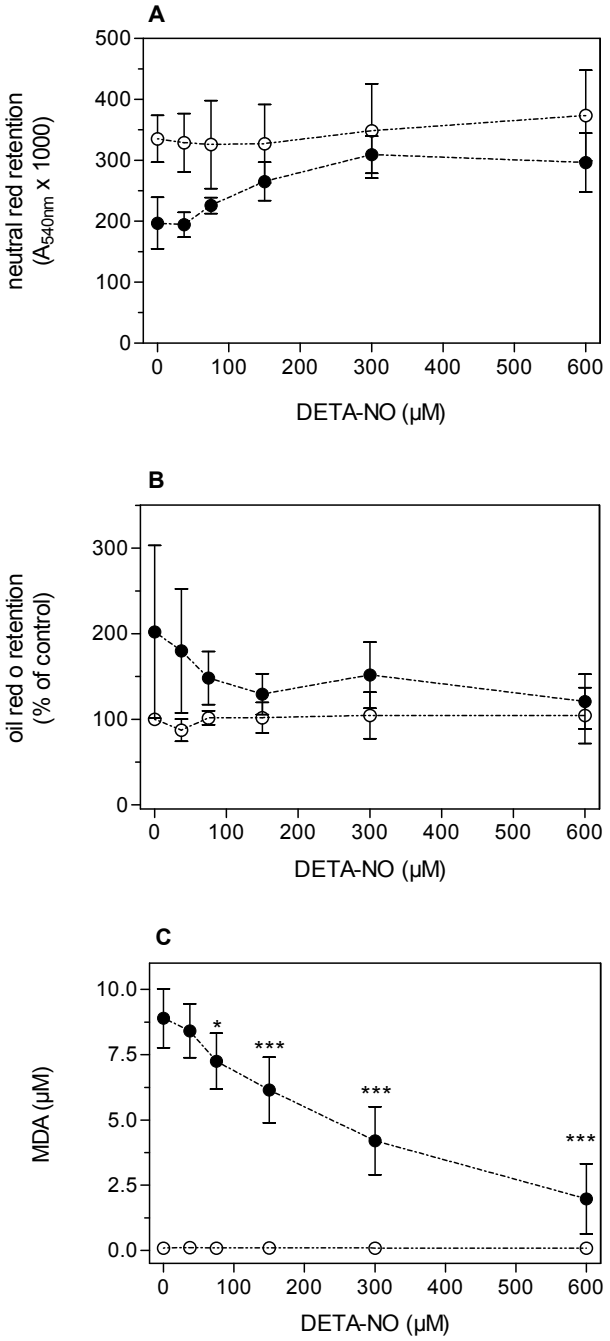


Figure 36: Effect of DETA-NO on oxLDL-induced changes in cell viability (A), lipid deposits (B) and malondialdehyde release (C) in THP-1 macrophages.

THP-1 macrophages were incubated with Cu^{2+}-oxidized LDL (100 µg/ml, ●) or BSA (2 mg/ml, control, ○) together with increasing concentrations (37.5 - 600 µM) of DETA-NO for 18 h at 37°C. Neutral red assay was performed to measure cell viability (A), (mean ± SD, n=3). Accumulation of lipids within the cells was monitored with oil red o staining (B) and the amount of MDA release was measured spectrophotometrically within the cell supernatant (C), (mean ± SD, n=4).

Incubation with DETA-NO alone had no effect on cell viability of THP-1 macrophages. Treatment with oxLDL (100 µg/ml) for 18 hours at 37°C decreased cell viability compared to the control (2 mg/ml BSA) to about 60%. Co-incubation of Cu^{2+}-oxidized LDL (100 µg/ml) together with DETA-NO (37.5 - 600µM) reversed the oxLDL-induced loss of cell viability in THP-1 macrophages dose-dependently almost to control levels (Fig. 36A).

Treatment with oxLDL (100 µg/ml) for 18 hours at 37°C increased the lipid deposits in differentiated THP-1 cells compared to the control (2 mg/ml BSA). However, this effect was not significant due to a high standard deviation. Incubation with DETA-NO alone had no effect on the accumulation of cholesteryl esters and triglycerides in THP-1 macrophages. Co-incubation of Cu^{2+}-oxidized LDL (100 µg/ml) together with DETA-NO (37.5 – 600 µM) decreased the oxLDL-induced accumulation of lipids in THP-1 macrophages dose-dependently, again not significantly due to high variation of the results (Fig. 36B).

Incubation with DETA-NO alone had no effect on TBARS formation in THP-1 macrophages. Treatment with oxLDL (100 µg/ml) for 18 hours at 37°C resulted in high malondialdehyde accumulation (8.9±1.1 µM) in cell supernatant compared to the control (2 mg/ml BSA). Co-incubation of Cu^{2+}-oxidized LDL (100 µg/ml) together with DETA-NO (37.5 – 600 µM) decreased the oxLDL-induced accumulation of TBARS in THP-1 macrophages dose-dependently ($p<0.05$, $p<0.001$) to 4.2±1.3 µM MDA at a concentration of 300 µM DETA-NO and 2±1.3 µM MDA with 600 µM of DETA-NO (Fig. 36C).

Taken together, NO released from DETA-NO decreased oxLDL-induced lipid deposits and malondialdehyde formation and improved cell viability, which decreased to about 60% in the presence of oxLDL.
Therefore, we aimed to investigate the influence of DETA-NO on oxLDL mediated HIF-1α protein stabilization and SR-BI protein reduction.

5.5.3 Effect of DETA-NO on HIF-1α and SR-BI protein expression in oxLDL treated THP-1 macrophages

THP-1 macrophages were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours at 37°C together with increasing DETA-NO concentrations (37.5 - 600 µM). The effect of DETA-NO on HIF-1α and SR-BI protein levels in Cu^{2+}-oxidized LDL treated THP-1 macrophages was investigated performing Western blot analyses with whole cell lysates.

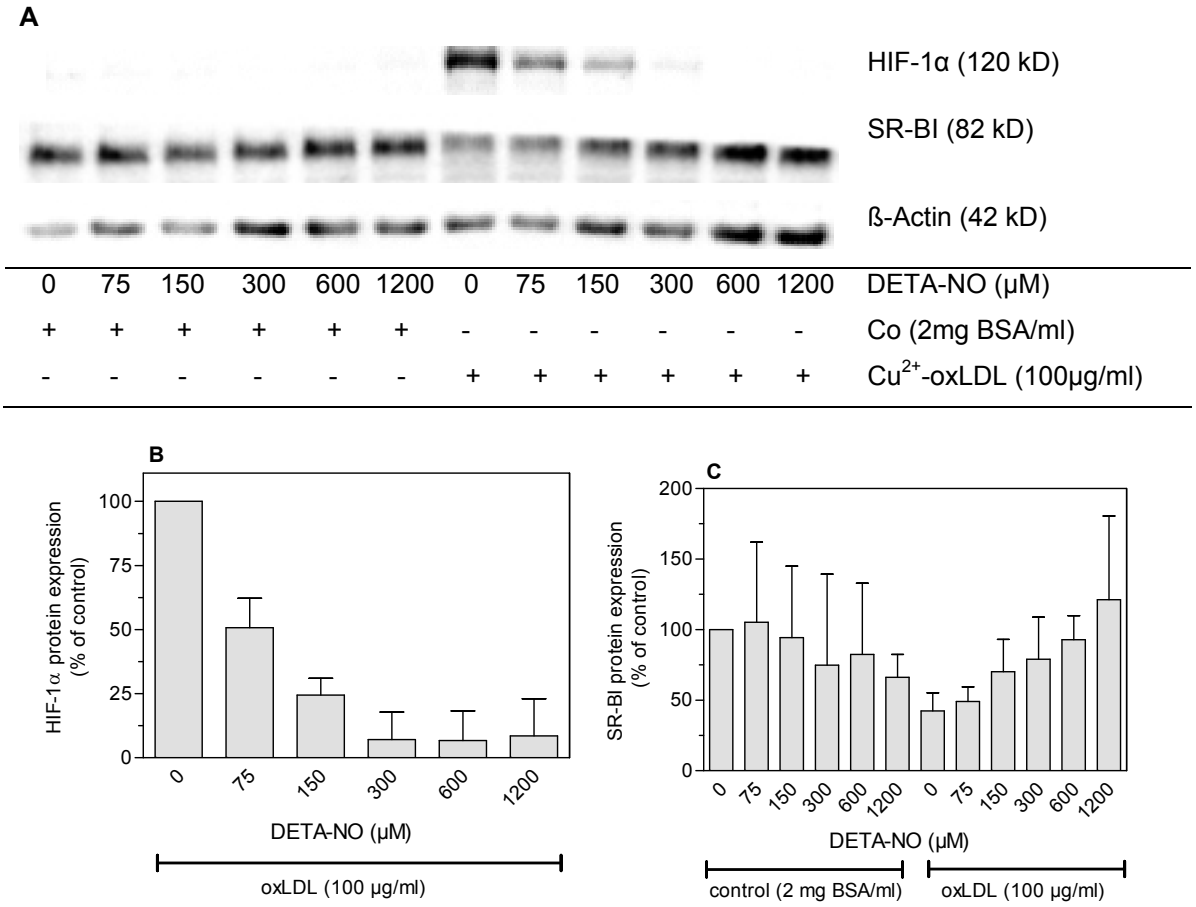


Figure 37: Effect of DETA-NO on HIF-1α and SR-BI protein levels in oxLDL treated THP-1 macrophages.

Cells were treated with Cu^{2+}-oxidized LDL (100 µg/ml) and increasing concentrations of DETA-NO (37.5 - 600 µM) for 18 h at 37°C. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed (A). ß-Actin was used as loading control. Graph (B) shows normalized intensity of HIF-1α protein expression in % of oxLDL-treatment alone. Graph (C) shows normalized intensity of SR-BI protein expression in % of control without DETA-NO, (mean ± SD, n = 3).

A concentration of 75 µM DETA-NO dropped oxLDL-induced HIF-1α protein accumulation (100%) down to 50%, 150 µM DETA-NO to 25%. With a concentration of 300 µM DETA-NO and higher HIF-1α protein was nearly undetectable (Fig. 37B). Therefore, a concentration of 300 µM DETA-NO was used for further experiments. DETA-NO per se showed no effect on HIF-1α protein stabilization (Fig. 37A).

As described before (cpt. 5.3.6), SR-BI protein decreased to about 50% after treatment with Cu^{2+}-oxidized LDL in THP-1 macrophages compared to the control. Co-incubation of oxLDL with DETA-NO reversed oxLDL-induced SR-BI attenuation dose-depently to a level comparable to the control (Fig. 37C).

To summarize, Cu^{2+}-oxidized LDL induced HIF-1α protein accumulation in THP-1 macrophages under normoxic conditions was attenuated by DETA-NO co-incubation dose-dependently, while oxLDL-induced loss of SR-BI protein in THP-1 macrophages was detained.

5.6 Effect of DETA-NO, oxLDL and DHA in THP-1 macrophages during a short-time incubation under normoxic and hypoxic conditions

Previous experiments (cpt. 5.5.3) clearly showed that DETA-NO (300 µM) is able to attenuate oxLDL-induced HIF-1α protein accumulation and SR-BI depletion in THP-1 macrophages during an 18 hours incubation.
It was published earlier that incubation with nitric oxide under normoxia for four hours provokes an accumulation of HIF-1α protein, whereas under hypoxia it attenuates cell enrichment in HIF-1α protein [SOGAWA et al. 1998; HUANG et al. 1999; SANDAU et al. 2001]. So far, we found no stabilization of HIF-1α protein when cells were incubated with up to 1.2 mM of DETA-NO for 18 hours under normoxic conditions (Fig. 37A).
Assuming, that an incubation time of 18 hours might be too long and all NO released from DETA-NO diffused into the medium, the effects of oxLDL and DETA-NO on TBARS formation and HIF-1α and SR-BI protein expression in THP-1

macrophages after a four-hour treatment under normoxia and hypoxia were analysed and incubation under normoxic and hypoxic conditions were compared.
THP-1 macrophages were preloaded with or without DHA (100 µM) for 30 minutes, and were incubated afterwards with Cu^{2+}-oxidized LDL (100 µg/ml) at 37°C for 4 hours in the presence or absence of DETA-NO (300 µM). This experimental setting was carried out both under normoxic and hypoxic conditions (1% O_2).

5.6.1 Effect of DETA-NO and DHA on oxLDL-induced TBARS formation in THP-1 macrophages during a short-time incubation under normoxic or hypoxic conditions

First, TBARS formation within the cell supernatant was measured spectrophotometrically at 534 nm.

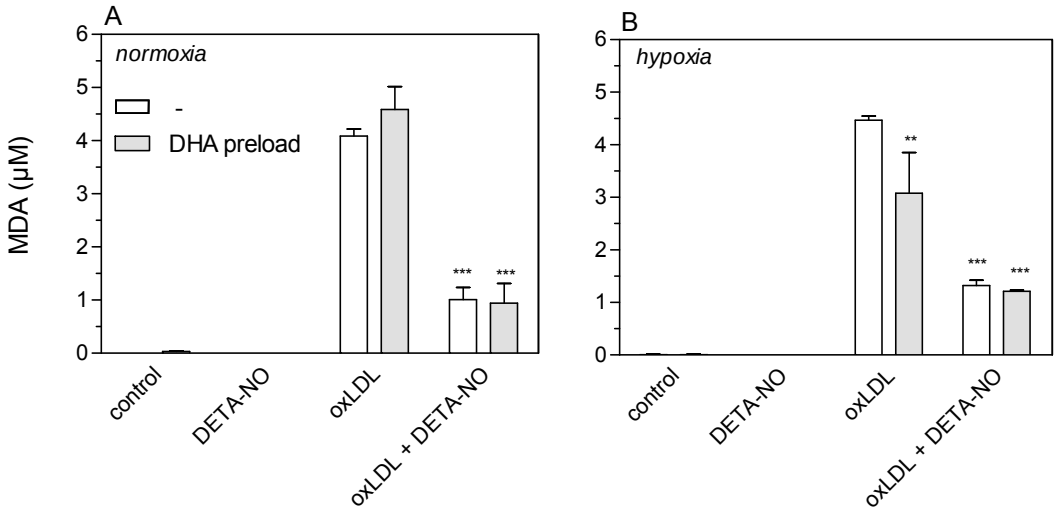


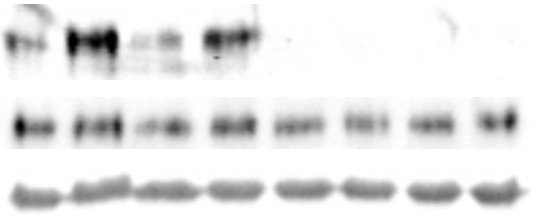
Figure 38: Effect of DETA-NO and DHA on oxLDL-induced accumulation of TBARS in THP-1 macrophages under normoxic and hypoxic conditions, measured as concentration of malondialdehyde (µM).
THP-1 macrophages were incubated in 6-well plates for 4 h at 37°C with BSA (2 mg/ml, control), Cu^{2+}-oxidized LDL (100 µg/ml) and/or DETA-NO (300 µM) with (grey bars) or without (white bars) DHA preload (100 µM, 30 minutes) under normoxic (A) or hypoxic (B) conditions. The amount of TBARS within the cell supernatant was measured spectrophotometrically. ** $p<0.01$, *** $p<0.001$ vs. oxLDL treatment alone, (mean ± SD, n = 3).

Treatment of THP-1 macrophages with Cu^{2+}-oxidized LDL (100 µg/ml) led to a release of malondialdehyde into cell supernatant. After 18 hours of incubation THP-1 macrophages accumulated about 9-12 µM of malondialdehyde (cpt. 5.3.3, 5.4.2, 5.5.2) within the cell supernatant, 4 hours of incubation led to about 4-5 µM MDA, both under normoxic and hypoxic conditions (Fig. 38). Neither DHA nor DETA-NO treatment (300 µM) resulted in measurable TBARS formation. Preloading of the cells with DHA (100 µM, 30 minutes) did not affect oxLDL-induced MDA accumulation in differentiated THP-1 cells under normoxia (see also cpt. 5.4.2), but decreased the MDA release under hypoxia from 4.5±0.1 to 3±0.8 µM ($p<0.01$). DETA-NO, applied together with oxLDL, attenuated the oxLDL-induced TBARS formation to 1±0.2 µM malondialdehyde under normoxia, which was also seen at an 18 hours incubation (cpt. 5.5.2), and to about 1.3±0.1 µM under hypoxia.

5.6.2 Effect of DETA-NO, oxLDL and DHA on SR-BI and HIF-1α protein expression in THP-1 macrophages during a short-time incubation under normoxic and hypoxic conditions

To reflect the dimished lipid peroxidation by NO, Western blot analyses were performed with whole cell lysates.

