CHARLES UNIVERSITY IN PRAGUE
1ST FACULTY OF MEDICINE

THE ROLE OF NAD(P)H OXIDASE IN SIGNAL TRANSDUCTION

THESIS

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MARTIN VEJRAŽKA
tutor: STANISLAV ŠTÍPEK

Introduction

Reactive oxygen species (ROS) are intensively studied because of their role in pathogenesis of variety of diseases. Database MEDLINE lists more than 160,000 entries with terms "*reactive oxygen species*", "*ROS*" or "*free radical*" and most of these studies deal with toxic effects of ROS. Concept of free radical toxicity explains, however, only one aspect of ROS function in the body.

Growing body of evidence shows that low, subtoxic amounts of reactive oxygen species play indispensable physiological role, mainly in cell signalling. The concentration of ROS is a result of their production on one side and decay on the other side. Although there is couple of sources of ROS, NAD(P)H oxidases seem to be most important for signalling. Peroxiredoxins and thioredoxins are key players in scavenging of low levels of ROS.

NAD(P)H oxidase

NADPH oxidase is a well described enzyme of professional phagocytes. Oxidising NADPH, it reduces molecular oxygen to superoxide $O_2^{\cdot -}$. Superoxide is than converted to hydrogen peroxide and further to other reactive oxygen species. ROS formed in this process serve as microbicidal agents, chemoattractants and mediators of inflammation. They are also responsible for collateral damage to tissues in inflammatory response.

Phagocyte NADPH oxidase is a multi-subunit enzyme. It consists of two membrane subunits, $gp91^{PHOX}$ (recently referred as Nox2) and $p22^{PHOX}$. Both membrane subunits are combined to cytochrome b_{558}. In order to activate NADPH oxidase, membrane subunits must be assembled with cytosolic subunits p47 PHOX, $p40^{PHOX}$, $p67^{PHOX}$ and a small G-protein rac. Assembly of NADPH oxidase is triggered by fosforylation of $p47^{PHOX}$ dependent on proteinkinase C.

Non-phagocyte NAD(P)H oxidase

NADPH oxidase activity was found in many non-phagocyte cells. Indeed, many subunits of this enzyme were found in plenty of cell types from various tissues. Contemporarily, seven isoenzymes of NADPH oxidase are known. They are usually denoted according to $gp91^{PHOX}$-like membrane subunit.

Nox1

Nox1 was the first discovered homologue of phagocyte NADPH oxidase. It is expressed in epithelial cells of colon, smooth muscle cells, uterus, prostate, kidneys, stomach and osteoclasts. Assembly with protein homologous to $p47^{PHOX}$ denoted as NOXO1 and another protein homologous to $p67^{PHOX}$ (NOXA1), as well as with $p22^{PHOX}$ and Rac is required for activity of Nox1. The physiological significance of Nox1 probably differs in various organs. It is probably a part of immune barrier of bowel. In other tissues it forms ROS rather for signaling. Nox1 also plays a role in angiotensin II induced arterial hypertension. Its activity is induced by some growth factors.

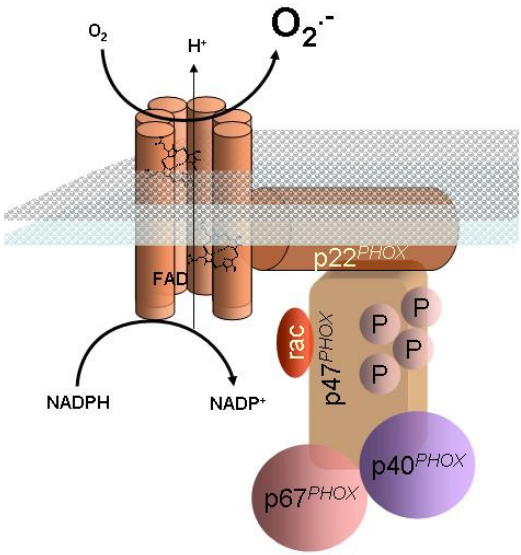

Fig. 1: **NADPH oxidase of phagocytes**

Nox2 is a new name for $gp91^{PHOX}$

Nox3
Nox3 is required for function of vestibular and cochlear system. ROS produced by this enzyme are probably involved in formation of otoconia. Activity of this isoenzyme depends on $p22^{PHOX}$; cytosolic subunits are not required, but increase its activity.

Nox4
Nox4 was originally described as "renal oxidase", but it is present also in fetal liver, smooth muscle cells, osteoclasts, hematopoetic cells, adipocytes, and umbilical epithelial cells. In contrast to other isoenzymes it was found in the intracellular space. Nox4 forms a complex with $p22^{PHOX}$ but is probably independent on cytosolic subunits. Its activity is probably controlled by regulation of expression. Nox4 produces superoxide with simultaneous oxidation of not only NADPH, but also (and predominantly) NADH. it is also capable of direct reduction of molecular oxygen to hydrogen peroxide. Nox4 is involved in regulation of erythropoetin, in pathogenesis of diabetic nephropathy and in insulin-induced ROS production. It is probably the main source of ROS in endothelium.

Nox5
Nox5 was found due to its homology to cDNA of Nox2. It was detected in spleen, testis, in fetal tissues, and in lower amounts in ovarium, placenta, pancreas, smooth muscle cells, bone marrow and uterus. Its activity is regulated by calcium. The physiological role of Nox5 is unclear.

Duox1 and 2
Dual oxidases Duox1 and Duox2 are enzymes of thyroid gland. These proteins are much bigger than other NAD(P)H oxidases and except of NADPH oxidase activity posses also peroxidase activity. Both Duox1 and 2 reduce oxygen directly to hydrogen peroxide and are regulated by Ca^{2+}. Duox1 was found also in respiratory tract, pancreas, placenta, prostate and testis. Duox2 is present in gastrointestinal tract and respiratory epithelium.

