



MEDICAL UNIVERSITY OF GDAŃSK

**The role of NADPH oxidase in the switch mechanism of the cell
death mode from apoptosis to necrosis *.**

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Ph.D. Thesis

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LIST OF ABBREVIATIONS

AIF - apoptosis inducing factor

AntmA – antimycin A

AP - apocynin

Apaf-1 – apoptosis activating factor-1

APS – ammonium persulphate

ATP – adenosine triphosphate

BSA – bovine serum albumin

CAD – caspase activated DNase

$\Delta\Psi_m$ – mitochondrial inner transmembrane potential

DDSA – Dodecenylsuccinic anhydride

DHE – dihydroethidium

DMEM – Dulbecco's Modified Eagles Medium

DMP-30 – 2,4,6-Tri(dimethylaminomethyl) phenol

DNA – deoxyribonucleic acid

DPI – diphenyliodonium chloride

EDTA – ethylenediaminetetraacetic acid

EGF – epithelial growth factor

EtBr – ethidium bromide

EtOH – ethanol

FADD – Fas-associated death domain

FBS – fetal bovine serum

FITC – Fluorescein Isothiocyanate

GSH – reduced glutathione (γ -glutamyl-cysteine-glycine)

GSSG – oxidized glutathione

H₂DCFDA – dichlorodihydrofluorescein diacetate

HPLC – High Performance Liquid Chromatography

IAP – inhibitors of apoptosis proteins

ICAD – inhibitor of CAD

MEN – menadione

MetOH – methanol

mtDNA – mitochondrial DNA

NADH – reduced nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate
NMA – Methyl-5-Norbornene-2,3-Dicarboxylic Anhydride
N-VNA – *N*-vanillylnonanamide
O₂^{•-} - superoxide radical anion
PARP – poly-ADP ribose polymerase
PAGE – polyacrylamide gel electrophoresis
PBS – phosphate buffered saline
PI – propidium iodide
PMSF – phenylmethylsulfonyl fluoride
PPP - pentose phosphate pathway
ROS – reactive oxygen species
RNS – reactive nitrogen species
rpm – rotations per minute
RT – room temperature
SDS – sodium dodecyl sulfate
Smac/DIABLO – second mitochondria derived activator of caspases/ direct IAP-binding protein with low isoelectric point
SOD – superoxide dismutase
TEMED – N, N, N', N'- tetramethyl-ethylenediamine
TRADD – TNF-R-associated death domain
TRAIL – TNF-related apoptosis inducing ligand
TNF – tumor necrosis factor
Tris – Tris- [hydroxymethyl]amino-methane
UV – ultraviolet
VK – vitamine K

INTRODUCTION

1. Human osteosarcoma tumors

Osteosarcoma is the most common primary malignant tumor of bone, including a class of osteoid – producing neoplasms that vary in clinical behaviour and responses to therapeutic regimens (Meyers and Gorlick, 1997; Bramwell 2000).

Up to now it is known that the classic high-grade osteosarcoma primarily afflicts individuals in the second decade of life and is distinguished by its locally aggressive character and early metastatic potential. Metastatic disease is often not apparent at diagnosis and causes the overwhelming majority of deaths among patients with this disease.

Recurrent or metastatic tumors are significantly less sensitive if not resistant, to conventional chemotherapy (Burns et al., 2001; Ferguson and Goorin, 2001).

The clinical management of osteosarcoma faces two serious dilemma: a) although preoperative and postoperative chemotherapy have improved the 5-year survival rate, the toxic and adverse effects associated with chemotherapy can significantly reduce the quality of a patient's life because of the young age of the afflicted individuals (Haydon et al., 2002); and b) osteosarcoma possesses a characteristically high rate of recurrence and metastasis, which causes the overwhelming majority of osteosarcoma-related mortality (Meyers and Gorlick, 1997; Bramwell, 2000).

Osteosarcoma is very resistant to the treatment. Surgical removal of the primary tumor is followed by distant recurrence in 80-90% of cases (Ferguson and Goorin, 2001). Unfortunately, osteosarcoma is not particularly sensitive to chemotherapy. Drug like vincristine is ineffective and the others used against solid tumors like: actinomycin-D, dacarbazine, cyclophosphamide, show only modest response rates (Ferguson and Goorin, 2001). The agents most commonly used in the treatment of osteosarcoma include doxorubicin, high-dose methotrexate and cisplatin. Among them cisplatin is very active against osteosarcoma.

2. Menadione (MEN)

Menadione (2-methyl-1,4-naphthoquinone) is a synthetic member of the vitamin K family and is described as vitamin K3. It possesses the most simple structure among vitamin K family, with no aliphatic chain prosthetic group at position 3 of naphthoquinone skeleton (Fig.1). The best-known naturally occurring members of the vitamin K family are: vitamin K1 (phylloquinone), which was found in many higher plants as well as algae (Thompson, 1971) and vitamin K2 (menaquinone) which is produced by intestinal bacteria from exogenous naphthoquinones (Seegers and Bang, 1967). Vitamin K3 treatment was applied in various types of rodent- and human-derived neoplastic cell lines *in vitro*, such as oral epidermal carcinoma, breast carcinoma, leukemia and hepatocellular carcinoma (HCC) cell lines (Chlebowski et al., 1985; Markovits et al., 2003; Chen et al., 2002; Nutter et al., 1991; Lamson and Plaza, 2003; Verrax et al., 2003). Although the mechanisms of antitumor effects of vitamin K have been investigated intensively, they still remain unclear. Most of the data come from *in vitro* experiments and there are only small number of reports demonstrating *in vivo* antitumor activity of vitamin K3. There are suggested two mechanisms of antitumor effects of menadione. It can act as the oxidative stress inducer via redox-cycling of the quinone or it can arrest cell cycle at G1 phase (Kuriyama et al., 2005).

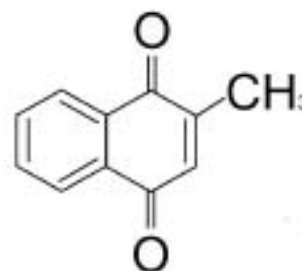


Fig.1. Menadione (Vitamin K3)

Historically, it was proposed that the menadione anti-cancer activity was due to oxidative stress via redox-cycling of the quinone to produce reactive oxygen species (ROS), such as the superoxide anion radical, hydroxyl radical, and hydrogen peroxide (Gant et al., 1988). Quinones can undergo either one-electron reduction, producing semiquinone radicals, or two-electron reduction, resulting in hydroquinones (Fig.2). The cytotoxicity of menadione may depend on direct arylation of nucleophiles such as glutathione and initiation one- or two-electron redox cycling (Lamson and Plaza, 2003). Redox cycling is defined as the ability to elicit a disproportionate NAD(P)H oxidation or oxygen utilization in such a biological system when compared with the quantity of quinone present, and involves the transfer of one electron to oxygen from the semiquinone intermediate. Redox cycling, together with the generation of reactive oxygen species and the subsequent oxidative stress induced, has been proposed as the mechanism by which quinones may cause toxicity and subsequently apoptosis (Gant et al., 1988; Criddle et al., 2006). The cytotoxicity of menadione displayed by redox cycling results

in thiols depletion accompanied by GSSG formation, NADPH oxidation and perturbation of calcium ion homeostasis (Thor et al., 1982).

Menadione possesses the ability to directly arylate thiols, depleting in this way the pool of glutathione and sulfhydryl-containing proteins, which comprises another aspect of an oxidative mechanism (Nishikawa et al., 1995). Menadione reacting directly with nucleophiles such as GSH by Michael addition (Nickerson et al., 1963) results in oxygen consumption, H₂O₂ and GSSG formation as well as production of a menadione-GSH conjugate (Ross et al., 1985; Dimonte et al., 1984). The decrease of sulfhydryl groups in treated cells suggests that vitamin K3 might also decrease the activities of other critical sulfhydryl-containing enzymes such as protein tyrosine phosphatases as well as p34Cdc2 protein associated with cell growth (Juan et al., 1996). There is another possibility that menadione inhibits glutathione reductase (GR) (Bellomo et al., 1987), what may prevent the reduction of GSSG to GSH.

Reduced glutathione (GSH), a tripeptide (γ -L-glutamyl-L-cysteine-glycine), is an important intracellular redox buffer that exists as a reduced predominant form, as a disulfide form (GSSG) or as mixed disulfide (GSSR) with protein thiols (Deneke and Fanburg, 1989). It is the most important antioxidant agent in the cells where it is present in mM concentrations. During the oxidative stress, GSH is oxidized instead of lipids, proteins or nucleic acids. Glutathione reductase participate in regeneration of GSH and it uses the NADPH produced in PPP as a reducing cofactor. The ability of the cell to diminish oxidative stress may be impaired due to decreased potential of the PPP (Riganti et al., 2004). The GSH/GSSG ratio reflects the redox status within the cell (Cotgreave and Gerdes, 1998) and this is responsible for the regulation of pro-inflammatory genes (Rahman and MacNee, 2000). A decreasing GSH/GSSG ratio inhibits the binding activity of NF- κ B in endothelial and alveolar epithelial cells (Chen et al., 2000; Haddad et al., 2000). Significant reduction of intracellular GSH levels is reflected by a low GSH/GSSG ratio, a possible result of the formation of glutathionyl adducts between GSH and quinone which is also due to the glutathione *S*-transferases (GST), which marks these adducts for export from the cell (Awad et al., 2002). These compounds alkylate thiol groups, mainly through the formation of thioether derivatives of cysteine (Bolton et al., 1997).

Menadione-induced oxidative stress is associated with a perturbation of intracellular Ca²⁺ homeostasis (Bellomo et al., 1982; Thor et al., 1982). During the metabolism of menadione, Ca²⁺ is released from intracellular stores, and the ability of mitochondria and microsomes to sequester Ca²⁺ is impaired. In addition, the metabolism of menadione results in the inhibition of plasma membrane Ca²⁺-ATPase activity. The critical involvement of protein

sulfhydryl group oxidation in the menadione-mediated inhibition of the Ca²⁺-ATPase is suggested by the finding that GSH was able to restore the impaired ATPase activity (Nicotera et al., 1985). NAD(P)H oxidation by menadione, results in inhibition of aerobic glycolysis (Rossi and Zatti, 1964), stimulation of pentose phosphate pathway activity (Rossi and Zatti, 1964; Rossi and Zoppi, 1966), and depletion of the mitochondrial ATP pools (Bellomo et al., 1982). Further studies revealed that menadione induced depletion of NAD(P)H results in depletion of mitochondrial ATP and loss of control of the flux of ionized calcium across mitochondrial and cellular membranes (Bellomo et al., 1982). Loss of control of ionized calcium flux, a process influenced by reduced glutathione, may be one mechanism by which depletion of reduced glutathione pools result in cytotoxicity (Bellomo et al., 1982).

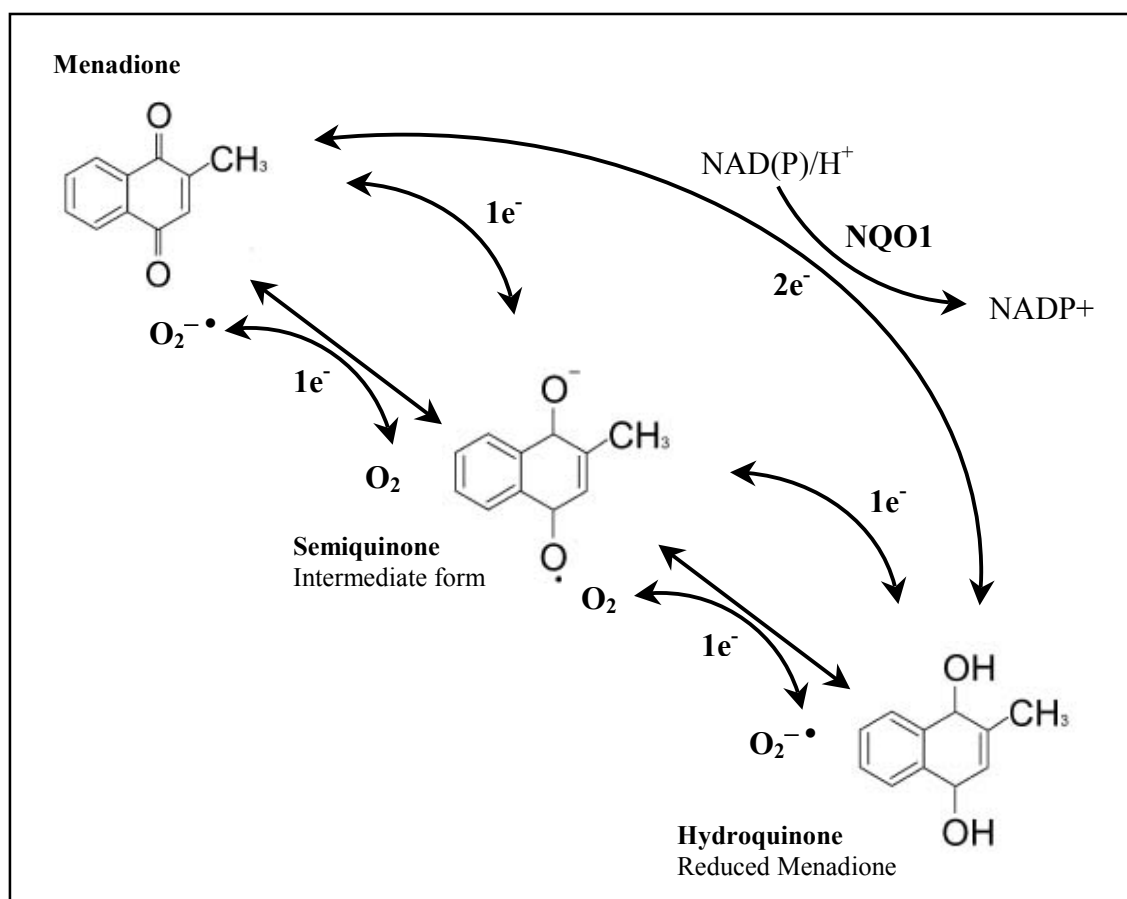


Fig.2. Redox cycling of menadione. (adapted and modified from Lamson and Plaza, 2003)

2.1. Non-oxidative model of menadione antitumor activity

Apart from oxidative mechanism, menadione exerts antitumor effects by affecting the key molecules of G1 phase cell cycle regulation (Kuriyama et al., 2005). Cell cycle molecules

play essential roles in carcinogenesis and tumor development. G1 phase-related molecules are especially important because they are required for the entry into the cell cycle from the quiescent state. Cell cycle molecules are divided into 3 groups, namely cyclins, Cdks and Cdk inhibitors. Among cyclins, the D-type (D1, D2 and D3), specifically cyclin D1, serve as a critical regulator of the cell cycle (Hanahan and Weinberg, 2000). Cyclin D1 forms complexes with Cdk4 and these proteins are responsible for driving cell cycle from G1 to S phase. Cdk inhibitors, especially of the INK family, are G1-phase specific and consist of p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}. These inhibitors are active only on Cdk4- or Cdk6-containing complexes. Moreover, binding of the INK family proteins to Cdk4 or to Cdk6 is independent of cyclin D (Chan et al., 1995; Hirai et al., 1995; Serrano et al., 1993). Because members of this family are known to bind and inhibit Cdk4 and Cdk6 without affecting other Cdks (Sherr, 1996), they are G1 phase-specific. p16^{INK4a} inhibits the turnover of cell cycle and makes cells stay at G1 phase.

Retinoblastoma (Rb) is the ultimate substrate of cyclin D1/Cdk4 and cyclin D1/Cdk6 complexes in the pathway leading to transition from G1 to S phase (Sherr, 1996). Rb protein controls gene expression mediated by a family of heterodimeric transcriptional regulators, described as E2Fs, which can transactivate genes which products are essential for S phase entry (Kuriyama et al., 2005). In its phosphorylated form, Rb protein binds to a subset of E2F complexes, converting them to repressors that constrain expression of E2F target genes. Phosphorylation of Rb protein frees these E2Fs, enabling them to transactivate the same genes, a process initially triggered by cyclin D1/Cdk4 and cyclin D1/Cdk6 complexes, and then accelerated by cyclin E/Cdk2 complexes (Kuriyama et al., 2005). Kuriyama et al. (2005) demonstrated *in vivo* that vitamin K3 exert antitumor actions by regulating the expression of cell cycle-related molecules. Their research on human hepatocellular carcinoma (HCC) cells revealed that menadione reduced the mRNA expression of Cdk4, but not that of cyclin D1 and increased mRNA expression of p16^{INK4a} and Rb. Therefore, reduced cyclin D1/Cdk4 kinase activities induced by vitamin K3 cause reduced proliferative activity of HCC cell, resulting in retarded HCC development (Kuriyama et al., 2005). On the other hand, increased p16^{INK4a} expression in HCC tumors suppressed cyclin D1/Cdk4 and cyclin D1/Cdk6 kinase activities, resulting in *in vivo* antitumor effects of menadione on HCC. This case suggest that menadione antitumor activity is at least in part due to cell cycle arrest at G1 phase of HCC cells (Kuriyama et al., 2005). Jamison et al. (2004) demonstrated that human bladder tumor cells exposed to combined treatment of vitamin K3 and C also results in cell cycle arrest. In addition, cells that were in G1 phase at the time of vitamin treatment are arrested in G1, while

those which have passed the G1 checkpoint progress through the S phase and become arrested in G₂/M. The G₂/M arrest is believed to depend on the regulation of cyclin B1 and p34^{cdc2} (Clopton and Saltman, 1995).

2.2. Synergistic antitumor chemotherapeutic action of MEN

Menadione was found to act as antitumor drug synergistic with cisplatin, 5-fluorouracil (5-FU), dacarbazine, and bleomycin in human oral epidermoid carcinoma cell culture. Synergistic action between vitamin K3 and doxorubicin, vinblastine, and 5-FU was also demonstrated in nasopharyngeal carcinoma cells (Liao et al., 2000). Synergistic effect of menadione and mitomycin C treatment was observed in lung cancer (Tetef et al., 1995) and advanced gastrointestinal cancers (Tetef et al., 1995).

Many clinical useful antitumor agents have a quinone group in their structure. Menadione possesses a broad spectrum of antitumor activity including multidrug-resistant human cancer cell lines. This compound may not exhibit serious toxic side effects in humans, in particular, cardiac toxicity, such as seen after in patients treated with doxorubicin, and may be a useful candidate in combination chemotherapy (Nutter et al., 1991; Thompson, 1971).

Synergistic cytotoxic activity of the combination of vitamins C and K3 possesses the features of cell death which is described as autoschizis (from the Greek *autos*, self, and *schizein*, to split, as defined by Gilloteaux et al., 1998). Ultrastructural studies of vitamin-treated tumor cells undergoing autoschizis revealed exaggerated membrane damage and an enucleation process in which the pericaria separate from the main cytoplasmic body by self-excision. These self-excisions continue until all that remained is an intact nucleus surrounded by a narrow rim of cytoplasm that contains damaged organelles, including SER, RER, mitochondria, membrane whorls, lysosomes, and lipid droplets (Gilloteaux et al., 1998). In the self-excising cells, all organelles surround the nucleus as a tight mass of membranes, vacuolated mitochondria, and mitochondria with intramatrix deposits, as well as apparently intact pieces of RER cisterns (Gilloteaux et al., 2001). The nucleus exhibits nucleolar segregation and chromatin decondensation followed by nuclear karyohexis and karyolysis (Gilloteaux et al., 1998; Gilloteaux et al., 1998; Gilloteaux et al., 2001; Gilloteaux et al., 2001; Jamison et al., 2002).

3. Apoptosis and necrosis

3.1. Apoptosis in physiology and pathology

The multicellular organism is very complicated in structure thus to keep it in homeostasis the nature developed very useful mechanism which Kerr et al. in 1972 named 'apoptosis'. The term apoptosis comes from the Greek word, meaning falling off (Kerr et al., 1972).

Apoptosis is a programmed cell death which is a physiological process occurring during embryogenesis and hormone dependent atrophy (Dockrell, 2001). Apoptosis is an evolutionary conserved process essential in the shaping of organs during development and the maintenance of tissue homeostasis in adult life (Bellany et al., 1995; Wyllie et al., 1980). Defects in apoptosis can cause a number of disease or may participate in some aspects of their pathology.

Because of its biological importance the programmed cell death occurs in all kind of metazoans (Tittel and Steller, 2000) such as in mammals, insects (Richardson and Kumar, 2002), nematodes (Liu and Hengartner, 1999) and cnidaria (Cikala et al., 1999). Programmed cell death is essential process for mammalian development. During the development there are produced many superfluous or potentially harmful cells. In most cases they die due to the process of apoptosis. In adults, billions of senescent cells undergo apoptosis every day and they are replaced by newly generated cells.

3.2. Apoptosis versus necrosis-difference in ultrastructural features and biochemistry

Apoptosis contrary to necrosis is active and ATP-dependent process. Apoptosis can be induced by a variety of stimuli including growth factor withdrawal ("death by neglect"), UV- or γ -irradiation, chemotherapeutic drugs and by triggering of the so called death receptors (Baumann et al., 2002). The most characteristic features for apoptosis are shrinkage of cells and cell-cell contact lost. Blebbing or budding of the plasma membrane (zeiosis) and formation of apoptotic bodies with cytosol and the condensed cell organelles (Baumann et al., 2002), karyorrhexis (Hail et al., 2006), chromatin condensation and DNA cleavage at internucleosomal linker regions result in cellular fragmentation (Fig.3). The mitochondrial inner transmembrane potential is decreased. Phosphatidylserine translocates from the inner surface to the outer surface of cell membranes. At the beginning, the cell membrane integrity

is preserved but efflux pumps become less effective. There is no induction of inflammation in the surrounding tissue. Apoptotic bodies are removed by phagocytes (Krammer, 1999) and protecting the neighboring cells from the release of the content into the environment. *In vivo*, most apoptotic cells are rapidly removed by phagocytosis (Hengartner, 2001).

On the other hand, necrotic cell death is a passive energy-independent process and results from non-physiological signals. Necrosis is an accidental cell death occurring in response to tissue injury (Wyllie, 1997). Nuclei do not condense and DNA degradation is rather random and induced by different set of enzymes as during apoptosis (Dong et al., 1997). During necrosis cells swell and cell membrane lose its integrity early in the process (Fig.3). Due to the disruption of the cell membrane, the cellular content is released and may induce an inflammatory reaction in the surrounding tissue (Baumann et al., 2002). This tissue inflammation is a significant feature of necrosis whereas phagocytosis of apoptotic cell bodies limits tissue inflammation (Savill and Fadok, 2000). Apoptotic bodies express phosphatidylserine and other cell surface molecules which can be recognized by macrophages using a series of receptors including CD14, scavenger receptors and integrins.

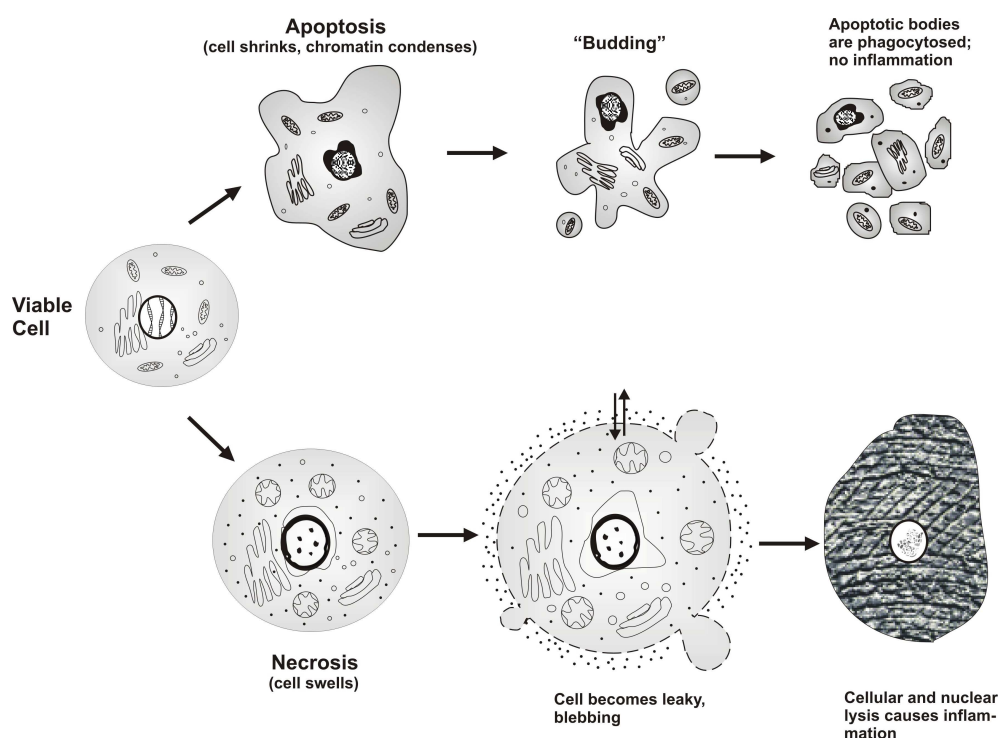


Fig.3. Characteristic features of apoptotic and necrotic cell death (modified from Van Cruchten, 2002).

3.3. Apoptosis stimulation

Apoptosis can be divided into three phases: initiation, effector and degradation (Green and Kroemer, 1998). The initiation phase mostly depends on the cell type and the apoptotic stimulus (e.g., oxidative stress, DNA damage, ion fluctuations, and cytokines). There is evidence that the initiation phase may have further influence on the efficacy of the effector and/or degradation phases. During the effector phase occurs the activation of proteases, nucleases, and other diffusible intermediaries that participate in the degradation phase. The final result of the effector and degradation phases are the apoptotic ultrastructural features (Ferri and Kroemer, 2000; Ferri and Kroemer, 2001; Hengartner, 2000; Thornberry and Lazebnik, 1998).

Among the variety of stimuli of apoptosis there are death receptors. The subfamily of these receptors belong to the TNF/NGF-receptor superfamily. They have an intracellular death domain (DD), important for transduction of the apoptotic signal. So far, there were discovered six members of this subfamily, namely TNF-R1 (CD120a), CD95 (APO/Fas), DR3 (APO-3/LARD/TRAMP/WSL1), TRAIL-R1 (APO-2/DR4), TRAIL-R2 (DR5/KILLER/TRICK2) and DR6 (Schulze-Osthoff et al., 1998; Ashkenazi and Dixit, 1999). Up to now, among those receptors, CD95 is the best-characterized (Krammer, 1999; Schmitz et al., 2000).

Death receptors are activated through their natural ligands. The ligands co-evolved with the death receptors as a death ligand family, called the TNF family. The death ligands belong to the type II transmembrane proteins, except for LT α .

There were conducted observations of morphological changes by Kerr et al. (1972) which were mainly caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These death proteases belong to a large protein family known as the 'caspases' and they are homologous to each other (Hengartner, 2000). The term caspases is derived from **c**ysteine-dependent **a**spartate-specific proteases (Gewies, 2003). Caspases are highly conserved through evolution, and can be found from humans all the way down to insects, nematodes, and hydra (Cikala et al., 1999; Budihardjo et al., 1999; Earnshaw et al., 1998). So far, there have been 7 different caspases identified in *Drosophila* (Richardson and Kumar, 2002), and 14 caspases identified in mammals (Nicholson, 1999), with caspase-11 and caspase-12 discovered only in the mouse (Denault and Salvesen, 2002). According to a unified nomenclature, the caspases are referred to in the order of their publication: caspase-1

is ICE (Interleukin-1 β -Converting Enzyme), the first mammalian caspase described to be a homologue of Ced-3 (Creagh and Martin, 2001; Miura et al., 1993). Caspase-1 together with caspases-4, -5, -11 and -12 seem to be mainly involved in the proteolytic maturation of proinflammatory cytokines such as pro-IL-1 β and pro-IL-18 so their contribution to the apoptotic cell death remains questionable (Denault and Salvesen, 2002).

All known caspases cleave substrates at Asp-Xxx bonds (that is, after aspartic acid residues) (Hengartner, 2000; Thornberry et al., 1997).

Caspases may be divided into two functional subfamilies: non-apoptotic caspases (caspase-1, -4, -5) and proapoptotic caspases. The proapoptotic caspases can be divided into the group of initiator caspases, which are responsible for upstream regulatory events during apoptosis, and effector caspases, which are directly responsible for cell disassembly events. Initiator caspases (caspase-2, -8, -9 and -10) are capable of trans- or autoactivation after they have been aggregated by adaptor molecules at death receptors. The downstream effector caspases (caspase-3, -6, -7) are substrates of the initiator caspases. The effector caspases possess only short prodomains whereas the initiator caspases possess long prodomains, containing death effector domains (DED) in case of procaspases-8 and -10 or caspase recruitment domains (CARD) as in the case of procaspase-2 and -9 (Gewies, 2003). Activation of downstream caspases results in a protease cascade that ensures widespread cleavage of multiple substrates and ultimately cell death (Baumann et al., 2002). Apoptosis can be slowed down or completely prevented by blocking the caspases' activity through mutations or pharmacological inhibitors (Earnshaw et al., 1998). Apart from this, many of these caspases may also participate in homeostatic cellular functions (*i.e.*, cytokine production, terminal differentiation, and proliferation) that are not associated with cell death.

3.3.1. Caspase activation

There are three general mechanisms of caspase activation. Most of the caspases are activated by proteolytic cleavage of downstream, effector caspases by an upstream caspase (Fig. 4). It is suggested that this is a kind of autocatalytic activation, with cleavage sites at Asp-X sites, which are candidate caspase substrate sites (Thornberry et al., 1997). The simplest way to activate a procaspase is an exposition to another, previously activated caspase molecule referred as 'caspase cascade'. 'Caspase cascade' is good for amplification and integration pro-apoptotic signals. This strategy of caspase activation is used by cells for the activation of the three short prodomain caspases, caspase-3, -6 and -7.