A normoxia



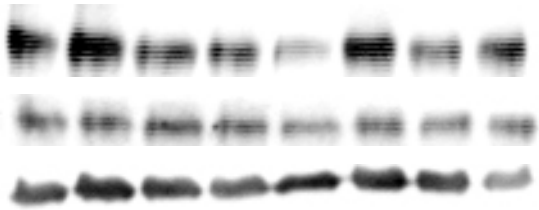
HIF-1α (120 kD)

SR-BI (82 kD)

ß-Actin (42 kD)

-	-	-	-	+	+	+	+	DHA preload (100 µM, 30 min)
-	+	-	+	-	+	-	+	DETA-NO (300 µM)
-	-	+	+	-	-	+	+	Cu^{2+}-oxLDL (100µg/ml)

B hypoxia

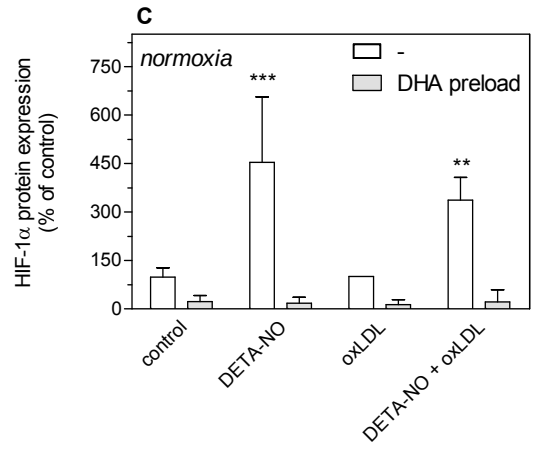


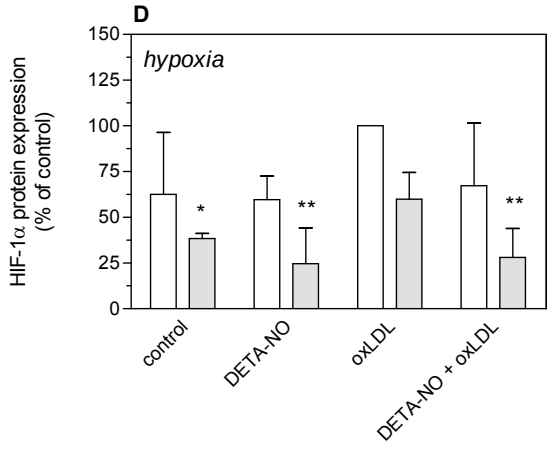
HIF-1α (120 kD)

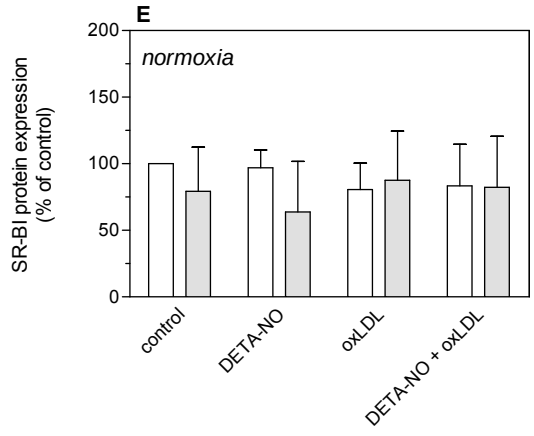
SR-BI (82 kD)

ß-Actin (42 kD)

-	-	-	-	+	+	+	+	DHA preload (100 µM, 30 min)
-	-	+	+	-	-	+	+	DETA-NO (300 µM)
-	+	-	+	-	+	-	+	Cu^{2+}-oxLDL (100µg/ml)







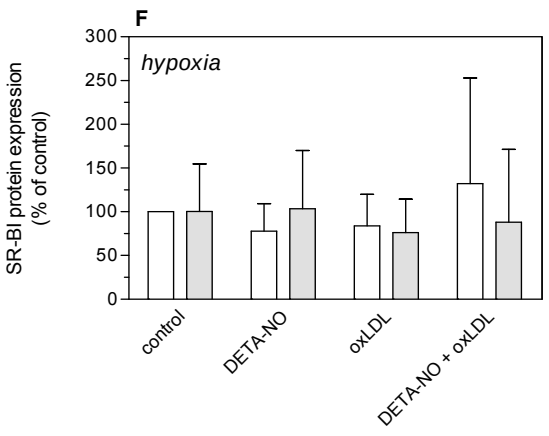


Figure 39: Effect of a short-time incubation with DETA-NO and DHA on HIF-1α and SR-BI protein levels in oxLDL treated THP-1 macrophages under normoxic and hypoxic conditions.
THP-1 macrophages were incubated for 4 h at 37°C with BSA (2 mg/ml, control), Cu^{2+}-oxidized LDL (100 µg/ml) and/or DETA-NO (300 µM) with (grey bars) or without (white bars) a previous DHA preload (100 µM, 30 minutes) under normoxic (A, C, E) or hypoxic (B, D, F) conditions. After cell lysis Western blot analyses were performed with whole cell lysates showing representative Western blots of 3 performed (A, B). ß-Actin was used as loading control.
Graphs show normalized intensity of HIF-1α protein under normoxic (C) and hypoxic (D) conditions in % of oxLDL without any other treatment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. oxLDL treatment (100%), (mean ± SD, n = 3).
Graphs show normalized intensity of SR-BI protein under normoxic (E) and hypoxic (F) conditions in % of control, (mean ± SD, n = 3).

HIF-1α protein was accumulated about 4.5 times higher ($p< 0.001$) in the presence of DETA-NO (300 µM) compared to oxLDL (100 µg/ml) after a short-time treatment of THP-1 macrophages for four hours under normoxia. DETA-NO induced accumulation of HIF-1α protein was attenuated in the presence of oxLDL. Preloading of the cells with DHA (100 µM, 30 min) decreased HIF-1α protein levels to hardly detectable levels, even in the control (Fig. 39C).
It is known that HIF-1α protein is stabilized under hypoxia [SEMENZA 2007] and this stabilization is diminished by NO. We could show that DETA-NO (300 µM) was not able to mitigate hypoxia-induced stabilization of HIF-1α protein. Treatment of THP-1 macrophages with oxLDL (100 µg/ml) for four hours under hypoxic conditions increased the hypoxia induced HIF-1α protein levels to about 140%, but not significantly due to a high variation of the results. Again, preloading of the cells with 100 µM DHA for 30 minutes attenuated HIF-1α protein levels (Fig. 39D).

None of the compounds used affected SR-BI protein levels in THP-1 macrophages, neither under normoxia nor hypoxia (Fig. 39E+F).

5.7 Effect of ascorbate and dehydroascorbate (DHA) preloading in DETA-NO treated HUVECs on HIF-1α protein expression

We could show that incubation with nitric oxide under normoxia for four hours provokes an accumulation of HIF-1α protein in THP-1 macrophages (cpt. 5.6.2). Ascorbate has been reported to decrease hypoxia-induced HIF-1α protein levels in human primary and cancer cell lines [KNOWLES et al. 2003; VISSERS et al. 2007]. Therefore, the next aim was to investigate the effect of vitamin C on DETA-NO-mediated HIF-1α protein expression in HUVECs, an endothelial cell model. Confluent HUVECs were incubated with increasing concentrations of DETA-NO (250, 500, 1000 µM) for 4 hours with or without a previous preload of either ascorbate (200 µM, 18 h) or DHA (200 µM, 30 min).

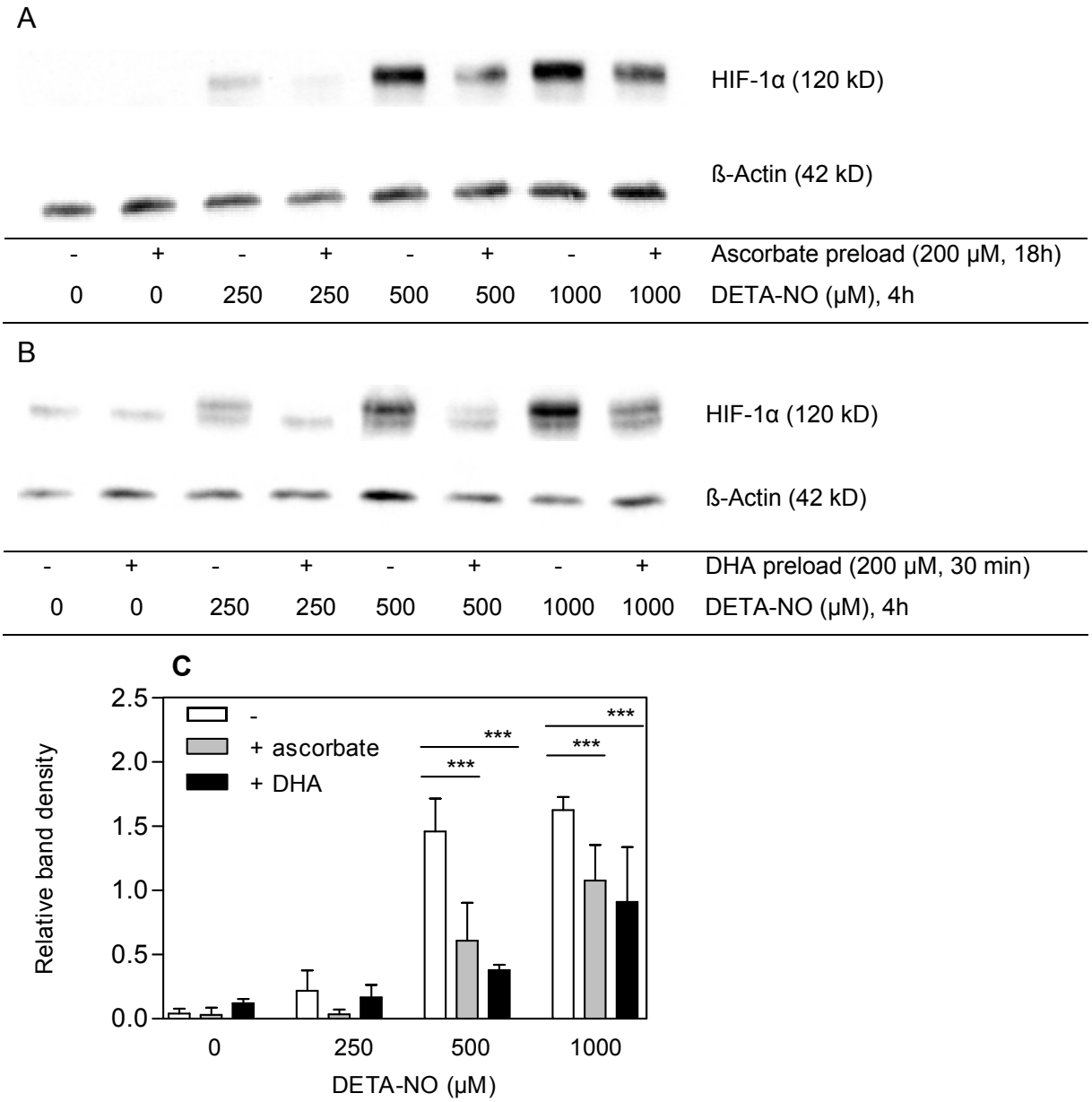


Figure 40: Effect of vitamin C on DETA-NO induced HIF-1α protein accumulation in HUVEC cells. HUVECs were incubated at 37°C with increasing concentrations of DETA-NO for 4 h with or without a previous ascorbate (200 µM, 18 h) (A) or DHA (200 µM, 30 min) (B) preload. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed. ß-Actin was used as loading control.
Graph (C) shows relative band density of HIF-1α protein. *** $p<0.001$ vs. DETA-NO treatment alone, (mean ± SD, n = 3).

DETA-NO triggered HIF-1α protein accumulation dose dependently (Fig. 40A+B) in HUVECs under normoxic conditions after 4 hours of incubation. This accumulation was attenuated when the cells were preloaded overnight with ascorbate (200 µM) (Fig. 40A) or for 30 minutes with DHA (200 µM) (Fig. 40B).

In the presence of 500 µM of DETA-NO, an inhibition of about 60% to 70% of HIF-1α protein accumulation was observed after ascorbate or DHA preloading ($p<0.001$). Pretreatment with ascorbate or DHA of cells, which were incubated with 1000 µM of DETA-NO, led to a decrease of about 30% of DETA-NO (1000 µM) induced HIF-1α protein accumulation ($p<0.001$).
Over night (18 hours) treatment of HUVECs with DETA-NO had no effect on HIF-1α protein (data not shown).

5.8 Effect of DETA-NO and HDL co- and pre-incubation on oxLDL-induced modifications in THP-1 macrophages

So far, we reported that DETA-NO attenuates oxLDL-induced HIF-1α protein stabilization, lipid accumulation, TBARS formation and reverses oxLDL-induced cytotoxicity in THP-1 macrophages (cpt. 5.5).
HDL inhibits oxLDL-induced HIF-1α protein accumulation, co- and pre-incubation of HDL circumvents oxLDL-induced loss of cell viability and accumulation of lipids in the cells (cpt. 5.4).
The next aim was to investigate whether there is a difference between pre-incubation and co-incubation of DETA-NO together with HDL on TBARS formation and HIF-1α and SR-BI protein expression in oxLDL treated THP-1 macrophages under normoxia.
THP-1 macrophages were incubated with DETA-NO (300 µM) and/or HDL (150 µg/ml) for 18 hours at 37°C in the presence or absence of Cu^{2+}-oxidized LDL (100 µg/ml), or 8 hours before Cu^{2+}-oxidized LDL (100 µg/ml) was added for 18 hours. These onsets were performed with or without preloading of the cells with DHA (100 µM) for 30 minutes.

5.8.1 Effect of DETA-NO and HDL co- and pre-incubation on TBARS formation in oxLDL treated THP-1 macrophages

TBARS formation was measured within the cell supernatant.

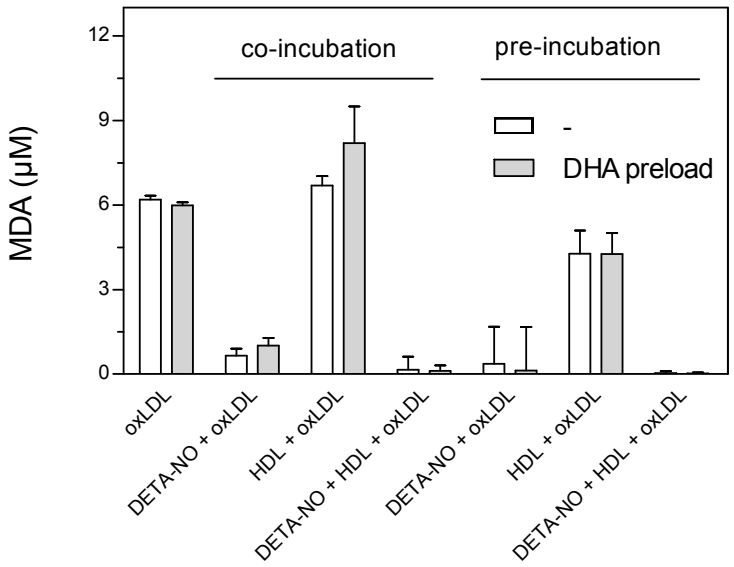


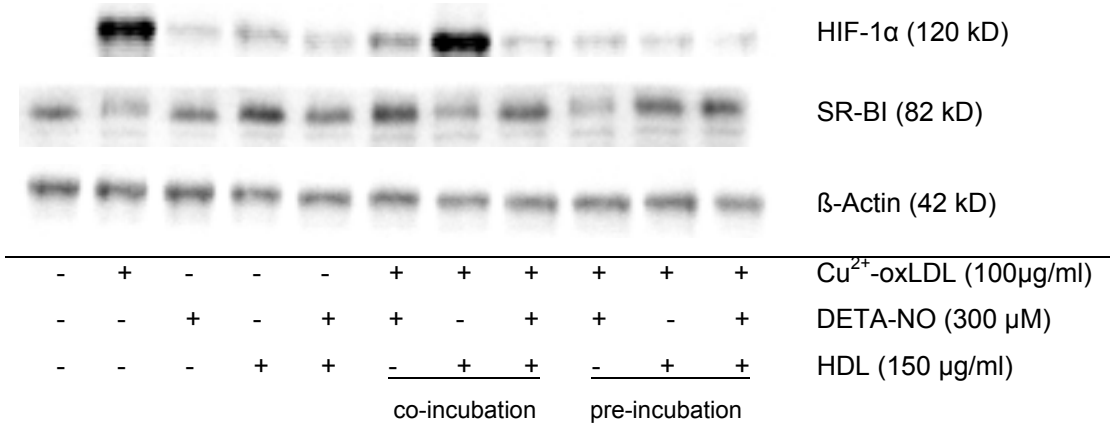
Figure 41: Effect of DETA-NO, HDL and vitamin C on oxLDL-induced accumulation of TBARS, measured as concentration of malondialdehyde (µM) in THP-1 macrophages.
THP-1 macrophages were incubated with DETA-NO (300 µM) and/or HDL (150 µg/ml) in the presence or absence of oxLDL (100 µg/ml) (co-incubation) or 8 h before oxLDL was added (pre-incubation) for 18 h at 37°C, with (grey bars) or without (white bars) DHA (100 µM) preloading for 30 minutes. The amount of TBARS was measured spectrophotometrically within the cell supernatant, (mean ± SD, n=2).

Incubation with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours led to an accumulation of 6 µM of malondialdehyde within the cell supernatant. Co-incubation with HDL (150 µg/ml) had no effect on MDA accumulation, but when HDL was given 8 hours before oxLDL was added (pre-incubation), TBARS formation was attenuated to about 4 µM. Co- or pre-incubation (8 hours) with DETA-NO (300 µM) reduced oxLDL-induced TBARS formation to concentrations below 1 µM. This effect was even more pronounced when both HDL and DETA-NO were incubated together with oxLDL, where MDA-levels were hardly detectable. DHA preload showed no effect on oxLDL-induced malondialdehyde accumulation (Fig. 41).

5.8.2 Effect of DETA-NO and HDL co- and pre-incubation on HIF-1α and SR-BI protein in oxLDL treated THP-1 macrophages

Western blot analyses were performed to investigate the effect of DETA-NO and/or HDL pre-incubation on HIF-1α and SR-BI protein levels in comparison to a co-incubation in differentiated THP-1 cells.

