[Frontiers in Bioscience 14, 4546-4556, January 1, 2009]

NADPH oxidase activity: In the crossroad of neutrophil life and death

Maria Augusta Arruda, Christina Barja-Fidalgo

Departamento de Farmacologia, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

TABLE OF CONTENTS

1. Abstract
2. Introduction
2.1. Born to kill
2.2. Born to die
3. Bargaining at the skid row: The role of pro-inflammatory, antiapoptotic molecules
4. NADPH oxidase: a double-edge sword in neutrophil survival
5. Perspectives
6. Acknowledgements
7. References

1. ABSTRACT

Neutrophils are terminally differentiated leukocytes, specialized in detecting and annihilating possible pathogens. For this function, neutrophils contain a number of cytotoxic systems, which can both kill the intruder or promote extensive tissue injury. The most stereotyped neutrophil cytotoxic mechanism is the extracellular and intra-phagosomal production of high amounts of superoxide (O_2^-) and other reactive oxygen species (ROS) via the activation of the complex NADPH oxidase (NADPHox). It has been proposed that the short neutrophil lifespan would be a mechanism of counter-regulating the indiscriminate release of its cytotoxic content, as well as aborting the excessive production of ROS. Studies performed in the last decades point out the role of NADPHox activity as one of the major systems involved in the up-regulation of neutrophil apoptosis. However, a growing number of evidence suggests that NADPHox-derived ROS are involved in the activation of signaling pathways that may lead to increased neutrophil survival. In this review, we evaluate the implication of NADPHox activity in the control of neutrophil's life and death, highlighting the signaling pathways modulated by NADPHox-derived ROS.

2. INTRODUCTION

2.1. Born to kill

Usually recognized as a mobile device of destruction, polymorphonuclear neutrophils are the first leukocytes recruited to the inflammatory loci, where they exert their effector activity against pathogens. These granulocytes are derived from pluripotent bone marrow cells that, under the influence of different growth factors such as colony stimulating factors (CSF) can give rise to distinct myeloid cells such as monocytes and eosinophils (1). Mature neutrophils are characterized by the presence of several cytoplasmatic granules where proteases, cationic proteins and receptors among other proteins are stocked (1).

Traditionally, it was thought that, once reaching the site of acute inflammation, neutrophils would indiscriminately release their cytotoxic arsenal that could result in extensive tissue injury. However, the recent advances regarding neutrophil biology have revealed that, depending on the combination and concentration of stimuli, neutrophils can display different patterns of activation, from just a mild activation profile, a process usually called priming, or the release of pre- or neo-synthesized proinflammatory molecules (e.g. proteases, eicosanoids, cytokines),

enhancement of the phagocytic activity and the production of reactive oxygen (ROS) or nitrogen (RNI) species, mainly by NADPHox and nitric oxidase synthase, respectively (1, 2). In some instances, as a last attempt to control infections, neutrophil extracellular traps (NET) can be released (3). These NET comprehend a complex mixture of neutrophil proteases associated to their own chromatin and other neutrophil components (*e.g.* cytoplasmatic proteins, membrane fragments). Their release involves a peculiar cell death program, as will be briefly depicted later (4).

The control of neutrophil responsiveness involves the activation of several classes of receptors, including G-protein-coupled receptors (GPCR), Fcγ receptors, cytokine receptors, and integrins, among others (5). In turn, these receptors lead to the activation of a myriad of intracellular signaling pathways, involving the activation of protein and lipid kinases such as protein kinase C (PKC), mitogen-associated protein kinases (MAPK), Akt, and phosphatidylinositol-3 kinase (PI3K), as well as phosphatases as SH2-containing inositol-5'-phosphatase (SHIP) and protein phosphatase 2A (PP2A), transcription factors like nuclear factor kappa B (NF-κB), activating protein 1 (AP-1) and Elk-1, and adaptor proteins related to the small GTPases Rho and Rac. These signaling components act synergistically in order to promote the adequate onset of an efficient inflammatory response and the removal of possible pathogens, preserving tissue homeostasis (6).

