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DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

Investigation of molecular mechanisms of nicotinic acid in the treatment of atherosclerosis

*Studium molekulárních mechanismů účinku kyseliny nikotinové v léčbě ateroskleróz*y

Diploma Thesis

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Fair declaration

I declare that I made this diploma thesis myself and mentioned every used sources and used literature.

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Thank you all

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1 ABBREVATION LIST

CETP	cholesterylester transfer protein
DMEM	dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EDTA	ethylene diamine tetraacetic acid
FBS	foetal bovine serum
FMLP	N-formyl-methionyl-leucyl-phenylalanine
GM- CSF	granulocyte- macrophage colony stimulating factor
GPR109A	G protein coupled receptor 109A
H_2O_2	hydrogen peroxide
HBSS	hank´s balanced salt solution
IL- 8	interleukin- 8
k.o.	knock out mice
LDLR	low-density lipoprotein receptor
MCP-1	macrophage/monocyte chemotactic protein-1
MIP-1-α	macrophage inflammatory protein-1-α
NA	nicotinic acid
NGS	normal goat serum
PBS	phosphate buffered saline
PFA	paraformaldehyde
PU-REP	pumaG reporter

PUMA-G	protein upregulated in macrophages by interferon- gamma
RANTES	regulated upon activation, normal T cell expressed and presumably secreted
RPMI 1640	Roswell park memorial institute tissue medium 1640
TG 4 %	thioglycolate brewer medium 4 %
WT	wildtype mice

Abstract

To investigate molecular mechanism of action of nicotinic acid in the treatment of atherosclerosis, we have used genetically modified mice, LDLR k.o. mice and PumaG double deficient mice. Mice were fed high fat diet for sixteen weeks and treated with or without 0.3% nicotinic acid in drinking water. Afterwards mice were sacrificed and the hearts were used for determination of the atherosclerotic lesions by defining the plaque area and areas immunostained for macrophages.

Further we aimed at clearing the mechanism of action of nicotinic acid. By using fluorescence microscopy we found area with expression of gene GPR109A in peritoneal exudates cells. Next, by setting the limit of intracellular calcium I was measuring the response of peritoneal cells to the nicotinic acid with subsequent addition of chemokines – mcp-1, rantes, il-8, mip-1- α. The goal was to lower the level of chemokines and thus to reduce the inflammation.

Abstrakt

Ke studiu molekulárního mechanismu účinku kyseliny nikotinové v léčbě aterosklerózy jsme použili geneticky modifikované myši, LDLR k.o.a PumaG deficientní myši. Myši byly krmeny vysokotukovou dietou po dobu šestnácti týdnů a zároveň jim byla podávána 0,3% kyselina nikotinová v pitné vodě. Poté byly myši usmrceny a srdce použita k určení plochy aterosklerotického plátu a vyjádření imunochemicky zbarvených makrofágů.

Dále jsme se zaměřili na objasnění mechanismu účinku kyseliny nikotinové. Pomocí fluorescenční mikroskopie byla nalezena oblast exprese genu GPR109A v buňkách peritoneální zánětlivé tekutiny. Následně pomocí stanovení hladiny intracelulárního kalcia byla měřena odpověď peritoneálních buněk na kyselinu nikotinu s následným přidáním chemokinů- mcp-1, rantes, il-8, mip-1-α. Záměrem bylo snížení hladiny chemokinů a tím snížení zánětu.

2 INTRODUCTION

2.1 Atherosclerosis

2.1.1 General patterns about atherosclerosis

Atherosclerosis is characterized by the thickening of the arterial wall and causes coronary artery disease and cerebrovascular disease, the most common causes of illness and death worldwide. The efforts to understand how risk factors such as high blood pressure, dysregulated blood lipids and diabetes contribute to atherosclerotic disease. [1] Intensive study of the cellular and molecular mechanism that underlies the formation of atherosclerotic plaques and plaque rupture has led to a consensus view of these processes. [2]

At present, the main conceptual approaches to therapy for atherosclerosis are manipulation of plasma lipoprotein metabolism or cellular cholesterol metabolism, and manipulation of inflammatory processes. [1]

Studies of genetically modified mice are commonly used to identify and validate potential therapeutic targets. But there is important difference between mice and human with respect to process lipoprotein metabolism and to inflammatory pathways. [3]

2.1.2 Initiation and progression of atherosclerosis

THE NORMAL ARTERY

Usually a normal artery has three layers (the intima, the media and the adventitia). The intima is lined by monolayer of endothelial cells in contact with the blood; it contains resident smooth muscle cells incorporated in extracellular matrix. The internal elastic lamina forms the border of the intima with the media. The media contains layers of smooth muscle cells invested with a collagen- and elastin-rich extracellular matrix (the aorta contains concentric lamellae of smooth muscle cells which have between dense bands of elastin). The elastic lamina forms the border of the media with the adventitia. The adventitia contains nerves and some mast cells

and is the origin of the vessel which supplies blood to the outer two thirds of the tunica media.

Accumulation of lipoprotein particles

Lipoprotein particles associate with constituents from the extracellular matrix and separate from some antioxidants within the intima and in this way can oxidative modify. This is a factor that may trigger a local inflammatory response that could be responsible for the next steps in the lesions formations.

Adhesion, penetration and accumulation of leukocytes

The luminal endothelial can occur early with adhesion of nuclear leukocytes in hypercholesterolemia. The expression of various adhesion molecules for leukocytes probably triggers the first step in the recruitment of white blood cells to the sight of further lesions. After the adhesion some white blood cells will migrate into the intima. The direct migration of leukocytes probably depends on chemoattractant factors including modified lipoprotein particles and chemoattractant cytokines such as the chemokine macrophage, chemoattractant protein-1 produced by vascular wall cells in response to macrophage proteins. Leukocytes are seating permanently in the evolving fatty streak and can divide and exhibit augmented expression of receptors for modified lipoproteins-scavenger receptors. These mononuclear phagocytes soak the lipids and transform them into foam cells whose cytoplasm is filled with lipid droplets.

Formation of the fibrous cap and lipid core

As the fatty streak extends into more complicated atherosclerotic lesions, smooth muscle cells accumulate within the expanding intima and the amount of extracellular matrix increases. The fibrous cap, formed of extracellular matrix elaborated by the smooth muscle cells in the intima, characteristically overlies a lipid core field with macrophages. In addition to dividing, these cells in the lipid core can die and release their lipid contents into the extracellular space. [4] (Fig. 1)

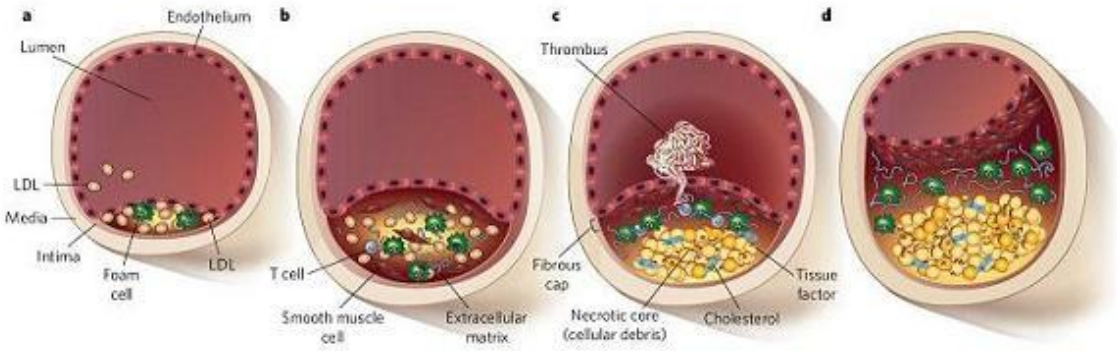

Fig. 1 Initiation and progression of atherosclerosis. Atherosclerosis occurs at sites in the arterial tree where laminar flow is disrupted. A lesions begins as a fatty streak (a) and can develop into an intermediate lesion (b), and then into a lesion what is vulnerable to rupture (c) and, finally, into an advanced obstructive lesion (d). 1

An integral view of experimental results in animals and study of human atherosclerosis indicate that the fatty streak represents the initial lesion of atherosclerosis. This accumulation of lipoprotein particles may not simply come from an increased permeability of the overlying endothelium. These lipoproteins can be collected in the intima of arteries because they are bound to constituents of the extracellular matrix, increasing the residence time of the lipid-rich particles within the arterial wall. Lipoproteins accumulating in extracellular area of the intima of arteries often associate with proteoglycan molecules in the arterial extracellular matrix. This accumulation supports a pathogenic role for such modifications of lipoproteins in atherosclerosis.

2.1.3 Leukocyte Recruitment

After the accumulation of extracellular lipid, recruitment of leukocytes occurs as a second phase in formation of the fatty streak. The white blood cell types that are typically found in the evolving atheroma comprise primarily cells of the mononuclear lineage-monocytes and lymphocytes. An amount of adhesion molecules or receptors for leukocytes expressed on the surface of the arterial endothelial cell likely participates in the recruitment of leukocytes to the nascent fatty streak. Adhesion molecules of particular importance include VCAM-1 vascular cell adhesion molecule and ICAM-1 intercellular adhesion molecule and P-selectin. Expression of the gene encoding

the adhesion molecule CD44 is upregulated specifically in atherosclerotic lesions, and deletion of this gene results in reduced monocyte recruitment and less development of atherosclerosis. Laminar shear forces can also suppress the expression of leucocyte. Sites of predilection for forming atherosclerotic lesions often have disturbed laminar flow. Ordered laminar shear of normal blood flow extends the production of nitric oxide by endothelial cells. After the adhesion to the surface of the arterial endothelial cell through interaction with a receptor such as VCAM-1, the monocytes and lymphocytes penetrate the endothelial layer and settle down in the intima. In addition to products of modified lipoproteins, cytokines could regulate the expression of adhesion molecules involved in leukocytes recruitment. Modified lipoproteins could induce cytokine release from vascular wall cells; this pathway could provide an additional connection between accumulation and modification of lipoproteins and leukocyte recruitment. The regulated migration of leukocytes into the arterial wall could also result from the actions of modified lipoprotein. For example, oxidized LDL can promote the chemotaxis of leukocytes as well as oxidatively modified lipoproteins can elicit the production by vascular wall cells of chemoattractant cytokines such as monocyte chemoattractant protein 1(Mcp- 1).

2.1.4 Foam- cell formation

Once the mononuclear phagocytes are settled in the intima they begin to divide into macrophages and transform into foam cells. Transformation of mononuclear phagocytes into foam cells requires the uptake of lipoprotein particles by receptor-mediated endocytosis. Supposedly it is recognized receptor for LDL that mediates this lipid uptake. Alternative receptors that can mediate lipid- loading of foam cells include a growing number of macrophage scavenger receptors, which primarily endocytose modified lipoproteins and other receptors for oxidized LDL or β-VLDL, a type of lipoprotein commonly encountered in certain hypercholesterolemic states. By sucking in lipids from the extracellular place, the mononuclear phagocytes carrying such scavenger receptors could remove lipoproteins from the developing lesions. Lipid accumulation is a significant supporter to creation of the atheroma. Macrophages could thus play a vital role in the dynamic economy of lipid accumulation in the arterial wall during atherogenesis. Macrophages taking up modified lipoproteins could design

cytokines and growth factors that can further signal some of the cellular events in lesions complication. An amount of growth factors or cytokines worked out by mononuclear phagocytes can stimulate smooth- muscle cell proliferation and production of extracellular matrix, which accumulates in atherosclerotic plaques. Examples of cytokines that can induce local production of growth factors that can play an important role in plaque evolution and complication are IL-1 and TNF-α. Other cytokines, namely interferon- γ derived from activated T-cells within lesions, can inhibit smooth- muscle proliferation and synthesis of interstitial forms of collagen. [4]

2.2 Lipoprotein metabolism

Lipoprotein transport lipids in the blood and their metabolism are closely interrelated with the initiation and progression of atherosclerosis. [5] Primarily are concerned LDLs and HDLs. HDL particles have anti-atherogenic properties due their ability to take up cholesterol from non hepatic cells and transport them back to the liver through the reverse cholesterol transport pathway. [6] HDL seems to have anti-inflammatory and anticoagulatory properties. [7]

The intestine absorbs dietary fat and packages it into chylomicrons, which are then transport to peripheral tissues through the blood. In adipose tissues and muscles, the enzyme lipoprotein lipase breaks down chylomicrons, and fatty acids enter these tissues. The rest of chylomicrons are subsequently taken up by the liver. The liver loads lipids onto apoB and secretes VLDLs, which undergo lipolysis by lipoprotein lipase to form LDLs. These LDLs are taken up by the liver through binding to the LDL receptor. HDLs are generated by the intestine and the liver through the secretion of lipid free apoA-I, which recruits cholesterol from these organs through the action of the transporters ABCA1, forming nascent HDLs, and this protects apoA-I from being rapidly degraded in the kidneys. Nascent HDLs promote the efflux of cholesterol from tissues through the actions of ABCA1. Mature HDLs promote this efflux through the actions ABCG1. In macrophages, the nuclear receptor LXR up-regulates the production of both ABCA1 and ABCG1. The free cholesterol in nascent HDLs is esterified to cholesteryl ester by the enzyme lecithin cholesterol acyltransferase LCAT, which creating mature HDLs. The cholesterol in HDLs is returned to the liver directly

through uptake by the receptor SRBI and indirectly by transfer to LDLs and VLDLs through the cholesteryl ester transfer protein CETP. The lipid involves of HDLs is altered by the enzymes hepatic lipase and endothelial lipase and by the transfer proteins CETP and phospholipid transfer protein PLTP, affecting HDL catabolism. [1] (Fig. 2)

The lowering of LDL-cholesterol levels is one of the most important therapeutic targets that have been used to reduce cardiovascular morbidity and mortality. [8] Optimization of strategies have recently focused to innovate approaches how to raise HDL-cholesterol levels. [9,10] The strong HDL- cholesterol elevating effect has nicotinic acid, which is unique among the drugs currently approved for clinical use. [11]

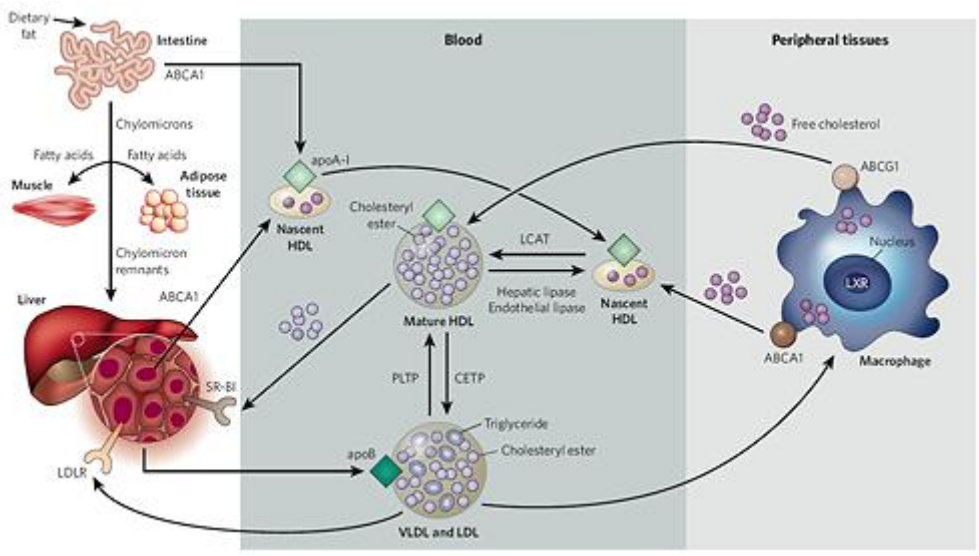

Fig. 2 Metabolism of lipid particles. Lipoprotein metabolism has a key role in atherogenesis; it involves the transport of lipids, particularly cholesterol and triglycerides, in the blood. 1

2.3 Nicotinic acid

Nicotinic acid and nicotinamide serve as precursors of coenzymes NAD and NADP and are water soluble vitamins of the vitamin B complex. [12] Pharmacological doses of nicotinic acid induce a profound change in the plasma levels of various lipids and lipoproteins. The ability of nicotinic acid to strongly increase

the plasma concentrations of HDL and reduce the risk of cardiovascular events has in recent years led to an increased interest in the pharmacological potential of nicotinic acid. The clinical use of nicotinic acid is, however, hindered by harmless but unpleasant side effects, especially by a strong cutaneous vasodilatation called flushing. [13,14]

2.3.1 Mechanism of action of nicotinic acid

The recent discovery of the G-protein-coupled receptor GPR109A, also referred HM74A in humans and PUMA-G in mice, as a receptor for nicotinic acid has allowed for better understanding of the mechanisms underlying the metabolic and vascular effects of nicotinic acid. Through the use of mice lacking the gene coding for the receptor GPR109A was shown to be the receptor mediating the lipid-lowering effects of nicotinic acid. G-protein mediates many effects of nicotinic acid on adipocytes and immune cells. [15,16,17]

2.3.2 GPR109A

GPR109A is a member of the class A rhodopsin-like GPCRs. GPR109A is also expressed at significant levels in the spleen and immune cells, such as monocytes, macrophages, neutrophils. [15, 16, 17, 18] Expression of GPR109A in immune cells appears to be regulated by various cytokines and can be upregulated by GM-CSF in neutrophils. [19]

GPR109A has been shown to couple to members of the G_i family of G proteins, activation of G_i- type leads to different cellular effects depending on the type of cells. In immune cells G_i activation primarily results in the stimulation of β-isoform of phospholipase C or of phosphoinositide-3-kinase γ via G protein β, γ-subunits released from activated G_i, in other cells for example adipocytes, activation of G_i preferentially results in the inhibition of the activity of adenyl cyclase, resulting in a decrease in intracellular cyclic AMP levels. Protein kinase A phosphorylates a number of proteins especially hormone- sensitive lipase and perilipin, which are required for triacylglycerol hydrolysis. Phosphorylation of perilipin activates access to the TAG containing lipid droplets by the now activated hormone sensitive lipase and adipose triacylglycerol lipase, which hydrolyzes the triacylglycerols in to free fatty acids and glycerol. [20]

2.3.3 Effect of nicotinic acid on lipid metabolism

Oral administration of nicotinic acid causes a decrease in free fatty acids plasma levels, followed by a reduction in the concentration of VLDL triglycerides. [21]

2.4 Immune cells in atherosclerosis

Atherosclerotic plaques contain blood-borne inflammatory and immune cells, mainly macrophages and T cells, then vascular endothelial cells, smooth muscle cells, extracellular matrix, lipids and acellular lipid-rich debris. [22] Accumulation of immune cells and lipid droplets in the intima occurs during the first phase of plaque formation.

Other cell types present in mature plaques include dendritic cells, mast cells, a few B cells and probably natural killer T cells. [22, 23, 24]

Many of these immune cells activate and produce proinflammatory cytokines such as interferon-γ and tumour- necrosis factor. [25]

2.5 Chemokines

Chemokines belong to a large group of structurally related chemotactic cytokines and they can be divided into 4 families based on the position of the first two cystein residues-CC, CXC, CX_3C, and XC.

Within atherosclerotic plaques is their function as messengers to direct leukocytes to sites of inflammation and they can control homeostasis and other activities of emigrated cells. By signalling through G protein chemokines govern a variety of cell responses including cell activation and transmigration in leukocytes. [26,27]

In addition to the expression of adhesion molecules, several chemokines produced by vascular cells guide the recruitment of immune cells.

The key role in the initiation of atherosclerosis plays CC-chemokine ligand 2 also known as MCP-1 and its receptor CC-chemokine receptor 2. [28, 29]

3 AIM OF WORK

We have focused on:

Development of atherosclerosis in LDLR (low density lipoprotein receptor) deficient and LDLR, PumaG double deficient mice treated or untreated with nicotinic acid.

In the second part of this work we have focused on:

Analyse of GPR109A expression in leukocyte with PU- REP mice by using methods bloodsmear and diff-quick staining.

PUMA-G expression in vitro by stimulating thioglycolate, casein solution for induce of inflammation

Concentration response relationship of cell signalling on the:

- nicotinic acid (NA)
- macrophage/monocyte chemotactic protein-1 (MCP-1)
- macrophage inflammatory protein-1-α (MIP-1-α)
- regulated upon activation, normal T cell expressed and presumably secreted (RANTES)
- interleukin-8 (IL-8)

by measuring intracellular calcium levels in the cell lines expressing of primary cell culture, cell culture line.

