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DIPLOMARBEIT

Titel der Diplomarbeit

Influence of Indirubin-3´-monoxime on Redox Systems in PDGF-activated Vascular Smooth Muscle Cells

Verfasser

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angestrebter akademischer Grad

Magister der Pharmazie (Mag. Pharm.)

Wien, 2010

Studienkennzahl lt. Studienblatt:	A 449
Studienrichtung lt. Studienblatt:	Pharmazie
Betreuerin / Betreuer:	Univ. Prof. Dr. Verena Dirsch

A Introduction

1 Indirubin

1.1 Chemical properties, occurrence and history

Indirubin is a red coloured, structural isomer of the blue dye indigo. It is found in small amounts together with other indigoids. Plants like *Isatis Indigofera*, *Indigofera tinctoria*, *Indigofera suffrutticosa* and some others contain the precursors Indican or Isatan B which are transformed by oxidation and dimerization to Indirubin and its isomers during the procession of the plant material [1].

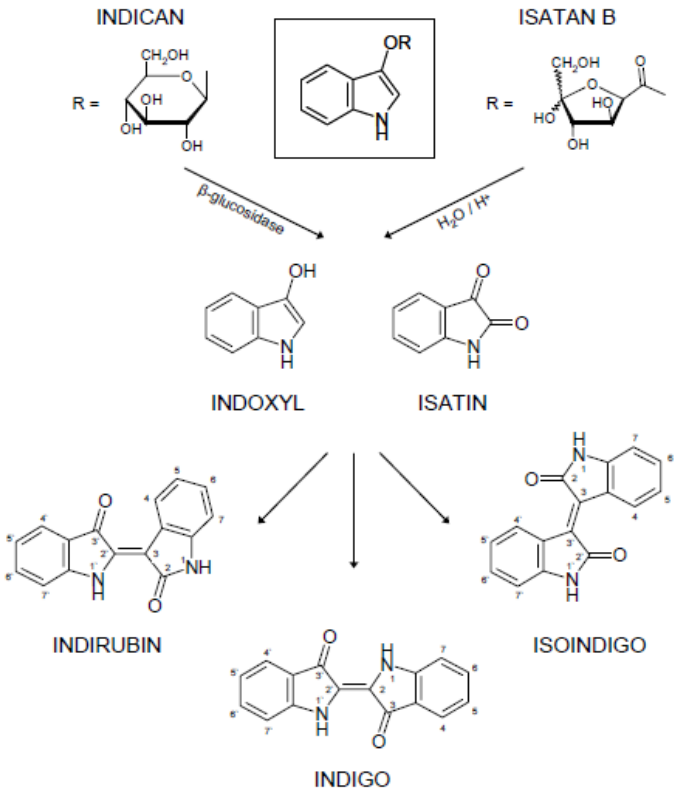


Figure I

Formation of Indigo and Indigoids in plant material

The colourless precursors Indican and Isatan B are deglycosylated by enzymatic or acidic hydrolysis into Indoxyl. Further oxidation in the presence of oxygen leads to Isatin. By spontaneous, non enzymatic dimerization Indigo, Isoindigo and Indirubin are formed.

Scheme taken from Schwaiberger, A. [2]

Indirubin was also found in the ancient dye "Tyrian blue", extracted from molluscs [3] Indirubin was found among other indigos as a product of Cytochrome P450 oxidation. CYP 2A6 was able to oxidise Indol to Isatin and Indoxyl, precursors of Indigoids. Indigo was also found at random in bacterial cultures, modified for expression of human Cytochrome P450. In this case, Indigo is believed to be syntesized as a breakdown product of tryptophan [4, 5]. Indirubin can be found in the urine of leukemia patients [6] and patients suffering from purple urine bag syndrome [7]. Indigo and Indirubin occur physiologically in small amounts as side product of tryptophan metabolism [8].

The name Indirubin was originally given to red coloured sideproducts of the Indigo synthesis in the 19th century. In western chemistry it was used in the dying industry to variate the intensity of indigo colour [9, 10].

1.2 Medicinal use

Indirubin was found to be the active ingredient of Danggui Longhui Wan, a recipe in Traditional Chinese Medicine (TCM) composed of 11 herbal medicines [11, 12].

In TCM praxis Danggui Longhui Wan has been used for a long time to treat cancer especially chronic myelotic leukaemia. Clinical trials with the mixture showed promising results also for several other forms of leukaemia. The active principle of this mixture was found in Qing Dai, a dark blue powder, prepared from the leaves of *Baphicacanthus cusia* (Acanthaceae), *Poligonum tinctorium* (Polygonaceae), *Isatis indigofera* (Brassicaceae), *Indigofera suffrutticosa* (Indigoferaceae) and *Indigofera tinctoria* (Fabaceae) [13] and could finally traced back to indirubin.

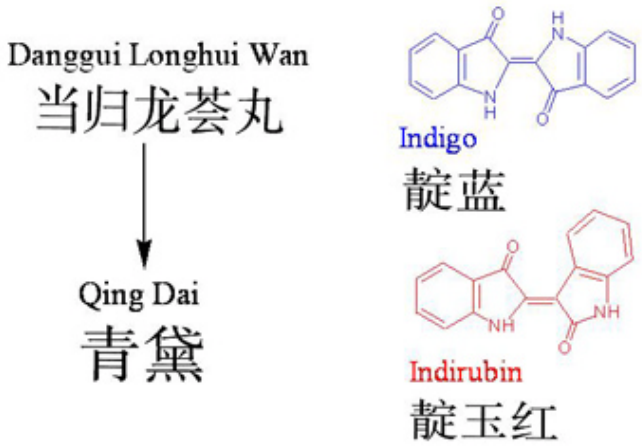


Figure II

Use of Indigoids in Traditional Chinese Medicine

Indigo naturalis (Qing Dai) is one component of the TCM recipe Danggui Longhui Wan, commonly used to treat chronic myelotic leukaemia. It contains Indigo as well as Indirubin which was investigated to be the active ingredient.

Scheme downloaded from http://www.sb-roscoff.fr/ indirubin/intro.html

1.3 Derivatives

Because of the poor solubility of Indirubin itself [13], several derivatives were designed. Among them there are 5-halogeno-indirubin, N-ethyl-indirubin and Indirubin 3´-monoxime [14-16]. Indirubin 3´-monoxime (I3MO), one of the few commercially availiable indirubin derivatives, shows better solubility and good antitumor activity together with low toxicity in animal models [13]. I3MO is the indirubin derivative used in this study.

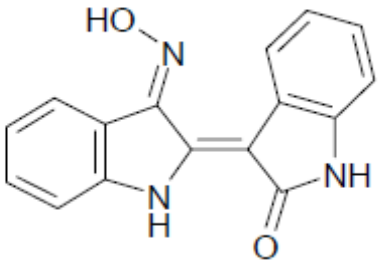


Figure III

Chemical Structure of Indirubin 3´monoxime

Scheme taken from Schwaiberger,A. [2]

1.4 Reported bioactivities of Indirubins

Indirubin and its derivatives show a wide range of biological activities, many of them cell type specific. Most prominent in the literature are inhibition of kinases, anti-inflammatory properties and activation of the aryl hydrocarbon receptor (AhR) by indirubins.

1.4.1 Inhibition of Kinases

Indirubin and derivatives can competitively replace ATP from its binding pocket and hereby inhibit kinase activity [13].

I3MO is reported to be an inhibitor for **cyclin dependent kinases (CDK)**, namely CDK 1,2, 4 and 5 [13]. CDK inhibition by I3MO leads to often cell-type specific effects blocking the cell cycle either in late G1 or G2/M phase. In HBL 100 cells for example, I3MO leads to cell cylce arrest and endoreplication followed by necrotic cell death [17]. The potential to interfere with the cell cyle has drawn attention onto indirubin and its derivates for a possible use in health disorders with aberrant proliferation such as cancer. Inhibition of CDK5/p25 by I3MO may be beneficial in Alzheimer´s disease due to reduced phosphorylation and thus reduced activation of tau protein [18]. In this context, I3MO-mediated inhibition of another tau-kinase, namely GSK3β may be also of interest [18-20].

The **glycogen synthase kinase (GSK)3-β** is a widespread enzyme, first discovered in the regulation of insulin-induced glycogen synthesis [21]. It acts in the Wnt pathway, as explored in Xenopus laevis and Drosophila, via regulation of β-catenin and its downstream gene expression [22]. Also the apoptosis of dopaminergic neurons in Parkinson´s disease could be reduced by I3MO due to inhibiton of GSK3β [23].

7-Bromo-Indirubin-oxime is a potent inhibitor of **Aurora Kinase B and C**, members of a family of serine/threonine kinases involved in mitotic cell division processes. Since these kinases are overexpressed in most human cancer cells, their inhibition by indirubins may explain the successful use of indirubins in cancer therapy [24, 25].

Indirubin-3´-monoxime is also known as inhibitor of **Jun-N-terminal kinase** (JNK), reducing c-Jun phosphorylation which has been reported to play a role in neuronal apoptosis [26].
The proliferation of NIH/3T3 cells is blocked by indirubin derivatives which was explained by specific inhibition of autophosphorylation of the receptor tyrosine kinase FGFR1 (**Fibroblast growth factor receptor1**) [27].

1.4.2 Anti-inflammatory activity

Indirubin and its derivates show anti-inflammatory qualities in several studies. In human myelomonocytic HBL-38 cells, I3MO inhibited the production of IFNγ and in murine splenocytes it abolished the synthesis of IL-6, both known as inflammatory cytokines [28]. Studies using the human bronchial epithelial H292 cell line infected with influenza virus could demonstrate that Indirubin and Indirubin-3´monoxime reduced the production of the chemokine RANTES (regulated on activation, normal t-cell expressed and secreted). The virus-induced phosphorylation of the NFκB-regulatory protein IκBα and the phosphorylation of the MAPK p38 was reduced in response to I3MO treatment [29].
Studies suggest that at least a part of Indirubins anti-inflammatory effect is mediated via inhibition of the NFκB pathway [30].

1.4.3 Ligand of Aryl Hydrocarbon Receptor (AhR)

Indirubin has further been reported to act as ligand and activator of the aryl hydrocarbon receptor [8]
Although the endogenous ligand of AhR is not found yet, activated AhR (e.g. by exogenous ligands) has been identified to be involved in cellular differentiation [31] and expression of xenobiotic metabolizing enzymes like cytochromes P450 in response to e.g. dioxin or PCB´s [31-33]. Indirubin is known to induce CYP 1A1 and 1B1 in human breast cancer cells through AhR [34].
Studies with indirubin derivatives, only capable of binding to AhR and not to kinases have shown that binding to AhR can suffice to induce a G1 phase arrest due to increased p27 expression, whereas cytotoxic effects of Indirubins are more related to kinase inhibition than to AhR activation [35].

1.4.5 Other

Indirubin derivatives are also reported to block **STAT** (Signal transducers and activators of transcription) signaling, which is found constitutively active in cancer cells. Treatment with Indirubin leads to decreased expression of antiapoptotic proteins Survivin and Mcl-1, inducing apoptosis [36].

Recently Indirubin-3´monoxime was shown to inhibit mitochondrial oxidative phosporylation. This indicates new mechanism of Indirubin on inhibiting the cell function [37].

2 Atherosclerosis

2.1 General overview

Multiple processes may lead to the manifestation of atherosclerosis. Although the complete mechanisms are not fully understood yet, genetic predisposition as well as dietary and life-style habits, like smoking and alcohol are believed to be responsible for atherosclerotic diseases.

Atherosclerosis describes abnormal indurations and lesions in the large (elastic) as well as in the medium (muscular) arteries. It is characterized as thickening of the intima in response to vascular intimal injury.

Atherosclerosis is a disease that starts at an early age with clinically silent lesions, occurring at predisposed areas in large and medium sized arteries. Its later complications contribute to mortality more than any other desease in western world [38].

2.2 Physiology

The typical arterial wall can be divided in three distinct tissues (tunicas): intima, media and adventitia. The innermost layer, next to the lumen is named the intima, consisting of a monolayer of endothelial cells, laid on a basement membrane, the internal elastic lamina. The media is composed of smooth muscle cells, providing the vasomotor tone. The vessel is completed by the adventitia, which is separated from the media by the external elastic lamina. The adventitia consists of loose connective tissue, fibroblasts and autonomic nerve endings.

There are three types of arteries, which can be divided by their size and compostition.

1. The large (or elastic) arteries contain a high amount of elastic fibers in the media, which are arranged in compact layers together with VSMCs.

2. The medium (or muscular) arteries contain fewer elastic material in their media and are smaller in size.

3. The arterioles and the capillares together represent the smallest type of arterial vessels mainly found in tissue and organs.

Atherosclerosis affects only the large and the medium sized arteries. It seems that there are predominant arteries like the aorta or the corona, which are very prone to atherosclerotic events, whereas other vessels are relatively safe. This might be in relation to shear forces and turbulent blood flow which are events that expose parts of the vessels to vascular injury [38].

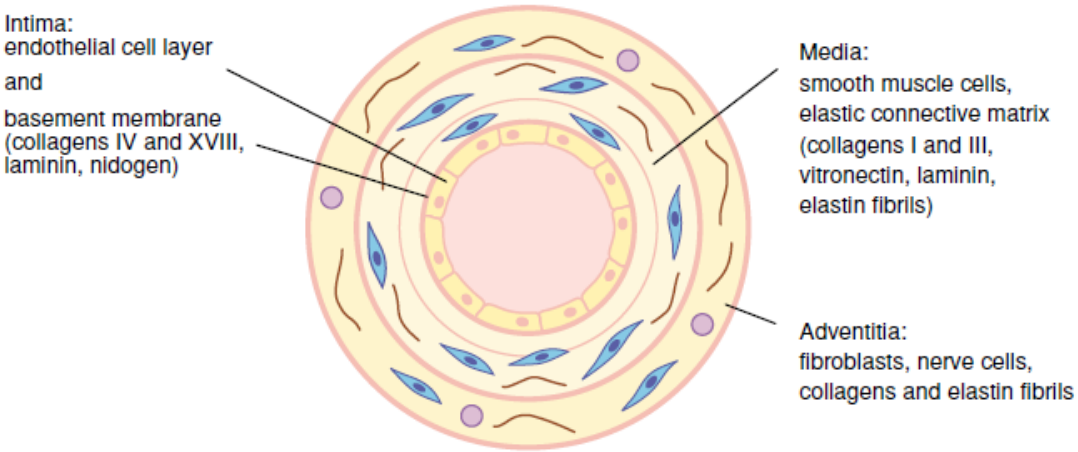


Figure IV

Anatomy of the Arterial wall

Scheme downloaded from:

http://journals.cambridge.org/fulltext_content/ERM/ERM4_01/S1462399402004039sup005.pdf

2.3 Pathological events that lead to atherosclerosis

At an early stage of the disease, it is believed that the monolayer of endothelial cells, which belong to the intima of the arteric vessel becomes injured e.g. by hypertension, aberrantly high lipids, metabolites of cigarette smoke or shear forces. This results in inflammation, endothelial cell activation and increased endothelial permeability leading to lipoprotein accumulation as well as adhesion of monocytes and of platelets. Monocytes then begin to emigrate into the intima, develop into macrophages and engulf accumulated lipids, leading to the formation of “foam” cells. The result is a so called "fatty streak", an early stage of an atherosclerotic lesion which is clinically silent.

Vascular smooth muscle cells from the media then begin to migrate towards the intima and begin to proliferate, a process induced by growth factors and cytokines. Platelet-derived Growth Factor (PDGF) is the most important stimulus for proliferation and migration of VSMCs [39]. Also FGF is reported to act as a mitogen and chemoattractant and to a smaller extent IGF-1, TGFβ and EGF [40]. These mitogens are derived from accumulating platelets, macrophages and lymphocytes on the site of the inflammation as well as from VSMCs themselves and intimal endothelial cells. VSMCs also engulf lipids and synthesize extracellular matrix. This process contributes to the narrowing of the vessel.