Vascular oxidase

NAD(P)H oxidases are the major source of superoxide in vessels. Nox1, 2, 4 and 5 were found in all cellular types of vascular wall. All these enzymes contribute to total ROS production in vessels; therefore, the term "vascular oxidase" was introduced. Most important properties of vascular oxidase include:

- Low activity. ROS production by vascular oxidase is about 1 – 10% of phagocytic one.
- Non-stimulated activity. In contrast to phagocytic enzyme, vascular oxidase is characterised by basal activity. Regulation of vascular oxidase differs from phagocytic oxidase.
- Subcellular localisation. A great part of vascular oxidase is found in intracellular space. Superoxide is however not release directly to cytosol but rather to vesicles and organells, including nucleus.
- Early and late activation. Vascular oxidases may be activated in time period of several minutes to several hours. Early response is due to phosphorylation and enzyme assembly, late response is regulated by gene expression.
- Role in signal transduction. Vascular oxidase produces low, subtoxic concentrations of ROS and plays a role in signal transduction. It stimulates some transcription factors and protein kinases and inhibits protein phosphatases.
- Substradte specifity. Vascular oxidase uses not only NADPH but also NADH (especially Nox4).

Expression of vascular oxidase differs in various vessels. Nox4 is generally found in all vascular cells in rather high concentrations. Nox2 is also expressed in all vascular cells except of media of large arteries: it is replaced by Nox1 there. Nox5 was found in human vascular smooth muscle cells, Duox2 was proved in media of human aorta.

Vascular oxidase and signaling

Proteinkinase C is probably the most important activator of vascular oxidase. Activity of this enzyme is also increased by phosphatidic acid, phospholipase D and arachidonic acid. Function of G-protein Rac (one of cytosolic subunits regulating several isoenzymes of vascular oxidase) depends on isoprenylation and is therefore influenced by activity of HMG-CoA reductase. Upstream to these signals are cytokins, growth factors and vasoactive peptides that mostly stimulate vascular oxidase. Activity of vascular oxidase is also increased by shear stress. Angiotensin II is one of most effective activators of vascular oxidase.
Interestingly, ROS produced by vascular oxidase may further activate this enzyme in a pathway dependent on EGF. A stimulatory loop is closed in this way suggesting that vascular oxidase may act as a “switch” between two functional levels.

Vascular oxidase and nitric oxide

Superoxide produced by NAD(P)H oxidases effectively reacts with NO. Peroxynitrite is formed in this reaction. Bioavailability of NO is decreased with inreasing superoxide production in this way. Important consequences for pathogenesis of arterial hypertension, atherosclerosis, cardial failure, endothelial dysfunction, restenosis of arteries of hypertrophy of arterial media were suggested.

Aims

Originally, this study aimed to describe the role of NAD(P)H oxidase in signal transduction, especially with respect to blood pressure regulation and to pathogenesis of cardiovascular disease. Following tasks had to bee solved:

1. Techniques of cell culture had to be implemented. Cultures of individual cell types of vascular wall were to be introduced.
2. To determine the differences in NAD(P)H oxidase activity in cells from normotensive and hypertensive individuals.
3. Using specific inhibitors and activators, to study of the signal cascade controlling the NAD(P)H oxidase activity and differences of regulatory mechanisms between normotensive and hypertensive individuals.

During these studies we encountered unexpected behavior of apocynin, a widely-used inhibitor of phagocyte NADPH oxidase that we intended to use also for studies on non-phagocyte NAD(P)H oxidase. Because of importance of these findings we studied the function of apocynin in a more detailed way. Results were published and attracted notable attention of other research groups; they create a major part of presented thesis.

Original aims lost their importance in the meantime because most questions were answered by other teams. We therefore continued with studies on potential links between NAD(P)H oxidase and cardiovascular disease. Concretely, we have studied the effect of uric acid, an important risk factor of cardiovascular disorders but also a significant scavenger of ROS.

Methods

Cell culture

Vascular fibroblasts were isolated from rat aortas using explanting culture, routinely fed twice a weak with F12K supplemented with 10% FBS and subcultured approximately once a weak, dilution factor 1:4. Cultures were prepared from normotensive rat (*Wistar*, WKY, and *Brown norvergicus*, BN). and from spontaneously hypertensive rat (SHR). Cells from passage 7 to 12 were used for experiments.
Vascular smooth muscle cells were isolated from rat (WKY, SHR) aorta cleared of endothelium and adventitia by enzymatic digestion. Cells were grown on thin layer collagene I and routinely fed twice a week with DMEM supplemented with 10% FBS and ECGS. Subcultures were performed once a week in ratio about 1:10. Cells from passage 5 to 12 were used for experiments. Contractibility of cultured cells was regularly assessed in order to check authenticity of culture.
We tried several protocols for establishing culture of endothelial cells. Best results were achieved with cultivation of heart muscle microvascular endothelium, however we fail to scale up the culture so that it could be used for experiment.

Isolation of peripheral leucocytes

Leucocytes from peripheral blood were isolated by means of isopycnic centrifugal sedimentation using Histopaque or Percoll density gradients.

Isolation of plasma membranes

Plasma membrane enriched suspension was prepared using osmotic and ultrasonic disruption of cells followed by sequential ultracentrifugation.

Reagents

Serum treated zymosan was prepared from zymosan A from *Saccharomyces cerevisiae*. Swelled zymosan was repetitively incubated with pooled rat serum.

2',7'-dichlorofluorescein (DCFH) was prepared by hydrolysis of 2',7'-dichlorofluorescein diacetate (DCFH-DA) in alkaline environment.

Pre-activated apocynin was prepared from apocynin by incubation with hydrogen peroxide and horse-radish peroxidase. Excess of hydrogen peroxide was then removed by action of catalase. Remaining peroxidase and catalase were removed by boiling and centrifugation.

Assays for ROS production

Assay with DCFH-DA. 2',7'-dichlorofluorescein diacetate is a cell permeable probe for ROS, especially hydrogen peroxide and hydroxyl radical. It freely enters intact cells. Then, it is deesterified to DCFH by cytoplasmic esterases. DCFH is trapped in cells. ROS oxidise DCFH to fluorescent DCF. Fluorescence of DCF was measured at excitation wavelength 501 nm and emission wavelength 521 nm.