As long as most of the inflammatory reactions occur in the extravascular environment, the migration of neutrophils from the vascular lumen towards the inflammatory site is also essential to the success of the inflammatory response. This process, called chemotaxis, requires a gradient of chemotactic factor (s) and is tightly coordinated by a number of adhesion molecules present in both leukocyte and endothelial cell. As succinctly discussed below, the engagement of these molecules with their counter-receptors triggers the formation of complex signaling cascades, which in turn modulate not only neutrophil migration but also other neutrophil's effector functions and survival (1).

2.2. Born to die

When compared to monocytes that are able to live 24 to 48 h in the blood, neutrophils have an extremely short life span, with a half-life of about eight hours in circulation (7, 8). Aged neutrophils undergo spontaneous apoptosis (programmed cell death) in the absence of cytokines or other pro-inflammatory agents prior to their removal by macrophages (9). These processes, as discussed later, have a major impact in the resolution phase of inflammation and are exquisitely regulated, involving a plethora of inter-crossing signaling pathways.

It has been assumed for several years that the inflammatory site is a dead-end road to neutrophils, but their vanishing from the inflamed area remained obscure until the 1980's, when it was observed in *in vitro* experiments that aged neutrophils, bearing intact plasma membrane, were ingested by tissue macrophages (10). Later, this process was monitored *in vivo* and characterized as the phagocytosis of apoptotic neutrophils (9). In fact, it dates from the 19th century with the seminal observations of Metchnikoff, describing the engulfment of microphages (neutrophils) by macrophages in irritated tissues (11). This classical report was followed by a number of descriptions of neutrophil-engulfing macrophages in sites of inflammation, being the Reiter's cells (synovial macrophages engulfing apoptotic neutrophils), which are abundant in the inflamed joints of arthritis patients, an archetypal example of this phenomenon (9).

When cultured *in vitro*, neutrophils undergo apoptotic spontaneously (50% - 80% of apoptotic cells after being incubated for 20 h), displaying the classical morphological and biochemical features such as: (a) cytoplasmatic condensation; (b) phosphatidylserine (PS) exposure in the outer leaflet of the plasma membrane; (c) internucleosomal DNA cleavage; (d) fragmentation and compartmentalization of the intracellular content in apoptotic bodies (12); and the activation of the caspases. The caspases are cysteine proteases that trigger proteolysis cascades that ultimately resulting in the functional and structural dismantling of the cell ((13), and other articles in this issue). The caspases were highly conserved throughout evolution and are classified as initiator (caspase-1, -2, -4, -5, -8, -9, -10 and -14) and effector caspases (caspases-3, -6 , -7) (13).

These severe morphological and biochemical alterations are accompanied by a dramatic change in the repertoire of cell surface molecules which enable their recognition and engulfment by professional phagocytes, such as macrophages, or amateur ones, such as fibroblasts and mesangial cells (14, 15). As already mentioned, PS is exposed in the outer plasma membrane leaflet, leading to the apoptotic cell recognition by a specific receptor, possibly involving thrombospondin (TSP) or CD36 as co-factors (15). PS exposure also causes opsonization of apoptotic cells by the complement factor iC3b which is recognized by phagocytes' integrins (complement receptors CR3 and CR4) (16). Apoptotic cells can also express calreticulin which can be bound to CD91, whereas oxidized low-density lipoprotein (ox-LDL)-like sites can bind to other scavenger receptors, including scavenger receptor A and oxidized low-density lipoprotein-1 (15). Putative molecules expressed in the dying cell that could positively modulate apoptotic cell uptake are annexin-1 (17) and intercellular adhesion molecule-3 (18).

The engagement of the ligands expressed in the apoptotic bodies to the receptors expressed on the phagocytic cells is essentially non-phlogistic, and leads to the synthesis and release of anti-inflammatory mediators, such as tumor growth factor (TGF)-β, and also to the decrease of pro-inflammatory molecules secretion, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8 (19). This sequence of events is essential to the resolution phase of inflammation (19, 20).