4 MATERIAL

4.1 Material

Biozym pipette 20-200µl	Biozym, Hessig Oldendorf, GE
Coverslip glasses, 24x60mm	Hartenstein, Heidelberg, GE
Delimiting pen, Dako pen	Dako, USA
Eppendorf pipettes	Eppendorf, Hamburg, GE
Eppendorf tubes 1.5ml, 2ml	Eppendorf, Hamburg, GE
Falcon tubes 15ml, 50ml	Becton Dickinson Labware, USA
Falcon cell strainer 40 µm	Becton Dickinson Labware, USA
Microtube 2ml	Sarstedt, Nümbrecht
Needle 21, 27G	Braun, Melsungen, GE
Pasteur capillary pipettes, 230mm	WU Mainz, GE
Pipette tips	Brand, Wertheim, GE
Polysine slides	Menzel, GE
Scissors	FineScience Tools, Heidelberg, GE
Sterile pipette 2ml, 5ml, 10ml, 25ml	Sarstedt, Nurmbrecht, GE
Syringe 1ml, 20ml, 60ml	Becton Dickinson Labware, USA
Tweezers	FineScience Tools, Heidelberg, GE

4.2 Instruments

Analytical balance	Scaltec, Göttingen, GE
Autoclave- H1Clave-HV-85	HMC, Hong Kong

Cryotome Leica CM 3050S	Leica, GE
Centrifuge 5810R	Eppendorf, GE
Freezer -80°C	Brunswick, USA
Incubator Hera cell 240	Heraeus, GE
Laminar flow cabinet	Heraeus, GE
Liquid nitrogen tank LS 4800	Taylor- Wharton, Husum, GE
Mikroscope Zeiss Axiovert 25	Hitachi, USA
Mikroscope Leica DM LS2	Leica, GE
Ultracentrifuge Himac CS 100fx	Hitachi, USA
Vortex	Heidolph genie 2, Heidelberg, GE
Waterbath	Eppendorf, Hamburg, GE

4.3 Chemicals

Aceton	Zentralbereich Neuheimer Feld, GE
Adsorbed rabbit anti- mouse macrophage AB	Accurate, USA
Biotinylated anti- rabbit IgG	Vector, CA
Brewer thioglycollate medium	Sigma, GE
Calcium chloride dihydrate	J.T.Baker, Deventer, Holland
Casein sodium salt, from bovine milk	Sigma, GE
D(+)- Glucose- monohydrate	Merck, Darmstadt, GE
Dimethyl sulfoxide	AppliChem, Darmstadt, GE
DMEM 1x	Gibco, GE

DPBS 1x, 10x	Gibco, GE
Ethylene diamine tetraacetic acid	Roth, Karlsruhe, GE
Foetal bovine serum	Gibco, GE
Fura-2/AM	Molecular Probes, Invitrogen, GE
FMLP	Sigma, GE
Glycerol gelatine	Merck, Darmstadt, Ge
GM-CSF	Sigma, GE
Hematoxylin	Zentralbereich Neuheimer Feld, GE
HEPES	Roth, Karlsruhe, GE
Hydrogen peroxide 30 %	Sigma, GE
Il-8	Sigma, GE
Insulin, human recombinant zinc, solution	Gibco, GE
L- Glutamine 200mM	Gibco, GE
Magnesium chloride hexahydrate	AppliChem, Darmstadt, GE
Mcp-1	Sigma, GE
Mip-1-α	Sigma, GE
Nicotinic acid	Sigma, GE
NGS	Vector, CA
Pen Strep, Penicilin Streptomycin	Gibco, GE
Potassium chloride	AppliChem, Darmstadt, GE
Rantes	Sigma, GE
RPMI 1640	Gibco, GE

Sodium pentobarbital- Narcoren	Rhone Merieux, Laupheim, GE
Sodium pyruvate 100mM	Gibco, GE
Tissue freezing medium	Leica, GE
Triton	Zentralbereich Neuheimer Feld, GE

4.4 Kits

ABC kit	Vector, CA
AEC staining kit	Sigma, GE
Diff quick staining set	Medion Diagnostics, GE

4.5 Software

Adobe Photoshop 7.0

Adobe Photoshop CS3

Microsoft Office

Stereo Investigator

Till Vision

5 METHODS

5.1 In vivo experiments

5.1.1 Animal experiments and development of atherosclerosis

Eight weeks old male LDLR deficient mice (group 012) and LDLR, PumaG double deficient mice (group 014) were used to study atherosclerosis. Animals of each strain were divided into two experimental groups and underwent high fat diet which contained 21% butter fat and 1.5% cholesterol ad libitum with or without 0.3% nicotinic acid in drinking water for 16 weeks. Mice were weighed and controlled every week.

5.1.2 Dissection of the heart

Mice were anesthetized with 100 µl of pentobarbital i.p. and the depth of narcosis was controlled by pressing the sciatic nerve. After blood withdrawal by cardiac puncture the chest was opened, small incision was made in the right ventricle and the mouse was perfused through the left ventricle with 20ml PBS followed by 20 ml fixative 4% PFA, 20mM EDTA, 5% sucrose in PBS by using a perfusion pump. The upper half of the heart was cut and incubated in fixative and 30% sucrose at 4°C overnight, respectively. Afterwards the hearts were frozen in tissue medium and stored at -80°C until we cut them in a cryostat in sections of 8 µm.

Starting with the section where all three aortic valves were visible the sections were sequentially collected in a series of five slides. Slides were stored at -80°C until they stained.

5.1.3 Immunostaining

Slides were dried at room temperature for 30 minutes and made a circle around all sections with a delimiting dako pen. Sections were fixed on ice cold acetone for two minutes and permeabilized in PBST (PBS + 0.1% TRITON-X-100) for two minutes to remove freezing medium. Slides were washed two times for two minutes in PBS. To quench endogenous peroxidase slides were incubated in 0.3% H_2O_2 in PBS and then rinsed three times in PBS for 2 minutes. Sections were blocked with 5% NGS for 45 minutes at RT and then incubated with rabbit anti- mouse

macrophage antibody at 1:100 dilution in 5% NGS at 4°C overnight. Afterwards slides were washed three times for two minutes in PBS and incubated with biotinylated goat secondary anti- rabbit IgG antibody in 1:200 dilution in 5% NGS for 30 minutes at RT. Slides were washed three times for 2 minutes in PBS. To localize peroxidase in sections ABC reagent, which consists of 2.5 ml PBS with 1 drop A + 1 drop B, was used and slides were incubated for 30 minutes in the humid chamber. Then slides were rinsed two times in PBS for two minutes and then were slides dipped in AEC reagent for five minutes, which consists of 4 ml H_2O with 2 drops Acetate and 1 drop AEC + 1 drop H_2O_2. After that slides were immersed in distillated H_2O for five minutes, then in acidic water for 20 seconds, counterstained in hematoxylin for five minutes and finally slides were rinsed under cold tap water for five minutes. Then I dried slides and covered them with warmed up glycerol gelatin.

Negative control underwent the same treatment except of incubation with primary antibody which was replaced by 5% NGS.

5.1.4 Quantification of atherosclerosis

Stained sections in 50x magnification were photographed in a microscope. To evaluate the percentage of lesion/plaque area in the aortic valve and the percentage of immunostained macrophages in lesion/ plaque lesion area measurements were performed on each slide with image analysis software Adobe Photoshop CS3 by using a magnetic lasso tool. There was manually traced the outline of the region of the aortic valve, of the plaque, respectively and red stained macrophages. The number of pixels was. Data were imported into Microsoft Excel which was used for statistical evaluation.

5.2 In vitro experiments

5.2.1 Animals

Mice lacking PUMA-G–knockout mice and wildtype mice were taken from breed of our institute. Mice were injected with 4% thioglycolate and treated from two to five days or with casein solution over night.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is growth factor, stimulating proliferation, which was used to enhance cells adherence to glass cover slips.

5.2.2 Peritoneal lavage

Mouse was euthanized by carbon dioxide to asphyxiation. After cleaning the abdomen with 70% ethanol, was made a ventral midline incision and retracted abdominal skin to expose peritoneal wall. Mice were injected with 10 ml 5mM EDTA in PBS into the peritoneum. The abdomen was massaged and slowly withdrew the peritoneal fluid. This step was repeated with 5 ml dPBS. Peritoneal fluid was filtrated through 40 μm cell strainer into a 50 ml conical tube and was centrifuged ten minutes at 1000 rpm at 4°C. The supernatant was removed and cell pellet was resuspended cell in PBS medium for subsequent staining with Diff Quick or in RPMI 1640 medium for subsequent measurement of intracellular calcium.

5.2.3 Staining with Diff Quick

It was made a drop of 5-10 μl of cell suspension on the lower part of a slide. Then the cells were smeared with using spreader slide under the angle of 45°C to disperse the cells over the slide's length. The slides were left to air dry. To fix the smear the methanolic fixative solution was used to stabilize cellular components. Solutions I and II were then applied individually to the fixed smear to differentially stain specific cellular components. Every slide was dipped in fixative solution five times, one second each time. It was allowed to excess to drain. Then was every slide dipped in solution I five times, one second each time and finally the same process was repeated with solution II. Then all slides were washed with distilled water and left

to dry. Stained slides in 400x magnification were photographed in a microscope with normal vision and fluorescence to find expression of gene.

5.2.4 Isolation of peritoneal neutrophils

Mouse was injected with 1 ml of casein solution (9 g of casein compound with stirring 10 ml PBS 80^{o} C to 90^{o} C, pH 7.2 containing 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$). Solution was brought to a boil and then autoclaved one hour at 125^{o} C) into peritoneal cavity of mouse, to induce inflammatory response. The injection of 1 ml casein solution was repeated next morning. Three hours after the second injection, the mouse was euthanized by carbon dioxide asphyxiation. It was made a ventral midline incision with scissors and then retracted abdominal skin with forceps to expose the intact peritoneal wall. After cleaning abdomen with 70% ethanol was injected 5 ml of sterile 5mM EDTA in PBS solution into the peritoneum. The abdomen was massaged gently to distribution of the harvest solution. The peritoneal fluid was slowly withdrawn and transferred into a 50 ml conical tube. It was injected another 5 ml of sterile 5mM EDTA in PBS solution and repeated the procedure to remove the remaining cells from the peritoneum and transfer them to the tube. Peritoneal fluid was filtrated through 40 μm cell strainer into a tube and it was centrifuged ten minutes at 200 rcf at room temperature. Then the supernatant was removed and the peritoneal exudates cells were washed three times, each time were the cells resuspended in 10 ml of PBS and centrifuged. The washed pellet was resuspended in 1 ml sterile PBS, the number of viable cells was counted by trypan blue. It was mixed 3-5x 10^7 peritoneal exudates cells with 9 ml Percoll gradient solution(5 ml sterile PBS, ph 7.2 in 45 ml sterile Percoll) at room temperature in a 5 ml Beckman ultracentrifuge tubes. Peritoneal fluid was ultracentrifuged 20 min at 26 000 rpm at 4^{o} C. Then the thin, faint upper layer was discarded, which contained macrophages and lymphocytes and it was collected the PMN- polymorphonuclear leukocytes, which were found in the second opaque layer. The bottom layer contained erythrocytes. Cells were washed by adding 10 ml of PBS and centrifuged five minutes at 200 rcf at room temperature. The supernatant was removed, the pellet was resuspended in 1 ml medium DMEM, containing 2mM Glutamine + 10% FBS.

5.2.5 Isolation of neutrophils from peripheral blood

Mouse was anesthetized by injecting 100 µl Phenobarbital, it was drawn 5 µl of EDTA into 1 ml syringe with 21-G needle, then syringe was used to bleed mice by cardiac puncture, blood was transferred to 1.5 ml Epi. The blood was centrifuged ten minutes at 200 rcf in a tabletop centrifuge at room temperature, then the whitish buffy coat layer, which appears at the plasma interface was collected, cells were counted, it was resuspended 3-5 x 10^7 cells in 1 ml of PBS added 9 ml Percoll gradient solution at room temperature in a 5 ml Beckman ultracentrifuge tubes. Cells were ultracentrifuged the 20 min at 26 000 rpm at 4^{o}C. Then the thin, faint upper layer, which contained macrophages and lymphocyte, was discarded and the PMN-polymorphonuclear leukocytes, which were found in the second opaque layer, were collected.

5.2.6 Primary cell culture

The glass cover slips (24-mm round #1 glass) were placed in 6-well culture plates. After the isolation from mice were peritoneal exudates cells, peritoneal neutrophils, blood neutrophils diluted in medium. The drops of cells were made on an average around 150 µl and were localized to the middle of the cover slips. The medium contained 20nM GM-CSF. Cells were incubated overnight. In the morning the cells were washed with used medium.

5.2.7 Cell culture lines

THP-1(cell type: human acute monocytic leukemia)

Developed from the peripheral blood of 1 year old boy with acute monocytic leukemia at relapse in 1978, the cells were described to produce lysozyme and to be phagocytic.

The cells were cultivated at 37^{o}C with 5% CO_2 in medium 90% RPMI 1640, containing 10% FBS, supplemented with 50 units/ml penicillin and 50 mg/ ml

streptomycin. The cells were maintained at 0.1-1.0 10^6 cells/ml, splitted them 1:2 every 3-4 days. The cells were round, single cells in suspension, partly in clusters.

Cells defrosting

The ampoule with cells was thawed in a water bath (37°C) and the cells were transferred into a 15 ml centrifuge tube with adding 10 ml prewarmed medium and mixed gently. The cells were centrifuged at 1000 rpm at 24°C for five minutes. The supernatant was decanted, the cells were resuspended in a defined volume of medium, there was removed a small sample in order to determine cell number and viability. For the first couple of days after recovery was used higher concentration of FBS (15%).

Cell counting

Neubauer counting chamber

To determine the number of living cells, a probe from cells solution was taken, diluted 1:10 in trypan blue and counted in Neubauer counting chamber. Trypan blue deposits in cell protein structures, but can enter only permeable cell membranes and thus stains dead cells only. The final cell count can be calculated:

Cells/ ml = cell count from four small quadrants x dilution x chamber constant (10^4).

Cell frosting

Eukaryotic cells were stored in liquid nitrogen. To freeze the cells, pellets were placed in freeze medium and resuspended to 1 ml of $5x10^6$ –$1x10^7$ cells. The especially microtubes were transferred into liquid nitrogen tanks.

5.2.8 Measurement of intracellular calcium

For the measurement of intracellular calcium cells were seeded on cover slip glasses (24 x 60 mm) placed in 6-well plate with medium consisting 20nM GM-CSF overnight to induce differentiation into macrophages and thereby adherence. The no adherent cells were removed by washing with Hank´s balanced salt solution (HBSS)

containing calcium ions. All experiments were performed at temperature 37^{o}C. The cells placed on cover slips were incubated in modified HBSS solution containing reduced calcium (2mM CaCl2) and pluronic F-127 (0.01%) as a detergent and Fura-2 AM (5mM) for 60 min in the dioxide incubator. After loading, preparations were superfused with HBSS solution, containing 2mM $CaCl_2$. Cover slips were then removed from the culture plates and mounted into a chamber with adding 600 µl buffer HBSS with Ca ions. Buffer HBSS consists of 120mM NaCl, 5.4 mM KCl, 0.8mM $MgSO_4.7H_20$, 10mM HEPES, 2mM $CaCl_2.2H_2O$. The agonists were added after 50-100sec. Negative control experiments were performed by using buffer, positive control experiments using FMLP. Used agonists were NA, MCP-1, MIP-1-α, IL-8, RANTES. Fluorescence excitation was measured at 340 and 380 nm light (F340/380), emission recorded at 510 nm. Fluorescence emission from a region of the loaded cells was recorded at 30 Hz, with a photometry system T.I.L.L. Photonics and pCLAMP 8 software Axon Instruments. (Fig. 3)

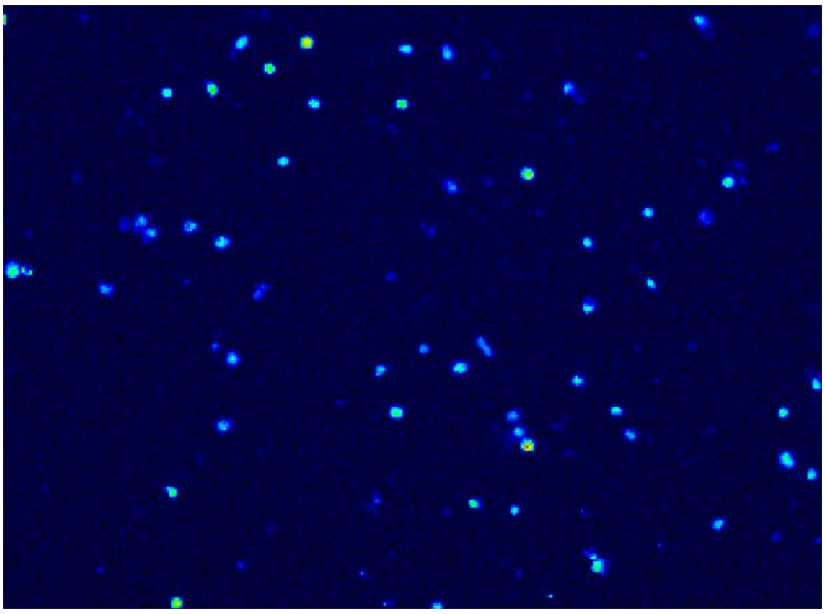

Fig. 3 Peritoneal exudates cells stained with Fura-2 before stimulation of agonists

6 RESULTS

6.1 In vivo experiments

6.1.1 Heart section

The following pictures show (Fig.4-Fig.21) 8 μm sections of heart immunostained with antibody against macrophages.

The difference between two following sections is 40 μm, usually. The aortic valve opens up gradually and quantity of plaque increases.

Images were made in the program Stereo investigator by using Leica microscope, by 50x magnification, intensity 5.

Hearts were divided into two groups:

- 012 – LDLR k.o. - hearts 11-15 only high fat diet- control

- hearts 16-20 high fat diet + 0.3% nicotinic acid

- 014- PumaG double deficient - hearts 1-3 only high fat diet- control

- hearts 4-6 high fat diet + 0.3% nicotinic acid

We compared percentage area of plaque and macrophages

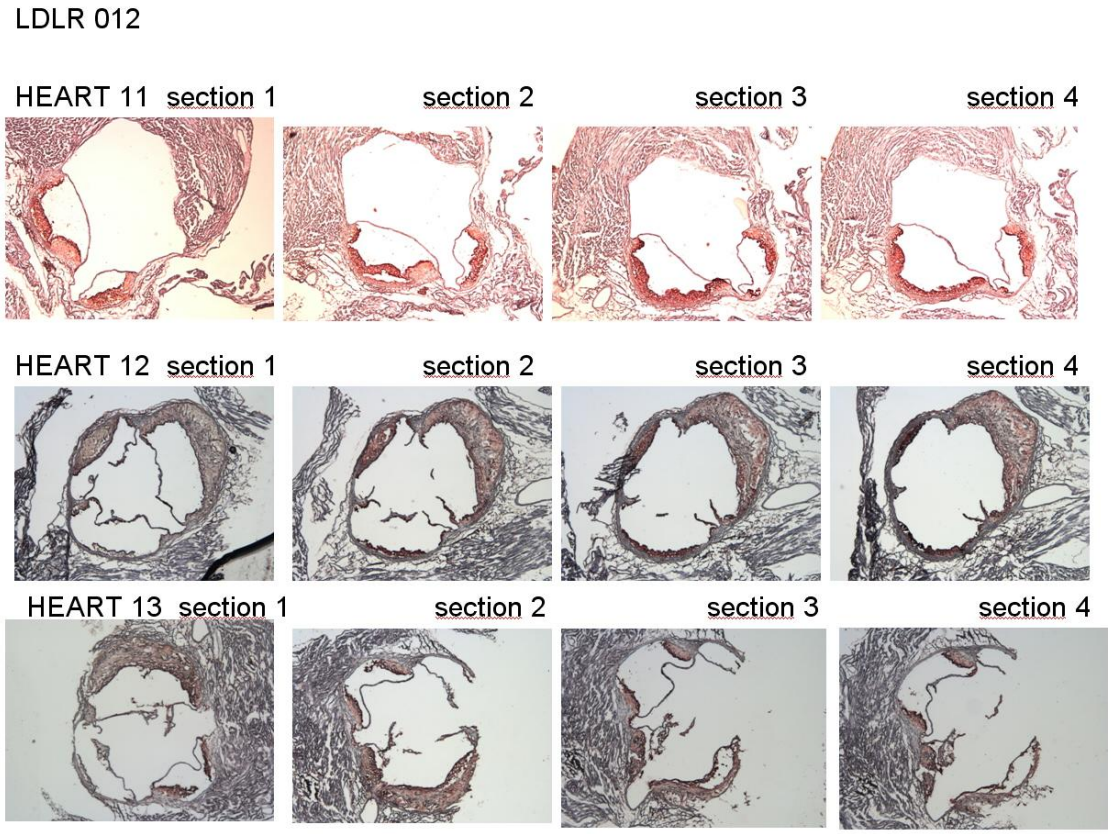

Fig. 4 Heart section 1-4 of LDLR deficient mice with high fat diet without nicotinic acid

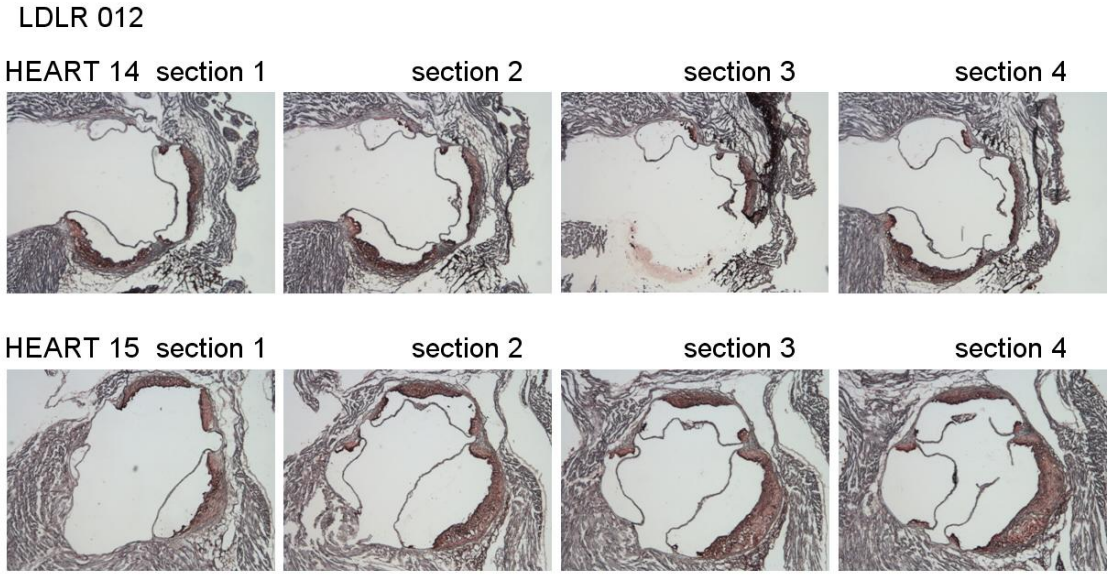

Fig. 5 Heart section 1-4 of LDLR deficient mice with high fat diet without nicotinic acid