Assay with WST-1. WST-1 is a water soluble tetrazolium derivative that is reduced by superoxide to a yellow formazan with absorption maximum at 450 nm.WST-1 is not cell permeable. NAD(P)H oxidase activity was assessed as NADH / NADPH dependent reduction of WST-1 by cells or plasma membranes. The reaction was fully abandoned by superoxide dismutase and tiron (a specific synthetic scavenger of superoxide) proving that superoxide is the reducing species. WST-1 was not back-oxidised by hydrogen peroxide.

Results and discussion

Activity of NAD(P)H oxidase in vascular fibroblasts of normotensive and hypertensive rat. Effect of angiotensin II.

Activity of both NADH and NADPH oxidase was significantly higher in cultured vascular fibroblasts from hypertensive animals compared to normotensive ones (Fig. 2). No difference was found between fibroblasts from both used normotensive strands (WKY and BN).

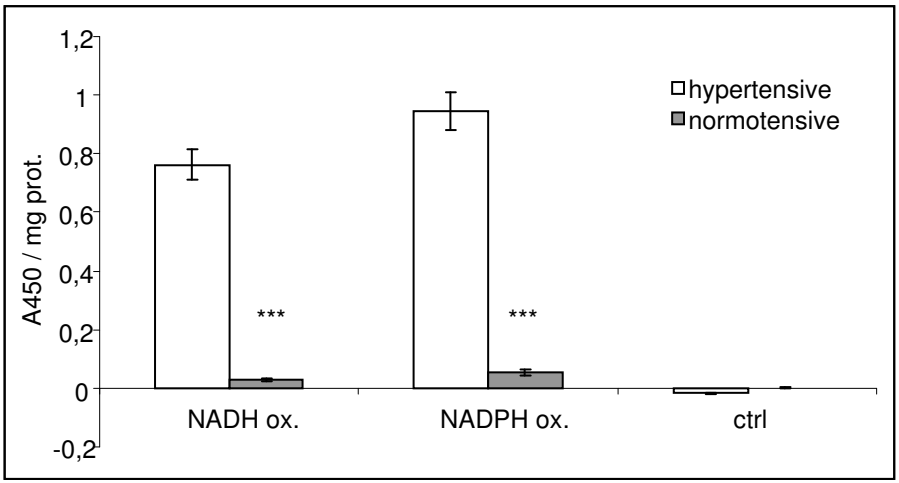

Fig. 2: **Activity of NAD(P)H oxidase in vascular fibroblasts from rat aorta.** Cultured fibroblasts from hypertensive (SHR) rat had higher activity of both NADH oxidase and NADPH oxidase compared to normotensive (BN) fibroblasts. Ctrl: WST-1 reduction in absence of NADH and NADPH. *** p<0,001 (hypertensive vs. normotensive)

Incubation of vascular fibroblasts with angiotensin II (100 nmol·l^{-1}, three hours) hours lead to small but statistically significant increase of both NADH oxidase and NADPH oxidase activity in normotensive fibroblasts. In cells from hypertensive rat, both activities were rather lowered but the decrease was not statistically significant (Fig. 3).

In next experiments we intended to describe the signaling pathway from angiotensin II to NAD(P)H oxidase activity. First measurements with apocynin, an inhibitor of phagocyte NADPH oxidase, gave surprising results and therefore we decided to study this inhibitor in more detail.

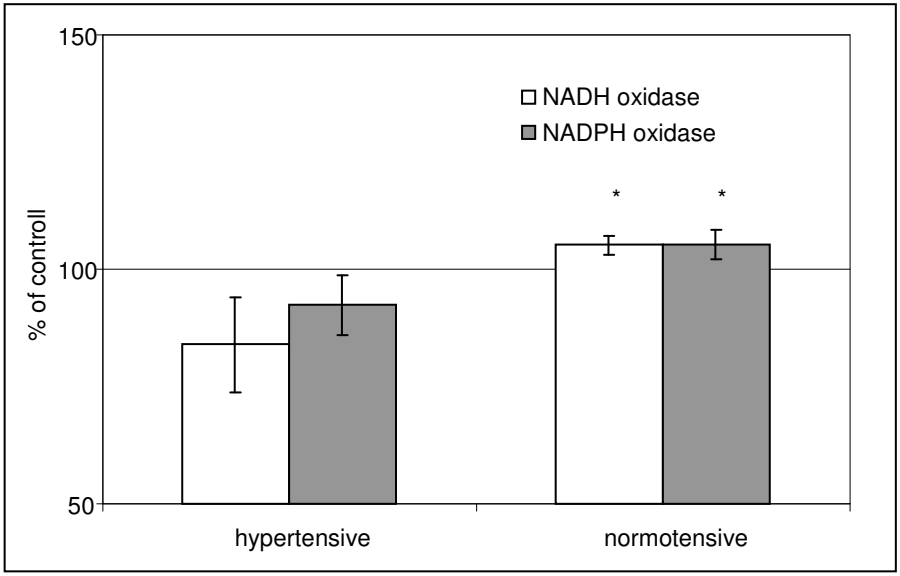

Fig 3: **Effect of angiotensin II on NAD(P)H oxidase activity in vascular fibroblasts of normotensive (BN) and hypertensive (SHR) rat.**
Fibroblasts were incubated with angiotensin II (100 nmol·l^{-1}) for three hours. Activity of NAD(P)H oxidases is represented as % of controls untreated with angiotensin. * $p<0,05$.

Effects of apocynin on ROS production by phagocyte and non-phagocyte cells

Apocynin (4-hydroxy-3-methoxyacetophenone) is a methoxy-substituted catechol originally extracted from roots of *Picrorrhiza kurroa*, a small perennial herb that grows in the Himalayas. Like other methoxy-substituted catechols, it can decrease the production of superoxide by activated neutrophils and macrophages while the ability of phagocytosis remains unaffected. This compound is investigated as a possible anti-inflammatory drug
Apocynin is also used as an experimental tool to inhibit phagocyte NADPH oxidase. We intended to use this compound, like other authors, to inhibit NADPH oxidase in non-phagocytes. The addition of apocynin, however, did not lead to a decrease in ROS formation but surprisingly to a significant increase.

