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Neutrophil apoptosis and hypoxia

Neutrophils are the most abundant population of leukocytes, which constitute the defense against pathogens. Released by neutrophils the proteolytic enzymes and reactive oxygen species help in eliminating infections, but also cause extensive tissue damage. Neutrophil apoptosis plays an essential role in cell homeostasis and resolution of inflammation. It is mediated by a complex network of intracellular apoptotic/survival signaling pathways and can be modulated by a variety of extracellular stimuli such as hypoxia. Here, we review recent studies on the mechanisms of neutrophil death and survival accentuating on neutrophil apoptosis under hypoxic conditions. Neutrophils possess components of both extrinsic and intrinsic apoptotic routes. However, in neutrophils this mechanism has special features. The involvement of death receptors, caspases, mitochondria, and Bcl-2 proteins are discussed. Both the transcription factor NF-κB and p38MAPK regulate the neutrophil apoptotic program. Despite that reactive oxygen species (ROS) can directly promote and/or adjust apoptosis, there is no consensus about the role of ROS on neutrophil lifespan. Thus both the type of ROS involved and the site of their generation may be important for neutrophil apoptosis. Finally, hypoxia can activate several signaling pathways. The possible differences between the effects of sustained and intermittent hypoxia are also addressed.
Key words: neutrophils, apoptosis, hypoxia.

TAKE-HOME MESSAGES

Neutrophil apoptosis is a central process for homoeostasis and successful resolution of inflammation, but in neutrophils it has special features because neutrophils are committed to cell death.

Similar to other cells neutrophil apoptosis possesses components of extrinsic death receptor and intrinsic mitochondrial apoptotic pathways in which NF-κB and p38MAPK controlled proteins such as Bcl-2 family members and caspases are involved.

ROS generation is involved in neutrophil apoptosis of activated or infected cells but is not absolutely required as a mediator of neutrophil apoptosis under physiological conditions.

In contrast to other cells, in which hypoxia induces apoptosis, in neutrophils hypoxia causes a profound inhibition of apoptosis both in vitro and in vivo. The survival effect of intermittent hypoxia was much more prominent then sustained hypoxia.

AN OVERVIEW OF NEUTROPHIL APOPTOSIS

Neutrophils are the most common type of leukocytes in the circulation which constitute the first line of defense against pathogens. They are bone marrow derived, terminally differentiated, short lived (8-20 hrs) inflammatory cells that are released to the circulation continuously. Senescent neutrophils are cleared from the blood by liver, spleen and bone marrow in direct contact with flowing blood [1]. Neutrophils can exist in the circulation in one of three functional states: quiescent, primed or activated [2]. When quiescent neutrophils encounter a stimulus they are left in a primed state. Upon encountering a second stimulus, they proceed to activation, releasing reactive oxygen species (ROS), proteolytic enzymes and inflammatory mediators, which are implicated in clearance of infections [2]. However, an uncontrolled release of formi-

dable array of toxic substances may inflict damage to surrounding tissues and propagate inflammation. Neutrophil apoptosis (NA) is a fundamental mechanism involved in maintaining a normal level of neutrophils and ensuring the rapid resolution of inflammation [3, 4]. NA triggers the phagocytosis of apoptotic neutrophils by macrophages and is vital for limiting of tissue damage in vivo [3]. If neutrophil viability is prolonged, destruction of surrounding cells will take place. When this process, is initiated in the vasculature it is implicated in cardiovascular diseases. Importantly, mature neutrophils can undergo apoptosis even without requiring any apparent inductive stimuli. It suggests that the apoptotic program may already have been initiated in circulating neutrophils [5].

NA is mediated by a complex network of intracellular death/survival signaling pathways and can be modulated by a variety of extracellular stimuli such as cytokines and hypoxia. NA can be initiated by the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. The last one may play a pivotal role in the control of spontaneous NA [6, 7]. The caspase cascade represents the main mechanism which is activated by both pathways. Caspases are synthesized as inactive zymogens and are activated by proteolysis, leading to enzyme cleavage and nuclear DNA fragmentation. Caspase-8 is the initiator caspase triggered by death receptors, whereas initiator caspase-9 cleavage is the signature of the mitochondrial pathway. Caspase-3, an effector caspase, is activated by the caspases-8 and -9 [8]. Figure 1 illustrates the sequence of events of NA. The data describing NA pathways are summarized in a number of recent reviews [3–6, 9, 10].