As the atherosclerotic process continues, cells in the center of the atherosclerotic plaque get necrotic and form a core consisting of disorganized lipds and dead cell material. VSMCs form a fibrous cap that covers this core, resulting in a fibrofatty atheroma.

Ongoing inflammation, shear forces as well as activity of metalloproteases and collagenases, however, render the atheroma prone to rupture. The break can lead to formation of a thrombus which on the one hand can be carried away by the blood stream to cause ischaemic events elswhere, or on the other hand contribute to the deteriorated narrowing of the vessel. [38]

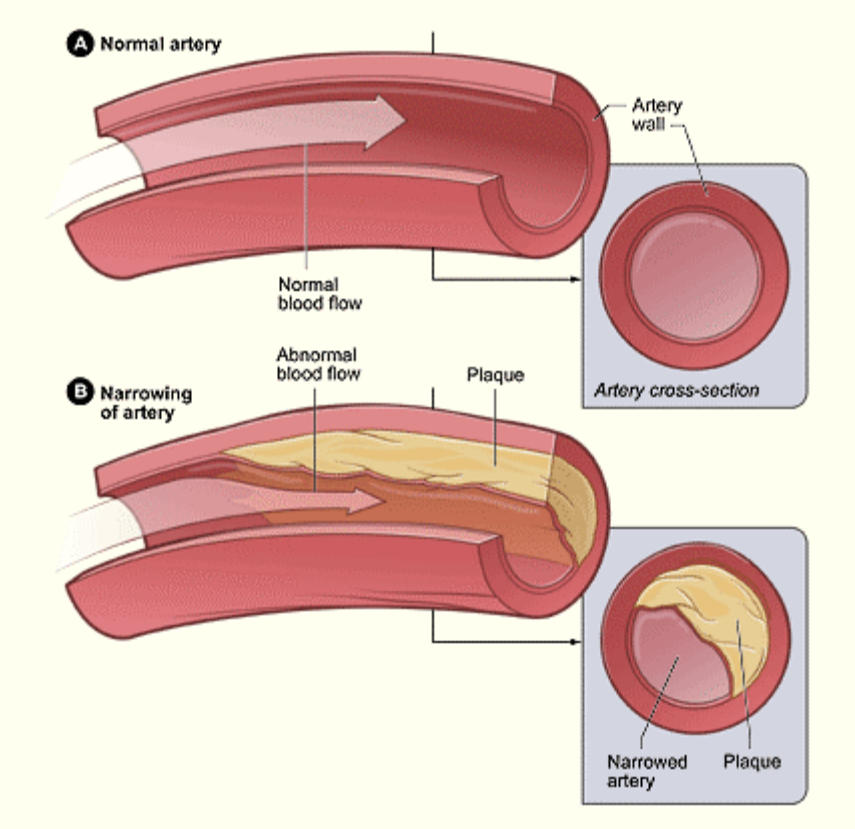


Figure V

Formation of a plaque

Through inflammation, migration and proliferation of VSMCs, accumulation of platelets, macrophages and lymphocytes and incorporation of lipids a plaque is forming which leads to narrowing of the vessel and reduction of blood flow.

Scheme downloaded from:
http://www.web-books.com/eLibrary/Medicine/Cardiovascular/Atherosclerosis.htm

2.4 Therapeutic options

The best therapy of atherosclerotic diseases is primary prevention of avoidable risk factors. Physical workout/exercise and dietary measures can reduce lesions at an early stage. Also several drug-mediated interventions, e.g. to lower the cholesterol level, a major risk factor for atherosclerosis, are implicated [39]. After a plaque has been formed, several surgical methods like balloon angioplasty (also PCTA) accompanied by stent placement, transplantation of vessels and bypasses can be done, but ironically these procedures themselves lead to restenosis (see next chapter) and inflammation of the vessel [41].

2.5 Restenosis

Restenosis, i.e. the renarrowing of the vessel, is a major problem for patients undergoing a PTCA (percutaneus transluminal coronary angioplasty). About 20% of all patients develop it. The processes that lead to restenosis are similar to those, involved in the genesis of atherosclerosis. Due to inflammatory processes, driven by endothelial injury during balloon inflation or stent placement, proliferation of VSMCs and therefore narrowing of the vessel is caused. In contrast to the atherosclerotic plaque, restenosis shows a diffuse and symmetrical narrowing, affecting long parts of the vessel [39].

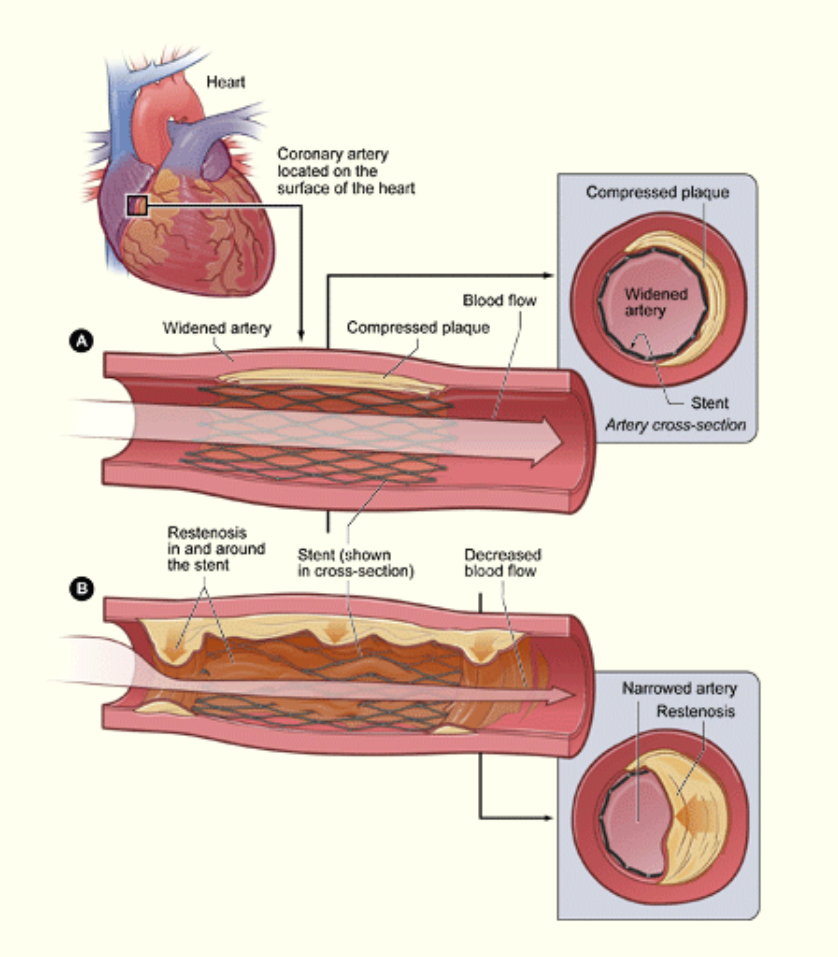


Figure VI

Illustration of PTCA and of Restenosis

The processes that lead to restenosis are almost the same involved in the genesis of atherosclerosis. Restenosis is a major problem in therapy of vascular narrowing, attending 20% of all patients.

Scheme downloaded from
http://www.heart.org.in/dieseases/images/stent_restenosis.gif

In current restenosis therapy, drug eluting stents are in use. In contrast to common metal stents, they are coated with a polymer, eluting antiproliferative drugs like Sirolimus or Paclitaxel. These coated stents show a reduction in restenosis rate in animal models and in clinical trials [42, 43].

Although these stents are of great advantage, several drug-related side effects shorten the therapeutic benefit. Sirolimus, a immunmodulating drug, is reported to cause thrombosis and reduce wound healing ability [44]. Paclitaxel shows cytotoxic effects common for anti-mitotic agents [45, 46].

3 PDGF

The platelet-derived growth factor (PDGF) was originally found in platelets, but all vessel cells seem capable of expressing it. It is a cytokine that was primarily discovered as the active factor leading to proliferation and migration of smooth muscle cells of apes [47]. PDGF and its elicited downstream signalling is now accepted to be the most predominant stimulus involved in the activation, migration and proliferation of vascular smooth muscle cells. PDGF is related to diseases like atherosclerosis [48] but several other vascular effects of PDGF stimulation are reported. Its importance may be highlighted by the fact that the embryonal development of mice can be severely influenced when knocking out either the A chain of PDGF or its α receptor subunit or the B chain or β receptor subunit [49-51]. Also an effect on wound healing [52], regulation of the fluid pressure [53] and a PDGF-BB-specific effect on NO-mediated relaxation of blood vessels [54] were reported. PDGF from platelets may play a role in a feedback mechanism controlling platelet aggregation [55, 56].

3.1 Structure and binding properties

The PDGF molecule consists of two isomeric protein chains, which are synthesized as precursor molecules and processed via proteolysis. The mature chains consist of about 100 amino acids residues and have approximately 60% of their residues in common [57-61]. There are several isoforms of these chains, with the A and the B isomer being the most predominant, which can form homo- (AA, BB) or heteromeric (AB) dimers. Recently, a PDGF C and a PDGF D isomer were discovered, but yet little is known about their activity and distribution [62]. The PDGF AB heterodimer was only found in human platelets so far. It seems, that homodimers are the dominant appearance form of PDGF dimers.

The detailed structure of PDGF was revealed by crystal structure analysis, showing that 6 cysteine residues form disulfid bonds. Three of these cysteine residues are involved in intrachain bonds forming a knot structure, while the remaining two cysteine residues form interchain bonds connecting the subunits of PDGF. PDGF shows similarity to the vascular growth factor [60]. The knot structure of the PDGF is

surrounded by two long β-sheet chains (loop 1 and 3) pointing in one direction and a smaller chain (loop 2) pointing to the other direction. The subunits of PDGF are assembled in an antiparallel manner and therefore loop 1 and 3 of the first subunit lie next to loop 2 of the other subunit [63, 64].

3.2 Structure of the PDGF receptor (PDGFR) and ligand-induced autophosphorylation

PDGF binds to its own receptor, which again exists in two isomers, the alpha (PDGFRα) and the beta (PDGFRβ). In the inactive state the PDGFR subunits are separated from each other. Like for other members of this family of tyrosine kinase receptors, PDGF-R undergoes dimerization upon ligand binding. The monomers in the membrane are then bound together by the extracellular ligand. Thereby the α isoform is capable of binding the A or the B monomer of PDGF while the β subunit is only capable of binding the B monomer. Like this, the AA and the AB isomers of the PDGF are only capable of binding and activating the αα receptor and only the BB isomer can activate all isomers of the PDGF receptor. These data are mainly based on in vitro studies, but yet few information is available about the real in vivo situation.

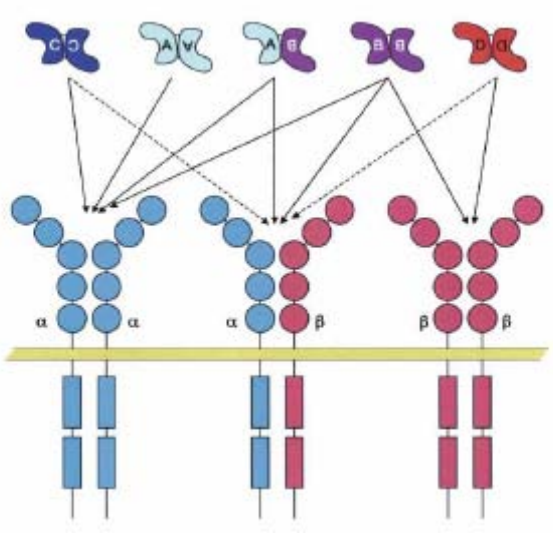


Figure VII

In vitro ability of PDGF dimers to bind to the different PDGF Receptor isoforms

The α chain of the PDGFR is able to bind PDGF A,B,C,D whereas the β chain of the PDGF is only capable of binding the PDGF B and D.

Scheme taken from Andrae [47]

When bound together on the PDGF molecule the receptor monomers are brought into a close proximity to each other, resulting in mutual autophosphorylation on several tyrosine residues. Thereby the intrinsic kinase activity of the assembled receptor plays a major role. Mutation of the Y^{857} residue, located in the kinase domain of the PDGF receptor leads to reduced kinase activity. The intercellular part of PDGF contains 15 tyrosine residues and 11 of them are known as autophosphorylation sites [65, 66].

3.3 Signal transduction

The phosphotyrosines on the cytosolic part of the PDGF receptor constitute specific docking sites for effector molecules with a Src-homology (SH) domain. Once recruited to the receptor these effector molecules can regulate the kinase activity of the receptor, recruit other effector molecules via SH3 or PH (pleckstin homology) domains and like this stepwise transduce the signal further downstream.

3.4 Important Intracellular Pathways, activated by PDGF

3.4.1 MAPK Kinase pathway

The activation of the MAP Kinase pathway starts with the activation of RAS [67-69]. Ras is a small GTPase and underlies a tight regulation through the GAP (GTPase activating protein) on the one hand and the GEF (guanine nucleotide exchange factor) SOS1/Grb2 complex on the other hand. Sos1 forms a complex with Grb2, a SH2 domain protein that binds to the phosphorylated PDGFR directly or via adaptor proteins, and converts RAS GDP to RAS GTP and therefore activates it [67]. The activation of RAS leads to activation of Raf-1 a serin/threonin kinase and the first kinase in a line of kinases followed by MEK 1,2 and then by the MAP kinases ERK1,2. Further signalling leads to various cellular effects, among them the expression of several gene products like c-myc, c-fos, c jun [70-73]. After docking to the PDGF receptor on Y^{771}, GAP hydrolyses RAS GTP to RAS GDP, therefore inactivates RAS and terminates the signal.

Similar pathways lead to the activation of p38 MAPK and JNK Kinases. It has been shown that the activation of p38MAPK is related to vascular injury [74].

Downregulation of JNK prevented sucessfully neointima formation in ballon catheter injured arteries of rats [75].

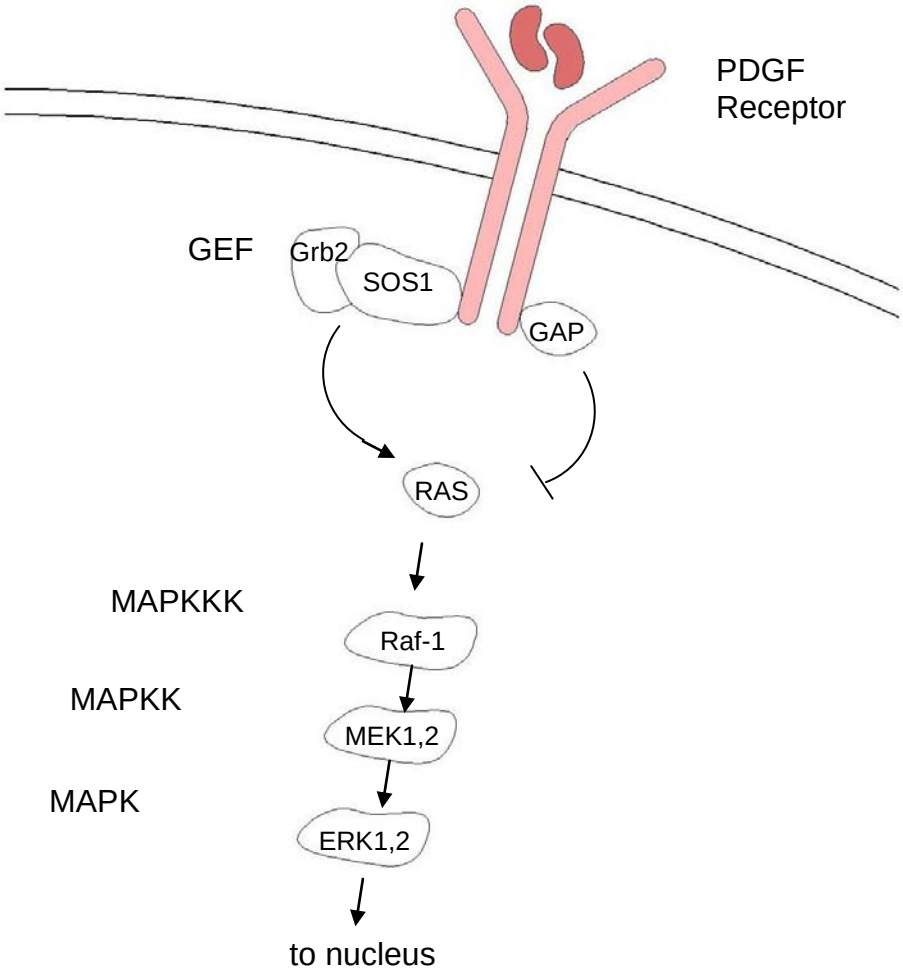


Figure VIII
MAPK Kinase pathway

3.4.2 PI3K/AKT Pathway

PDGF stimulation leads to activation of the PI3K/AKT pathway [76, 77] that is linked to cell cycle progression and proliferation [78].