A



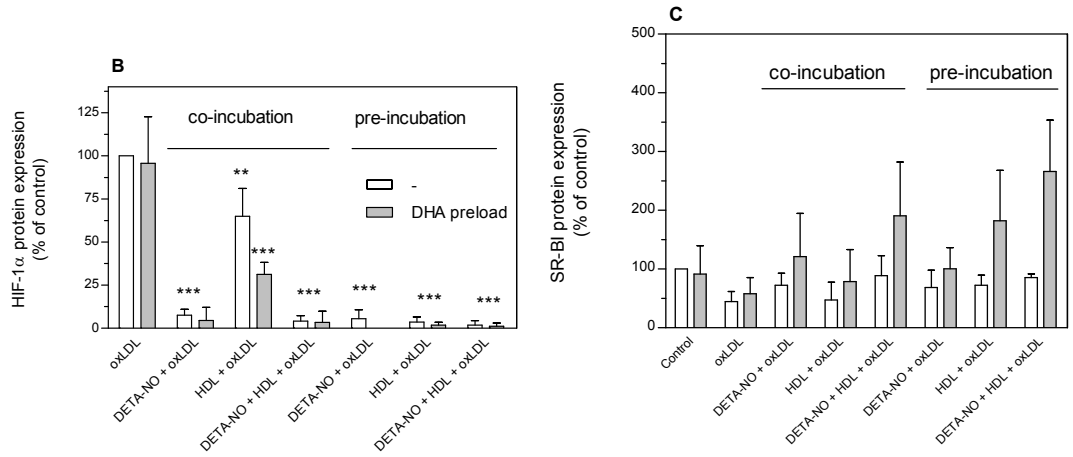


Figure 42: Effect of DETA-NO, HDL and vitamin C on HIF-1α and SR-BI protein levels in oxLDL treated THP-1 macrophages.

THP-1 macrophages were incubated with DETA-NO (300 µM) and/or HDL (150 µg/ml) in the presence or absence of oxLDL (100 µg/ml) (co-incubation) or 8 h before oxLDL was added (pre-incubation) for 18 h at 37°C, with (grey bars) or without (white bars) DHA preloading (100 µM, 30 minutes). After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed (DHA preload blot not shown). ß-Actin was used as loading control (A). Graph (B) shows normalized intensity of HIF-1α protein expression in % of oxLDL treatment alone. *** $p<0.001$ vs. oxLDL treatment without DHA preloading (100%), (mean ± SD, n = 3). Graph (C) shows normalized intensity of SR-BI protein expression in % of control (without DHA preloading), (mean ± SD, n = 3).

Again, Cu^{2+}-oxidized LDL (100 µg/ml) strongly induced HIF-1α protein accumulation and diminished SR-BI protein in THP-1 macrophages. Co- or pre-incubation with DETA-NO (300 µM) and/or HDL (150 µg/ml) attenuated oxLDL-induced HIF-1α protein accumulation and loss of SR-BI protein (Fig. 42A).

OxLDL-induced HIF-1α accumulation was attenuated significantly ($p<0.001$) to 8% in the presence of DETA-NO (300 µM) (co-incubation). This effect was even more pronounced when DETA-NO was given 8 hours before oxLDL was added (pre-incubation), in particular after DHA preloading (100 µM, 30 min).
HDL (150 µg/ml) diminished oxLDL-induced HIF-1α protein accumulation significantly ($p<0.01$) to 65%, with a higher decrease (to 30%) after DHA preloading ($p<0.001$) (co-incubation). This effect was enforced when HDL was given 8 hours before oxLDL was added ($p<0.001$) (pre-incubation), where HIF-1α protein levels were detected below 10% of oxLDL treatment alone (100%). HIF-1α protein accumulation was attenuated almost completely when HDL and DETA-NO were applied together 8 hours before oxLDL was added ($p<0.001$) (Fig. 42B).

Treatment with oxLDL (100 µg/ml) impaired SR-BI protein in THP-1 macrophages. This attenuation was reversed when DETA-NO (300 µM) and HDL (150 µg/ml) were given together with (co-incubation) or 8 hours before oxLDL was added (pre-incubation). This effect was even more pronounced when the cells were preloaded with DHA (100 µM) for 30 minutes (Fig. 42C).

5.9 Effect of Cu^{2+}-oxidized LDL, DETA-NO and HDL on cholesterol efflux in THP-1 macrophages

Cu^{2+}-oxidized LDL increased lipid deposits in THP-1 macrophages, DETA-NO and HDL were shown to inhibit this accumulation of lipids (cpt. 5.4.2, 5.5.2). Therefore we were interested to measure its impact on cholesterol efflux in THP-1 macrophages which were incubated with Cu^{2+}-oxidized LDL.

After trace-labeling the cholesterol pool of differentiated THP-1 cells with ^{3}H-cholesterol (2 µCi) and pre-incubating them with or without DETA-NO (300 µM) and/or HDL (150 µg/ml), new media with DETA-NO and/or HDL in the presence or absence of Cu^{2+}-oxidized LDL (100 µg/ml) was given to the cells. Pure medium and native LDL were given to the cells as controls. After 16 hours of incubation the cells were equilibrated and afterwards efflux was initiated with HDL (10 µg/ml) for 6 hours. The counts of cell medium and cell lysates were measured and efflux was expressed as percentage of radioactivity released from cells into the medium relative to the total counts measured in cell lysates plus medium.

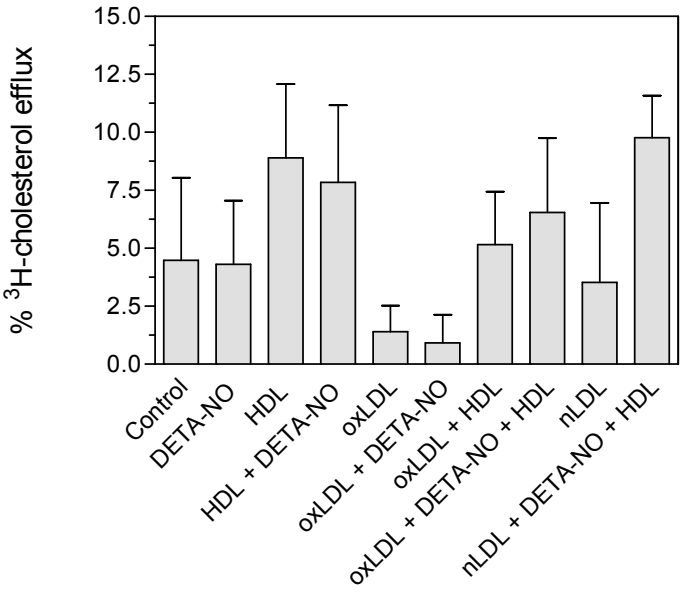


Figure 43: Effect of Cu^{2+}-oxidized LDL in the presence or absence of DETA-NO and/or HDL on cholesterol efflux in THP-1 macrophages.

After 48 h of differentiation ^{3}H-cholesterol (2 µCi) was added to trace-label THP-1 cells, 16 h later DETA-NO (300 µM) and/or HDL (150 µg/ml) were added to the cells (pre-incubation). Cell supernatant of THP-1 macrophages (72 h of differentiation) containing PMA, ^{3}H-cholesterol, DETA-NO and HDL was removed and after washing the cells new media with DETA-NO and/or HDL in the presence or absence of Cu^{2+}-oxidized LDL (100 µg/ml) was given to the cells for 16 h. Pure media and native LDL were given to the cells as controls. After washing cells were equilibrated twice with fresh media and cholesterol efflux was initiated with HDL (10 µg/ml) for 6 h. Then the counts of trace-label were measured within cell supernatant and cell lysats in a ß-counter and efflux (% of total ^{3}H-cholesterol) was calculated thereafter, (mean ± SD, n = 4).

DETA-NO (300 µM, 4.3±2.8) alone had no effect on cholesterol efflux from THP-1 macrophages compared to the control without any treatment (4.5±3.6% efflux). Incubation of the cells with HDL (150 µg/ml), given alone (8.9±3.2% efflux) or together with DETA-NO (7.8±3.3% efflux) increased the subsequent cholesterol efflux. When the cells were treated with oxLDL (100 µg/ml) cholesterol efflux decreased to about 1.4±1.1%, pre-incubation with DETA-NO did not alter this oxLDL-induced reduction in cholesterol efflux. However, pre-incubation of oxLDL treated THP-1 macrophages with HDL and HDL together with DETA-NO enforced the subsequent efflux to 5.2±2.3 and 6.5±3.2%, respectively. Incubation with nLDL (100 µg/ml) had no influence on cholesterol efflux (3.5±3.4%), except when cells were pre-incubated with HDL and DETA-NO (9.8±1.8% efflux) (Fig. 43).

Taken together, oxLDL decreased and HDL increased cholesterol efflux, but not significantly. DETA-NO showed no influence on cholesterol efflux from THP-1 macrophages.

5.10 **Effect of DETA-NO post-incubation in oxLDL treated THP-1 macrophages**

DETA-NO (300 µM) attenuates oxLDL (100 µg/ml) induced HIF-1α protein accumulation and TBARS formation in THP-1 macrophages when given together or 8 hours before oxLDL is added (cpt. 5.8). The next step was to investigate the effect of DETA-NO when it is applied to differentiated THP-1 cells after an 18 hours incubation with Cu^{2+}-oxidized LDL.
In detail, differentiated THP-1 cells treated with oxLDL (100 µg/ml) for 18 hours were washed twice with serum-free media before DETA-NO (300 µM) was added for another 18 hours, or DETA-NO was added for further 18 hours to the cells after an 18 hours oxLDL incubation without washing, resulting in a total of 36 hours of oxLDL incubation.

5.10.1 Effect of DETA-NO post-incubation on TBARS formation in oxLDL treated THP-1 macrophages

Formation of TBARS within the cell supernatant was measured spectrophotometrically after reaction with thiobarbituric acid.

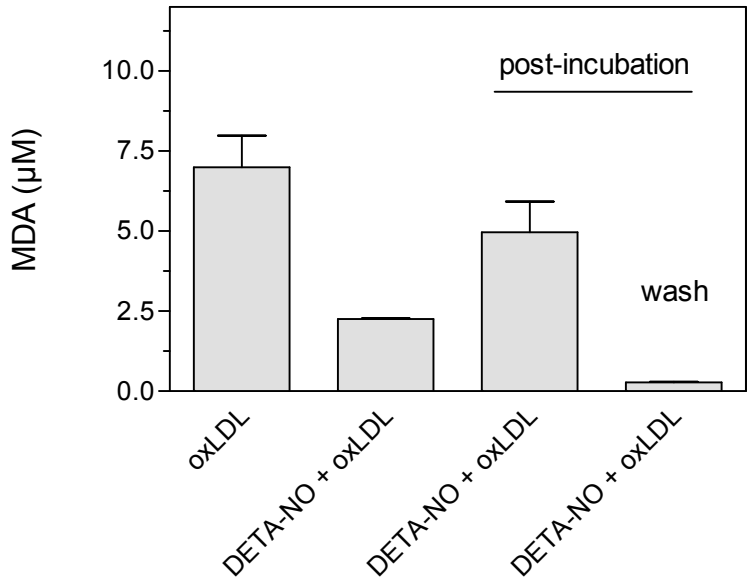


Figure 44: Effect of DETA-NO post-incubation on oxLDL-induced accumulation of TBARS, measured as concentration of malondialdehyde (µM) in THP-1 macrophages.
THP-1 macrophages were incubated at 37°C with oxLDL alone (100 µg/ml), together with DETA-NO (300 µM) for 18 h (co-incubation) or with DETA-NO after an 18 h oxLDL treatment for further 18 h, with or without an intermediate washing step (post-incubation). The amount of TBARS was measured spectrophotometrically within the cell supernatant, (mean ± SD, n=2).

Simultaneous addition of DETA-NO (300 µM) decreased oxLDL (100 µg/ml) induced malondialdehyde formation from 7±1 µM (oxLDL treatment alone) to 2.3±0.01 µM of MDA. DETA-NO addition to the cells for 18 hours after 18 hours of oxLDL incubation reduced TBARS formation to about 5±1 µM (Fig. 44).

5.10.2 Effect of DETA-NO post-incubation on HIF-1α and SR-BI protein in oxLDL treated THP-1 macrophages

Differentiated THP-1 cells were lysed and Western blot analysis was performed to investigate the influence of DETA-NO co- or post-treatment in oxLDL treated cells.

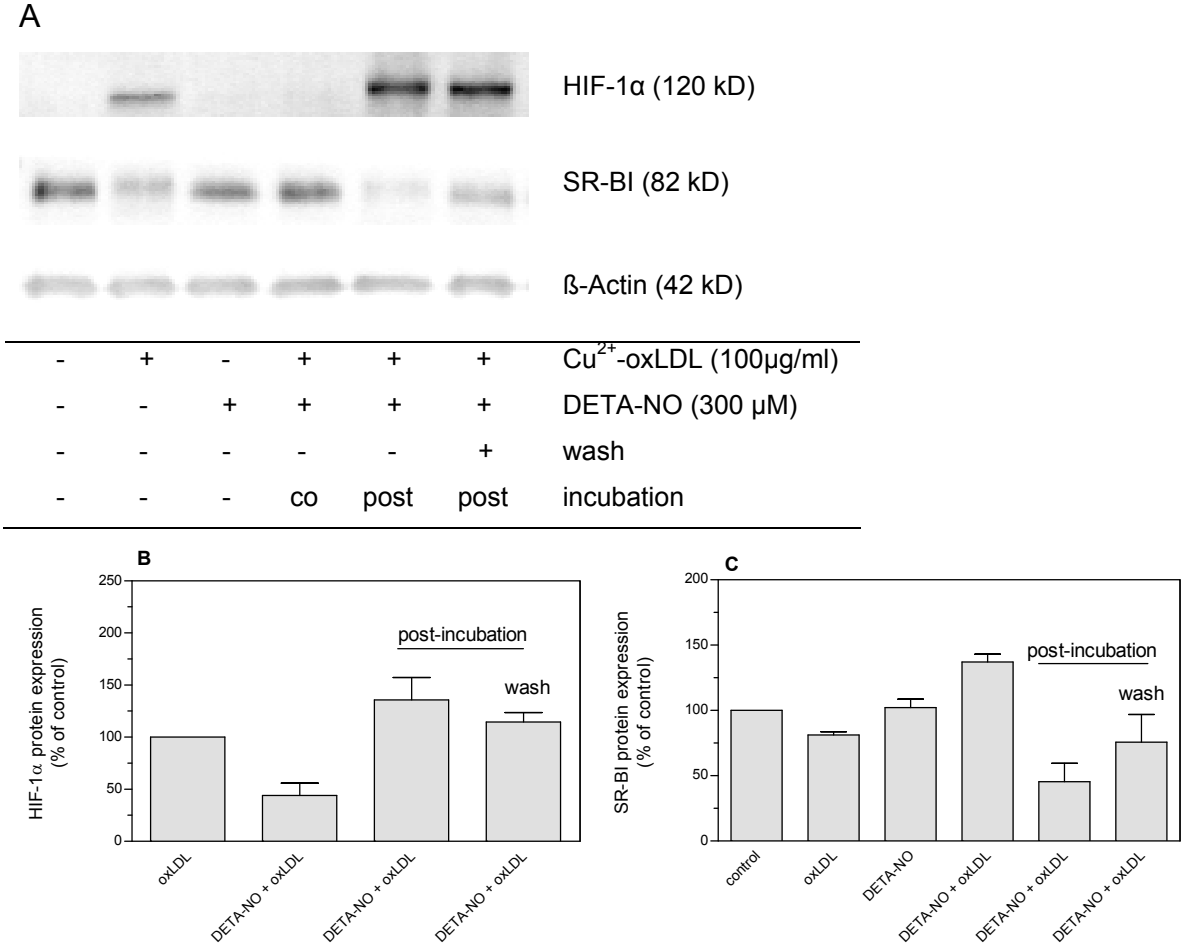


Figure 45: Effect of DETA-NO post-incubation on HIF-1α and SR-BI protein levels in oxLDL treated THP-1 macrophages.

THP-1 macrophages were incubated with oxLDL alone (100 µg/ml), with DETA-NO (300 µM) alone or together with oxLDL for 18 h at 37°C (co-incubation) or with DETA-NO after an 18 h oxLDL treatment for further 18 h (post-incubation), with or without an intermediate washing step. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 2 performed. ß-Actin was used as loading control (A).

Graph (B) shows normalized intensity of HIF-1α protein expression in % of oxLDL treatment alone, (mean ± SD, n = 2).

Graph (C) shows normalized intensity of SR-BI protein expression in % of control (no treatment), (mean ± SD, n = 2).

DETA-NO (300 µM) was not able to attenuate oxLDL (100 µg/ml) induced HIF-1α protein accumulation and SR-BI protein depletion in THP-1 macrophages, when it was given after an 18 hours oxLDL treatment (post-incubation), compared to an oxLDL - DETA-NO co-incubation, which is shown in Fig. 43A. DETA-NO alone had no effect on HIF-1α or SR-BI expression.

OxLDL-induced HIF-1α protein accumulation was reduced about 50%, when the cells were treated with DETA-NO together with oxLDL (co-incubation). When THP-1 macrophages were incubated with DETA-NO for 18 hours after 18 hours of oxLDL treatment (post-incubation), regardless if with or without an intermediate washing step, HIF-1α protein levels were even higher than in cells with oxLDL treatment alone. These findings indicate that DETA-NO is able to attenuate oxLDL-induced HIF-1α protein accumulation in THP-1 macrophages, but only when it is applied together with or before addition of oxLDL (Fig. 43B).