Effects of apocynin on ROS production by vascular fibroblasts

Apocynin increased fluorescence of DCFH-DA treated fibroblasts (Fig. 4). The increase of fluorescence may be due to the oxidation of DCFH trapped in cells by ROS. However, such observation does not correspond to the fact that apocynin was assumed to reduce ROS formation by cells as it acts as an inhibitor of NADPH oxidase.

We therefore tested whether the increase in fluorescence is not caused by a direct interaction of apocynin with DCFH or whether a fluorescing metabolite of apocynin is not formed. In a cell-free system, apocynin was mixed with DCFH deesterified in vitro (Fig. 5). Compared with previous experiments, the background of the probe was higher (in experiments with cells, only probes trapped in the cells gave the signal); however, the addition of apocynin did not cause any increase in fluorescence comparable with the previous experiment. This means that apocynin could not enhance fluorescence by direct interaction with the probe. Apocynin itself showed no fluorescence as well.

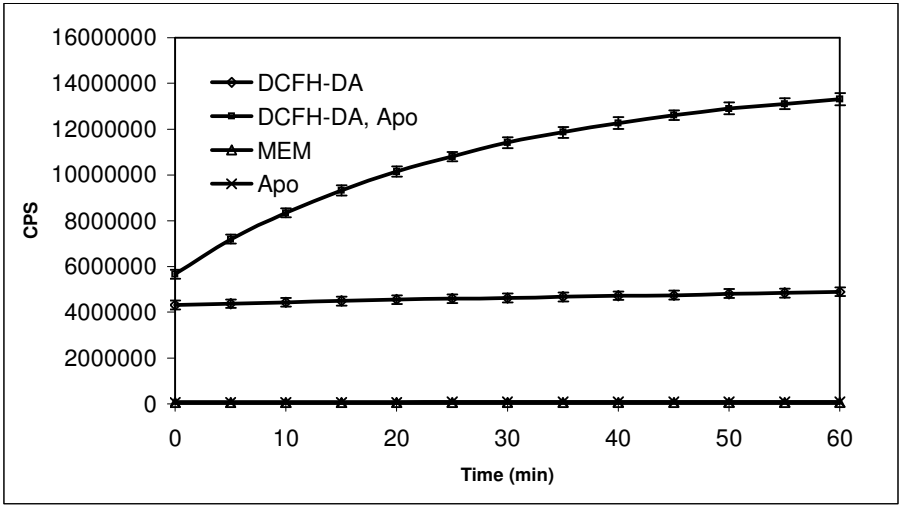

Fig. 4: **Effect of apocynin on ROS formation by vascular fibroblasts.** Vascular fibroblasts (5×10^4 cells per millilitre) were treated with DCFH-DA (10 $\mu mol.l^{-1}$) or culture medium (MEM) for 30 minutes and washed with MEM. Fluorescence of DCF in counts per second (CPS) was then measured in presence or absence of apocynin (Apo, 100 $\mu mol.l^{-1}$).

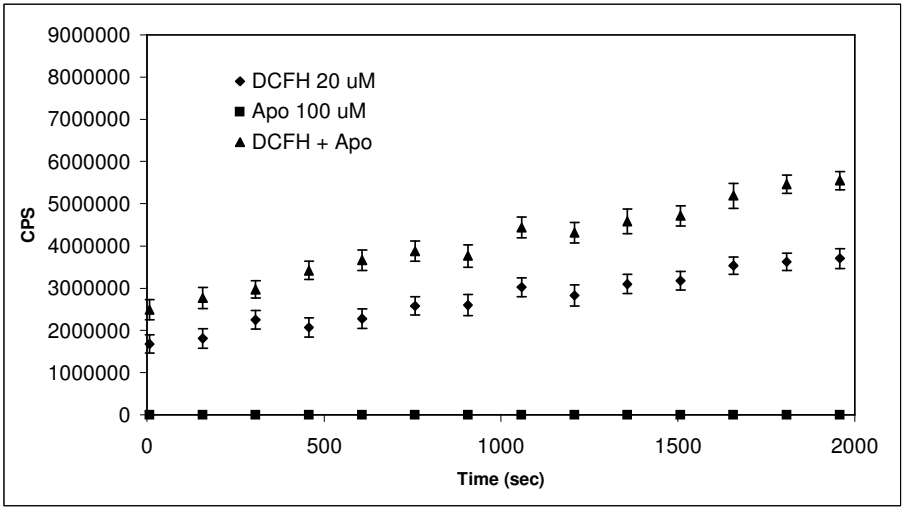

Fig. 5: **Effect of apocynin on fluorescence of DCFH.**
2‘,7‘-dichlorofluorescin (DCFH) was prepared in vitro by de-esterification of DCFH-DA. Fluorescence of DCFH (20 μmol.l^{-1}) in presence or absence of apocynin (Apo, 100 μmol.l^{-1}) was measured in counts per second (CPS).

In cells that were not treated with DCFH-DA, no increase in fluorescence after treatment with apocynin was observed (Fig. 4). Apocynin alone does not fluoresce in the examined range of spectrum and no fluorescing metabolite of it was formed under used conditions.

Effects of apocynin on ROS formation by immune-stimulated phagocytes
The findings stated above contradict the assumption that apocynin, as an NADPH oxidase inhibitor, should decrease ROS formation and consequent DCF fluorescence. In the literature, apocynin is well described as an inhibitor of respiratory burst in immune-stimulated macrophages. We therefore tested whether the respiratory burst of phagocytes is suppressed by apocynin under the conditions used in the previous experiment.
When apocynin was added to macrophages immune-stimulated with serum-treated zymosan (STZ), ROS production increased in the first minutes. Then it slowed down rapidly and after 15 minutes, ROS formation was lower than during absence of apocynin (Fig. 6). The addition of fresh apocynin led to a

new increase of ROS formation; therefore, a decrease in the rate of ROS production could not be caused by reaching the detection limit of the method or by exhausting macrophages.