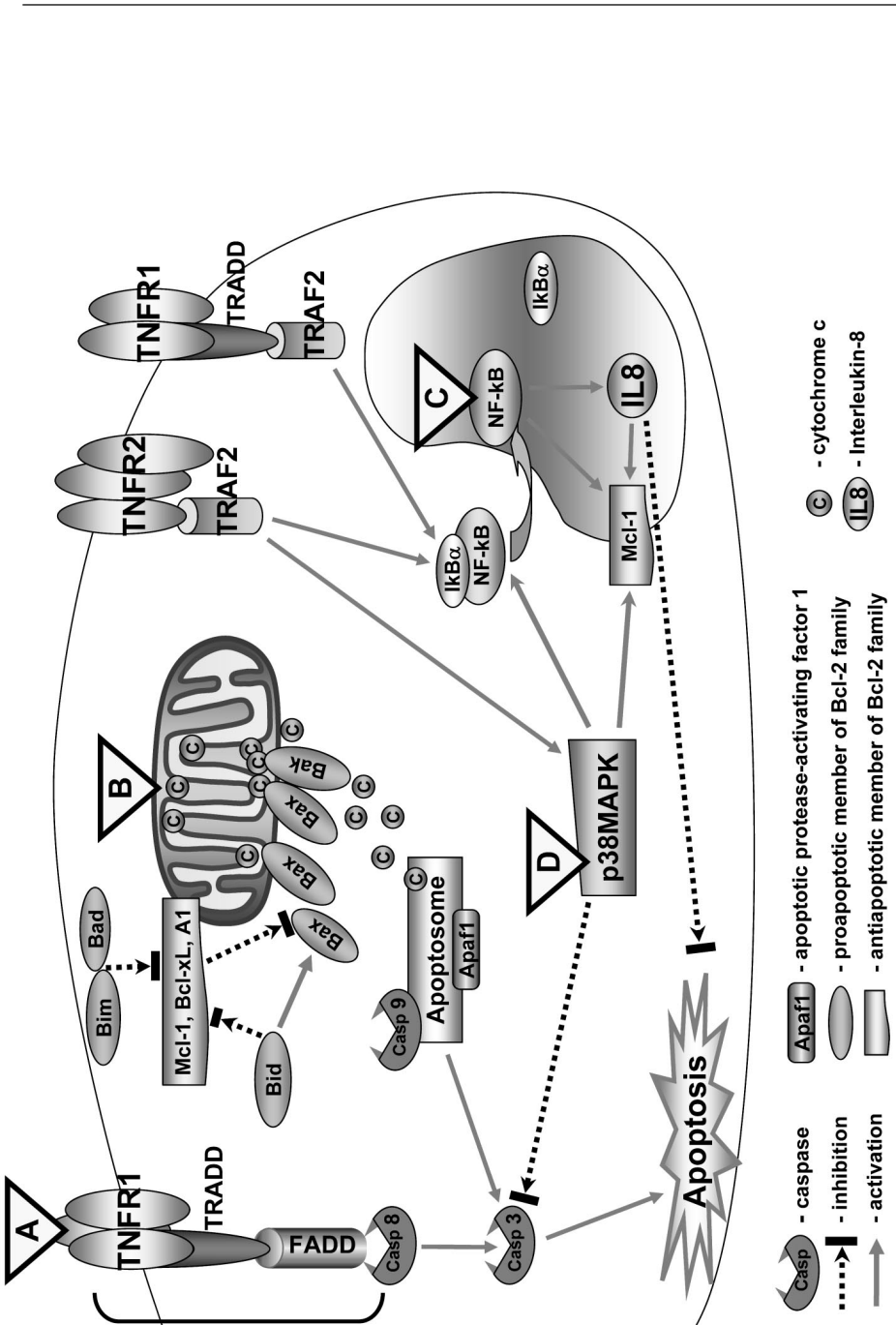
THE DEATH RECEPTOR PATHWAY

In the extrinsic pathway, ligation of a death receptor such as tumor necrotic factor receptor 1 (TNFR1) or CD95 induces the formation of a death-inducing signaling complex (DISC). DISC consists of the death receptor, TNF receptor associated death domain-containing proteins (TRADD), Fas-associated death domain (FADD) adaptor protein, and an initial caspases (A in Fig.1). Clustering of death receptors following ligation promotes aggregation of pro-caspase-8 molecules within the DISC, inducing their autoproteolysis and generation of active caspase-8, which activates the downstream caspase-3, that are the terminal effectors of apoptosis [5, 6, 9]. Importantly, in human neutrophils DISC may form spontaneously [5]. TNFR1 signaling is also known to promote neutrophil survival through the nuclear transcription factor (NF-κB) activation, which can be induced by recruitment of TNF receptor associated factor 2 (TRAF2). In both signals the TRADD may act as a platform adaptor that recruits TRAF2 or FADD and thus activate distinct signaling cascades including activation of NF-κB-induced survival pathway or caspase-dependent proapoptotic route. In contrast to TNFR1, TNFR2 does not contain a TRADD motif [4, 11] and recruits TRAF2 in NF-κB activation directly. Additionally, TNFR2 may promote survival by mitogenactivated protein kinases (MAPK) activation.

THE MITOCHONDRIAL PATHWAY

Mitochondria are the site of oxidative phosphorylation in the cells and classically defined as organelles highly specialized in ATP generation [12]. It is now generally assumed that alteration of mitochondrial function is an early feature of NA [4–7]. In viable cells, these organelles are organized as a diffuse tubular network that clusters during apoptosis. Critically, the mitochondrial route of apoptosis connects caspases and Bcl-2 proteins pathways (B in Fig.1).