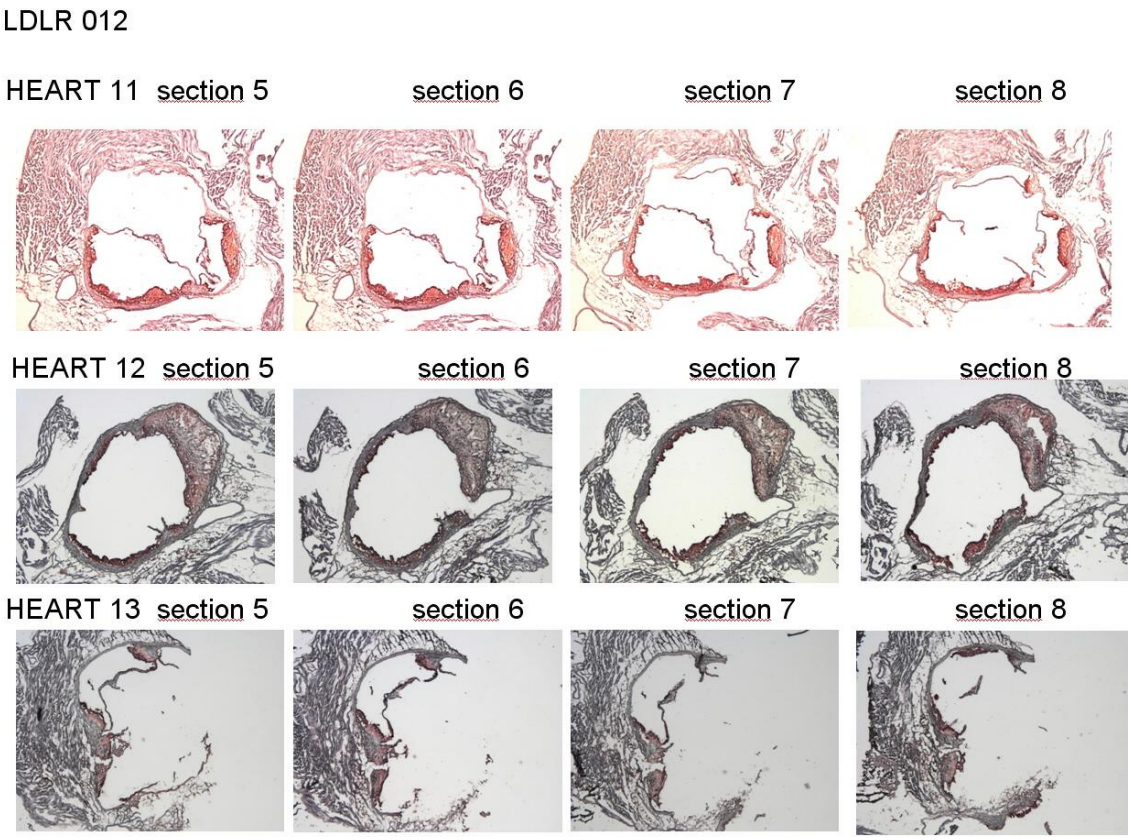

Fig. 6 Heart section 5-8 of LDLR deficient mice with high fat diet without nicotinic acid

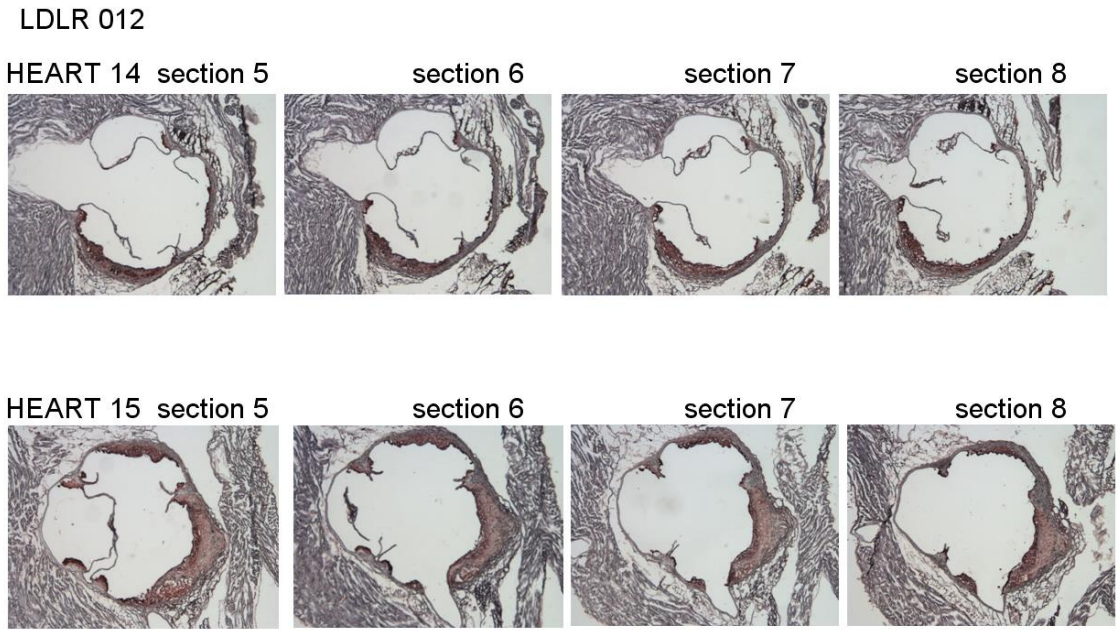

Fig. 7 Heart section 5-8 of LDLR deficient mice with high fat diet without nicotinic acid

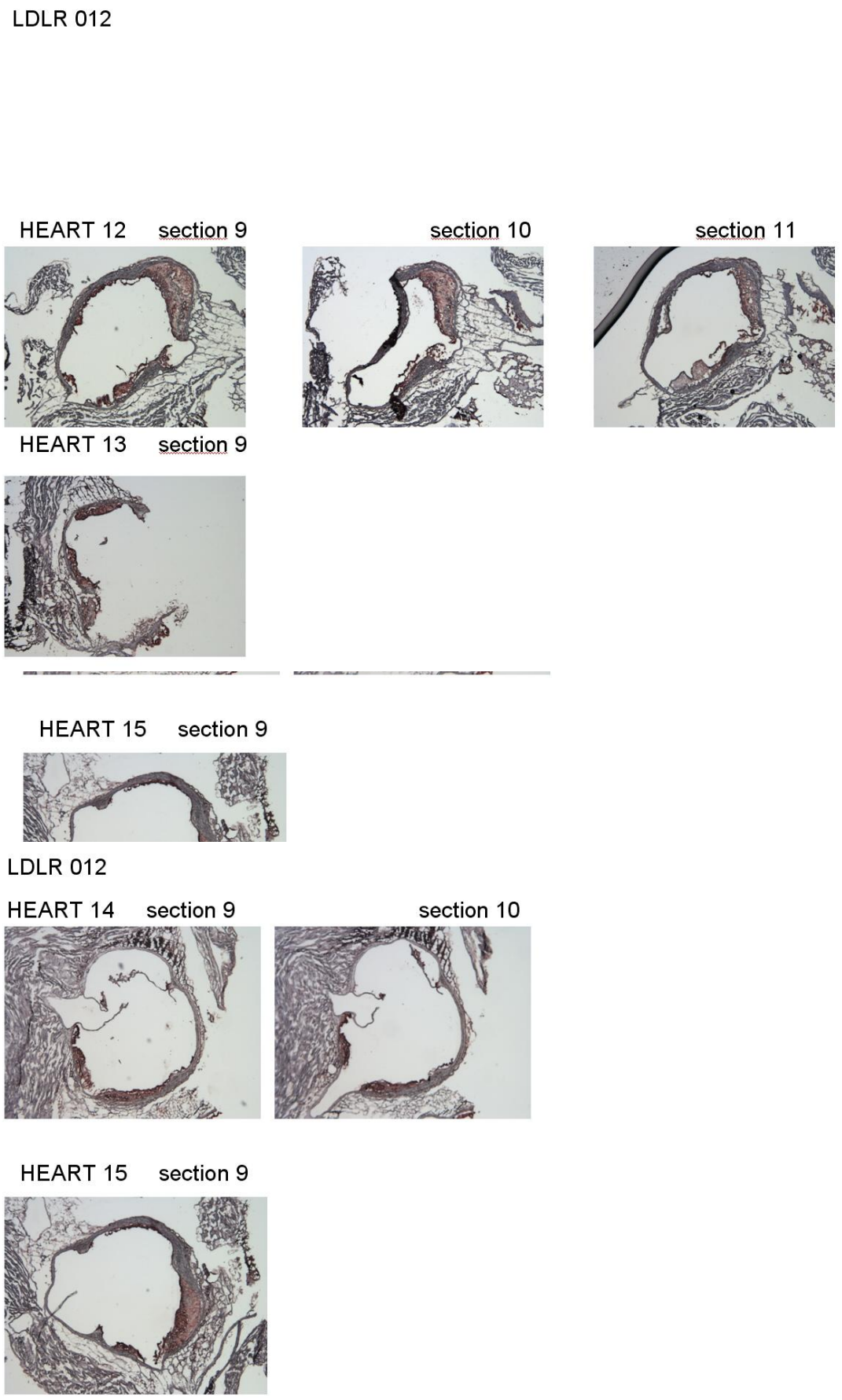

Fig. 9 Heart section 9-10 of LDLR deficient mice with high fat diet without nicotinic acid

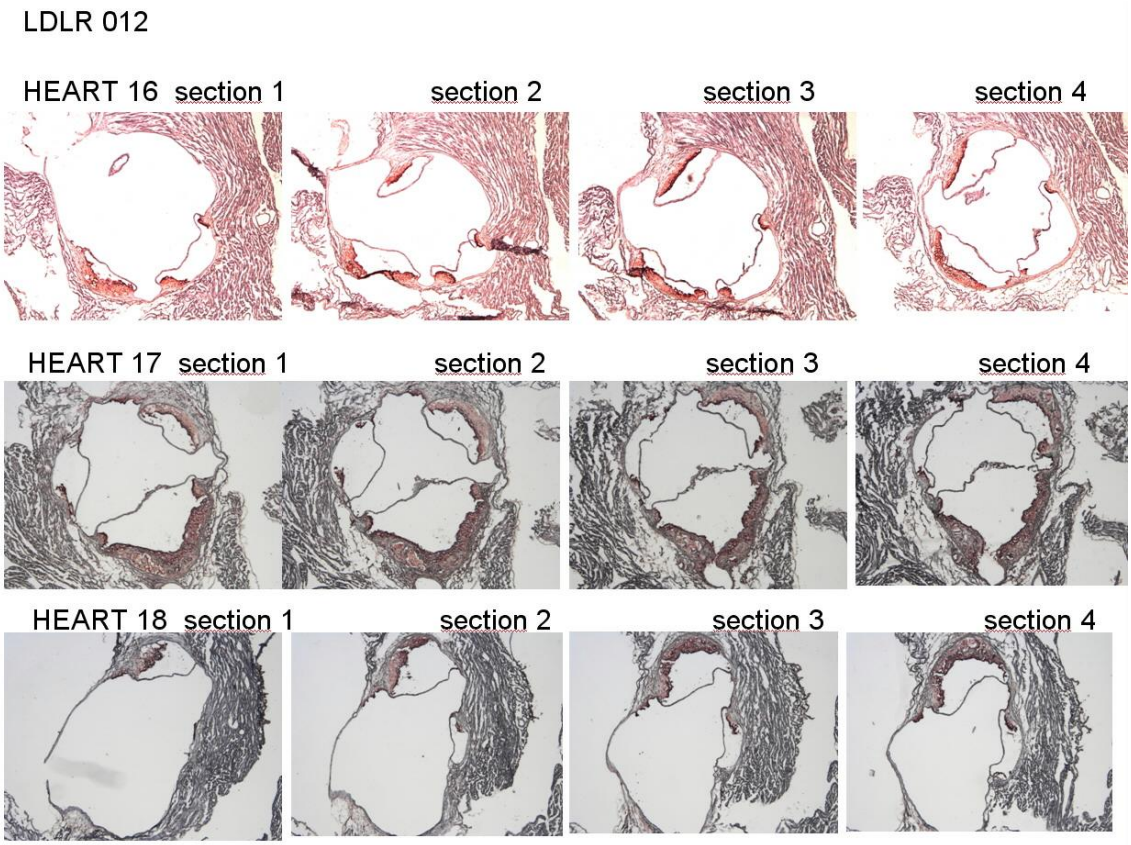

Fig. 10 Heart section 1-4 of LDLR deficient mice with high fat diet with 0,3% nicotinic acid

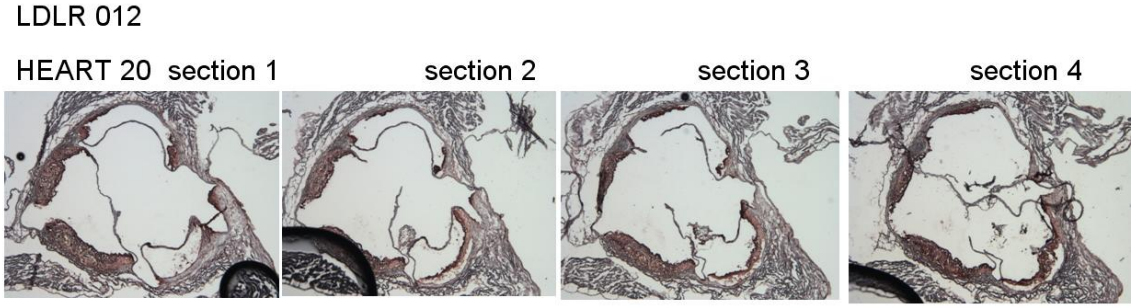

Fig. 11 Heart section 1-4 of LDLR deficient mice with high fat diet with 0,3% nicotinic acid

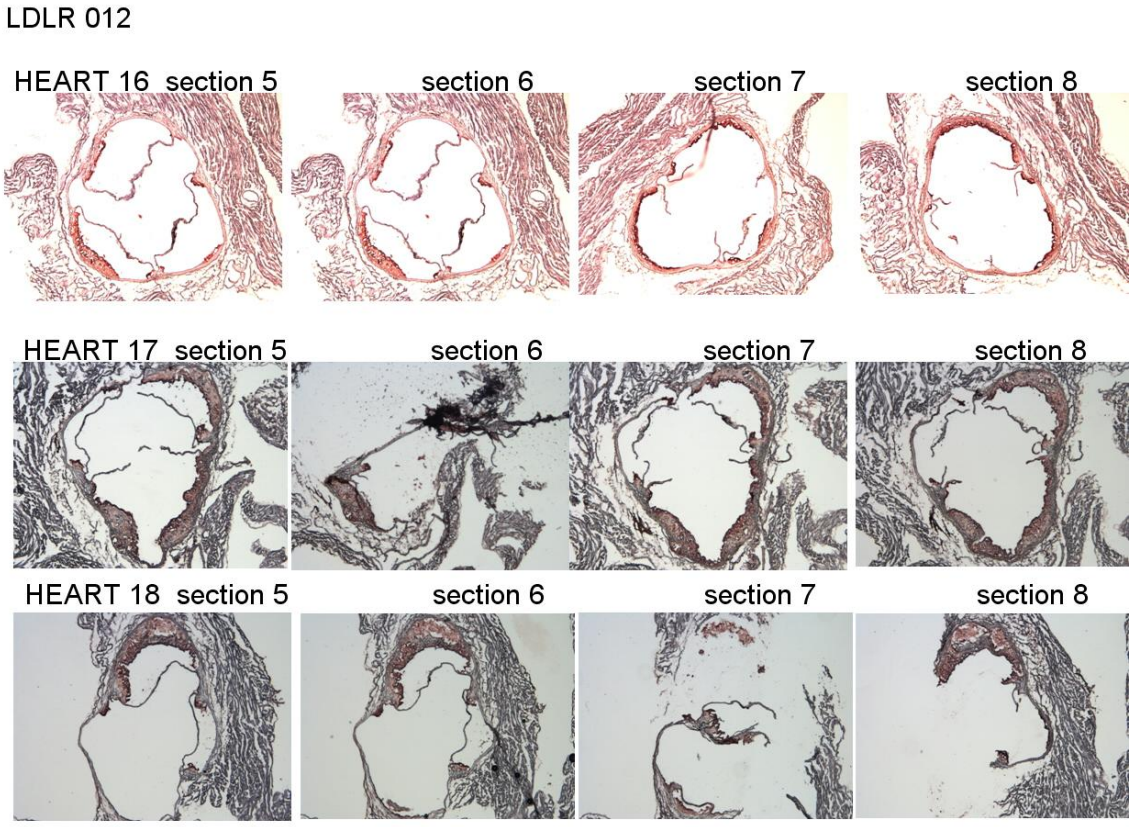

Fig. 12 Heart section 5-8 of LDLR deficient mice with high fat diet with 0,3% nicotinic acid

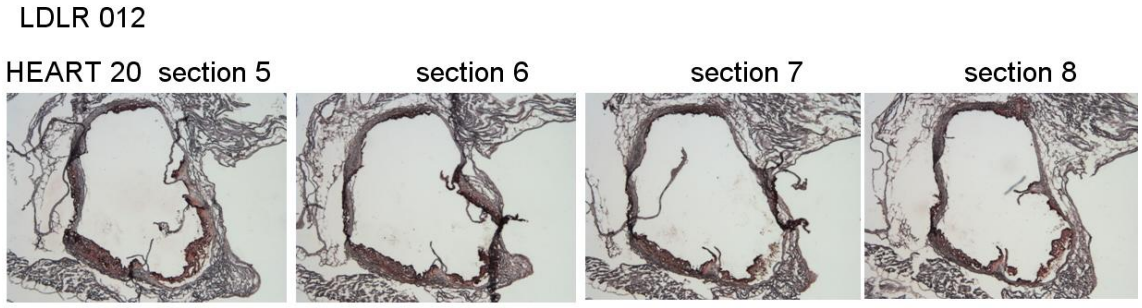

Fig. 13 Heart section 5-8 of LDLR deficient mice with high fat diet with 0,3% nicotinic acid

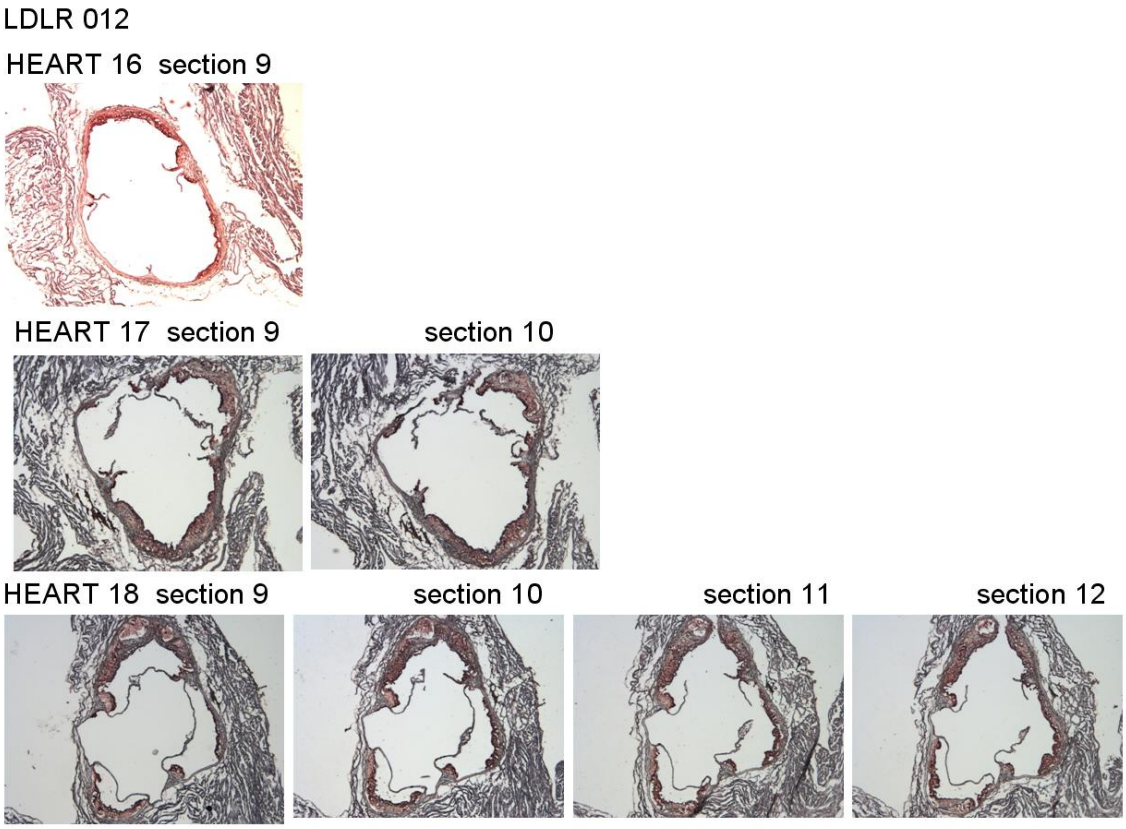

Fig. 14 Heart section 9-12 of LDLR deficient mice with high fat diet with 0,3% nicotinic acid