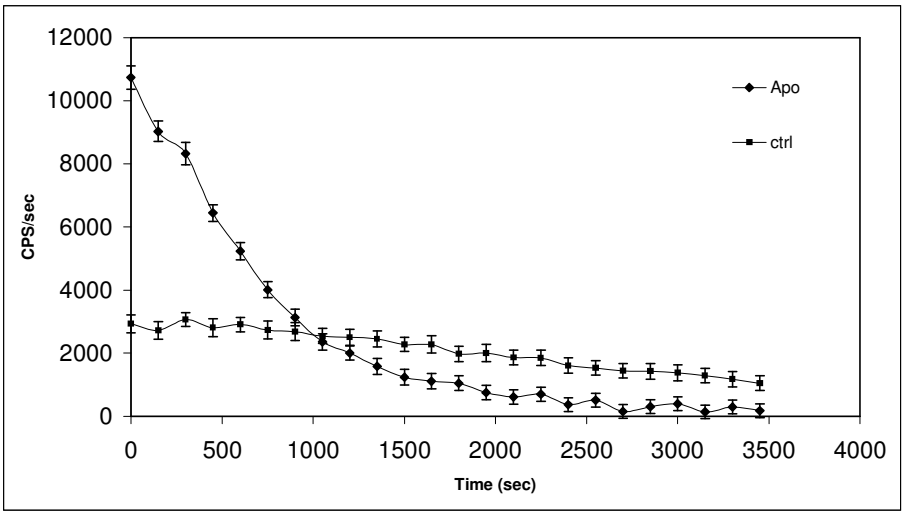

Fig 6: **Effect of apocynin on oxidation of DCFH-DA by macrophages stimulated with STZ.**
Macrophages pre-treated with DCFH-DA were immune-stimulated with STZ (1 $mg.ml^{-1}$) in presence or absence of apocynin (Apo, 100 $\mu mol.l^{-1}$). Rate of ROS formation was measured as rate of increase in DCF fluorescence per second.

These results suggest that fresh apocynin does not act as an NADPH oxidase inhibitor but is, in contrast, capable of stimulating ROS production. However, it can be metabolized to another compound that suppresses ROS formation.

Effect of pre-activated apocynin on ROS production
As stated above, apocynin did not inhibit ROS production in fibroblasts. In macrophages, inhibition was delayed most probably because apocynin had to be converted to its dimer through reaction with products of immune stimulated phagocytes. We further tested the effect of apocynin pre-activated in vitro with horseradish peroxidase and hydrogen peroxide. It had an immediate inhibitory effect on the oxidation of DCFH-DA by both vascular fibroblasts (Fig. 7) and STZ stimulated macrophages.

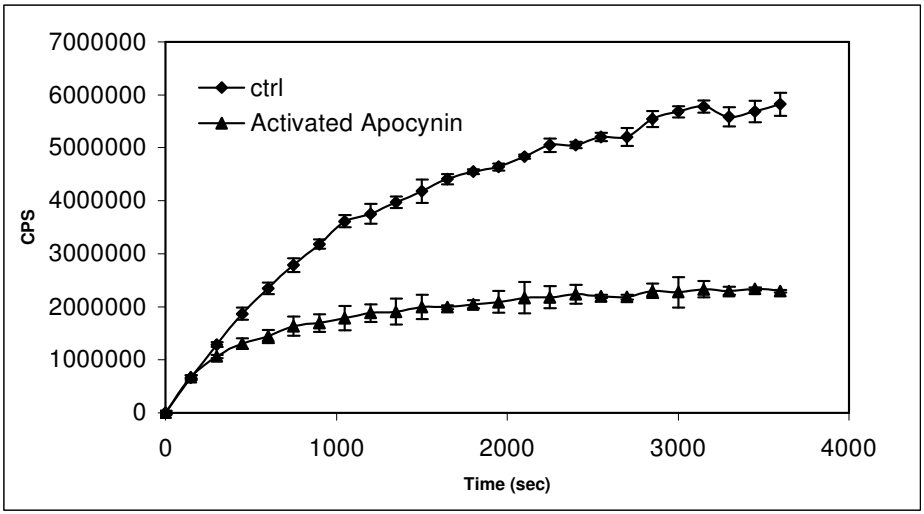

Fig. 7: **Effect of apocynin pre-activated with HRP and hydrogen peroxide on ROS formation by vascular fibroblasts.**
Vascular fibroblasts were treated with DCFH-DA, washed and resuspended in pre-activated apocynin or MEM. Fluorescence of DCF was measured in counts per second (CPS).

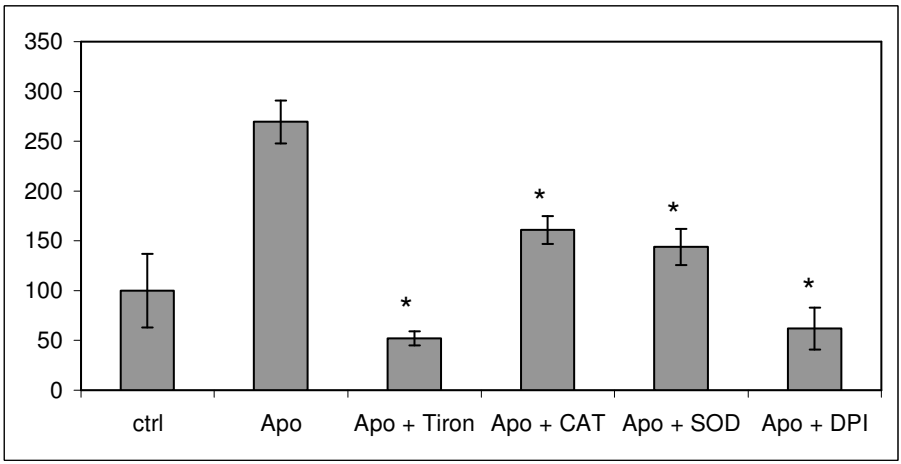

Fig. 8: **Effect of scavengers on apocynin stimulated ROS production.**
Fluorescence of controls without apocynin and inhibitors is 100%. * $p<0,05$ compared to apocynin.

Effect of ROS scavengers and NADPH-oxidase inhibitors
Tiron, a specific cell-permeable superoxide scavenger, almost completely abolished the influence of apocynin on oxidation of DCFH by vascular fibroblasts. Superoxide dimutase (SOD) and catalase also at least partially suppressed the effect of apocynin (Fig. 8).
The stimulatory effect of apocynin on ROS formation by vascular fibroblasts was significantly diminished by diphenylene iodinium (DPI), too. DPI is an inhibitor of flavoproteins and lacks scavenging properties. All these findings strongly support the hypothesis that apocynin enhanced fluorescence corresponds to increased ROS production. The species increased after apocynin treatment is most probably superoxide that consequently dismutes to form hydrogen peroxide.