Another way of caspase activation is induced proximity. In this so called extrinsic apoptosis pathway, members of the death-receptor superfamily (CD95 (Apo1/Fas) and tumor necrosis factor receptor I) upon ligand binding, they aggregate and form membrane-bound signaling complexes. These multimolecular complexes of proteins are called the death-inducing signaling complex (DISC). During stimulation the serine phosphorylated adapter molecule FADD/Mort-1 (Boldin et al., 1995; Chinnaiyan et al., 1995) is recruited to oligomerized, most likely trimerized CD95 receptors. These complexes recruit, through the adapter molecule FADD (Fas-associated death domain protein), several molecules of procaspase-8 (two isoforms of caspase-8, caspase-8/a [FLICE/Mach- α 1/Mch5 β] and caspase-8/b [Mach- α 2]) (Boldin et al., 1996; Muzio et al., 1996) and caspase-10, and CAP-3, a molecule that contains the N-terminal death effector domains [DED] of caspase-8 and a yet uncharacterized C-terminus (Kischkel et al., 1995), resulting in a high local concentration of zymogen.

Caspase-9 is using the most complex mechanism of activation which is described as intrinsic apoptosis pathway. In this case its activation is mediated by means of conformational change, not proteolysis. In this process caspase-9 associates with a regulatory subunit Apaf-1. Both cytochrome c and Apaf-1 protein are required for caspase-9 activation (Li, P. et al., 1997; Zou et al., 1997). For the holoenzyme formation, Apaf-1 is oligomerized in the obligatory presence of cytochrome c and ATP. This process allows recruitment of procaspase-9 into the very large apoptosome complex. To summarize, the activation of effector procaspases-3, -6 and -7 are usually done by proteolysis by an upstream caspase, whereas initiator caspases are activated through regulated protein-protein interactions.

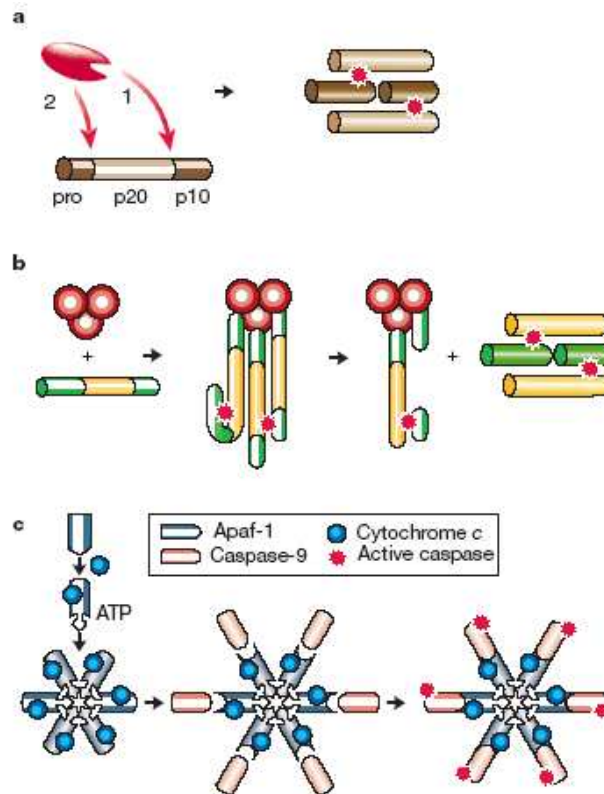


Fig. 4. Mechanisms of caspase activation: **a)** proteolytic cleavage by an upstream caspase, **b)** induced proximity, **c)** holoenzyme formation (adapted from Hengartner, 2000).

3.3.2. Apoptotic pathways

The extrinsic apoptosis pathways can be divided into type I and II. The “death receptors”, present at the cell surface, are activated to mediate apoptosis signaling. These receptors after ligation with specific ligands transmit apoptotic signals. Death receptors belong to the **tumor necrosis factor receptor (TNFR)** gene superfamily, where the members are TNFR-1, Fas/CD95 and the TRAIL receptors DR-4 and DR-5 (Ashkenazi, 2002). Further signaling pathway is mediated by the cytoplasmic part of the death receptor which contains a conserved sequence termed the **death domain (DD)**. Adapter molecules like FADD or TRADD themselves contain their own DDs by which they are attached to the DDs of the activated death receptor forming in this way the so-called **death inducing signaling complex (DISC)**. In addition to its DD, the adaptor FADD also contains a death effector domain (DED) which through homotypic DED-DED interaction sequesters procaspase-8 to the DISC. As it was described before, the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Subsequently active

caspase-8 induce downstream effector caspases which finally cleave specific substrates resulting in cell death. Summarizing, cells which possess the capacity to induce such direct and mainly caspase-dependent apoptosis pathways were classified to the so called type I cells (Scaffidi et al., 1998).

In the type II cell the signal which comes from the activated receptor does not generate a caspase signaling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be enhanced by the apoptotic pathway going through mitochondria. The small protein Bid from the Bcl-2 family is the link between the caspase signaling cascade and the mitochondria. Caspase-8 cleaves Bid and this truncated form - tBid translocates to the mitochondria where it acts together with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol (Luo et al., 1998). Cytochrome c binds to monomeric Apaf, which in presence of ATP changes the conformation and oligomerizes to assemble the apoptosome that triggers the activation of procaspase-9 (Acehan et al., 2002). Activated caspase-9 promotes caspase cascade initiation involving downstream effector caspases such as caspase-3, -7 and -6, which results in the cell death (Slee et al., 1999).

The Bcl-2 family is another set of apoptotic regulators acting through the regulated protein-protein interactions. Its name originates after a gene involved in B-cell lymphoma (hence the name *bcl*) (Tsujimoto et al., 1985). This family has been divided into three groups, based on structural similarities and functional criteria (Adams and Corry, 1998; Antonsson and Martinou, 2000). Members of group I possess anti-apoptotic activity and protect the cells from death, whereas members of group II and III promote cell death. Considering this fact it is logical that cells with more pro-death proteins are sensitive to death while cells with an excess of protective family members are usually resistant (Hengartner, 2000). The members of the first group, such as Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl-1, possess four short, conserved Bcl-2 homology (BH) domains (BH1-BH4). Their C-hydrophobic tail allows to localize the proteins to the outer surface of mitochondrial membrane, outer leaflet of the nuclear membrane and endoplasmic reticulum. The bulk of the protein faces the cytosol. Bcl-2 is localized to mitochondria, ER and nuclear membranes whereas Bcl-x_L is cytosolic. Upon apoptotic stimuli, Bcl-x_L translocates to the various membranes (Prasad and Prabhakar, 2003). The group II includes Bax, Bak and Bok, contains the hydrophobic tail and BH1-BH3 domain but it doesn't contain the BH4 domain (Adams and Corry, 1998; Antonsson and Martinou, 2000). Bak is localized to the ER and nuclear membranes whereas Bax is cytosolic and localizes to the mitochondria during apoptosis induction. Although there are not direct data

indicating that Bcl-2 or Bcl-x_L heterodimerize with Bax or Bak *in vivo*, however both neutralize each other functionally (Prasad and Prabhakar, 2003). Group III is the most variable, but the only common feature for these members is the presence of the ~ 12-16-amino-acid BH3 domain (Adams and Corry, 1998) (Fig. 5). This so called the BH3-only proteins consist of members like: Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, BNIP3 and Spike (Cory and Adams, 2002; Mund et al., 2003). Regulation of the release of pro-apoptotic factors like cytochrome c from the mitochondrial intermembrane compartment into the cytosol seems to be the key function of Bcl-2 family members (Adams and Corry, 1998; Antonsson and Martinou, 2000).

According to the Bcl-2 family members function, they can be divided into anti-apoptotic (e.g. Bcl-2, Bcl-x_L) and pro-apoptotic (e.g. Bax, Bak) proteins (Gross et al., 1999; Vander Heiden and Thompson, 1999). The characteristic feature for the anti-apoptotic group is the presence of the BH4 domain, whereas for apoptosis induction the BH3 domain is crucial. So far data illustrate that the members of the pro-apoptotic Bcl-2 family contain only BH3 domain proteins (e.g. Bid, Bad, Bim).

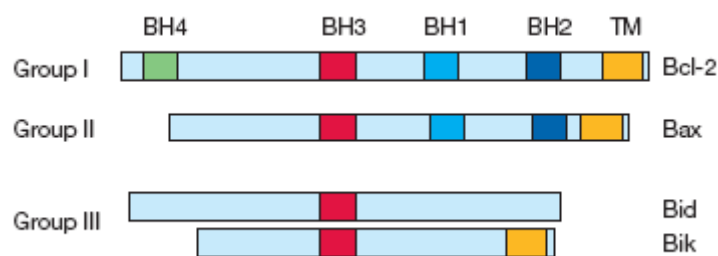


Fig. 5. Bcl-2 family members. (adapted from Hengartner, 2000).

In mitochondria there is a cocktail of pro-apoptotic proteins such as cytochrome c, apoptosis-inducing factor (AIF) (Susin et al., 1999), the endonuclease endoG (Li et al., 2001), Smac/DIABLO (Verhagen et al., 2000) and Htr/Omi (Verhagen et al., 2002). Among them the most important is cytochrome c, the humble electron carrier.

There are three basic models proposed for the regulation of cytochrome c exit by the Bcl-2 family members: 1. Bcl-2 members form channels that facilitate protein transport, 2. they interact with other proteins to form channels and 3. they induce rapture of the outer mitochondrial membrane.

In the first model, it is suggested that, Bcl-2 proteins after conformational change, it might act by inserting into the outer mitochondrial membrane, where they could form

channels or even large holes. Bcl-2 family members indeed can insert into synthetic lipid bilayers, oligomerize and form channels (Reed, 1997).

In the second model it is suggested that Bcl-2 family members interact with many proteins (Reed, 1997). There is a possibility that a large pore channel can be formed by pro-apoptotic family members that recruit other mitochondrial outer membrane proteins. For such reason the **voltage-dependent anion channel (VDAC)** is a protein to which several Bcl-2 family members can bind and regulate its channel activity (Shimizu et al., 1999). Upon binding VDAC undergoes conformational change but the pore size of VDAC channel is too small to allow proteins to pass through (Hengartner, 2000).

In the last proposed model it is considered that the Bcl-2 family members control homeostasis of the mitochondria. In this case, apoptotic signals alter mitochondrial physiology (for example, ion exchange or oxidative phosphorylation) so in the result the organelle swells and undergo physical rupture of the outlet membrane releasing intermembrane proteins into the cytosol.

There are two possibilities to disturb the mitochondrial homeostasis either directly by the Bcl-2 family members (for example, through the proposed intrinsic ion-channel activity mentioned above) or indirectly, through modulation of other mitochondrial proteins. Again in this regulation VDAC protein plays a major role together with **adenine nucleotide translocase (ANT)**, as they are core components of mitochondrial **permeability transition pore (PTP)**, a large channel whose opening results in rapid loss of membrane potential and organellar swelling. ANT is the most abundant protein of the inner mitochondrial membrane and as the transmembrane channel is responsible for the export of ATP in exchange with ADP (antiport). VDAC also called porin is the most abundant protein of the outer mitochondrial membrane, which forms a non-selective pore through the outer membrane. The so called 'contact sites' are composed of VDAC-ANT complexes which probably connect inner and outer mitochondrial membrane allowing a close association of the two membranes and thereby constituting the PT pore (Beutner et al., 1998). Upon opening the PTP the cytochrome c is released and the apoptotic cell death occurs.

There are several other proteins present in mitochondria and released during apoptosis like AIF (a flavoprotein with apoptotic activity) (Lorenzo et al., 1999), Smac/DIABLO (Verhagen et al., 2000; Heckman et al., 2002), Omi/HtrA2 (Prasad and Prabhakar, 2003) and several pro-caspases, including procaspase-2, -3 and -9 (Loeffler and Kroemer, 2000). The Smac (for **second mitochondria-derived activator of caspases**) (Du et al., 2000) or DIABLO (Verhagen et al., 2000) (for **direct IAP-binding protein with low pI**) and Omi (Prasad and

Prabhakar, 2003) are known as a mammalian IAP inhibitors which act by direct blocking caspase activity (Fig. 6). Smac/DIABLO binds to IAP family members and neutralizes their anti-apoptotic activity. Smac/DIABLO is normally a mitochondrial protein, but it is released into the cytosol in cells induced to die, presumably following the same exit route as cytochrome c.

IAPs are a family of antiapoptotic proteins among which eight human IAP homologues have been identified, so far. They consist of NAIP, c-IAP1, c-IAP2, XIAP and survivin. It is believed that the antiapoptotic activity of IAPs depends on the interaction between the BIR domains and caspases. It was described that c-IAP1, c-IAP2 and XIAP directly inhibit caspases-3, -7, -9 (Salvesen and Duckett, 2002a).

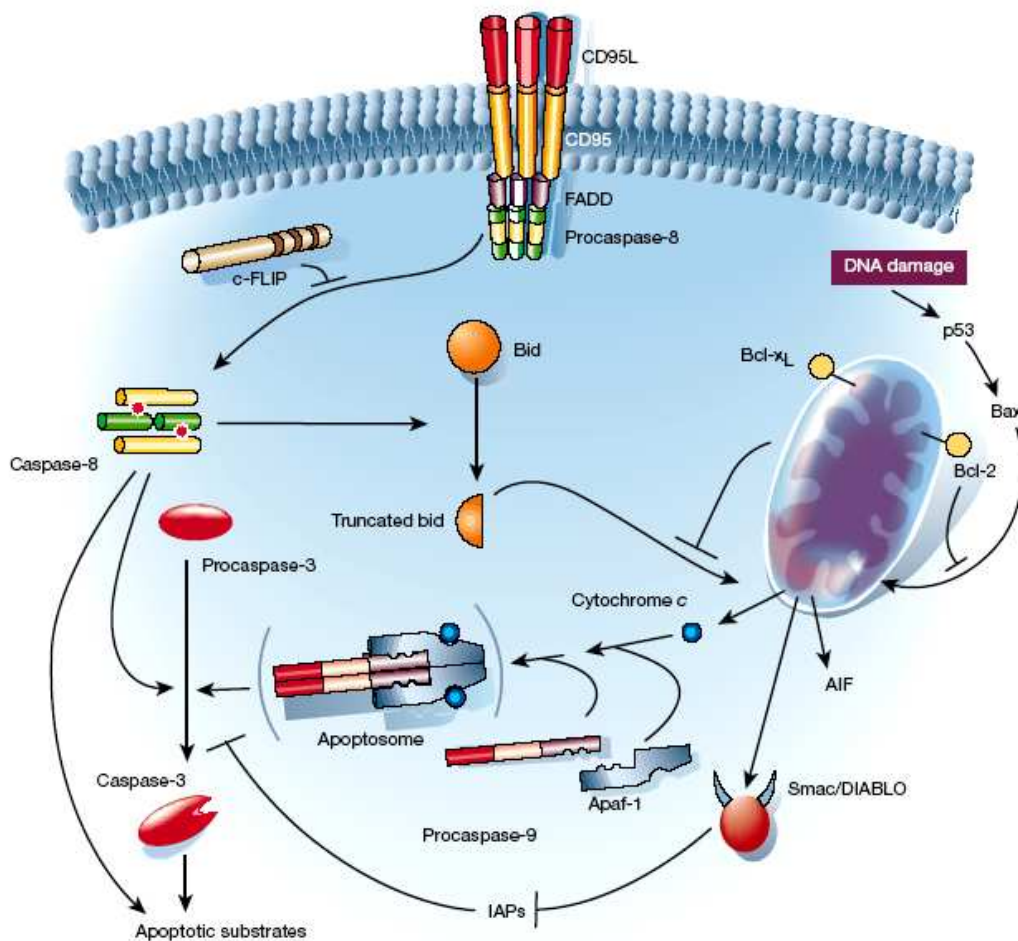


Fig. 6. Two major apoptotic pathways in mammalian cells. (adapted from Hengartner, 2000).

DNA damage is another source of the signal for apoptosis as a result of irradiation, drugs and other sort of stress. In most cases, p53 transcription factor is activated in response to DNA damage which promotes expression of proapoptotic Bcl-2 members and suppresses

antiapoptotic Bcl-2 and Bcl-X_L. Apart from mitochondria and the nucleus, ER and lysosomes are also implicated in apoptotic signaling pathways.

p53 is the apoptosis factor which takes part in cancer because it is inactivated in presumably more than 50% of all human cancers (Hainaut and Hollstein, 2000). p53 is a tumor suppressor protein which is activated as a transcription factor in response to e.g. DNA damage or oncogene activation, resulting in growth arrest or apoptosis by stimulating the expression of various p53 target genes such as p21, Bax, Noxa, Puma, Apaf-1, Fas, and DR5 (Vousden and Lu, 2002) or by suppression the expression of antiapoptotic proteins, e.g. Bcl-2, Bcl-X_L or surviving (Hoffman et al., 2002; Wu et al., 2001).

In non-stressed, normal growing viable cells p53 is present in the cytosol at low cellular concentrations and prevented to enter the nucleus, and its transactivation domain is inactivated (Chene, 2003). The central regulator of p53 is the oncogene Mdm2, which is a ubiquitin-ligase. It binds to p53 protein and marks it for proteasomal degradation. In this way, p53 levels are kept low in normal cells (Kubbutat et al., 1997). p53 can be rescued from degradation by the deubiquitinating enzyme HAUSP (Fig.7).

p53 is stabilized and activated in response to cellular stress e.g. DNA damage, which provides the phosphorylation of p53 at specific serine/threonine residues which prevents the Mdm2-p53 interaction (Schon et al., 2002). Stabilized and activated p53 can translocate into the nucleus where it activates the transcription of proapoptotic genes and suppresses the transcription of antiapoptotic genes what under certain conditions can result in the induction of apoptosis. Protooncogenes such as c-myc, adenovirus E1A, and ras induce p53-mediated apoptosis signalling as well as the depletion of pRb retinoblastoma tumor suppressor (Henriksson et al., 2001). E2F-1 can promote cell cycle progression and proliferation but at the same time directly triggers expression of the tumor suppressor ARF which leads to stabilization and activation of p53 (Ginsberg, 2002) (Fig. 7).

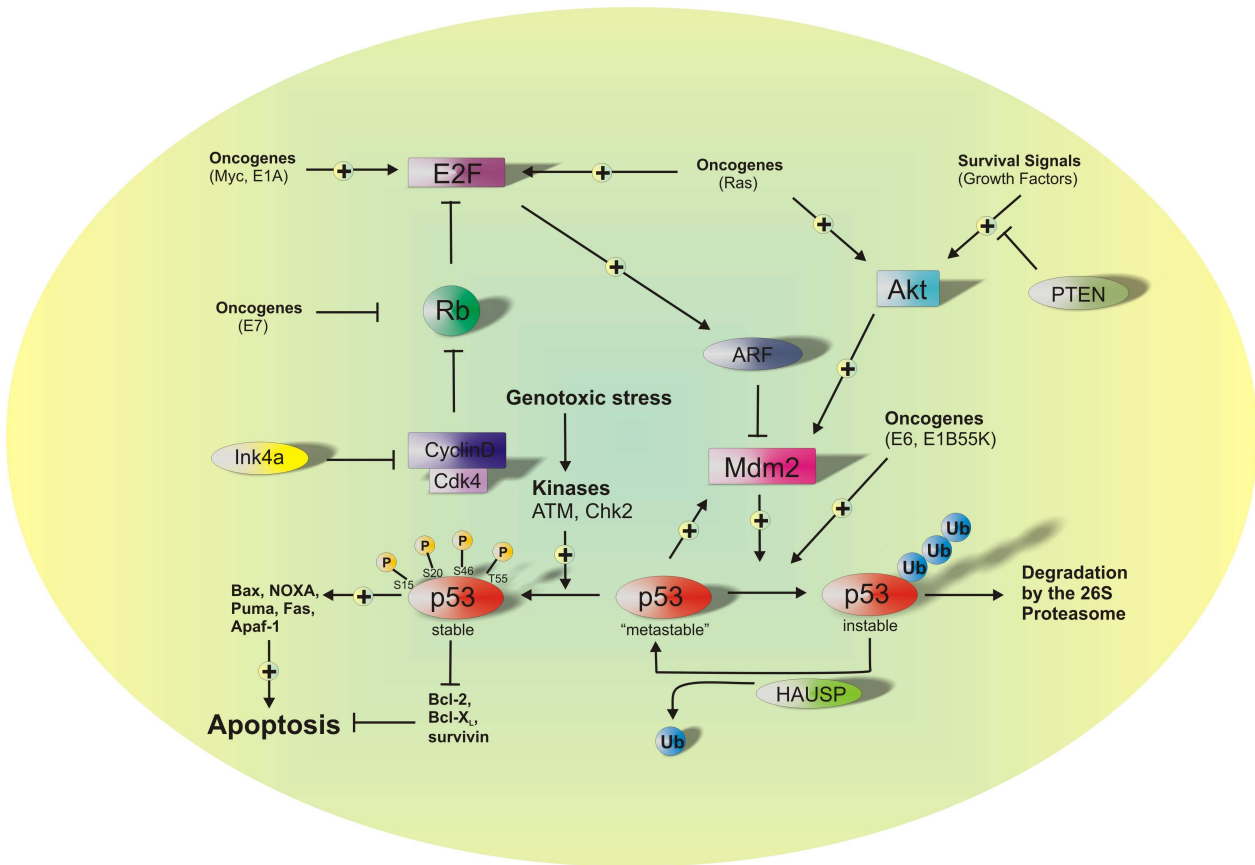


Fig.7. The p53 network of apoptosis regulation. (modified from Gewies, 2003)

3.4. Apoptosis in the immune system development

Apoptosis is crucial for tissue homeostasis in multicellular organisms. It plays a very important role in development and in the immune system (Krammer, 1999; Vaux and Korsmeyer, 1999), regulating the maturation of T- and B-lymphocytes, and in keeping their homeostasis. Apoptosis plays role in elimination of autoreactive lymphocytes. The failure in apoptotic signaling pathways can result in the development of autoimmune disorders (Prasad and Prabhakar, 2003). Many diseases are connected with either too much or too little apoptosis, such as AIDS, cancer and autoimmunity, respectively (Krammer, 1999). Death by apoptosis is essential for function, growth and differentiation of T-lymphocytes.

The lymphocyte population can be controlled by keeping the balance between newly matured lymphocytes released from the central lymphoid organs (bone marrow and thymus) and the constant removal of lymphocytes due to death by neglect and activation induced cell death. Any disturbance in this homeostasis may result in the development of autoimmune disease (Prasad and Prabhakar, 2003).

Lymphocytes are principal mediators of the immune response. Before they achieve their final state during maturation and subsequent release into the periphery they are subjected to several checks at various stages (Sebzda et al., 1999). A major checkpoint in developing lymphocytes, in the central lymphoid organs, is at the initial stages when the lymphocyte precursors fail to express a functional unique antigen receptor. If these cells fail to receive survival signals, they undergo apoptosis (death by neglect) (Melchers et al., 2000; Baird et al., 1999). The number of T-cells that leave the thymus and enter the peripheral T cell pool is only about 2-3% of the number initially generated. Thus, apoptosis in the thymus plays a key role in the selection of T-lymphocytes (Baumann et al., 2002).

3.5. The switch of the cell death mode from apoptosis to necrosis

One of the hot topics in the research field of apoptosis is the switch mechanism of the cell death mode from apoptosis to necrosis. There is a body of evidence describing the switch of the cell death mode from apoptosis to necrosis using various experimental conditions (Ledda-Columbano et al., 1991; Sata et al., 1997; Sun et al., 1997).

Clarification of the mechanism of the switch of the cell death mode is of vital importance especially for clinical medicine since necrosis causes local and general inflammatory reactions while apoptosis does not. If the treatment of cancer causes massive necrosis instead of apoptosis, it may become lethal in extreme cases. There have been proposed three mechanisms for the switch of the cell death mode: 1. a burst in the intracellular generation of reactive oxygen species (ROS) (Bonfoco et al., 1995; Nobel et al., 1997); 2. inactivation of caspases (Lemaire et al., 1998; Melino et al., 1997; Samali et al., 1999) and 3. depletion of intracellular ATP (Eguchi et al., 1997; Leist et al., 1997; Ferrari et al., 1998; Ha and Snyder, 1999).

4. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Reactive Oxygen Species (ROS) are a family of small but highly reactive molecules, including free radicals, such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), and non-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen (1O_2) (Fig. 8). Reactive Nitrogen Species (RNS) consist of nitric oxide and peroxynitrite. ROS and RNS production, is associated with normal cellular metabolism (Li, Y. et al., 1997). While low levels of ROS

and RNS stimulate cell growth, increased levels of ROS is responsible for apoptosis or necrosis stimulation in various cells (Luczak et al., 2004).

Oxidative stress is considered to be a pathogenic factor of many diseases, such as inflammatory diseases, cardiovascular diseases (hypercholesterolemia, atherosclerosis), diabetes, Alzheimer's disease, smoking-related diseases, cancer, and aging etc. (Cai and Harrison, 2000; Rueckschloss et al., 2003; Halliwell and Gutteridge, 1999).

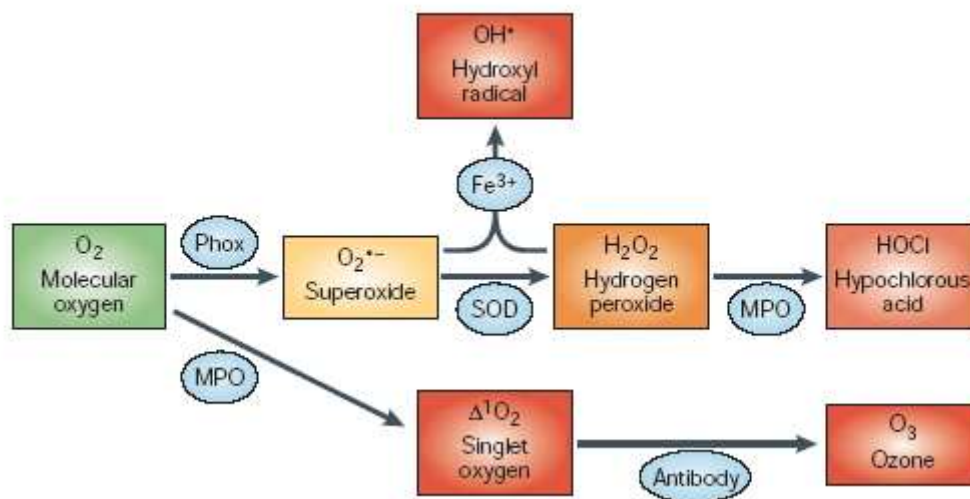


Fig. 8. Reactive oxygen species. NADPH oxidase (NOX) enzymes (such as the phagocyte NOX, Phox) are one of the sources of superoxide generation. Hydrogen peroxide (H_2O_2) is generated in the dismutation reaction of superoxide, accelerated by the enzyme superoxide dismutase (SOD). In the presence of iron, superoxide and H_2O_2 react to generate hydroxyl radicals. In inflamed areas, neutrophils form hypochlorous acid (HOCl) from H_2O_2 and chloride by the phagocyte enzyme myeloperoxidase (MPO). In areas of inflammation, singlet oxygen is formed from oxygen through the action of Phox and MPO-catalyzed oxidation of halide ions. Ozone can be generated from singlet oxygen by antibody molecules. The colour coding indicates the reactivity of individual molecules (green: relatively unreactive; yellow: limited reactivity; orange: moderate reactivity; red: high reactivity and non-specificity. (adapted from Lambeth, 2004).

ROS can be produced by both endogenous and exogenous sources. Endogenous sources consist of oxidative phosphorylation, *P*-450 metabolism, peroxisomes, and inflammatory cell activation (Table 1) (Klaunig and Kamendulis, 2004).

Table 1. Reactive oxygen and nitrogen species generation and removal in cell [adapted and modified from Klaunig and Kamendulis, 2004].

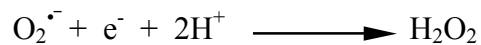
Cellular oxidants	Source	Oxidative species
Endogenous	Mitochondria	$O_2^{\cdot-}$, H_2O_2 , $\cdot OH$
	NADPH oxidases	$O_2^{\cdot-}$
	Cytochrome P-450	$O_2^{\cdot-}$, H_2O_2
	Peroxisomes	H_2O_2
	Macrophage/inflammatory cells	$O_2^{\cdot-}$, $\cdot NO$, H_2O_2 , OCI^-
Exogenous	Redox cycling compounds	$O_2^{\cdot-}$
	Metals (Fenton reaction)	$\cdot OH$
	Radiation	$\cdot OH$
Cellular antioxidants		
<u>Enzymatic</u>		<u>Nonenzymatic</u>
Superoxide dismutase		Vitamin E
Catalase		Glutathione
Glutathione peroxidase		Vitamin C
Glutaredoxin		Catechins
Thioredoxin		
Oxidants > Antioxidants \rightarrow Oxidative damage		

Neutrophils, eosinophils, and macrophages are an additional endogenous source and are major contributors to the cellular reactive oxygen species (Klaunig and Kamendulis, 2004). ROS are generated by stimulated polymorphonuclear neutrophils (PMNs) in host defense mechanism against invading microorganisms (Van den Worm et al., 2001). Upon triggering, neutrophils start to consume large amount of oxygen which is converted into ROS, a process which is described as the respiratory or oxidative burst (Babior, 1978; Babior, 1995). This process depends on the activity of the enzyme NADPH oxidase. This oxidase can be activated by both receptor-mediated and receptor-independent process. Receptor-dependent stimuli consist of complement components C5a, C3b and iC3b (Ogle et al., 1988), the bacterium-derived chemotactic tripeptide N-formyl-Met-Leu-Phe (fMLP) (Williams et al., 1977), the lectin concanavalin A (Weinbaum et al. 1980), and opsonized zymosan (OPZ) (Whitin et al., 1985). Receptor-independent stimuli include long-chain unsaturated fatty acids and phorbol 12-myristate 13-acetate (PMA) (Schnitzler et al., 1997). Upon activation, the oxidase accepts

electrons from NADPH at the cytosolic side of the membrane and donates these to molecular oxygen at the other side of the membrane or in the phagosomes containing ingested microorganisms (van den Worm, 2001). In this way, there occurs a one-electron reduction of oxygen to superoxide anion as presented in the following equation:



Most of the oxygen consumed in this way will not be present as superoxide radical ($\text{O}_2^{\bullet -}$) but will be dismutated to hydrogen peroxide (Hampton et al., 1998; Roos et al., 1984). Superoxide was shown to act as a small second messenger molecule in many of different cellular processes (Rosen and Freeman, 1984; Mayer and Schmitt, 2000).