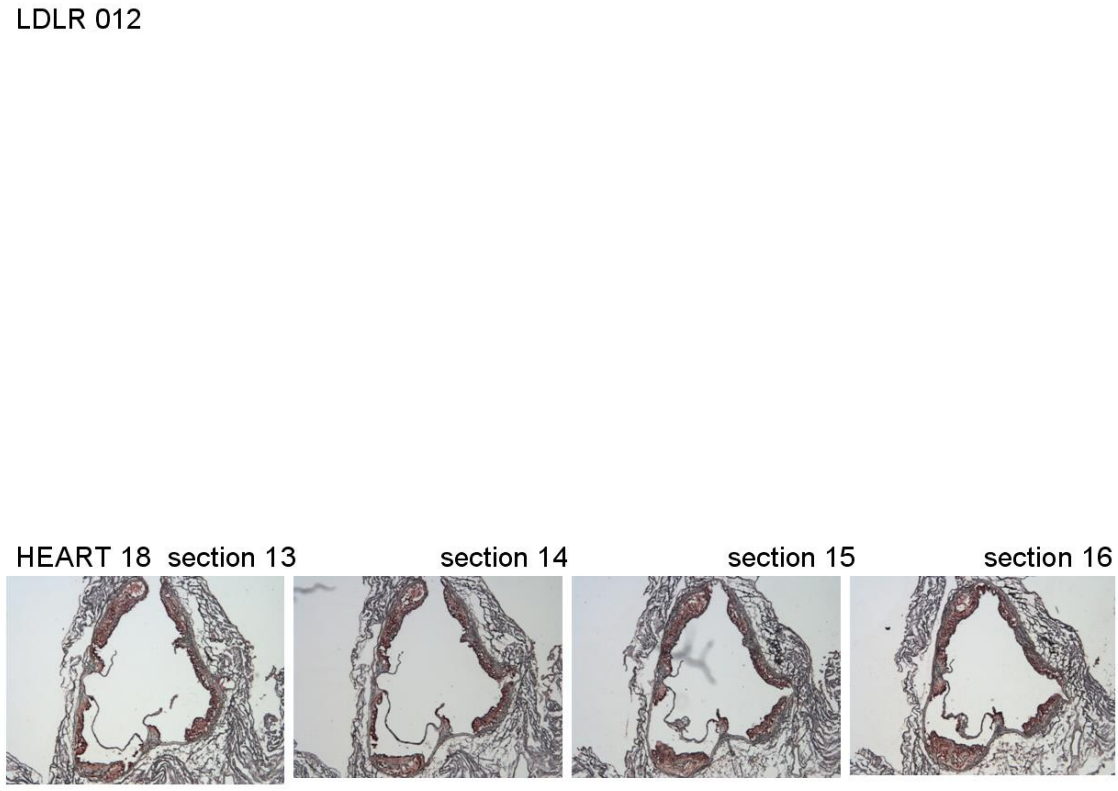

Fig. 15 Heart section 13-16 of LDLR deficient mice with high fat diet with 0,3% nicotinic acid

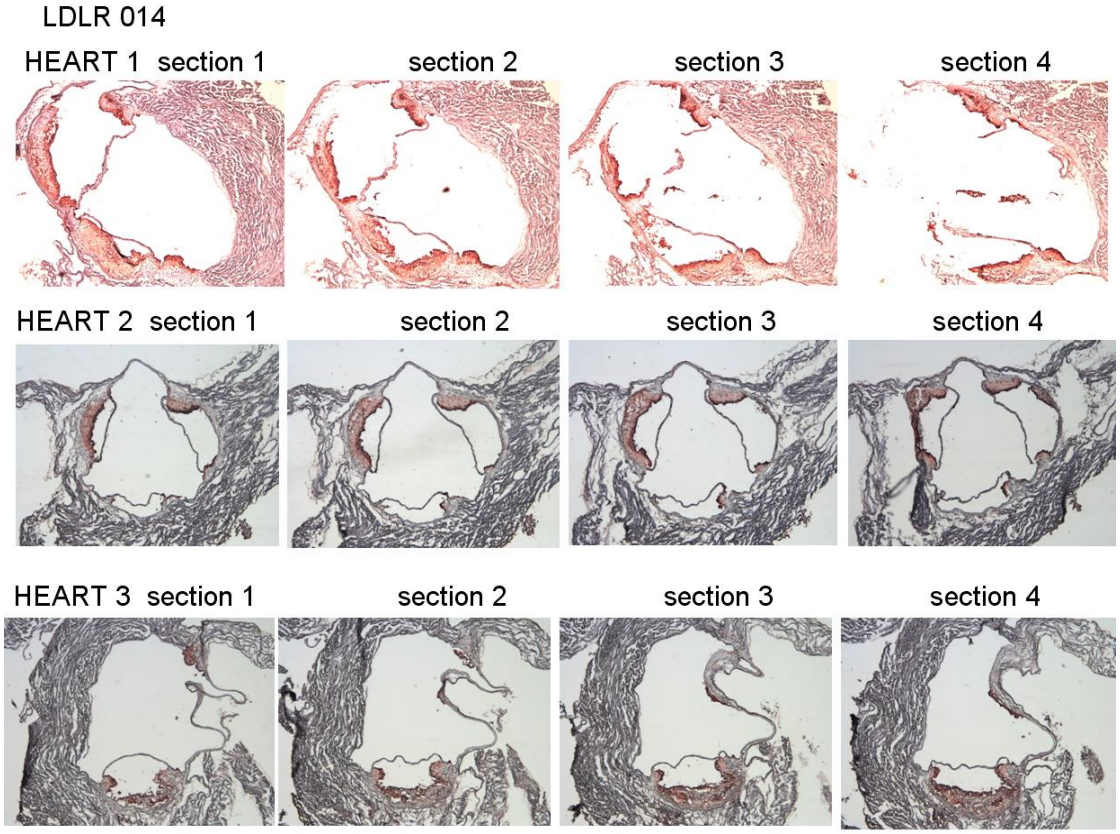

Fig. 16 Heart section 1-4 of LDLR PumaG double deficient mice e with high fat diet without nicotinic acid

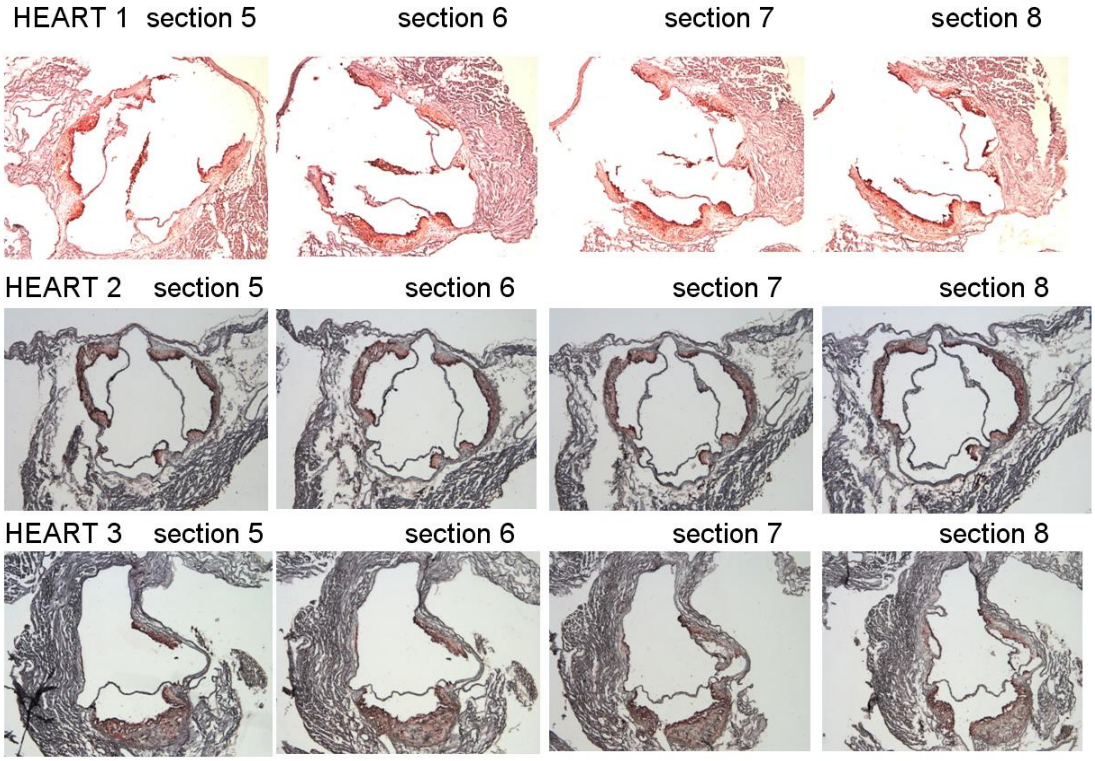

Fig. 17 Heart section 5-8 of LDLR PumaG double deficient mice e with high fat diet without nicotinic acid

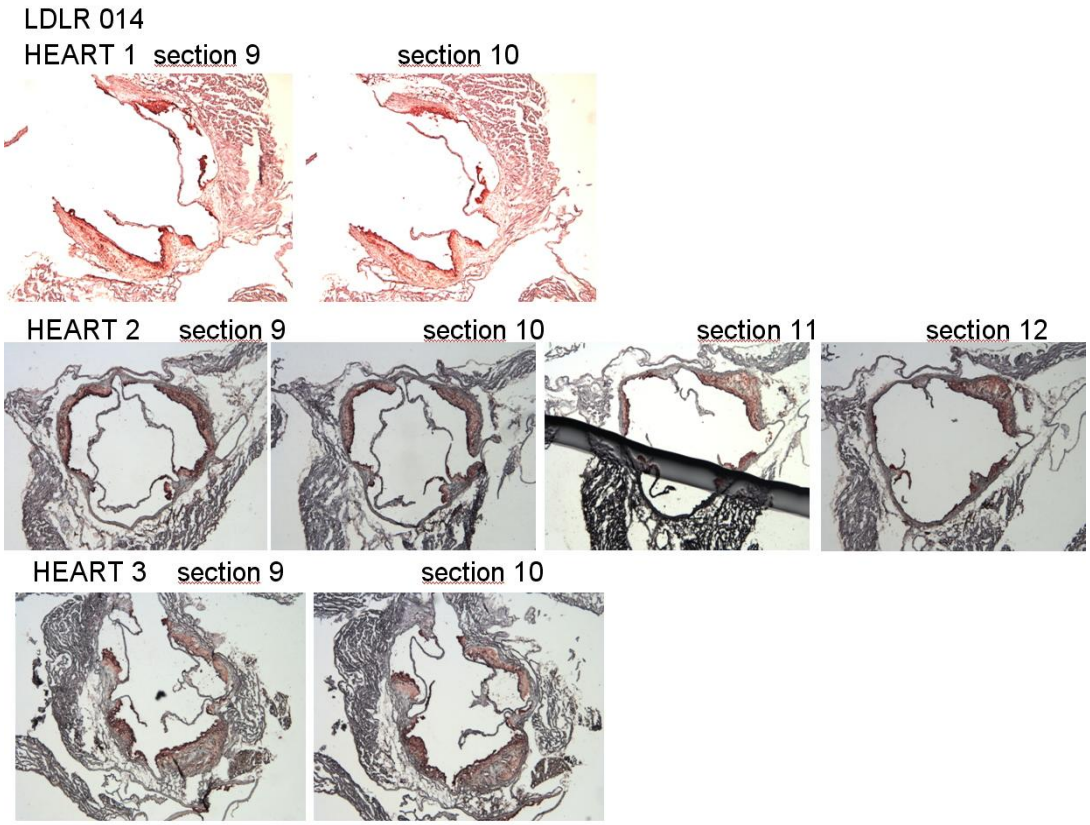

Fig. 18 Heart section 9-12 of LDLR PumaG double deficient mice e with high fat diet without nicotinic acid

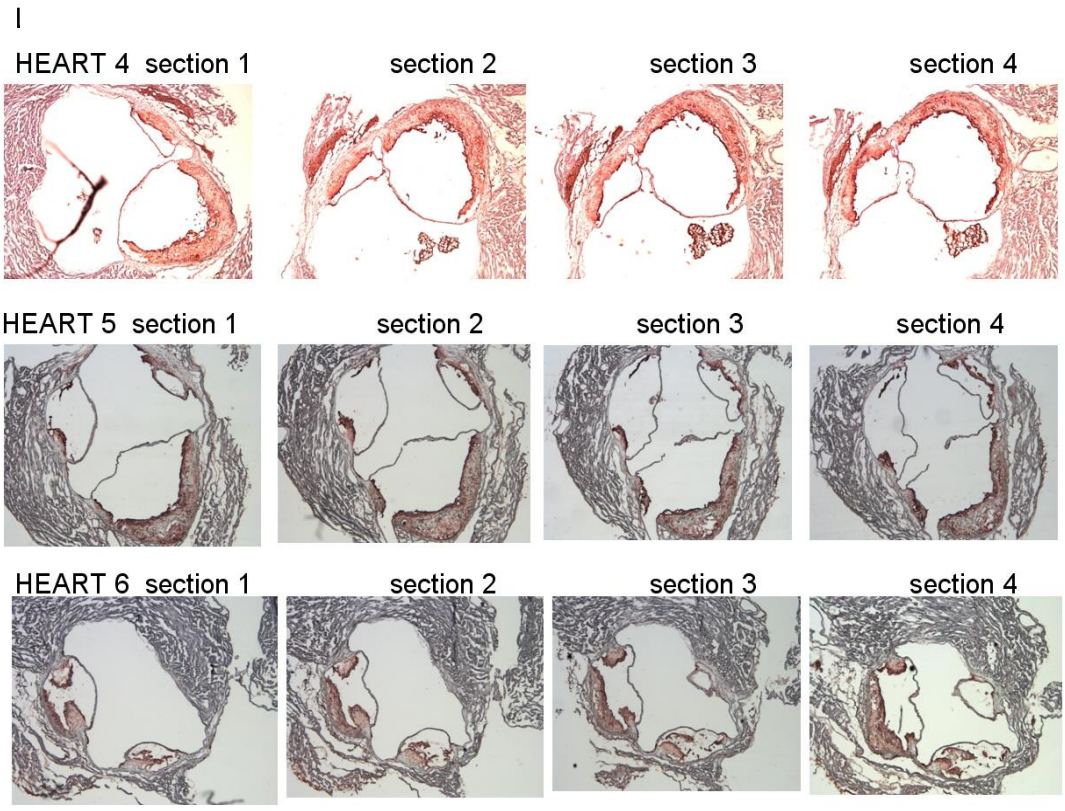

Fig. 19 Heart section 1-4 of LDLR PumaG double deficient mice e with high fat diet with 0,3% nicotinic acid

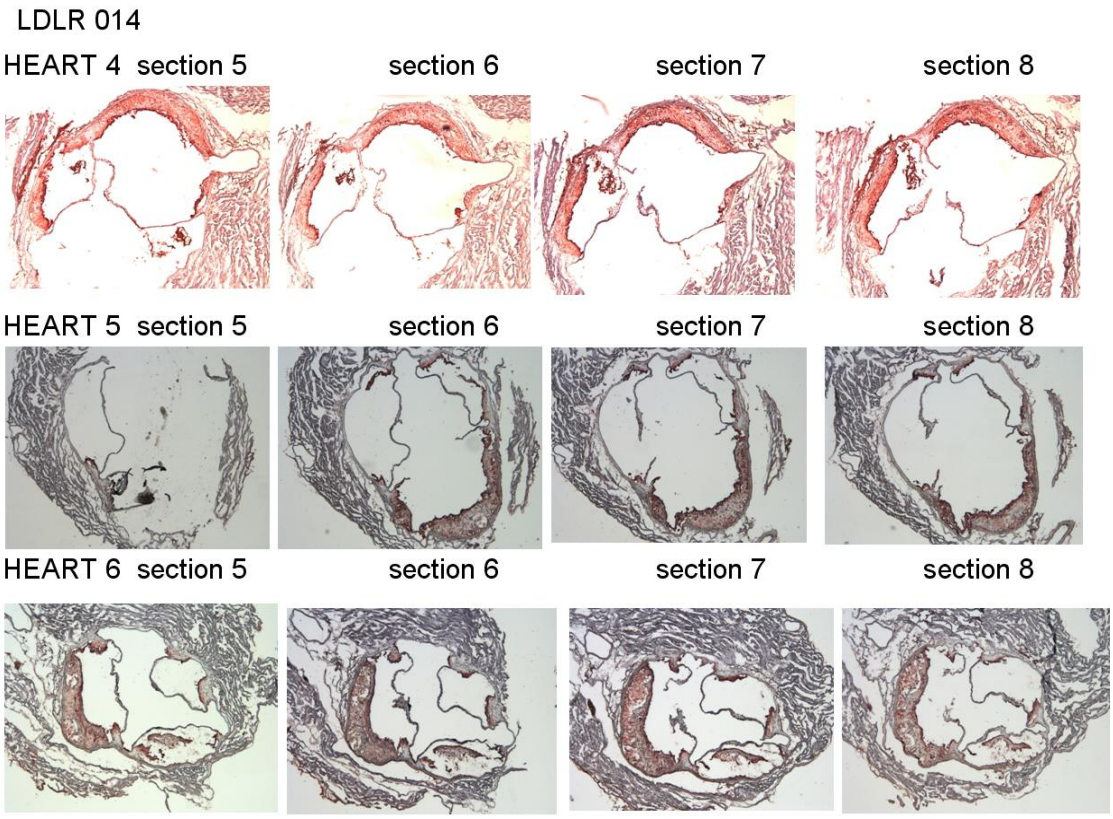

Fig. 20 Heart section 5-8 of LDLR PumaG double deficient mice e with high fat diet with 0,3% nicotinic acid

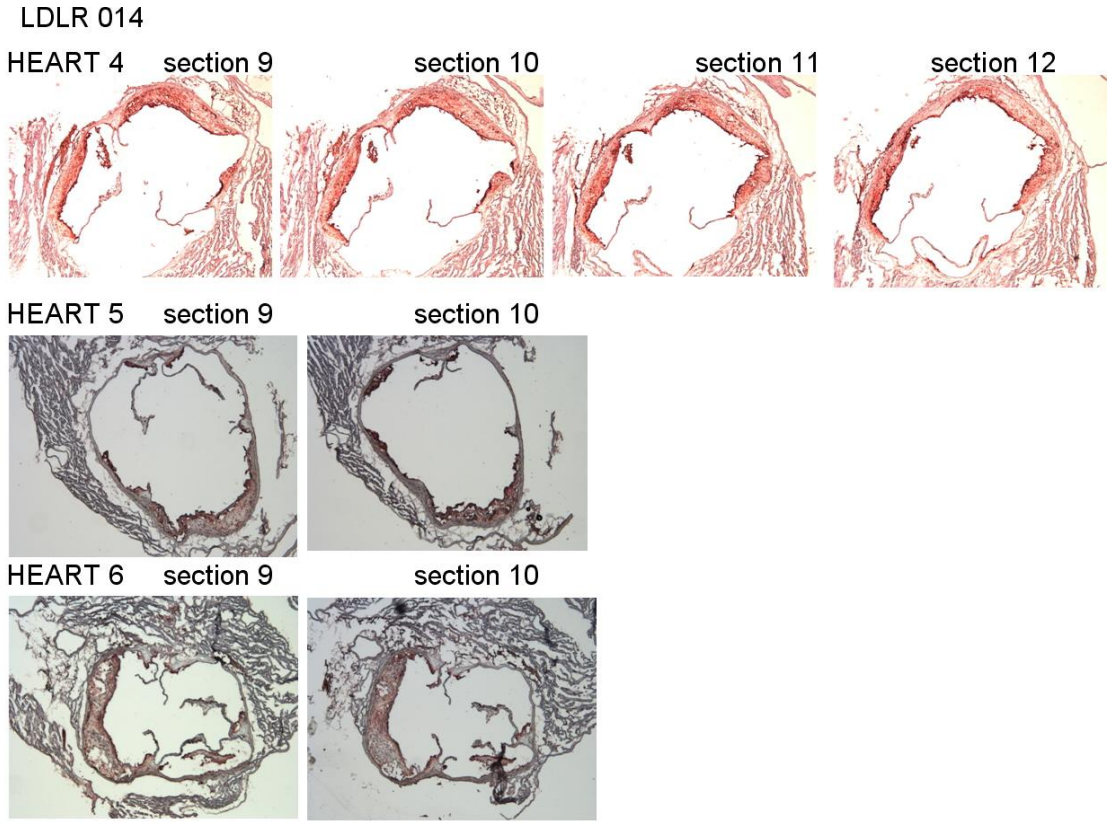

Fig. 21 Heart section 9-12 of LDLR PumaG double deficient mice with high fat diet without nicotinic acid