Cu^{2+}-oxidized LDL induced loss of SR-BI protein was prevented in the presence of DETA-NO (co-incubation) where SR-BI protein levels were about 35% higher than in the control. But when differentiated THP-1 cells were incubated with oxLDL for 18 hours with subsequent addition of DETA-NO (post-incubation), regardless if with or without an intermediate washing-step, to remove oxLDL, DETA-NO was not able to reverse the oxLDL-induced decrease in SR-BI protein (Fig. 43C).

These results confirm that oxLDL and its products have to be present to stabilize HIF-1α protein and reduce SR-BI protein, respectively.

5.11 Effect of $NaNO_2$ and Vitamin C on oxLDL-induced modifications in THP-1 macrophages

The inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) can be recycled *in vivo* to form NO. It was shown that for example nitrite reduction to NO is elevated through reducing agents like vitamin C [LUNDBERG et al. 2008]. Therefore, we examined the effect of $NaNO_2$ in the presence of vitamin C on oxLDL-induced cell toxicity, lipid deposits, TBARS formation, HIF-1 α protein accumulation and SR-BI protein depletion.

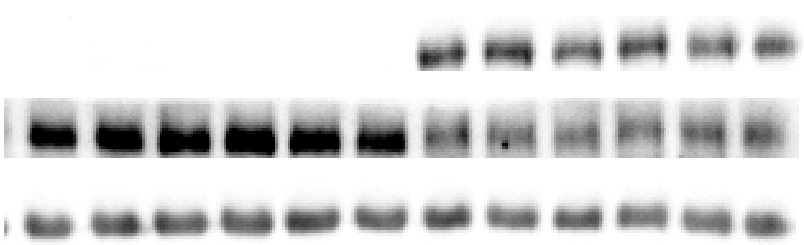
THP-1 macrophages were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours at 37°C with increasing concentrations of $NaNO_2$ (0.1 - 100 µM) and ascorbate (100 µM).

Neutral red assay was performed to examine the effect of $NaNO_2$ in the presence of vitamin C on the celltoxic events of Cu^{2+}-oxidized LDL.
As mentioned before (cpt. 5.3.5), cell viability of THP-1 macrophages was reduced about 40% after treatment with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours. Unlike DETA-NO (cpt. 5.5.2), a co-incubation with ascorbate (100 µM) and/or $NaNO_2$ could not attenuate this loss of cell viability (data not shown).

Triglycerides and cholesterylester within the cells were stained with oil red o and after extraction with isopropanol the amount of the dye was measured spectrophotometrically.
Treatment with Cu^{2+}-oxidized LDL (100 µg/ml) increased lipid deposits in THP-1 macrophages of about 30% (see also cpt. 5.3.2). Co-incubation with ascorbate (100 µM) and/or $NaNO_2$ had no effect on stainable lipids in the cells (data not shown). Hence $NaNO_2$ and ascorbate were not able to inhibit accumulation of lipid deposits induced by oxLDL treatment of THP-1 macrophages.
While DETA-NO (300 µM) could inhibit foam cell formation (cpt. 5.5.1, 5.5.2), $NaNO_2$ was ineffective, either with or without ascorbate (100 µM) (data not shown).

THP-1 macrophages accumulated malondialdehyde and other aldehydes (about 9 µM) after treatment with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours. $NaNO_2$ (0.1 - 100 µM) and ascorbate (100 µM) were not able to attenuate this process (data obtained by spectrophotometry and not shown).

In differentiated THP-1 cells oxLDL-induced HIF-1α protein accumulation and loss of SR-BI protein could be attenuated by DETA-NO (cpt. 5.5.3). Hence the effect of $NaNO_2$ in the absence and presence of ascorbate on HIF-1α and SR-BI protein in oxLDL treated THP-1 macrophages was investigated.



0	0.1	0.5	1	10	100	0	0.1	0.5	1	10	100	$NaNO_2$ (µM)
+	+	+	+	+	+	+	+	+	+	+	+	Ascorbate (100 µM)
+	+	+	+	+	+	-	-	-	-	-	-	Co (2mg BSA/ml)
-	-	-	-	-	-	+	+	+	+	+	+	Cu^{2+}-oxLDL (100µg/ml)

Figure 46: Effect of $NaNO_2$ and ascorbate on HIF-1α and SR-BI protein levels in oxLDL treated THP-1 macrophages.

THP-1 macrophages were incubated with Cu^{2+}-oxidized LDL (100 µg/ml) or BSA (2 mg/ml, control) together with increasing concentrations (0.1 - 100 µM) of $NaNO_2$ in the presence or absence of ascorbate (100 µM) for 18 h at 37°C. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed. ß-Actin was used as loading control.

Fig. 46 depicts that $NaNO_2$ (0.1 – 100 µM) and ascorbate (100 µM) did neither affect the oxLDL (100 µg/ml)-induced HIF-1α protein accumulation nor the oxLDL-induced SR-BI protein reduction in THP-1 macrophages.

5.12 Effect of ebselen on oxLDL-induced modifications in THP-1 macrophages

Ebselen, an antiinflammatory seleno-organic antioxidant, is able to reduce hydroperoxides to their corresponding hydroxy compounds [SCHEWE 1995]. It was also shown that pretreatment of oxLDL with this seleno-organic compound, which is known to have GPx-like activity [SIES 1993], inhibits oxLDL-induced depletion of intracellular glutathione (GSH) levels. Pretreatment did also slightely reduce MDA levels [SHEN and SEVANIAN 2001].

Therefore, the effect of ebselen on oxLDL-induced TBARS formation, HIF-1α protein accumulation and SR-BI protein depletion in THP-1 macrophages was investigated. THP-1 macrophages were incubated with either BSA (2 mg/ml, control) or Cu^{2+}-oxidized LDL (100 µg/ml) together with increasing concentrations of ebselen (0.1 – 20 µM) for 18 hours at 37°C. TBARS formation was measured within the supernatant and the cells were lysed and analysed by Western blot analysis.

5.12.1 Effect of ebselen on TBARS formation in oxLDL treated THP-1 macrophages

The formation of malondialdehyde was measured spectrophotometrically after reaction with thiobarbituric acid within the cell supernatant of THP-1 macrophages.

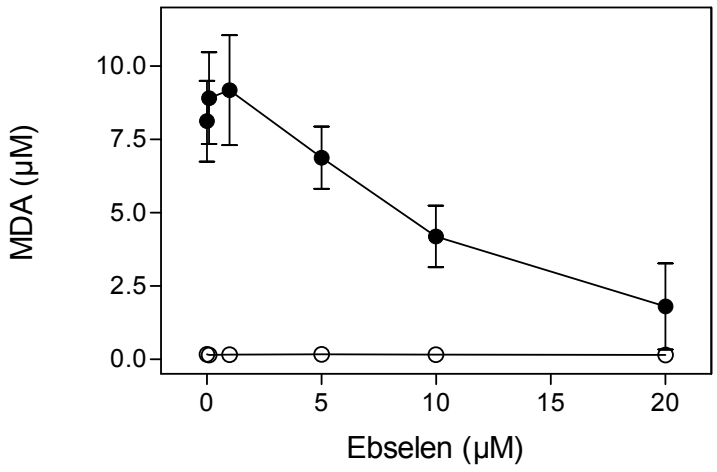


Figure 47: Effect of ebselen on oxLDL-induced accumulation of TBARS, measured as concentration of malondialdehyde (µM) in THP-1 macrophages.
THP-1 macrophages were incubated with BSA (2 mg/ml, Control, ○) or Cu^{2+}-oxidized LDL (100 µg/ml, ●) together with the indicated ebselen concentrations (0.1 – 20 µM) for 18 h at 37°C. The amount of TBARS was measured spectrophotometrically within the cell supernatant, (mean ± SD, n=2).

Fig. 47 depicts that ebselen decreased oxLDL (100 µg/ml) induced TBARS formation in THP-1 macrophages dose dependently. 10 µM of ebselen led to a decrease of about 50% of oxLDL-induced malondialdehyde formation, 20 µM of ebselen to a decrease of about 80%.

5.12.2 Effect of ebselen on oxLDL-induced HIF-1α protein accumulation and SR-BI depletion in THP-1 macrophages

THP-1 macrophages were lysed after 18 hours of incubation with the indicated amounts of ebselen (0.1 – 20 µM) in the presence or absence of Cu^{2+}-oxidized LDL (100 µg/ml). Afterwards, the expression levels of HIF-1α and SR-BI protein were measured by Western blot analyses.

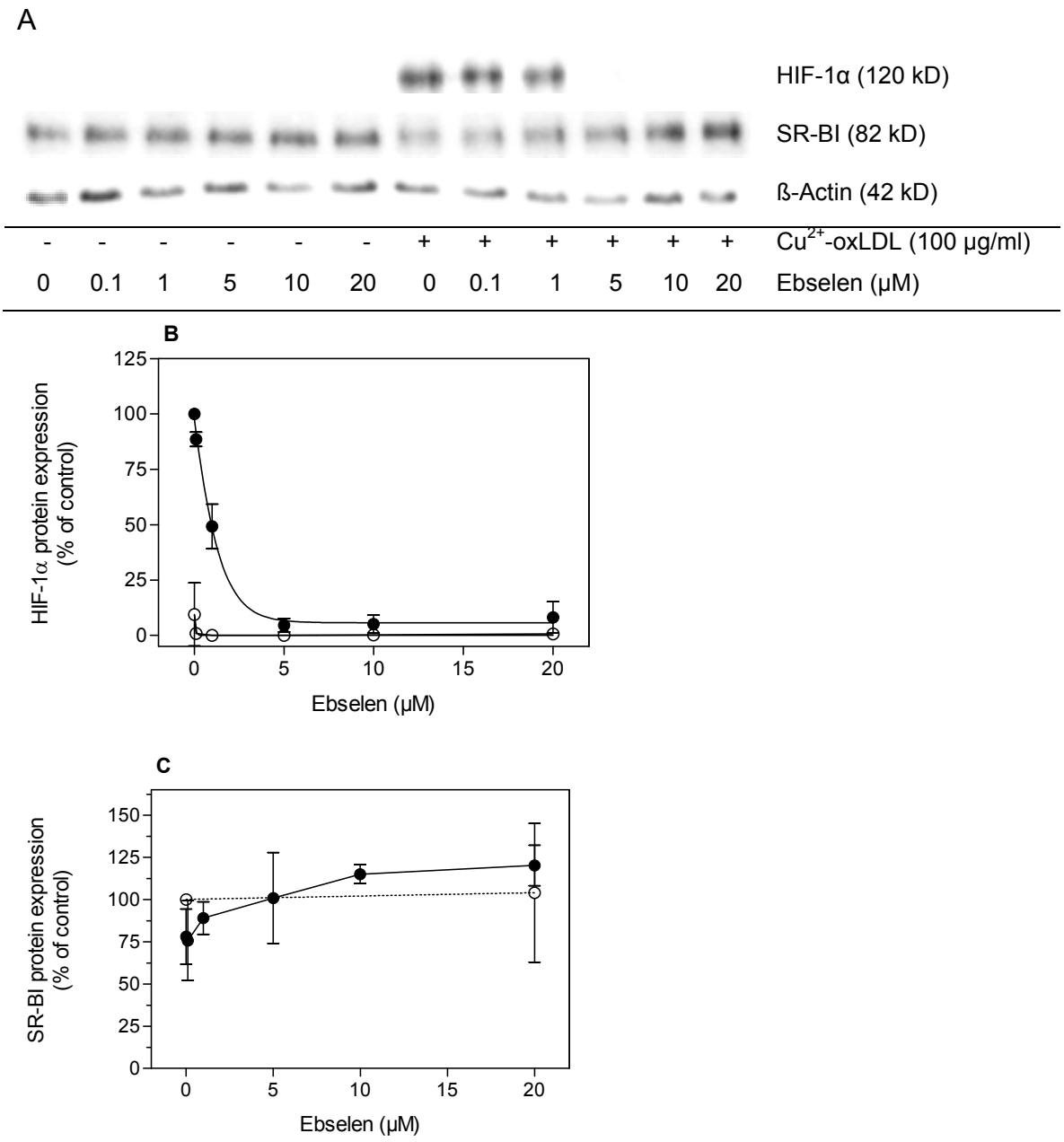


Figure 48: Effect of ebselen on HIF-1α and SR-BI protein levels in oxLDL treated THP-1 macrophages.

THP-1 macrophages were incubated at 37°C with BSA (2 mg/ml, Control, ●) or Cu^{2+}-oxidized LDL (100 µg/ml, ○) together with the indicated ebselen (0.1 – 20 µM) concentrations for 18 h. After cell lysis Western blot analyses were performed with whole cell lysates showing one representative Western blot of 3 performed. ß-Actin was used as loading control (A).

Graph (B) shows normalized intensity of HIF-1α protein expression in % of oxLDL treatment alone, (mean ± SD, n = 3).

Graph (C) shows normalized intensity of SR-BI protein expression in % of control, (mean ± SD, n = 3).

The presence of ebselen (0.1 – 20 µM) resulted in a dose dependent mitigation of oxLDL (100 µg/ml) induced HIF-1α protein accumulation and loss of SR-BI protein in THP-1 macrophages, with a complete inhibition of HIF-1α protein at a concentration of 5 µM of ebselen or more (Fig. 48A).

Co-treatment of Cu^{2+}-oxidized LDL with 1 µM of ebselen led to 50% decrease of HIF-1α protein, and from a concentration of 5 µM of ebselen on HIF-1α protein accumulation was inhibited to hardly detectable levels (Fig. 48B).

Treatment of THP-1 macrophages with Cu^{2+}-oxidized LDL led to a loss of SR-BI protein of about 25%. When the cells were co-incubated with ebselen, SR-BI protein levels increased dose-dependently to levels comparable to SR-BI protein levels of the control without oxLDL treatment (Fig. 48C).

These data were confirmed by photomicrographs, which showed again that concomitant incubation of oxLDL treated THP-1 macrophages with ebselen abolished foam cell formation dose-dependently (data not shown).

5.13 mRNA expression of CD36, SR-BI, HIF-1α and VEGF during differentiation of THP-1 cells

THP-1 cells were incubated with 160 nM PMA for 72 hours to determine mRNA levels of the differentiation marker CD36, the scavenger receptor SR-BI, the transcription factor HIF-1α and its target gene VEGF during macrophage differentiation. Every 24 hours RNA was isolated and after cDNA synthesis RT-PCR was performed. GAPDH was used as loading control.
Fig. 49A shows representative bands of the indicated genes of interest.

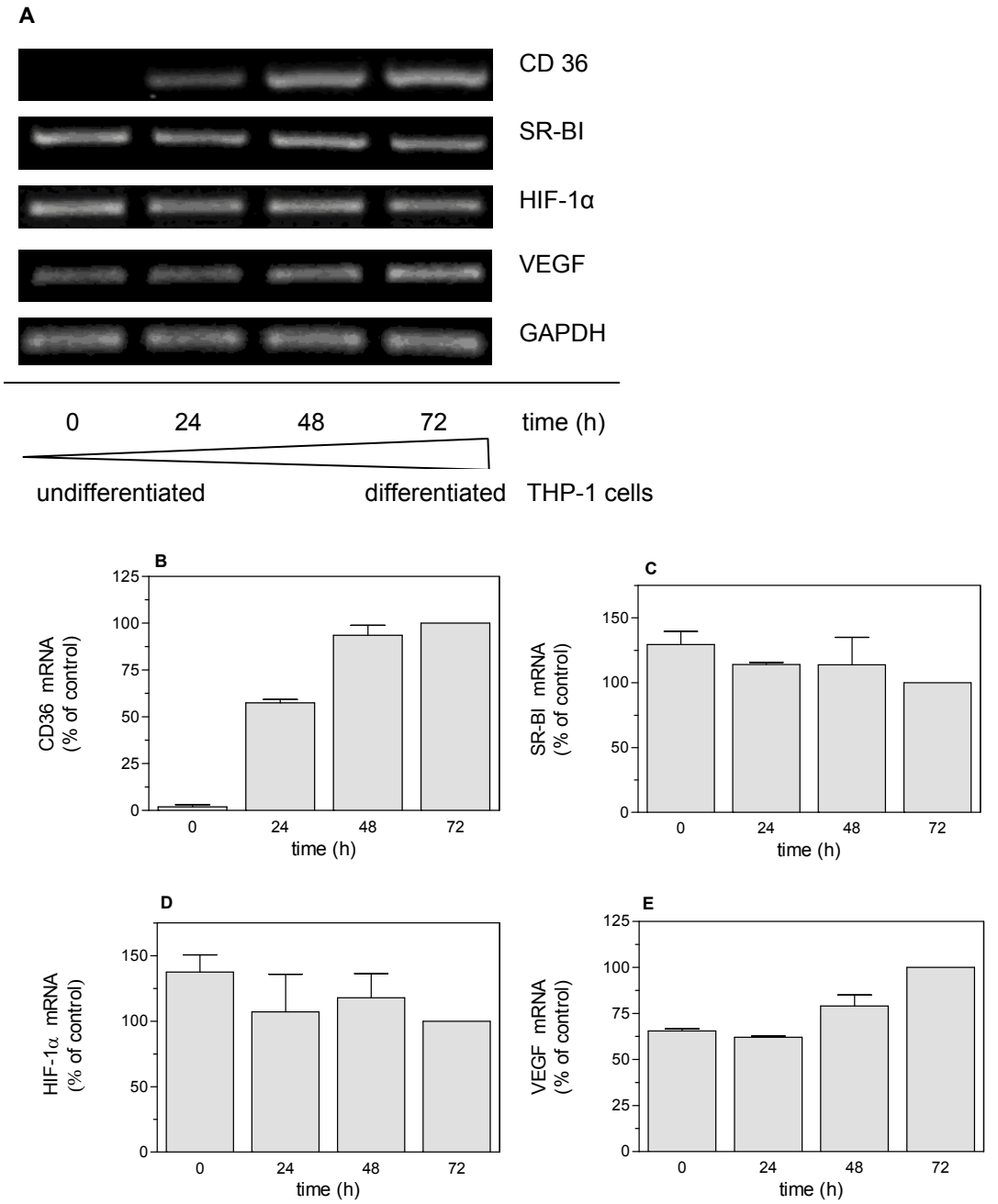


Figure 49: Influence of THP-1 cell differentiation on expression of CD36, SR-BI, HIF-1α and VEGF mRNA.