Phosphoinositide kinases (PI3K) are a family of enzymes, capable of phosphorylating phosphatidyl inositole bisphosphate in their 3´position. PI3K binds to the activated PDGF receptor with its regulatory subunit p85 on tyrosine residues Y^{740} and Y^{751}. After binding to the receptor, PI3 Kinase is phosphorylated at Y^{508} in the regulatory subunit. PI3K is in complex with a catalytic subunit p110 which is responsible for converting phosphatidylinositole (4,5) bisphosphate into phosphatidyl inositole (3,4,5) triphosphate = $PI_{(3,4,5)}P_3$ [79].

$PI_{(3,4,5)}P_3$ is able to interact with proteins bearing a PH (pleckstin homology) domain [76, 77]. $PI_{(3,4,5)}P_3$ is interacting with the PH domain on AKT (also referred as Protein Kinase B), leading to recruitment of AKT to the inner cell membrane [80-82].

The activation of AKT via PI3K is also regulated by $PI_{(3,4,5)}P_3$. It activates PDK1 and PDK2 kinases which in turn phosphorylate AKT on a threonine T^{308} and a serine S^{473} residue. Phosphorylation of both residues is required for maximal activation of AKT leading to detachment from the inner membrane towards the nucleus. [79, 83] In contrast, the phosphatases PTEN and SHIP are responsible for degrading $PI_{(3,4,5)}P_3$ into $PI_{(4,5)}P_2$ and $PI_{(3,4)}P_2$ and therefore acting as negative controllers of this pathway [84-86].

AKT itself is involved in the regulation and activation of a great number of pathways leading to cell growth and proliferation. Phosphorylation by AKT leads to activation or often inhibition of its target proteins. Examples of AKT targets are the Forkhead-related transcription factors and the glycogen synthase kinase-3 (GKS-3) and the CDK inhibitors p21CIP1 and p27Kip1 [84, 87].

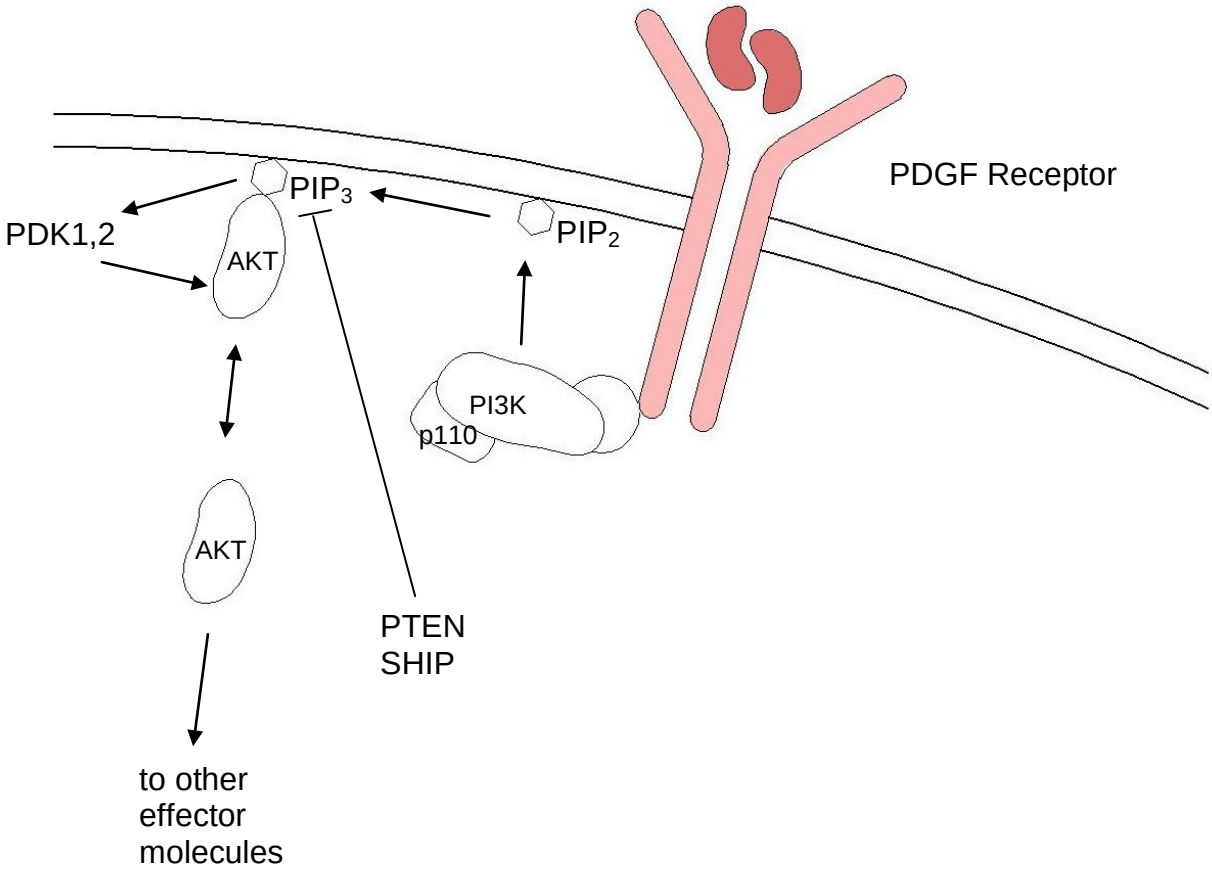


Figure IX
PI3K / AKT Kinase pathway

3.4.3 SRC Kinase pathway

The Src family of tyrosine kinases, consisting of nine members in total, is well known to be involved in transducing the mitogenic PDGF signal, as several studies with blocking of Src showed [88-90].

The Src Kinase structure shows an N-Terminal half with a membrane targeting sequence, a unique domain (which differs among the members of SRC family) and a SH2 and SH3 domain. The C-Terminal half consists of a catalytic domain, and a short amino acid sequence, involved in regulation of activity. Y^{527} in the C-Regulatory region are important for regulation of activity. If Y^{527} is phosphorylated, the SH2 domain on the N-Terminus is binding intramolecularly to pY^{527} (autoinhibition). Dephosphorylation of Y^{527} by a phosphatase is leading to activation, the SH2 domain

is free to interact with other phosphotyrosines such as in the activated PDGF-R. Autophosphorylation of Y^{416} stabilizes the active state [88].

Src Kinase is part of many promitogenic signalling cascades. Interruption of this cascades by the Src inihibitor SU6656 [88] stops PDGF induced protein synthesis and proliferation.

3.4.4 JAK/STAT Pathway

In contrast to other signalling pathways like the MAPK cascade, only two intracellular molecules are involved in transmitting the signal from the membrane to the DNA in the JAK/STAT pathway. It can be activated by cytokines like interferons or interleukines as well as growth factors like PDGF or EGF. Oxidative stress is also in discussion to activate Jak/Stat signalling [91-93]

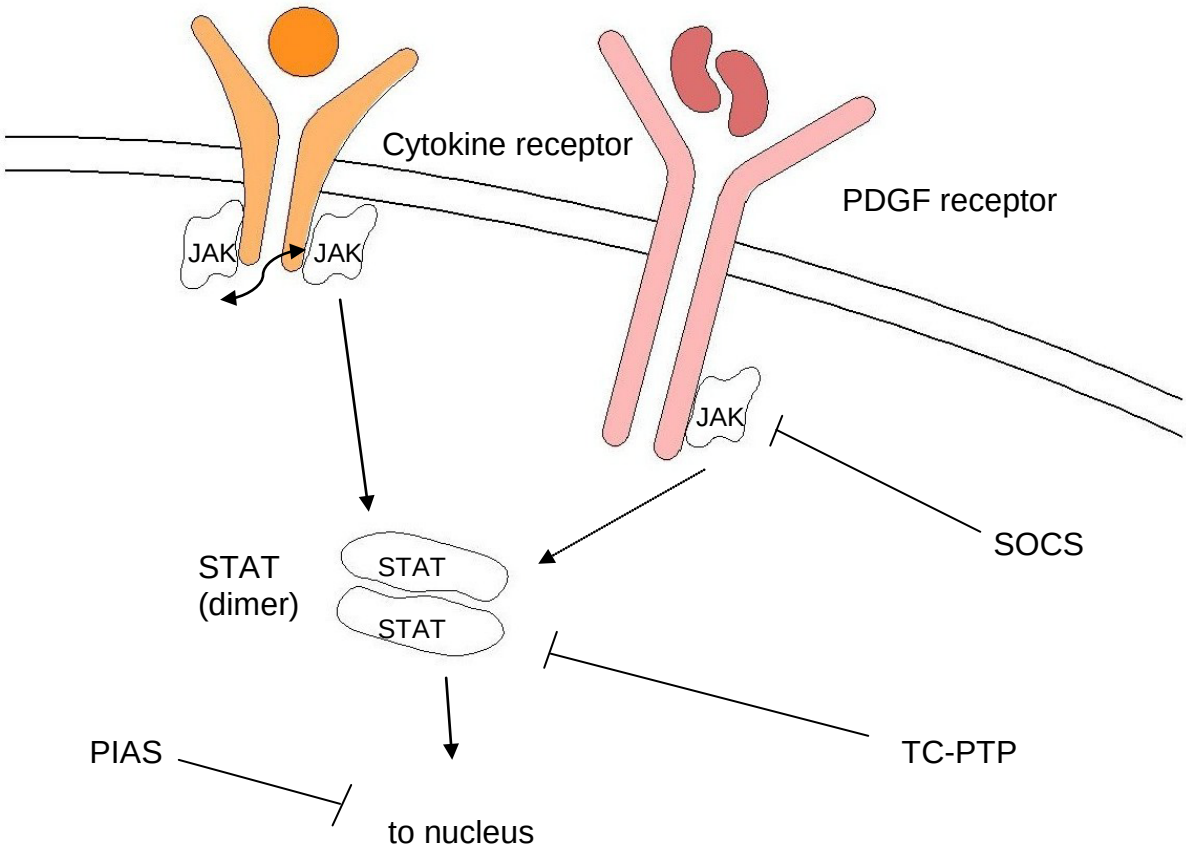


Figure X
JAK / STAT Pathway

3.4.4.1 JAK Kinases

The family of JAK consists of 4 members, the JAK1, JAK2, JAK3 and TYK2. They are involved in cytokine signalling. In contrast to growth factor signalling, cytokine receptors lack an intrinsic tyrosine kinase domain. Cytokines binding on a cytokine receptor lead to dimerization and receptor bound JAK get close enough to trans-phosphorylate each other. This provokes an intramolecular change in JAK molecule, enabling the JAK to phosphorylate STAT transcription factors [91, 92, 94]. JAK is also reported to be activated by PDGF tyrosine kinase, but it is unclear if this is of importance in activating the STAT signal [93, 95, 96].

JAK are negatively controlled via supressors of cytokine signalling (SOCS). The synthesis of these proteins is cytokine-induced and they act negatively on signalling pathways in different ways. Described actions are binding to phosphorylated tyrosine residues via SH2 domains and therefore inhibition of signalling, or blocking signal molecules via binding to their N-Terminus. Also a induction of proteasome-associated degradation is reported [97]. Also SHP-1, a phosphatase is involved in dephosphorylation of JAK and receptor tyrosin kinases.

3.3.4.2 STAT Transcription factors

The signal transducers and activators of transcription (STAT) represents a family with at least seven members: STAT 1, STAT 2, STAT 3, STAT 4, STAT 5A, STAT 5B, STAT 6, STAT 1β, STAT 3β. The expression and the function are highly tissue-specific. They were first discovered in the context of interleukin signalling [98].

STAT transcription factors are activated by phosphorylation of a highly conserved tyrosine Y^{705} residue in the C-terminal near transactivation domain [99, 100]. There is evidence that also phosphorylation of a Serin S^{727} residue, present in some members of the STAT family is necessary to provide full activity of STAT [91].

STAT molecules were also found to bind to the PDGF receptor with their SH2 domain. [101].

After activation, STAT molecules form homo- or heterodimers in a way that the phosphotyrosine of the transactivation domain of one molecule lies next to a N-terminal SH2 domain of the second molecule of the dimer complex. STAT dimers translocate into the nucleus and bind to specific promotor regions on the DNA [98].

There is evidence, that the process of dimerization, activation and internalisation into the nucleus differs within the family of STAT. Some STAT isoforms are constitutively present in the nucleus, others already exist as stable homodimers before being activated. However, the resulting change in DNA transcription and protein synthesis upon activation remains [102-106].

3.4.4.2 Regulation of STAT transcription factors

Negative regulation of STAT-mediated signalling is done by protein inhibitors of activated STAT (PIAS). They act either on the DNA binding region of the STAT dimer or indirectly by suppressing DNA transcription. Another mechanism of regulating the STAT activity is dephosphorylation of the STAT dimer by protein phosphatases like TC-PTP [106].

3.5 Distribution

Distribution and concentration of PDGF is variable in different tissues. As mentioned above, in embryonal tissue PDGF plays a crucial role in growth signalling. In normal tissue, there is often almost no detectable amount of PDGF, but e.g. inflammation can lead to a high increase.

Cause	increased PDGF isoform
high blood pressure	PDGF A ↑
nervous contraction of vessels	PDGF A ↑
shear stress	PDGF A/B ↑
hypercholesteremia	PDGF A/B ↑

Table I: Causes of increased level of PDGF in tissue [107]

4 ROS

Reactive oxygen species (ROS) are highly reactive chemical derivates of oxygen, which have different functions in cells. The highest concentration of ROS can be found in phagocytes, where they act in immunodefense by destruction of incorporated bacteria and germs, but in lower doses ROS can be found in almost every cell, where they act as second messengers for extracellular stimuli [108-110].

4.1 Oxidative Stress and Antioxidants

Due to their high reactivity, ROS are a latent danger for the cell itself. Therefore several defense mechanisms are present to transform ROS into non dangerous species. An imbalance between the ROS level and the capability of degrading them, leads to oxidative stress, leading to damage of DNA as well as of intracellular structures [111].

Several enzymatic mechanisms are able to degrade and to detoxify ROS. Among them are the superoxide dismutase (SOD), catalase and glutathion peroxidase [108, 112, 113]. Furthermore a large number of nonspecific substances like vitamin C, vitamin E, polyphenols are able to bind and inactivate ROS. As these substances are found in a broad variety of food, an appropriate diet can relieve oxidative stress.

4.2 ROS Species

There are several forms of oxygen derivates. Among them are radical species like the superoxide anion and stabile oxygen derivatives like hydrogen peroxide.

Type of Oxygen Forms	Formula	Name
radical species	$O_2^{\cdot -}$	superoxide radical
	$OH^{\cdot}$	hydroxyl radical
	$ROO^{\cdot}$	peroxy radical
	$RO^{\cdot}$	alkoxy radical
stabile oxygen derivates	H_2O_2	hydrogen peroxide
	ROOH	hydroperoxide
	O_3	ozone
	OCl-	hypochlorous anion

Table II: Types of Oxygen molecules

Among them, the **$O_2^{\cdot -}$ - superoxide radical** is the primal source of ROS, from which other species can be derived.