6.1.2. Quantification of atherosclerosis

	control	0.3% NA
LDLR	18.5	17.5
PumaG.LDLR	17.5	18.1

	Control	0.3% NA
LDLR	9.4	7.9
PumaG.LDLR	3.4	1.8

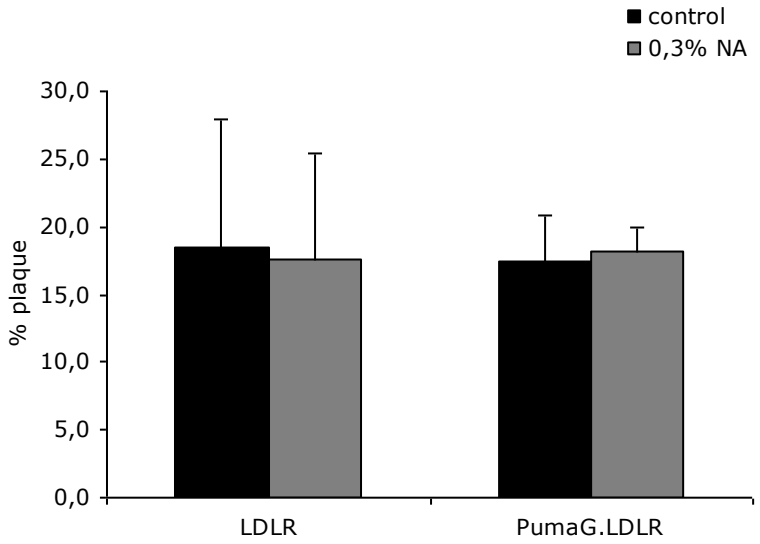

Fig. 22 Quantification of plaque all sections

First we evaluated the total area of plaque in the aortic valve at all slides and compared control group with the group, who were added nicotinic acid in the drinking water and then red stained macrophages in the plaque.

	control	0.3% NA
LDLR	29.3	28.9
PumaG.LDLR	27.3	24.4

	Control	0.3% NA
LDLR	10.5	6.0
PumaG.LDLR	2.5	2.9

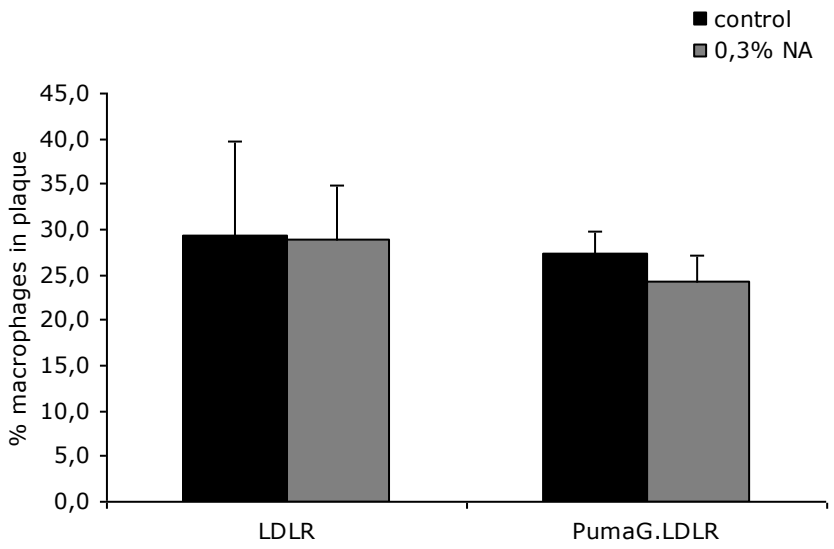

Fig. 23 Quantification of macrophages all sections

As one can see from the diagrams (Fig.22, Fig.33) as expected, unfortunately we didn't observe the difference between untreated group (control) and treated group with 0.3% nicotinic acid, which is ultimately not so surprising because between both groups we didn't see neither difference between the plaque.

In the summary this experiment the groups of interest were too small and it is to see higher values of standard deviation. The experiment should be repeated with a larger number of mice in group.

6.2 In vitro experiments

6.2.1 Cell smear

BLOOD CELLS

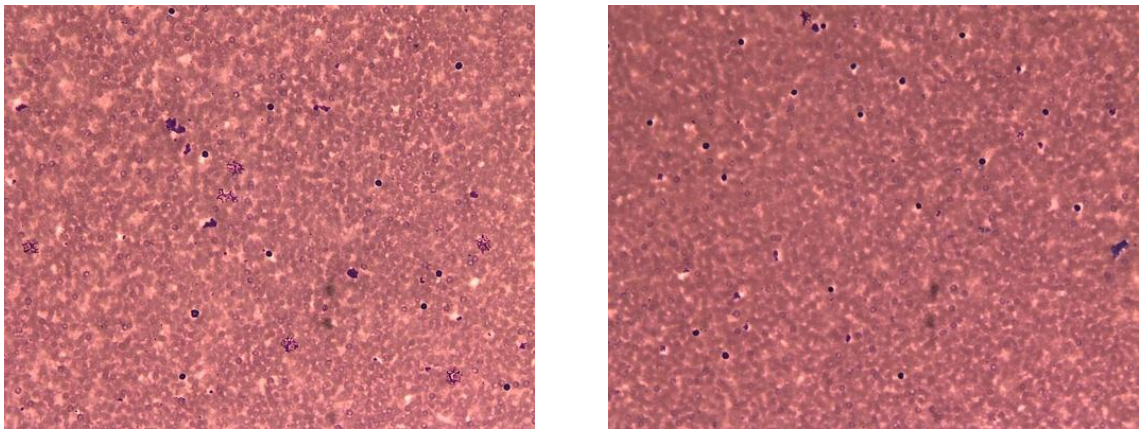

Fig. 24 The effect of blood cells obtained per eye, the retro-orbital bleeding from wildtype mice, directly stained with Diff- Quick staining.

Blood cells (Fig.24) obtained per eye, the retroorbital bleeding placed on glass slides were stained with Diff Quick method as described 5.2.3. There occurred number of: Erythrocytes, Lymphocytes, Monocytes, Neutrophils, Basophils, Eosinophils and Platelets.

When we changed conditions for example. without staining, erythrocyte lysis, we have never seen positive fluorescence. Result was no expression of GPR109A in blood.

To determine the optimal timing for imaging fluorescence images were obtained maximal one hour past staining and protected before light.

PERITONEAL EXUDATE CELLS

Peritoneal macrophages 400x magnification (Fig. 25- Fig.29)

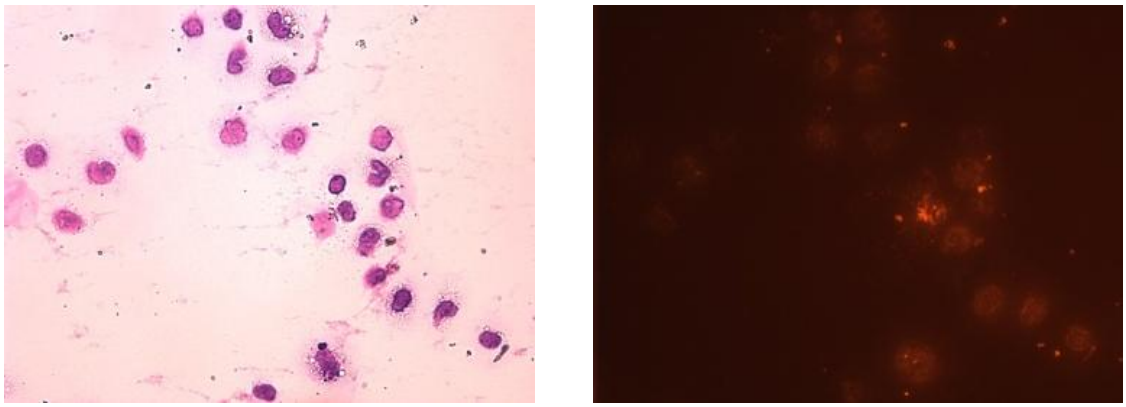

Fig. 25 The effect of peritoneal exudates cells obtained from knockout mice PU- REP, which was 4 days before injected with 4% thioglycolate i.p. to evoke inflammation. This figure shows positive fluorescence after thioglycolate stimulation.

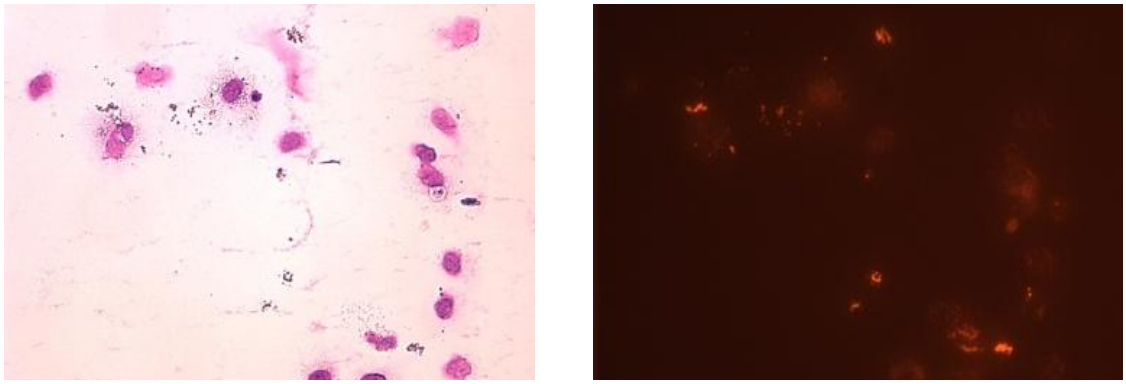

Fig. 26 The effect of peritoneal exudates cells obtained from knockout mice Puma-reporter, which was 4 days before injected with 4% thioglycolate i.p. to evoke inflammation. This figure shows positive fluorescence after thioglycolate stimulation.

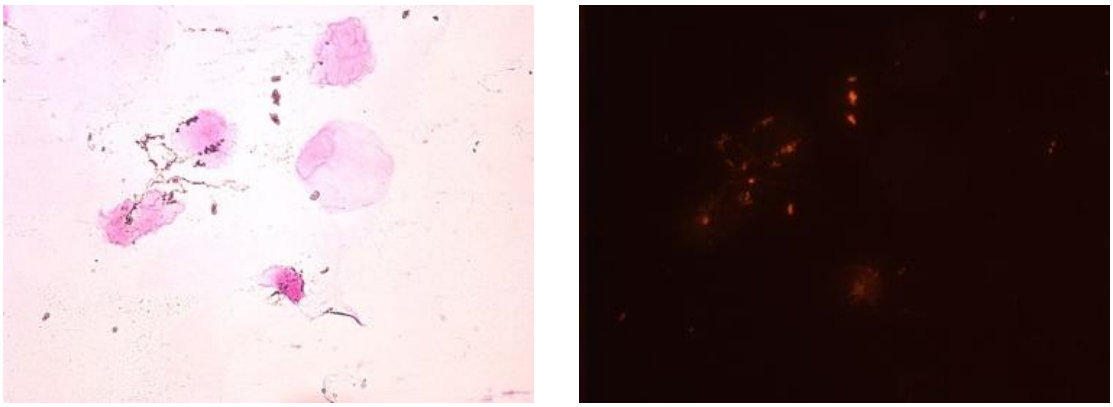

Fig. 27 The effect of peritoneal exudates cells obtained from knockout mice Puma-reporter, which was 4 days before injected with 4% thioglycolate i.p. to evoke inflammation. This figure shows positive fluorescence after thioglycolate stimulation.

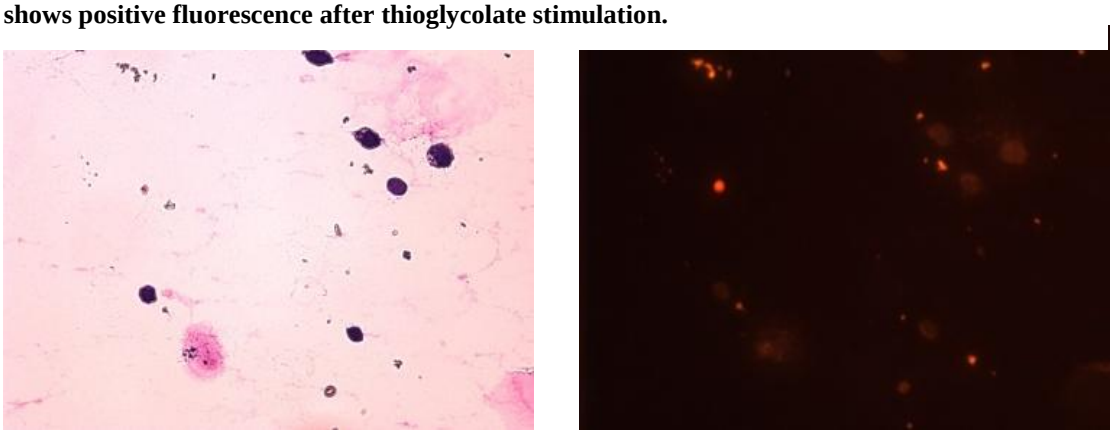

Fig. 28 The effect of peritoneal exudates cells obtained from knockout mice Puma-reporter, which was 4 days before injected with 4% thioglycolate i.p. to evoke inflammation. This figure shows positive fluorescence after thioglycolate stimulation.

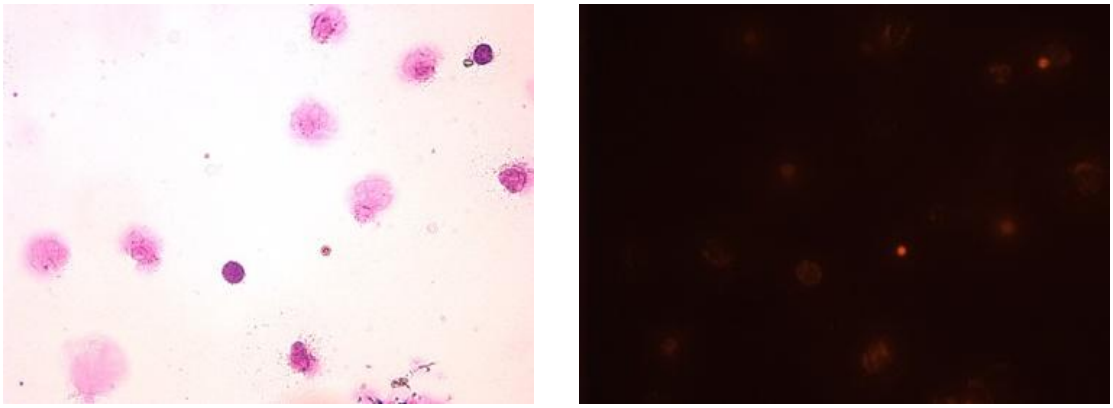

Fig. 29 The effect of peritoneal exudates cells obtained from knockout Puma-reporter mice, naïve without inflammation. This figure shows less expressed cells as during inflammatory state.

As one can see from the picture above (Fig. 25- Fig.29) gene GPR109A is expressed in peritoneal exudates, by inflammation state are more cells expressed as shown fluorescence microscopy.

Blood .NEUTROPHILS

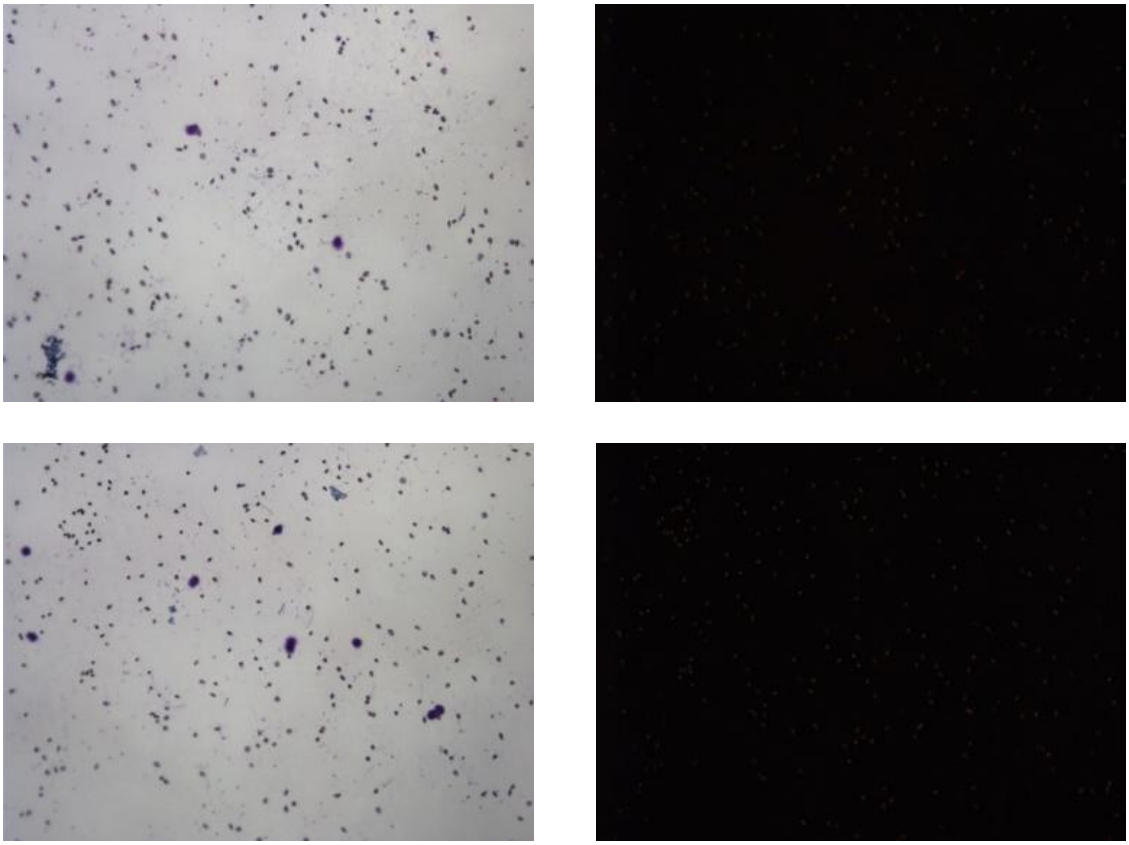

Fig. 30 Blood neutrophils obtained from Puma-reporter mice injected with casein solution to evoke inflammation. On the left side evaluating under microscope by 200x magnification, in the right side under fluorescence, no expression of gene GPR109A.

The neutrophils were prepared as described in the Methods 5.2.5 and stained with Diff- Quick staining, subsequently. The cells were observed directly under a fluorescence microscope. As one can see from the picture (Fig.30) this data show no expression of gene GPR109A in the blood.

6.2.2 Calcium measurement

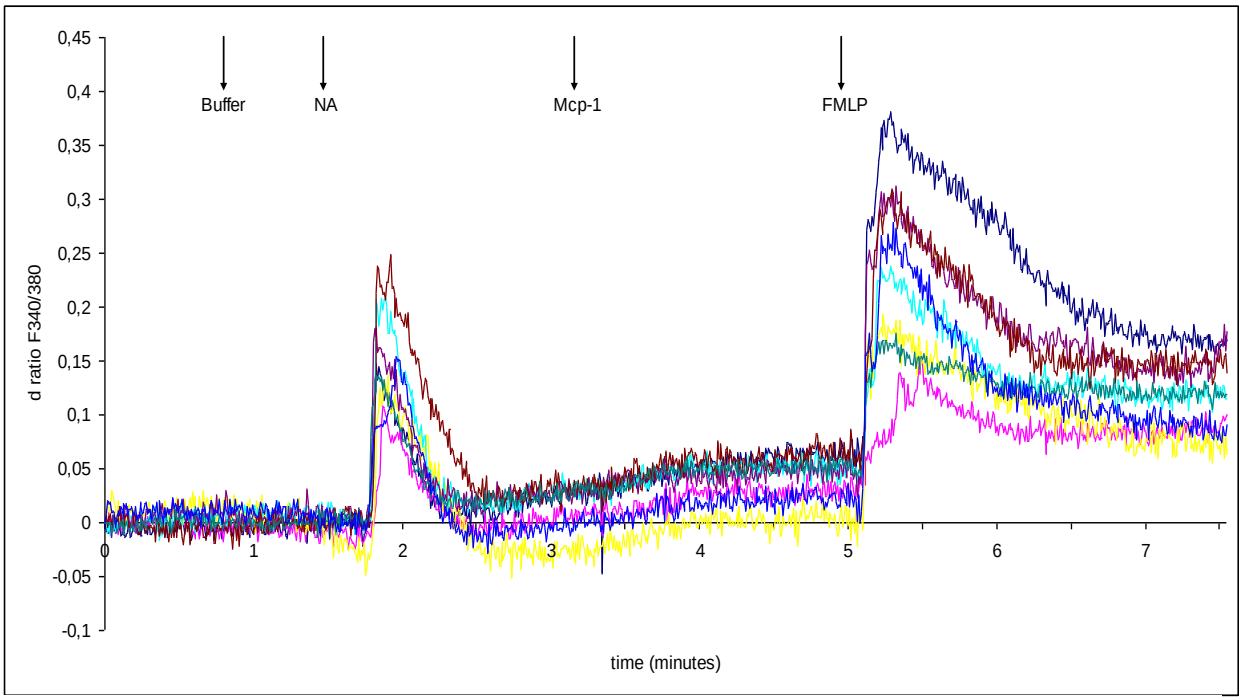

Fig. 31 The effect of 100µM nicotinic acid and 10nM macrophage/ monocyte chemotactic protein-1, subsequently on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The Fura-2 interacted with cells 45 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. Nicotinic acid increased intracellular calcium by all marked cells, no changes past adding mcp-1.