Discussion on experiments with apocynin
Apocynin is a widely used inhibitor of phagocyte NADPH oxidase. NADPH oxidase is also present in non-phagocyte cells where it plays an important role in ROS production for cell signaling. As phagocyte and non-phagocyte NADPH oxidases share most subunits and properties, we tried to use apocynin as an NADPH oxidase inhibitor in experiments with vascular cells. Surprisingly, apocynin had no inhibitory effect on ROS production but even enhanced ROS formation.
The reactive oxygen species formed after apocynin treatment is most probably superoxide. Increased ROS production was completely abolished by superoxide-specific scavenger tiron and diminished by superoxide dismutase. Catalase reduced the effect of apocynin as well. It may be supposed that the originally formed superoxide dismutes to oxygen and hydrogen peroxide that consequently oxidizes DCFH to fluorescent DCF. Interestingly, DPI, a non-scavenging inhibitor of NADPH oxidase, diminished the effect of apocynin as well suggesting that NADPH oxidase could be the source of superoxide. However DPI is not a specific inhibitor of NADPH oxidase but acts on all flavoproteins. It seems likely that apocynin stimulates ROS production by an enzyme that contains the flavine group or is regulated by a flavoprotein.
In immune-stimulated phagocytes, the role of apocynin is more complex. First, it stimulates ROS formation, however, after a certain period its behavior changes and inhibition of ROS yield occurs. When apocynin is “pre-activated” with hydrogen peroxide and a peroxidase prior to use, it immediately acts as an inhibitor both in phagocytes and non-phagocytes. It seems that the activation of apocynin consists of its oxidation by H_2O_2 in presence of a peroxidase activity, either externally added or produced by macrophages.
On the other hand, there are studies that prove the inhibitory effects of

apocynin on ROS formation in non-phagocytes as well. The conditions under which these experiments were performed, however, are different: cells or tissues were treated with apocynin several hours or even several days before measurement was begun. It can be assumed that slower production of H_2O_2 by non-phagocytes was sufficient in these cases and peroxidase activity was probably replaced by pseudo-peroxidase effect of iron and other transition metals present as ubiquitous contaminants.
The capability of apocynin to inhibit ROS formation first after activation by peroxidase and hydrogen peroxide might be of pharmacological importance. Apocynin is activated in the locus of inflammation only where there is both free leukocyte myeloperoxidase and hydrogen peroxide. In this case apocynin acts as an anti-inflammatory agent. Phagocytes that are not immune activated cannot be inhibited in this way.
The question remains whether the increase in ROS formation after treatment with fresh apocynin might play any role. Cell produced ROS have important signaling properties that are associated with many cytokines and growth factors. Apocynin could act in this manner not only as an anti-inflammatory drug by suppressing microcide and cytotoxic agent formation but might at the same time support cell proliferation and tissue reparation.

Results of our experiments with apocynin were published in *Biochimica et Biophysica Acta, General Subjects* in 2005. This paper was awarded as one of top-ten most downloaded articles of the year 2005. Several authors extended our study. Reinehr *et al.* studied the effect of apocynin on hepatocytes. These cells posses a high peroxidase activity and so inhibitory effect of apocynin prevailed. Riganti *et al.* confirmed that apocynin increases oxidative stress and production of hydrogen peroxide.Ximenes *et al.* suggested that the inhibitory derivative of apocynin is its radical formed by peroxidase catalysed oxidation.

Effects of uric acid on vascular oxidase activity

Increased serum uric acid (UA) is linked to higher cardiovascular risk. It is an independent risk-factor for arterial hypertension, especially for essential hypertension in young patientsand its complications. Relationships between increased serum UA and coronary arterial disease, atherosclerosis, cerebral stroke, endothelial dysfunction and renal disease have also been established. Interestingly, oxidative stress plays a role in pathogenesis of all of disorders mentioned above. On the other hand, UA possesses important antioxidant properties: it is a potent scavenger of superoxide, hydroxyl radical and singlet oxygen, and acts as an iron chelator. UA is even believed one of the most important antioxidants of blood plasma.

Association of increased UA and cardiovascular disease could seem to be contradictory to antioxidant properties of UA. We tested the hypothesis that UA could stimulate NAD(P)H oxidases of vascular wall and thus increase production of reactive oxygen species.

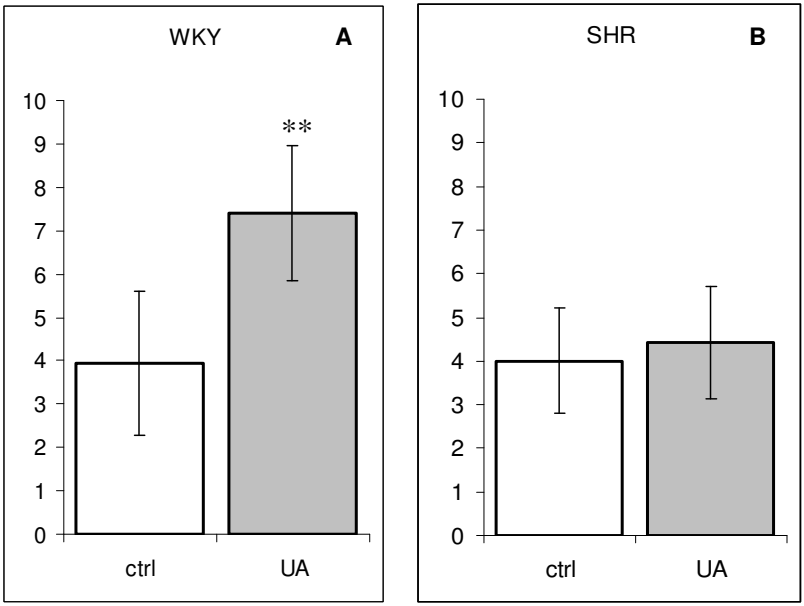

Fig. 9: **NADH oxidase activity in plasma membranes of vascular smooth muscle cells isolated from aortas of normotensive (WKY) and spontaneously hypertensive (SHR) rat.**
Cells were treated with high concentration of uric acid (1 mM, UA) for 48 hours. ** $p<0.01$