4.3 Genesis of ROS

In VSMCs, ROS are produced intracellularly mainly by the NADPH oxidase enzyme complex [108, 109], which is located at the cell membrane and assembled out of several subunits. The catalized reaction is a transfer of electrons from NADPH to elementar oxygen.

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\cdot -}$$

In the resting state NADPH oxidase consists of a membrane bound subunit, the b^{558} complex, and three cytosolic compounds: $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. The b^{558} membrane bound complex consists of two different subunits, $gp91^{phox}$(=NOX2) and $p22^{phox}$ [108, 109, 111].

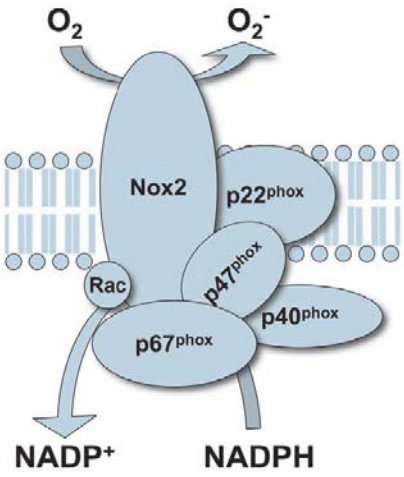


Figure XI

Assembly of a representative NADPH Oxidase containing the NOX 2 protein

The NADPH oxidases form superoxide by transferring an electron from NADPH coenzyme to Oxygen.

Scheme taken from Dworakowski, R. [114]

The activation of NADPH oxidases, e.g. upon stimulation with a growth factor, starts in most cases with phosphorylation of $p47^{phox}$ and the recruitment of the cytosolic subunits towards the membrane where they assemble with the b^{558} complex. Furthermore Rac, a small GTPase binds to the complex [115].

The NOX family consists of several isoforms that are differentiated by the $gp91^{phox}$, part of the b^{558} Complex. $Gp91^{phox}$ found in phagocytes is named NOX2.

There are also some other sources of ROS in the cell like mitochondrial respiratory and enzymatic reactions, but in these reaction chains, ROS are only sideproducts.

4.3.1 Distribution of NOX

NOX proteins were first found in phagocytes, where they provide the highly oxidative environment, necessary for immunodefence, but later several other Nox isoforms (isoforms of $gp91^{phox}$ subunit) were discovered. Their distribution varies:

Nox Isoforms (gp91^{phox})	Found in:
NOX 1	Colon, Smooth Muscle Cells, Uterus, Prostate, Kidney, Stomach, Osteoclasts
NOX 2	Phagocytes
NOX 3	Inner ear
NOX 4	Kidney, Smooth Muscle Cells, Endothelial cells
NOX 5	Testis, spleen, Smooth Muscle Cells

Table III: Distribution of NOX isoforms [108, 116]

NOX 5 has a different structure lacking p22^{phox}, and is therefore only activated by Ca^{2+} release [111].

The exact function of each NOX isoform is not clearly known yet, but the different tissue distribution and activity suggests a highly specified function of each subtype in cell signalling [108].

In vascular cells the most predominant isoforms are NOX1 (VSMC), NOX2 (endothelial cells) and NOX4 (VSMC and endothelial cells). The amount and activity of NOX proteins in vascular cells is sensitive to the influence of growth factors like EGF, PDGF, angiotensin II and insulin, mechanical forces like shear stress and the presence of metabolic factors like free fatty acids or lipids.

4.4 Impact of ROS levels on cellular processes

Aside of their well known use in host defense, Nox-derived ROS act in signal transduction and have been discovered to have multiple and often contradicting effects on cells, tissues and physiologic parameters. ROS are usually associated with inflammation, but also influence anti inflammatory processes.

An important crosstalk between the nitrogenous system and ROS is the inactivation of NO by superoxide. The reaction of superoxide with NO leads to the formation of peroxinitrite, causing a diminished vascular relaxation and higher tonus [108].

ROS furthermore interact with redox-sensitive cysteine residues on proteins and enzymes and hereby modulate their activity. Cysteine-oxidation of protein tyrosine phosphatases, a class of enzymes that inactivate intracelluar messengers by removing phosphate residues, gives ROS a wide influence on activation and inactivation of intracelluar signalling cascades such as MAPK (see also next chapter). It also has an influence on the gene expression, by altering the phosphorylation status of transcription factors, leading to altered expression of e.g. TNFα, TGFβ1, AngII.

ROS are reported on the one hand to trigger senescence and/or apoptosis either directly by damaging proteins, DNA and lipids or more indirectly by altering phosphorylation of apoptotic signalling molecules.

On the other hand proliferation, induced by mitogens such as PDGF and angiotensin, depends on an increased level of ROS which is needed to activate kinases or inactivate phosphatases and hereby to transmit the signal further downstream. PI3K plays a crucial role in mediating the PDGF-induced ROS production [115], acting on Rac1, a GTPase downstream of PI3K and subunit of Noxes [110].

The question remains, why there are so many and also contradicting effects of ROS. It is believed that the different species of ROS, their distinct localization within tissues, cells and compartments, their different sources as well as duration and the strength of the ROS signal lead to this great variety of effects.

4.5 Regulation of PDGF-R signalling by ROS and phosphatases

As mentioned above, PDGF-R phosphorylation and subsequent signalling are controlled by the phospho-tyrosine phosphatases (PTP). This protein family is known to de-phosphorylate specific tyrosine residues and therefore inhibit the recruitment of effector molecules to the activated receptor. Therefore, after the binding of a ligand on the PDGF receptor, the activity of the PTPs has to be transiently suppressed in order to allow initiation of the signalling cascade. Hereby ROS play a crucial role.

In PDGF-related signalling, PTP 1β, PTEN, SHP-1 and SHP-2, DEP and TC-PTP are involved [112]. All of these PTPs bear a cysteine residue in their catalytic center which is sensitive to oxidation. After dimerization of the PDGF receptor, PI3K is docking on phosphorylated tyrosines with its p85 subunit, activating Rac and thus increasing Nox –derived ROS production. Thus, upon PDGF stimulation there is an immediate increase of ROS which leads to inactivation of phosphatases. Like this, receptor phosphorylation can be maintained till the initial signalling steps have occurred.

5 Specific background and aim of this work

Previous work in the lab had identified I3MO as promising compound to interfere with PDGF-mediated proliferation of VSMC in vitro. In vivo, I3MO could also reduce proliferation in a cuff induced neointimal formation model. I3MO could thus serve as a novel lead structure for stent-coating drugs.
Concerning the molecular mode of action I3MO had been found to selectively block phosphorylation of STAT3 without affecting MAPK and AKT upon PDGF stimulation and to dampen PDGF-receptor phosphorylation (PhD thesis Andrea Schwaiberger) [2]. The detailed molecular mechanism how I3MO is mediating these effects remained unresolved.

The aim of this work was to:

1) confirm previous key findings, that

    a. Indirubin 3´monoxime is not affecting the PDGF induced phosphorylation of MAPK Kinases ERK1,2 and AKT.
    b. Indirubin 3´monoxime has an influence on PDGF-induced receptor phosphorylation.

2) investigate the possible role of ROS in the action of I3MO on PDGF signalling since STAT3 phosphorylation is highly redox sensitive.

3) check whether antioxidants can mimic the effect of I3MO.

B MATERIALS AND METHODS

1 MATERIALS

All chemical substances were obtained from Sigma Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland) or Carl Roth (Karlsruhe, Germany) unless otherwise noted.

1.1 Cell culture media and reagents

Name	Provider
Dulbecco´s Modified Eagle Medium (DMEM) 4.5 g/L glucose, w/o L-glutamine, w/o phenol red	Lonza Group Ltd. (Basel, Switzerland)
Penicillin/streptomycin mixture (10,000 U/ml potassium penicillin/ 10,000 µg/ml streptomycin sulphate)	Lonza Group Ltd. (Basel, Switzerland)
L-glutamine 200 mM	Lonza Group Ltd. (Basel, Switzerland)
Trypsin (1:250)	Invitrogen (Carlsbad, CA, USA)
Calf serum	Lonza Group Ltd. (Basel, Switzerland)

Table IV: Cell culture reagents

Calf serum was heat-inactivated (30 min, 56°C), aliquoted and stored at -20°C.

1.2 Growth factors and cytokines

Name	Provider
Recombinant PDGF-BB, human	Bachem (Weil am Rhein, Germany)

Table V: Growth factors and cytokines

Lyophilized PDGF-BB was dissolved in sterile aqua bidest. to a concentration of 0.1 µg/µl and further diluted in sterile PBS + 0.2 % BSA to a final concentration of 10 ng/µl. Aliquots with 20 µl were stored at -20°C and prior to experiments diluted with DMEM to a final concentration of 1 ng/µl. For stimulations 20 ng/ml PDGF-BB were used.

1.3 Indirubin-3´monoxime

I3MO was provided by Laurent Mejer (CNRS, Roscoff, France). It was dissolved in DMSO and stock solutions of 10 mM were stored in aliquots at -80°C.

1.4 Antibodies

Target	Source	Dilution	Provider
Phospho Akt S^{473}	Rabbit	1:1000	New England Biolabs (Beverly, MA, USA)
Phospho Erk1/2 T^{202}/Y^{204}	Rabbit	1:1000	New England Biolabs (Beverly, MA, USA)
Phospho p38 T^{180}/Y^{182}	Rabbit	1:1000	New England Biolabs (Beverly, MA, USA)
Phospho PDGF-R β $Y^{579}/^{581}$	Rabbit	1:1000	Biosource (Camarillo, CA, USA)
phosphoSrc Y^{418}	Rabbit	1:1000	Biosource (Camarillo, CA, USA)
Phospho STAT3 Y^{705}	Rabbit	1:1000	New England Biolabs (Beverly, MA, USA)
α-Tubulin	Mouse	1:1000	Santa Cruz, CA, USA
Phospho tyrosine (P-Tyr-100)	Mouse	1:1000	New England Biolabs (Beverly, MA, USA)

Table VI: Primary antibodies

Target	Source	Dilution	Provider
Rabbit IgG	Goat	1:2500	New England Biolabs (Beverly, MA, USA)
Mouse IgG	Goat	1:2500	Upstate (Charlottesville, VA, USA)

Table VII: Secondary, HRP-linked antibodies

Antibodies were diluted as stated above in TBS-T and if recommended by the provider, prepared with 5 % BSA or 5 % milk powder.

1.5 Solutions

Cell Culture solutions		Used Reagents
Growth Medium	500 ml	DMEM
	10%	Calf Serum
	100 U/ml / 100µg/ml	Penicillin/Streptomycin
	2 mM	L- Glutamine
CS-free medium	500 ml	DMEM
	100 U/ml / 100µg/ml	Penicillin/Streptomycin
	2 mM	L- Glutamine
PBS	8.0 g	NaCl
	0.2 g	KCl
	1.15 g	Na_2HPO_4
	0.2 g	KH_2PO_4
	0.1 g	$MgCl_2 . 6H_2O$
	0.1 g	$CaCl_2 . 2H_2O$
	ad 1000 ml	Aqua dest.
Trypsin/EDTA in PBS	0.05%	Trypsin
	0.02%	EDTA

Solutions Western Blotting		
Lysis buffer (stock solution)	50 mM	HEPES
	50 mM	NaCl
	50 mM	NaF
	10 mM	$Na_4P_2O_7 . 10H_2O$
	5 mM	EDTA
	1 mM	Na_3VO_4
	The reagents were dissolved in 325 ml H_2O (4° C), adjusted to pH 7.5 with NaOH and filled up to a final volume of 430 ml.	in Aqua dest.
- prior to use	4.3 ml	Stock Solution
	50 µl	PMSF 0.1M
	202 µl	Complete™ 25x
	500 µl	TRITON X-100 (10%)
SDS Sample Buffer (stock solution)	37.5 ml	TRIS-HCl Solution 0.5M pH 6.8
	6.0 g	SDS
	30.0 g	Glycerol
	15.0 g	Bromophenol blue
	ad 1000 ml	Aqua dest.
-prior to use (3x solution)	85%	Stock solution

	15%	2-Mercaptoethanol
	For 1x solution the appropriate amount of H_2O was mixed with 3x solution.	
Electrophoresis buffer 5x	15.0 g	TRIS-base
	72.0 g	Glycerine
	5.0 g	SDS
	ad 1000 ml	Aqua dest.
Blotting buffer 5x	15.17 g	TRIS-base
	72.9 g	Glycine
	ad 1000 ml	Aqua dest.
- prior to use	200 ml	5x Blotting buffer
	200 ml	MeOH
	600 ml	Aqua dest.
TBS-T pH 8.0	3.0 g	TRIS-base
	11.1 g	NaCl
	1 ml	Tween-20 (add after adjusting pH)
	ad 1000 ml	Aqua dest.
PMSF	0.1 M	PMSF in Isopropanol
Complete™ 25x (Roche Diagnostics, Penzberg, Germany)	one tablet 2ml	Aqua dest.
Resolving gel 10%	5 ml	PAA solution 30%/0.8% bisacrylamid
	3.75 ml	TRIS-base pH 8.0
	0.15 ml	SDS-10%
	6.1 ml	Aqua dest.
	15 µl	TEMED
	75 µl	APS 10%
Stacking gel	1.7 ml	PAA solution 30%/0.8% bisacrylamide
	1.0 ml	TRIS-HCl pH 6.8
	0.1 ml	SDS 10%
	7.0 ml	Aqua dest.
	20 µl	TEMED
	100 µl	APS 10%
Coomassie staning solution	1.5 g	Coomassie brilliant blue R250
	50 ml	Acidic acid (100%)
	225 ml	EtOH (96%)
	ad 500 ml	Aqua dest.

Coomassie destaining solution	100 ml	Acidic Acid (100%)
	333 ml	EtOH (96%)
	ad 1000 ml	Aqua dest.
Luminol (stock solution)	0.44 g	Luminol
	ad 10 ml	DMSO
p-coumaric acid (stock solution)	0.15 g	p-coumaric acid
	ad 10 ml	DMSO
ECL (homemade)	4.5 ml	Aqua dest.
	0.5 ml	TRIS-base, 1M pH 8.5
- prior to use	25 µl	Luminol (stock solution)
	11 µl	p-coumaric acid (stock solution)
	1.5 µl	H_2O_2 (30%)
Membrane stripping solution	0.11 M	Glycine
	1% (v/v)	SDS in 0.1M HCl

Solution for flow cytometry		
FACS buffer pH 7.37	8.12 g	NaCl
	0.26 g	KH_2PO_4
	2.35 g	Na_2HPO_4
	0.28 g	KCl
	0.43 g	LiCl
	0.2 g	Na_2N_3
	0.36 g	Na_2EDTA
	ad 1000 ml	Aqua dest.
HBSS buffer pH 7.4	8.18 mg	NaCl
	400 mg	Kcl
	19.75 mg	Na_2HPO_4
	50 mg	KH_2PO_4
	180 mg	$CaCl_2 . 2H_2O$
	97.5 mg	$MgSO_4$
	1000 mg	Glucose
	4760 mg	HEPES
	ad 1000 ml	Aqua dest.