The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The measurement was performed in 6-well plate. Individual cover slips were placed into chamber heated on 37°C with 600 µl HBSS medium contains Ca ions.

Negative control experiments were performed using buffer, positive control experiments using FMLP.

Values on the x axis indicate agonist concentration and values on the y axis indicate the measured 340/380 nm fluorescence ration as an indicator for the intracellular-free $[Ca^{2+}]_{i.}$

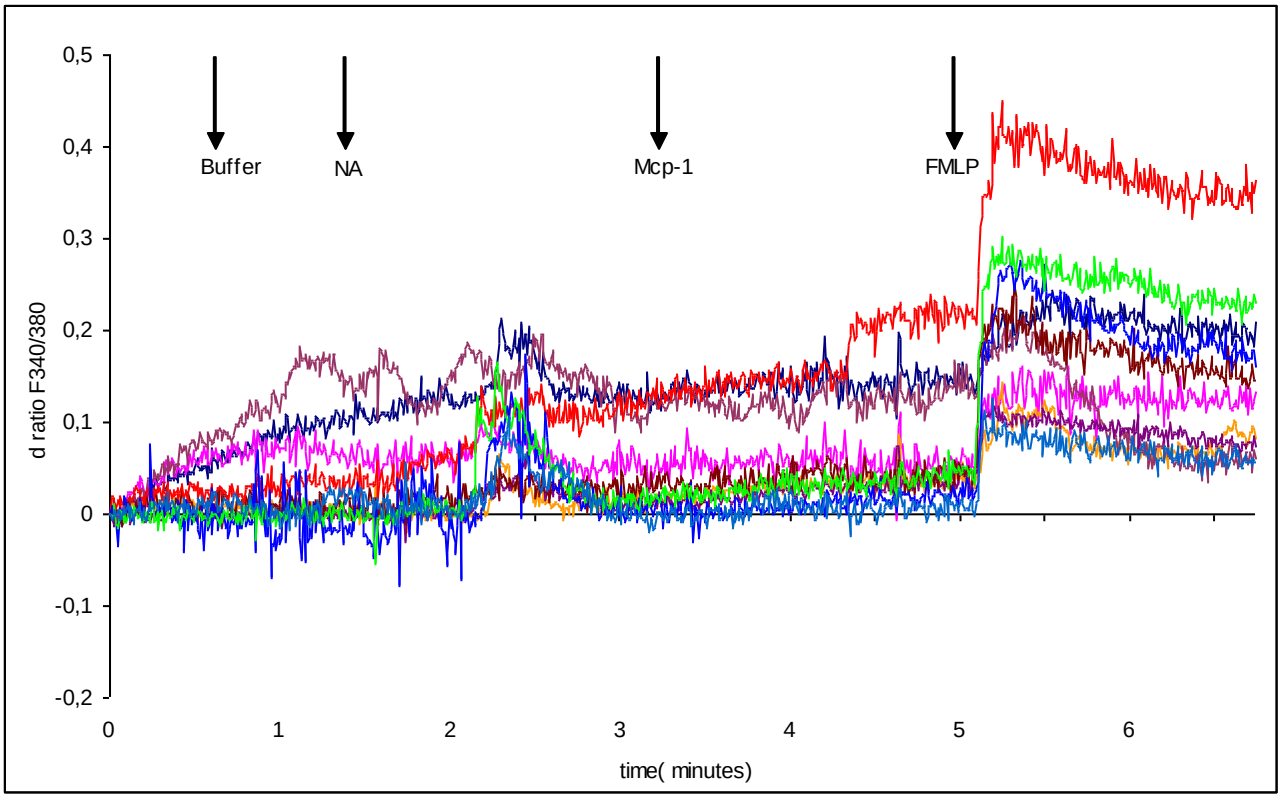

Fig. 32 The effect of 100µM nicotinic acid and 10nM macrophage/ monocyte chemotactic protein-1, subsequently on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 90 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. All cells marked cells reacted on positive control.

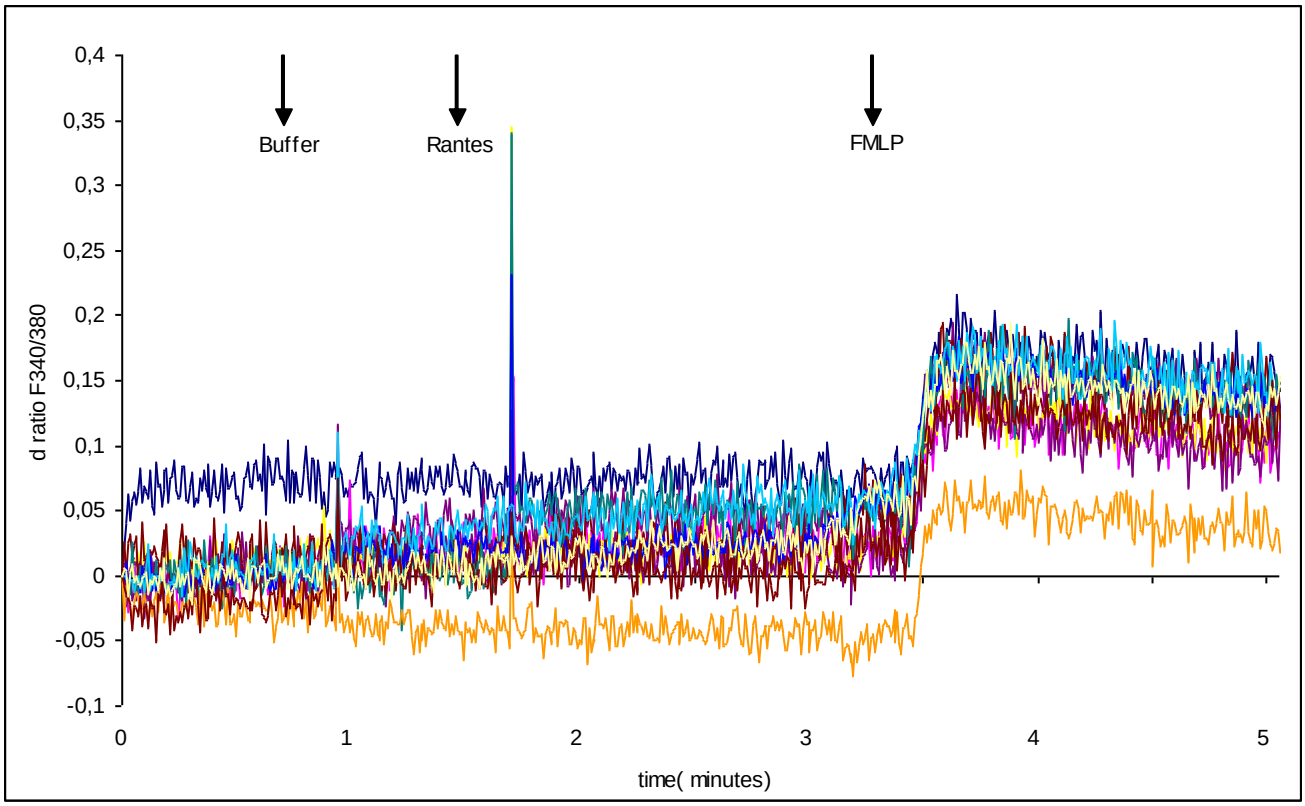

Fig. 33 The effect 1 ng/ml RANTES on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 60 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. All marked cells reacted on positive control, no one on chemokine RANTES; in this figure is quiet large noise.

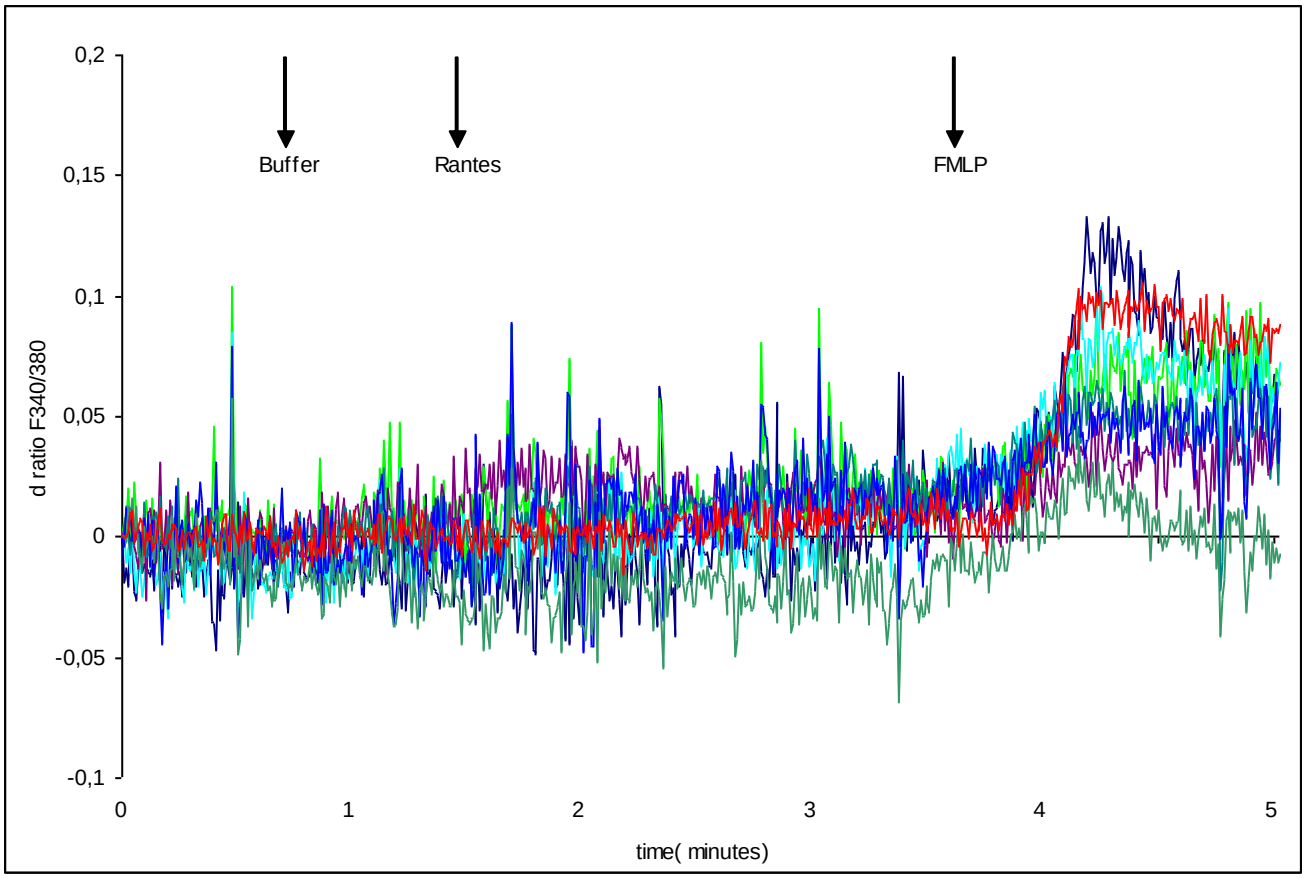

Fig. 34 The effect of 1 ng/ml RANTES on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 70 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. In this figure is opinion of reaction on positive control, but the noise is large and cells are not detectable.

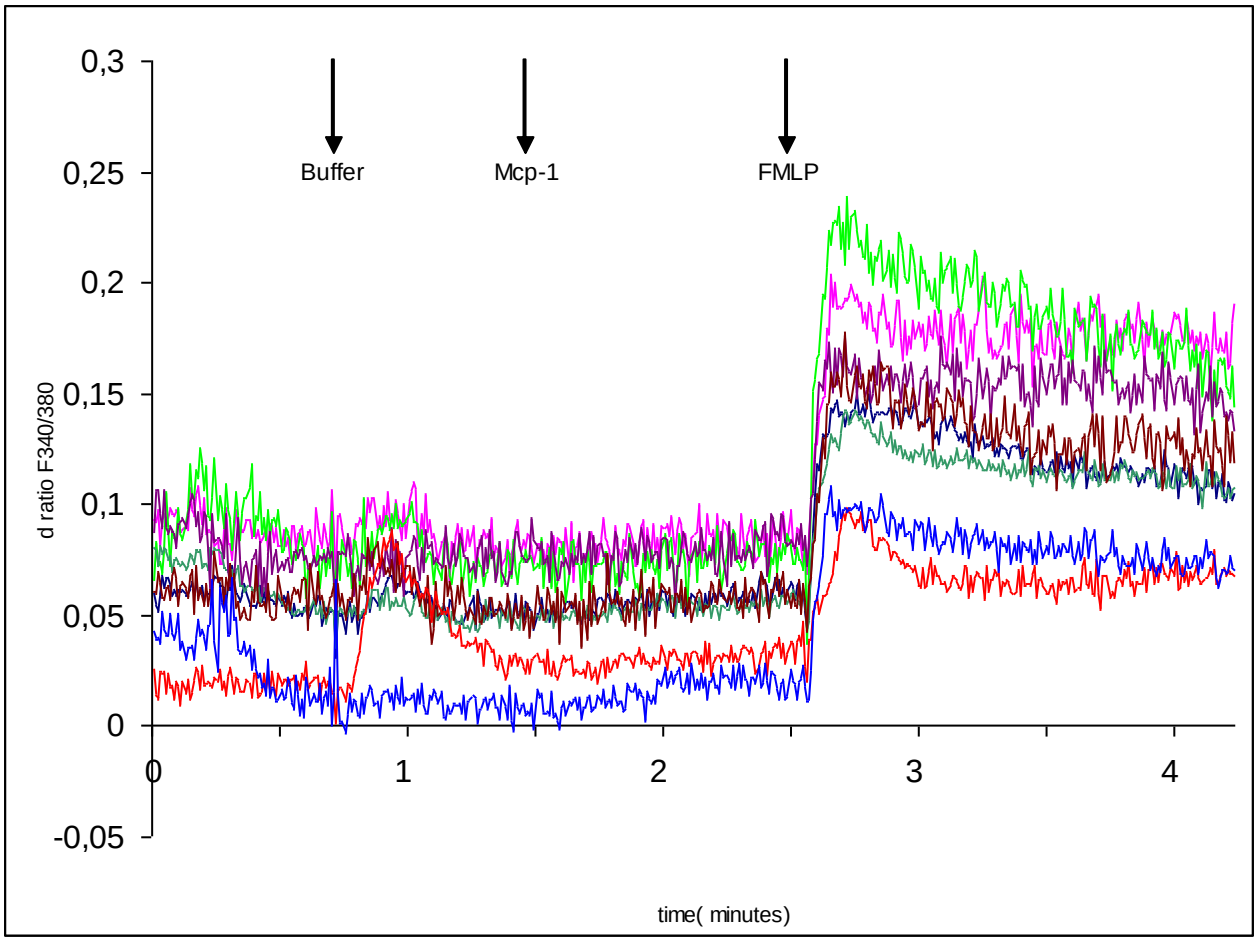

Fig. 35 The effect of 10nM macrophage/ monocyte chemotactic protein-1 on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 70 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. All cells marked cells reacted on positive control, no one cell reacted on chemokine mcp-1. The red line could not be taken for detectable because gives response on negative control.

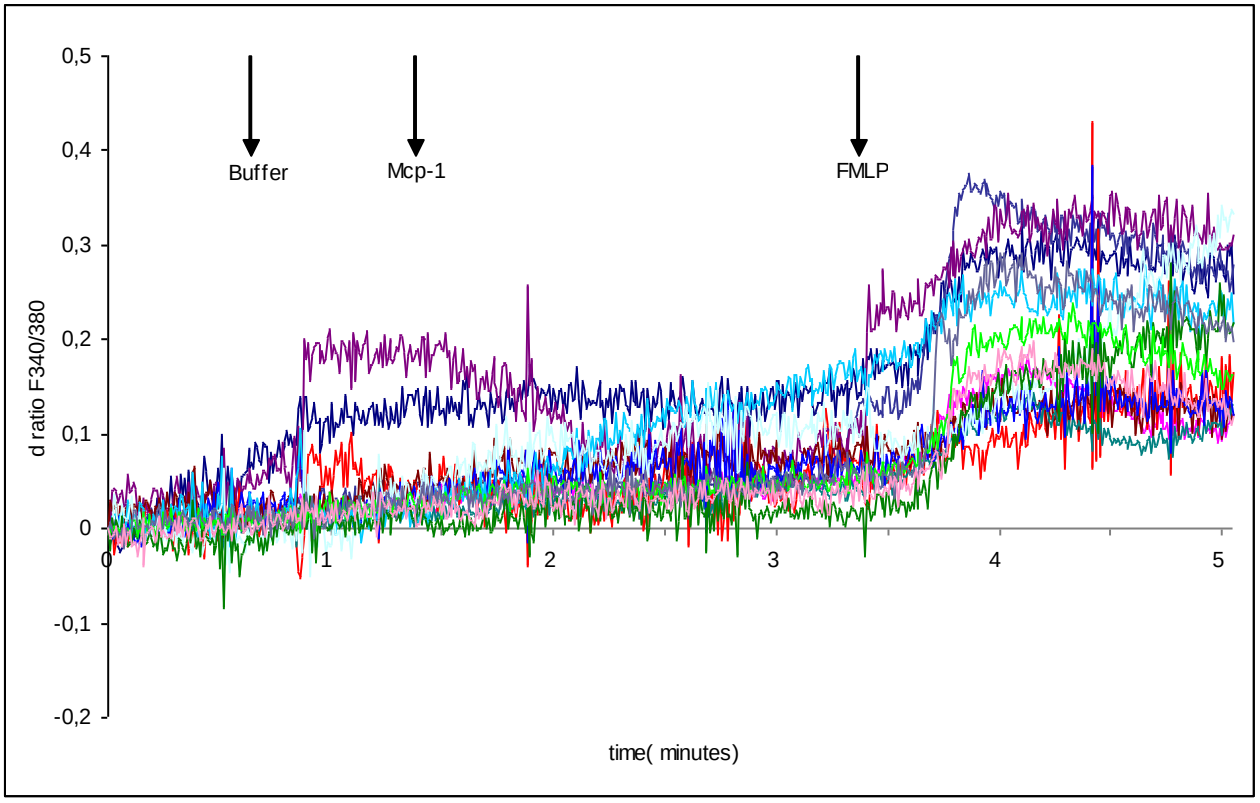

Fig. 36 The effect 10nM macrophage/ monocyte chemotactic protein-1 on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 90 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. Most of marked cells reacted on positive control.

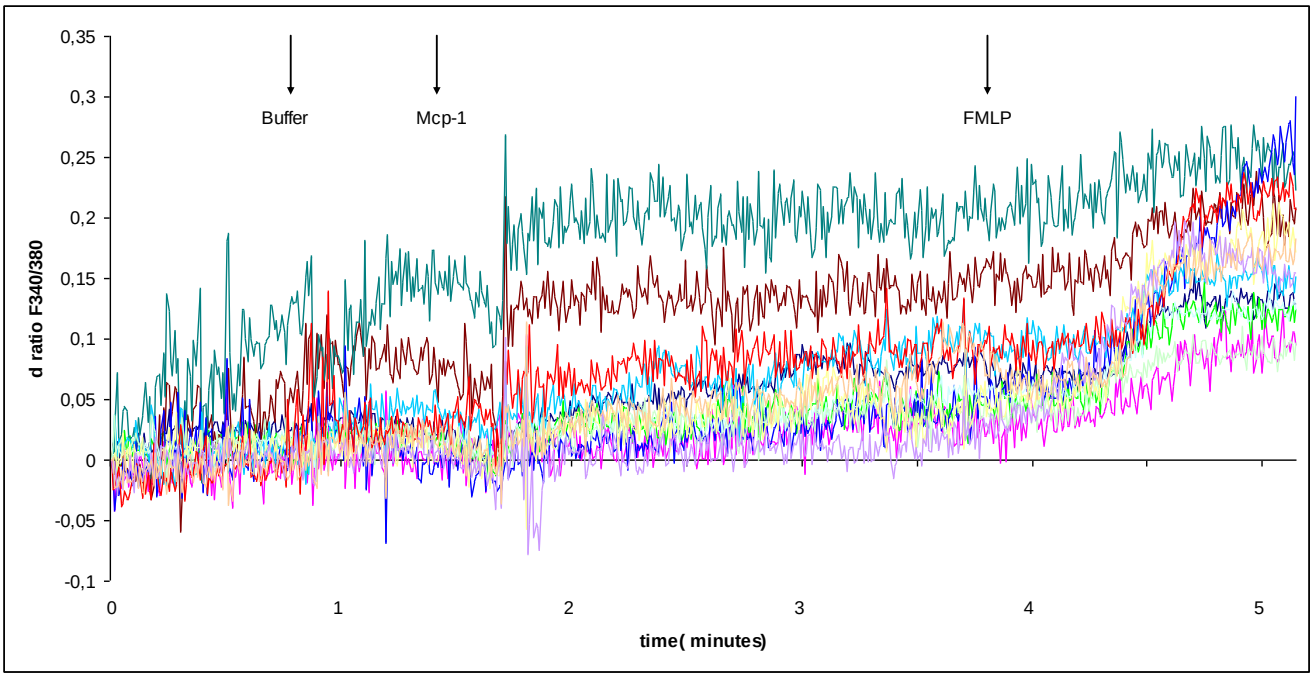

Fig. 37 The effect of 10nM macrophage/ monocyte chemotactic protein-1 on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 80 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. All marked cells didn't give response on positive control, it is possible they were contaminated or death during measurement.