However, hydrogen peroxide (H_2O_2) is bactericidal only at high concentrations (Hyslop et al., 1995) while exogenously generated superoxide does not kill bacteria directly (Babior et al., 1975; Rosen and Klebanoff, 1979), because of its limited membrane permeability. There are proposed several secondary oxidants which participate in destructive mechanism of PMNs (Fig.9.).

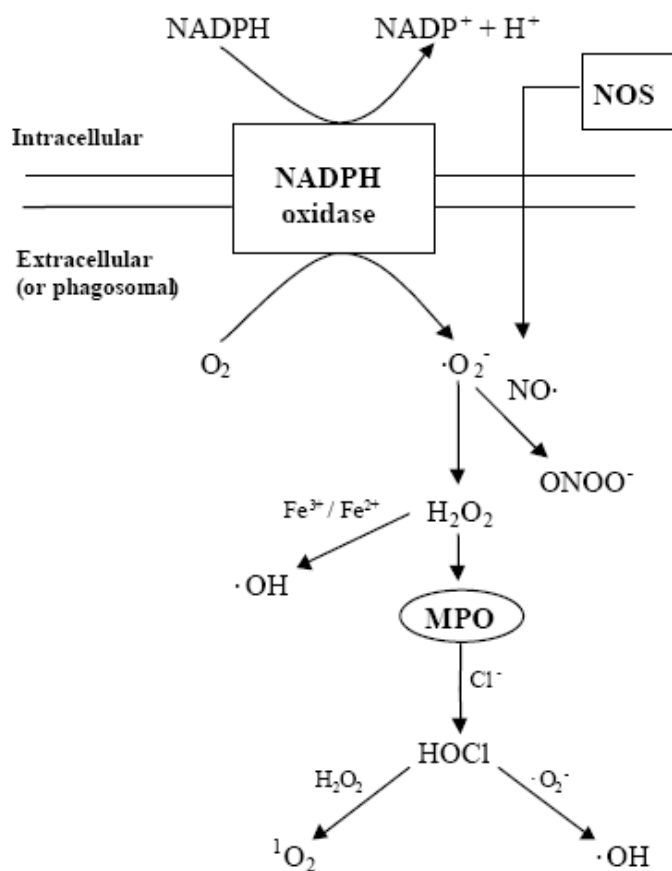
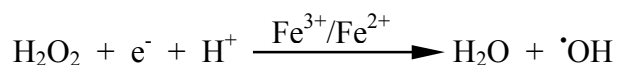


Fig. 9. Reactive oxygen species production and reactions in stimulated neutrophils.

[NOS: nitric oxide synthase, MPO: myeloperoxidase; adapted from Hampton et al., 1998]

Hydroxyl radical ($\cdot\text{OH}$), is formed in Fenton reaction catalyzed by the iron. It is extremely reactive with most biological molecules like nucleic acids, lipids, and proteins (Samuni et al., 1988; Betteridge, 2000).



Singlet oxygen ($^1\text{O}_2$), is considered as the electronically excited state of oxygen and may react with membrane lipids initiating peroxidation (Halliwell, 1978). Most of the H_2O_2 generated by PMNs is consumed by myeloperoxidase (MPO), an enzyme released by stimulated PMNs (Kettle and Winterbourn, 1997; Klebanoff, 1999; Nauseef, 1988; Zipfel et al., 1997). This heme-containing peroxidase is a major constituent of azurophilic granules and is unique in using H_2O_2 to oxidize chloride ions to the strong non-radical oxidant hypochlorous acid (HOCl) (Harrison and Shultz, 1976). There are also other substrates of

MPO such as iodide, bromide, thiocyanite, and nitrite (Van Dalen et al., 1997; Vliet et al., 1997).



HOCl is the most bactericidal oxidant known to be produced by the PMNs (Klebanoff, 1968), and many species of bacteria are killed readily by the MPO/ H₂O₂/ chloride system (Albrich and Hurst, 1982).

Together with ROS there are also RNS produced by inflammatory cells, such as nitric oxide (NO) and NO-derived peroxynitrite (ONOO⁻) with cytotoxic mechanism of action (Harald et al., 1994; Moncada et al., 1991; Bredt and Snyder, 1994). Peroxynitrite is relatively stable oxidant (Fukuyama et al., 1996) with properties similar to those of hydroxyl radical. This anion is suspected to play a pivotal role in a number of disorders such as acute lung injury (Kooy et al., 1995), human asthma (Saleh et al., 1998), inflammatory bowel disease (Singer et al. 1996), idiopathic pulmonary fibrosis (Saleh et al., 1997), and animal models for septic shock (Szabo et al., 1994).

ROS regulate multiple cell functions and gene expression (Barbieri et al., 2004). There are several enzyme systems which participate in cellular ROS generation including NADPH oxidase (Babior, 1999; Bonizzi et al., 1999), 5-lipoxygenase (Morre and Brightman, 1991), xanthine oxidase (Hille and Nishino, 1995), NADH oxidase (DeLeo and Quinn, 1996), cyclooxygenases and others (Bonizzi et al., 1999). Under physiological conditions, the mitochondrial respiratory chain is the major site for ROS production in cells (Barja, 1999; Boveris and Chance, 1973).

5. NADPH oxidase

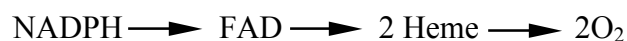
NADPH oxidase was considered as characteristic only for phagocytes (neutrophils (Batot et al., 1995), eosinophils, monocytes and macrophages (Segal et al., 1981)). Upon activation during phagocytosis NADPH oxidase generates a large quantity of superoxide anion and plays a pivotal role in non-specific host defence against pathogens (Lambeth, 2004). However, the recent researches have revealed that membrane NADPH oxidase can be also found in other type of tissue like: cardiovascular system (vascular smooth muscle cells (VSMCs) (Griendling et al., 2000), endothelial cells (Bayraktutan et al., 2000), adventitial and cardiac fibroblasts (Pagano et al., 1998) and cardiomyocytes (Bendall et al., 2002)),

kidney (Cheng et al., 2001; Geiszt et al., 2000), spleen (Cheng et al., 2001; Banfi et al., 2001), colon (Geiszt et al., 2003), thyroid (De Deken et al. 2000), osteoclasts (Cheng et al., 2001), ovary (Cheng et al., 2001), sperm (Banfi et al., 2001), mammary glands (Banfi et al., 2001), cerebellum (Lambeth et al., 2000), eye (Cheng et al., 2001), etc. The level of production of superoxide by this enzyme in other tissues is much decreased and its role is supposed to be involved in signaling processes. The function of NADPH oxidase in professional phagocytes is to kill invading microorganisms.

NADPH oxidase is a multicomponent enzyme with a redox center that transfers electrons from cytoplasmic NADPH onto extracellular molecular oxygen, thereby generating superoxide anion, which serves as a precursor for a variety of toxic oxygen metabolites (including hydrogen peroxide, hypochlorous acid, peroxynitrite and ozone) that contribute to the resolution of infection (Lambeth, 2004). The production of superoxide from oxygen and NADPH by this enzyme occurs according to the following reaction (Roos et al., 2003):



The electron transfer from NADPH oxidase to oxygen is a multistep process, during which the electrons are transported sequentially along several moieties of the oxidase (Roos et al., 2003):



FAD and the two heme groups are the part of the redox center of this enzyme which is cytochrome *b*₅₅₈. However, the electron transfer can take place only after NADPH binding to this protein, which occurs when the complete enzyme has assembled during activation (Roos et al., 2003).

The structure of phagocytes NADPH oxidase is quite complex. The oxidase consists of the catalytic subunit gp91^{phox} (known also as NOX2) and regulatory subunits p22^{phox}, p67^{phox}, p47^{phox}, p40^{phox} and a low-molecular-weight GTP binding protein Rac (either Rac1 in macrophages and nonphagocytic cells or Rac2 in neutrophils) (Fig. 10) (Babior, 1999; Lambeth, 2004; Roos et al., 2003; Sumimoto et al., 1994). During 1990s, there were searched databases for homologues of gp91^{phox} that might take part in ROS generation. These investigations revealed several sequences encoding portions of new gp91^{phox} homologues (Lambeth, 2004). In 1999, the first of the NOX homologues of gp91^{phox} was described, NOX1

(Suh et al., 1999). Subsequent examining provided with 6 additional enzymes in this family (Table 2).

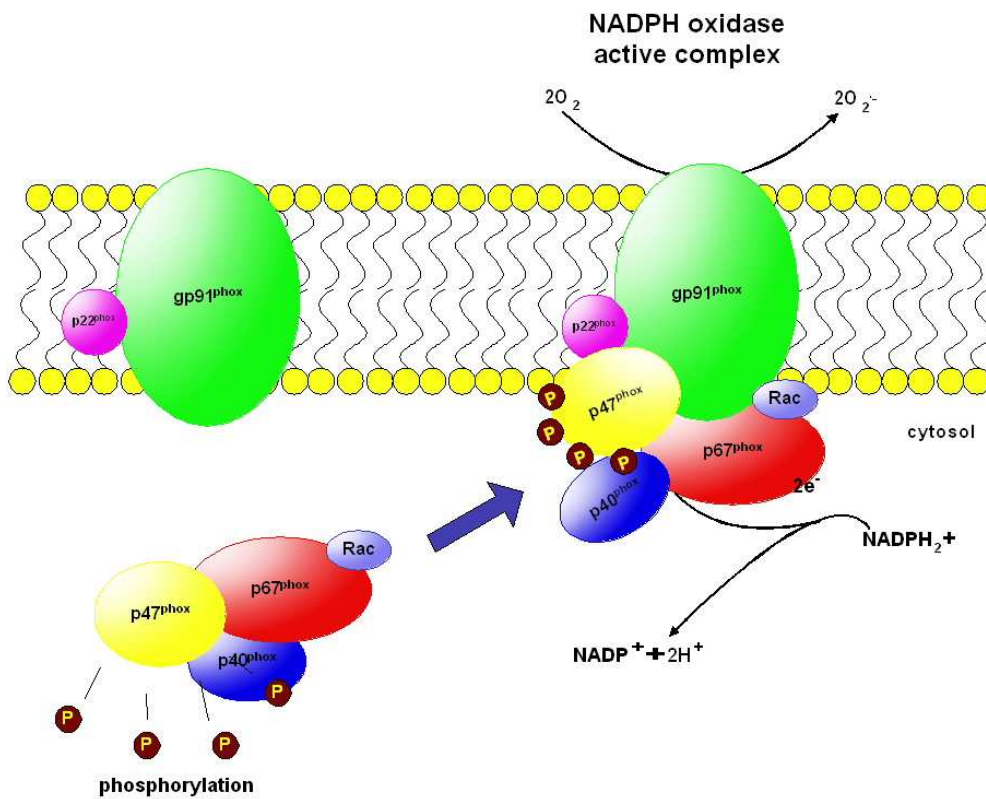


Fig. 10. Structure and activation of Nox2 NADPH oxidase (adapted and modified from Dworakowski et al., 2006).

The expression of these enzymes in various tissues provides evidence that ROS generation might be a general feature of many or perhaps all cells (Lambeth, 2004).

The NOX enzymes are classified into three main groups, according to the presence of domains in addition to the gp91^{phox} domain (Lambeth et al., 2000) (Fig. 11). NOX1, NOX3 and NOX4 are nearly identical in size and structure to gp91^{phox}. They have up to 60% homology and are predicted to contain six transmembrane α -helices and similar to gp91^{phox}, they contain the electron transfer centers that are required to pass electrons from NADPH to molecular oxygen to form superoxide (Lambeth, 2004; Dworakowski et al., 2006). According to the predicted topology of this sub-group by Lambeth, 2004, NADPH is oxidised on the cytosolic side of the membrane and the oxygen is reduced across the membrane to generate superoxide. In a secondary reaction, two molecules of superoxide can then react to generate H₂O₂, which can function as a substrate for peroxidases such as myeloperoxidase in neutrophils. NOX5 structure is based on gp91^{phox} with an additional N-terminal calmodulin-

like domain that contains four binding sites for calcium (Fig. 11). In calmodulin, calcium binding results in a conformational change, exposing hydrophobic residues that bind to and regulate target enzymes (Lambeth, 2004). Considering this fact, in cells where the NOX5 is expressed, it is activated by the calcium ionophore ionomycin (Banfi et al., 2001). The third group comprises the DUOX enzymes which is based on the NOX5 structure, containing additionally an amino-terminal peroxidase-homology domain (Fig. 11). Between the peroxidase-homology domain and the calcium-binding domain there is predicted an extra transmembrane α -helix. The peroxidase domain is placed on the extracellular face of the plasma membrane (Lambeth, 2004). Although the sequence of peroxidase domain of DUOX is homologous to myeloperoxidase, it has an amino-acid replacement at residues that are thought to be crucial for myeloperoxidase activity (Zeng and Fenna, 1992). Concluding this group, it seems that DUOX plays dual function generating reactive oxygen and using it for its own peroxidase domain. This role allows to oxidase an extracellular co-substrate such as extracellular matrix proteins (Lambeth, 2004).

Nox isoforms expression varies in a cell-specific manner (Table 2) (Bendall et al., 2002; Cucoranu et al., 2005; Bayraktutan et al., 1998; Ago et al., 2004; Byrne et al., 2003). However, interesting is that several cell types can co-express more than one Nox subunit (Ago et al., 2004; Byrne et al., 2003; Dworakowski et al., 2006).

Nox2 isoform previously termed gp91^{phox} was for the first time identified in phagocytes. It is the best understood active NADPH oxidase complex. Nox2 is utilized as a catalytic core protein in the plasma membrane, critically depending on p22^{phox}, which not only stabilizes Nox2 but also serves as a docking site for other binding proteins, p47^{phox} and p67^{phox} (Opitz et al., 2007). Upon phosphorylation by PKC, the complex of p47^{phox} and p67^{phox} migrates from the cytosol to the membrane, where this interaction is strengthened by cytosolic adaptor protein, p40^{phox}, which stabilizes the p47^{phox} and p67^{phox} interaction (Suh et al., 2006; Kuribayashi et al., 2002). The small GTPase Rac protein translocates independently of this process to the plasma membrane (Heyworth et al., 1994).

Nox1 is the first described homologue of gp91^{phox}/Nox2 and also forms a heterodimer with p22^{phox}, which is activated in the presence of NoxO1 and NoxA1 that are the most likely physiological regulators (Banfi et al., 2003; Geiszt et al., 2003; Takeya et al. 2003). Also Rac1 supports Nox1 oxidase activity (Cheng et al., 2006; Opitz et al., 2007; Miyano et al., 2006; Ueyama et al., 2006).

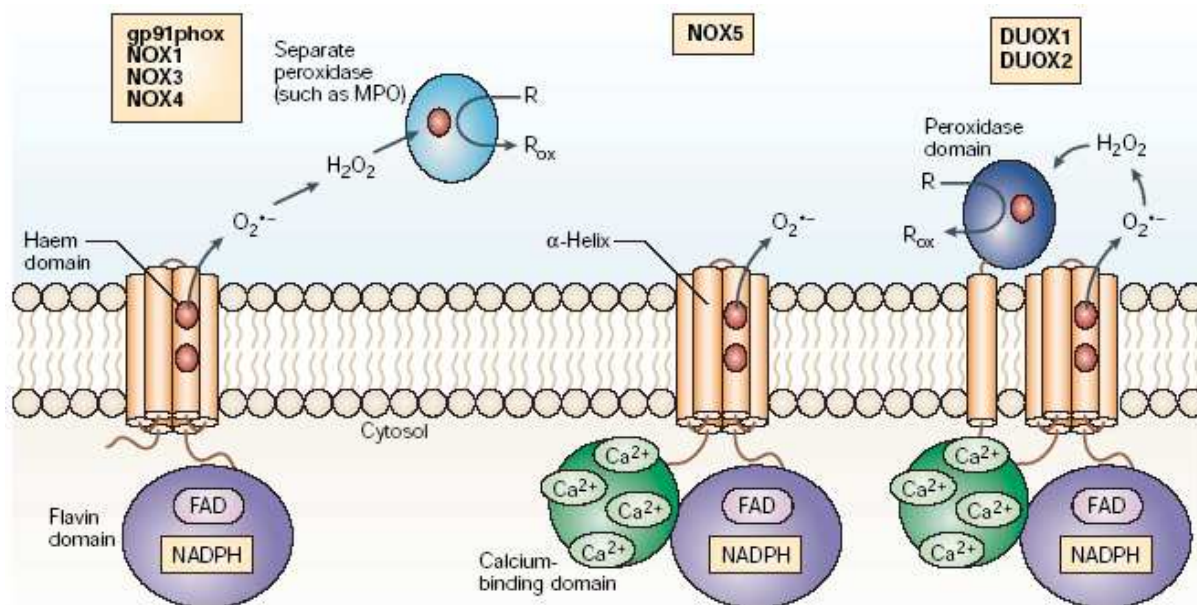


Fig. 11. Transmembrane topology and domain structure of NOX and DUOX enzymes (adapted from Lambeth, 2004).

Nox3 is expressed in a tissue-specific manner in the inner ear and fetal kidney (Cheng et al., 2001; Banfi et al., 2004). All mRNA for NoxO1, NoxA1, p47^{phox}, p67^{phox} and p22^{phox} are coexpressed with Nox3 in the inner ear of mice (Cheng and Lambeth, 2005). Also Nox3 forms a complex with p22^{phox}, however this complex appears to be capable of producing O₂^{•-} even in the absence of NoxO1 and NoxA1 (Banfi et al., 2004). The role of Rac in regulating Nox3 activity is controversial (Ueno et al., 2005; Ueyama et al., 2006).

Nox4 was identified in a variety of tissues and cell types (Table 2). Like Nox1-3, Nox4 forms a heterodimer with p22^{phox} for full activity and stabilization of the enzyme complex (Ambasta et al., 2004). However, unlike for Nox1 and Nox2, p47^{phox}, p67^{phox}, NoxO1, NoxA1, nor Rac appear to regulate ROS production in Nox4 overexpression systems (Martyn et al., 2006). These data suggest that activation of Nox4 does not depend on any of the known regulatory subunits and it may serve as a constitutive ROS generating oxidase (Opitz et al., 2007). Although there is some evidence in mesangial cells that Nox4 activity is regulated by angiotensin II (Gorin et al., 2003). However, the mechanism underlying this effect is unclear. This Nox unlike other Nox isoforms produces H₂O₂ instead of O₂^{•-} and intracellularly versus extracellularly (Martyn et al., 2006).

Nox5 is, according to the latest phylogenetic analysis by David Lambeth's team, the evolutionary most ancient one of the mammalian NADPH oxidases (Opitz et al., 2007). Nox5 expression was detected in plenty of type of cells and tissues (Table 2). Unique feature of

Nox5 is that this isoform does not require p22^{phox}, or indeed any of the other known regulatory subunits, for activation (Kawahara et al., 2005). This Nox contains a cytosolic N-terminal Ca²⁺-binding domain with calmodulin-like EF-hand motifs. This makes Nox5 sensitive to Ca²⁺ similar to the Duox family (Lambeth, 2004). An increase of intracellular levels and subsequent binding of Ca²⁺ to the EF-hand motifs lead to a conformational change and activation of Nox5 (Banfi et al., 2001; Banfi et al., 2004). However the intramolecular mechanism is unknown.

Among regulatory subunits there is p22^{phox} subunit that together with gp91^{phox} form a mutually stabilizing, membrane-associated complex. This complex has been biochemically isolated and is referred to as flavocytochrome *b*₅₅₈ (Lambeth, 2004).

Activation of NADPH oxidase can occur after translocation of cytosolic components: p67^{phox}, p47^{phox}, p40^{phox} and a low-molecular-weight GTP binding protein Rac (either Rac1 or Rac2) to the cell membrane and their assembly with the membrane subunits - flavocytochrome *b*₅₅₈ (Lambeth, 2004). Nox4 seems to be unusual as several studies revealed that it does not require either p67^{phox}, p47^{phox} (or analogues) or Rac for its activity. It suggests that its mode of activation may be significantly different (Ambasta et al., 2004; Martyn et al., 2006). In case of neutrophils and other phagocytic leukocytes the activation of NADPH oxidase is strictly regulated in order to minimize the risk of damage to the organism (Roos et al., 2003).

Table 2. Human NOX/DUOX enzymes (Lambeth, 2004)

Enzyme	Highest level of expression	Known regulatory factors	References
gp91 ^{phox} (NOX2)	Phagocytes, endothelial cells, cardiomyocytes and fibroblasts	p47 ^{phox} , p67 ^{phox} , p40 ^{phox} and RAC1/RAC2	Vignais, 2002
NOX1	Inducible: colon epithelium and vascular smooth muscle cells, stomach, uterus, prostate	NOXO1, NOXA1 and p22 ^{phox}	Ago et al., 2003; Banfi et al., 2003; Geiszt et al., 2003; Krause, 2004; Suh et al., 1999
NOX3	Fetal kidney, adult inner ear	N.D.	Banfi et al., 2004; Cheng et al., 2001; Lambeth et al., 2000
NOX4	Kidney, osteoclasts, skeletal muscle, ovary and testis, eye, placenta, endothelial cells, fibroblasts, VSMCs, cardiomyocytes; widespread	N.D.	Banfi et al., 2004; Cheng et al., 2001; Geiszt et al., 2000
NOX5	Spleen, adult testis, ovary, mammary glands, placenta foetal tissues, pancreas and cerebrum, B- and T-lymphocytes	Calcium	Banfi et al., 2001; Cheng et al., 2001
DUOX1	Thyroid, cerebellum and lungs	Calcium	De Deken et al., 2000; Lambeth et al., 2000
DUOX2	Thyroid, colon, pancreatic islets and prostate	Calcium	De Deken et al., 2000; Edens et al., 2001

DUOX - dual oxidase; N.D.- not determined; NOX - NADPH oxidase; NOXA1 – NOX activator 1; NOXO1 – NOX organizer 1

Cytochrome *b*₅₅₈ is a flavo-hemoprotein composed of two subunits, gp91^{phox} and p22^{phox}, in a 1:1 stoichiometry (Lambeth et al., 2000). The membrane-bound gp91^{phox} subunit includes flavine adenine dinucleotide (Han and Lee, 2000), which is in the cytosolic tail of the component and a pair of hemes that are located in the membrane-associated portion of the component (Cross et al., 1995). Both the hemes and the flavin are involved in electron transfer by NADPH oxidase. p22^{phox} has a proline - rich sequence at its carboxyl terminus which is in the cytosol that serves as a targeting site for the two SRC-homology 3 (bis-SH3) domains of p47^{phox} (Ago et al., 2003; Dinauer et al., 1990; Groemping et al., 2003). This tail binds cytosolic phosphorylated subunit p47^{phox}, which carries the cytosolic proteins to the membrane proteins to assemble the complete and active form of oxidase. This subunit is very essential to form active complex of NADPH oxidase because in patients whose neutrophils

are deficient in p47^{phox}, they have chronic granulomatous disease (described below), a disease in which neutrophils are unable to produce superoxide. Chronic granulomatous disease can occur also in patients with deficiencies in p67^{phox} and deficiencies in the two membrane components of the oxidase: gp91^{phox} and p22^{phox} (Babior, 2004). p47^{phox} is encoded by *NCF1* (*Neutrophil Cytosolic Factor 2*) gene on chromosome 7q11.23. It contains nine serine phosphorylation sites, two SH3 domains, one PX (Phox homology) domain involved in targeting membranes by binding to phosphoinositides, and one proline-rich region (Roos et al., 2003).

Under resting conditions, an autoinhibitory region in p47^{phox} combines with the bis-SH3 domain, preventing its binding to p22^{phox} (Ago et al., 2003).

p67^{phox} is encoded by *NCF2* gene, on chromosome 1q25. It is thought as an ‘accessory protein’, which possesses a (higher affinity) binding site for NADPH, four tetratricopeptide repeat (TPR) motifs involved in binding of Rac1 and Rac2, two SH3 domains and one proline-rich region. And this subunit is required for the activity of the oxidase (Roos et al., 2003).

p40^{phox} is another cytosolic subunit which seems to play an adaptor protein role, which stabilizes the p47^{phox} and p67^{phox} interaction (Suh et al., 2006; Kuribayashi et al., 2002). Its exact role in the cell is poorly defined, however, there are data which state that this subunit is constitutively associated with p67^{phox} in the cytosol of resting phagocytes (Wientjes et al., 1993; Tsunawaki et al., 1994). p40^{phox} can also interact with p47^{phox} (Ito et al., 1996). It is 40kDa protein (Wientjes et al., 1993) and p40^{phox} polypeptide is encoded by *NCF4* gene on 22q13.1 chromosome (Zhan et al., 1996). The protein contains one SH3 and one PX domain (Roos et al., 2003).

The cytosolic subunits have to be phosphorylated to translocate to membrane subunits. The phosphorylation occurs on serine residues of p47^{phox} mostly carried by protein kinase C (PKC) (El Benna et al., 1996; Nauseef et al., 1991) and also by Akt (protein kinase B), whose activation depends on phosphatidylinositol 3-kinase (PI3-kinase) (Hoyal et al., 2003).

5.1. Regulatory subunits domain organization

Regulatory subunits of NADPH oxidase-NOX1 possess a domain structure (Fig. 12). p47^{phox} subunit as well as NOX Organizer 1 (NOXO1) have a similar domain organization, except for the absence of the autoinhibitory region (AIR) in NOXO1. The same situation is in case of p67^{phox} and NOX Activator 1 (NOXA1), they have almost identical domain

organization, except that NOXA1 lacks one of the SH3 domains present in p67^{phox}. SRC - homology 3 (SH3) domains typically bind to proline - rich (PR) sequences (Lambeth, 2004). In the case of p42^{phox}, the SH3 domains work together as a single domain, and here are described as a bis - SH3 domain.

In NOXA1 and p67^{phox} there are four tricodecapeptide repeat (TPR) domains (Lambeth, 2004). The Phox (Phagocyte NOX) homology (PX) domain originally was identified in Phox proteins such as p47^{phox} and p40^{phox} and recognized as a novel class of conserved domains in a variety of eukaryotic proteins implicated in cell signaling (Ponting, 1996; Sato et al., 2001). Many signaling proteins were discovered to possess this PX domain and it plays role in lipid binding. In p47^{phox} subunit, the carboxy-terminal SH3 domain seems to interact with the Phox - homology (PX) domain, probably through a proline - rich sequence in the PX domain (Hiroaki et al., 2001). This interaction inhibits the binding of the PX domain to membrane lipids (Ago et al., 2003). The PX domain is probably also involved in the protein stabilization of p47^{phox} (Heyworth and Cross, 2002). In case of NOXO1, four variants (α , β , γ , and δ) are generated by alternative splicing of exon 3, which encodes PX domain (Cheng and Lambeth, 2005; Takeya et al., 2006).

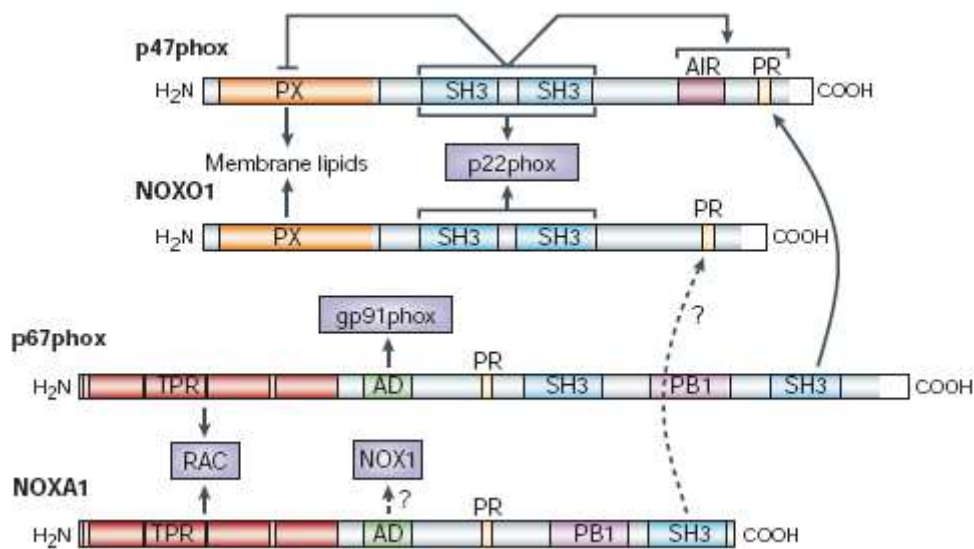


Fig. 12. Domain structure of regulatory proteins for NOX enzymes (adapted from Lambeth, 2004).

Recently, Ueyama et al. (2007) have presented that the β and γ isoforms, despite similar phospholipid binding properties, display distinct subcellular localization patterns, NoxO1 β being mainly localized to the plasma membrane, NoxO1 γ predominantly in the nucleus, and NoxO1 α and δ (whose phospholipid-binding properties were not investigated) within vesicles

or cytoplasmic aggregates. Similar to p47^{phox}, NOXO1 binds to p22^{phox}, which is required for NOX1 dependent activity (Takeya et al., 2003).

Chronic granulomatous disease (CGD) is a rare disorder in humans resulting from genetic defects in the NADPH oxidase that prevent oxidant production. CGD occurs in patients depleted of one of the four *PHOX* subunits (*PHOX* subunits are the subunits that comprise the NADPH oxidase) (Casimir et al., 1992; Clark et al., 1989; Curnutte et al., 1989). The feature of this illness is the inability in defeating severe infectious that are very hard to treat (Forrest et al., 1988; Johnston and Newman, 1977) because the cells are unable to produce superoxide (Curnutte et al., 1974). Patients suffer from an increased susceptibility to infection and granuloma production at inflammatory sites (Segal et al., 2000). These infections begin very early in the life and are frequently fatal (Babior and Woodman, 1990; Dinauer and Orkin, 1992). In the past, the greatest problems were with staphylococci. More recently, however, *Burkholderia cepacea* has been a major source of trouble causing a pneumonia that is difficult to treat (Speert et al., 1994). The most surprising in this human disease is that it is normally a pathogen of onion and the patients are infected by a wide variety of other microorganisms. The real problem is *Aspergillus* of various species (Corrado et al., 1980; Chang et al., 1998). This cause an intractable pneumonia and sometimes septicemia in chronic granulomatous disease patients, and are probably the most frequent cause of death in these patients.