As is summarized by [4, 6, 9], Bcl-2 is the prototype for a family of mammalian genes and the proteins they produce. They govern mitochondrial outer membrane permeabilization and can be either pro-apoptotic (Bax, Bak, Bim, Bid, Bad) or anti-apoptotic (Mcl-1, Bcl-X_L, A1/Bfl-1). In most cell types, the ex-



(A) The extrinsic pathway of NA is initiated upon ligation of death receptor TNFR1, which induces formation of a death-inducing signaling complex (DISC) consisting of the death receptor, TNF receptor associated death domain-containing proteins (TRADD), Fas-associated death domain (FADD), and an initiator caspase-8, which activates the terminal effector of apoptosis caspase-3. (B) The intrinsic pathway involves mitochondria. Under normal condition neutrophils express high levels of the pro-apoptotic molecules of Bcl-2 family (Bax, Bak, Bim, Bid and Bad) and low levels of anti-apoptotic members (A1, Bcl-xL, Mcl-1). During apoptosis cytosolic Bax and Bak are translocated into the outer membrane of mitochondria and induced cytochrome c release. In the cytoplasm, cytochrome c complexes with apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9 to form a protein complex the 'apoptosome', which is involved in caspase-3 activation. (C) Neutrophil survival pathway induced by NF-κB activation. In the cytoplasm NF-κB is held by inhibitory proteins IkBα. The release of IkBα from the NF-κB complex allows active NF-κB translocate into the nucleus. TNFR1 and TNFR2 are involved in NF-κB activation by recruitment of TNF receptor associated factor 2 (TRAF2). NF-κB regulates the synthesis of IL-8 and activates the anti-apoptotic Mcl-1 proteins. (D) Involvement of the p38MAPK in NA. p38MAPK may induce NF-κB activation, activate the anti-apoptotic Mcl-1 proteins and can directly phosphorylate and inhibit caspase-3 activity, thereby hinder NA.