THP-1 monocytes were differentiated towards macrophages with 160 nM PMA for 72 h at 37°C, every 24 h RNA was isolated and after cDNA synthesis RT-PCR was performed, showing representative bands of the genes of interest (A). GAPDH was used as loading control. Graphs show normalized (GAPDH) intensity of CD36 (B), SR-BI (C), HIF-1α (D) and VEGF (E) mRNA in % of band intensity after differentiation for 72 h, which was set to 100%, (mean ± SD, n = 2).

CD36 is known as a marker for macrophage differentiation, in concordance with the literature [ALESSIO et al. 1996] we could show that in THP-1 monocytes there was nearly no CD36 mRNA expression detected. CD36 mRNA levels, like protein levels (cpt. 5.1), increased time-dependently during macrophage differentiation in THP-1 cells. After 24 hours of differentiation 60% and after 48 hours about 95% of macrophage's expression level of CD36 (72 hours of differentiation) was reached (Fig. 49B).

In contrast to increasing SR-BI protein levels during macrophage differentiation (cpt. 5.1), a slight decrease in SR-BI mRNA expression in THP-1 was observed. SR-BI levels in THP-1 monocytes were about 25% higher than in macrophages (Fig. 49C).

HIF-1α mRNA expression of THP-1 monocytes was about 30% higher than in THP-1 macrophages, showing a time-dependent slight decrease during differentiation (Fig. 49D).

VEGF mRNA levels rised time-dependently during 72 hours of differentiation. In THP-1 monocytes 66±1.1% of the VEGF mRNA levels of THP-1 macrophages was reached, with no further rise during the next 24 hours. After 48 hours the VEGF mRNA levels were about 20% lower than in THP-1 macrophages (72 hours of differentiation with 160 nM PMA, 100%) (Fig. 49E).

5.14 Effect of copper and HOCl-modified LDL on mRNA levels of CD36, SR-BI, HIF-1α and VEGF in THP-1 monocytes and macrophages

Next, we investigated the mRNA levels of the indicated genes of interest in THP-1 macrophages in comparison to THP-1 monocytes after treatment with modified LDL. Therefore THP-1 monocytes or macrophages were incubated in the presence or absence of copper or HOCl-modified LDL (100 µg/ml) for 18 hours. After RNA isolation and cDNA synthesis RT-PCR was performed. Figure 50A shows representative bands of the indicated genes of interest.

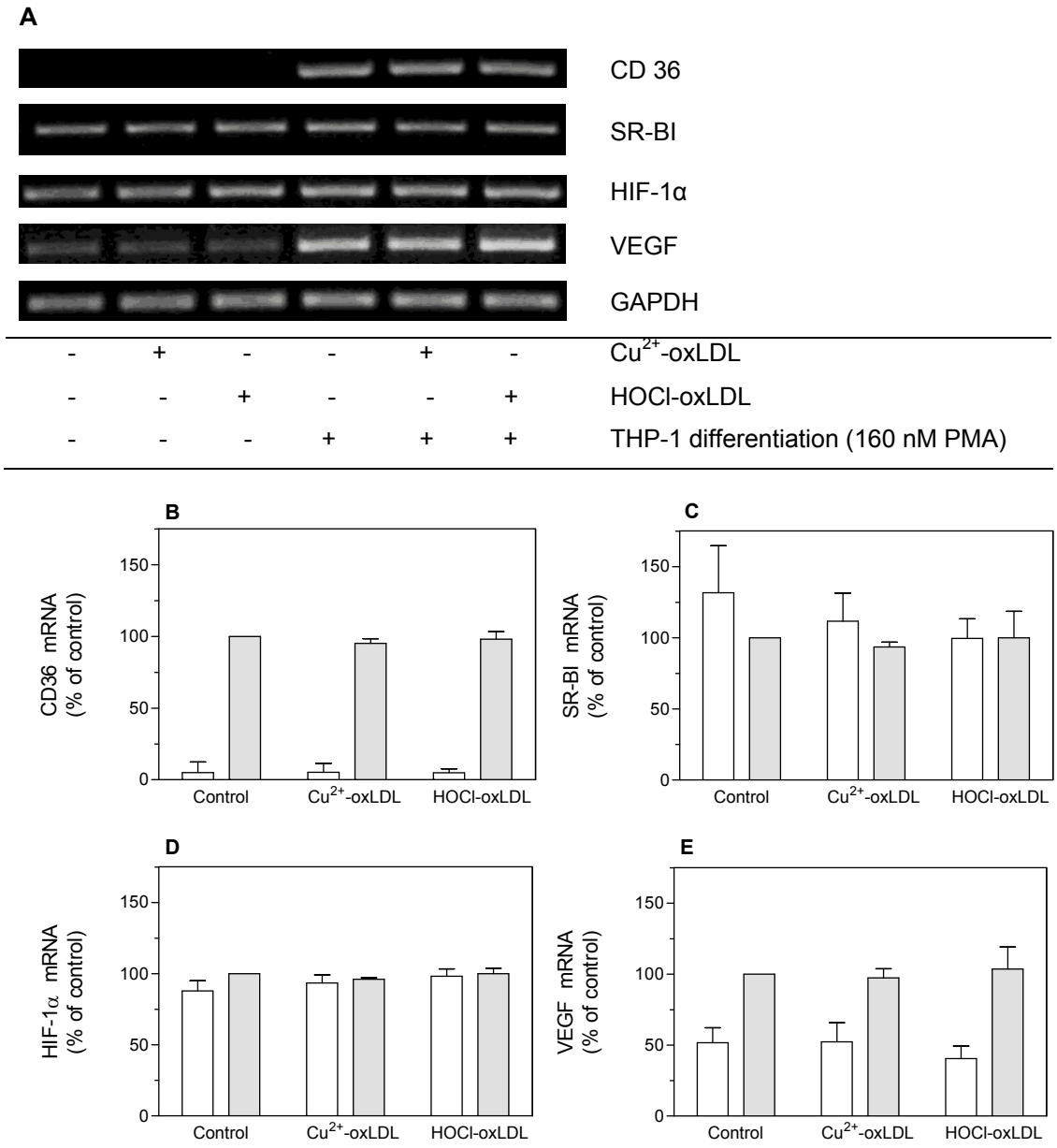


Figure 50: Influence of modified LDL on expression of CD36, SR-BI, HIF-1α and VEGF mRNA in undifferentiated and differentiated THP-1 cells.

THP-1 monocytes and macrophages were treated with copper- or HOCl-modified LDL (100 µg/ml) for 18 h at 37°C. After RNA isolation and cDNA synthesis RT-PCR was performed, showing representative bands of the genes of interest in (A). GAPDH was used as loading control. Graphs show normalized (GAPDH) intensity of CD36 (B), SR-BI (C), HIF-1α (D) and VEGF (E) mRNA in % of band intensity of control after differentiation for 72 h, which was set to 100%, (mean ± SD, n = 4).

THP-1 monocytes expressed 5% of the CD36 mRNA levels of THP-1 macrophages, confirming that CD36 levels increase during differentiation with phorbol ester. Incubation of THP-1 monocytes or macrophages with copper or HOCl-modified LDL (100 µg/ml) had no effect on CD36 mRNA expression levels compared to untreated controls (Fig. 50B).

SR-BI mRNA levels of THP-1 monocytes were about 30% higher than in THP-1 macrophages, which could also been shown previously during differentiation studies. When THP-1 monocytes were treated with Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours, SR-BI levels decreased about 20% compared to THP-1 monocytes without treatment. Incubation with HOCl-modified LDL (100 µg/ml, 18 hours) decreased SR-BI mRNA expression levels about 30% in THP-1 monocytes compared to these cells without any treatment reaching similar SR-BI expression levels like THP-1 macrophages. In THP-1 macrophages treatment with either Cu^{2+}- or HOCl-modified LDL for 18 hours had no effect on SR-BI mRNA expression compared to THP-1 macrophages without any treatment (Fig. 50C).

Neither Cu^{2+} nor HOCl-modified LDL had an effect on HIF-1α mRNA levels compared to the control (Fig. 50D). Therefore, the enormous accumulation of HIF-1α protein after incubation with Cu^{2+}-oxidized LDL (cpt. 5.3.6) seems to be due to posttranscriptional stabilization.

VEGF mRNA levels in THP-1 macrophages were about 45% higher than in THP-1 monocytes, which confirms previous results (cpt. 5.13). Copper or HOCl-modified LDL had no effect on VEGF mRNA levels compared to cells without any treament (Fig. 50E).

Taken together, treatment of THP-1 monocytes and macrophages with native LDL (100 µg/ml) for 18 hours had no effect on these genes of interest (data not shown).

5.15 Effect of HDL, native and Cu^{2+}-oxidized LDL on SR-BI and HIF-1α mRNA levels in THP-1 macrophages

THP-1 cells were differentiated towards macrophages, incubated with HDL (150 µg/ml), native (100 µg/ml) or Cu^{2+}-oxidized LDL (100 µg/ml) for 18 hours, RNA was isolated and Northern blot analyses were performed.

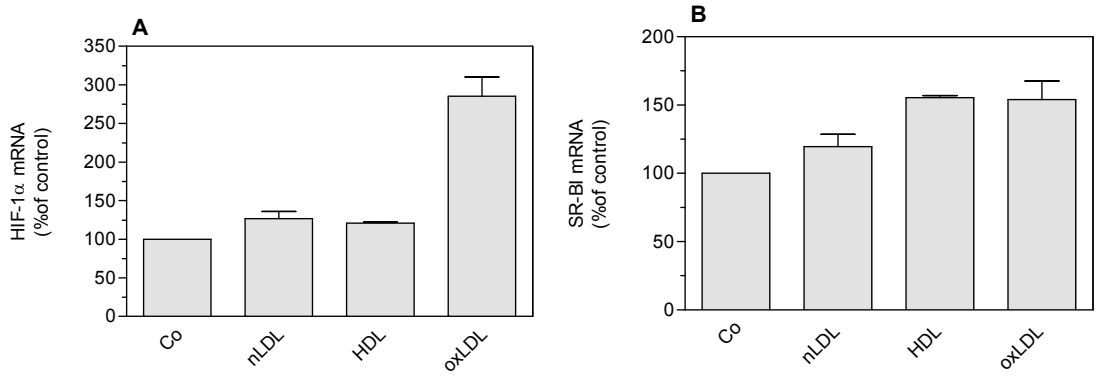


Figure 51: Effect of native, Cu^{2+}-oxidized LDL and HDL on HIF-1α and SR-BI mRNA levels in THP-1 macrophages.
Cells were treated with native LDL (100 µg/ml), HDL (150 µg/ml) or Cu^{2+}-oxidized LDL (100 µg/ml) for 18 h at 37°C and after RNA isolation Northern blot analyses were performed. Graphs show normalized (28S) intensity of HIF-1α (A) and SR-BI (B) mRNA in % of control (100%), (mean ± SD, n = 2).

Contrary to our RT-PCR results (cpt. 5.14), where Cu^{2+}-oxidized LDL did not affect HIF-1α mRNA levels in THP-1 macrophages after incubation with Cu^{2+}-oxidized LDL, performing Northern blot, there was an increase detected, namely a HIF-1α mRNA expression 3 times higher than in control macrophages without any treatment.
Native LDL and HDL had no effect on HIF-1α mRNA levels in THP-1 macrophages (Fig. 51A).

Administration of HDL (150 µg/ml) and Cu^{2+}-oxidized LDL (100 µg/ml) to THP-1 macrophages for 18 hours led to an increase of about 50% of SR-BI mRNA expression compared to the control without any treatment. However, there is an unexplainable discrepancy between the RT-PCR results (cpt.5.14), where Cu^{2+}-oxidized LDL caused a slight decrease of SR-BI mRNA levels, and this oxLDL-induced rise in SR-BI mRNA. Treatment of THP-1 macrophages with native LDL for 18 hours had no effect on SR-BI mRNA levels (Fig. 51B).

6 Discussion

Atherosclerosis is among the major causes of morbidity and mortality in westernized societies [WITZTUM and STEINBERG 2001]. It is a progressive disease characterized by lipid accumulations and fibrous elements in the large arteries followed by endothelial damages and resulting in clinical complications like stroke or myocardial infarction [LUSIS 2000]. The oxidative modification of the lipid as well as the apolipoprotein B (apoB) moiety of native LDL plays an important role, as it is taken up rapidly by macrophages resulting in foam cell formation and early fatty streaks [STEINBERG 1997; LUSIS 2000; GLASS and WITZTUM 2001; MALLE et al. 2006]. However, the underlying mechanisms are still not completely understood.

In this study we aimed to get further insights into the mechanisms apparently involved in foam cell formation, one of the initial steps of atherosclerosis due to its role in atherosclerotic plaque formation in the large arteries [STEINBERG et al. 1989; BERLINER et al. 1995; LUSIS 2000; GLASS and WITZTUM 2001; LIBBY 2006; PACKARD and LIBBY 2008].

THP-1 cells are commonly used as *in vitro* models for macrophages in atherosclerosis-related investigations [NGUYEN-KHOA et al. 1999; WANG et al. 2001; TSUKAMOTO et al. 2002; VINALS et al. 2005; MARCIL et al. 2006; RUBIC and LORENZ 2006; KANG et al. 2008]. These cells are kept as monocytes and are differentiated towards macrophages with 160 nM of phorbol 12-myristate 13-acetate (PMA). Differentiated THP-1 cells behave like human monocyte-derived macrophages in several aspects [AUWERX 1991]. It was shown earlier that these cells are able to accumulate free cholesterol and cholesteryl esters and build foam cells after treatment with modified LDL [VIA et al. 1989; BANKA et al. 1991].

Due to sex-specific differences in the functions of HDL we exclusively used lipoproteins isolated from healthy young women for our experiments. Association of estrogen with HDL in premenopausal women may be responsible for their superior

cardioprotection. It is known that HDL-associated 17ß-estradiol enforces cholesterol efflux from macrophages through the enhancement of cholesteryl ester cycle [SULISTIYANI and ST CLAIR 1997; NAPOLITANO et al. 2001; MENDELSOHN and KARAS 2005; BADEAU et al. 2009].

The reactions inducing LDL modification *in vivo* are still seen controversial. The heme enzyme myeloperoxidase (MPO), elevated levels of which have been detected in human atherosclerotic plaques [DAUGHERTY et al. 1994; MALLE et al. 2000], utilizes H_2O_2 to oxidize halides like Cl^-. HOCl, one of the major reaction products of the MPO/H_2O_2/Cl^--system in human plasma [MALLE et al. 2006], has been found to modify LDL, and HOCl-modified LDL has also been found in atherosclerotic lesions supporting the possible role of the system *in vivo* [HAZEN and HEINECKE 1997]. The *in vivo* relevance of copper ion-modified LDL is discussed controversially. However, transition metal ions like iron and copper were found in atherosclerotic lesions, which can then promote oxidative damage of lipoproteins or extracellular matrix components [EVANS et al. 1995; STADLER et al. 2004; RAMAN et al. 2008] and Cu^{2+}-oxidized LDL exhibits similar physical and biological characteristics like oxLDL *in vivo* [STEINBRECHER et al. 1987].
In our experiments HOCl modified LDL showed a higher relative electrophoretic mobility than native LDL, which is comparable to Cu^{2+}-oxidized LDL. Protein carbonyls, a marker for protein oxidation, were also increased in HOCl-modified LDL, but not as high as in Cu^{2+}-oxidized LDL.
These results are in contrast to the results of Nguyen-Khoa et al. [NGUYEN-KHOA et al. 1999], who showed that HOCl, but not copper modification results in an elevation of protein carbonyls within the LDL particle, altough the molar oxidant to LDL ratios were comparable to ours. Additionally, they incubated LDL 8 hours longer compared to our protocol. However, the group of Lester Packer, who developed the method to examine protein carbonyls after LDL oxidation, could also show a dose dependent increase in protein carbonyls after treatment of LDL with copper [YAN et al. 1995].
With light microscopy images we could show that in THP-1 macrophages the first foam cells are built after 8 hours of treatment with Cu^{2+}-oxidized LDL (100 µg/ml)

and after 18 hours nearly all cells were transformed to foam cells. Treatment of THP-1 monocytes also led to foam cell formation, documented with light microscopy, whereas treatment of THP-1 cells, both undifferentiated and differentiated, with HOCl-modified LDL did not affect shape and appearance of the cells.

Unlike Cu^{2+}-oxidized LDL, HOCl-modified LDL significantly increased lipid deposits as demonstrated by oil red o retention of the cells only at a concentration of 200 µg/ml and it did not decrease cell viability or initiate TBARS formation. This lack of TBARS formation can be well explained by the fact hat NaOCl is known to modify the protein fraction of the LDL particle [HAZELL and STOCKER 1993; MALLE et al. 2006].

Cell viability decreased after treatment with Cu^{2+}-oxidized LDL. The cytotoxic effects of oxLDL are well known and are thought to cause the death of macrophages and smooth muscle cells within atherosclerotic plaques and therefore lead to the formation of a necrotic core region [HEGYI et al. 1996; SALVAYRE et al. 2002].