Treatment of chronic granulomatous disease is based on prophylaxis (Forrest et al., 1988; Fischer et al., 1993) and it requires big doses of antibiotics, specifically a mixture of sulphonamides. This treatment resulted in decrease in mortality and in days of hospitalization. Another method of treatment was applying IFN- γ (Ahlin et al., 1997), which improved the clinical status of the patients. Also the effects of antifungal agents are checked and the results are promising (Gallin et al., 2003). Occasionally, in some patients, it is necessary to carry out bone marrow transplantation. This model of treatment has been successful in several cases, resulting in the cure of the disease, but it leaves the patients with the immunological problem of the transplant (Rappeport et al., 1983; Seger et al., 2002).

Approximately 80% of the patients with chronic granulomatous disease are male. This is because of the localization of the various *PHOX* genes on the chromosomes. Especially the gene for gp91^{phox} is on the X chromosome, and accounts for ~60% of cases. There is a wide range of genetic defects in these patients, including missense mutations, nonsense mutations and splicing defects, amongst others (Bolscher et al., 1991; Bu-Ghanim et al., 1995; Newburger et al., 1994). The rest of the *PHOX* proteins are autosomal and can occur in equal

amount of cases both in male and female. About 30% of patients have defects in p47^{phox} (Roos et al., 1996). In almost all of these patients with p47^{phox} deficiency have the same mutation- the elimination of a guanine-thymine dinucleotide in exon 2 (Casimir et al., 1991). It has place because of the presence of pseudogene nearby. Very rarely it happens that the pseudogen and p47^{phox} gene recombine, causing the loss of the guanine-thymine dinucleotide and the inactivation of p47^{phox}, resulting in chronic granulomatous disease due to a functional deficiency of p47^{phox} (Roesler et al., 1995).

6. Inhibitors of NADPH oxidase

As for now there are described 3 inhibitors of NADPH oxidase: apocynin, diphenyleneiodonium, *N*-vanillylnonanamide.

6.1. Apocynin (AP)

Apocynin (4'-hydroxy-3'-methoxy-acetophenone or acetovanillone) (Fig.13) is naturally occurring methoxy-substituted catechol (Stolk et al., 1994). It is present in the perennial herb *Picrorhiza kurroa*, which grows in the Himalayan mountains (Atal et al., 1986; *Picrorhiza kurroa*. Monograph., 2001). It has been used in Ayurvedic medicine by primitive cultures for centuries in the treatment of inflammatory diseases. Recent research has focused on the hepatoprotective, antioxidant and immune-modulating activity of its active component (*Picrorhiza kurroa*. Monograph., 2001). It was

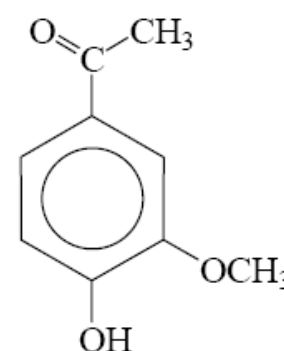


Fig.13. Apocynin

proven that apocynin possesses anti-inflammatory activity (Wang et al., 1994; Weber et al., 1994; Stolk et al., 1994; Lafeber et al., 1999; Muller et al., 1999; Muijsers et al., 2000) in ischemia-reperfusion lung injury (Dodd-O and Pearse, 2000), in airway hyperresponsiveness (Muijsers et a., 2001), atherosclerosis (Meyer and Schmitt, 2000), in rat models for colitis (Palmen, 1996) and arthritis ('t Hart et al., 1990). Growing interest in apocynin as an anti-inflammatory agent is combined with its very low toxicity (LD₅₀: 9g/kg upon oral administration to mice) (Gajewska et al., 1981). It is suggested that these features of apocynin can put this chemical in a novel series of non-steroidal anti-inflammatory drugs (NSAIDs) (Van den Worm et al., 2001). The studies on neutrophils and eosinophils show that apocynin, after metabolic conversion (Stolk et al., 1994), inhibits the assembly of NADPH oxidase by

reducing translocation of p47^{phox} subunit to the plasma membrane (Barbieri et al., 2004; Stolk et al., 1994). Obtained results from those cells present that apocynin is a unique inhibitor of superoxide production. It was observed that, together with ROS, myeloperoxidase (MPO) (Stolk et al., 1994), horseradish peroxidase (Simons et al., 1990) and probably other peroxidases can preactivate methoxy-substituted catechols including apocynin. In this way it is converted into a symmetrical dimer by means of the formation of a 5,5'-carbon-carbon bound (Fig.14). This dimer-diapocynin is a potent active compound that perform inhibition of NADPH oxidase assembly (van den Worm, 2001; Johnson et al., 2002; Holland and Johnson, 1999). This metabolic conversion of apocynin by peroxidases prevents NADPH oxidase assembly by suppressing the translocation of p47^{phox} and p67^{phox} through conjunction to essential thiol groups (Stolk et al., 1994). This oxidated by peroxidases apocynin, converted to a variety of oligophenolic and quinone-type compounds, may be inhibitors of the small G protein Rac1 that controls cell migration (Klees et al., 2006). Proteins which are the members of Rho family, possess the ability to remodel the actin cytoskeleton (Etienne-Manneville and Hall, 2002; Raftopoulou and Hall, 2004). It is also an evidence that NADPH oxidase associates with the actin cytoskeleton (Quinn et al., 1989). Rac1 and Cdc42 are the small G-proteins that can modulate and rearrange the actin cytoskeleton. They regulate signal transduction pathways that mediate distinct cytoskeletal rearrangements required for the production of lamellipodia and filipodia and then subsequent cell migration (Sahai and Marshall, 2002). Active Rac1 is necessary for the translocation of p47^{phox} and p67^{phox}. The role of Rac1 in NADPH oxidase activation is not well understood but this protein is able to bind to p67^{phox} subunit, and this binding may be what causes the final formation of the active NADPH oxidase complex (Quinn and Gauss, 2004). Klees, et al. (2006) discovered that apocynin derivatives cause a significant rearrangement of actin cytoskeleton, cell rounding, and decrease levels of active Rac1 and its related G protein Cdc42. In this way apocynin derivatives inhibits migration of the breast cancer cells. They suggest that apocynin may be a source for inhibitors of Rac1-mediated tumor cell migration. Like other therapeutic anticancer drugs of plant origin (e.g. taxol, paclitaxel, perillyl alcohol) (Martin, 1993; Crowell, 1999), apocynin is promising potential anticancer compound.

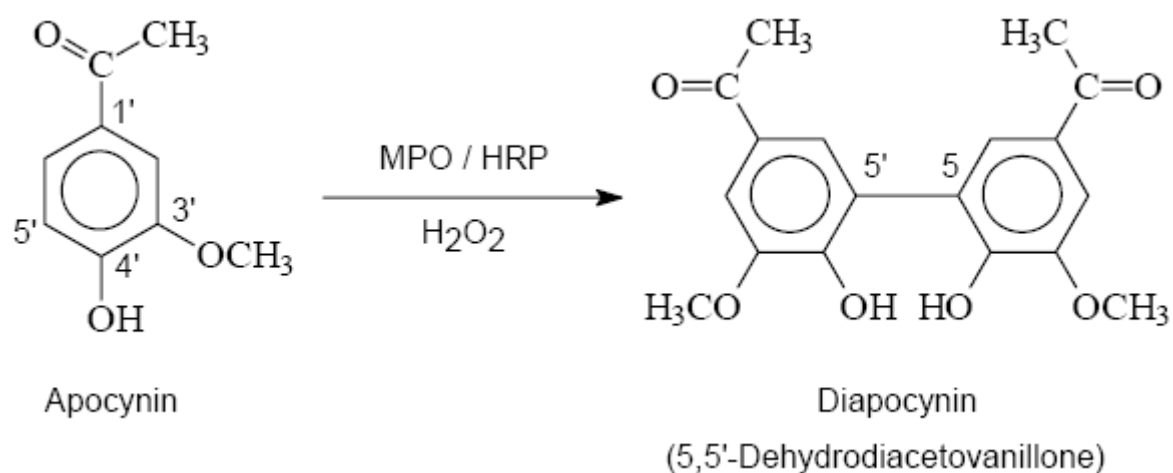


Fig.14. Peroxidase-mediated conversion of apocynin into diapocynin. [adapted from *van den Worm*, PhD thesis, Chapter 5, 2001]

Apocynin ability to inhibit NADPH oxidase activity appears to depend on the type of cell stimulus (Stolk et al., 1994). It is suggested that there are different intracellular signaling pathways for NADPH oxidase assembly after phorbol 12-myristate 13-acetate (PMA) and receptor-mediated stimulation (Stolk et al., 1994). Van den Worm et al. (2001) in their research project stimulated human neutrophils with opsonized zymosan or PMA. Opsonized zymosan (OPZ) mimics opsonized microorganisms and consists of the cell walls of baker's yeast coated with IgG, mannose-binding lectin, and C_{3b(i)} complement fragments (Roos et al., 1981). Phorbol 12-myristate 13-acetate activates neutrophils directly at the level of protein kinase C (PKC), which also leads to the activation of the respiratory burst (Burnham et al., 1989). Both of these compounds stimulate the superoxide generation by NADPH oxidase however, there is significant difference in their transductional mechanism (McPhail and Snyderman, 1983). Opsonized zymosan, stimulating human neutrophils, leads to a substantial release of the enzyme myeloperoxidase from primary granules inside the cell (Niessen et al., 1991). Myeloperoxidase release occurs only in stimulated cells, so this makes an apocynin a selective inhibitor of NADPH oxidase-mediated reactive oxygen production by activated human neutrophils (Van den Worm et al., 2001). Additionally, it was discovered that when apocynin was preexposed to hydrogen peroxide generated by xanthine oxidase from hypoxanthine, the oxygen consumption was inhibited (Stolk et al., 1994).

Barbieri et al. (2004) discovered that apocynin markedly decreased the intracellular reduced/oxidized glutathione ratio (GSH/GSSG) in stimulated monocytes but the GSH/GSSG ratio was not influenced by apocynin in resting monocyte. Similar results were obtained by Riganti et al. (2006) on mouse glial cells, however they also observed that an extracellular level of GSH and GSSG significantly increased after incubation with apocynin. Appearance

of extracellular GSSG very often is due to intracellular oxidation of GSH and subsequent efflux of GSSG (Schafer and Buettner, 2001). The efflux of GSH is a sensitive index of apoptosis (Hammond et al., 2001). After an oxidative stress, intracellular GSH is rapidly regenerated by glutathione reductase, which oxidases NADPH to NADP⁺ during the reaction. Oxidative stress is responsible for stimulation of the pentose phosphate pathway (PPP) through activation of the regulatory enzyme glucose-6-phosphate dehydrogenase (G6PD) which is sensitive to the decrease of the NADPH/NADP⁺ ratio (Luzzatto, 1967; Eggleston and Krebs, 1974). After apocynin treatment in glial cells, a H₂O₂ concentration was significantly increased, the PPP and the tricarboxylic acid cycle were induced. In cells exposed to apocynin, the alteration of PPP seems to be a consequence, and not a cause of the oxidative stress. In glial cells as well as in human erythrocytes and epithelial cells, in a dose dependent way, apocynin induced a significant increase of both malonyldialdehyde (MDA) level (index of lipid peroxidation) and lactate dehydrogenase (LDH) release (index of membrane damage, a cytotoxic effect) in the extracellular medium. On the other hand, the same experiment repeated together with apocynin and glutathione resulted in the blockade of the PPP and tricarboxylic acid cycle as well as in the prevention of MDA generation and LDH leakage. In this system GSH express protective role against lipoperoxidative and cytotoxic effects of an oxidative stress (Riganti et al., 2006).

The activation of tricarboxylic acid cycle is a response to the oxidative stress, which causes cell damage. In this cycle, there is increased turnover of ATP which is necessary in reparation process (Riganti et al., 2006). Cytotoxic effect of apocynin could be partly responsible for a further decrease of intracellular GSH (Riganti et al., 2006). Apocynin can induce under longer times of exposure an oxidative damage and a cytotoxic effect (Fig.15). It contains a phenolic structure and several phenolic molecules have shown to be cytotoxic. It has been suggested that the one-electron oxidation of phenolic compounds by cell oxidoreductases, results in the generation of phenoxyl radicals. These radicals would be readily reduced to phenols by intracellular reductants such as ascorbate and thiols, triggering a redox cycling (Kagan et al., 1994; Shvedova et al., 2000).

There is also suggestion that copper can be involved in triggering the apocynin-induced production of ROS and the consumption of GSH (Riganti et al., 2006).

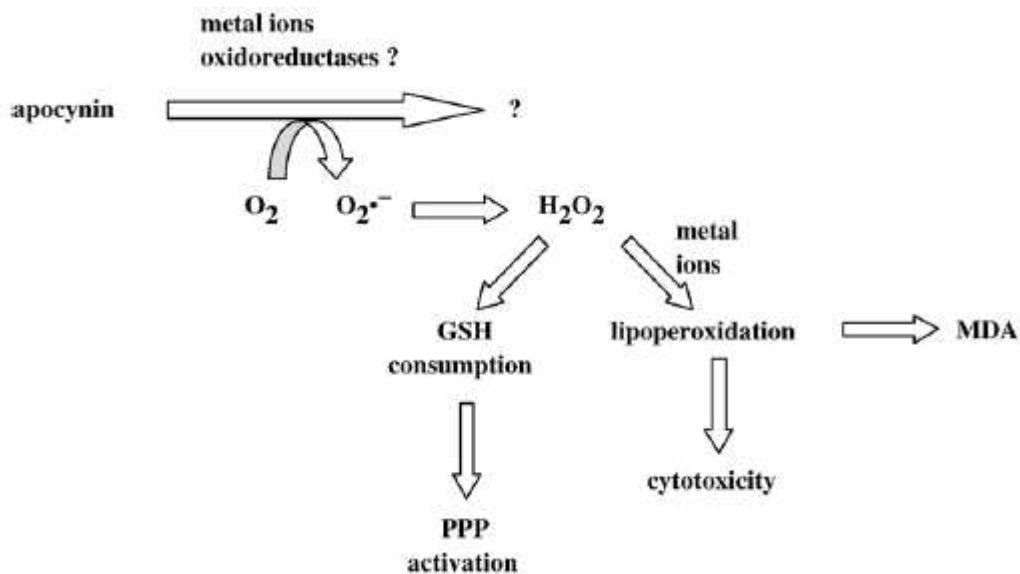


Fig.15. Pathway of oxidative stress induction by apocynin. (adapted from Riganti et al., 2006)

Together with apocynin there were other vanillins described as effective inhibitors of NADPH oxidase including protocatechuic, vanillic and caffeic acids (Boveris and Chance, 1973; Stolk et al., 1994). There were performed structure-activity relationship studies to test a number of apocynin analogs, creating several theories considering the impact of different substitutions on the benzene ring (Dorsch et al., 1994; Stuppner et al., 1995). Van den Worm et al. (2001) reported that several analogs of apocynin that differ at positions C-1 and C-5 possess the ability to inhibit reactive oxygen species production by human neutrophils induced by OPZ and PMA. They revealed in these cells the correlation between the substitution of a methoxy group at the position C-5 of apocynin, vanillin and vanillic acid, and their ability to interfere with generation of ROS. The anti-oxidant activity of these compounds may be increased due to the rise in electronic density of the aromatic ring at the methoxy group substitution at C-5 position.

Table 3. Structures of apocynin, vanillin, and vanillic acid and their C-5 methoxylated derivatives. (Van den Worm et al., 2001)

		R ₁	R ₂	
Apocynin	(APO)	-COCH ₃	H	
Vanillin	(VAN)	-CHO	H	
Vanillic acid	(VAC)	-COOH	H	
Acetosyringone	(ACS)	-COCH ₃	-OCH ₃	
Syringaldehyde	(SAL)	-CHO	-OCH ₃	
Syringic acid	(SAC)	-COOH	-OCH ₃	

Vejrazka et al. (2005) discovered that apocynin acts as an inhibitor of NADPH oxidase in neutrophils and macrophages and as a stimulator ROS formation in non-phagocyte cells. According to these data, they hypothesize that among ROS, the most probably formed, is the superoxide, after apocynin treatment. Increased superoxide production was completely abolished by tiron, a superoxide-specific scavenger or diminished by superoxide dismutase. The effect of apocynin was also reduced by catalase. When apocynin is preactivated with hydrogen peroxide and a peroxidase prior to use, it acts as an inhibitor both in phagocytes and non-phagocytes (Vejrazka et al., 2005). Babior, B.M. (1999) created a hypothesis that, since sulfhydryl groups are important for the function of the leukocyte NADPH oxidase, the oxidant effect of apocynin could participate to the mechanism of enzyme inhibition.

Apocynin is considered to stimulate ROS production by an enzyme that contains the flavine group or is regulated by a flavoprotein. In the locus of inflammation, phagocytes secrete hydrogen peroxide as well as myeloperoxidase, however in non-phagocyte cells, they are practically absent (Vejrazka et al., 2005). Nevertheless, there are studies that prove the inhibitory effects of apocynin on ROS formation in non-phagocytes as well (Thabut et al., 2002; Hamilton et al., 2001; Beswick et al., 2001; Holland et al., 2001). Because angiotensin II-mediated apoptosis has been implicated in the progression of heart failure, the inhibition of NADPH oxidase activation may be beneficial in the treatment of heart failure (Qin et al., 2006).

Apocynin was observed to express other effects beside its ability to inhibit NADPH oxidase: for instance, it interference with actin polymerization and cytoskeletal rearrangement in polymorphonuclear granulocytes (Muller et al., 1999), modulates the arachidonic acid metabolism through a not yet clarified mechanism (Engels et al., 1992), and inhibits cytochrome P-450 activity in endothelial cells (Pietersma et al., 1998).

There are many controversial data about the apocynin activity and still its mechanism of action remains not clearly defined. Thus further studies on this drug must be performed.

6.2. Diphenyleneiodonium (DPI)

Diphenyleneiodonium (Fig. 16) is a irreversible inhibitor of flavin-containing enzymes including the NADPH oxidase (O'Donnell et al., 1993; Cross and Jones, 1986), cytochrome P-450 reductase (McGuire et al., 1999), xanthine oxidase (O'Donnell et al., 1994), oxidoreductases of the mitochondrial respiratory chain, including NADH:ubiquinone oxidoreductase (Li and Trush, 1998;

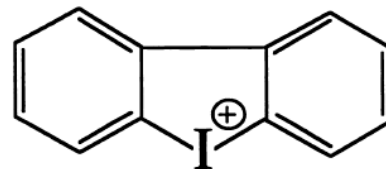


Fig. 16. Diphenyleneiodonium (DPI)

Ragan and Bloxham, 1977) and nitric oxide synthase (Stuehr et al. 1991). Firstly, DPI was identified as a hypoglycemic agent which was able to block gluconeogenesis and respiration in rat liver (Holland et al., 1973). DPI is reduced to its diphenyleneiodonyl radical form due to electron transport through the flavin moieties of these flavoenzymes and irreversible phenylation of flavin or adjacent amino acid and heme groups of the proteins (Doussiere et al., 1999). Flavoproteins, such as glutathione reductase, glucose oxidase and amino acid oxidases, are not involved in the generation of free radicals and they are not inhibited by DPI. This demonstrates that inhibition by iodonium derivatives has a radical mechanism of function of flavoproteins enzymes (O'Donnell et al., 1994). Although DPI, as a flavoprotein inhibitor, is expected to decrease the cellular production of ROS and RNS, results of these studies are controversial. There has been reported both stimulation and inhibition of ROS and RNS generation (Li and Trush, 1998; Li et al., 2003). Li et al. (2003) presented in HL-60 cells that DPI could induce mitochondrial ROS production and apoptosis. Mitochondrial ROS generation induced by DPI occurs through the mitochondrial respiratory chain inhibition. Furthermore, the elevated mitochondrial ROS cause the induction of apoptosis by initiating loss of mitochondrial membrane potential, cytochrome c release, and caspase activation. On the other hand, they proved that DPI was not able to induce superoxide production in mitochondrial DNA-deficient HL-60 (ρ^0) cells. This result indicates the involvement of mitochondria in DPI-induced superoxide production (Li et al., 2003). Balcerczyk et al. (2005) demonstrated in endothelial cells that DPI inhibits ROS production and that the induction of ROS generation may be an artifact. They explain that DPI may directly oxidize fluorescent probes used to detect ROS.

Riganti et al. (2004) have shown that DPI and structurally related compound diphenyliodonium are able to inhibit both pentose phosphate pathway (PPP), and tricarboxylic acid cycle, leading to increased generation of ROS, accumulation of lipoperoxidation products and decreased glutathione/ glutathione disulfide (GSH/GSSG) ratio, and exerting a cytotoxic effect. It is suggested that the inhibition of glucose metabolism mediated by DPI, through PPP and tricarboxylic acid cycle can undergo via several NAD(P)-dependent enzymes, such as glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and lactate dehydrogenase (Riganti et al., 2004). The cells are much more susceptible to oxidative stress when PPP inhibition occurs, because the ROS which are produced during the normal metabolism are not neutralized by the activation of PPP and the subsequent redox cycling of NADPH and GSH (Riganti et al., 2006).

There are also data which present that modulation by DPI the cellular oxidant-antioxidant homeostasis may result in apoptosis. It has been demonstrated that DPI sensitizes carcinoma cells to Fas-mediated apoptosis by induction of the efflux of GSH from T24 bladder carcinoma cells to extracellular medium via a specific transport channel. Fas receptor after binding its ligand, Fas-L, induce apoptosis by activation of caspase-8 and caspase-3. In this way DPI changes the redox state of cells not only by modulation of the level of ROS and RNS production (Pullar and Hampton, 2002). However another mechanism of the apoptotic action of DPI involves impairment of cyclin B1 accumulation and cell arrest at G2 (Scaife, 2004). Balcerczyk et al. (2005) presented in endothelial cells that DPI has a proapoptotic action however this might be due to disturbance of the generation of ROS necessary for cell proliferation. Li et al. (2003) demonstrated different apoptotic mechanism of DPI action. They suggest that DPI has the potency to decrease a mitochondrial membrane potential and subsequently release cytochrome c and activate caspase-3, and that both of these processes occurred after mitochondrial ROS production.

Similarly to apocynin, DPI prevents NF- κ B activation in adherent monocytes by inhibition of DNA binding of NF- κ B after exposition to PMA (Phorbol Myristate Acetate) (Barbieri et al., 2004).

DPI can act both as an antioxidant and an inducer of superoxide production. However, its mechanism of plasma membrane NADPH oxidase inhibitory action still is under investigation.

6.3. *N*-vanillylnonanamide (*N*-VNA)

Capsaicin is an active component in *Capsicum* species (Fig. 17). Various tissues of *Capsicum* species (Solanaceae) have been traditionally used as medical agents in Mesoamerica (Molina-Torres et al., 1999). They are

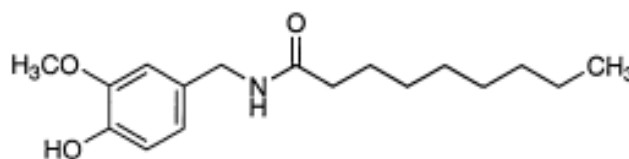


Fig. 17. *N*-vanillylnonanamide

one of the major ingredients in Mayan therapeutic remedies. Capsaicin is pungent component in hot chilli peppers (Cordell and Araujo, 1993). The medical value of capsaicin has been evaluated in the treatment of painful conditions such as rheumatic diseases, cluster headache, painful diabetic neuropathy, postherpetic neuralgia, etc. (Cordell and Araujo, 1993; Bevan and Szolcsányi, 1990; Markovits and Gilhar, 1997).

N-vanillylnonanamide described also as pseudocapsaicine is structural analogue of capsaicin.

Capsaicinoids commonly have both the polar part (4-hydroxyl group in *N*-vanillyl moiety) and the lipophilic structure (hydrocarbon chain), potentially producing the interdigitation of phospholipids to rigidify membranes with an increase in their relative concentrations. Tsuchiya (2001) demonstrated that pseudocapsaicine change the membrane fluidity. Di Marzo et al. (1998) presented another mechanism of action of *N*-vanillylnonanamide where it possesses the ability to bind selectively to cannabinoid receptor-CB1. Zhang et al. (2003) shown that *N*-vanillylnonanamide possesses the ability to inhibit C₂-ceramide-stimulated p47^{phox} translocation.

7. ρ^0 cell lines

Cells lacking mitochondrial DNA (mtDNA) are described as ρ^0 cells. They can be derived from normal cells by a long term exposure to ethidium bromide (EtBr) or other reagents that inhibit replication of mitochondrial genes such as ditercalinium, rhodamine, bromodeoxyuridine or dideoxycytidine (Inoue et al., 1997; Schubert and Jacob, 1970). Within the time incubation in (EtBr) containing medium, cells gradually deplete mtDNA. This compound in relatively low concentration, preferentially inhibits synthesis of mtDNA by half after each division of the cell, without affecting nuclear DNA (King and Attardi, 1989).

mtDNA is responsible for coding all subunits of protein complexes involved in electron transport and oxidative phosphorylation (Jazayeri et al., 2003) thus cells devoid of mtDNA have altered metabolic requirements. Glycolysis is for these cells the only one source of energy and they require both uridine and pyruvate for growth (Delsite et al., 2002; Buchet and Godinot, 1998). ρ^0 cells may serve as a model to investigate the role of mitochondria in the cell death machinery (Jiang et al., 1999; Chandel and Schumacker, 1999; Marchetti et al., 1996) as they are one of a key component in this process. In addition, cells that lack mitochondrial DNA retain apparently normal apoptotic signaling (Jiang et al., 1999; Marchetti et al., 1996; Park et al., 2004). Thus in this project ρ^0 cells were used in order to demonstrate if the intracellular level of superoxide together with ATP level are related to the switch mechanism from apoptosis to necrosis.

AIM OF THE PROJECT

The aims of this project are:

1. Detection of NADPH oxidase in human osteosarcoma 143B cell line,
2. Demonstration of a possible role of intracellular level of superoxide as a key factor directly related to the switch mechanism of the cell death mode from apoptosis to necrosis.
3. Clarification of a possible role of NADPH oxidase in the switch mechanism of the cell death mode from apoptosis to necrosis,
4. Clarification of the correlation between changes in the intracellular level of ROS and ultrastructural changes of 143B cells.

APPARATUS AND EQUIPMENT

Bunsen burner,

Bürker Hematocytometer,

Cell scraper, sterile (Sarstedt, Germany),

Cell culture incubator, 5% CO₂ in air atmosphere (Lab-line, Barnstead, Germany),

Centrifuge (1.5 ml vials) (Force 16, Denver Instruments, USA),

Centrifuge (15ml and 50ml tubes) (2200A, Hermle Labortechnik, Germany),

Centrifuge Sigma 3K30 (Polygen, Poland),

Confocal microscope:

Confocal system Radiance 2100 (Bio-Rad, UK) equipped with Krypton/Argon lasers,

Contrast-phase light microscope (Eco-vision, Ecotone, Poland),

Dewar flask for liquid nitrogen storage (MVE SC 20/20, Planner, UK), tube racks,

Diamond knife (MJO-DIATOME CO., Fort Washington, PA, USA),

Dounce Homogenizer, 1ml capacity (Wheaton, NJ, USA),

Electric pipette-aid Pipetus-akku (Hirschmann Laborgerate, Germany),

Electron microscope Hitachi H-7000 (Hitachi Co., Japan),

Electronic analytic balance (Sartorius, Germany),

Electronic technical balance (Sartorius, Germany),

Exposure cassette Kodak[®] BioMax (Sigma, USA),

Flow cytometer BD LSR II System (Becton Dickinson, USA),

Freezers: 4°C/-20 °C (CZP 236, CZB2316, Polar, Poland),

-80 °C (Polar 530V, Angelantoni Industrie Spa, Perugia, Italy),

Frosted microscope slides and coverslips (Erie Scientific, USA),

HPLC chromatographic system:

- pump Merck-Hitachi Li Chromatograph 6200 (BDH Instruments, UK),
- variable length UV detector Merck-Hitachi Li Chromatograph 4000 (BDH Instruments, UK),
- sample injection port Reodyne 7125 (Berkley, USA),
- equipped with software Turbochrom (Perkin-Elmer Nelson, UK) and personal computer,
- HPLC analytic column 150x4.6 mm containing 3 µm-diameter packing fraction Hyperosil ODS (Hichrom Reading, UK),

– HPLC pre-column 20x2 mm containing 10 µm-diameter packing fraction Spherisorb ODS2 (Phase Separation, UK),
Labo-Stirrer LR 51B (Yamato, Japan),
Laminar chamber Aura 2000 B.S. (Bio Air Instruments, Italy),
Magnetic stirrer (M&S Instruments, Japan),
Metalware (spatulas),
Multi-plate reader Spectra 200 (Jupiter, ASYS Hitech GmbH, Austria),
pH-meter F-22 (Horiba, Japan),
Pipetors and tips,
Plasticwares for cell cultures: sterile cell culture dishes with 60 mm, 100 mm and 150 mm diameter (BD Falcon, USA); cell culture multiwell plates, 6 and 96 well flat bottom, with lid (Sarstedt, Germany); polystyrene 1.5 ml tubes (Sarstedt Germany); sterile 2 ml cryogenic vials; polystyrene, sterile 2 ml, 10 ml, 25 ml pipettes (BD Falcon, USA), 15 ml, 50 ml tubes, polypropylene (BD Falcon, USA),
Polyacrylamide gel electrophoresis apparatus (Sigma, USA),
Semi-dry blotter (Sigma, USA),
Sonifier Cell Disruptor 200 (Branson Ultrasonics Corporation, USA),
Stabilizer power supplier,
Water Deionization System TKA GenPure (TKA, Germany),
Vortex MT-31 (Yamato, Japan)

SOFTWARE:

BD FACSDiva Software v.4.1.2
CorelDRAW Graphics Suite 11
LaserSharp 2000 v.4.0 (Bio-Rad, UK)
Turbochrom (Perkin-Elmer Nelson, UK)
WinMDI v.2.8

MATERIALS

8. Cell culturing and manipulations

8.1. human osteosarcoma 143B cell line

Thymidine kinase-deficient human osteosarcoma 143B cell line were obtained from the American Type Culture Collection (ATCC number: CRL 8303). Properties: tissue-osteosarcoma (bone); morphology-fibroblast; age stage-13 years old; growth properties-adherent, passage 2-3 times per week.