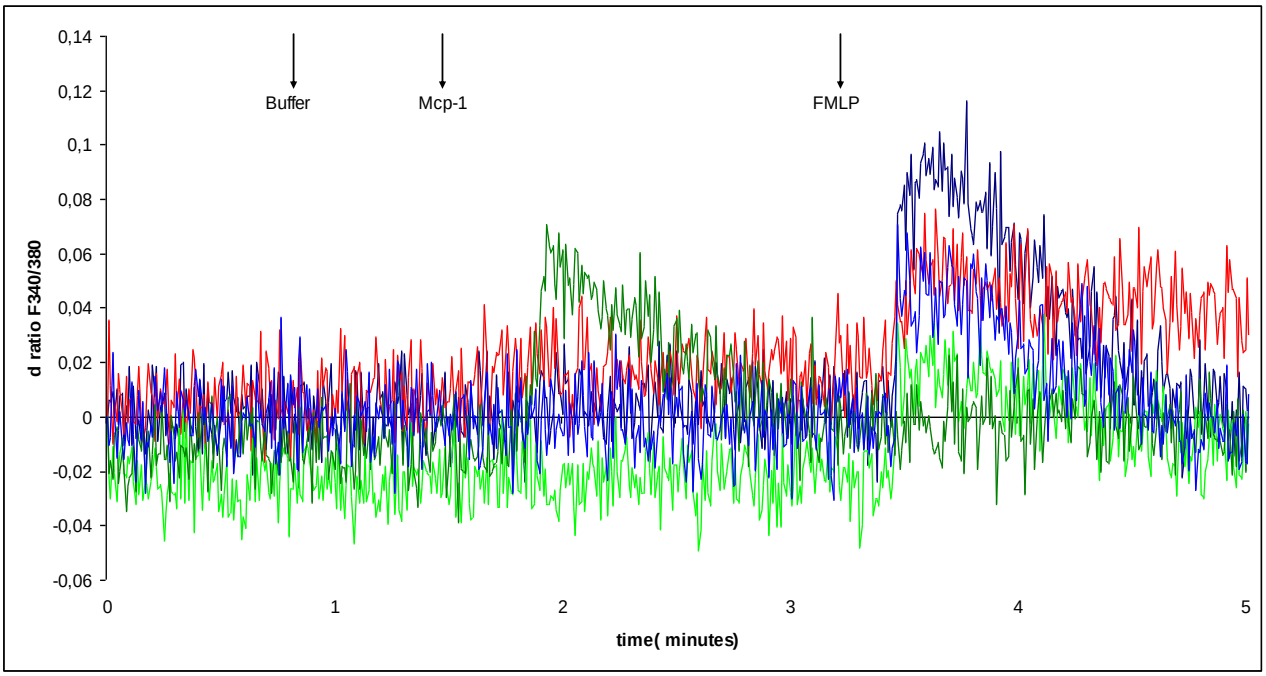

Fig. 38 The effect of 10nM macrophage/ monocyte chemotactic protein-1 on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 3 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 45 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. The cell- dark green line responded on mcp-1, but didn't react on positive control, other cells reacted on positive control, and the noise is also large.

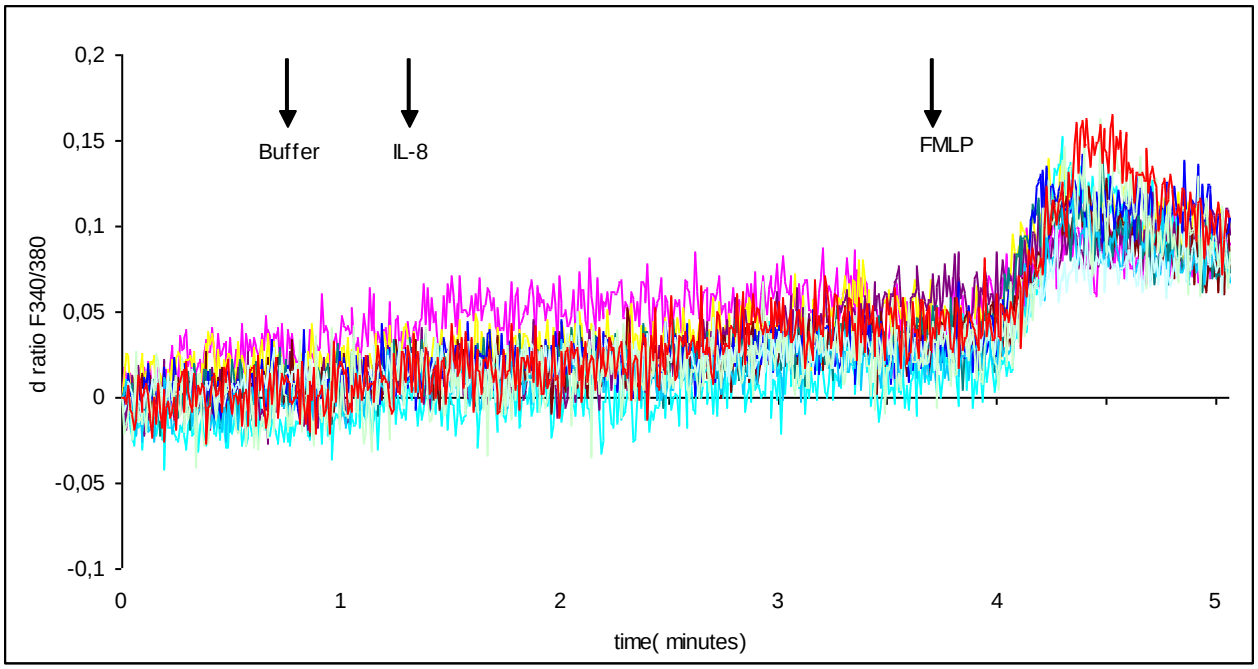

Fig. 39 The effect of 1µg/ml Interleukin-8 on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 3 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 55minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. All marked cells responded on positive control, no one on chemokine interleukin-8.

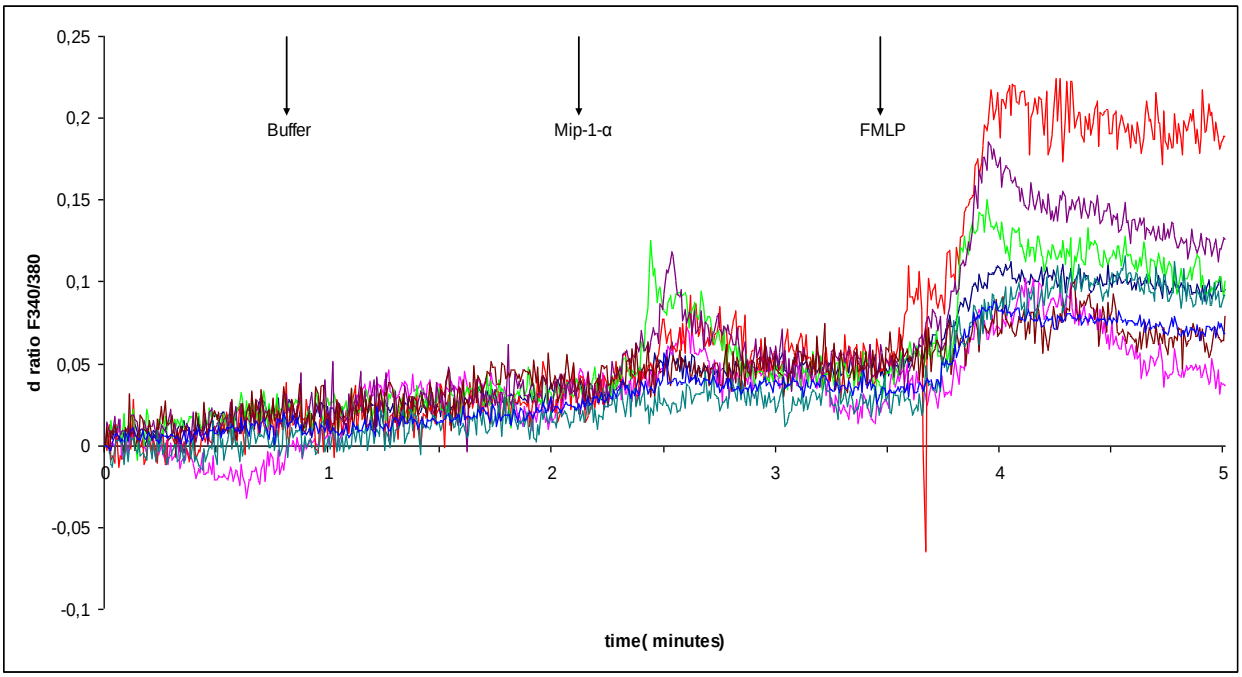

Fig. 40 The effect of 500ng/ml macrophage inflammatory protein-1-α on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 3 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 80minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. The several number of cells increased concentration of intracellular calcium past adding mip-1-α and also reacted on positive control.

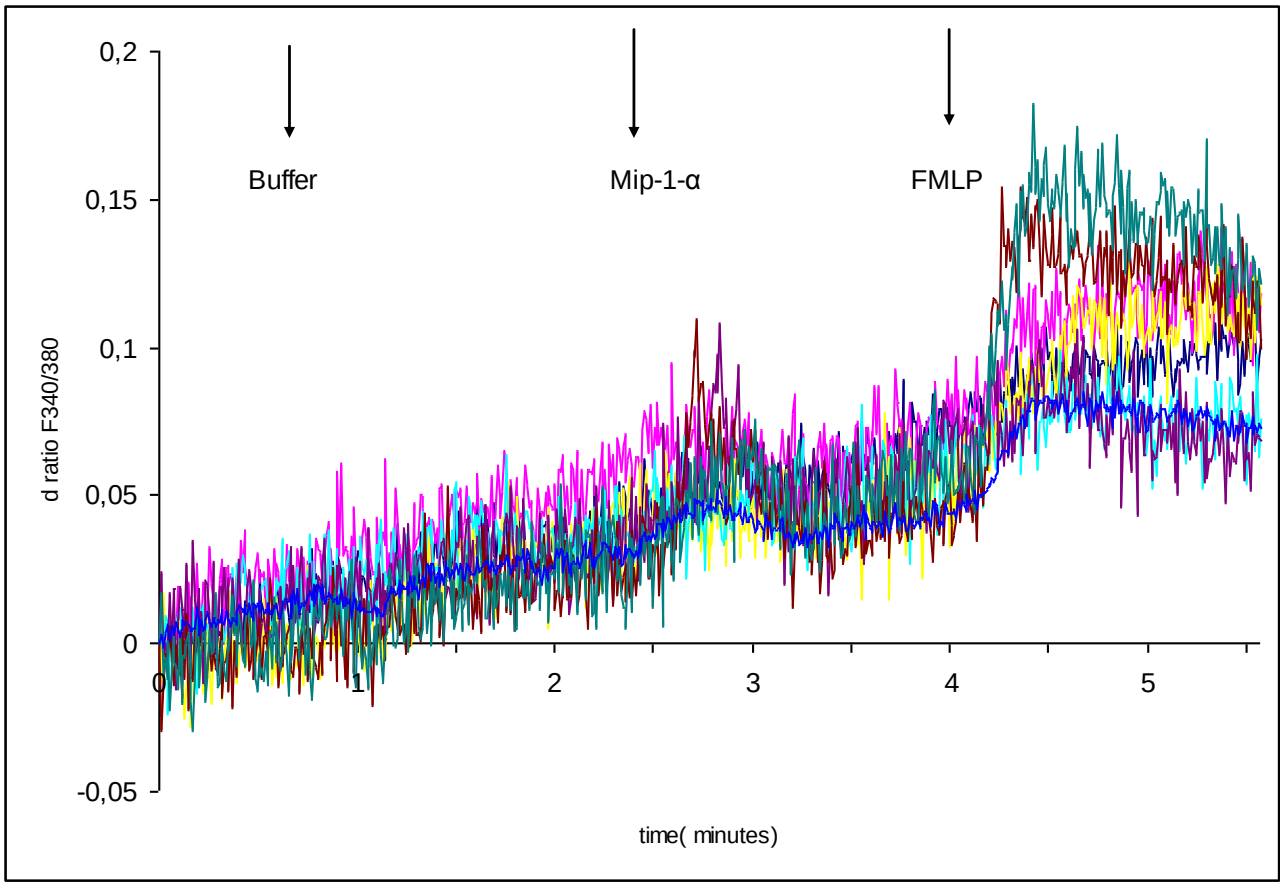

Fig. 41 The effect of 500 ng/ml macrophage inflammatory protein-1-α on calcium signal in Fura-2-loaded peritoneal exudates cells from wildtype mice, which was 3 days injected with 4% thioglycolate to induce inflammation. The cells were loaded in medium containing 20nM GM-CSF overnight to enhance their adherence. The Fura-2 interacted with cells 90 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. All marked cells reacted on chemokine mip-1-α and also on positive control.

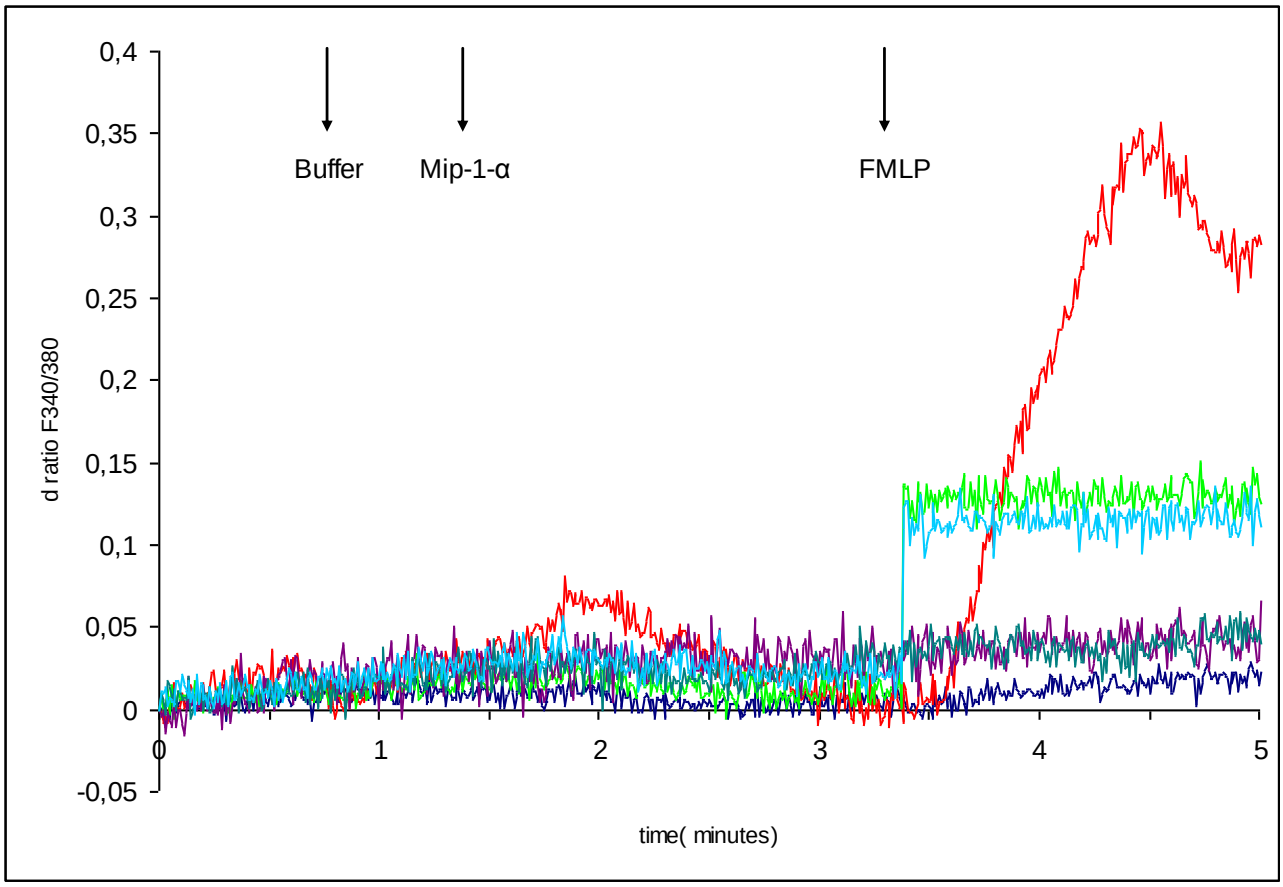

Fig. 42 The effect of 500ng/ml macrophage inflammatory protein-1-α on calcium signal in Fura-2-loaded THP-1 cell lines. The cells were loaded in medium containing 20nM GM-CSF for 6hours to enhance their adherence. The Fura-2 interacted with cells 80 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. One cells- the red line reacted on chemokine mip-1-α and also on positive control.

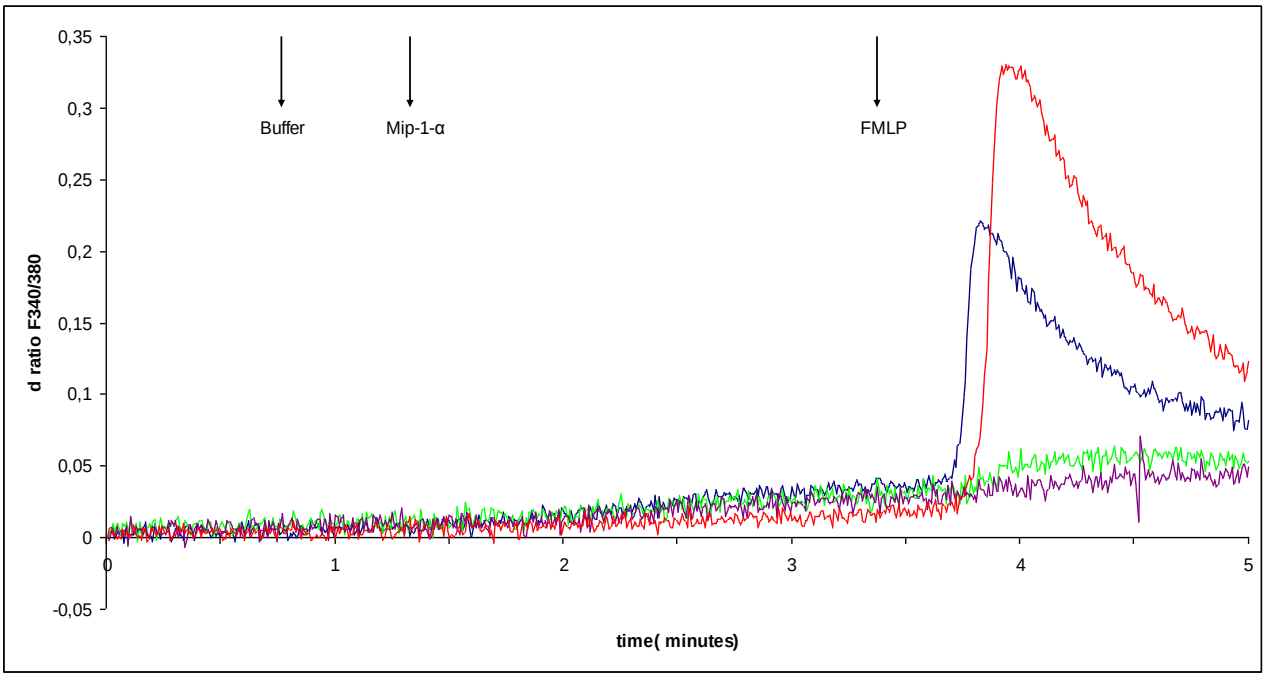

Fig. 43 The effect of 500 ng/ml macrophage inflammatory protein-1-α on calcium signal in Fura-2-loaded THP-1 cell lines. The cells were loaded in medium containing 20nM GM-CSF for 6hours to enhance their adherence. The Fura-2 interacted with cells 95 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. No cells reacted on chemokine mip-1-α and two of them increased intracellular concentration of calcium on positive control.