Table VIII : Solutions

1.6 Other Equipment

Miscellaneous material	
Name	Provider
Precision Plus Protein™ Standard	BIO-RAD Laboratories (Hercules, Ca, USA)
Gel blotting paper	Whatman plc (Kent, UK)

Table IX: Miscellaneous material

Technical Equipment	
Name	Provider
Vi-Cell™ XR Cell Viability Analyzer	Beckman Coulter (Fullerton, Ca, USA)
Tecan GENios™ Pro, Tecan Sunrise™	Tecan (Mannedorf, Switzerland)
LAS-3000™ Luminescent Image Analyzer	Fujifilm (Tokyo, Japan)
Power Supply Power Pack™ HC	BIO RAD Laboratories (Hercules, Ca, USA)
Mini Trans-Blot™ Electrophoretic Transfer Cell	BIO RAD Laboratories (Hercules, Ca, USA)
FACSCalibur™	BD Biosciences Pharmingen (San Diego, Ca, USA)
Light Microscope Olympus CKX 31	Olympus Europa GmbH (Hamburg, Germany)

Table X: Technical Equipment

EDV Software	
Name	Provider
AIDA™ (Advanced Image Data Analyzer) version 4.06	raytest GmbH (Straubenhardt, Germany)
GraphPad PRISM™ version 4.03	GraphPad Software, Inc. (San Diego, Ca, USA)
Image Reader LAS-3000™ version 2.0	Fujifilm (Tokyo, Japan)
Vi-Cell™ XR 2.03	Beckman Coulter (Fullterton, Ca, USA)

Table XI: EDV Software

2 Methods

2.1 Cell Culture

2.1.1 Cultivation of cells

Vascular Smooth Muscle Cells (VSMC), extracted from male Sprawgue Dawley rat thoracic aortas were used for all experiments.
Cells were cultivated in 75 cm^2 flasks at +37º C and 5% CO_2. Phenol-red free Dulbecco´s modified Eagle´s medium with 10% calf serum, 2 mM Glutamine, 100 U/ml Penicillin and 100 μm/ml Streptomycin was used as growth medium. Cells were grown to nearly 90% confluence.

2.1.2 Passaging the cells

After reaching a confluence of about 90%, cells were harvested with Trypsin/EDTA, pelleted by centrifugation (10 min, 1100 rpm, 4º C) and resuspended in growth medium. Afterwards cells were counted in a ViCell™. With Trypan blue staining, the level of viable cells was determined. Approximately 2 x 10^6 cells were seeded in a 75 cm^2 flask.
For experiments, only cells between passage 7 and 14 were used.

2.1.3 Light microscopy

Cells were checked during the growth period regularly via light microscopy for status of confluence and abnormal varieties.

2.1.4 Preparing cells for experiments

Stimulation of cells was carried out either in 6-well plates or in 60 mm dishes. Cells were seeded after passaging, reaching a confluence of approx. 80-90% on the day of

the experiment. 24 h before the experiment, cells were rendered quiescent by incubation with DMEM without CS.

2.1.5 Stimulation of cells

Before stimulating the cells the CS-free DMEM was replaced by fresh, CS-free medium, then the preincubation with Indirubin 3´monoxime (I3MO) was done at concentrations, indicated in the results section. Control cells, which represent the quiescent status, were treated the same way, containing only the vehicle (DMSO 1%). After 30 minutes of incubation, a time course of PDGF (20 ng/ml) stimulation was performed. In defined time intervals from 2 minutes to 10 minutes PDGF was added to the dishes (2´ 5´ 7´ 10´ minutes).

To terminate the stimulation, cells were washed twice with ice cold PBS and then lysed with lysis buffer containing phosphatase and protease inhibitors for 30 min at 4º C. The cell residues were scrapped off from the plate and transferred into pre-cooled eppendorf-tubes, then centrifuged at 13000 rpm for 10 min at 4º C.

A small amount of the cell-lysate was put aside for determination of the protein level in each plate with the Bradford Assay. The rest was diluted with 3x SDS sample buffer containing bromphenol blue and boiled at 95º C for 5 minutes. Then it was stored at -20º C.

2.2 Preparation of cell lysates for Western Blotting

2.2.1 Protein level quantification

The quantification of the protein level in the obtained cell lysates was examined with the method of Bradford. This method is based on the fact that the maximal absorbance of a Coomassie blue solution shifts from 470 nm to 595 nm when binding to basic, aromatic amino acid residues.

The small probes of the cell lysates kept aside for protein quantification were diluted 1:10 with aqua bidest. 10 µl of each lysate sample were put in triplicate on a 96 well plate and filled up to 200 µl with Bradford Solution. Together with the samples, 10 µl of a calibration standard with an increasing concentration of BSA was brought up on the plate, consisting of 50-500 µg/ml Bovine Serum Albumine (BSA) in aqua bidest. Then the wells were filled up with 190 µl Bradford Solution.

After applying the cell lysates and the calibration standards, the 96-well plate was incubated for 5 minutes. Then the absorbance was measured with a Tecan Sunrise™ microplate reader at 595 nm.

2.2.2 SDS PAGE

Cell Lysates were separated by electrophoresis on a discontinous SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), according to Laemmli.

This method uses SDS, an anionic detergent to destroy the 3D structure of the proteins and polypeptides by binding to hydrophobic regions of the molecule. Furthermore, disulfid bonds are cleaved by treatment with reducing agents such as DTT (Dithiothreitol). SDS is added in surplus, so that the detergent covers the whole protein with a negative charge. Therefore the final separation is based on the molecular weight of the proteins and peptides.

Two kinds of gel layers were prepared to carry out the SDS-PAGE. The first layer concentrates cell lysates to an ultrathin zone (stacking gel) while the second layer

(running gel), finally separates proteins. These gel layers differ in their amount of PAA and therefore in the density of the acrylamide mesh. Gels were formed out of a solution of 30% PAA/0,8% Bisacrylamide. The PAA concentration is chosen in a way that finally the proteins of interest are separated clearly in the middle of the running gel, while other proteins, lighter or heavier in their molecular weight, will not be separated or even run out of the gel.

Equal amounts of proteins were applied in preformed pockets in the stacking layer. Electrophoresis was driven then by a Mini-PROTEAN™ 3 cell system connected to a PowerPac™ HC Power supply at 110 V for 21 minutes to stack the proteins and then at 200 V for 36 minutes for separation.

2.3 Western blotting

2.3.1 Transfer to membranes and protein detection

After the separation of the proteins, the resulted protein bands were transferred to a PVDF membrane using western blot with tank blotting technique. Proteins were transferred for 90 min at 100 V with a Mini Trans-Blot™ electrophoretic transfer cell system.

After the transfer was finished, the PVDF membranes were blocked with 5% fat free milk powder in TBS-T for approx. 1 h. The blocking solution was then washed off and the primary antibody was applied for an overnight incubation at 4º C. To make sure that the proteins were loaded equally, the gel layers were stained seperately with Coomassie staining solution.

The next day the membrane was washed three times with TBS-T and the secondary horseradish-peroxidase conjugated antibody was applied for 3 hours at room temperature. The membrane was washed another tree times with TBS-T and then Protein detection was carried out with a LAS-3000™ luminescent image analyzer using ECL solution. Separated protein bands were quantified with AIDA™ software using α-Tubulin as a loading control.

2.3.2 Stripping of membranes

In order to detect more than one protein on one membrane, the used antibodies can be stripped off with membrane stripping solution.

First the membrane was washed in Aqua dest. for 10 min and then it was treated with membrane stripping solution for 15 min on a shaking platform at room temperature. Afterwards it was washed three times with TBS-T and then blocked with 5% fat free milk powder in TBS-T for one hour. New antibodies were brought up on the membrane as described above.

2.3.3 Staining of gels

To make sure that the proteins were loaded equally on the gel layers, the gel residues were stained with Coomassie blue after carrying out western blotting.
Resolving gel residues were put in a Coomassie blue dying solution on a shaking platform for 20 minutes at room temperature until the dying agent was penetrated into the gel layer. Afterwards the gel residue was washed in TBS-T until the protein bands appear as blue strips against a transparent ground.

2.4 Flow Cytometry

2.4.1 Technical and physical basics

Flow cytometry is based on the emission of optical signals by cells or cell particles when they pass a beam of laser light. Therefore cells are brought in a capillary, where they pass the beam one by one at high speed, so that detections can be made in real time. Flow Cytometry can distinguish cells by their relative size and their relative granularity. Detection can also be done by flourescent agents and the measurement of flourescence intensity. The technique is e.g. used for cell cycle analysis, cell death measurement and apoptosis, membrane potential measurement, expression of proteins and surface antigens and measurement of oxidative burst.

When a cell or a cell particle passes a laser beam it scatters the light in several ways. Hereby the amout of scattered light correlates with the size and the complexity of the cell.
The Forward Scatter (FSC) detects the light that is bended by the cell along the beam, representing a measure for the volume of the cell. The Sideward Scatter (SSC) detects the light fractions scattered at right angle to the laser beam, representing a measure for granularity. The gained signals are detected and processed by a computer system.

Parallel to the measurement of scattered light, flurescence measurement can be done. Therefore a flourescent agent is brought up on the cells, binding to specific cellular components and allowing quantitative measurement of these components. Common flourescent agents are DAPI (4´,6-diamino-2-phenylindole), used in virus detection, Propidiumiodide for detection of viability or H_2DCF-DA. Also antibodies, carrying flourescent groups are used. With different flourescenting dyes and filters and the use of lasers of different colours a big variety of gained information can be reached.

All flow cytometric investigations were performed on a FACSCalibur™ by illuminating the cells with an argon laser (488 nm). H_2DCF-DA was used as a flourescence agent.

2.4.2 ROS measurement

2´,7´-dichloro-dihydroflourescein-diacetate (H_2DCF-DA) was used as a flourescence dye to detect ROS generation in VSMC. The reagent is brought up on the cells in a non flourescent, non reactive diacetate form. After passing the cell membrane, the diacetate is cleaved via cellular esterases to H_2DCF. This molecule is prone to oxidation by reactive oxygen species and thereby transformed in DCF which is a flourescent agent.

Cells were seeded at 0.25 x 10^6 cells/well and kept in growth medium for two days. 24 h before the measurement, cells were set on medium without CS. Wells were washed with HBSS at 37 ºC and then equilibrated with HBSS. Cells were either preincubated with I3MO (3 μM), vehicle (1% DMSO) or left untreated. 15 minutes later, H_2DCF-DA was added to the cells and another 15 minutes later, cells were stimulated with PDGF-BB (20 ng/ml) for 10 minutes. Due to the light sensitivty of DCF, all of these processes had to be performed in a darkened laboratory. After sucking off the buffer and washing, the cells were trypsinised and the stimulation was stopped with BSA 2% in PBS. Immediately after harvesting, cells were brought to the flow cytometer and the geo mean of flourescence intensity was recorded.

2.5 Statistics

All experiments were performed at least three times. Data are expressed as means ± SEM. Statistical analysis was evaluated by one-way ANOVA followed by a Bonferroni multiple comparison test. In case of time courses, treatment with or without I3MO at each time point was compared using one-tailed paired t-test. P values < 0.05 were considered significant. All tests were performed using GraphPad PRISM™ software version.

C RESULTS

1 Impact of Indirubin 3´- monoxime on the phosphorylation of the Kinases ERK1,2 and AKT

1.1 Stimulation with PDGF

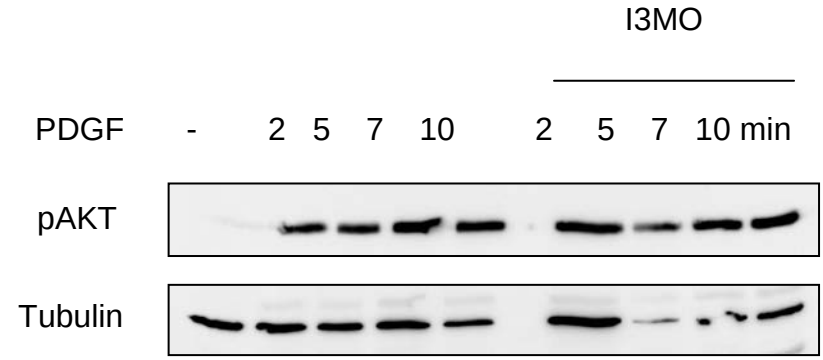
Stimulation of VSMC with PDGF leads to an increase in phosphorylation on Serine/Threonine Kinase AKT and on MAP-Kinases ERK1,2. These kinases are reported to be involved in PDGF-induced proliferation [101, 117, 118]. It is accepted that phosphorylation of these kinases is a sign of activation [119, 120]. We therefore stimulated cells in a time course and revealed the phosphorylation level of the kinases by western blotting with phospho-specific antibodies. For reference we left cells untreated in CS-free medium.

As shown in Figure XII and XIII, PDGF leads to a strong and fast increase in phosphorylation of AKT and ERK1,2 after 2 minutes which does not alter significantly during the whole time course up to 10 minutes.

1.2 Treatment with I3MO

Previous experiments in our lab showed a delay in phosphorylation of AKT and ERK1,2 in response to I3MO, but there was finally no marked reduction in activation of these kinases detectable [2]. In our experiments we could confirm that there is not any significant reduction in phosphorylation of the MAP Kinases ERK1,2 and AKT in the presence of I3MO, as seen in Figure XII and XIII.

Blots and Graphs for pAKT



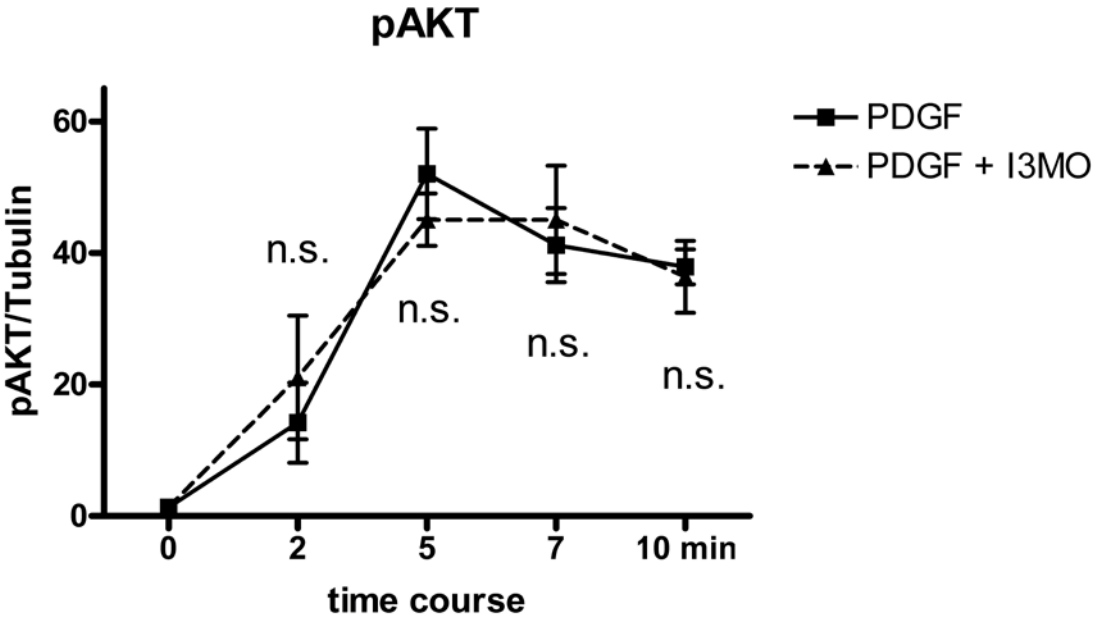
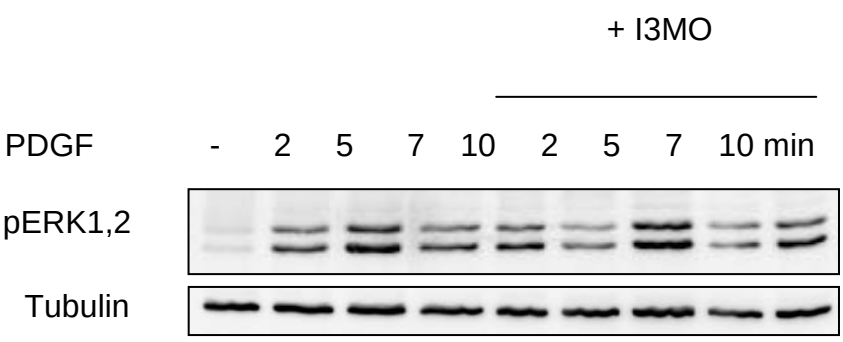


Figure XII

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were incubated with I3MO (3 µM) for 30 minutes or with DMSO (1%) only. After incubation a time course was driven by stimulating the cells for 2, 5, 7, 10 minutes with PDGF-BB (20 ng/ml). As reference cells were left untreated. After western blotting the protein level of pAKT was detected with a phospho-specific antibody as described in the methods section. Tubulin was used as a loading control.