However, HPLC-analyses of lipoprotein-treated (100 µg/ml each) THP-1 macrophages revealed the highest total cholesterol content after incubation with HOCl-modified LDL. Native LDL led to higher total cholesterol contents compared to control cells or cells, which were incubated with Cu^{2+}-oxidized LDL. These findings are in concordance with a study from Cader et al [CADER et al. 1997] who showed that both copper and HOCl-modified LDL led to an accumulation of total cholesterol in THP-1 cells compared to native LDL, whereas HOCl-modified LDL increased total cholesterol to a higher extent than Cu^{2+}-oxidized LDL. The authors also demonstrated that treatment with HOCl-modified LDL primarily resulted in a great increase of esterified cholesterol therefore leading to the observed high total cholesterol concentrations. Ryu et al. compared cholesteryl ester accumulation in mouse peritoneal macrophages, which were incubated with either copper or HOCl-modified LDL. They also could clearly demonstrate that macrophages accumulate more cholesterol after treatment with HOCl-modified LDL due to a greater cholesteryl ester accumulation [RYU et al. 1995]. Marsche et al. could show that

treatment with HOCl-modified LDL resulted in high accumulation of cholesteryl esters in THP-1 macrophages, but led in general only to a moderate lipid accumulation, which is likely due to the presence of amino acids within the cell medium [MARSCHE et al. 2003], which can inhibit lipoprotein aggregation [HAZELL and STOCKER 1993]. We separated unesterified from esterified cholesterol by HPLC only after incubation with native or Cu^{2+}-oxidized LDL. Cholesteryl esters were elevated in cells treated with native or Cu^{2+}-oxidized LDL compared to the control. We suppose that the great extent of cholesterol accumulation after HOCl-modified LDL treatment might be due to an elevation in cholesteryl esters. Further experiments are necessary to verify our assumption.

The expression of macrophage class B scavenger receptor CD36, a highly glycosylated 88 kDa integral plasma membrane protein, increases in human peripheral blood monocytes [HUH et al. 1996] as well as in PMA-treated THP-1 cells [ALESSIO et al. 1996] during monocytic maturation. We could confirm a trigger of CD36 expression in THP-1 cells during 72 hours of differentiation, both at mRNA and protein levels using RT-PCR, Western blot analysis and immunofluorescent staining. The expression of CD36 in THP-1 macrophages is essential during foam cell formation because besides the scavenger receptor A (SR-A I/II) it is responsible for the excessive uptake of modified LDL [STEINBERG et al. 1989; ENDEMANN et al. 1993; ACTON et al. 1994; HUH et al. 1996; KUNJATHOOR et al. 2002]. These two scavenger receptors are in charge for about 75 to 90% of the uptake and degradation of modified LDL [KUNJATHOOR et al. 2002]. There is evidence that about 50-60% of macrophage foam cell formation induced by oxLDL is mediated through CD36 [ENDEMANN et al. 1993; NICHOLSON et al. 1995; FEBBRAIO et al. 2000; YAMASHITA et al. 2007]. OxLDL binds to CD36 via its lipid moiety and to other scavenger receptors via its apoprotein part [COLLOT-TEIXEIRA et al. 2007]. CD36 was also shown to be highly expressed in macrophage foam cells in human atherosclerotic lesions [NAKATA et al. 1999] pointing out its role in foam cell formation *in vivo*.

More than 10 years ago Nakagawa et al showed that treatment of human monocyte-derived macrophages with oxLDL increases CD36 expression both at mRNA and

protein level [NAKAGAWA et al. 1998]. Recently, this was confirmed by another group, which showed that CD36 mRNA increased after treatment with oxLDL time dependently in THP-1 monocytes, which was significant from 24 hours on. They also demonstrated that oxLDL itself is able to induce differentiation of monocytes towards macrophages, but only to a small extent, namely 5 to 10% of the population [FUHRMAN et al. 2008]. After stimulation with oxLDL peroxisome proliferator-activated receptor-γ (PPARγ) is activated and heterodimerizes with the retinoid X receptor (RXR). Binding of this complex to the CD36 promotor region is responsible for the rise in CD36 expression [TONTONOZ et al. 1998].

In our case, we could not show any effect of Cu^{2+}-oxidized LDL on CD36 mRNA expression in both THP-1 monocytes and macrophages. This discrepancy can possibly be explained because of different experimental settings. Whereas we used THP-1 monocytes and macrophages and incubated them for 18 hours with 100 µg/ml oxLDL, the group of Nakagawa used primary human monocytes/macrophages, which were freshly isolated and differentiated for one week. They incubated with lower concentrations of oxLDL (up to 50 µg/ml) but for a longer period of time (24 hours) [NAKAGAWA et al. 1998]. Fuhrman et al. used mouse peritoneal macrophages and THP-1 cells, but they incubated these cells for up to 4 days with 20 µg/ml oxLDL. They also showed that THP-1 monocytes differentiated towards macrophages after treatment with oxLDL for 4 days [FUHRMAN et al. 2008].

Using light microscopy we could demonstrate that THP-1 monocytes transform to foam cells after treatment with oxLDL (100 µg/ml) for 18 hours. However, the cells remained in suspension and did not differentiate. Fuhrmann et al. used PMA (1 ng/ml) for all experiments to enforce the effect of oxLDL, which may explain the discrepancies between our results and theirs. Tontonoz et al. also showed an increase in CD36 and PPARγ expression in THP-1 cells after treatment with oxLDL (50 µg/ml) for 4 days. Contrary to our experiments they used undifferentiated THP-1 cells. We assume that our differentiated THP-1 cells express CD36 at maximal levels, and that there is no further increase in CD36 expression possible.

Besides CD36, we also measured the expression of the scavenger receptor class B type I (SR-BI) in THP-1 macrophages incubated with oxLDL. SR-BI is a glycoprotein with a large extracellular domain, anchored in the plasma membrane both at the N and the C terminus with short cytoplasmic end parts [RIGOTTI et al. 2003]. Besides its function in mediating high-affinity binding of HDL facilitating bidirectional flux of cholesterol across the plasma membrane [ACTON et al. 1996; JI et al. 1997], SR-BI is also known to take up modified lipoproteins, in particular in macrophages [ACTON et al. 1994]. During monocyte-to-macrophage differentiation the expression of SR-BI increases and SR-BI was also found in atherosclerotic lesions, predominantly expressed on the macrophage surface [HIRANO et al. 1999; CHINETTI et al. 2000]. However, its role in macrophage lipid metabolism during the pathogenesis of atherosclerosis remains unclear.

CD36 and SR-BI are known to function as receptors for Cu^{2+}-oxidized LDL [ACTON et al. 1994; HUH et al. 1996]. In 2003, Marsche et al. demonstrated that both CD36 and SR-BI also function as scavenger receptor for hypochlorite modified LDL in THP-1 and in chinese hamster ovarian (CHO) cells overexpressing CD36 and SR-BI [MARSCHE et al. 2003]. Whereas Westendorf et al. could show that hypochlorite modified LDL (30 µg/ml) upregulates CD36 mRNA expression and downregulates SR-BI mRNA expression in Raw264.7 cells during 24 hours of incubation, neither HOCl nor Cu^{2+}-oxidized LDL had any effect on mRNA expression of both CD36 and SR-BI in THP-1 monocytes and macrophages in our experiments. This discrepancy can be explained because of a stronger HOCl-mediated LDL oxidation in the study mentioned, although they used 30 µg/ml of modified LDL, while we used 100 µg/ml. Furthermore, we modified our LDL with NaOCl with a molar oxidant to LDL ratio of 500:1, the resulting molar ratio of the authors was 2716:1 [WESTENDORF et al. 2005].

Han et al. could demonstrate that 7-ketocholesterol, a prominent oxysterol formed during Cu^{2+}-modification of LDL [TANAKA and KANAMARU 1993], but not cholesterol, decreased SR-BI protein in Raw264.7 cells pointing out an important role of this oxysterol regarding regulation of SR-BI expression [HAN et al. 2001]. It was shown earlier from Cader et al. that copper ion but not HOCl modification of LDL led to an enormous accumulation of 7-ketocholesterol [CADER et al. 1997].

Thus, one can speculate that HOCl-modified LDL, which is devoid of 7-ketocholesterol, is not able to affect SR-BI protein expression.

We could demonstrate that expression of the scavenger receptor class B type 1 (SR-BI) protein was triggered in THP-1 cells after differentiation with PMA (160 nM) for 72 hours, whereas SR-BI mRNA expression remained unchanged during monocyte-to-macrophage differentiation. Incubation of the cells with Cu^{2+}-oxidized LDL reduced SR-BI protein expression. HOCl-modified LDL had no effect on SR-BI expression. Our data are consistent with the findings of Han et al., who could demonstrate a time- and dose-dependent decrease of SR-BI protein in Raw264.7 macrophages, after treatment with Cu^{2+}-oxidized LDL [HAN et al. 2001]. Contrary to the results of Han et al., Hirano et al. found an elevation of SR-BI mRNA after treatment with Cu^{2+}-oxidized LDL [HIRANO et al. 1999]. However, there are some differences concerning incubation conditions. Besides a longer incubation used by the latter group, they also used human monocyte-derived macrophages on day 7 after plating. Han et al. could demonstrate, that human monocytes trigger SR-BI expression at early stages of differentiation, but attenuate it, if these cells are fully differentiated. Therefore, they pointed out a putative role of monocyte/macrophage differentiation state concerning oxLDL regulation of SR-BI expression [HAN et al. 2001].
Furthermore, it was shown recently that oxLDL led to a time dependent increase of CD36 and SR-BI mRNA expression in THP-1 monocytes, which was significant from 8 hours of incubation on [FUHRMAN et al. 2008]. Our results regarding mRNA of SR-BI are not conclusive at all. Whereas we could reveal an increase in SR-BI mRNA after oxLDL treatment in THP-1 macrophages with Northern Blot analyses, no changes so far in CD36 and SR-BI mRNA have been detected performing RT-PCR, in agreement with Fuhrmann et al [FUHRMAN et al. 2008].

Changes in cellular oxygen partial pressure due to changes in oxygen supply have been regarded as a factor in atherosclerotic plaque formation. To keep the balance of oxygen and to avoid oxidative oxygen damage or oxygen deficiency [BRÜNE and ZHOU 2007], cellular oxygen conditions are partially controlled by the hypoxia

inducible factor HIF-1, a transcription factor which is composed of two subunits. The ß-subunit is constitutively expressed in many cell types. Whereas the α-subunit (HIF-1α) is undetectable under normoxia due to rapid proteasomal degradation, it is stabilized under hypoxic conditions. After HIF-1α/HIF-1ß heterodimerization and translocation to the nucleus it binds to promotor-specific hypoxia responsive element (HRE) sites [SEMENZA 2001]. Several target genes are transactivated by HIF-1, including those encoding erythropoietin, glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (VEGF). Thereby HIF-1 action contributes to increased angiogenesis, enhanced glycolysis, and other properties that promote adaption of the organism to an environment with low oxygen concentrations [SEMENZA 1999; SEMENZA 2000a; SEMENZA 2000b].

It has been reported that the mitochondrial electron transport is partially inhibited during hypoxia. Due to redox changes in the electron carriers ROS generation is elevated. ROS then can enter the cytosol and might function as second messengers in signaling pathways resulting in HIF-1α stabilization [CHANDEL et al. 1998]. It was demonstrated that HIF-1α can also be stabilized under normoxic conditions by the growth factor angiotensin II (ang II), which is able to increase ROS levels through NAD(P)H oxidase activation [RICHARD et al. 2000].

Within atherosclerotic plaques, hypoxia, correlating with HIF-1α protein and VEGF, was found predominatly in the macrophage-rich core of the lesions [SLUIMER et al. 2008]. Hypoxia itself was also found to induce accumulation of triglycerides in human macrophages, thereby leading to foam cell formation [BOSTROM et al. 2006].

Recently, the stabilization of HIF-1α in human Mono-Mac-6 (MM6) macrophages after oxLDL treatment has been demonstrated [SHATROV et al. 2003]. Consistent with these findings we could also show a dose- and time-dependent HIF-1α protein stabilization in THP-1 macrophages after incubation with Cu^{2+}-oxidized LDL, but not HOCl-modified or native LDL. There was no impact of lipoproteins on HIF-1α mRNA stabilization detected performing RT-PCR. Again, Northern blot analysis revealed conflicting data, because we could detect an increase of HIF-1α mRNA in THP-1 macrophages. So far, these discrepancies are not explainable.

The data of Shatrov et al. supported a putative role of redox-dependent mechanisms during oxLDL-induced HIF-1α protein accumulation, because they could demonstrate an inhibition of this accumulation after N-acetyl-L-cysteine (NAC) treatment, which decreases ROS, and after incubation with dipenyleniodonium (DPI), that blocks ROS generation by NADPH oxidase [SHATROV et al. 2003]. It has also been suggested that HIF-1α is involved in foam cell formation, because it was shown that HIF-1α-siRNAs block oxLDL-induced macrophage foam cell formation in U937 human monoblastic cells [JIANG et al. 2007]. The differentiation-dependent induction of the ABCA1 gene in monocytes could also be related to the activation of the HIF-1α pathway, which is contradictory to the results of Jiang et al., because ABCA1 mediates cholesterol efflux [SCHMITZ and LANGMANN 2005]. However, considering all these findings, describing the presence of hypoxia and accumulation of HIF-1α and VEGF in atherosclerotic lesions, induction of HIF-1α protein by oxLDL and involvement of HIF-1α protein in foam cell formation indicate a major contribution of HIF-1α protein in the pathogenesis of atherosclerosis. Furthermore, it was shown that HIF-1α RNA-interference inhibits foam cell formation in U937 cells [JIANG et al. 2007], pointing out an additional important role of this transcription factor in atherosclerotic diseases.
Taken together, our data suggest that stabilization of HIF-1α protein seems to contribute highly to the pathogenesis of atherosclerosis.

Under normoxia, the α-subunit of HIF-1 is degraded by the proteasome, a proteolytic multicatalytical complex which requires ubiquitinylated substrates for proteolytic action [GRUNE et al. 1997; CIECHANOVER 1998]. Whereas the ubiquitin activity remains unchanged in atherosclerotic plaques, proteasome activity decreases [VERSARI et al. 2006]. It was also shown earlier that oxLDL (200 µg/ml) impairs proteasome activity in the ECV-304 human endothelial cell line [VIEIRA et al. 2000]. By contrast, we could not detect any influence of copper or HOCl-modified LDL (100 µg/ml) on 20S proteasome activity in THP-1 macrophages. Vieira et al. hypothesize that at low, non-toxic oxLDL concentrations, the proteasome functions as cellular defense system by degrading proteins, which were altered by oxLDL. At high, toxic concentrations of oxLDL (above 200 µg/ml) proteasome activity is

impaired thus leading to an accumulation of altered and ubiquinated proteins [VIEIRA et al. 2000]. We presume that oxLDL had no impact on 20S proteasomal activity in THP-1 macrophages due to relatively low concentrations of the modified lipoprotein.

HIF-1α regulates classical hypoxia responsive genes like VEGF, which is important in the pathogenesis of atherosclerosis due to its influence on neovascularization [RIVARD et al. 2000; SCHMITZ and LANGMANN 2005]. However, it has to be noted that besides plaque neovascularisation VEGF is also involved in compensatory arteriogenesis in atherosclerosis and related diseases. VEGF and other growth factors like fibroblast growth factor 2 (FGF2) are able to induce thrombi bypassing by building a new vascular network [CHEN and WALTERSCHEID 2006], emphasizing the double-edged role of VEGF in the pathogenesis of atherosclerosis.
An induction of macrophage VEGF secretion in response to oxLDL was observed in some studies [RAMOS et al. 1998; INOUE et al. 2001; SALOMONSSON et al. 2002]. A positive association between HIF-1α staining and VEGF protein in atherosclerotic plaques was also demonstrated. In these plaques, HIF-1α protein was found predominatly in macrophages or macrophage derived foam cells [VINK et al. 2007; SLUIMER et al. 2008]. HIF-1α and VEGF protein, found within human carotid artery plaques, might also contribute to intraplaque angiogenesis, which can then result in an induction of plaque hemorrhage and progression [HIGASHIDA et al. 2008]. Hence, the expression of VEGF after oxLDL-treatment and in hypoxic plaques is supposed to be regulated by HIF-1α. We could demonstrate an increase of VEGF mRNA during THP-1 cell differentiation. We could also show an oxLDL-induced accumulation of HIF-1α protein. However, no impact of oxLDL on VEGF mRNA was detected.
It was shown earlier that RNA interference for HIF-1α in U937 human monoblastic cells attenuated VEGF expression [JIANG et al. 2007]. However, Oskolkova et al could demonstrate that induction of VEGF by oxidized phospholipids is unlikely to be mediated via a HIF-1/HRE-dependent transcription [OSKOLKOVA et al. 2008] arguing against a HIF-1α-mediated VEGF expression after treatment with oxLDL. Recently, Riazy et al. described an involvement of phosphatidylinositol 3-kinase

(PI3K) and the protein kinase C subtype zeta (PKCζ) activation during oxLDL-induced VEGF secretion in macrophages, which seems to be independent of an involvement of the scavenger receptors SR-A or CD36 and their uptake of modified lipoproteins [RIAZY et al. 2009]. Since the atypical PKCζ and other members of the PKC superfamily are known to be activated by PMA, which stimulates angiogenesis through this pathway [MONTESANO and ORCI 1985; LIU and HECKMAN 1998; TAYLOR et al. 2006], one can hypothesize that the increase of VEGF mRNA expression during THP-1 differentiation may be due to PMA-induced enhancement in PKC activity.