In most of the cell types, the apoptotic signaling can be both dependent on or independent of the engagement of death receptor (DR), which are classically defined as extrinsic or intrinsic (mitochondrial) apoptotic pathways, respectively (12). Both processes lead to the activation of the effector caspase-3 and to the release of proteins normally confined to the mitochondrial intermembrane space of the cytosol, as discussed below (13).

In neutrophils, however, spontaneous apoptosis has some unique characteristics compared to other cell types. For example, it seems that there is no autocrine or paracrine activation of DR (death receptors) in mature neutrophils, although the expression of both DR and their agonists has been reported in these cells (21-22). Neutrophil spontaneous apoptosis involves the agonist-independent DR activation, inducing a signaling complex (DISC), which is mediated by conformational changes in DR caused by the redistribution of these receptors in ceramide-rich lipid rafts (23). Moreover, Murphy and co-workers have described that the apoptosome formation in neutrophils occurs in a very particular stoichiometric fashion, requiring low amounts of cytochrome c for its assembly, suggesting that these cells display a very low threshold to the initiation of the apoptotic cascade, attesting once more the early and definitive commitment of these cells to the apoptotic cell death program (24).

The study of the apoptotic program in mature neutrophils has brought to light different aspects of the cell death program, particularly related to the involvement of mitochondria in this process (25, 26). Mitochondria, organelles specialized in generating ATP, have also emerged in the last two decades, as regulators of cell fate (27). These organelles keep locked in their intermembrane space, a number of proteins that, once released in cytosol can both initiate and amplify the apoptotic process. For example, cytochrome c normally transfers electrons from complex III to complex IV in the respiratory chain, but during apoptosis it is released into the cytoplasm where, participating in the apoptosome assembly, activates the caspase cascade. (15). The constitutive roles of other pro-apoptotic proteins, such as Smac/Diablo, AIF, and Omi/HtrA2, in neutrophil program of cell death are under intense investigation (28).

Notably, until few years ago, there was no consensus about the content and function of neutrophil's mitochondria. In fact, it was believed that neutrophils would have very few or no mitochondria at all, and that neutrophils would obtain energy not from oxidative phosphorylation but from the glicolysis (27). Moreover, transmission electronic microscopy was not able to assure the existence of mitochondria in these leukocytes. However, the development of selective fluorescent dies made possible the detection of mitochondria in mature neutrophils, revealing their organization in a tubular network distributed along the cytoplasm (28), which is formed during neutrophil differentiation process (29).

Whilst mitochondria uncoupling agents have no implications in neutrophil apoptosis, it was shown that they can interfere in neutrophil cytoskeleton dynamics and chemotaxis (30). Of note, mitochondria transmembrane potential ($\Delta\Psi m$) dissipation is an initial event in neutrophil spontaneous apoptosis and closely correlates to Bax (a pro-apoptotic Bcl-2 protein) translocation to the mitochondria and subsequent aggregation of these organelles (29).

The maintenance of the outer mitochondria membrane integrity is a key event in cell homeostasis, and is tightly regulated by anti- and pro-apoptotic Bcl-2 family members, a highly conserved group of proteins involved in the control of the apoptotic program (27). The anti-apoptotic Bcl-2 proteins (e.g. Bcl-2, Bcl-XL, Mcl-1, and A1) act stabilizing the mitochondrial outer membrane. They also inhibit the oligomerization of effector pro-apoptotic proteins such as Bax, Bik and Bak and their consequent insertion into mitochondria, what leads to the release of the pro-apoptotic intermembrane space proteins to the cytosol and the progress of apoptotic cascade (21). A second group of pro-apoptotic Bcl-2 proteins, including Bad, acts antagonizing anti-apoptotic proteins functions as well as positively modulating pro-apoptotic proteins activity (29).

In contrast with most cells, mature neutrophils present an atypical pattern of expression of anti- and pro-apoptotic proteins. In these leukocytes, the levels of pro-apoptotic Bcl-2 proteins are high, while the content of anti-apoptotic proteins is extremely low (31), characterizing their constitutive engagement with the apoptotic pathway. However, as we are going to discuss later, this pattern of expression can be dramatically altered when cells are exposed to pro-inflammatory mediators, such as IL-8 and leukotriene B_4 (LTB_4; 31).