n.s. = not significant ($P > 0.05$) Graphs show averaged data of three independent experiments. (paired t-test, PDGF versus PDGF + I3MO)

Blots and Graphs for ERK1,2



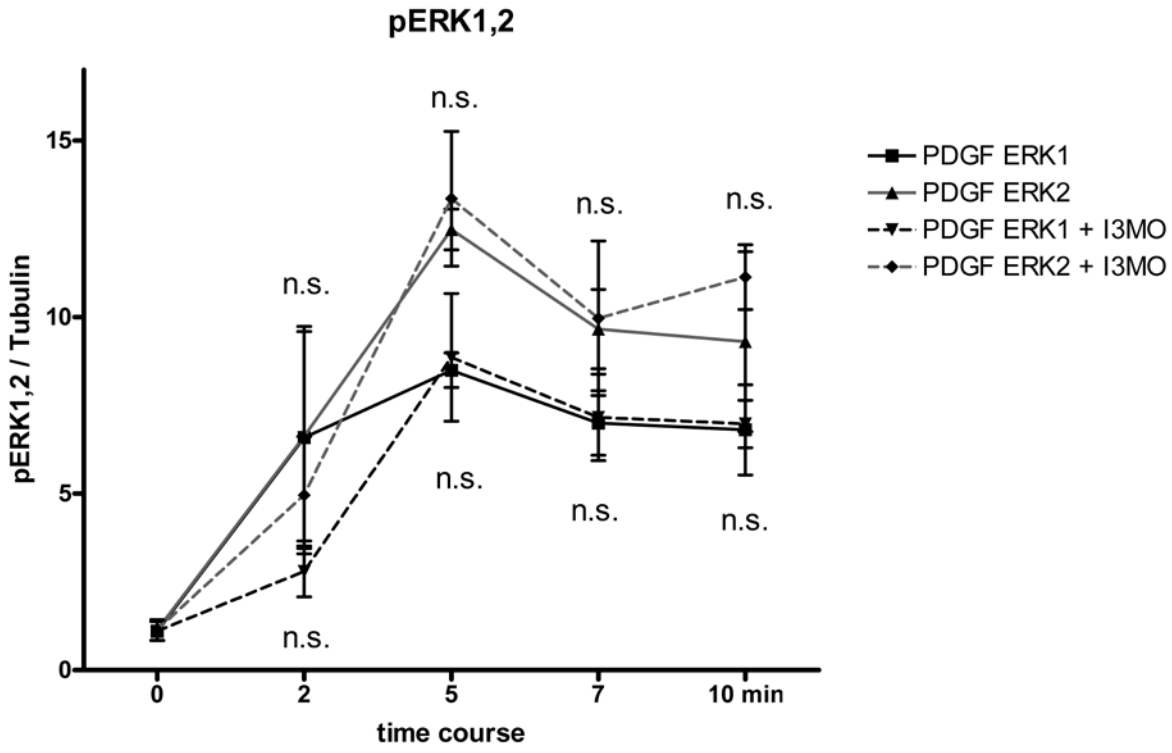


Figure XIII

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were incubated with I3MO (3 µM) for 30 minutes or DMSO (1%) only. After incubation a time course was driven by stimulating the cells for 2, 5, 7, 10 minutes with PDGF-BB (20 ng/ml). As reference cells were left untreated. After western blotting the protein level of pERK1 and pERK2 was detected with a phospho-specific antibody as described in the methods section. Tubulin was used as a loading control. n.s. = not significant ($P > 0.05$) Graphs show averaged data of three independent experiments. (paired t-test, PDGF versus PDGF + I3MO)

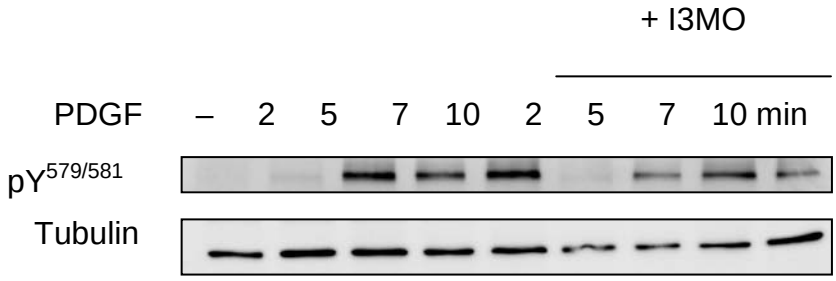
2 Influence of Indirubin on PDGF Receptor Phosphorylation

2.1 PDGF-induced autophosphorylation

It is generally accepted, that binding of the PDGF dimer leads to dimerization and further transphosphorlyation of two receptor monomers [101]. In our experiments we could show, according to previous tests, [2] that PDGF leads to a strong increase in phosphorylation on $Y^{579/581}$.

2.2 Treatment with I3MO

Previous experiments in our lab showed a reduction of phosphorylation of $Y^{579/581}$ in the presence of Indirubine 3-monoxime [2]. Our experiments could also show a significant reduction in phosphorylation at 5 min. However, in contrast to prior experiments other time points failed to be significant.



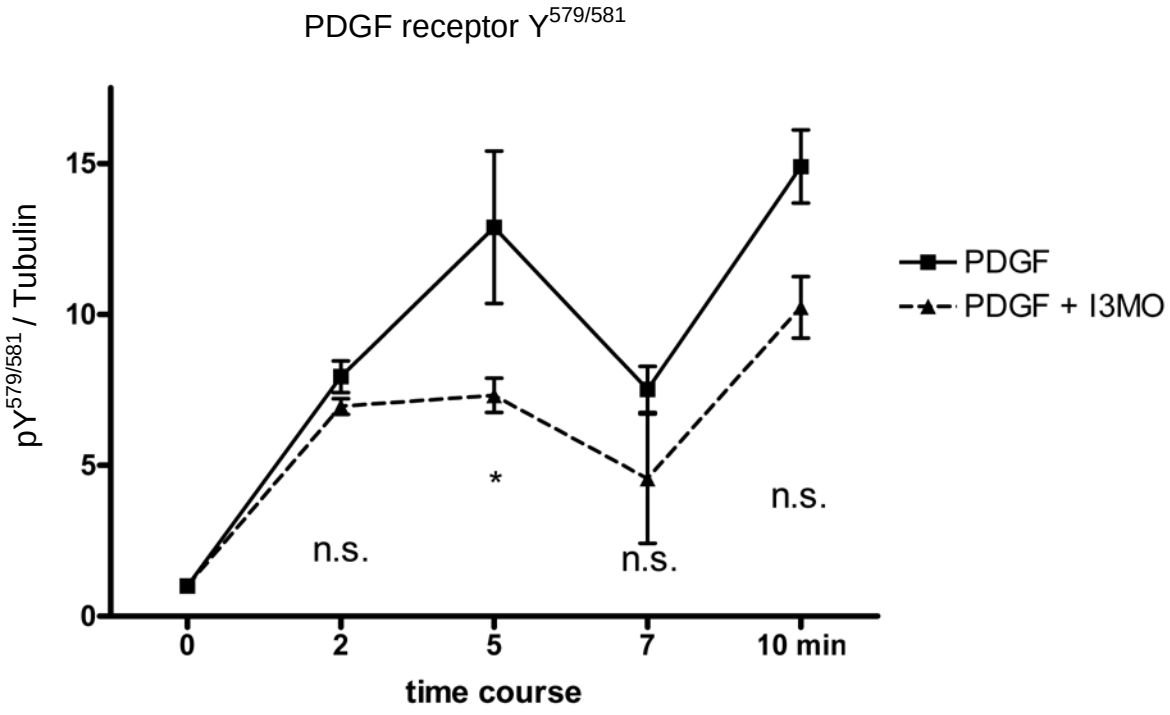


Figure XIV

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were incubated with I3MO (3 µM) for 30 minutes or with DMSO (1%) only. After incubation a time course was driven by stimulating the cells for 2, 5, 7, 10 minutes with PDGF-BB (20 ng/ml). As reference, cells were left untreated. After western blotting the phosporylation level of PDGF-R $Y^{579/581}$ was detected with a phospho-specific antibody as described in the methods section.Tubulin was used as a loading control.

* = $P < 0.05$ n.s. = not significant ($P > 0.05$) Graphs showa veraged data of three independent experiments. (paired t-test, PDGF-BB versus PDGF-BB + I3MO)

3 Influence of Indirubin 3´-monoxime on ROS mediated signalling

Prior experiments in our lab led to the hypothesis that Indirubin 3´-monoxime acts on STAT via reduction of intracellular ROS. Experiments with N-Acetyl Cystein (NAC), a precursor of glutathion -an important cellular antioxidant-, were performed to elucidate the influence of ROS on targets of several pathways involved in PDGF induced proliferation.

3.1 Impact of Indirubin 3´-monoxime on the kinases ERK1,2 compared to the Antioxidant NAC

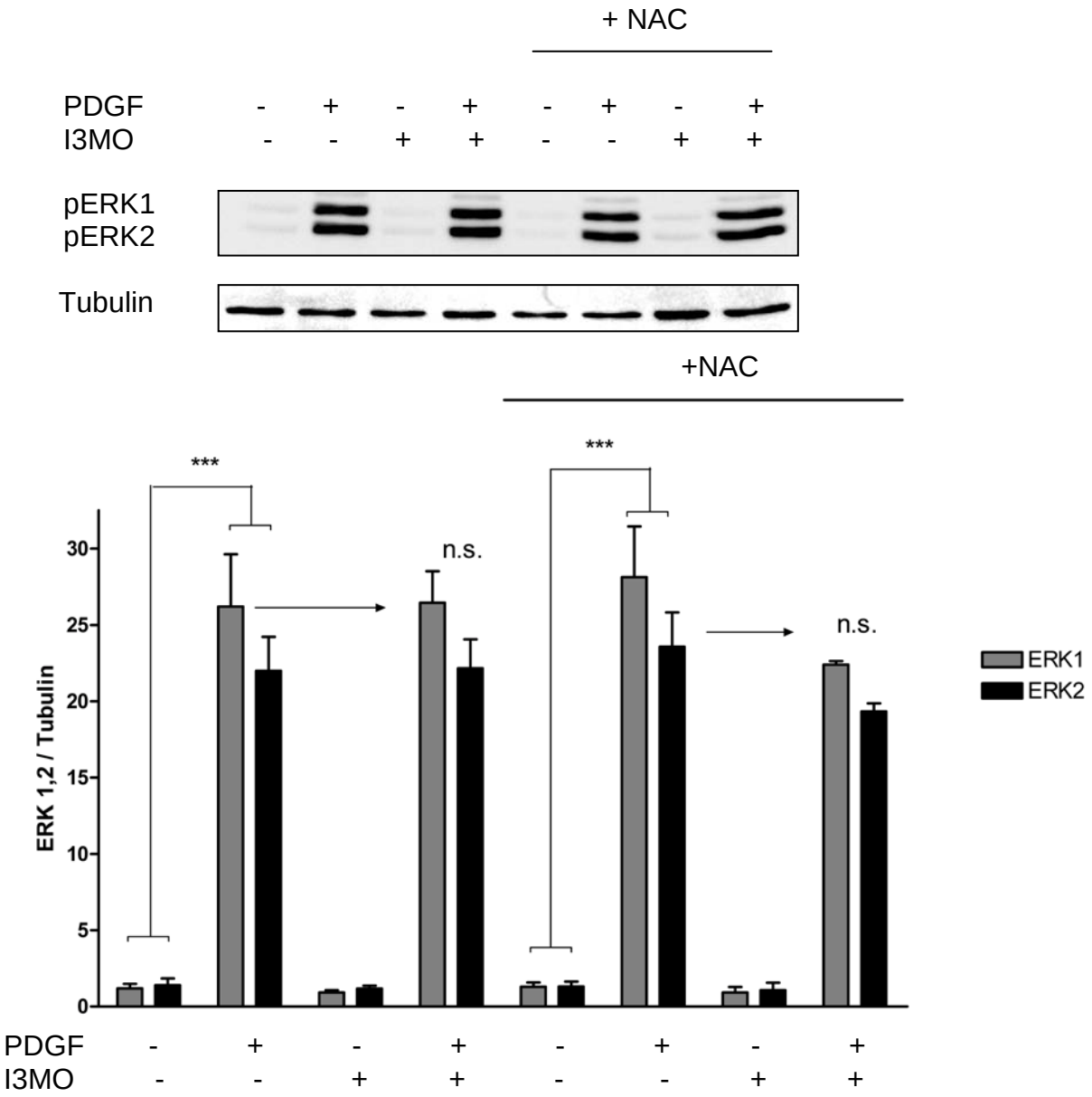


Figure XV

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were pretreated with NAC (10 mM) for one hour, giving time to synthesize glutathion as an antioxidant. As reference cells were left untreated. 30 minutes before stimulation I3MO (3 µM) was added. Stimulation was performed for 10 minutes with PDGF-BB (20 ng/ml). After western blotting the phosporylation level of Erk1,2 was detected with a phospho-specific antibody as described in the methods section. Tubulin was used as a loading control. Graphs show averaged data of three independent experiments. (one-way ANOVA versus PDGF-BB treatment) *** = $P<0.001$, n.s. = not significant ($P > 0.05$)

3.1.1 Impact of NAC on ERK1,2 activation

Treatment with PDGF leads to a strong phosphorylation of ERK Kinases which is not affected by the presence of NAC.

3.1.2 Impact of I3MO on ERK1,2 compared with NAC

In the presence of I3MO the induction of phosphorylation by PDGF is, according to previous findings, not significantly reduced. Also the presence of NAC together with I3MO does not influence the activation of ERK1,2.