Next, we aimed to investigate some putative antagonists of oxLDL and their effects on oxLDL-induced modifications in THP-1 macrophages.

The protective effect of HDL during atherosclerosis is commonly known and supposed to be founded mainly in its role in reverse cholesterol transport (RCT) by which HDL removes excess cholesterol from peripheral tissues and cells, including those in the artery wall and lipid laden macrophages, and delivers it back to the liver for biliary secretion [GLOMSET 1973; NOFER et al. 2002; RADER 2003; VILES-GONZALEZ et al. 2003; RADER and DAUGHERTY 2008].

SR-BI can bind HDL reversibly and mediates cholesterol efflux to phospholipid-containing acceptors [JI et al. 1997; JESSUP et al. 2006]. The transporter protein ABCA1 promotes cholesterol efflux of cholesterol from cells to HDL precursors (lipid-free or lipid-poor apolipoproteins), ABCG1 transports cholesterol to lipid-rich and phospholipid-containing acceptor particles as mature HDL [WANG et al. 2004; JESSUP et al. 2006]. Thus, both transporter proteins contribute to the efflux of cholesterol from macrophages and to general cholesterol transport *in vivo* [WANG et al. 2007]. The role of SR-BI concerning cholesterol efflux is seen controversial, efflux studies showed that in cholesterol-enriched cells about 50% of the efflux is attributable to ABCA1, and approximately 20% to ABCG1. SR-BI contribution to efflux was described to be rather small [ADORNI et al. 2007].

We could show that SR-BI protein and cholesterol efflux to HDL (10 µg/ml) was reduced in oxLDL treated THP-1 macrophages. Expression of ABCA1 or ABCG1 was not examined. Han et al. could also demonstrate that incubation of Raw264.7

macrophages with oxLDL led to a decrease of about 40% of cholesterol efflux to HDL and also to a loss of SR-BI expression [HAN et al. 2001]. Another group could demonstrate that treatment with oxLDL upregulated both ABCA1 expression and cholesterol efflux to apoA-1 in THP-1 macrophages. A significant increase was not found until 24 hours of oxLDL administration and 12 hours of efflux [TANG et al. 2004] whereas we incubated our cells with oxLDL for 16 hours and measured efflux to HDL after 6 hours, but not to apoA-1. However, these differences may not explain the discrepancies in our results.

In human umbilical vein endothelial cells (HUVEC) it was shown earlier that Cu^{2+}-oxidized LDL decreased both ABCA1 protein and mRNA expression dose-dependently, whereas in THP-1 macrophages ABCA1 mRNA expression rose after oxLDL administration, pointing out a tissue specific influence of oxLDL [ZHU et al. 2005]. This ABCA1 upregulation in THP-1 cells after oxLDL treatment was also repeated by other groups [CHAWLA et al. 2001; GEERAERT et al. 2007; RODE et al. 2008]. However, apart from that, Favari et al. could not demonstrate any impact of oxLDL on both ABCA1 protein expression and cholesterol efflux in J774 macrophages [FAVARI et al. 2005].

Since ABCA1 and ABCG1 seem to be upregulated after oxLDL treatment in THP-1 macrophages and the contribution of the downregulated SR-BI to cholesterol efflux seems to be rather small, we hypothesize that oxLDL might decrease the cholesterol efflux through other unknown mechanisms. Jessup et al. hypothesize that the relative contributions of SR-BI and ABCG1 to efflux may also depend on cellular loading conditions and the cholesterol gradient between cell and acceptor [JESSUP et al. 2006].

Incubation of oxLDL treated (100 µg/ml) and control THP-1 macrophages with HDL (150 µg/ml) increased cholesterol efflux, also in supplemental presence of DETA-NO. Additionally, we could also demonstrate that there is a slight, non-significant increase in SR-BI protein after treatment of oxLDL treated THP-1 macrophages with HDL.

SR-BI transfers cholesterol, preferentially free cholesterol, from HDL to cells. Almost all of the cholesterol entering via SR-BI is available for efflux, which indicates that most of the cholesterol transferred to cells remains at the plasma membrane and/or

in more accessible intracellular cholesterol pools [STANGL et al. 1999]. Some other data indicate a role of SR-BI in enhancing cholesterol efflux to various acceptors, probably due to elevating the local concentration of acceptors tethering to the cells surface and thus facilitating the bi-directional flux of cholesterol and phospholipids [JIAN et al. 1998]. A large part of SR-BI-enhanced cholesterol flux seems not to be a result of enhancend binding, but appears to be rather related to a change in plasma membrane lipid domain structure. SR-BI expression may both alter the distribution of membrane-free cholesterol to a caveolar fraction and the accessibility. It is also suggested that SR-BI expression results in a redistribution of cholesterol to membrane domains that serve to facilitate the flux between cells and lipoproteins [DE LA LLERA-MOYA et al. 1999; KELLNER-WEIBEL et al. 2000]. All of this data support our hypothesis, that treatment with HDL enhances cholesterol efflux from THP-1 macrophages to HDL as acceptor by increasing SR-BI protein levels.

HIF-1α protein stabilization in oxLDL treated THP-1 macrophages was attenuated dose-dependently in the presence of HDL. Therefore, we suppose that HDL acts in several aspects, on the one hand by increasing cholesterol efflux from macrophage foam cells and on the other hand through its antioxidative properties. Thus, both result in a depletion of ROS and therefore in an inhibition of HIF-1α protein stabilization.

However, oxLDL-induced TBARS formation was not attenuated in the concomitant presence of HDL. A slight decrease of MDA levels was detected only when HDL was given to THP-1 macrophages 8 hours before oxLDL was added.

Cell viability of THP-1 macrophages, measured with neutral red assay, decreased after treatment with oxLDL. This decrease was reversed in the concomitant presence of HDL. A protective effect of dehydroascorbate (DHA) on oxLDL-induced loss of cell viability was only seen at a concentration of 200 µM DHA or more (data not shown). The antagonistic effect of HDL may be due to its capability to protect macrophages from oxLDL-induced apoptosis by promoting cholesterol efflux, in particular of the highly cytotoxic oxysterol 7-ketocholesterol, most probably via ABCG1 [TERASAKA et al. 2007].

The next presumed antagonist of oxLDL-mediated actions in THP-1 macrophages we investigated was nitric oxide, which is known to have complex functions during atherogenesis [NAPOLI and IGNARRO 2001]. L-arginine-derived NO is an endogenous vasodilator [IGNARRO et al. 1999] and inhibits platelet and monocyte adherence to the vessel wall [ANDERSON et al. 1995; CANNON 1998]. Endothelial dysfunction due to a reduction of NO synthase activity is supposed to be one of the earliest steps in the pathogenesis of atherosclerosis [ANDERSON et al. 1995; IGNARRO et al. 1999; NAPOLI and IGNARRO 2001; HIGASHI et al. 2009] indicating an important role of nitric oxide in atherosclerosis related diseases.

We could demonstrate that DETA-NO, a synthetic donor of nitric oxide, which delivers a steady state concentration of NO about 1/2000 of the applied DETA-NO concentration [KÖHL et al. 2006], antagonized oxLDL-induced effects in THP-1 macrophages.

DETA-NO reversed both differentiation towards foam cells and lipid accumulation in oxLDL treated THP-1 macrophages. From a concentration of 300 µM on, hardly any foam cell was visible under the microscope. Contrary to our assumption that this effect may be due to stimulating cholesterol efflux, DETA-NO had no impact on oxLDL-induced reduction of cholesterol efflux in THP-1 macrophages. When DETA-NO was given together with HDL to the cells 8 hours before oxLDL was added, cholesterol efflux increased to even higher levels than in the control or in the oxLDL/HDL co-incubation. It was recently shown that HIF-1α RNA-interference inhibits foam cell formation in U937 cells [JIANG et al. 2007]. Therefore, we hypothesize that inhibition of oxLDL-induced HIF-1α protein stabilization by DETA-NO also leads to inhibition of foam cell formation without any influence in cholesterol efflux.

OxLDL-induced TBARS-formation, measured as MDA-levels in THP-1 macrophage cell supernatant, was inhibited dose-dependently in the presence of DETA-NO, with more than 75% inhibition at a concentration of 600 µM and about 50% at a concentration of 300 µM. This antagonizing effect of DETA-NO was even higher when it was given 8 hours before oxLDL was added. DETA-NO, added for further

18 hours after 18 hours of oxLDL incubation to THP-1 macrophages resulted in a slight reduction of aldehyde accumulation.

Administration of oxLDL to THP-1 macrophages led to a loss of cell viability. This viability deprivation was reversed in the concomitant presence of DETA-NO dose-dependently.

All of these results point to a beneficial role of nitric oxide during the pathogenesis of atherosclerosis presumably due to inhibition of oxLDL-induced ROS formation in THP-1 macrophages.

During oxLDL/DETA-NO co-incubation oxLDL-induced HIF-1α protein stabilization was inhibited dose-dependently. At a concentration of 300 µM DETA-NO, which delivers up to 150 nM of nitric oxide to the cells and has a half-life of about 20 hours, almost no HIF-1α protein was detectable. These findings are consistent with the data of Shatrov et al. who could demonstrate an inhibition of oxLDL-induced HIF-1α protein accumulation by the relatively best physiologically similar NO donor S-nitrosoglutathione (GSNO). They hypothesized that O_2^- may play a role in oxLDL-induced HIF-1α protein stabilization and the effect of NO donors may be explained by the diffusion controlled interaction of O_2^- with NO resulting in preventing HIF-1α protein accumulation [SHATROV et al. 2003].

Whereas a pre-incubation of oxLDL treated cells with DETA-NO (300 µM) revealed even higher inhibition of HIF-1α protein accumulation than a co-incubation, a post-incubation was not effective. Thus, DETA-NO has to be present before and during HIF-1α protein accumulation by oxLDL to antagonize this effect and can not function in diminishing this protein.

SR-BI protein depletion in differentiated THP-1 cells, caused by oxLDL (100 µg/ml), was rescued by DETA-NO dose-dependently, with expression levels comparable to the control at a concentration of 600 µM DETA-NO. This effect was even more pronounced when DETA-NO (300 µM) was given 8 hours before oxLDL was added. DETA-NO post-incubation could not reverse oxLDL-induced loss of SR-BI protein in THP-1 macrophages.

Taken together, we could show that NO released from DETA-NO reversed oxLDL-induced foam cell formation, accumulation of lipid deposits and malondialdehyde,

loss of cell viability, stabilization of HIF-1α and depletion of SR-BI protein dose-dependently.

Whereas NO is known to provoke HIF-1α protein accumulation under normoxia, it attenuates cellular enrichment in HIF-1α protein under hypoxia [SOGAWA et al. 1998; HUANG et al. 1999; SANDAU et al. 2001]. Under normoxia, NO presumably acts via binding to non-heme iron of the prolyl hydroxylases (PHD), and thus inhibits HIF-1α protein degradation [METZEN et al. 2003]. Under hypoxia, it is proposed that HIF-1α protein accumulation is blocked by NO via targeting the oxygen-dependent degradation domain (ODD) [HUANG et al. 1999], enhancement of HIF-1α and van Hippel-Lindau tumor suppressor protein (pVHL) interaction [WANG et al. 2002]. The ensuing block of mitochondrial complex I [AGANI et al. 2002] or the inhibition of cytochrome c oxidase provide more cellular oxygen and result in increased prolyl hydroxylase activity [HAGEN et al. 2003]. Thus, it is hypothesized that oxygen tension and resulting redox state of cells constitute the impact of nitric oxide on HIF-1 regulation [KÖHL et al. 2006].

Sandau et al. could show that the higher the NO concentration and the longer the incubation time under normoxia, the smaller is the HIF-1α protein stabilization. They demonstrated an increase of HIF-1α protein accumulation under normoxia with 100 µM of DETA-NO with the highest signal after 4 hours, which decreased time-dependently afterwards [SANDAU et al. 2001]. Concordantly, we found no stabilization of HIF-1α protein when THP-1 macrophages were incubated with up to 1.2 mM of DETA-NO for 18 hours under normoxic conditions, but DETA-NO provoked the accumulation of this transcription factor after 4 hours of incubation in these cells.

There are several reports that ROS as well as NO are able to trigger HIF-1α protein stabilization under normoxic conditions. Recently, Köhl et al could demonstrate that ROS, generated within the cells by the redox cycling agent 2,3-dimethoxy-1-naphthoquinone (DMNQ), are able to reverse DETA-NO-induced HIF-1α protein accumulation via inhancing PHD activity [KÖHL et al. 2006]. Whereas we could show an impact of DETA-NO on HIF-1α protein accumulation under normoxia after 4 hours of treatment in THP-1 macrophages, the impact of oxLDL during that short time of incubation was comparatively small.

Under hypoxia, we could demonstrate an inhibition of HIF-1α protein accumulation when THP-1 macrophages were preloaded with DHA (100 µM, 30 min). Administration of DETA-NO (300 µM) during 4 hours of hypoxic conditions could not alter hypoxic HIF-1α protein stabilization. OxLDL-treatment during hypoxia even potentiated the hypoxic HIF-1α protein signal, which was attenuated in the concomitant presence of DETA-NO.

All effects of DETA-NO were potentiated in the concomitant presence of HDL. HDL is known to activate the endothelial NO-synthase (eNOS) through SR-BI in endothelial cells which leads to a higher NO release within the endothelium. Whereas HDL had no effect in SR-BI−deficient cells, it caused a four-fold increase in eNOS activity in SR-BI-expressing cells, which was attenuated by antibodies to apoA-I or to the C-terminal cytoplasmic tail of SR-BI. This indicates that coupling of the HDL−SR-BI interaction to eNOS stimulation occurs in plasmalemmal caveolae [YUHANNA et al. 2001], where SR-BI and eNOS were found to collocate [SHAUL 2003], and that it requires apoA-I binding [YUHANNA et al. 2001]. It was then shown that HDL-induced stimulation of eNOS is due to estradiol associated with the lipoprotein. HDL and the associated estradiol bind to SR-BI and stimulate the production of nitric oxide [GONG et al. 2003]. However, we could not detect eNOS in THP-1 macrophages (data not shown). But taking into account that we used also estradiol-rich female HDL and that SR-BI protein expression increased after HDL and DETA-NO treatment one may hypothesize that HDL could also potentiate NO production in macrophages by a yet unknown mechanism.

It was shown recently that the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) can be recycled *in vivo* to form NO [LUNDBERG et al. 2008]. Ascorbate for example was shown to reduce NO_2^- to NO in mildly acidified nitrite-containing urine [CARLSSON et al. 2001]. Therefore, we hypothesized that incubation of oxLDL-treated THP-1 macrophages with $NaNO_2$ and ascorbate should have comparable effects like DETA-NO due to NO production in cell medium from NO_2^- through the reducing agent vitamin C. But no effect of $NaNO_2$ (0.1 – 100 µM) in the presence of ascorbate (100 µM) in oxLDL treated THP-1 macrophages on cell viability, TBARS formation, lipid deposits, HIF-1α and SR-BI protein levels was detected.

Nitrite plasma levels range from 50 to 300 nM [LUNDBERG et al. 2008]. Whereas in the study of Carlsson et al. 50 to 500 µM of $NaNO_2$ and 10 mM of ascorbate were used [CARLSSON et al. 2001], in our experimental setting $NaNO_2$ concentrations were rather comparable (0.1 – 100 µM) to theirs, but ascorbate concentration was much lower (100 µM) in our study approaching physiologic concentrations, which normally range from about 40 to 90 µM [POLIDORI et al. 2004].

Vitamin C, a well known water-soluble antioxidant in human blood plasma, is able to prevent oxidative modification of LDL by scavenging free radicals and other reactive species in the aqueous milieu. It prevents initiation of lipid peroxidation by trapping aqueous radicals before they can attack LDL lipids [FREI et al. 1989; STOCKER et al. 1991].

Therefore, we next aimed to investigate the effects of vitamin C, administered both as ascorbate over night or dehydroascorbate (DHA) for 30 minutes, on oxLDL treated THP-1 macrophages.
Time responses of either ascorbate or DHA administration to THP-1 macrophages during a period of 30 minutes were measured using HPLC. THP-1 macrophages showed about seven times higher intracellular ascorbate concentration after 30 minutes of DHA preload (100 µM) compared to those loaded with ascorbate (100 µM), whereas extracellularly there was nearly no dehydroascorbate detected. The rather small amount of extracellular DHA found in cell supernatant (5% after 30 minutes) is not due to excessive uptake (only 1 – 5% of DHA is taken up) but may be due to its instability at physiologic pH and temperature (half-life 5-7min) [DHARIWAL et al. 1990]. After incubation with 100 µM DHA for 30 min, THP-1 macrophages accumulate about 2 mM ascorbate within the cells. These concentrations are comparable with the findings of Levine et al. who report an ascorbate content of 1.5 mM for neutrophils and 3 mM for monocytes, respectively, in humans after a daily administration of about 100 mg vitamin C [LEVINE et al. 1996]. Hence, in our experimental settings, THP-1 macrophages accumulate intracellular vitamin C at levels which are comparable to blood cells *in vivo*.

No influence of vitamin C on TBARS formation or lipid accumulation was detected. Therefore, we concluded that ascorbate was not very effective in scavenging or inhibiting ROS formation in oxLDL treated THP-1 macrophages.

HIF-1α subunits are modified post-translationally by a series of oxygen-dependent enzymatic hydroxylations at proline residues resulting in binding of the van Hippel-Lindau tumor suppressor protein (pVHL), followed then by ubiquitination and subsequent degradation by the proteasome.