Noteworthy, other specialized cell death programs that operate in neutrophils have been also described. The phagocytosis-induced cell death (PICD) is a singular apoptotic program triggered by the engulfment and killing of opsonized microorganism mediated by a complex interplay between ROS, ERK and caspase-8 (32). Another alternative death pathway for activated neutrophils has been recently reported (4). This process, which is not related to necrosis or apoptosis, occurs concomitantly to the release of NETs, as previously mentioned (3, 4). A hallmark of this process is the fact that neutrophils retain the ability of efficiently killing pathogens after their death (3,4). On the other hand, the release of these extremely harmful structures in the extracellular milieu can lead to an extensive tissue injury, thus amplifying the inflammatory response.

3. BARGAINING AT THE SKID ROW: THE ROLE OF PRO-INFLAMMATORY, ANTI-APOPTOTIC MOLECULES

Neutrophil spontaneous apoptosis can be both up- and down-modulated by a number of inflammatory mediators, cytokines and chemokines that interfere on neutrophil accumulation at inflammatory sites. TNF-α, a known pro-inflammatory mediator has been demonstrated to display a dual role in regulating neutrophil survival, both accelerating (through its death receptor – TNFR1) and

repressing (through TNFR2) neutrophil death (33). However, most of the pro-inflammatory molecules are shown to down-regulate neutrophil spontaneous apoptosis. These mediators including free heme (34), lipopolysacharide (LPS; 35), LTB_4 (36), IL-8 (35), IL-1 (37), granulocyte/macrophage colony stimulating factor (GM-CSF) (38), the complement factor C5a (39), among others, are recognized by their ability of evoking or priming some of the neutrophil's effector functions previously mentioned such as chemotaxis, granules release, phagocytosis and NADPHox activation (40).

In addition, the interaction of neutrophils with the extracellular matrix (ECM) components during migration from blood towards the infected tissue can also modulate their survival. Neutrophil recruitment is supported by leukocyte adhesion molecules, chemokines and cytokines expressed in and around blood vessels at the sites of inflammation (41). Evidences have shown that the endothelial transmigration regulates neutrophil apoptosis, and that adhesion molecules can act as modulators of their programmed cell death (41). The classical leukocyte adhesion cascade is triggered by ligation of chemoattractants to chemotactic GPCRs and comprises the elements of tethering, rolling, activation and adhesion mediated by the selectins, integrins and their counter-receptors localized on endothelial cell surface and ECM (42).

Constitutively expressed on neutrophils, L-selectin (CD62L) controls leukocyte capture and rolling by strengthening the stability of the L-selectin tethers (43). L-selectin-ligand interaction leads to leukocyte activation, modulating the activation and affinity of integrins involved in neutrophils firm adhesion and transmigration through the endothelial barrier (42). Neutrophil activation is usually accompanied by L-selectin shedding, that modulates physiologic leukocyte rolling velocity (39-40). Interestingly, apoptotic neutrophils present a low expression of L-selectin and an increased expression of β2 integrins (CD18) (46, 47), although showing reduced adhesion to fibrinogen (46). Beta 2 integrins are specific expressed on leukocytes and are involved in the modulation of most neutrophil functions, such as the recruitment to inflamed sites, phagocytosis and production/release of ROS (48, 49). The role of $\alpha_M\beta2$ (Mac-1) in neutrophil apoptosis is in some points rather controversial (49, 50). Mac-1 is able to interact with a large variety of ligands and cooperates with a variety of other neutrophil surface receptors (50). Engagement of Mac-1 with soluble cross-linking antibodies (51), ICAM-1 (52) fibrinogen (53), C5a (54), or soluble selective ligands of neutrophil $\alpha_M\beta2$ integrin, as the disintegrin jarastatin (55), extend neutrophil life span. Most of these stimuli are described to delay *in vitro*, neutrophil apoptosis through the activation of integrin-associated intracellular signaling involving PI3K/Akt, ERK MAPK and NF-κB pathways (31). However, evidence has also shown that Mac-1-dependent phagocytosis is a key step for the process of phagocytosis-induced cell death (PICD) (32). Phagocytosis of complement-opsonized targets requires Mac-1, which works as a complement binding receptor (50). Mac-1-mediated phagocytosis results in the activation of the NADPH-oxidase system and consequently generation of ROS within the developing phagolysosomes (48). The sustained intracellular ROS has been associated with apoptosis, through the activation of caspases 8 and 3 (32). Accordingly, neutrophils from CGD patients, as well as normal neutrophils treated with the NADPH oxidase inhibitor, diphenylene iodonium (DPI), fail to undergo PICD (49). Furthermore, it was also demonstrated that β2-integrins are crucial signaling molecules in ROS-dependent apoptosis of neutrophils induced by microorganisms as diverse as *Mycobacterium tuberculosis*, *Trichomonas vaginalis*, *Escherichia coli*, *Entamoeba histolytica* among others (32, 56-58). Thus, the signaling mechanisms involved in Mac-1 engagement in PICD seem to be associated to the intracellular generation of ROS, derived from the activation of NADPHox (32).