3.2 Impact of Indirubin 3´-monoxime on AKT Kinase compared to the Antioxidant NAC

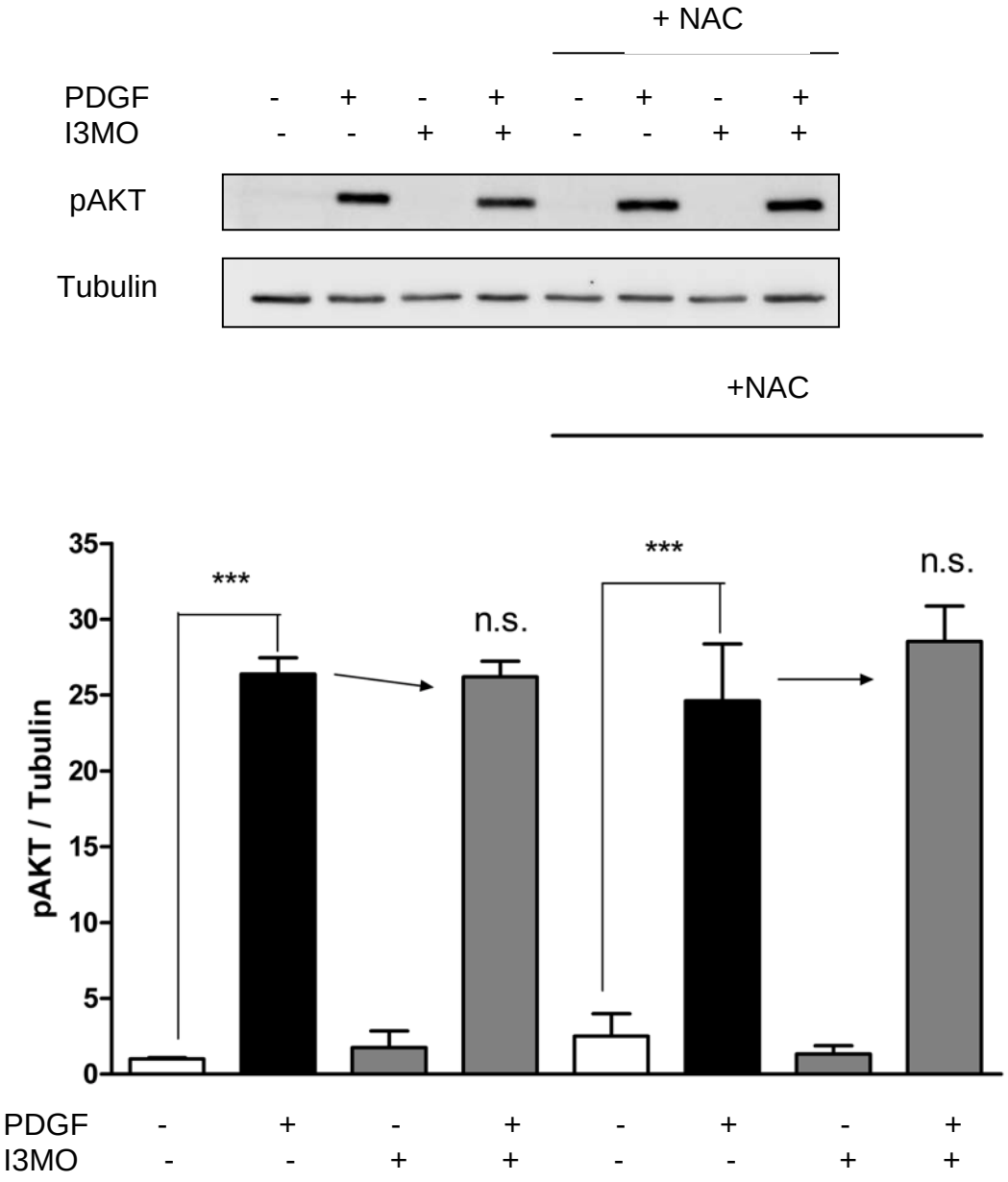


Figure XVI

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were pretreated with NAC (10mM) for one hour, giving time to synthesize glutathion as an antioxidant. As reference cells were left untreated. 30 minutes before stimulation I3MO (3 µM) was added. Stimulation was performed for 10 minutes with PDGF-BB (20 ng/ml). After western blotting the phosporylation level of pAKT was detected with a phospho-specific antibody as described in the methods section.Tubulin was used as a loading control. Graphs show averaged data of three independent experiments. (one-way ANOVA versus PDGF-BB treatment)

*** = $P < 0.001$, n.s. = not significant ($P > 0.05$)

3.2.1 Impact of NAC on AKT activation

Treatment with PDGF leads to a strong phosphorylation of AKT Kinase, which is not affected by the presence of NAC.

3.2.2 Impact of I3MO on AKT compared with NAC

In the presence of I3MO the induction of phosphorylation is, according to prior findings, not significantly reduced. Also the presence of NAC together with I3MO does not influence the activation of AKT.

3.3 Impact of Indirubin 3´-monoxime on STAT3 compared to the Antioxidant NAC

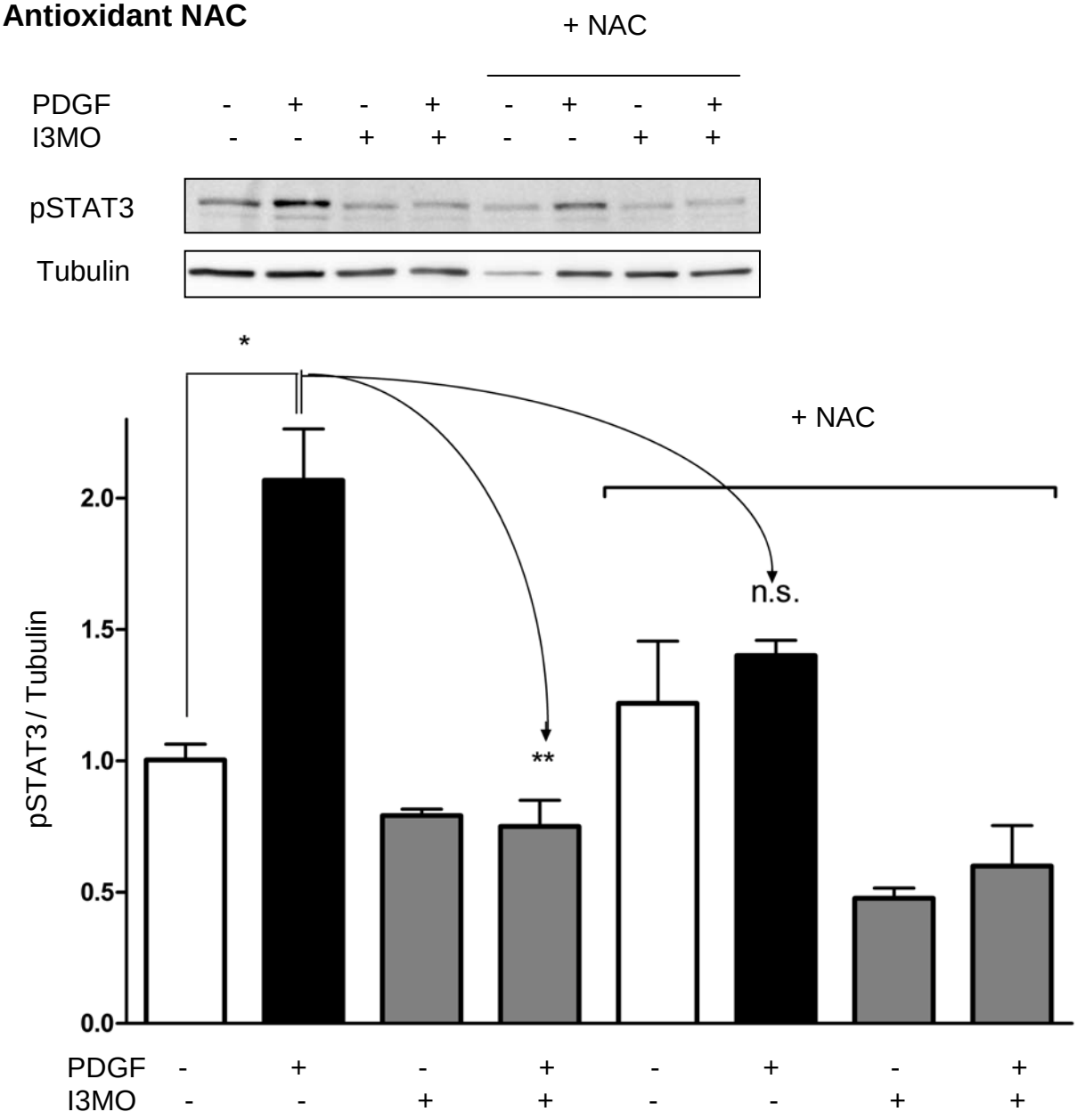


Figure XVII

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were pretreated with NAC (10mM) for one hour, giving time to synthesize glutathion as an antioxidant. As reference cells were left untreated. 30 minutes before stimulation I3MO (3 µM) was added. Stimulation was performed for 10 minutes with PDGF-BB (20 ng/ml). After western blotting the phosporylation level of pSTAT3 was detected with a phospho-specific antibody as described in the methods section.Tubulin was used as a loading control. Graphs show averaged data of three independent experiments. (one-way ANOVA versus PDGF-BB treatment)

* = $P < 0.05$, n.s. = not significant ($P > 0.05$)

3.3.1 Impact of NAC on STAT3 activation

Treatment with PDGF leads to a strong increase in STAT3 phosphorylation, which appears reduced in the presence of NAC. However, data are not statistically significant.

3.3.2 Impact of I3MO on STAT3 compared with NAC

In the presence of I3MO the phosphorylation of STAT3 in response to PDGF is, according to previous findings, significantly reduced. The presence of NAC does not reveal any significant synergistic effects in reducing phosphorylation level of STAT3.

3.4 Impact of Indirubin 3´-monoxime on PDGF receptor autophosphorylation compared to the Antioxidant NAC

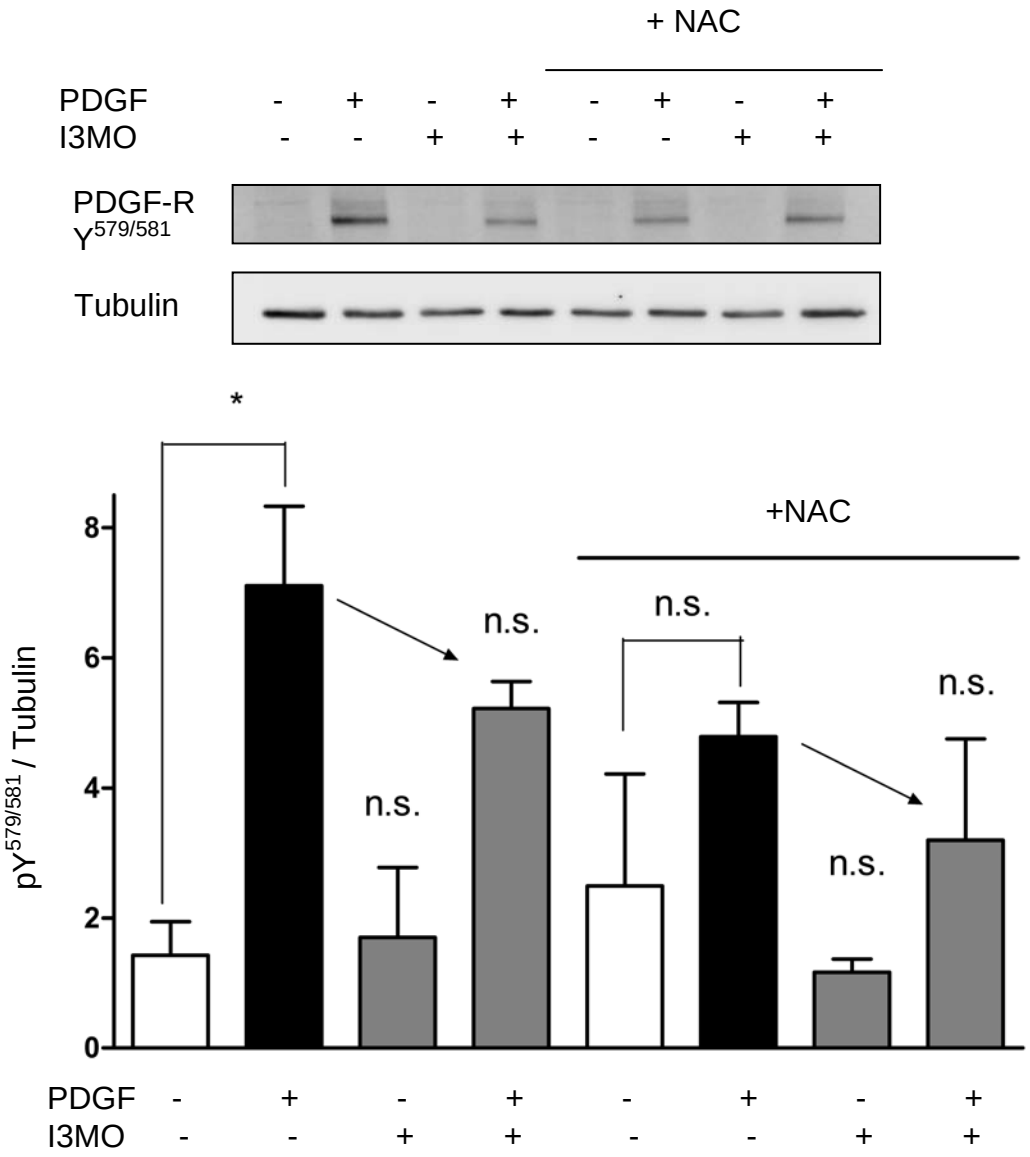


Figure XVIII

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were pretreated with NAC (10mM) for one hour, giving time to synthesize glutathion as an antioxidant. As reference cells were left untreated. 30 minutes before stimulation I3MO (3 µM) was added. Stimulation was performed for 10 minutes with PDGF-BB (20 ng/ml). After western blotting the phosporylation level of PDGF receptor $Y^{579/581}$ was detected with a phospho-specific antibody as described in the methods section.Tubulin was used as a loading control. Graphs show averaged data of three independent experiments (one-way ANOVA versus PDGF-BB treatment) * = $P < 0.05$ n.s. = not significant ($P>0.05$)

3.4.1 Impact of I3MO on PDGF receptor phosphorylation

Treatment with PDGF leads to a strong increase in PDGF receptor autophosphorylation, which appears reduced in the presence of NAC. However, data were not statistically significant.

3.4.2 Impact of I3MO on PDGF receptor phosphorylation compared to NAC

In the presence of I3MO the autophosphorylation of the PDGF receptor in response to PDGF is, in contrast to prior findings, not significantly reduced. The presence of NAC does not reveal any significant synergistic effects in reducing PDGF-R phosphorylation level, although the standard error of the mean at I3MO + NAC is too high to make clear statements.

3.5 Effect of Indirubin 3´-monoxime on the level of Reactive oxygen Species

Western Blot data from previous experiments suggested an influence of Indirubin 3´-monoxime on STAT3 (not shown) and PDGF Receptor phosphorylation. The influence of Indirubin 3´-monoxime could be mediated via protein tyrosin dephosphorylating phosphatases, which are regulated in their activity via ROS.

In order to check the Influence of Indirubin 3´-monoxime on ROS level, we set up a flow cytometric experiment in which reactive oxygen species were quantified by the flourescent agent H_2DCF. Although the data did not reach significance the PDGF-triggered flourescence after treatment with Indirubin 3´-monoxime appears to be reduced. (Figure XIX)

Previous experiments with Indirubin 3´-monoxime even showed a reduction of reactive oxygen species when I3MO is added at the time of PDGF stimulation instead of beeing added 15 minutes prior to stimulation [2].

3.5.1 Time-dependent Impact of I3MO on Reactive Oxygen Species Level

Using DCF and flow cytometry, we measured the flourescence in VSMC, which correlates to the level of intracellular ROS. Indirubin was again added at different timepoints with regard to PDGF stimulation in order to evaluate probable mechanisms of action.