- Medium- Dulbecco's Modified Eagles Medium (DMEM) (Sigma Chemicals, USA), containing 4500 mg/l glucose, L-glutamine, NaHCO₃, and pyridoxine, supplemented with:
 - 10% Fetal Bovine Serum (FBS) (Sigma Chemicals, USA),
 - 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma Chemicals, USA).

8.2. human osteosarcoma 143B ρ^0 cell line

143B ρ^0 cell line was established and kindly provided by Dr. M. Tanaka, Tokyo Metropolitan Institute of Gerontology, Japan.

- Medium- Dulbecco's Modified Eagles Medium (DMEM) (Sigma Chemicals, USA), containing 4500 mg/l glucose, L-glutamine, NaHCO₃, and pyridoxine, supplemented with:
 - 10% Fetal Bovine Serum (FBS) (Sigma Chemicals, USA),
 - 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma Chemicals, USA),
 - 1 mM sodium pyruvate (Sigma Chemicals, USA),
 - 50 µg/ml uridine (Wako Pure Chemicals Industries, Japan),
 - 2500 mg/l glucose (Sigma Chemicals, USA).
- Phosphate buffered saline, (PBS), cell culture tested, pH 7.4 (Sigma Chemicals, USA), containing 0,01M; NaCl 0,138 M; KCl 0,0027 M
- Trypsine-EDTA, 0,5g/ml, cell culture tested (Sigma Chemicals, USA)

8.3. Freezing conditions

- 5% DMSO, dimethyl sulfoxide, cell culture tested (Sigma-Aldrich, USA) in DMEM (Sigma Chemicals, USA)

9. Chemicals for cell culture treatment

- Menadione sodium bisulfide (MEN) (Sigma Chemicals, USA),
 - final concentration, 100 μ M,
- Diphenyleneiodonium chloride (DPI) (Sigma Chemicals, USA),
 - final concentration, 1 μ M,
- Apocynin (Lancaster, Lancaster Synthesis Ltd., UK),
 - final concentration, 500 μ M,
- *N*-vanillylnonanamide (Sigma Chemicals, USA),
 - final concentration, 250 μ M,
- Antimycin A (Antm A) (Sigma Chemicals, USA),
 - final concentration, 50 μ M,
- Oligomycin (a mixture of oligomycins A, B and C) (Sigma Chemicals, USA),
 - final concentration, 3,5 μ g/ml.

All reagents were prepared as 1000x-concentrated stock solutions dissolved in DMSO (AP, *N*-VNA, DPI, Antm A, oligomycin) or MiliQ water (MEN) and stored in -20°C .

10. Detection and measurement of ROS generation

- Dihydroethidium (DHE) (Molecular Probes, Inc., USA) – final concentration, 5 μ M, was prepared as 1000x stock solution in DMSO
- Phosphate buffered saline, (PBS), cell culture tested, pH 7.4 (Sigma, USA), containing 0,01M; NaCl 0,138 M; KCl 0,0027 M
- Trypsine-EDTA, 0,5g/ml, cell culture tested (Sigma, USA)

11. Assessment of changes in the cell membrane asymmetry and integrity

- Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, BD, USA) containing:
 - Annexin V-FITC in aqueous buffered solution containing BSA and 0,09% sodium azide
 - Propidium Iodide staining solution in PBS (pH 7.4)
 - Annexin V binding buffer

- Phosphate buffered saline, (PBS), cell culture tested, pH 7.4 (Sigma Chemicals, USA), containing 0,01M; NaCl 0,138 M and KCl 0,0027 M
- Trypsine-EDTA, 0,5g/ml, cell culture tested (Sigma Chemicals, USA)

12. Measurement of intracellular levels of ATP

- Sample preparation
 - 1,3 M HClO₄ ((POCh Gliwice, Poland),
 - 3 M K₃PO₄ (POCh Gliwice, Poland),
 - 0,5 M NaOH (POCh Gliwice, Poland),
 - Phosphate buffered saline, (PBS), cell culture tested, pH 7.4 (Sigma Chemicals, USA), containing 0,01M; NaCl 0,138 M and KCl 0,0027 M
 - Trypsine-EDTA, 0,5g/ml, cell culture tested (Sigma Chemicals, USA).

- Protein content measurement

BCATM Protein Assay Kit (Pierce, IL, USA) containing:

- BCATM Reagent A containing: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0,1 M sodium hydroxide,
- BCATM Reagent B containing: 4% cupric sulfate,
- Albumin Standard Ampules, 2 mg/ml, containing bovine serum albumin (BSA) at 2 mg/ml in 0,9% saline and 0,05% sodium azide.
- Standard solutions were prepared in the following concentrations: 25 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1mg/ml, 1,5 mg/ml, and 2 mg/ml.

- HPLC determination of intracellular ATP level

Eluents:

- A-150 mM KH₂PO₄ + 150 mM KCl, pH 6.0,
- B-15% acetonitrile in buffer A,
- ATP analytical grade (Sigma Chemicals, USA) as reference standard.

13. NADPH oxidase detection by Western blotting

13.1. Cell lysis

- Protein extracting buffer, content:
 - 220 mM Mannitol
 - 68 mM Sacharose
 - 50 mM HEPES/HCl (pH 7.4)
 - 50 mM KCl
 - 5 mM EGTA
 - 2 mM MgCl₂
 - 1 mM dithiothreitol (DTT)
 - protease inhibitor cocktail+ 10µM PMSF
- Protease Inhibitor Cocktail Set III (CALBIOCHEM, EMD Biosciences, Inc., Darmstadt, Germany)

Table 4. The components of protease inhibitor cocktail 1000x concentrated, solubilized in 1ml of DMSO solution.

Name	Concentration in the vial	Target protease
AEBSF, Hydrochloride	100 mM	Serine Proteases
Aprotinin, Bovine Lung, Lyophilized	80 µM	Broad spectrum, Serine Proteases
Bestatin	5 mM	Aminopeptidase B and Leucine Aminopeptidase
E-64, Protease Inhibitor	1,5 mM	Cysteine Proteases
Leupeptin, Hemisulfate	2 mM	Cysteine Proteases and Trypsin-like Proteases
Pepstatin A	1 mM	Aspartic Proteases

- Phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA), (stock solution 100mM in EtOH), final concentration, 10 µM (target proteases: serine proteases and acetylcholinesterase).

- Phosphate buffered saline, (PBS), cell culture tested, pH 7.4 (Sigma, USA), containing 0,01M; NaCl 0,138 M and KCl 0,0027 M
- Trypsine-EDTA, 0,5g/ml, cell culture tested (Sigma, USA)

13.2. Protein content determination

- BCA™ Protein Assay Kit (Pierce, IL, USA) containing:
 - BCA™ Reagent A containing: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0,1 M sodium hydroxide
 - BCA™ Reagent B containing: 4% cupric sulfate
 - Albumin Standard Ampules, 2 mg/ml, containing bovine serum albumin (BSA) at 2 mg/ml in 0,9% saline and 0,05% sodium azide.
- Standard solutions were prepared in the following concentrations: 25 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1mg/ml, 1,5 mg/ml, and 2 mg/ml.

13.3. Polyacrylamide gel electrophoresis (SDS-PAGE)

- 12% running gel (5ml):
 - 30% Acrylamide/bis-Acrylamide solution 2,0 ml
 - 1,5 M Tris-HCl (pH 8.8) 1,3 ml
 - 10% SDS (final 0,1%) 0,05 ml
 - 10% APS (final 0,1%) 0,05 ml
 - TEMED 0,002 ml
 - MilliQ H₂O 1,6 ml
- 5% stacking gel (3ml):
 - 30% Acrylamide/bis-Acrylamide solution 0,5 ml
 - 1,0 M Tris-HCl (pH 6.8) 0,38 ml
 - 10% SDS (final 0,1%) 0,03 ml
 - 10% APS (final 0,1%) 0,03 ml
 - TEMED 0,003 ml
 - MilliQ H₂O 2,1 ml

- SDS running buffer (pH 8,8)
 - 0,25M Tris-HCl
 - 1,92M Glycine
 - 1% SDS

- 5x Sample Buffer
 - 225 mM Tris-HCl, pH 6,8
 - 50% (v/v) Glycerol
 - 5% (w/v) SDS
 - 5% 2-Mercaptoethanol
 - 0,05% Bromophenol blue

- TriChromRanger™ Prestained Molecular Weight Marker Mix (Pierce Biotechnology, Inc., IL, USA) which consists of seven proteins with a molecular weight range from 16,5 kDa to 210 kDa.
- Electrophoresis conditions: 150 V/1h 30 min

Coomasie Brilliant Blue (CBB) staining and destaining

- Staining solution
 - 50% Methanol
 - 10% Acetic acid
 - 0,05% CBB R-250

- Destaining solution
 - 50% Methanol
 - 10% Acetic acid

13.4. Protein transfer

- PVDF membrane (Whatman®, Schleiser&Schuell, Whatman GmbH, Germany)
- Blotting papers (Whatman®, Schleiser&Schuell, Whatman GmbH, Germany)
- Semi-dry transfer buffer (pH 8.3):

- 25 mM Tris base
- 150 mM Glycine
- 0,02% SDS
- 10% Methanol
- Transfer conditions: 260mA /1h

13.5. NADPH oxidase subunits detection

- Antibodies against NADPH oxidase subunits:
 - anti-gp91^{phox} mouse monoclonal antibody IgG₁ (stock solution 250 µg/ml in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1,5 mM NaN₃, 1 mg/ml BSA) (BD Transduction Laboratories, USA)
 - anti-p67^{phox} mouse monoclonal antibody IgG_{2b} (stock solution 250 µg/ml in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1,5 mM NaN₃, 1 mg/ml BSA) (BD Transduction Laboratories, USA)
 - anti-p47^{phox} mouse monoclonal antibody IgG₁ (stock solution 250 µg/ml in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1,5 mM NaN₃, 1 mg/ml BSA) (BD Transduction Laboratories, USA)
 - rabbit anti-mouse IgG-horseradish peroxidase conjugated (stock solution 1 mg/ml in PBS, pH 7.2) (Molecular Probes, Inc., USA)
 - human cell lysate HL-60-positive control (stock solution 1 mg/ml in SDS-PAGE buffer (62mM Tris pH 6.8, 2% SDS, 0,9% β-mercaptoethanol, 0,003% bromophenol blue, 5% glycerol) (BD Transduction Laboratories, USA)
- BM Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH, Penzberg, Germany)
- Block Ace Reagent (Dai Nihon Seiyaku, Osaka, Japan)
- Tris buffered saline (TBS buffer):
 - 10mM Tris-HCl, pH 7.6
 - 150mM NaCl
- TBS-Tween 20 (TBS-T) buffer:
 - TBS buffer
 - 0,1% (v/v) Tween 20

- Exposure cassette Kodak[®] BioMax (Imaging Network, Inc., Canada)
- X-ray film (Kodak Imaging Network, Inc., Canada)
- Developer/replenisher (Kodak Imaging Network, Inc., Canada)
- Fixer/replenisher (Kodak Imaging Network, Inc., Canada)

14. Detection of NADPH oxidase subunits localization by confocal microscopy with Zenon Mouse IgG Labelling Kits (Molecular Probes, OR, USA)

- Zenon Mouse IgG Labelling Kits(Molecular Probes, OR, USA) labeled with:
 - Alexa Fluor[®] 488,
 - Alexa Fluor[®] 555,
 - Alexa Fluor[®] 647
- Antibodies against NADPH oxidase subunits:
 - anti-gp91^{phox} mouse monoclonal antibody IgG₁ (stock solution 250 µg/ml in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1,5 mM NaN₃, 1 mg/ml BSA) (BD Transduction Laboratories, USA)
 - anti-p67^{phox} mouse monoclonal antibody IgG_{2b} (stock solution 250 µg/ml in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1,5 mM NaN₃, 1 mg/ml BSA) (BD Transduction Laboratories, USA)
 - anti-p47^{phox} mouse monoclonal antibody IgG₁ (stock solution 250 µg/ml in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1,5 mM NaN₃, 1 mg/ml BSA) (BD Transduction Laboratories, USA)
- Block Ace Reagent (Dai Nihon Seiyaku, Osaka, Japan)
- 4% formaldehyde in PBS solution
- Phosphate buffered saline, (PBS), cell culture tested, pH 7.4 (Sigma Chemicals, USA), containing 0,01M; NaCl 0,138 M; KCl 0,0027 M
- Perma Fluor mounting solution (Immunon, Pittsburgh, PA, USA)

15. Detection of cell death mode with electron microscopy

- Fixative containing:
 - 4% glutaraldehyde, EM grade (PolySciences Inc., PA, USA),
 - 4% formaldehyde, EM grade (PolySciences Inc., PA, USA),
 - 0,2 M Na-cacodylate (Sigma Chemicals, USA) in water pH 7.4,
- 0,2 M Na-cacodylate (Sigma chemicals, USA) in water pH 7.4,
- Post-fixation with 1% osmium tetroxide (OsO₄), EM grade (Merck, Germany),
- Samples dehydration:
 - 25% EtOH + 1% uranyl acetate (PolySciences Inc., PA, USA),
 - graded series of ethanol: 50%, 80%, 90%, 96%, 100% EtOH, pure p.a. (POCh Gliwice, Poland),
 - 100% ethanol was prepared by dehydration of 99.8% EtOH, pure p.a. (POCh Gliwice, Poland) with copper sulphate anhydrous pure-CuSO₄ (Ubichem Ltd., UK),
 - Propylene oxide (PolySciences Inc., PA, USA),
- Embedding (Epon mixture):

Table 5.

Chemical	Amount (g)
NMA	6,13
DDSA	3,04
Epon	10,83
Total:	20
DMP-30	300-350 µl

DMP-30 was added after careful mixing of the first three compounds.

16. Other chemicals

- Water used in experiments, was deionized, ultrapure and sterilized with electronic conduction equal to 0,055 µS, aquired from Water Deionization System TKA GenPure (TKA, Germany),
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA)

METHODS

17. Cell culturing and handling

All the operations concerning handling the cells in the open vials, plates or dishes were carried out under strict aseptic conditions in the class 2 flow laminar chamber sterilized every time before usage with built-in UV lamp for at least 30 min. Any item transferred into clean bench was decontaminated by spraying 70% ethanol. Any manipulations of opening and closing vials, tubes and bottles inside the laminar flow chamber were carried out by the Bunsen burner on. All the reagents used during handling the cells as well as plastic wares were sterile and disposable.

17.1. Storage

Cryoprotective medium (DMEM containing 5% (v/v) DMSO) was used for the cells storage, in the in liquid nitrogen vapor phase.

17.2. Recovery of the cells – seeding

A cell vial was quickly thawed (in about 2 min) in 37°C water bath, washed with 70% ethanol and placed into the laminar flow chamber. 5 ml of the complete growth medium was used for the vial content dilution in a sterile 15 ml plastic tube. Centrifugation of the cells was carried out at 2000 rpm for 3 minutes at RT. After centrifugation the tube was sterilized with 70% ethanol before transferring to the clean bench. The supernatant then was discarded, 5 ml of the fresh complete growth medium was used to resuspended cell pellet by thirty cycles of pipetting in and out. Homogenous suspension was transferred to the 50 ml plastic tube containing 20 ml of the complete growth medium and pipetted for 30 cycles. After all, the suspension was poured onto the 75 cm² cell culture flask. The culture was incubated at 37°C in 5% CO₂ humidified atmosphere.

Complete growth medium for 143B line is Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 100 units/ml of penicillin and 100 µg/ml of streptomycin. Medium was stored in 4°C and prior to the usage, it was incubated in the water bath at 37°C for 30 minutes.

Complete growth medium for 143B ρ^0 cell line is Dulbecco's Modified Eagles Medium (DMEM) which was modified to contain: 1mM sodium pyruvate, 50 µg/ml uridine, 2500 mg/l glucose supplemented with 10% Foetal Bovine Serum (FBS) and 100 units/ml of penicillin

and 100 µg/ml of streptomycin. Medium was stored in 4°C and prior to the usage, it was incubated in the water bath at 37°C for 30 minutes.

17.3. The cell culture examination

The cell culture was optically examined for any evidence of microbial contamination, using an inverted microscope, equipped with contrast-phase lens. At the same time, there was processed the evaluation of cell confluence, adherence, shape and general morphology. Before placing the culture dish back to the incubator it was decontaminated by spraying 70% ethanol.

For the determination of growth rate of the cells, a Bürker hemacytometer was used. Cell suspension was diluted to such a degree that the cells did not overlap each other on the hemacytometer's grid and were equally distributed. The cells were counted in all 9 main squares of hemacytometer and the medium value for one square was calculated. Final number of cells was gained by multiplying medium number of cells per square times dilution factor 10000.

17.4. Subculturing procedures

The passage was performed when the cell culture confluency reached around 80%. The culture growth medium was removed by aspiration. The cell layer was rinsed with PBS, prewarmed to 37°C, to remove all traces of serum, containing trypsin inhibitor. Then the cells were treated with 1ml of Trypsin-EDTA solution until cell layer was dispersed. A 5 ml of complete growth medium was added onto the dish and cells were aspirated by pipetting and transferred into sterile 15 ml plastic tube. Tube was closed and centrifuged at 2000 rpm for 3 minutes at room temperature. Moving back to the flow laminar chamber, the tube was sterilized with 70% ethanol. Then the supernatant was gently discarded and the cell pellet was resuspended in 5 ml of complete growth medium by thirty cycles of pipetting in and out. 1 ml of homogenous suspension was transferred to 50 ml plastic tube containing 19 ml of complete growth medium and pipetted for 30 cycles. The suspension was transferred to the two 100mm diameter sterile culture dishes, 10 ml per dish giving subcultivation ratio of 1:10. The culture was incubated at 37°C in 5% CO₂ humidified atmosphere.

For experimental purposes the cell suspension was poured onto dishes of various sizes, but the subcultivation ratio 1:10 was always kept. All the experiments were always conducted in logarithmic phase of cell growth, in other words, at confluency equal to 50 – 60%.

17.5. Cell freezing

For cell freezing cells were cultured on two dishes of 100mm in diameter. When the confluency of cultured cells reached the maximum, the cells were processed for freezing. The growth medium was discarded by aspiration and the cell layer was rinsed with PBS prewarmed to 37°C. 1 ml of Trypsin-EDTA solution was added to each cell dish. When the cells became detached from the dish's bottom, 5 ml of complete growth medium was added and cells were aspirated by pipetting and transferred into sterile 15 ml plastic tube. Then the tube was centrifuged at 2000 rpm for 3 minutes at RT. Putting back to the clean bench, the tube was sterilized with 70% ethanol. Then the supernatant was gently discarded and the cell pellet was resuspended in 2 ml of ice cold cryoprotectant medium containing complete growth medium with 5% (v/v) DMSO. Cell suspension was transferred into 2 ml sterile, cryogenic vial and incubated at 4°C for 30 minutes. After that cryogenic vial was incubated at -20°C for another 30 minutes. As the last step, the vial was transferred to -80°C overnight. After -80°C incubation, the cryogenic vial was moved into a Dewar flask containing liquid vapour nitrogen phase (-196°C) for long term storage.

18. Treatment of cells with various chemicals

Cells were incubated for various length of time with chemicals specified below: menadione sodium bisulfide (MEN), (Sigma Chemicals, USA) (final concentration, 100 µM), apocynin (AP) (Lancaster, Lancaster Synthesis Ltd., UK) (final concentration, 500 µM), *N*-vanillylnonanamide (*N*-VNA) (Sigma Chemicals, USA) (final concentration, 250 µM), diphenyleneiodonium chloride (DPI) (Sigma Chemicals, USA) (final concentration, 1 µM), antimycin A (Antm A) (Sigma Chemicals, USA) (final concentration, 50 µM), Oligomycin (a mixture of oligomycins A, B and C) (Sigma Chemicals, USA), (final concentration, 3,5 µg/ml).

All reagents were prepared as 1000x-concentrated stock solutions dissolved in DMSO (AP, *N*-VNA, DPI, Antm A, Oligomycin) or MiliQ water (MEN) and stored in -20°C.

19. Measurement of ROS generation

ROS generation was detected in the culture cells using flow cytometry method according to the following steps:

- 19.1. Dihydroethidium (DHE) (Molecular Probes, Inc., USA) – final concentration 5 µM, was prepared as 1000x concentrated solution in DMSO (Sigma-Aldrich, USA). DHE is the compound that in the presence of superoxide anion converts into

- ethidium. DHE itself is blue fluorescent in cell cytoplasm while the oxidized form ethidium is red fluorescent upon DNA intercalation.
- 19.2. 143B cells growing on the 60 mm-culture dishes, treated with variety of chemicals for different length of time were stained with DHE at final concentration 5 μ M for 30 minutes at 37°C.
 - 19.3. Cells collected by trypsinization were centrifuged at 2000 rpm for 3 minutes at RT.
 - 19.4. Supernatant was discarded and cell pellet was washed with 2 ml PBS, pH 7.4. Cell suspension was then centrifuged at 2000 rpm for 3 minutes at RT.
 - 19.5. Step 19.4 was repeated.
 - 19.6. Cells were suspended in 1 ml of PBS and immediately submitted to flow cytometry analysis on BD LSR II System (BD, USA) flow cytometer using BD FACSDiva Software v.4.1.2 with following settings for DHE: excitation wavelength 518 nm, emission 605 nm.
 - 19.7. Acquired data were exported to WinMDI software and processed.

20. Assessment of changes in the cell membrane asymmetry and integrity

Changes in the cell membrane asymmetry and integrity were detected using Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, BD, USA) containing fluorescein (FITC) conjugated Annexin V and propidium iodide (PI) double staining and subsequent flow cytometry analysis. Annexin V is a small protein with high affinity for phosphatidylserine (PS), an element of plasma membrane, which is in physiological conditions localized exclusively on the inner plasma membrane leaflet. Externalization of PS is a common feature of the early apoptotic stages. PI is a membrane impermeable dye, which stains nuclei in the case of a necrotic plasma membrane integrity loss.

- 20.1. Cells growing on the 60 mm culture dishes were collected by trypsinization and centrifuged at 2500 rpm for 5 minutes at RT.
- 20.2. Cells were washed twice with PBS and centrifuged at 2500 rpm for 5 minutes at RT.
- 20.3. Cell pellet was suspended (app. 10^6 cells/ml) in the 100 μ l of Annexin V binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2,5 mM CaCl_2).
- 20.4. Then 5 μ l of Annexin V-FITC and 5 μ l of PI were added into suspension, and incubated for 15 min at RT (25°C) in the dark.
- 20.5. Sample volume was adjusted to 0,5 ml with the Annexin V binding buffer and submitted to flow cytometry analysis using BD LSR II System flow cytometer (BD,

USA) using BD FACSDiva Software v.4.1.2 with the following settings: PI excitation wavelength-535 nm, emission 617 nm; FITC excitation wavelength-494 nm, emission 518 nm.

20.6. Acquired data were exported to WinMDI software and processed.

21. Measurement of intracellular levels of ATP

21.1. Sample preparation (Kalsi et al., 1999)

Cells growing on 150 mm diameter culture dishes were collected by trypsinization. Both cells attached to the bottom of the culture dish and floating ones were centrifuged together at 4000 rpm for 4 minutes at RT. After centrifugation the cell pellet was washed with PBS. Then the cells were suspended in 0,3 ml of cold 1,3 M HClO₄ and incubated for 15 minutes on ice to extract cellular nucleotides. Acid extracts (0,3 ml each) were neutralized to pH 5-6 with cold 3 M K₃PO₄ and centrifuged at 13000 rpm for 3 minutes at 4°C. The supernatants were transferred to new vials and subjected for further analysis by HPLC as described below. Protein concentration was measured according to the protocol provided to the commercial BCATM Protein Assay Kit (Pierce, IL, USA) after dissolving the perchloric acid precipitates with 0,5 M NaOH.

21.2. HPLC determination of intracellular ATP level (Smoleński et al., 1990)

The assignment of ATP was accomplished in the Chair and Department of Biochemistry, Medical University of Gdańsk. The method applied was based on the one described by Smoleński et al. (348). Determination was performed using chromatographic system consisting of pump Merck-Hitachi Li Chromatograph 6200 (BDH Instruments, UK) and variable wavelength UV detector Merck-Hitachi Li Chromatograph 4000 (BDH Instruments, UK). Separation was accomplished using analytical column 150x4.6 mm containing 3 µm-diameter packing fraction Hyperosil ODS (Hichrom Reading, UK). Between injector and analytical column, pre-column 20x2 mm containing 10 µm-diameter packing fraction Spherisorb ODS2 (Phase Separation, UK) was inserted. Sample injection port Reodyne 7125 (Berkley, USA) was equipped with 20 µl capacity injection loop. Samples were eluted with solutions: A –150 mM KH₂PO₄ + 150 mM KCl, B - 15% acetonitrile in buffer A, with elution speed 0.9 ml/min. Elution gradient program was as follows: 0 min – 0% B; 0.1 – 3% B; 3.5 min – 9% B; 5 min – 100% B; 7 min – 100% B; 7.1 min – 0% B. Column was equilibrated after 4.9 minutes giving in conjunction 12 minutes between each injection.

The temperature of the column was kept in the interval of 17-19° C by water jacket. Obtained data were analyzed with Turbochrom software. ATP peak was identified according to comparison of retention times of a reference standard and samples (Fig. 18).

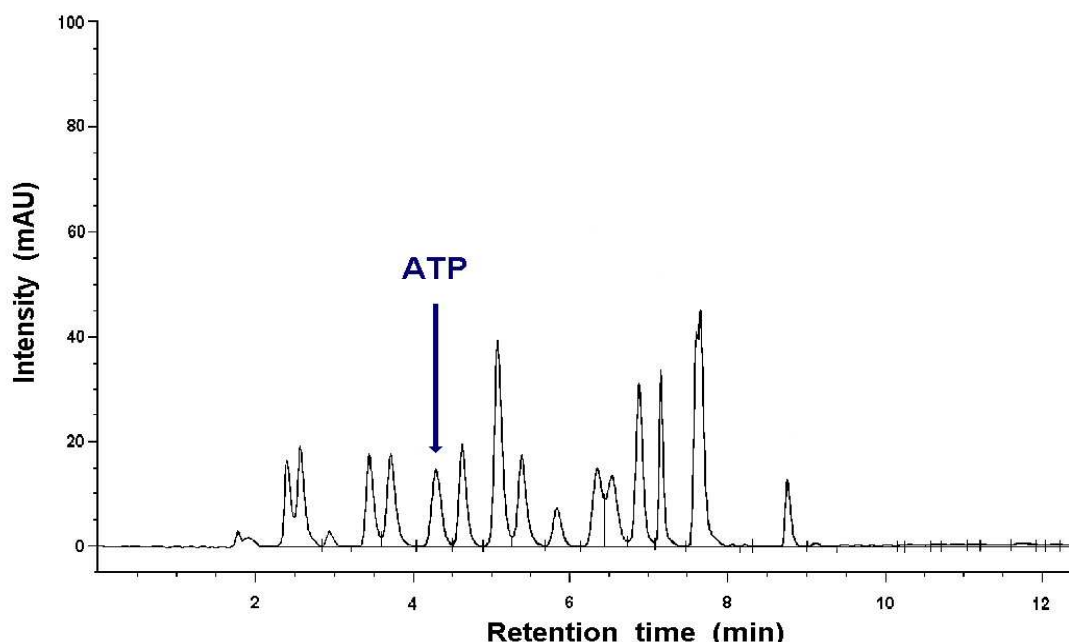


Fig. 18. Chromatogram of a reference standard at detection wavelength $\lambda=260$ nm. Retention time of ATP peak was 4.29 min.

22. NADPH oxidase detection by Western blotting

22.1. Cell lysates preparation

22.1.1. Cells were incubated on 150 mm diameter culture dishes, with 100 μ M MEN and co-incubated with NADPH oxidase inhibitors: 500 μ M AP, 250 μ M *N*-VNA, 1 μ M DPI for various lengths of time.

22.1.2. Cells were collected by trypsinization and centrifuged at 2000 rpm for 3 min at RT.

22.1.3. The supernatant was discarded over the cell pellet and cells were washed twice with cold PBS, pH 7.4 and transferred to the 2 ml Eppendorf test tube.

22.1.4. Cells were centrifuged at 3000 rpm for 5 min at 4°C.

22.1.5. Supernatant was discarded and cell pellet was suspended in 500 μ l of protein extracting buffer

22.1.6. Cells were incubated on ice for 30 minutes and homogenized with glass Dounce Wheaton homogenizer by 40 strokes.

22.1.7. Cells were centrifuged at 14000g for 15 minutes at 4°C.

22.1.8. Supernatant was collected and stored at -80°C.

22.2. Protein concentration measurement

Protein concentration of the sample was measured according to the commercial protocol provided to the BCA™ Protein Assay Kit (Pierce, IL, USA).

22.2.1. Standard solutions of Bovine Serum Albumine (BSA) in the protein extracting buffer, were prepared at the following concentrations: 25 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1 mg/ml, 1.5 mg/ml, and 2 mg/ml.

22.2.2. Working Reagent (WR) was prepared by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B in a sufficient volume based on the number of samples that were assayed.

22.2.3. 25 µl of each standard and measured samples were pipetted to the three different wells of 96-well plate.

22.2.4. To each well containing 25 µl of standard solution or measured sample 200 µl of working reagent was added.

22.2.5. Blank sample was prepared by adding 25 µl of protein extracting buffer to 200 µl working reagent.

22.2.6. Plate was thoroughly mixed on a plate shaker for 30 seconds.

22.2.7. Plate was covered and incubated at 37°C for 30 minutes.

22.2.8. Then the plate was cooled and the absorbance was read at 540 nm.

22.2.9. Mean values of absorption of standard solutions of BSA were used to draw standard curve and calculate linear regression equation.