PHDs contain a non-heme iron group, require oxygen and 2-oxoglutarate for the hydroxylation reaction and result in HIF-1α protein accumulation in the absence of any of these cofactors. These enzymes require also ascorbate for optimal activity *in vitro* [SCHOFIELD and RATCLIFFE 2004] and it was shown previously that vitamin C is able to attenuate hypoxia-induced HIF-1α protein accumulation in human primary and cancer cell lines dose-dependently with ascorbate concentrations ranging from 10 to 500 µM [KNOWLES et al. 2003; VISSERS et al. 2007].

Correspondingly, we could also demonstrate a decrease of oxLDL-induced HIF-1α protein accumulation after preloading THP-1 macrophages with 100 µM DHA for 30 minutes. Thus, we hypothesize that oxLDL-induced HIF-1α protein accumulation may also be due to an inhibition of the enzymatic hydroxylation of the α-subunit, because vitamin C is acting at this stage of the HIF-1α protein degradation process.

DETA-NO, given to human umbilical vein endothelial cells (HUVEC) for four hours, provoked HIF-1α protein accumulation dose-dependently. Preloading these cells with ascorbate or dehydroascorbate (200 µM) also attenuated this protein stabilization.

Vitamin C was also shown to stabilize tetrahydrobiopterin, a cofactor of eNOS. Because of its antioxidative properties, it is able to recycle oxidized tetrahydrobiopterin back to the reduced pterin and therefore prevents endothelial dysfunction resulting from NO deprivation [HUANG et al. 2000; HELLER et al. 2001]. Thus, ascorbate is supposed to have a dual role in endothelial cells. On the one hand it diminishes nitric-oxide induced HIF-1α protein accumulation by inhibition

of the enzymatic hydroxylation, on the other hand it is able to prevent loss of endothelial NO by stabilizing a cofactor of the endothelial NO synthase.

Next, we examined the role of ebselen, an antiinflammatory seleno-organic antioxidant, during oxLDL-induced HIF-1α protein accumulation and SR-BI depletion in THP-1 macrophages. Ebselen was shown to reduce hydroperoxides to their corresponding hydroxy compounds [SCHEWE 1995]. It was also shown that pretreatment of oxLDL with this synthetic glutathione peroxidase (GPx) mimetic [SIES 1993] inhibits oxLDL-induced depletion of intracellular glutathione (GSH) levels. Ebselen particularly scavenges lipidhydroperoxides (LOOH) and was shown previously to reduce LOOH content in lipoproteins in the presence of $CuSO_4$ resulting in an inhibition of lipoprotein oxidation [THOMAS and JACKSON 1991; SHEN and SEVANIAN 2001]. Pretreatment of LDL with this compound led also to a slight reduction in MDA levels [SHEN and SEVANIAN 2001]. This compound was also shown to attenuate ROS formation, HIF-1 DNA binding and VEGF mRNA expression during hypoxia [CHANDEL et al. 1998].
We could demonstrate a dose-dependent attenuation of oxLDL-induced TBARS accumulation, foam cell formation and HIF-1α protein stabilization in the concurrent presence of ebselen in THP-1 macrophages. As it was shown from the literature that ebselen can reduce LOOHs and ROS [THOMAS and JACKSON 1991; SHEN and SEVANIAN 2001], we hypothesize that concomitant administration of ebselen with oxLDL results in an inhibition of radical chain reactions. Aldehyde formation (measured as TBARS formation) and accumulation of ROS might be inhibited by ebselen, resulting in a decrease of HIF-1α protein stabilization and furthermore in an inhibition of foam cell formation.
SR-BI depletion in oxLDL treated THP-1 macrophages could also be dose-dependendly reversed in the presence of ebselen.

In summary, oxLDL was shown to stabilize HIF-1α protein under normoxic conditions and decreases SR-BI protein expression in THP-1 macrophages. Those effects were exerted exclusively by Cu^{2+}-oxidized LDL, whereas native and HOCl-modified LDL were ineffective. HDL was shown to decrease HIF-1α protein

accumulation, presumably by enhancing cholesterol efflux and/or reduction of ROS. This effect was potentiated in the concomitant presence of HDL and DETA-NO. DETA-NO alone was able to block the stabilization of this transcription factor completely during 18 hours of oxLDL-treatment. Also oxLDL-induced foam cell and malondialdehyde formation were inhibited by DETA-NO, presumably due to scavenging of oxidation products, but DETA-NO could not reverse oxLDL-induced mitigation of cholesterol efflux from THP-1 macrophages to HDL. $NaNO_2$, which was supposed to release nitric oxide in the presence of ascorbate, had no effect on oxLDL-induced events. The action of ebselen, which was able to reduce HIF-1α protein accumulation, foam cell and TBARS formation, may also be due to reduced ROS formation and lipid peroxidation. Vitamin C was shown to mitigate oxLDL-induced HIF-1α protein stabilization, most probably due to enhanced PHD activity, but could not act at the same extent like the other antagonists. With respect to the influence of these parameters on oxLDL-induced loss of SR-BI protein expression, it remains difficult to provide definitive answers due to diverging results and high variations throughout the experiments performed. However, it was shown that HDL, DETA-NO and ebselen were able to reverse oxLDL-induced decrease of SR-BI protein.

To summarize, there is growing evidence for HIF-1α to play an important role in the pathogenesis of atherosclerosis. HDL, DETA-NO, vitamin C and other antioxidative agents like ebselen might act as counterplayers and thus as protective factors during cardiovascular disease. Nevertheless, further investigations are necessary to elucidate the role and interaction of these factors in atherosclerosis and oxLDL-induced pathogenesis.

7 Summary

Cardiovascular diseases (CVD) are among the major causes of morbidity and mortality in westernized societies. The excessive uptake of oxidatively modified low density lipoprotein (LDL) by macrophages, which results in foam cell formation, is one of the initial steps in the pathogenesis of atherosclerosis. Since the transcription factor hypoxia inducible factor 1 (HIF-1) and the scavenger receptor class B type I (SR-BI) play a crucial role in this process, we aimed to investigate their expression in THP-1 macrophages under the influence of modified LDL in the presence or absence of the putative counterplayers high density lipoprotein (HDL), nitric oxide (NO) and vitamin C.

In contrast to the ß-subunit, which is constitutively expressed, the protein expression of HIF-1α is known to be abolished under normal oxygen conditions due to proteasomal degradation, whereas it is stabilized under hypoxia or in the presence of reactive oxygen or nitrogen species or of oxidatively modified LDL. We could verify this in THP-1 macrophages. Expression of SR-BI, a scavenger receptor for lipoproteins and a designated HDL-receptor, was shown to be downregulated in the presence of oxidatively modified LDL, which is consistent with our results.

HDL is known to act atheroprotective due to its influence on reverse cholesterol transport, its antioxidative potential and its positive impact on endothelial function. The antioxidant vitamin C and the gasotransmitter and vasodilator NO were shown to inhibit hypoxia-induced HIF-1α protein accumulation. Therefore, we investigated the impact of these factors on oxLDL treated THP-1 macrophages.

In this study we could demonstrate that HDL was able to increase SR-BI protein expression, diminish HIF-1α protein accumulation and enhance cholesterol efflux in oxLDL treated THP-1 macrophages.
DETA-NO, a well known synthetic NO donor, abolished HIF-1α protein stabilization completely and enhanced SR-BI protein expression in oxLDL treated cells. Although

DETA-NO had no impact on oxLDL-induced reduction of cholesterol efflux from cells to HDL, it could inhibit oxLDL-induced foam cell formation in THP-1 macrophages, supposedly by enhancing HIF-1α protein degradation.
While vitamin C was able to decrease oxLDL-induced HIF-1α protein accumulation, but not to the same extent like the other two counterplayers, it had hardly any impact on the other parameters measured.

The complex progression of atherosclerosis is determined by the balance of a multitude of several risk and protective factors. However, the underlying mechanisms of the pathogenesis and progression of this disease are still not completely understood in detail. There is growing evidence for HIF-1α playing a role in the pathogenesis of atherosclerosis. With our results we could underline the protective effects of the counterplayers HDL, nitric oxide and vitamin C in macrophages. Thus, further investigations are necessary to elucidate the role and interaction of these molecules in atherosclerosis and oxLDL-induced pathogenesis.

8 Zusammenfassung

Kardiovaskuläre Erkrankungen zählen zu den Hauptursachen von Morbidität und Mortalität in den westlichen Industriestaaten. Die exzessive Aufnahme von oxidativ modifiziertem Low Density Lipoprotein (LDL) in Makrophagen führt zur Bildung von sogenannten Schaumzellen, und dies stellt einen der ersten Schritte in der Entstehung der Atherosklerose dar.
Nachdem der Transkriptionsfaktor Hypoxie-induzierbarer Faktor 1 (HIF-1) und der Scavenger Rezeptor Klasse B Typ I (SR-BI) vermutlich in diesen Prozess involviert sind, war es das Ziel dieser Studie, deren Expression in THP-1-Makrophagen in der Anwesenheit von oxidativ modifiziertem LDL und dessen potentiellen Gegenspielern High Density Lipoprotein (HDL), Stickstoffmonoxid (NO) und Vitamin C zu untersuchen.

Im Gegensatz zur ß-Untereinheit, welche permanent exprimiert wird, verhindert proteasomaler Abbau die Akkumulierung von HIF-1α Protein unter normalen Sauerstoffbedingungen. Unter hypoxischen Bedingungen, in der Anwesenheit von reaktiven Sauerstoff- und Stickstoffspezies und oxidativ modifiziertem LDL wird der Abbau von HIF-1α Protein jedoch verhindert. Dies konnten wir mit unseren Untersuchungen an THP-1 Makrophagen bestätigen.
Es gibt Hinweise aus der Literatur, dass SR-BI, ein Scavenger-Rezeptor für Lipoproteine und HDL, in der Anwesenheit von oxidativ modifiziertem LDL vermindert wird, was wir ebenfalls zeigen konnten.

HDL ist für seine gefäßschützenden Eigenschaften bekannt, vor allem wegen seines Einflusses auf den reversen Cholesterintransport, seinen antioxidativen Eigenschaften und seiner positiven Wirkung auf die Endothelfunktion.
Von dem Antioxidans Vitamin C und dem Gasotransmitter und Vasodilator NO ist bekannt, dass sie eine Akkumulierung von HIF-1α Protein in hypoxischen Zuständen verhindern können.

Diese Tatsachen führten dazu, einen Einfluss dieser Faktoren auf THP-1 Makrophagen, die mit oxidativ modifiziertem LDL behandelt wurden, zu untersuchen.

In dieser Studie konnten wir zeigen, dass HDL die SR-BI Proteinexpression erhöht, die Stabilisierung von HIF-1α Protein verhindert und den Cholesterinefflux aus THP-1 Makrophagen erhöht.
DETA-NO, ein synthetischer NO-Donor, konnte die Akkumulierung von HIF-1α Protein verhindern und die SR-BI Proteinexpression in mit oxLDL behandelten Zellen erhöhen. Obwohl DETA-NO keinen Einfluss auf den durch oxLDL verminderten Cholesterinefflux zeigte, war es imstande, die oxLDL-induzierte Schaumzellenbildung zu verhindern. Dieser Effekt dürfte auf einen durch Stickstoffmonoxid vermittelten erhöhten Abbau des HIF-1α Proteins zurückzuführen sein.
Während Vitamin C die oxLDL-induzierte Akkumulierung von HIF-1α Protein abschwächen konnte, jedoch nicht in dem Ausmaß wie die beiden anderen Gegenspieler, hatte es kaum Wirkung auf die anderen untersuchten Parameter.

Das komplexe Fortschreiten der Atherosklerose ist von einer Balance zwischen einer Vielzahl von Risiko- und protektiven Faktoren abhängig. Die zu Grunde liegenden Mechanismen der Krankheitsentstehung und deren Fortschreiten sind jedoch bis heute nicht vollständig geklärt. Bei der Entstehung von Atherosklerose scheinen HIF-1α Protein und seine Gegenspieler HDL, Stickstoffmonoxid und Vitamin C eine zentrale Rolle zu spielen. Mit unseren Ergebnissen konnten wir die protektiven Funktionen dieser Faktoren bestätigen, jedoch sind weitere Untersuchungen notwendig, um deren Rolle und Interaktion in der Entstehung von Atherosklerose und oxLDL-induzierten Krankheiten aufzuklären.

9 References

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10 Curriculum vitae

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Career and practical trainings

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group	Ao. Univ. Prof.Dr. Witta Monika Strobl
title	Effect of uremia on cellular cholesterol efflux and plasma lipoprotein profile in children and young adults with chronic renal failure Else Kröner Fresenius Stiftung, Project # FA 608 A0501
since june 2005	*PhD-thesis at the Department of Medical Chemistry, Center of Physiology and Pathophysiology, Medical University of Vienna*
supervisor	Univ. Prof. Dr. Hans Goldenberg, Ao. Univ. Prof. Dr. Hildegard Laggner
title	Influence of oxidatively modified LDL on the transcription factor HIF-1α and the scavenger receptor SR-BI in THP-1 macrophages ÖNB Jubiläumsfonds, Project # 10537
1998-2004	*Diploma studies of Nutritional Sciences, University of Vienna* Diploma thesis at the Dept. of Nutritional Sciences, University of Vienna
supervisor	Prof. Dr. I. Elmadfa, Prof. Dr. D. Majchrzak
title	The status of lipid parameters, fat-soluble vitamins, carotenoids and vitamin C of vegetarians, vegans and non-vegetarians in Austria
02-05/2005	Department of Human Genetics, KIMCL, Medical University of Vienna, Prof. Dr. Rotraud Wieser Search for target genes of the leukemogenic transcription factor EVI1 and its putative antagonist, MDS1/EVI1 (RT-PCR, PCR, plasmide-cloning and cell-transfection)
08/2003	Department of Nutritional Sciences, University of Vienna Prof. Dr. I. Elmadfa, Dr. D. Majchrzak Vitamin (A,C,D,E) determination (HPLC, Test Kits, …)
7/2003	Department of Medical Chemistry, Medical University of Vienna Prof. Dr. H. Goldenberg, Dr. Barbara Scheiber-Mojdehkar Iron metabolism research (PCR, ELISA, …)

Publication list

- C. Roehrl, S. Fruhwürth, B. Stambera, S.M. Schreier, A. Dolischka, N. Zemann, M. Huettinger, M. Hermann, W. Strobl and H. Stangl (2009)
  Scavenger Receptor, Class B, Type I provides an alternative means for ß-VLDL uptake independent of the LDL receptor
  BBA Lipids, under revision

- M.K. Muellner, S.M. Schreier, B. Schmidbauer, M. Moser, S. Kapiotis, P. Quehenberger, H. Goldenberg, H. Laggner (2008)
  Vitamin C inhibits NO-induced stabilization of HIF-1α in HUVECs
  FEBS Journal, under revision

- S.M. Schreier, M.K. Muellner, H. Steinkellner, M. Hermann, H. Esterbauer, M. Exner, B.M. K. Gmeiner, S. Kapiotis, H. Laggner (2009)
  Hydrogen Sulfide Scavenges the Cytotoxic Lipid Oxidation Product 4-HNE
  Neurotox Res, Published online 2009 Aug 13

- M.K. Muellner, S.M. Schreier, H. Laggner, M. Hermann, H. Esterbauer, M. Exner, B.M.K. Gmeiner, S. Kapiotis (2009)
  Hydrogen sulfide, the third endogenous gasotransmitter, destroys lipid hydroperoxides in oxidized LDL
  Biochemical Journal, Volume 420, pages 277-281

- H. Laggner , M.K. Muellner , S. Schreier , B. Sturm , M. Hermann, M. Exner , B.M.K. Gmeiner and S. Kapiotis (2007)
  Hydrogen sulphide: a novel physiological inhibitor of LDL atherogenic modification by HOCl
  Free Radical Research, Volume 41, Issue 7 2007, pages 741 – 747

- H. Laggner, S. Schreier, M. Hermann, M. Exner, A. Muehl, B. Gmeiner, S. Kapiotis (2007)
  A benefical "side effect" of an OTC antidepressant drug?
  The main components contributing to the antidepressant action of St. John's Wort inhibit low-density lipoprotein atherogenic modification
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Abstracts:

- H. Laggner H, S.M. Schreier, M. Hermann, M. Exner, S. Kapiotis, B.M.K. Gmeiner (2009)
  S-Carbamoylation impairs the radical scavenging activity of cysteine
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- H. Laggner H, S.M. Schreier, M. Hermann, H. Esterbauer, M.K. Muellner, M. Exner, B.M.K. Gmeiner, S. Kapiotis (2008)
  The novel gaseous vasorelaxant hydrogen sulfide inhibits angiotensin-converting enzyme (ACE) activity of endothelial cells
  Free Radical Research, Volume 42, Pages S96-S96

- S. Schreier, M. Moser, H. Goldenberg, H. Laggner (2007)
  Vitamin C and HDL decrease oxLDL-induced accumulation of HIF-1α in macrophages
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- S. Schreier, H. Goldenberg, H. Laggner (2006)
  Vitamin C and HDL decrease oxLDL-induced accumulation of HIF-1α in macrophages
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- H. Laggner, S. Schreier, M. Hermann, M. Exner, B. Gmeiner, S. Kapiotis (2006)
  The main componenents contributing to the antidepressant action of St.John's wort are inhibitors of low-density lipoprotein atherogenic modifications
  ATHEROSCLEROSIS Supplements 7, (3), June 2006, 525-526, Th-P15:149

Awards:

- 2nd International Symposium, Human Nutrition & Metabolism Research and Training Center Graz, October 9 – 10 2006, Graz
  HNMRC Casa Medica Poster Prize 2006

- International Society for Free Radical Research, 13th Biennal Congress, August 15 – 19 2006, Davos, Switzerland
  Young Investigator Fellowship, talk