Other families of integrins, especially β1 and β3 are also expressed on neutrophils and their sequential activation is fundamental for neutrophil adhesive-related functions (46). Most of these integrins are constitutively expressed, being up-regulated upon cell activation (59). β1 integrins present on neutrophils are primarily responsible for their transmigration through endothelial monolayer (60) and their interaction with ECM proteins (61). The engagement of those adhesion molecules with their ligands can trigger the integrin-coupled intracellular signaling, inducing formation of focal adhesion complexes and phosphorylation and intracellular targeting of focal adhesion kinase (FAK) to the focal adhesion sites (62). Acting as a scaffold for other signaling molecules, FAK activation modulates different signaling pathways in neutrophils involved not only with cell mobility (63), but also in NADPHox-dependent ROS production (64), and modulation of cell survival (32, 46). Recently, it was shown that $\alpha9\beta1$-mediated interaction with VCAM-1 delays neutrophils apoptosis (65). This anti-apoptotic effect of $\alpha9\beta1$was further confirmed and the signaling mechanisms involved were identified by our group using a specific ligand of this integrin, the disintegrin VLO5, which is also a potent neutrophil activator that triggers integrin-associated intracellular pathways (R. Saldanha-Gama, J.A. Moraes, C. Marcienkewikz and C. Barja-Fidalgo, unpublished data).

Theoretically, the activation of signaling pathways as diverse as MAPK, PI3K/Akt and NF-κB triggers a pro-survival response in blood neutrophils activated by soluble mediators under regular culture conditions (31). The activation of these signaling pathways leads to a shift in the expression of pro- and anti-apoptotic proteins, mediating a sharp increase in anti-apoptotic Bcl-2 family proteins' expression and the degradation or sequestration of pro-apoptotic Bcl-2 members (31). However, if these activated neutrophils are committed to phagocytose opsonized microorganisms, that pattern in protein expression changes again, and cells undergo the apoptotic program PICD (32) (Figure 1).

It is quite reasonable to postulate that neutrophil accumulation at infected inflammatory loci leads to a self-