pression and activity of protective Bcl-2 members is higher than pro-apoptotic members. In contrast, mature neutrophils constitutively express pro-apoptotic proteins, whereas the expression of anti-apoptotic Bcl-2 members is very low or undetectable in resting cells [3, 13]. However, anti-apoptotic proteins are highly and transiently expressed when neutrophils are exposed to survival factors, such as heme, IL-8, GM-CSF or hypoxia. The balance between pro- and antiapoptotic members determines the fate of the cells [14]. Under physiological conditions, the mitochondrial membrane is polarized and has a membrane potential, the maintenance of which keeps proteins such as cytochrome c and ROS within the confines of the mitochondria. Proapoptotic Bcl-2 proteins exert their effects by activation of an inner mitochondrial permeability transition pore and by induction of apoptogenic factor cytochrome c release. In the cytosol cytochrome c is involved in the assembly of a multimolecular complex known as "apoptosome", which consists of cytochrome c, apoptotic protease-activating factor 1 (Apart 1), and caspase-9 (Fig.1, B). In the presence of ATP this complex induces the proteolitic cleavage and activation of procaspase-3 that triggers a downstream cascade of caspase-3 activity.

Bax is the best known pro-apoptotic soluble protein. In freshly isolated neutrophils Bax is found in the cytoplasm in a phosphorylated closed state, heterodimerized to Mcl-1. Under apoptosis Mcl-1 levels are markedly decreased by proteasome-mediated degradation. Waning levels of Mcl-1 release Bax from the heterocomplex Bax:Mcl-1 and allow Bax to translocate to the mitochondria where it is thought to form oligomers and exercise its pro-apoptotic function [15, 16]. Whereas the activated Bax and Bak would act as ion channels and adaptor proteins and mediate the release of cytochrome c, the anti-apoptotic Bcl-2 would block NA through inhibition of Bax and/or Bak, by promoting the stability of mitochondrial outer membrane and/or impairing insertion of pro-apoptotic proteins. Additionally, the second groups of pro-apoptotic proteins Bad and Bid can modulate negatively the anti-apoptotic Bcl-2 proteins and positively the pro-apoptotic ones.

Mcl-1 is represented a key anti-apoptotic protein. It is only member of anti-apoptotic Bcl-2 family that has been reliably and reproducibly measured at both the mRNA and protein level in human neutrophils [17] It's well documented that spontaneous apoptosis is accompanied by degradation of Mcl-1, but not other anti-apoptotic molecules [18]. Anti-apoptotic Mcl-1 transcripts are extremely unstable (near 3 hours half-life) [19]. Moreover, Mcl-1 is a subject to rapid turnover [20]. Such rapid changes in Mcl-1 function permit neutrophils to switch cell fate very rapidly from survival to death in response to external signals. Importantly, Mcl-1 is up-regulated in response to survival stimuli, thereby having a marked effect on NA [21].

INVOLVEMENT OF NF-κB AND P38MAPK IN NA

Both the transcription factor NF-κB [3, 12, 22, 23] and p38MAPK [12, 24, 25] regulate the NA program (C and D in Fig.1).

NF-κB comprises a family of transcription factors that act as regulators of genes involved in NA and its regulation is highly cell specific and redox sensitive. NF-κB is normally found in the cytoplasm held by inhibitory proteins called IkBα and is activated by various stimuli, which converge at the IKK (IkB kinase) complex. IKK phosphorylates IkBα leading to its ubiquitination, followed by proteosomal degradation. The release of IkBα from the NF-κB complex allows active NF-κB translocation into the nucleus and bind to consensus sites in the DNA of responsive genes.NF-κB activity in neutrophils is regulated by mechanisms clearly different from those in other cells. The most important

difference is that the newly synthesized IkBα can enter the nucleus, remove NF-κB from gene promoters and transport it back to the cytoplasm. Thus, nuclear accumulation of IkBα is associated with inhibition of NF-κB activity and the induction of NA [26, 27]. Using different NF-κB inhibitors it was shown that inhibition of NF-κB is a powerful inducer of NA [22], in contrast activators of NF-κB provides a strong survival signal [23]. NF-κB controls the expression of survival genes such as the Bcl-2 family members and regulates the synthesis of IL-8 [28], known as one of the most important survival proteins [29]. Anti-IL-8: IL-8 complex suppresses spontaneous NA. The survival effect is correlated with a decline in caspase-3 and caspase-9 activity, increase in anti-apoptotic protein (Bcl-XL) and decreased pro-apoptotic proteins (Bax, Bak) expressions [30].