1) Indirubin-3´monoxime added 15 minutes before stimulation with PDGF
2) Indirubin-3´monoxime added simultanously with stimulating PDGF
3) Indirubin-3´monoxime added 5 minutes after PDGF stimulation

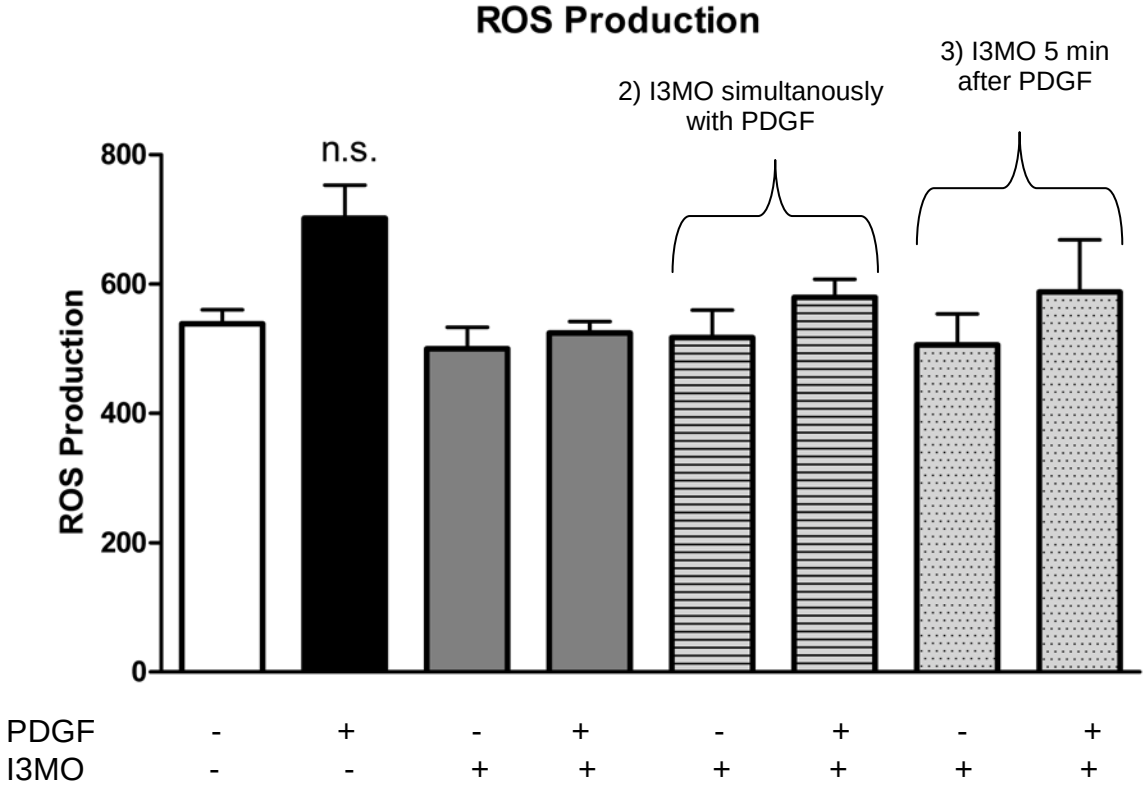


Figure XIX

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were pretreated with I3MO (3 µM) or vehicle (DMSO 1%) for 15 min. H_2DCF (20 µM) was added and cells were incubated for another 15 min. Stimulation was performed for 10 minutes with PDGF-BB (20 ng/ml). I3MO (3 µM) was also added simultanously with PDGF-BB stimulation or 5 minutes afterwards. Flow cytometry was performed right afterwards as described in the methods section. Graphs show averaged data of four independent experiments performed in triplicate. (one-way ANOVA versus PDGF-BB treatment) n.s. = not significant ($P > 0.05$)

Although the results did not reach significance, there was a tendency towards a reduced ROS level after I3MO incubation prior to PDGF stimulation. If Indirubin 3´-monoxime is added simultanously to PDGF, reduction holds on. Even if Indirubin is added 5 minutes after PDGF stimulation, a reduction of ROS is visible, possibly pointing to targets for Indirubin which are involved in signalling processes occurring up to 5 minutes after PDGF stimulation.

3.5.2 Impact of I3MO on ROS level compared to NAC

In the following setting, the measurement of ROS level in VSMC was performed in the presence of I3MO as well as in the presence of NAC. The aim of this experiments was to determine if the effect of Indirubin could also be achieved by adding NAC, a known antioxidant. Furthermore we added NAC together with I3MO in order to check synergistic effects. Previous experiments excluded a direct antioxidative, radical scavenging action of Indirubin on ROS [2].

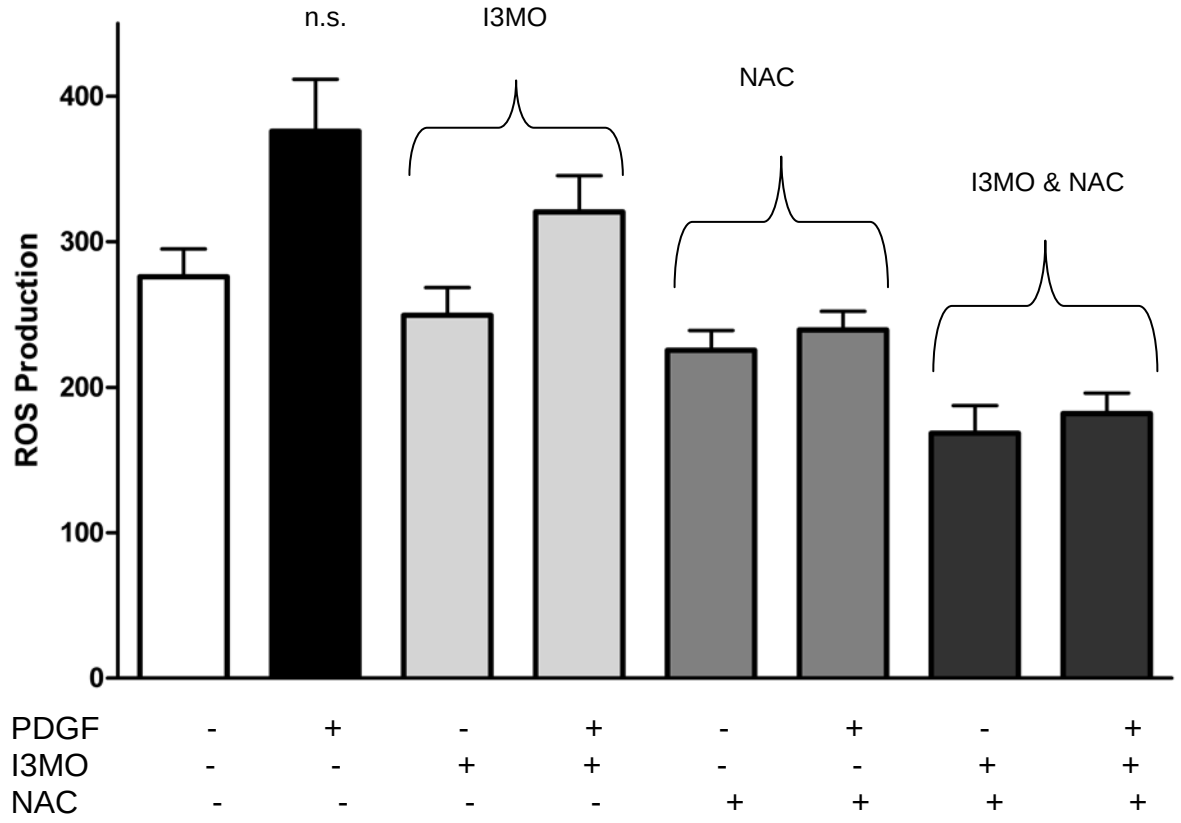


Figure XX

VSMC were grown to near confluence and kept in CS-free medium for 24 hours. They were pretreated with NAC (10mM) for one hour, giving time to synthesize glutathion. I3MO (3 µM) or vehicle (DMSO1%) was added 15 min before stimulation. Stimulation was performed for 10 minutes with PDGF-BB (20 ng/ml). Flow cytometry was performed right afterwards as described in the methods section. Graphs show averaged data of four independent experiments.

(one-way ANOVA versus PDGF-BB treatment) n.s. = not significant ($P > 0.05$)

D Discussion

1 Influence of I3MO on phosphorylation of Kinases AKT and ERK1,2

The stimulation of VSMCs with PDGF leads to an increase in the phosphorylation on Serine/Threonine kinase AKT and the MAP Kinase ERK1,2. It is accepted that phosphorylation of these kinases is a sign of activation [119, 120]. The activation of the PI3K/AKT pathway as well as the MAP-Kinase pathway is related to cell proliferation and migration [101, 117]. Prior experiments in our group showed that there is no influence of I3MO on the PDGF-mediated increase in phosphorylation of these kinases [2]. As shown in Figure XII and XIII, we could confirm previous results, showing that there is no influence on the phosphorylation of these kinases from I3MO.

I3MO seems not to mediate its negative effect on cell proliferation via inhibition of the phosphorylation of PDGF-activated Kinases AKT and ERK1,2.

2 Influence of I3MO on phosphorylation of STAT3 transcription factor

PDGF is also known to induce the activation of the JAK/STAT pathway [95]. Previous findings in our lab showed a significant reduction of the PDGF-induced phosphorylation of STAT3 by I3MO. We also wanted to confirm these findings, but for unknown reasons our experiments failed to produce usable results.

3 Influence of I3MO on phosphorylation of the PDGF receptor

It is generally accepted, that PDGF receptor activation leads to association of two PDGF receptor monomers and further transphosphorylation [63]. We examined the phosphorylation on PDGF receptor $Y^{579/581}$. As previous experiments in our group showed, I3MO seems to have a diminishing effect on phosphorylation of these tyrosin residues, as well as on the overall phosphorylation of the receptor [2].
As shown in Figure XIV, treatment with Indirubine 3´-monoxime leads to a visible and reproducible reduction of the phosphorylation on $Y^{579/581}$, although, in contrast to

previous findings, the results lack to be significant in most of the timepoints. Possible reasons for the unsignificant results could be the great variation between the individual experiments. This variance, which is not unusual for cell-based model systems, results in huge standard deviations.

4 Influence of Indirubin 3´-monoxime on ROS-mediated signalling

Reactive oxygen species as second messengers are known to be involved in PDGF signal transduction, because several PDGF-related pathways are reported to require ROS [111, 112, 121, 122]. This led to the hypothesis that I3MO acts on PDGF signalling via influencing the signal transduction by modulating levels of reactive oxygen species.

Experiments, done with N-Actetyl Cystein, a precursor of the intracellular antioxidant glutathion, should reveal, whether the effect of I3MO on kinase pathways, as well as on STAT3 transcription factor could be mimiked by an antioxidant. As shown in Figures XV and XVI, the presence of NAC does not have any inhibitory effect on the PDGF-mediated phosphorylation of the Kinases AKT and ERK1,2. This correlates with the previous findings, that I3MO has no influence on these kinases. (See figures XII and XIII)

The effect of NAC on PDGF-mediated activation of STAT3 is shown in Figure XVII. Although the results do not reach significance, there is a tendency towards reduced levels of phosphorylated STAT3 in the presence of NAC as well as in the presence of I3MO.

Taking into account, that I3MO affects the kinases AKT and ERK1,2 as well as the STAT3 transcription factor in the same way as NAC, the presumption can be raised, that I3MO indeed exerts its effect by influencing the level of reactive oxygen species. This is in line with previous findings, showing that inhibition of ROS by the NADPH oxidase inhibitor DPI reduces STAT3 phosphorylation [2].

The influence of NAC on PDGF receptor $Y^{579/581}$ transphosphorylation is also comparable to the influence obtained with I3MO. As Figure XVIII shows,

phosphorylation of $Y^{579/581}$ appears to be reduced in the presence of I3MO as well as in the presence of NAC (although not significant). This datas together with previous results [2], suggests an influence of ROS on regulation of PDGF receptor phosphorylation, as well as a possible mechanism of I3MO similar to NAC. Potential targets influenced by modulated ROS levels are phosphotyrosine phosphatases.

5 Influence of I3MO on ROS production

Previous flow cytometric experiments to examine the influence of I3MO on PDGF-triggered elevation in intracellular ROS production were repeated [2]. In order to put light on probable mechanisms of action of I3MO on PDGF receptor-signalling. I3MO was added at three different timepoints. In all experiments cells were treated with I3MO 15 minutes prior to, simultaneously with and 5 minutes after PDGF stimulation, respectively.

Figure XIX shows, a visible reduction in PDGF-triggered ROS production after incubation with I3MO. In all settings, even application of I3MO simultaneously and 5 minutes after stimulation led to a visible decrease of ROS production. However, the results did not reach significance. This is maybe due to the low increase in ROS after PDGF treatment in comparison to the basal level of ROS.

6 Influence of I3MO on ROS production in the presence of NAC

Further we wanted to determine, wether the effect of I3MO on PDGF-triggered ROS production could also be achieved by an antioxidant and if this effect is similar to I3MO. Prior experiments excluded a direct antioxidative, radical scavenging action of indirubin [2].

In these flow cytometric experiments we incubated cells with I3MO, NAC and also applied both substances together, in order to reveal any synergistic effect. As seen in figure XX, the antioxidant NAC implemented almost the same effect on PDGF-treated cells like NAC. Applying NAC and I3MO together revealed no synergistic effect.

7 Concluding remarks and outlook

Overall, we could confirm previous data, that I3MO does not affect PDGF-induced ERK1,2 and AKT phosphorylation, but inhibits STAT3 phosphorylation and dampens PDGF receptor phosphorylation. I3MO may decrease PDGF-induced ROS formation up to 5 minutes after stimulation. I3MO mimics the effect of NAC, a known antioxidant, with regard to MAPK-, AKT- and STAT phosphorylation and ROS production, which highlights ROS as a potential target of I3MO. Synergistic action of NAC and ROS could not be observed.

Indirubin and its derivatives inhibit proliferation of VSMC and could be used in various therapeutic applications. In a cuff-induced neointimal formation mouse model for example could be shown, that I3MO reduces the formation of a neointima in mouse arteries [2]. In order to optimize the chemical structure or find derivatives with a better pharmacodynamic profile than Indirubin 3´-monoxime, it is necessary to elucidate the molecular mechanism of its antiproliferative action. Further experiments should determine how Indirubin 3´-monoxime acts on intracellular ROS production, and how this influence is linked with the STAT3 phosphorylation and the cell cycle machinery.

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APPENDIX

A Abbreviations

In alphabetical order

A

AhR	Aryl hydrocarbon Receptor
ATII	Angiotensin II

C

c-Fos	DNA binding transcription factor
c-Jun	DNA binding transcription factor
c-Myc	DNA binding transcription factor
CYP P450	Cytochrome P450 Enzyme Family

D

DEP	Phosphotyrosin Phosphatase
DTT	Dithiothreitol

E

EGF	Epidermal growth factor
ELK1	Ets Like gene1 – transcription factor

F

FGF	Fibroblast growth factor

G

GAP	GTPase activating proteins
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide exchange factor
Grb2	Adaptor protein involved in Cytokine signalling
GSK-3	Glycogen Synthase Kinase 3
GTP	Guanosine Triphosphate

I

I3MO	Indirubine 3´-monoxime
IFNγ	Interferon gamma
IGF1	Insuline-like growth factor

IL-6	Interleukin (-6)

J

JAK	Tyrosin kinase – Just Another Kinase/Janus Kinase
JNK	c-Jun N-terminal kinases

M

MAPK	Mitogen Activated Protein Kinase

N

NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFκB	Nuclear Factor κB
NOX	NADPH Oxidase

P

PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PCB	Polychlorinated biphenyls
PCTA	Percutaneus transluminal coronary angioplasty
PDGF	Platelet derived Growth factor
PIAS	Protein Inhibitors of activated STAT
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog – a phosphatase
PTP1β	Phosphotyrosin Phosphatase
PVDF	Polyvinylidenflouride

R

Rac	small GTPases – Subfamily of Rho family of GTPases
Raf1	Serine/Threonine specific kinase
Ras	small GTPase
ROS	Radical Oxigen Species

S

SH	Src homology Domain
SHIP	Phosphatase
SDS	sodium dodecyl suphate
SDS-Page	sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SOCS	Supressor of Cytokine signalling
SOD	Superoxide dismutase
SOS1	“Son of Sevenless” protein
Src	Tyrosin kinase –named after similar kinases found in “Rous Sarcoma Virus”
STAT	Signal Transducers and activator of transcription

T

TBS-T	Tris-buffered saline containing – Tween 20
TC-PTP	T-Cell Phosphotyrosin Phosphatase
TGFβ	Transforming growth factor
TNFα	Tumor necrosis factor

V

VSMC	Vascular Smooth Muscle Cells

B Sources of Graphics

Figure I	Thesis Andrea Schwaiberger [2]
Figure II	Download from http://www.sb-roscoff.fr/indirubin/intro.html
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Figure VI	Download from http://www.heart.org.in/diseases/images/stent_restenosis.gif
Figure VII	Andrae et al. [47]
Figure XI	NADPH oxidase-derived reactive oxygen species in the regulation of endothelial phenotype Rafa Dworakowski, Sara P. Alom-Ruiz, Ajay M. Shah

All other graphics were created by the author.

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PC/ Mac Erfahrung
MS Office
AVS - Apothekerverlags Software

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D Acknowledgments

First, I want to thank Univ.-Prof.Dr. Verena Dirsch for the opportunity to work at this diploma thesis and Dr. Andrea Schwaiberger who introduced me to the work in the cell culture, guided my experiments and helped me on writing.

For good cooperation and their friendliness I want to thank Elke Heiss who helped me writing my work and was always availiable for advice.

A special thank to Helge for tea and talks and to Christoph for cell donations and advice. Further I want to thank the whole team of the department for support and guidance.

For their support and encouraging words at any time, I want to thank my parents and my brother, who helped me in doing the graphics.