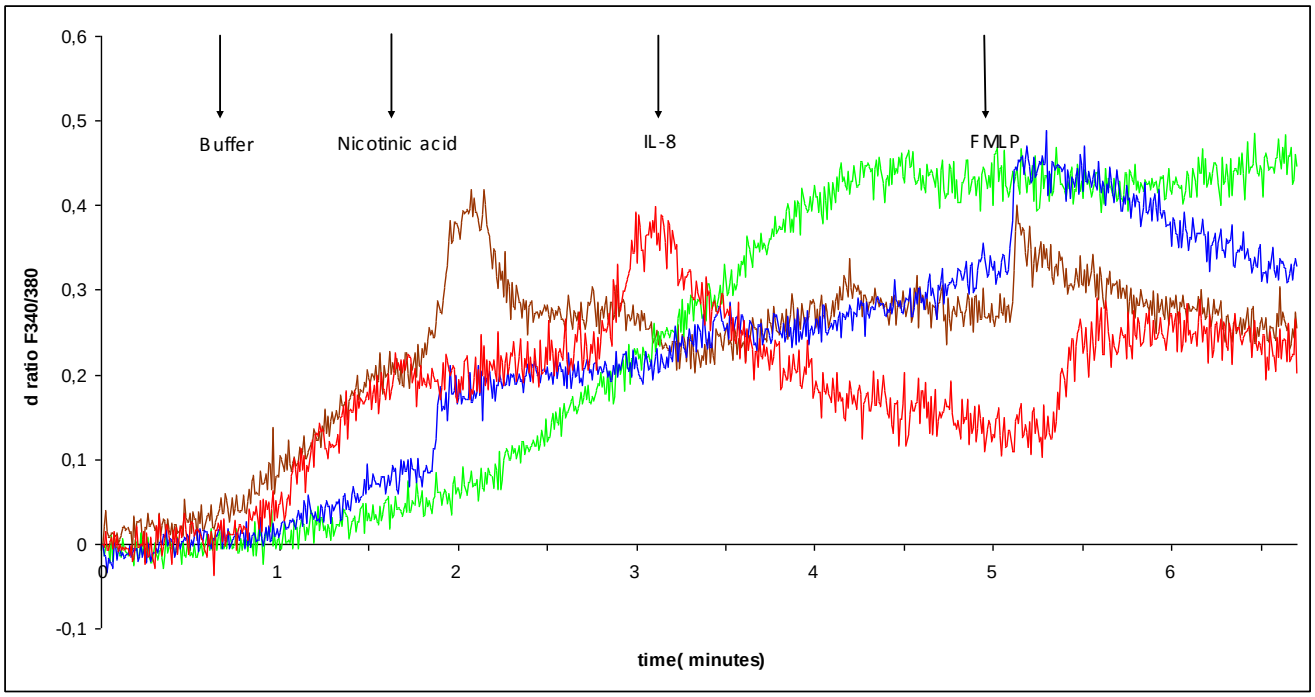

Fig. 44 The effect of 100µM nicotinic acid and 1 µg/ml Interleukin-8, subsequently on calcium signal in Fura-2-loaded blood neutrophlis from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded on polylysine coated cover slips to enhance their adherence. The Fura-2 interacted with cells 55 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. The cells- brown and blue line showe response on nicotinic acid and also on positive control, the cell- red one response in chemokine interleukin-8.

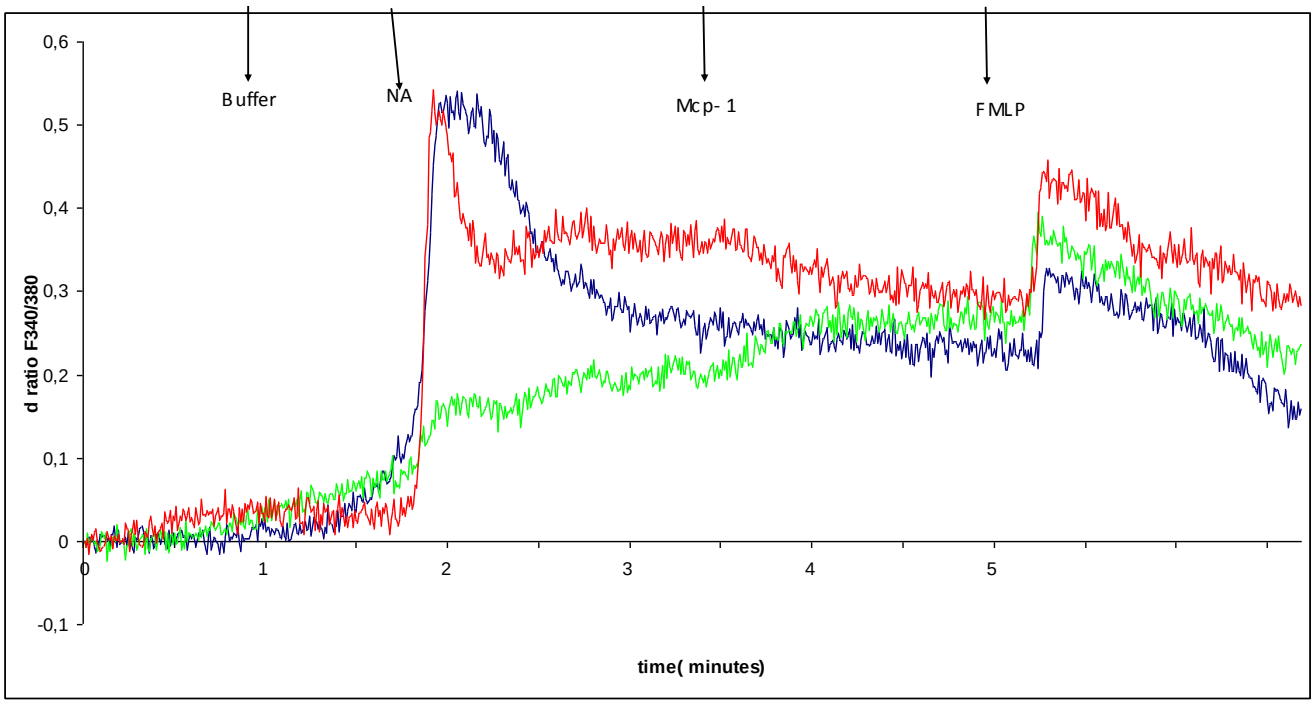

Fig. 45 The effect of 100µM nicotinic acid and 10nM macrophage/ monocyte chemotactic protein-1, subsequently on calcium signal in Fura-2-loaded blood neutrophlis from wildtype mice, which was 2 days injected with 4% thioglycolate to induce inflammation. The cells were loaded on polylysine coated cover slips to enhance their adherence. The Fura-2 interacted with cells 70 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. The cells- red and blue line showed response on nicotinic acid and also on positive control, the cell- green one couldn't be taken as detectable.

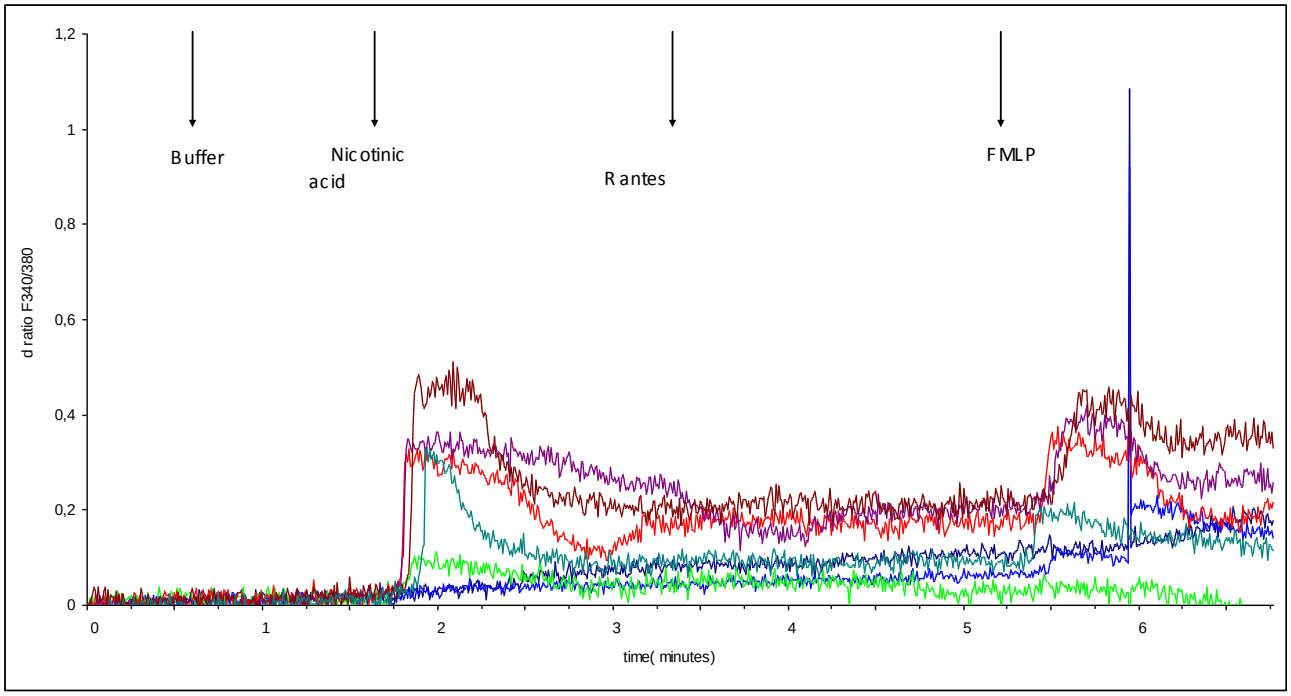

Fig. 46 The effect of 100µM nicotinic acid and 1 ng/ml RANTES, subsequently on calcium signal in Fura-2-loaded peritoneal neutrophils obtained from wild type mice. The fura-2 interacted with cells 80 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. Four marked cells reacted on nicotinic acid, no cells on RANTES.

The cells were prepared as described in the Methods 5.2.5. The cells were loaded with Fura-2 on polylysine coated cover slips to enhance their adherence and directly measured. The viability of neutrophils is short.

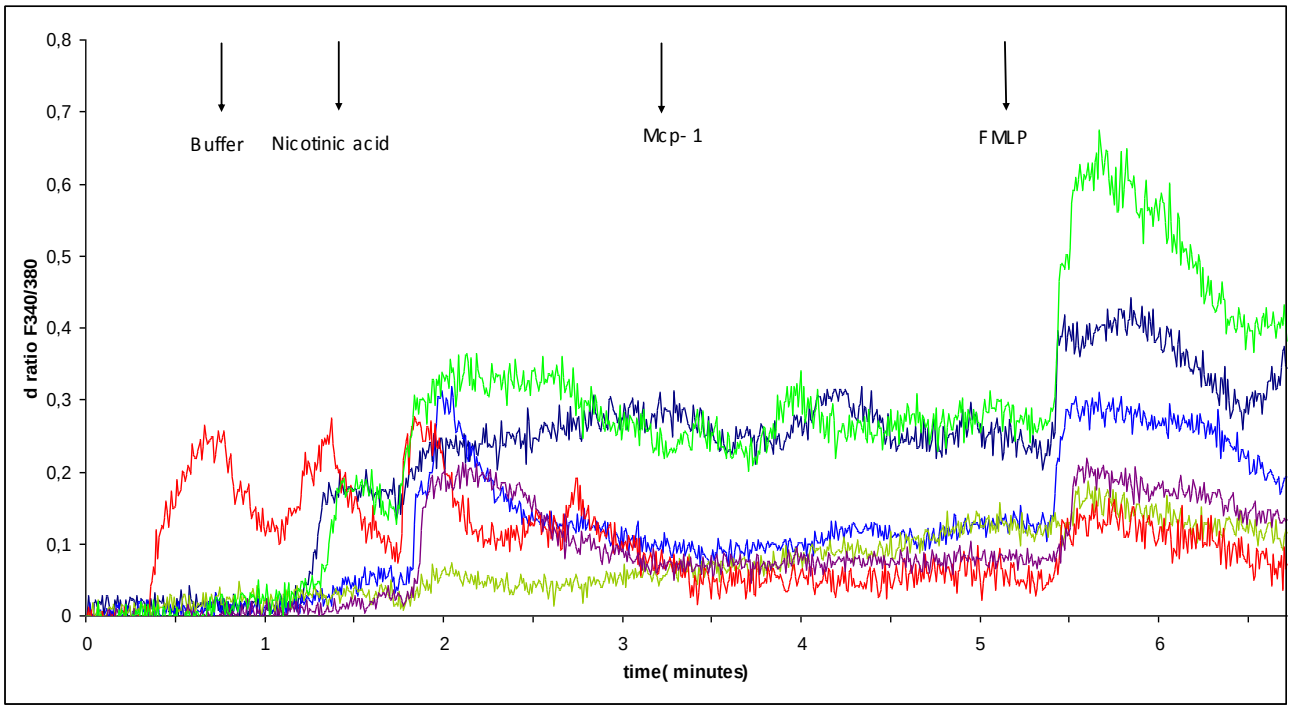

Fig. 47 The effect of 100µM nicotinic acid and 10nM macrophage/ monocyte chemotactic protein-1, subsequently on calcium signal in Fura-2-loaded peritoneal neutrophils obtained from wild type mice. The Fura-2 interacted with cells 90 minutes. The figure shows the time course of a response at a fluorescence ratio of 340/380. Nicotinic acid increased concentration of calcium and is visible some response to mcp-1 (the green line), it is also possible it cloud be some interruptions during measurement.

The cells were prepared as described in the Methods 5.2.5. The cells were loaded with Fura-2 on polylysine coated cover slips to enhance their adherence and directly measured. The viability of neutrophils is short.

As one can see from the charts above (Fig. 31- Fig. 47), used cells whether the peritoneal cells, neutrophils or cell lines THP-1 had reproducible response on nicotinic acid and positive control. We never confirmed repeatable response of any chemokine. We expected decreased concentration of used chemokine past adding nicotinic acid, which was no proven.

7 DISCUSSION

Atherosclerosis of the coronary arteries commonly causes angina pectoris and myocardial infarction. Atherosclerosis of the arteries supplying the CNS frequently provokes strokes and transient cerebral ischemia. Atherosclerosis causes intermittent claudication and gangrene. Also it can jeopardize limb viability, in the peripheral circulation. Atherosclerotic lesions often form regions of disturbed blood flow at branching points of arteries. [4]

The increase in HDL-cholesterol levels, especially in patients with low levels, is one of the major strategies currently pursued. The oldest antidyslipidemic drug, nicotinic acid, remains the best choice for increasing HDL-cholesterol plasma levels. The recent discovery of a receptor for nicotinic acid has allowed new insight into the mechanisms underlying the pharmacological effects of nicotinic acid. [13]

Most notably, nicotinic acid is able to raise plasma HDL cholesterol levels. Using a mouse mutant lacking the newly discovered receptor study research groups in Institute of Pharmacology in Heidelberg, Germany. They are currently analysing its role as a potential mediator of wanted and unwanted pharmacological effects of nicotinic acid like HDL cholesterol increases, anti-atherosclerotic effects as well as peripheral vasodilatation called flushing. [13]

The idea that elevated HDL protects against coronary heart disease comes primarily from epidemiological studies.

Novel agents being developed for regulating HDL metabolism include inhibitors of CETP and stimulators of the ABCA1 transporter CETP inhibitors can increase plasma HDL-cholesterol by 60 % and lower LDL-cholesterol by 30 %.

The epidemiological, experimental and clinical trial evidence suggests that there is a good rationale for raising HDL-cholesterol in these and other high-risk patients. The protective effect of HDL-cholesterol against atherosclerosis and cardiovascular disease is

mediated by both enhanced reverse cholesterol transport and by direct anti-atherosclerotic mechanisms. The first approach to raising HDL-cholesterol in at risk patients is lifestyle modification aimed at improving body weight and insulin resistance. The currently available statins will often require adjunctive therapy, such as gemfibrozil or fish oil, to raise HDL-cholesterol. Statin-fibrate combinations need to be monitored closely for risk of myositis. However, there is still more research to be done, including, for example, elucidating the precise molecular mechanism of the athero-protective effect of HDL and the developing and testing of new clinical therapies for improving HDL metabolism. The incremental benefit of adding a fibrate, fish oils or 'pipeline agents', such as CETP inhibitors (cholesterylester transfer protein), to a statin in low HDL patients with the metabolic syndrome are clearly burning questions for future clinical endpoint trials. [30]

Nicotinic acid is the most effective agent available for increasing high-density lipoprotein-cholesterol, but its use is associated with side effects that negatively affect patient compliance: these appear to arise largely as a result of production of prostaglandin D(2) and it is subsequent activation of the DP(1) receptor. Desire to reduce the side effects (and improve pharmacokinetic parameters) has led to the development of a number of agonists that have differing effects, both in terms of clinical potency and the severity of adverse effects. [31]

Niacin was found to inhibit lipolysis in adipocytes and reported as early as 1960 by Butcher, who investigated its effect on cyclic AMP. The investigators also found that niacin inhibits cyclic AMP production in epinephrine (adrenaline)-exposed adipocytes. Later work suggested the presence of a G-protein-coupled cell surface receptor of the G_{ig}/G_o type. In 2003, the nicotinic acid receptor was identified as the human orphan receptor HM74 or the highly homologous, higher affinity HM74A, as well as the mouse homolog PUMA-G (protein upregulated in macrophages by interferon-[gamma]). HM74A (GPR109A) and PUMA-G are also identified as the pharmacologically active niacin receptors. The receptors mediate inhibition of adenyl cyclase in adipocytes and thereby block lipolysis, accounting for the marked reduction of free fatty acids caused by niacin. Furthermore, there was an approximately 30% reduction in triglycerides in fat-fed normal mice. In receptor-deficient mice, no reductions in plasma free fatty acid and triglyceride levels were seen.

Substantial mRNA expression of HM74A/PUMA-G was found in brown and white adipose tissue, spleen, adrenal, and lung, but expression was essentially absent in liver. [32]

Nicotinic acid has been used for almost 50 years as a lipid-lowering drug. Its primary action is to decrease lipolysis in adipose tissue by inhibiting hormone-sensitive triglyceride lipase. This anti-lipolytic effect of nicotinic acid involves the inhibition of cyclic adenosine monophosphate (cAMP) accumulation in adipose tissue through a G(i)-protein-mediated inhibition of adenylyl cyclase. A G-protein-coupled receptor for nicotinic acid has been proposed in adipocytes. G-protein-coupled receptor, 'protein upregulated in macrophages by interferon-gamma' (mouse PUMA-G, human HM74), is highly expressed in adipose tissue and is a nicotinic acid receptor. Binding of nicotinic acid to PUMA-G or HM74 results in a G(i)-mediated decrease in cAMP levels. In mice lacking PUMA-G, the nicotinic acid-induced decrease in free fatty acid (FFA) and triglyceride plasma levels was abrogated, indicating that PUMA-G mediates the anti-lipolytic and lipid-lowering effects of nicotinic acid in vivo. The identification of the nicotinic acid receptor may be useful in the development of new drugs to treat dyslipidemia. [15]

Extended-release nicotinic acid)/laropiprant is a once-daily fixed-dose combination tablet that has been evaluated (with or without an HMG-CoA reductase inhibitor [statin]) in the treatment of adults with dyslipidaemia or primary hypercholesterolaemia. Nicotinic acid is a lipid-modifying drug and laropiprant is an anti-flushing agent, which reduces flushing induced by niacin. [33]

Genetically modified mouse models have been instrumental in elucidating the underlying molecular mechanisms in lipid metabolism. [34]

Wildtype mice are quite resistant to atherosclerosis as a result of high levels of antiatherosclerotic HDL and low levels of proatherogenic LDL and VLDL.

LDL receptor deficient mouse ($LDLr^{-/-}$ mice), in which atherosclerosis develops especially when fed a lipid-rich diet. In humans, mutation in the gene for the LDL receptor cause familial hypercholesterolemia. Mice lacking the gene for LDL receptor develop atherosclerosis. [35]

Experiment with two monitored groups of mice- LDLR (012) treated with 0.3% nicotinic acid and untreated control group and PumaG LDLR (014) treated with 0.3% nicotinic acid and untreated control.

We have tested the effect of nicotinic acid in the earlier mentioned groups of mice and found that the antiatherosclerotic effect was gone even tough in wildtype mice that was preserved and the lipid profile was not changed. The groups of mice were too small and it is to see higher values of standard deviation. The experiment should be repeated with a larger number of mice in group.

To investigate the physiological expression of the PumaG receptor, a PumaG reporter mouse was developed. It expresses the mRFP (monomeric red fluorescent protein) under the GPR109A promoter.

In the second part of this work, we have focused to find receptor for gene by using fluorescent microscopy. First step was isolation of blood cells per eye, the retro-orbital bleeding method and various modification of cells (without peritonitis, with peritonitis past incubation with 4% thioglycolate 2-5 days, protection before coagulation by using EDTA, erythrocyte lysis). We made drop with cells on microscope slide and made cell smear and stained with Diff-Quick. It was hard to identify expression in blood cells, we have seen more erythrocytes than other cells, which can express the receptor GPR109A. Therefore we focused on isolation of blood and subsequently peritoneal neutrophils. In the forward step we removed peritoneal cells by using peritoneal lavage method. Peritoneal exudates cells expressed receptor GPR109A which is to see in fluorescence microscopy.

Expression of a functional GPR109A receptor was assessed to specifically induce increases in calcium concentrations. We were interested on the effect of nicotinic acid and chemokines. The cells were incubated over night with 20 nM GM-CSF to differentiation immature monocytes into macrophages, GM-CSF was washed in the morning. Addition of nicotinic acid to fura-2 loaded peritoneal exudates cells led to transient changes. Peak calcium levels were observed. All cells didn’t express detectable levels of GPR109A surface protein, didn’t responded with increases in calcium ions. In contrast, addition of FMLP demonstrated that the cells were properly loaded and responsive, in principle. These data suggested that if positive control worked then the cells didn’t have functional receptors

of chemokines. This may be due for example long time of incubation overnight. Therefore we tested also with cell lines THP-1.

It is necessary to repeat more experiments with various conditions; it depends on duration of inflammation caused by injection 4% thioglycolate, on duration incubation, on concentrations added agonists, on time of measurement and lot of other interventions.

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