Treatment of vascular smooth muscle cells from normotensive rat (Wistar) with uric acid (1mmol/L) stimulated superoxide production by both NADH and NADPH oxidase in isolated plasma membranes (Fig. 9A). NADH oxidase activity increased to 190% of controls. Basal activity of NADPH oxidase was much lower (Fig. 10A); after uric acid treatment it increased to 180%.
In vascular smooth muscle cells from hypertensive rat (SHR), the activity of both oxidases and effect of UA differed. Generally, UA had no significant effect on the NADH or NADPH oxidase activity (Fig. 9B and 10B). Interestingly, activity of NADPH oxidase in SHR was the same as uric acid-stimulated

activity in normotensive cells. On the other hand, NADH oxidase activity of VSMC from SHR was close to the activity of non-stimulated normotensive cells.

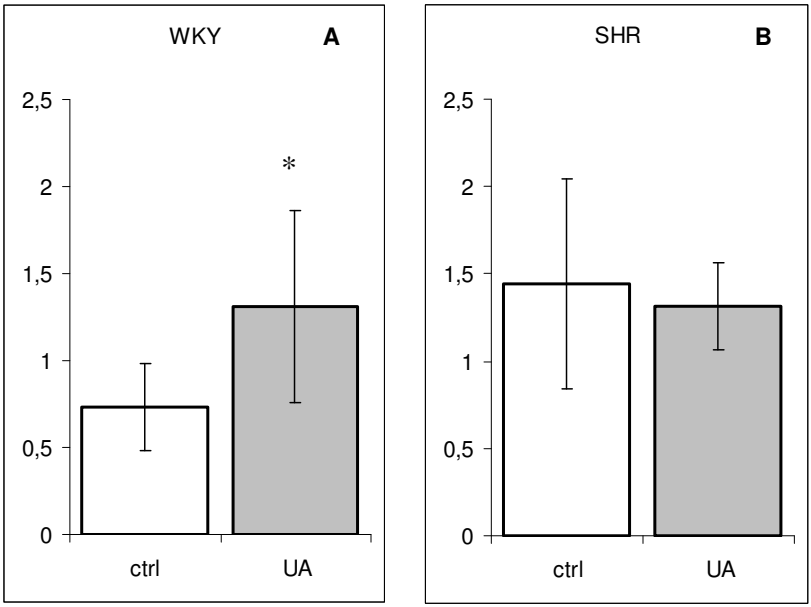

Fig. 10: **NADPH oxidase activity in plasma membranes of vascular smooth muscle cells isolated from aortas of normotensive (WKY) and spontaneously hypertensive (SHR) rat.**
Cells were treated with high concentration of uric acid (1 mM, UA) for 48 hours. ** $p<0.01$

Both uric acid free environment and medium with 1 mmol.l-1 of uric acid do not correspond to physiological situation: plasmatic concentration of UA is 150 $\mu mol \cdot l^{-1}$ in rat. Therefore, we assessed the effect of uric acid in various concentrations between these values on NAD(P)H oxidase activity. Superoxide production by NADH oxidase was lowest for UA concentrations near to physiological values (Fig. 11). NADPH oxidase activity was much lower in general; least superoxide was formed when cells were grown in medium lacking UA (Fig. 12).

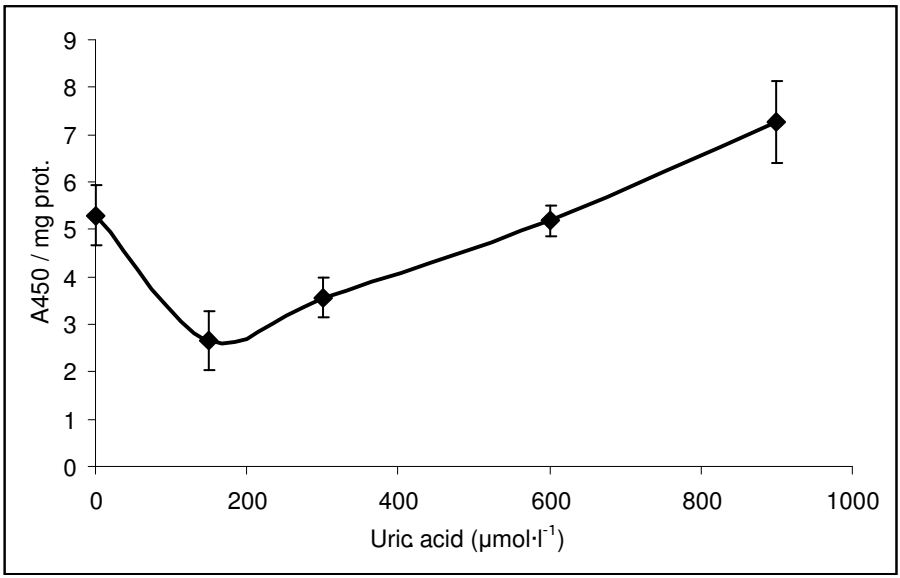

Fig. 11: **Effect of uric acid on NADH oxidase activity in VSMC.**
Cells were treated with indicated concentration of uric acid. Activity of NADH oxidase was assessed in isolated plasma membranes.