The p38MAPK activation is part of a general stress response that mediates survival in neutrophils [24, 31]. Given the observation that p38MAPK is implicated in the activation of NF-κB [32] it is conceivable that this might lead to expression of survival genes of the Bcl-2 family and IL-8. Moreover, p38MAPK can directly phosphorylate and inhibit the activities of caspases-8 and caspase-3 and thereby hinder neutrophil apoptosis [24].

ROS AS INTRACELLULAR MEDIATORS OF NEUTROPHIL APOPTOSIS

During the last decade, ROS molecules (superoxide anion - O_2^-, hydrogen peroxide - H_2O_2, and the hydroxyl radicals - OH^-) moved from a category of merely unwanted side products of oxidative metabolism to important messenger molecules. Among all cell types neutrophil possess the most powerful system of ROS [33]. ROS are generated in cells as a consequence of normal mitochondrial oxidative metabolism and also as part of the respiratory burst, that participate in microbial killing [34]. The mitochondria serve as the primary source in the quiescent state whereas in activated neutrophils the primary ROS generated by the nicotinamide adenine dinucleotide phosphate oxidase (NADPHox) system [6, 7, 35, 36]. The latter is a multi-enzymatic complex responsible for the generation of high amounts of $O_2^{\cdot}$ – through the reduction of molecular oxygen. In resting neutrophils, about 95% of the inactive NADPHox is found in the membranes of subcellular granules and vesicles, and the rest resides in the plasma membrane or distributed among cytosol. The cell activation results in phosphorylation of NADPHox cytosolic subunit and translocation of the granule pool to plasma or phagosomal membrane. The activation of the granule pool of NADPHox induces intracellular ROS production, while the stimulation of the membranebound oxidase mainly generates extracellular release of ROS. Importantly, intracellular generation, but not extracellular release of ROS, leads to NA [29].

Among the ROS activated molecular targets are the caspases, the phosphoinositol PI3K/Akt pathway molecules and NF-κB [13]. Moreover, ROS can mediate death receptor clustering [37] and rapidly (during minutes) activate p38MAPK systems [34]. As was discussed by [38], ROS may be involved in NA by various ways as direct oxidation of DNA or/and modification of proteins and enzymes. Additionally, lipid peroxidation by ROS may contribute to membrane rupture, eliciting release of the contents of intracellular compartments [39]. Finally, H_2O_2 could be an intermediate in the intracellular signaling mechanism of NA, and its oxidized products, such as OH- (the most toxic of the oxygen intermediates resulting in DNA damage), may be crucial for NA [38].

The functional role of ROS in NA is controversial and the precise signal transduction pathways are not fully understood. However, most reports affirm that ROS directly cause NA [35]. Increased production of H_2O_2 was noted in neutrophils cultured for 4 hours

in the absence of any external stimulus [40]. The neutrophil incubation with H_2O_2 resulted in concentration-dependent increase in the rate of NA [41]. Both ionizing and ultraviolet radiation are capable of inducing NA, and both generate ROS. Catalase, which decreases the intracellular H_2O_2 levels in cultured neutrophils, inhibits NA [38, 42] and increases IL-8 expression [36]. Similarly, prolonged survival of neutrophils was detected in patients with chronic granulomatous disease with hereditary defect in ROS production [42], which was associated with enhanced IL-8 levels [43]. In contrast, it was shown that ROS is also associated with activation of survival signaling routes, in which NF-κB activation could be involved [12]. Critically, the type of ROS molecules involved could be important for NA. For instance, increased intracellular levels of superoxide in neutrophils lead to activation of NF-κB, whereas exposure of neutrophils to hydrogen peroxide inhibits nuclear translocation of NF-κB [44]. All these data, however, do not imply that ROS are absolutely required as mediators of NA, especially under physiologic conditions [45] and the apparent contradictory ROS effects on NA could be as a result of the activation status of cells [13]. Thus, several groups have demonstrated that ROS generation does not affect the rate of spontaneous [45, 46] and Fas/APO-1 triggered NA [37] or underlie the pro-apoptotic effect of TNF-α, but promote apoptosis in PMA-activated neutrophils [45]. H_2O_2 does not affect nuclear translocation of NF-κB in resting cells, but decrease it in LPS or TNF stimulated neutrophils [28, 40]. Moreover, the types of activating stimuli (different cytokines, infection and phagocytosis, PMA or LPS activation and hypoxia) may be crucial for ROS effects on NA. For example, NADPHox-derived intracellular ROS that is generated during phagocytosis induces NA via caspase activation, whereas treatment of the same neutrophils with fMLP results in oxidative burst that is almost entirely extracellular, and apoptosis in these cells is slightly reduced [39].