22.2.10. Final protein concentration was calculated basing on linear regression equation.

22.3. Polyacrylamide gel electrophoresis (SDS PAGE)

Preparation of gel:

22.3.1. Glass plates and spacers (1 mm thick) were assembled.

22.3.2. Running gel and stacking gel solutions were prepared according to the instruction involved in Methods 13.3. section.

22.3.3. The running gel was poured to about 1 cm below the wells of the comb (around 5 ml).

22.3.4. The gel was sealed with 1 ml MilliQ water.

22.3.5. When the running gel had set the water was removed and the stacking gel was poured (around 3 ml). The comb was immediately inserted.

22.3.6. When the stacking gel had set, it was placed in gel rig and immersed with SDS running buffer.

22.3.7. Prior to running the gel, the wells were washed out thoroughly with running buffer.

Samples preparation:

22.3.8. Homogenates of cells were mixed with 5x concentrated sample buffer.

22.3.9. Samples were boiled for 5 minutes and after that cooled for 5 minutes at RT.

22.3.10. Samples were loaded on the gel using 20 µg of protein per lane.

22.3.11. 5 µl of TriChromRanger™ Prestained Molecular Weight Marker Mix (Pierce Biotechnology, Inc., IL, USA) was loaded into the first lane.

22.3.12. 5 µl of positive control sample, obtained from HL-60 cells lysates (BD Transduction Laboratories, USA) was loaded after the Molecular Weight Marker (Pierce Biotechnology, Inc., IL, USA)

22.3.13. Samples were run at constant current with voltage set at 150 V for 1 hour and 30 minutes.

22.4. Protein transfer

22.4.1. After electrophoresis the stacking gel was separated from running gel and removed.

22.4.2. Pieces of blotting paper (Whatman®, Schleiser&Schuell, Whatman GmbH, Germany) and PVDF membrane (Whatman®, Schleiser&Schuell, Whatman GmbH, Germany) were prepared in size 0,5 cm greater than the size of the gel.

22.4.3. Both the blotting papers and PVDF membrane were soaked in Semi-dry transfer buffer.

22.4.4. Transfer “sandwich” was constructed onto the anode(+) plate of Semi-dry blotter (Sigma, USA) as follows: one sheet of blotting paper, one piece of PVDF membrane, the gel and one sheet of blotting paper. The cover with cathode(-) plate of Semi-dry blotter (Sigma, USA) was placed on the transfer “sandwich”.

22.4.5. The unit was connected to a suitable power supply and the transfer was performed at constant current with voltage set at 260 mA for 1 h at RT.

22.5. NADPH oxidase subunits detection

The following incubation and washing steps were carried out at RT on an orbital shaker platform.

22.5.1. Primary antibodies (mouse mAb against p47^{phox}, p67^{phox} and gp91^{phox}) (BD Transduction Laboratories, USA) and secondary antibodies (Molecular Probes, Inc., USA)

were diluted in 1x concentrated Block Ace Reagent (Dai Nihon Seiyaku, Osaka, Japan) in sterilized and MilliQ water.

22.5.2. PVDF membrane was removed from the transfer “sandwich” and rinsed with TBS buffer to remove methanol.

22.5.3. Subsequently PDVF membrane was put to the blocking buffer -10x concentrated Block Ace Reagent (Dai Nihon Seiyaku, Osaka, Japan) and kept at 4°C overnight.

22.5.4. The blocking buffer was removed and the membrane was overlaid with 10 ml of primary antibody at an appropriate dilution (anti-p47^{phox} 1:250; anti-p67^{phox} and anti-gp91^{phox} 1:500) (stock solution 250 µg/ml). PVDF membrane was incubated with primary antibody at RT for 1 h on shaker.

22.5.5. PVDF membrane was washed twice for 10 minutes each, with sufficient volume of TBS-T buffer.

22.5.6. Then the PVDF membrane was incubated with the blocking buffer-1x concentrated Block Ace Reagent (Dai Nihon Seiyaku, Osaka, Japan) with Tween 20 at final concentration 0,2% (v/v).

22.5.7. The PVDF membrane was incubated with secondary antibody rabbit anti-mouse IgG-horseradish peroxidase conjugated (Molecular Probes, Inc., USA) (stock solution 1 mg/ml in PBS, pH 7.2) diluted in the blocking buffer-10x concentrated Block Ace Reagent (Dai Nihon Seiyaku, Osaka, Japan) at 1:5000 dilution.

22.5.8. Step 22.5.5. was repeated.

22.5.9. The PVDF membrane was washed with TBS once for 10 minutes.

22.5.9. Detection reagent of BM Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH, Penzberg, Germany) was prepared by mixing 40 µl of Starting solution B with 4 ml of Luminescence substrate solution A.

22.5.10. PVDF membrane was incubated with detection reagent for 1 minute in the dark.

22.5.11. PVDF membrane was covered with transparent plastic bag and inserted into a film cassette-Exposure cassette Kodak[®] BioMax (Sigma, USA).

22.5.12. In the dark room, using a red safety light, the sheet of film was placed onto the blot and the film cassette was closed.

22.5.13. The film was exposed for an appropriate length of time.

22.5.14. The exposed film was developed in the presence of a red safety light, as follows: film was developed in developer/replenisher (Kodak Imaging Network, Inc., Canada), the reaction was blocked by dipping in the water and fixed by incubation in fixer/replenisher (Kodak Imaging Network, Inc., Canada).

23. Detection of NADPH oxidase subunits localization by confocal microscopy with Zenon Mouse IgG Labelling Kits (Molecular Probes, OR, USA)

23.1. Labelling of the cell samples with Zenon Mouse IgG Labeling Kits

Cell samples were prepared according to the provided protocol by Molecular Probes for Zenon Mouse IgG Labeling Kits (OR, USA). This method is based on a fluorophore-labeled Fab fragment directed against the Fc portion of an intact IgG primary antibody in order to form a labeling complex.

23.1.1. Cells were cultured on the 20x20 mm glass coverslips (Erie Scientific, USA).

- Steriled glass coverslips (Erie Scientific, USA) were obtained by three times for 5 minutes sonification of coverslips submerged in deionized water followed by autoclave sterilization.

23.1.2. Cells were washed three times for 5 minutes with pre-warmed PBS, pH 7.4.

23.1.3. Cells were then fixed with 4% formaldehyde solution in PBS for 15 min at 37°C under 5% CO₂.

23.1.4. Then the samples were washed twice with PBS.

23.1.5. Permeabilization of the cells were provided by incubation in PBS containing 0,1% Triton[®] X-100 for 5 min at RT.

23.1.6. The detergent solution was removed and the nonspecific binding sites in the cell sample were blocked in Block Ace Reagent (Dai Nihon Seiyaku, Osaka, Japan).

23.1.7. The cell samples were incubated with Zenon labeling reagents conjugated with primary antibody for 1h at room temperature (RT) in the dark.

23.1.8. After staining cells were washed two times with PBS, for 5 minutes each.

23.1.9. Second fixation of the cell samples was performed in 4% formaldehyde solution in PBS for 15 min at RT in the dark.

23.1.10. When the fixation was completed the samples were washed twice with PBS. This second fixation protects the Zenon Fab fragments from dissociation with time because the Fab fragments are not covalently coupled to the primary antibody and thus providing better signal intensity.

23.1.11. Step 23.1.8. was repeated.

23.1.12. Coverslips with cells were placed on a glass slides in Perma Fluor mounting solution (Immunon, Pittsburgh, PA, USA) and analyzed by Bio-Rad Laserssharp MRC 1024 scanning confocal microscope. Alexa Fluor[®] 488 excitation wavelength is 495 nm, emission 519 nm;

Alexa Fluor[®] 555 excitation wavelength is 554 nm, emission 567 nm; Alexa Fluor[®] 647 excitation wavelength is 652 nm, emission 667 nm.

23.2. Preparation of Zenon complexes

Zenon labeling complexes prepared according to the producer's protocol were diluted to the desired working concentration in Block Ace Reagent and a sufficient volume was applied to immerse the cell sample. Primary antibodies mouse IgG isotype against p47^{phox}, p67^{phox} and gp91^{phox} subunits of NADPH oxidase (stock solutions 250 µg/ml) were labeled with the Zenon Mouse IgG Labeling Kit to obtain a 3:1 molar ratio of Fab to antibody target. In this way:

23.2.1. 1 µg of primary antibody sustained in Block Ace Reagent were mixed with 5µl of the Zenon mouse IgG labeling reagent and incubated for 5 min at RT.

23.2.2. Then 5 µl of the Zenon blocking reagent was added to the reaction mixtures.

23.2.3. The solutions were incubated for 5 min at RT. The complexes were ready to be applied to the cell samples.

24. Visualization of cell death mode with electron microscopy (Karbowski et al., 1999a)

This method was based on Karbowski et al. (1999a) protocol.

24.1.1. An equal volume of fixative containing 4% glutaraldehyde, 4% formaldehyde and 0,2M Na-cacodylate (pH 7.4) was added to the culture medium to fix detached and attached cells.

24.1.2. Cells were fixed with formaldehyde solution at 4°C overnight.

24.1.3. After fixation, cells were scraped and transferred into 1,5 ml Eppendorf tubes.

24.1.4. Samples were centrifuged at 10000xg for 5 minutes at 4°C.

24.1.5. Supernatant was discarded and cells were suspended in 1 ml of 0,1 M Na-cacodylate solution (pH 7.4) and kept at 4°C for 1h.

24.1.6. Cells were then centrifuged at 10000xg for 5 min at 4°C.

24.1.7. Samples were post-fixed with 500 µl of 1% osmium tetroxide in 0,1 M Na-cacodylate solution (pH 7.4) and incubated at 4°C for 1h.

24.1.8. Samples were centrifuged at 10000xg for 5 min at 4°C.

24.1.9. Dehydration of the samples was initiated with 25% ethanol containing 1% uranyl acetate at 0°C for 30 min. After incubation, samples were centrifuged at 10000xg for 10 min at 4°C.

24.1.10. Cells were then incubated with 50% EtOH at 0°C for 10 minutes. After incubation, samples were centrifuged at 10000xg for 10 min at 4°C.

24.1.11. Pellets were suspended in 80% EtOH at 0°C for 10 minutes. After incubation, samples were centrifuged at 10000xg for 10 min at 4°C.

24.1.12. Pellets were suspended in 90% EtOH at RT for 10 minutes. After incubation, samples were centrifuged at 10000xg for 10 min at RT.

24.1.13. Pellets were suspended in 95% EtOH at RT for 10 minutes. After incubation, samples were centrifuged at 10000xg for 10 min at RT.

24.1.14. Pellets were suspended in 100% EtOH at RT for 10 minutes. After incubation, samples were centrifuged at 10000xg for 10 min at RT.

24.1.15. Step 24.1.14. was repeated twice.

24.1.16. Pellets were suspended in propylene oxide and immediately centrifuged at 10000xg for 10 min at RT.

24.1.17. Step 24.1.16. was repeated.

24.1.18. Epon mixture was prepared according to the Table 5.

- Propylene oxide-epon mixture 1:1 (v/v) was prepared.

24.1.19. Pellets were suspended in propylene oxide-epon mixture and incubated at RT for 1h.

24.1.20. Samples were centrifuged at 10000xg for 20 minutes at RT.

24.1.21. Supernatant was discarded and pellets were left to dry in RT.

24.1.22. Dry pellet was covered with small amount of epon mixture and left overnight.

24.1.23. Using preparation needles pellets were transferred onto capsule bottom end embedded with epon mixture.

24.1.24. Capsule forms were then incubated at 60°C for 1 week.

24.1.25. Thin sections were cut on a Porter-Blum ultramicrotome using a diamond knife (MJO-DIATOME CO.) and stained with uranyl acetate and lead citrate.

24.1.26. Acquired thin sections were processed and examined in a Hitachi H-7000 electron microscope (Hitachi, Japan) operated at 100 kV.

RESULTS

In the present study the role of plasma membrane NADPH oxidase in the switch mechanism of the cell death mode from apoptosis to necrosis in human osteosarcoma 143B cell line was investigated. MEN-induced cell injury processes in ρ^+ cells were investigated in comparison to those in ρ^0 cells.

25. Changes in the intracellular levels of superoxide in MEN-treated 143B cells

In the present study menadione (MEN) was applied as an inducer of oxidative stress and cell death. Enhanced generation of superoxide anion is an important factor which is implicated in the different steps of apoptosis and the switch of the cell death mode from apoptosis to necrosis. To observe changes in overall intracellular level of superoxide in response to MEN treatment, ρ^0 and ρ^+ cells were stained with dihydroethidium (DHE). Conversion of non-fluorescent DHE into ethidium was measured by flow cytometry. Time dependent changes in the intracellular level of superoxide anion in 143B ρ^0 and ρ^+ cells were observed (Fig. 19). ρ^0 and ρ^+ 143B cells were treated with 100 μ M menadione (MEN) for 2, 4, 6, 9 hours. The maximum of superoxide anion production was observed at 4 hours of MEN treatment in ρ^+ cells. Further treatment with this compound diminished the level of superoxide anion generated in these cells to the level five times higher than of the control. On the other hand, incubation of ρ^0 with 100 μ M MEN for the same length of time resulted in the continuous increase in the intracellular level of superoxide. After 9 h of treatment with MEN the amount of superoxide was 25 times higher than that of the control.

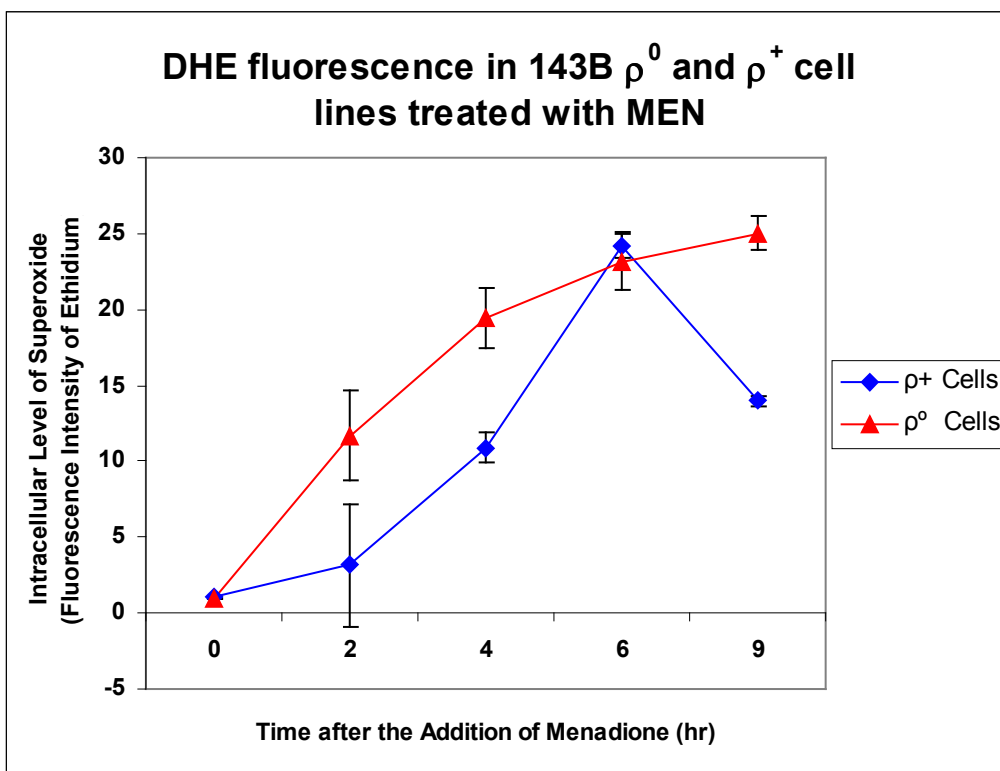


Fig. 19. Changes in the intracellular level of superoxide anion in 143B ρ^0 and ρ^+ cells. 143B ρ^0 and ρ^+ cells were cultured for up to 9 hours in the presence of 100 μ M MEN, and the level of DHE fluorescence corresponding to an intracellular level of superoxide was plotted against the duration of the time of incubation. Data are the averages and standard error (mean \pm SE) of six different experiments.

25. 1. Effects of NADPH oxidase inhibitors on MEN-induced changes in the intracellular level of superoxide

143B ρ^+ cells were treated with MEN for 6 and 9 hours, respectively, in the presence of membrane NADPH oxidase inhibitors: apocynin (AP), *N*-vanillylnonanamide (*N*-VNA) and diphenyleneiodonium (DPI), and stained with dihydroethidium (DHE) to detect intracellular level of superoxide. Comparisons were made between the data obtained from the cells treated with menadione alone for 6 h or 9 h and those treated with menadione in the presence of inhibitors of NADPH oxidase (Fig. 20). Intracellular level of superoxide in 143B ρ^+ cells treated for 6 hours with MEN became distinctly higher than that of the control cells (Fig. 20A). On the other hand, the pre-treatment of cells with inhibitors of NADPH oxidase, specified above, invariably suppressed such increases in the intracellular level of superoxide induced by MEN. Intracellular level of superoxide in 143B ρ^+ cells treated for 9 hours with MEN was definitely lower than that treated with MEN for 6 hours, and yet distinctly higher than that of the control, and inhibitors of NADPH oxidase had no effects on the intracellular level of superoxide in the cells treated for 9 hours with MEN. Typical examples of flow cytometric charts obtained from experiments demonstrated in Fig. 20A are shown in Fig. 20B.

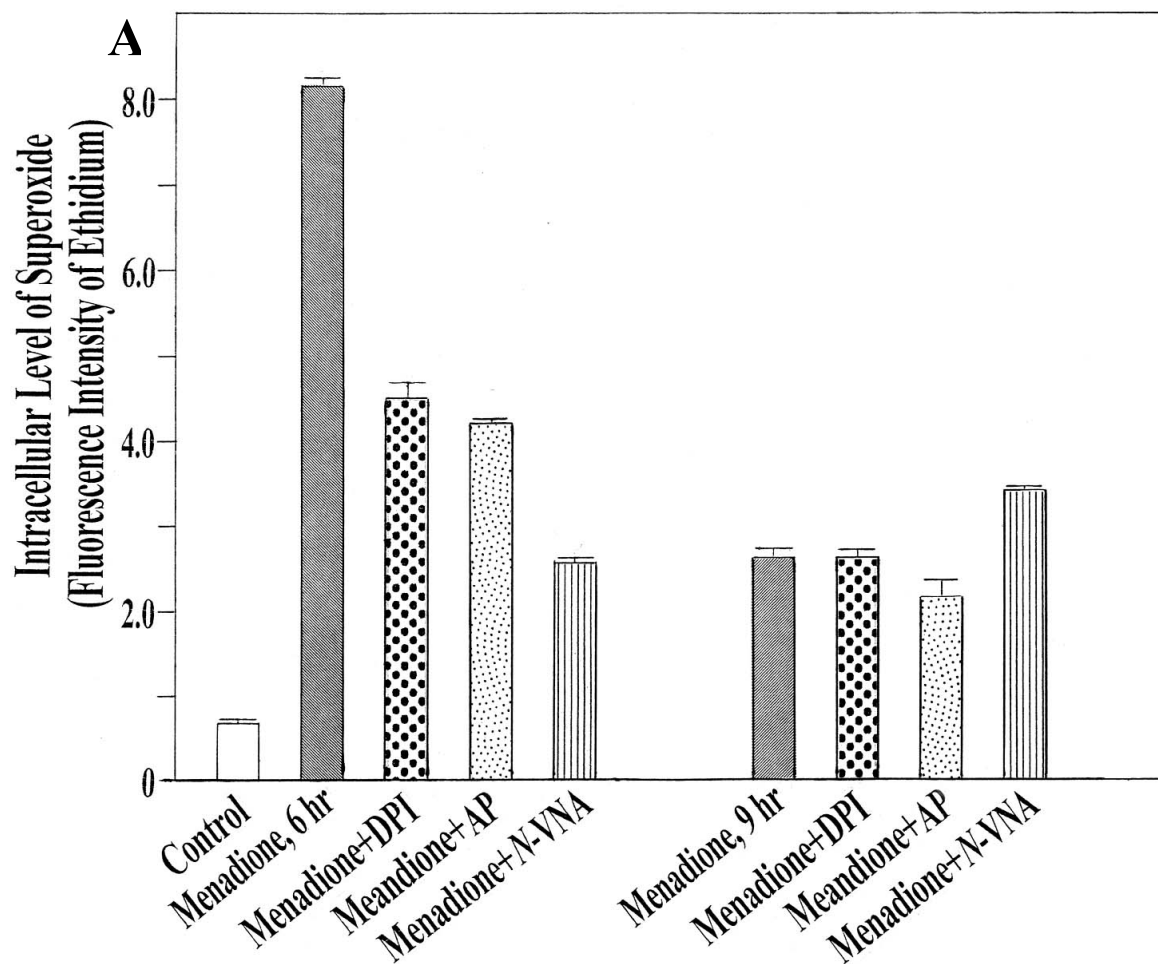


Fig. 20A. Effects of NADPH oxidase inhibitors on MEN-induced changes in the intracellular level of superoxide. Cells treated with MEN for 6h or 9h in the presence and absence of the pretreatment with inhibitors of NADPH oxidase; Data are the averages and standard error (mean \pm SE) of six different experiments.

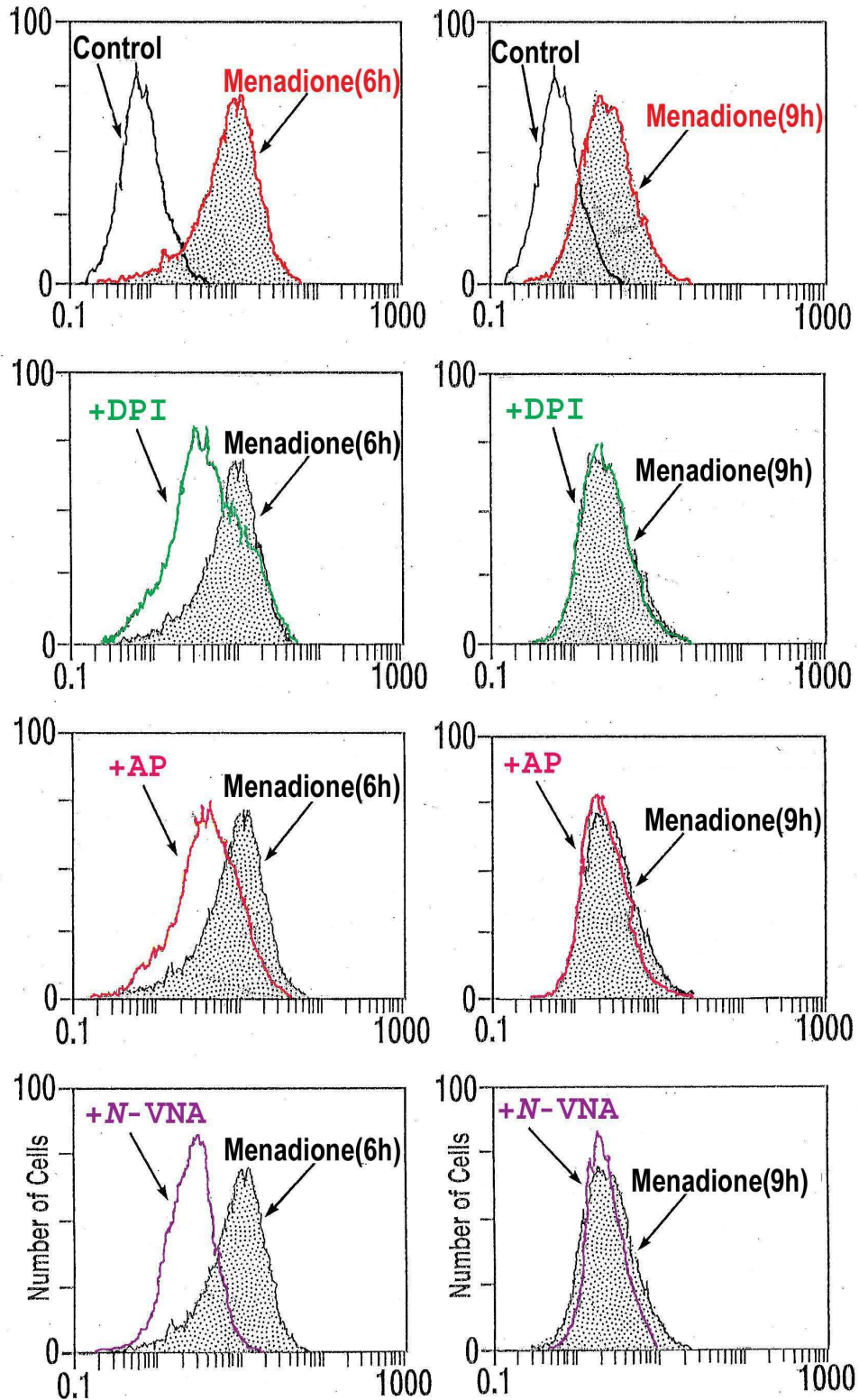
B

Fig. 20B. Flow cytometric charts demonstrating intracellular levels of superoxide in the cells treated with MEN in the absence and presence of the pretreatment with inhibitors of NADPH oxidase. Experimental conditions are the same as those shown in Fig. 21A.

26. MEN-induced cell death mode in 143B cells

In order to analyze the effects of the incubation time with MEN on the cell death mode, cells treated with MEN for up to 9 hours were double-stained with Annexin V-FITC and PI, and submitted to flow cytometry. Comparisons were made between ρ^0 and ρ^+ cells.

In physiological conditions, phosphatidylserine (PS) is localized in the inner membrane leaflet of the plasma membrane. PS is constantly transported by special ATPase from the outer to the inner leaflet of the plasma membrane, thus maintaining the asymmetric distribution of PS. Oxidation of PS, due to oxidative stress, apparently inactivates this ATPase, or the ATPase does not recognize the oxidized form of PS, thus PS remains in the outer leaflet of the plasma membrane (Skulachev, 2001). It is well recognized that one of the earliest features of apoptotic changes of the cell is the exposure of PS on the surface of the cell membrane. PS exposed to the outer leaflet of the cell membrane can be detected by annexin V conjugates (Haugland, 2003). On the other hand, necrotic cells are characterized by the loss of the membrane integrity. Thus, the membrane impermeable dye - propidium iodide (PI) cannot pass through the intact or early apoptotic cells but can pass through necrotic or late apoptotic cells (Fig. 21B). Typical flow cytometric charts obtained from 143B ρ^0 and ρ^+ cells are shown in Fig. 21A. In the case of ρ^+ cells, the largest population of apoptotic cells seems to be obtained after 6 hours of the MEN treatment. It should be noticed that both apoptotic and necrotic changes were accelerated in the case of ρ^0 cells compared to ρ^+ cells. Namely, a certain population of ρ^0 cells became necrotic after 4 hours' treatment with MEN (Fig. 21A). Fig. 21 summarizes time-dependent changes in the cell death mode in MEN-treated 143B ρ^0 and ρ^+ cells.

We have already shown that the population of apoptotic cells reaches maximum at 6 hours of the MEN treatment followed by an abrupt decrease in the case of 143B ρ^+ cells (Wochna et al., 2005). This was reproducible in the present study (Fig. 22A). The population of necrotic cells reached about 80% at 9 hours treatment with MEN with distinct decreases in the population of apoptotic cells indicating that the change in the cell death mode from apoptosis to necrosis did occur between 6 hours and 9 hours of the MEN treatment. In the case of ρ^0 cells, both apoptotic and necrotic changes were induced in much shorter incubation times with MEN compared to the case of ρ^+ cells (Fig. 22B). About 75% of the population of ρ^+ cells remained intact at 4 hours of the MEN treatment while the population of intact cells decreased to 42% in the case of ρ^0 cells. The population of apoptotic cells in MEN-treated ρ^0 cells reached the maximum (17%) at 2 hours of the treatment and decreased to 3% at 9 hours.

The population of necrotic cells was observed even in such a short incubation time with MEN as 2 hours (9,5%) reaching maximum (51%) at 9 hours. Acceleration of MEN-induced cell injury processes especially of apoptotic changes in ρ^0 cells suggest that the intracellular level of ATP may be essential for MEN-induced apoptosis.

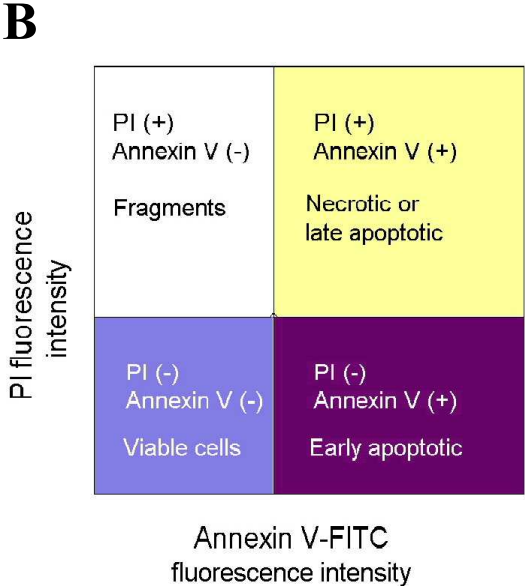
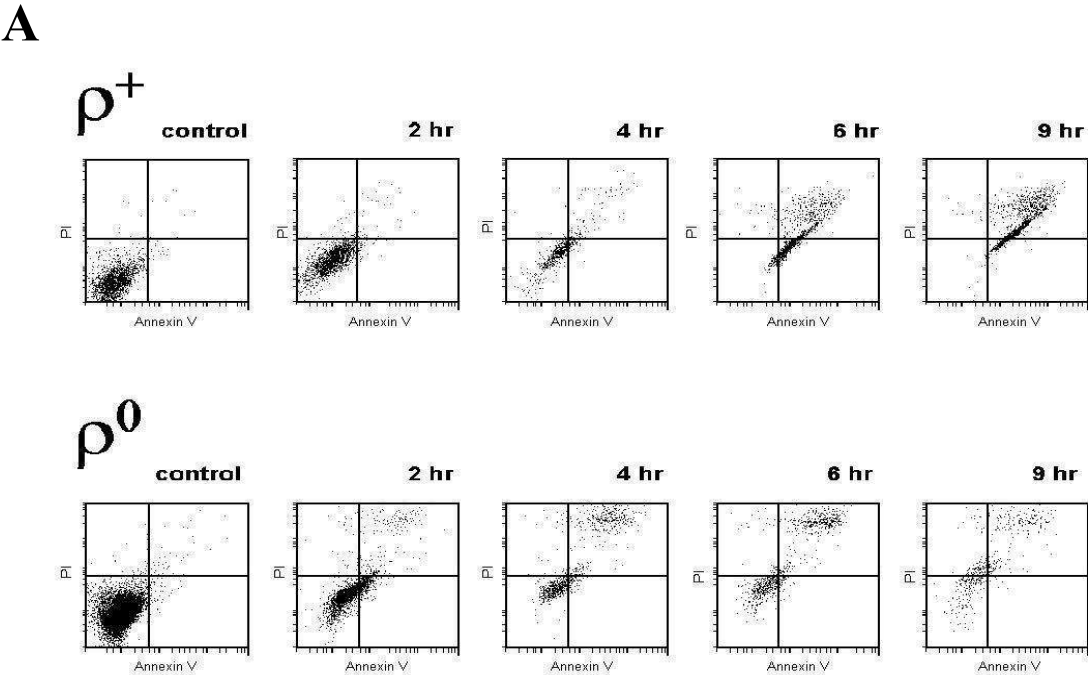


Fig. 21. Time dependent changes in the cell death mode in 100 μM MEN –treated 143B ρ^0 and ρ^+ cells detected by flow cytometry. A) Both ρ^0 and ρ^+ cells were treated with 100 μM MEN for 2, 4, 6 and 9 hours, and double-stained with Annexin V-FITC and PI for flow cytometric analysis. The representative results of at least three independent experiments are presented. B) Diagram representing interpretation of obtained results.