Discussion on the effect of uric acid on HAD(P)H oxidase
Uric acid is linked to increased cardiovascular risk despite the fact that it is a potent antioxidant and therefore might be rather protective. We showed that UA significantly stimulates superoxide production by plasma membrane NAD(P)H oxidase in vascular smooth muscle cells of normotensive rat aorta. Nox4 which prevails in VSMC has higher NADH oxidase than NADPH oxidase activity. This is in agreement with prevailing NADH oxidase activity in our results.
In hypertensive VSMC, UA did not stimulate NAD(P)H activity. Interestingly, the basal activity of NADH oxidase in hypertensive cells was comparable with non-stimulated normotensive cells while NADPH oxidase activity of hypertensive cells was similar to UA-stimulated normotensive cells. This strongly suggests that NADH and NADPH oxidase activities are carried by (at least) two different enzyme entities with distinct regulatory mechanisms.
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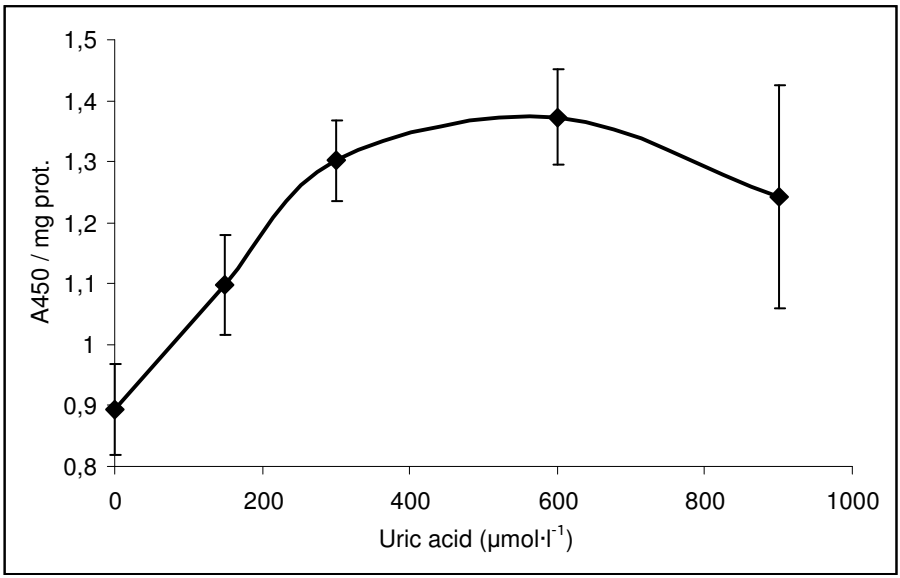

Fig. 12: **Effect of uric acid on NADPH oxidase activity in VSMC.** Cells were treated with indicated concentration of uric acid. Activity of NADH oxidase was assessed in isolated plasma membranes.

Several authors have already suggested that low concentrations of UA have antioxidant properties while high concentrations are rather pro-oxidant. It has even been found in some epidemiological studies that the relation between UA and cardiovascular risk is a J-shaped curve. This is in concordance with our *in vitro* model (Fig. 11). An important property of ROS producing NAD(P)H oxidase is that ROS further stimulate its activity. Thus, it may be suggested that low concentrations of UA decrease NAD(P)H oxidase activity due to its scavenging effect while high concentrations of UA stimulate NAD(P)H oxidase.

Conclusions

1. Experimental models for studies on NAD(P)H oxidase in cultured vascular smooth muscle cells and fibroblasts were introduced. Cultures of vascular cells from normotensive and hypertensive rat were established. We failed to introduce similar model with endothelial cells.

2. Activity of NAD(P)H oxidase in cultured fibroblasts from rat aorta was higher in cells from hypertensive animal (SHR) compared to normotensive ones (WKY, BN).
3. NAD(P)H oxidase of vascular fibroblasts was stimulated by angiotensin II in cells from normotensive rat. Angiotensin II had no significant effect on cells from hypertensive rat.

4. Apocynin, literally well described as inhibitor of phagocyte NADPH oxidase, increases ROS production (namely production of superoxide) in some cells. Increase of ROS production appears in cells lacking peroxidase activity and/or low production of hydrogen peroxide. Superoxide producing system is inhibited with DPI, an inhibitor of flavoenzymes.
5. Inhibiting of phagocyte NADPH oxidase is not a property of apocynin itself. A product of apocynin raising by action of hydrogen peroxide in presence of peroxidase is responsible for NAD(P)H oxidase inhibition.
6. Different effecr of apocynin on ROS production in phagocytes and non-phagocyte cells may be of pharmacological importance for intended use of apocynin as an antiinflammatory drug.

7. Uric acid influences NAD(P)H oxidase activity in vascular smooth muscle cells. Low concentrations of uric acid (lower than physiological) inhibit superoxide production by NAD(P)H oxidase while high concentrations stimulate ROS production.
8. Uric acid had no significant effect on VSMC from hypertensive rat.
9. Antioxidant effect of low and pro-oxidative effect of high concentrations of uric acid could explain current controversies between scavenging properties of uric acid and the fact that uric acid is a risk factor for cardiovascular disease.

Summary

Reactive oxygen species are usually assumed as dangerous, cytotoxic substances included in pathogenesis of variety of diseases due to their ability to damage biomolecules. However, ROS also play an indispensable role in many physiological processes. Low concentrations of ROS are involved in signal transduction as a part of pathways regulating protein phosphorylation, gene expression, NO availability or intracellular Ca^{2+}. They are important for cell cycle control and apoptose. In the last years, the role of ROS in regulation of vascular tonus is intensively studied.

NAD(P)H oxidases are supposed the major source of ROS. These are enzymes similar to phagocyte NADPH oxidase, a key enzyme of phagocyte respiratory burst in immune response. We have studied NAD(P)H oxidase of vascular wall in normotensive and hypertensive rat. Activity of superoxide production by NAD(P)H oxidase differ between cells from normotensive and hypertensive rat. Control of NAD(P)H oxidase activity is also changed in hypertensive animals, at least with respect to the effect of angiotensin II and uric acid.

In our experimental work, we encountered unexpected properties of apocynin, a known inhibitor of phagocyte NADPH oxidase. We proved that apocynin can increase ROS production. The reason is that NAD(P)H oxidase is not inhibited directly by apocynin but rather by a metabolite of this compound. Apocynin is converted to the active inhibitor by action of hydrogen peroxide and a peroxidase.

We also performed pilot studies on the effect of uric acid on NAD(P)H oxidase activity. Uric acid in low concentrations decreases while in high concentrations increases superoxide production in vascular smooth muscle cells. This finding could explain current controversies between scavenging properties of uric acid and increased cardiovascular risk in hyperuricemia.

List of publications

Publications on the topic of presented thesis:

Original paper:

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