Finally, NA could be partially related to the different levels and types of cellular antioxidant defenses. Thus, the toxic potential of ROS can be limited by intracellular powerful antioxidant, such as glutathione [5, 37]. Apparently, changes in redox status are the earliest event in NA. The intracellular antioxidant defenses of neutrophils may rapidly degrade H_2O_2, thus preventing the formation of by-products such as $HO^{\cdot}$.

HYPOXIA-INDUCED NEUTROPHIL SURVIVAL

Hypoxia, i.e. decreased availability of oxygen occurs under a variety of physiologic and pathologic conditions. Hypoxia activates a number of genes which are important in the cellular adaptation to low oxygen environment. Generally hypoxia induces apoptosis in different cell types. However, in contrast to other cells in neutrophils hypoxia causes a profound concentrationdependent and reversible inhibition of apoptosis in vitro [41, 47]. Also in vivo work [48], demonstrated prolonged neutrophil survival in healthy subjects exposed to acute hypoxemia.

The hypoxic survival effect was associated with marked stabilization of hypoxia-inducible factor (HIF-1) [22, 47], a master regulator of oxygen homeostasis that controls more than 70 target genes including erythropoietin, VEGF, and proteins associated with glucose and energy metabolism [49]. The ability of hypoxia to increase NF-κB p65 transcript abundance and activity, the ablation of hypoxic survival by the NF-κB inhibitors (gliotoxin and parthenolide), and the inhibition of hypoxic induction of NF-κB in HIF-1α knockout murine neutrophils suggests HIF-1α-dependent regulation of the NF-κB pathway in NA [22]. Additionally, it was documented that hypoxia activates p38MAPK, leading to Mcl-1 activation and a subsequent

delay in NA [21]. Similar to many antiapoptotic stimuli, long exposure to hypoxia decreases ROS generation in neutrophils [41]. Interestingly, short hypoxemia in vivo appears to effects the primed state of the neutrophils for ROS production without significant effect on the stimulated/activated state [48].

EFFECTS OF INTERMITTENT HYPOXIA ON NA

While some diseases involve episodes of sustained hypoxia (SH), diseases like vascularized tumors or Obstructive Sleep Apnea Syndrome (OSAS) are associated with intermittent hypoxic (IH) events. OSAS, in particularly, is characterized by intermittent and recurrent pauses in respiration during sleep. The various signaling pathways, caspase-mediated with IH and caspase-independent with SH, were described for PC-12 cells [50]. Moreover, IH leads to HIF-1α accumulation that persists significantly during re-oxygenation. In contrast HIF-1α levels in PC-12 cells exposed to SH were markedly reduced immediately after re-oxygenation [49]. It was also showed that in endothelial cells, IH induced a modification in HIF-1α phosphorylation pattern with progressive increase in HIF-1α phosphorilated form during hypoxic period, which could lead to cell survival and adaptation to hypoxia [51]. In contrast, Ryan et al. using HeLa cells found that HIF-1α is more sensitive to activation by SH than IH and that NF-κB is more sensitive to activation by IH than SH [52]. Using endothelial cell models they also found that IH activates NF-κB at least in part via p38 MAPK activation [53]. However what kind of response is true for neutrophils is unknown.