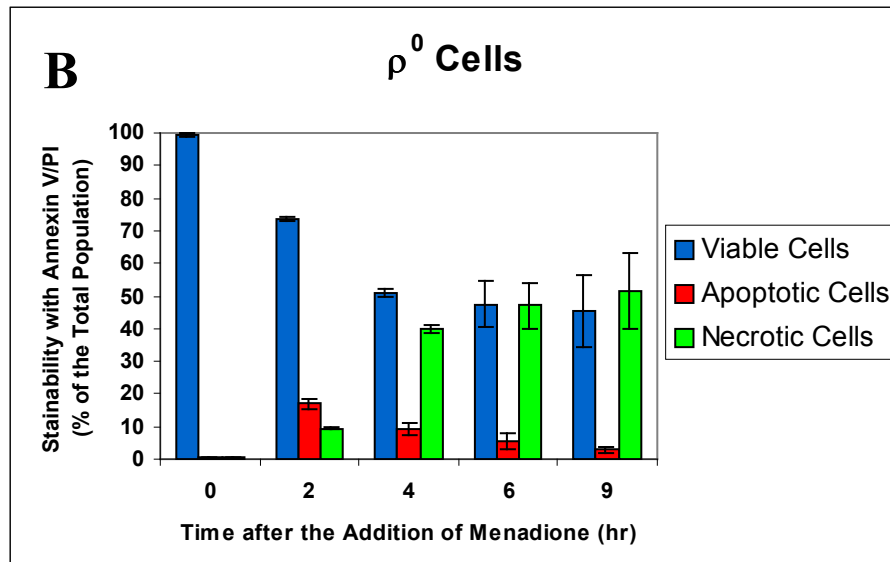
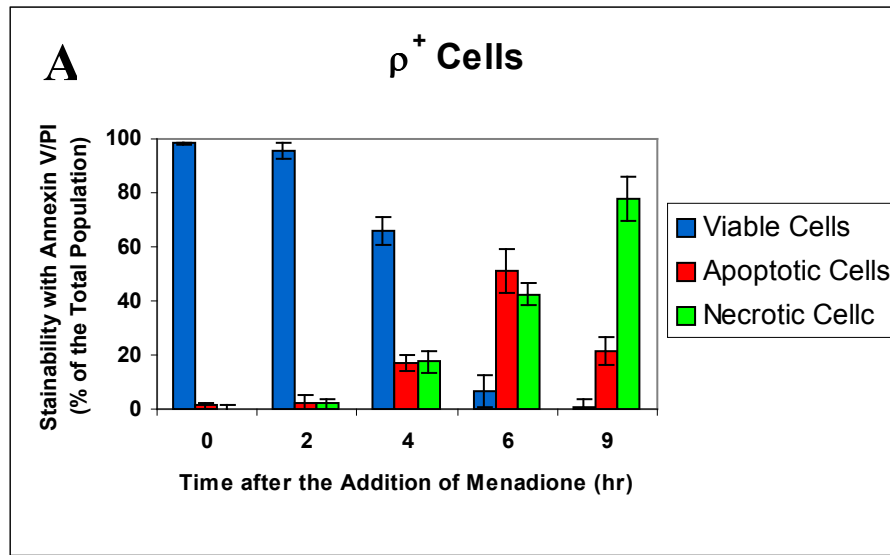
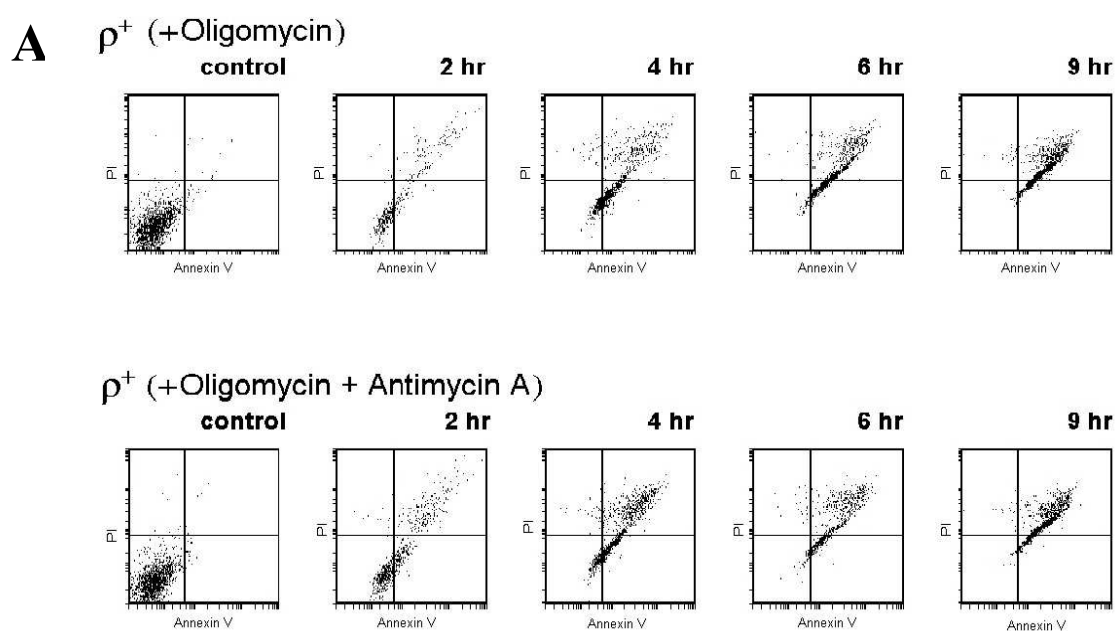


Fig. 22. Time-dependent changes in the cell death mode in MEN-treated 143B ρ^0 and ρ^+ cells detected by flow cytometry. ρ^+ (A) and ρ^0 (B) cells were treated with 100 μ M MEN for 2, 4, 6 and 9 hours, double-stained with Annexin V-FITC and PI for flow cytometry analysis. Data are the averages and standard error (mean \pm SE) of eight different experiments.

In order to determine the role of the cellular level of ATP in the switch mechanism of the cell death mode from apoptosis to necrosis, 143B ρ^+ cells were incubated with MEN in the presence of oligomycin or oligomycin plus antimycin A (AntmA), to mimic ρ^0 cells (Fig. 23) (Wochna et al., 2005). Oligomycin is an inhibitor of ATP synthase, while antimycin A inhibits the respiratory chain complex III. Cells were double-stained with Annexin V-FITC and PI and submitted to flow cytometry. Typical flow cytometric charts obtained from ρ^+ cells treated with MEN in the presence of oligomycin or oligomycin plus antimycin A are shown in Fig. 23A. It is evident from these charts that MEN-induced cell injury processes were distinctly accelerated compared to those of the control (compare to Fig. 21A). In the presence of oligomycin (Fig. 23B) and oligomycin plus antimycin A (Fig. 23C), ρ^+ cells treated with MEN for 2 hours became apoptotic by 20% and 24%, respectively. The population of apoptotic cells reached maximum at 4 hours of the MEN-treatment in both cases. Population of necrotic cells in the ρ^+ cells treated with MEN for 2 hours in the presence of the pre-treatment with oligomycin and oligomycin plus antimycin A were 3% and 24%, respectively, reaching 79% for the former and 77% for the latter, at 9 hours of MEN-treatment. These results indicate that data obtained from ρ^+ cells treated with MEN under the condition where ATP synthesis in mitochondria is restricted or suppressed, mimic those obtained from MEN-treated ρ^0 cells.



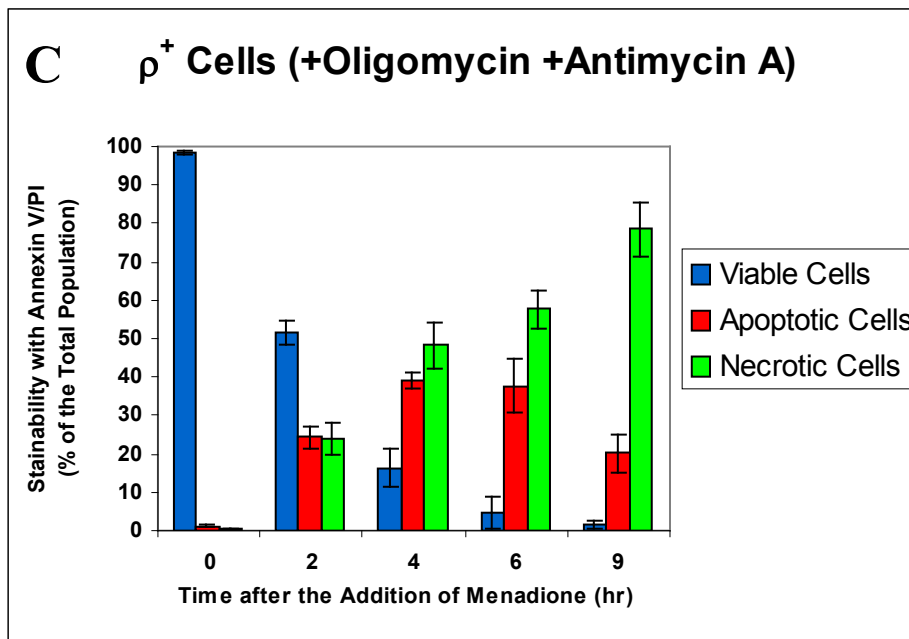
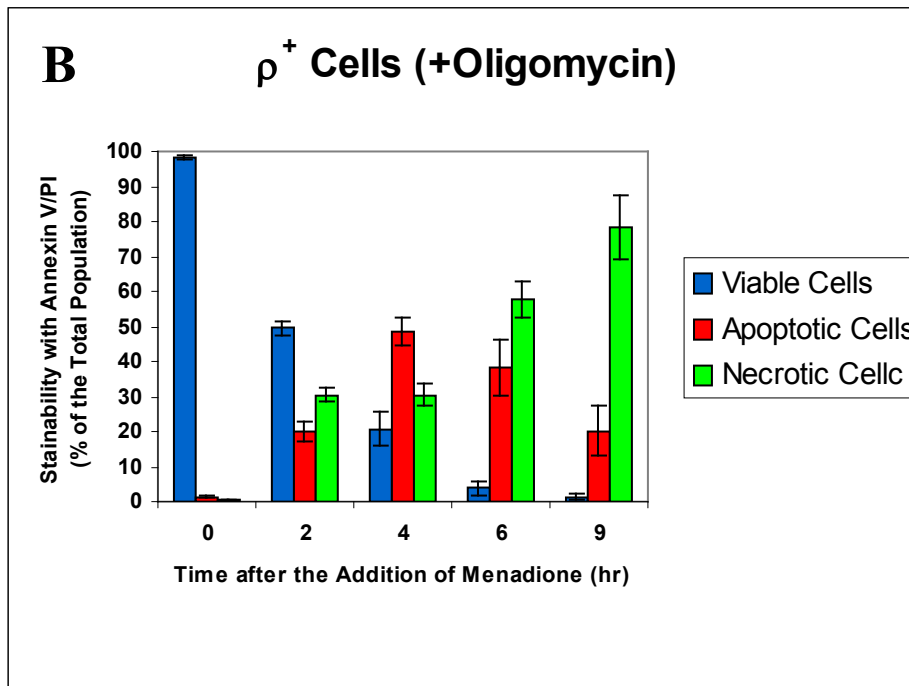
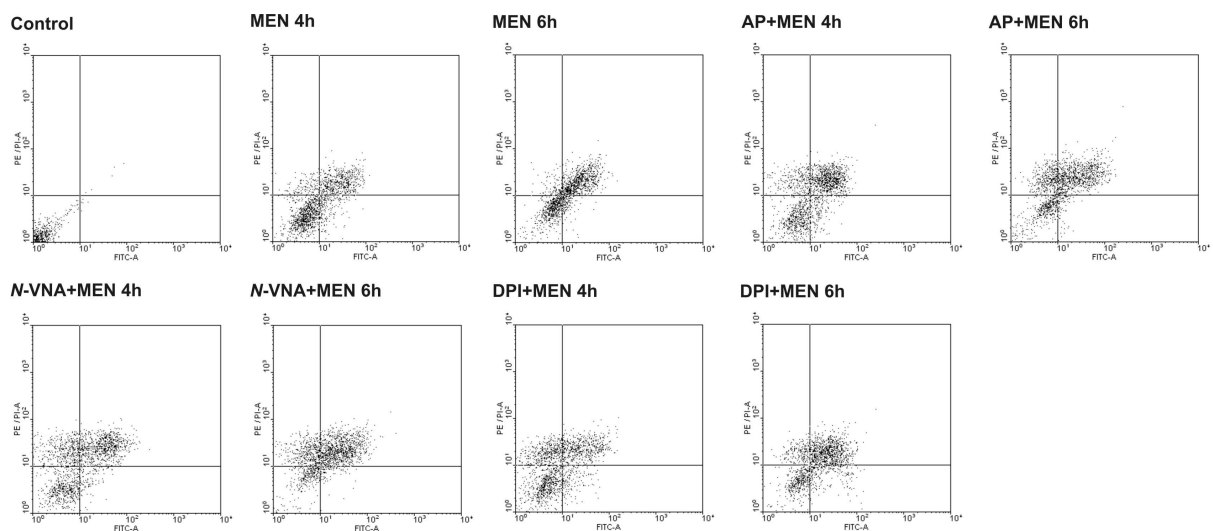


Fig. 23. Time-dependent changes in the cell death mode in MEN-treated 143B ρ^+ cells in the presence of oligomycin (3,5 $\mu\text{g/ml}$) or oligomycin plus antimycin A (50 μM) detected by flow cytometry. ρ^+ cells were treated with 100 μM MEN for 2, 4, 6 and 9 hours, double-stained with Annexin V-FITC and PI and analyzed by flow cytometry. (A) Demonstration of typical flow cytometric charts. (B) ρ^+ cells treated with MEN in the presence of oligomycin (3,5 $\mu\text{g/ml}$). (C) ρ^+ cells treated with MEN in the presence of oligomycin plus antimycin A (50 μM). Data shown in (B) and (C) are the averages and standard error (mean \pm SE) of eight different experiments.

Next, effects of NADPH oxidase inhibitors on cell injury process in MEN-treated 143B ρ^+ cells were examined. 143B ρ^+ cells were treated with MEN in the presence of AP, *N*-VNA, or DPI, stained with Annexin V and PI, and applied to flow cytometry.

In Fig. 24A, typical flow cytometric charts obtained from ρ^+ cells treated with MEN for 4 and 6 hours in the presence of inhibitors of NADPH oxidase are shown. Population of apoptotic cells apparently became increased in the cells treated with MEN for 6 hours in the presence of inhibitors of NADPH oxidase. These tendency were demonstrated more clearly in Fig. 24B. Namely, 30% of ρ^+ cells treated with MEN alone for 6 hours became apoptotic while the presence of AP, *N*-VNA, DPI caused increases in apoptotic cells to 54%, 44% and 58%, respectively. On the other hand, 55% of ρ^+ cells treated with MEN alone for 6 hours became necrotic while the presence of AP, *N*-VNA or DPI caused decreases in the population of necrotic cells to 18%, 26% and 14%, respectively. However, more than 90% of ρ^+ cells treated with MEN for 9 hours in the presence and absence of inhibitors of NADPH oxidase became necrotic. These data strongly suggest that inhibitors of NADPH oxidase were effective in putting off the switch of the cell death mode from apoptosis to necrosis in MEN-treated ρ^+ cells.

A



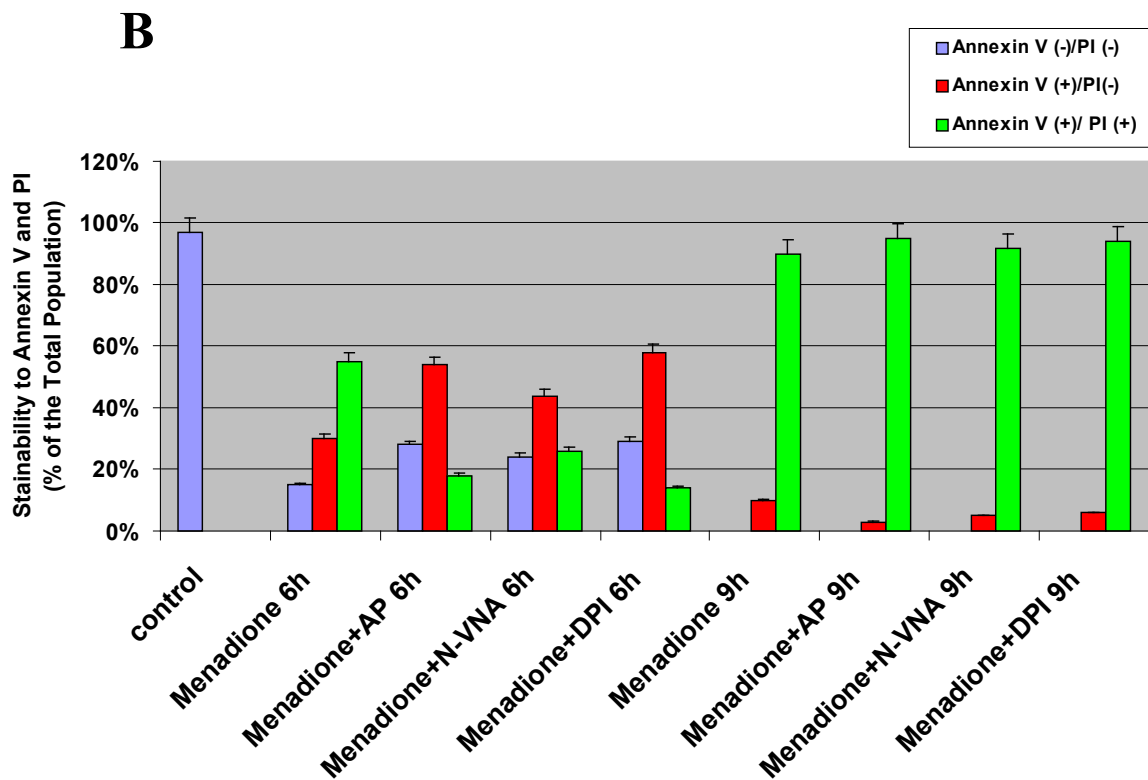


Fig. 24. Effects of NADPH oxidase inhibitors on cell viability of MEN-treated 143B cells. Cells were treated with 100 μ M MEN in the presence of inhibitors of NADPH oxidase (AP-500 μ M, N-VNA-250 μ M, DPI-1 μ M). (A) Demonstration of typical flow cytometric charts. (B) Blue bars represent the population of viable cells [AnnexinV(-)/PI(-)], red bars represent that of apoptotic cells [AnnexinV(+)/PI(-)] and green bars correspond to necrotic cells [AnnexinV(+)/PI(+)]. Data are the averages and standard error (mean \pm SE) of six different experiments.

27. Changes in the intracellular levels of ATP in MEN-treated 143B ρ^+ and ρ^0 cells

Results obtained in the present study from MEN-treated 143B ρ^0 cells as well as MEN-treated 143B ρ^+ cells co-treated with oligomycin or oligomycin plus antimycin A suggested a possible participation of decreased cellular ATP levels in the difference in the cell death mode. Thus, changes in the intracellular levels of ATP in MEN-treated 143B ρ^+ and ρ^0 cells were plotted against the duration of the time of the treatment (Wochna et al., 2005; Wochna et al., 2007). 143B ρ^+ and ρ^0 cells were treated with 100 μ M MEN for up to 9 hours, and the intracellular levels of ATP were measured using HPLC. At 2 hours of the MEN-treatment, the intracellular level of ATP in 143B ρ^+ cells decreased almost half of the control, and remained in the same level for up to 6 hours followed by an abrupt decrease thereafter (Fig. 25). The intracellular level of ATP in 143B ρ^0 cells was distinctly lower than that in 143B ρ^+ cells, and remained essentially in the same low level after MEN-treatment.

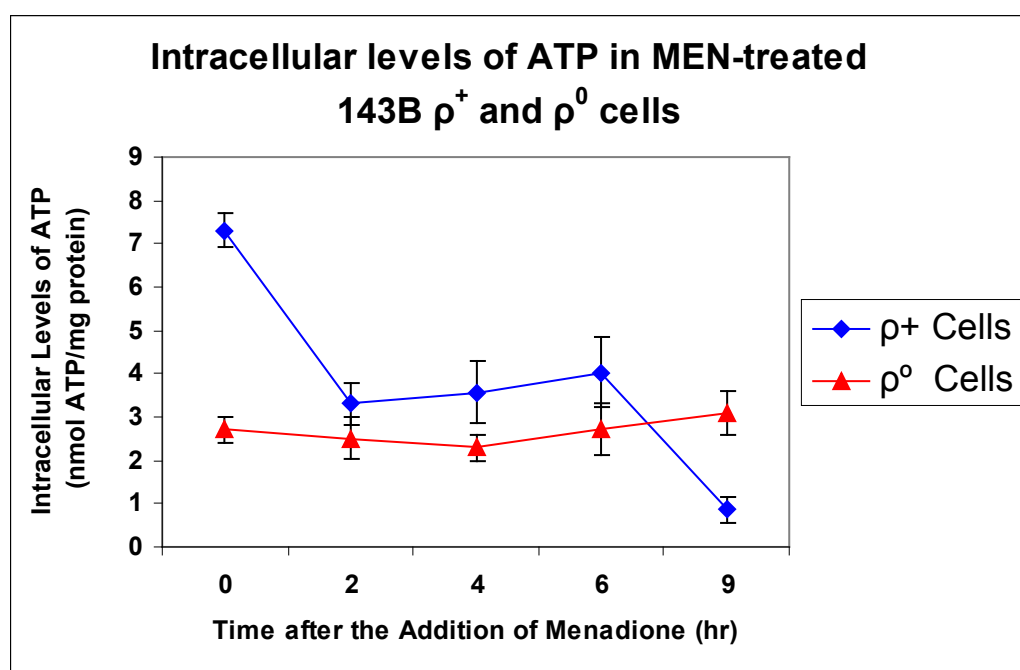


Fig. 25. Time-dependent changes in the intracellular level of ATP measured in 143B ρ^0 and ρ^+ cells after 100 μ M MEN treatment for various time 2, 4, 6 and 9 hours. Data are the averages and standard error (mean \pm SE) of three different experiments.

28. Electron microscopic changes of MEN-treated 143B cells

Effects of MEN on structural changes of 143B cells were examined electron microscopically (Fig. 26). 143B cells were characterized by the presence of nuclei with distinct nucleolei (Fig. 26A). When they were treated with MEN for 6 hours, two types of cells were discerned clearly: cells with dense nuclei and dense cytoplasm-apoptotic cells, and those with condensed chromatin with swollen cytoplasm-“intermediate cells” (Kamiński et al., 2003) (Fig. 26B). The latter cells are transitional state from apoptosis to necrosis. When the treatment of cells with MEN was prolonged to 9 hours, intermediate cells became predominant, and apoptotic cells were rarely seen (Fig. 26C).

Next, effects of inhibitors of NADPH oxidase on MEN-induced ultrastructural changes in 143B cells were examined. In Fig. 27, cells were treated with MEN for 6 and 9 hours, respectively in the presence and absence of the co-treatment with DPI (1 μ M). In the cells treated with MEN alone for 6 hours a mixed population of apoptotic and intermediate cells co-existed (Fig. 27B) whereas apoptotic cells were predominant in the cells treated with MEN for 6 hours in the presence of co-treatment with DPI (Fig. 27C). Intermediate cells were exclusively detected in the cells treated with MEN for 9 hours in the absence (Fig. 27D) and presence of DPI (Fig. 27E). AP (500 μ M) and *N*-VNA (250 μ M) demonstrated similar results with those obtained with DPI (electron micrographs are not shown). These data confirmed results obtained with flow cytometric analysis.

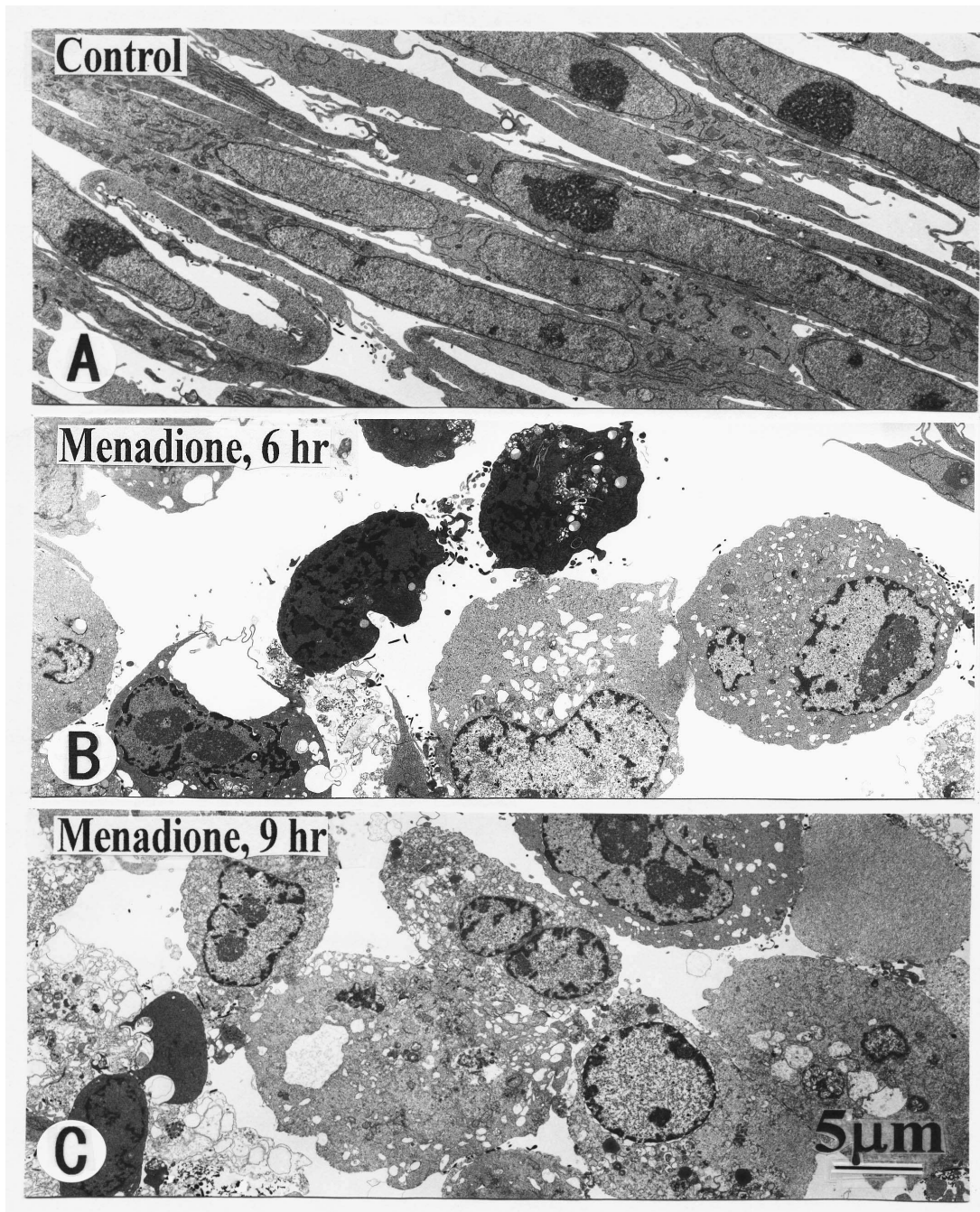


Fig. 26. Ultrastructural changes in MEN-treated 143B cells. Cells were treated with MEN for 6h and 9h. (A) Control 143B cells; (B) Cells treated with menadione for 6h; (C) Cells treated with MEN for 9h. Magnification: x 3500.