We compared the effects of IH and SH on NA using a unique computer-controlled incubation chamber which is attached to an external O_2-CO_2 computer-driven controller (BioSpherix OxyCycler C42 system, Redfield, NY). Chamber O_2, N_2, and CO_2 levels were continuously monitored and adjusted according to the desired programmed profile. Additionally a fiber-optic dissolved oxygen electrode was immersed below medium level to accomplish identical specific experimental profiles and to monitor dissolved oxygen concentrations. Using several IH cycles (3-6 and 10 cycles) and oxygen profiles ranging from 5 to 0.1% O_2 we established that the effects of IH were doze- and time-dependent. Importantly, NA was already significantly decreased after three cycles of IH at 5% oxygen concentrations as compared with normoxia, indicative of a relatively fast neutrophil activation over a period of 3 hours. Moreover, under all IH conditions NA was significantly lower compared to SH both in whole blood and in purified neutrophil cultures [54]. This trend was seen in each subject individually, but values were slightly higher in purified neutrophils compared with whole blood, due to neutrophil purification. Also in patients with OSAS NA was significantly attenuated [54]. This was verified by flow cytometry, morphological features of apoptosis as nuclear and chromatin condensation and a significant reduction in caspase-3 activity. Critically, the percentage of apoptotic neutrophils was negatively correlated with the severity of hypoxia [54]. Whether SH and IH trigger a common signaling pathway in NA is currently unclear. We found that similar to SH the anti-apoptotic effects of IH are mediated via p38MAPK signaling pathway, since the survival effects of hypoxia are lost with inhibition of p38MAPK (unpublished observations). We also determined that NF-κB activity is required for IH survival (article in preparation). Thus, treatment of neutrophils with structurally and mechanistically discrete NF-κB inhibitors gliotoxin, parthenolide, and IMD-0354 under IH resulted in significant increase of NA. Such increased NA was caspase-3 dependent and was accompanied with decreased IL-8 expression. NF-κB activity was found increase in nuclear fractions of neutrophils

treated with IH in vitro. Similar, IH activates NF-κB in neutrophils of OSAS patients [55].

Does ROS also mediate IH-induced decrease in NA? Similar to SH [41], we found that cytoplasmatic ROS generation was decreased by 90-92% in neutrophils exposed to IH as compared to neutrophils maintained in normoxia. Interestingly, the same levels of basal ROS production were detected in resting neutrophils of both OSAS patients and control subjects [56]. In contrast, after PMA stimulation significant increases in ROS generation were detected in OSAS patients compared to control [56]. This suggests that IH may induce neutrophil priming for ROS production after challenge, which is critical for the clearance of infections but may be dangerous to surrounding tissues. Onset of apoptosis in neutrophils is much more complex than the simple mechanisms we have presented here and the role of ROS molecules and oxidative stress needs to be further elucidated in NA.

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АПОПТОЗ НЕЙТРОФИЛОВ И ГИПОКСИЯ

В обзоре литературы представлены данные современной литературы о механизмах выживания и гибели нейтрофильных лейкоцитов. Рассматриваются вопросы регуляции активности апоптотической программы для этих клеток и роль в указанных процессах транскрипционных факторов NT-κB и p38 MAPK. Также дискутируются сегодняшние сведения об участии свободных радикалов в реализации апоптоза нейтрофилов. Особое внимание уделяется анализу роли гипоксии и различиям во влиянии на отдельные звенья апоптоза постоянной и периодической гипоксии.
Ключевые слова: нейтрофилы, апоптоз, гипоксия.

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АПОТЕОЗ НЕЙТРОФІЛІВ І ГІПОКСІЯ

В огляді літератури представлені дані сучасної літератури про механізми виживання і загибелі нейтрофільних лейкоцитів. Розглядаються питання регуляції активності апоптотичної програми цих клітин та роль у зазначених процесах транскрипційних факторів NT-кв та p38 MAPK. Також дискутуються сьогоднішні відомості про участь вільних радикалів у реалізації апоптозу нейтрофілів. Особлива увага приділяється аналізу ролі гіпоксії та відмінностям впливу на окремі ланки апоптозу постійної та періодичної гіпоксії.
Ключові слова: нейтрофіли, апоптоз, гіпоксія.

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