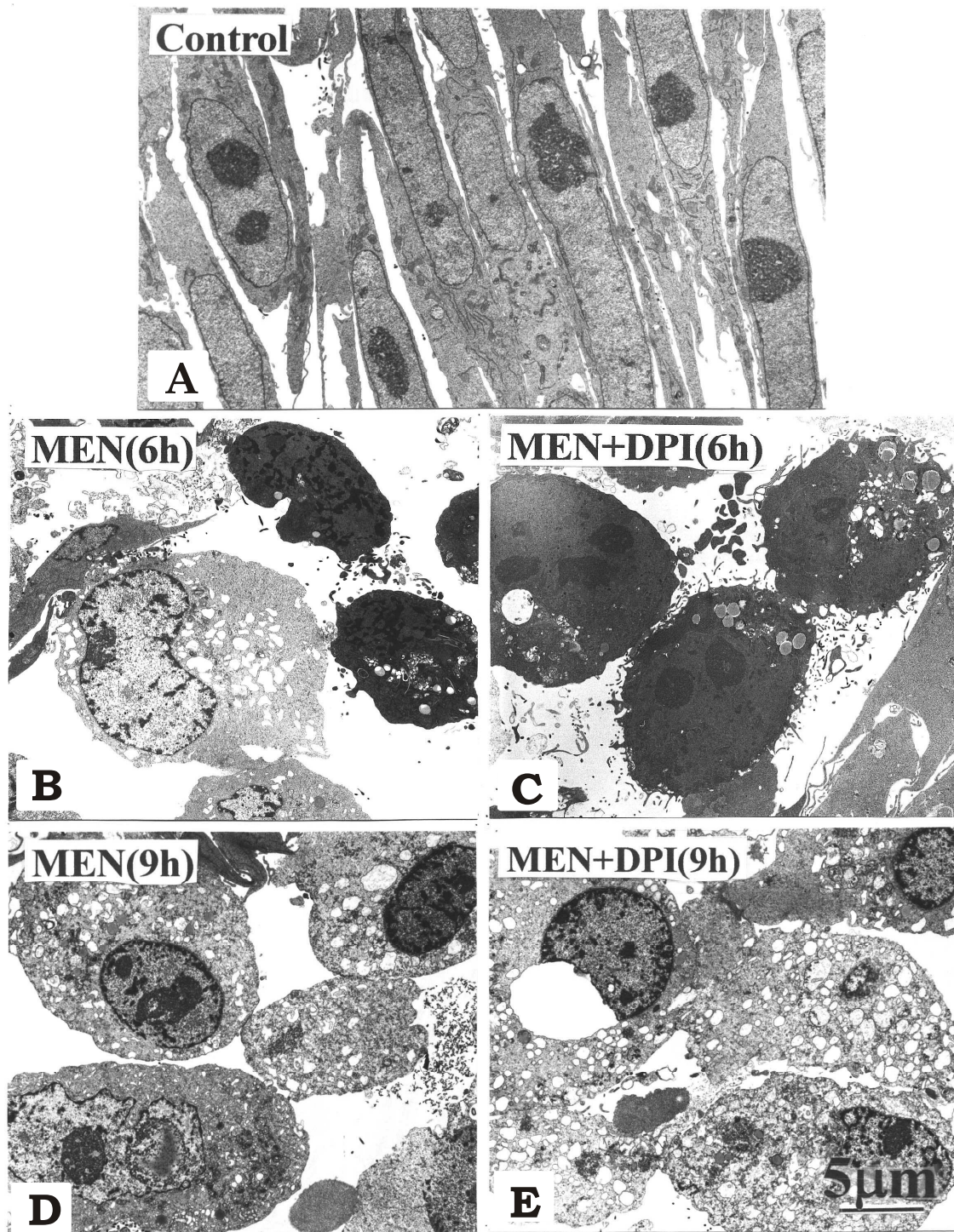


Fig. 27. Ultrastructural changes in MEN- and DPI-treated cells. (A) Control 143B cells; (B) Cells treated with MEN for 6h; (C) Cells treated with MEN and DPI for 6h; (D) Cells treated with MEN for 9h; (E) Cells treated with MEN and DPI for 9h. Magnification: x 3500.

29. Confocal microscopic localization of NADPH oxidase subunits in 143B cell

Data shown above strongly suggested the presence of NADPH oxidase in 143B cells. Thus, attempts have been made to localize NADPH oxidase in 143B cells using antibodies against subunits of the enzyme.

143B cells were incubated with MEN for 3 hours to activate NADPH oxidase. The localizations of particular subunits of NADPH oxidase: p47^{phox}, p67^{phox} and gp91^{phox} were detected by specific monoclonal antibody conjugated with fluorescent dyes, and visualized by confocal microscopy. In control 143B cells, the existence of each subunit was confirmed, but the intensity of fluorescence of each subunit was very weak (Fig. 28). On the other hand, in the cells treated with MEN for 3 hours, three different subunits of NADPH oxidase were definitely localized. They were localized predominantly in the perinuclear region of the cytoplasm extending to the cell membrane (Fig. 28).

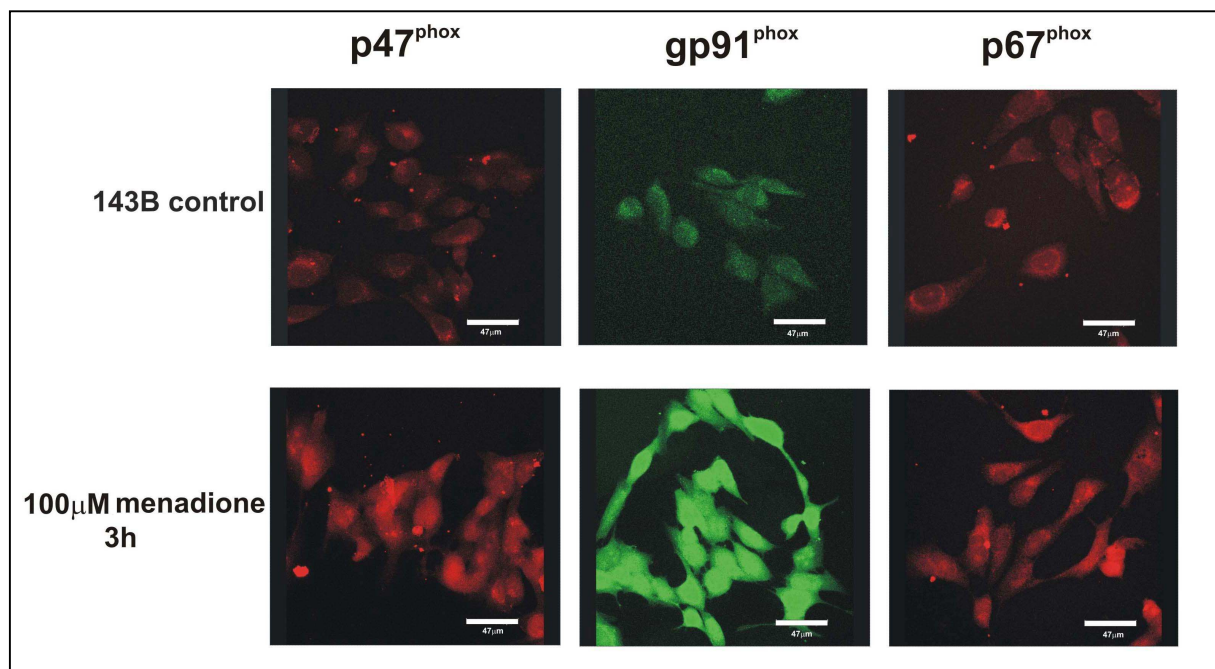


Fig. 28. Localization of NADPH oxidase subunits p47^{phox}, p67^{phox} and gp91^{phox} detected by specific monoclonal antibody against particular subunits conjugated with fluorescent dyes and visualised in confocal microscopy.

30. Detection of NADPH oxidase subunits in 143B cells by Western blotting method

In the present study efforts have been made to demonstrate the presence of NADPH oxidase in 143B cells by Western blotting besides confocal microscopic detection of the enzyme subunits. Both p47^{phox} and p67^{phox} subunits were detected in control 143B cells (Fig. 29).

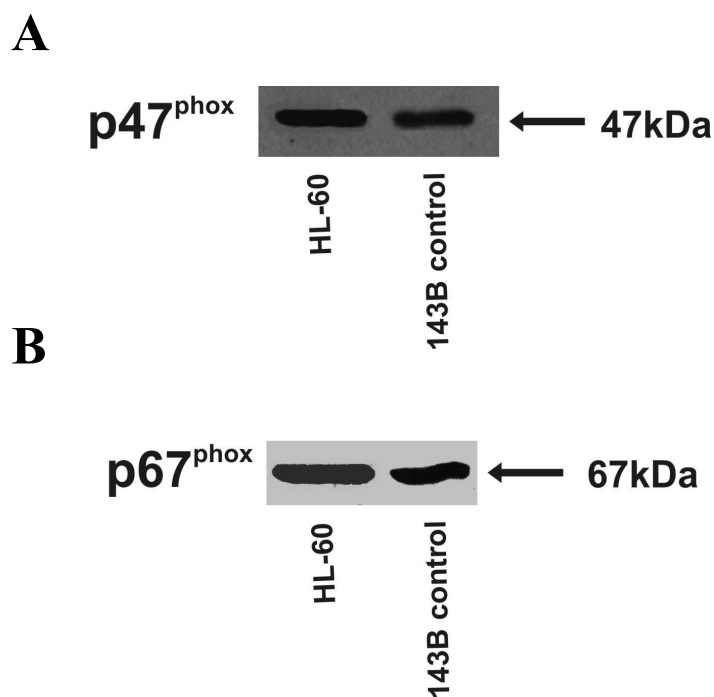


Fig. 29. Detection of NADPH oxidase subunits in 143B ρ^+ cells. (A) Determination of p47^{phox} subunit with mAb against this compound and (B) Determination of p67^{phox} subunit with mAb against this compound.

DISCUSSION

The purpose of the present study was to clarify the switch mechanism of the cell death mode from apoptosis to necrosis using MEN as an inducer of cell injury and osteosarcoma 143B ρ^+ and ρ^0 cells as experimental models. Special attention was paid to a possible role of NADPH oxidase in this mechanism. Major data demonstrated in the present study are summarized as follows:

1. The population of apoptotic cells in ρ^+ cells reaches maximum at 6 hours of the MEN treatment followed by an abrupt decreases. Apoptotic and necrotic changes were accelerated in MEN-treated ρ^0 cells and ρ^+ cells treated with MEN in the presence of oligomycin or oligomycin plus antimycin A compared to MEN-treated ρ^+ cells.
2. Intracellular level of superoxide in MEN-treated ρ^+ cells reaches maximum at 6 hours of the treatment with abrupt decreases thereafter while that in MEN-treated ρ^0 continued to increase during the course of the experiment.
3. Intracellular level of ATP in ρ^+ cells immediately became decreased after MEN-treatment reaching to half of the control level at 2 hours of the treatment. It kept the same level for up to 6 hours of MEN-treatment with abrupt decreases thereafter. Intracellular level of ATP in ρ^0 was distinctly lower than that in ρ^+ cells, and it remained essentially in the same level before and after the MEN-treatment.
4. NADPH oxidase was localized to 143B cells for the first time in the literature using antibodies against subunits of the enzyme by confocal microscopy and Westren blotting method.
5. MEN-induced various changes were suppressed or modified by inhibitors of NADPH oxidase: MEN-induced remarkable increases in the intracellular level of superoxide at 6 hours of the treatment were invariably suppressed by inhibitors of NADPH oxidase; NADPH oxidase inhibitors partially suppressed transition of the cell death mode from apoptosis to necrosis in MEN-treated cells. Namely, the population of apoptotic cells in the cells treated with MEN for 6 hours in the presence of inhibitors of NADPH oxidase was invariably larger than that treated for 6 hours with MEN alone. On the other hand, the population of intermediate cells (necrotic cells) in the former was smaller than the latter.

31. Time-dependent changes in MEN-induced cell death mode

The present study indicates strongly that the determination of the type of cell death mode under given experimental conditions should be done paying special attentions to the following points: 1) Time-dependent studies on the cell death mode given under any experimental condition are essential. The present study has confirmed the previous studies done by Kamiński et al. (Kamiński et al., 2003, 2004) that the population of apoptotic cells becomes predominant at 6 hours of the MEN-treatment. Thus, we may conclude that MEN induces apoptosis if we treat cells with MEN for 6 hours only or for up to 6 hours. On the other hand, apoptotic cells are no more detected and only intermediate (necrotic) cells can be detected at 9 hours of the MEN treatment. Thus, if we obtain data only from cells treated for 9 hours or longer, we may conclude that MEN induces necrosis in 143B cells. A survey of the literature concerning the correlation between MEN-induced cell injury and the cell death mode shows that reports are controversial. MEN induces apoptosis: nasopharyngeal carcinoma cells (Felicia et al., 1993), Jurkat human T-cells (Yaguchi et al., 1997; Derfuss, et al., 1998), rat osteoclasts (Sun et al., 1997), cardiac muscle cells (Turner, et al., 1998), glial cells (Hollensworth et al., 2000), pancreatic cells (Gerasimenko et al., 2000), rat hepatocytes (Jones, et al., 2000); MEN induces necrosis: HepB2 cells (Samali et al., 1999); MEN induces apoptosis and necrosis depending on its concentrations: RINm5F cells (Dypbukt et al., 1994), hepatocytes (McConkey et al., 1988), Jurkat cells (Ma, et al., 2002), pancreatic acinar cells (Sata et al., 1997). Differences in the reports on the cell death mode, described above, should be re-evaluated in the light of the length of the incubation time with MEN. 2) Electron microscopic studies on cell injury processes, especially of the cell death mode, is routinely carried out in many laboratories with the application of flow cytometry to culture cells stained with Annexin V and PI. Viability and severeness of cell injury are judged by stainability of cell to two dyes, specified above: viable cells: Annexin V(-)/PI(-); apoptotic cells: Annexin V(+)/PI(-); late apoptotic or necrotic cells: Annexin V(+)/PI(+). The present study has confirmed the previous studies done by Kamiński et al. (Kamiński et al., 2003, 2004) that cells with Annexin V(+)/PI(+) are “intermediate” cells in that they are in a transitional state from apoptotic to necrotic cell injury processes. Without electron microscopic studies on MEN-induced cell injury processes it was impossible to give such conclusion. Survey of the literature shows that reports on cell injury processes using electron microscopic techniques are few. However, the present study strongly suggest that application of electron microscopy to cell injury processes is essential together with that of flow cytometry. 3) A possible

involvement of “Secondary necrosis” in the judge of the cell death mode in cultured cells must be taken into account. The distinction between apoptosis and necrosis can be confused because of the lack of scavenging cells in culture models. Namely, necrotic cells detected in culture models have three origins: cells that became primary necrotic while they still attach to the culture dishes without going through apoptosis; those that become necrotic via foregoing apoptosis while they attach to the culture dishes; and those that become necrotic due to secondary necrosis after they float into the culture media via foregoing apoptosis (Kamiński et al., 2004). When the viability of the cells treated with MEN for up to 10 hours was compared between cells attached to the culture dishes and those attached to the culture dishes plus floating cells in the culture media, there was practically no difference in the population of apoptotic or necrotic cells between them since quite few cells became detached from the culture dishes after such relatively short period of time of incubation with MEN (Niemczyk and Wakabayashi, unpublished observations). However, in cases where a large number of cells detaches from the culture dishes after certain period of time of incubation with chemicals, a possible involvement of secondary necrosis must be avoided using cells attached to the culture dishes free from floating cells in the culture media.

Flow cytometric analysis of MEN-treated cells showed that the populations of Annexin V(+)/PI(+) cells and that of Annexin V(+)/PI(-) cells begin to increase at 2 hours after MEN treatment, the former exceeding the latter. The population of Annexin V(+)/PI(-) cells reaches a maximum at 6 hours after the MEN treatment and rapidly decreases thereafter while Annexin V(+)/PI(+) cells continuously increase thereafter (Kamiński, et al., 2003; Kamiński, et al., 2004). These results indicated a possibility that two different cell death mode, apoptosis and necrosis, proceeded at the same time. However, electron microscopy of these cells revealed that the presence of apoptotic cells and intermediate cells, and pure necrotic cells (cells with swollen nuclei and swollen cytoplasm) were rarely seen. This may suggest that MEN-induced cell death is essentially apoptotic in nature, at least under the present experimental conditions. Several reports are available in the literature describing that MEN at low concentrations induces apoptosis while at higher concentrations it induces necrosis (Sata, et al., 1997; McConkey, et al., 1988). However, apoptosis and necrosis co-existed in the present study. The present study strongly indicates a necessity of a careful studies on the cells death mode using both flow cytometric and electron microscopic techniques.

32. A burst in the intracellular level of superoxide as a possible mechanism for the transition of the cell death mode from apoptosis to necrosis in MEN-treated 143B cells

The present study has shown that the intracellular level of superoxide reaches maximum at 6 hours of MEN-treatment followed by abrupt decreases thereafter. The generation of massive reactive oxygen species (Nobel et al., 1997; Hampton and Orrenius, 1997), caspase inactivation (Melino, et al., 1997; Lemaire, et al., 1998), and decrease in intracellular levels of ATP (Leist, et al., 1997; Ferrari, et al., 1998; Ha and Synder, 1999) are among those factors proposed to be crucial for the switch mechanism from apoptosis to necrosis. Since apoptosis is an energy-requiring process for the activation of caspases, the depletion of intracellular ATP and the burst in ROS generation were considered possible two major mechanisms for the transition of the cell death mode from apoptosis to necrosis in MEN-treated 143B cells in the present study. Time-dependent changes of superoxide and ATP levels in MEN-treated ρ^+ and ρ^0 cells were presented. In the case of ρ^+ cells, intracellular level of superoxide reached maximum at 6 hours of the MEN-treatment with distinct decreases thereafter whereas it continued to increase during the course of the experiment in the case of ρ^0 cells. In the case of ρ^0 cells, intracellular level of ATP remained essentially in the same level during the course of MEN-treatment, while it became distinctly decreased immediately after the treatment with MEN, and remained in the same level for up to 6 hours with distinct decreases in ρ^+ cells. The switch from apoptosis to necrosis did occur to ρ^0 cells treated with MEN despite essentially no changes in the intracellular level of ATP. Thus, these data strongly suggest that intracellular level of ATP may not be related directly to the switch mechanism of the cell death mode from apoptosis to necrosis (Wochna et al., 2005; Wochna et al., 2007). It was also shown that results obtained from ρ^+ cells treated with MEN in the presence of oligomycin or oligomycin plus antimycin A were similar to those obtained with MEN-treated ρ^0 cells indicating that apoptotic and necrotic changes in the former cells were distinctly faster than MEN-treated cells in the absence of inhibitors, specified above. These data again suggest that intracellular level of ATP may not be related at least directly to the switch from apoptosis to necrosis. Decreases in intracellular level of ATP may accelerate both apoptotic and necrotic changes.

33. A possible contribution of NADPH oxidase to the switch from apoptosis to necrosis in MEN-treated 143B cells

If the burst in ROS generation is the primary mechanism for the switch from apoptosis to necrosis in MEN-treated 143B cells, then a question arises: what is the source of ROS to cause such burst in MEN-treated 143B cells?

Intracellular major sources of ROS generation are: mitochondria via complex I, II and III (Boveris, et al., 1972; Cadenas, et al., 1977; Turrens and Boveris, 1980), endoplasmic reticulum via cytochrome *P*-450, and cytoplasmic xanthine oxidase via the degradation of nucleic acids. It has been accepted that mitochondria are the main intracellular source of ROS (Chance, et al., 1979; Forman and Boveris, 1982).

On the other hand, data have been accumulated to demonstrate that NADPH oxidase, originally detected in the plasma membrane of leukocytes, is widely distributed among mammalian cells and superoxide generated from the enzyme has important role in some cases of apoptosis (Kim, et al., 2002; Arroyo, et al., 2002; Hu, et al., 2002). It is well known that NADPH oxidase is localized in a variety of the cells derived from different tissues like: vascular smooth muscle cells (VSMCs) and vascular endothelial cells (Griendling, et al., 1994; Griendling, et al., 2000; Hohler, et al., 2000), human placenta trophoblasts (Matsubara and Sato, 2001), human glomerular mesangial cells (Jones, et al., 1995), a hepatoma cell line (Ehleben, et al., 1997), kidney (Cheng, et al., 2001; Geiszt, et al., 2000), spleen (Cheng, et al., 2001; Banfi, et al., 2001), colon (Geiszt, et al., 2003), thyroid (De Deken, et al., 2000), ovary (Cheng et al., 2001), osteoclasts (Cheng et al., 2001), sperm (Banfi, et al., 2001), etc. In neurons, the rate of the generation of superoxide from NADPH oxidase has been reported to be 6-10-fold higher than that from mitochondria (Martin-Romero, et al., 2002). Thus, a possible contribution of NADPH oxidase to MEN-induced switch of cell death mode from apoptosis to necrosis was examined in the present study. Some data are already available in the literature to demonstrate that MEN stimulates intracellular ROS generation via activation of NADPH oxidase from hepatocytes (Thor, et al., 1982), yeast (Yamashoji, et al., 1991), and Jurkat cells (Suzuki and Ono, 1999).

The present study is the first in the literature to demonstrate that NADPH oxidase does exist in 143B cells. Some of subunits of NADPH oxidase were localized to 143B cells using confocal microscopic technique and Western blotting method. Data are not available in our hand to visualize the whole feature of NADPH oxidase in 143B cells at the moment, and further studies are definitely required to characterize NADPH oxidase in 143B cells.

Finally, I would like to discuss briefly about the possible contribution of superoxide generated from NADPH oxidase to the switch of cell death mode from apoptosis to necrosis in MEN-treated 143B cells focussing on the effects of NADPH oxidase inhibitors on the enzyme activities. The present study has demonstrated that the intracellular level of superoxide reaches the maximum at 6 hours of the MEN-treatment followed by an abrupt decrease thereafter. The population of apoptotic cells reaches maximum at 6 hours of the MEN treatment with distinct decreases thereafter in ρ^+ cells. Inhibitors of NADPH oxidase were invariably effective in suppressing intracellular level of superoxide enhanced by MEN-treatment. These data strongly suggest that increases in the intracellular level of superoxide originated from mitochondrial electron transfer chain and microsomal cytochrome P-450 via the metabolism of MEN are further enhanced by superoxide generated from NADPH oxidase activated by MEN, resulting in a burst in superoxide generation. This, in turn, inactivates caspases and mitochondria are seriously damaged, and the switch from apoptosis to necrosis takes place. The present study showed that intracellular level of superoxide remarkably elevated by MEN treatment in ρ^+ cells was suppressed by the combined treatment with MEN and NADPH oxidase inhibitors, and at the same time transition of cell death mode from apoptosis to necrosis was partly suppressed. These data also strongly indicate a possible involvement of NADPH oxidase in the switch of cell death mode from apoptosis to necrosis. Intracellular level of superoxide in ρ^+ cells treated for 9 hours with MEN was definitely lower than that in those treated for 6 hours with MEN although it was still much higher than that of the control. Inhibitors of NADPH oxidase had no improving effects on intracellular level of superoxide in the cells treated with MEN for 9 hours. Since ρ^+ cells treated with MEN for 9 hours become predominantly intermediate (necrotic) with extremely swollen cytoplasm and swollen mitochondria, the rate of generation of superoxide from mitochondria may be decreased compared to that in apoptotic cells. Deterioration of plasma membrane may be severe enough in intermediate (necrotic) cells so that NADPH oxidase may no longer be active.

In the case of ρ^0 cells, intracellular level of superoxide became increased continuously with the time of incubation with MEN, and apoptotic and necrotic changes, especially the latter, became distinct much faster than the case of MEN-treated ρ^+ cells. There was no distinct peak in the population of apoptotic cells in MEN-treated ρ^0 cells. Similar results to MEN-treated ρ^0 cells, described above, were obtained with ρ^+ cells treated with MEN plus oligomycin or MEN with oligomycin plus antimycin A. These results indicate that the transition of the cell death mode in ρ^0 cells continuously takes place after the MEN treatment. Since intracellular

level of ATP in ρ^0 cells is extremely low compared to that in ρ^+ cells, apoptotic cells may become necrotic much more easily and faster than the case of ρ^+ cells.

SUMMARY

Human osteosarcoma is very malignant tumor with high resistance to chemotherapy. 143B cell line was used in this project in order to find out proper therapy which will allow to omit the resistance of these cells to the drugs. I proposed in this project menadione as a compound which may be very effective in osteosarcomas treatment. As it was mentioned in introduction menadione is under clinical trials of many other tumors such as: advanced gastrointestinal cancers (Tetef et al., 1995), lung cancer (Tetef et al., 1995), human prostate carcinoma (Zhang et al., 2001), etc.

Apoptosis and necrosis are two distinct forms of cell death, with morphological and biochemical features different from each other. However apoptosis is superior to necrosis in the way that it participates in the organogenesis, cell differentiation and various disease conditions. Apoptotic cells are phagocytosed by neighbouring cells while necrotic cells cause local and general inflammatory reactions resulting in side effects during the disease treatment. So it would be of great importance for clinical medicine to control the switch mechanism from apoptosis to necrosis.

In the present study we attempted to clarify the role of membrane NADPH oxidase in the switch mechanism of the cell death mode using human osteosarcoma 143B cell line. MEN-induced injury process was examined in both ρ^+ and ρ^0 cells. Data on Annexin V and PI in MEN-treated ρ^+ cells show that the population of apoptotic cells reached the maximum at 6 h of the treatment followed by an abrupt decrease thereafter. On the other hand, the frequency to find apoptosis in MEN-treated ρ^0 cells was much smaller than in the case of ρ^+ cells. Necrotic population of the cells was the majority throughout the course of the experiment.

Time-dependent changes in the cellular level of ATP in MEN-treated ρ^0 cells suggest that the low level of ATP may play a role in the acceleration of apoptotic changes of the cell but it is not a triggering factor for the transition of the cell death mode from apoptosis to necrosis since the intracellular level of ATP in ρ^0 cells remained essentially at the same level before and after the MEN-treatment. Despite the ATP level apoptosis became necrosis continuously. This is also supported by the data obtained with ρ^+ cells where apoptotic and necrotic changes were distinctly accelerated after MEN-treatment in the presence of oligomycin or oligomycin plus antimycin A. These data suggested that the burst in the intracellular level of superoxide may be a triggering factor for the switch mechanism.

Time-dependent changes in the intracellular level of superoxide anion were determined in 143B ρ^+ and ρ^0 cells demonstrating that in ρ^0 cells the level of this anion was higher than in ρ^+

cells. When ρ^+ cells were treated with MEN, the intracellular level of superoxide reached the maximum at 6 h of the treatment, and decreased thereafter, while that in MEN-treated ρ^0 cells continued to increase during the course of the experiment. It suggests that the massive generation of superoxide causes the opening of megachannels of mitochondria resulting in the release of cytochrome c into the cytoplasm. Cytochrome c activates caspases resulting in apoptotic changes of ρ^0 cells.

It has been reported that in certain experimental conditions superoxide generated from NADPH oxidase plays a major role in the induction of apoptosis rather than that generated from mitochondria (Arroyo et al., 2002; Hu et al., 2002; Kim et al., 2002). The effects of inhibitors of NADPH oxidase such as: apocynin (AP), *N*-vanillylnonanamide (*N*-VNA) and diphenyleneiodonium chloride (DPI) on the MEN-induced changes in the intracellular level of superoxide were examined. 143B ρ^+ cells were incubated with MEN alone or with MEN plus inhibitors of NADPH oxidase. The intracellular level of superoxide anion was measured in the presence of DHE. Obtained data present that NADPH oxidase may contribute to the remarkable increase in the intracellular level of superoxide in the cells treated with MEN for 6 h. These assumptions were supported by the data obtained with inhibitors of NADPH oxidase which significantly suppressed the superoxide production after MEN-treatment for up to 6 h, although the intracellular level of superoxide remained higher than that of the control cells. The intracellular level of superoxide in the cells treated with MEN for 9 h was distinctly lower than that at 6 h and the inhibitors of NADPH oxidase had practically no effect. Ultrastructural characterization of the cells by electron microscopy, treated with MEN for 9 h revealed condensed nuclei and swollen cytoplasm and thus could be designated as 'intermediate cells' indicating that the cells were in a transitional state from apoptosis to necrosis (Kamiński et al., 2003). Mitochondria in the intermediate cell were often extremely swollen with the rupture of the outer membrane. This allow to conclude that cells treated with MEN for 9 h were often seriously damaged so the ability of mitochondria to synthesize ATP using molecular oxygen became decreased resulting in lowering of the rate of superoxide generation by mitochondria.

Then the effects of pretreatment with inhibitors of NADPH oxidase on viability of MEN-treated cells shown that population of apoptotic cells treated with AP, *N*-VNA and DPI for 6 hours was distinctly higher in comparison to the population of apoptotic cells treated with MEN alone. At 9 h of the MEN-treatment 90% of cells became necrotic, and pretreatment with NADPH oxidase inhibitors caused no change. Thus it might be reasonable to assume that the ability of mitochondria in the cells treated with MEN for 9 h to use molecular oxygen for

ATP synthesis is lowered compared to that of mitochondria in control cells. Although inhibitors of NADPH oxidase are partially effective in suppressing the transition of the cell death mode from apoptosis to necrosis, mitochondria are damaged when the incubation time with MEN exceeds 6 h and the cells become necrotic even in the presence of NADPH oxidase inhibitors.

Menadione has been shown to stimulate intracellular ROS generation *via* activation of NADPH oxidase from hepatocytes (Thor et al., 1982), yeast (Yamashoji et al., 1991) and a human B-lymphoma cell line and Jurkat cells (Suzuki and Ono, 1999). The present study strongly suggests that the burst in superoxide anion production by membrane NADPH oxidase might be the triggering factor for the transition of the cell death mode from apoptosis to necrosis.

Although NADPH oxidase was revealed in variety types of tissues, nobody demonstrated the presence of this enzyme in 143B cell line. We are the first who present by Western blotting method and confocal microscopy that NADPH oxidase does exist in this cell line. Although the results are not complete and demand further investigations we could demonstrate that two subunits: p47^{phox} and p67^{phox} among six are present in 143B cells. Another fact is that gp91^{phox} subunit possesses 5 different homologues which were described in INTRODUCTION and according to the literature NOX4 isoform seems to be present in this investigated cell line (Cheng et al., 2001).

Data obtained in this project suggest that the increase in the cellular level of superoxide anion mainly originates from NADPH oxidase and ROS may be the key factor that trigger the transition of the cell death mode from apoptosis to necrosis, and that decreases in the cellular level of ATP accelerate MEN-induced changes of the cells.

CONCLUSIONS

1. NADPH oxidase was localized in the plasma membrane of human osteosarcoma 143B cells for the first time in the literature.
2. Inhibitors of NADPH oxidase suppress MEN-induced remarkable elevation of intracellular levels of superoxide, and at the same time partly suppressed the transition from apoptosis to necrosis.
3. The present data together with those on ρ^0 cells strongly suggest that a burst in the intracellular levels of superoxide may be the key factor for the transition of the cell death mode from apoptosis to necrosis.

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