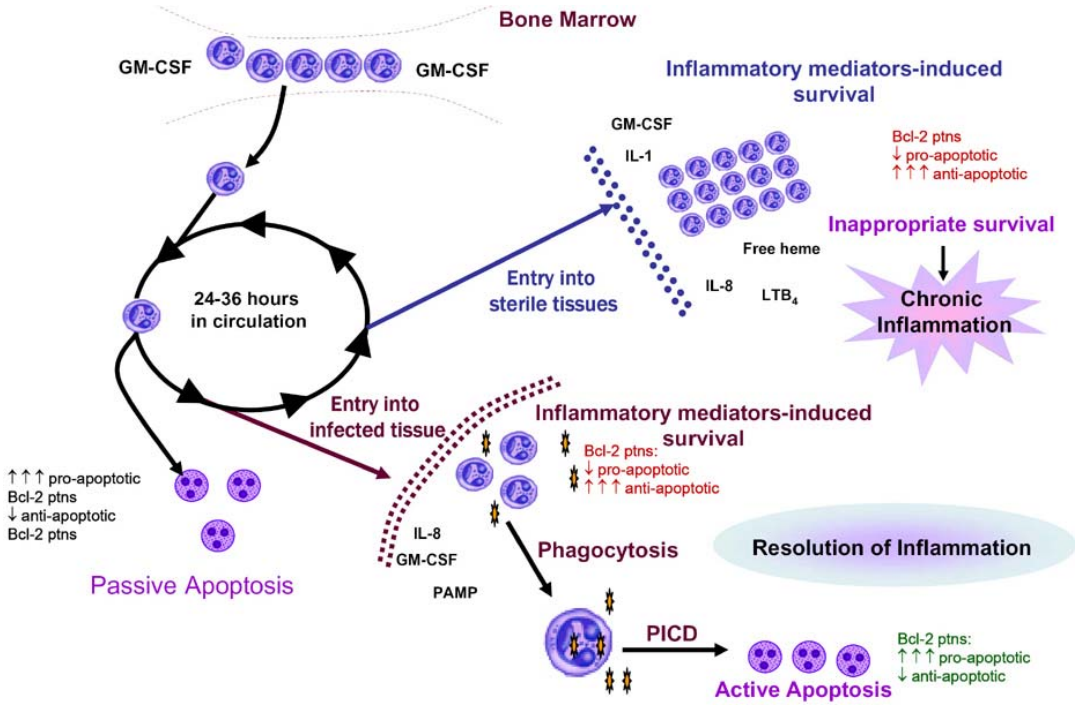

Figure 1. Neutrophil apoptosis: a key mechanism to modulate acute inflammatory response. Mature neutrophils leave the bone marrow gaining circulation where they remain for about 24-36 hours before undergoing spontaneous apoptosis followed by non-phlogistic phagocytosis in specialized site. Recruitment of neutrophils to either non-infected (sterile) or infected sites includes their engagement to adhesion molecules and the contact with pro-inflammatory molecule, leading to an immediate delay in neutrophil spontaneous apoptosis. This change in neutrophil phenotype is coordinated with a dramatic increase in the ratio of expression of anti-apoptotic/pro-apoptotic Bcl-2 family proteins. Once in an infected site neutrophils are able to phagocyte the pathogens and rapidly undergo a specialized apoptotic program (PICD), being ingested by tissue phagocytes. This process is essential to a proper resolution of an acute inflammatory response. On the other hand, into a sterile inflammatory response, neutrophils can display impaired apoptotic program, prolonging their life span and undergoing necrosis. The releasing of the cytotoxic content from necrotic cells can cause tissue injury and account for the chronic inflammation.

limiting process once their apoptotic program is accelerated as soon as they ingest and kill the invading pathogen, leading to a successful resolution of inflammation (49). On the other hand, sterile, pathogen-free inflammatory sites (as occurs in rheumatoid arthritis) offer a perfect environment for neutrophil accumulation, once there is a massive accumulation of soluble inflammatory mediators that impair neutrophil apoptotic program concomitant to the lack of counter-regulatory signals that down modulate neutrophil lifespan, as those evoked by pathogen ingestion during PICD. Under these aseptic conditions, aged neutrophils die by necrosis, leading to the amplification of the inflammatory response through the release of their highly cytotoxic content, causing tissue injury and consequent recruitment of neutrophils from circulation. An exponentially increase in neutrophil number at the inflammatory sites is observed in chronic inflammatory conditions, as rheumatoid arthritis (66) (Figure 1).

4. NADPH-OXIDASE: A DOUBLE-EDGE SWORD IN NEUTROPHILS SURVIVAL

Traditionally defined as deleterious cytotoxic molecules, which need to be scavenged at any cost, ROS have emerged to the status of signaling mediators, and it has become a consensus that the balance between ROS production and antioxidant actions plays a key role in cell homeostasis (67). ROS are involved in a myriad of cellular processes through the modulation of different kinases, phosphatases, and other signaling components, what may lead to changes in cell phenotype (68). There are many cellular sources of ROS, such as the xanthine-xanthine oxidase system, lipo- and cyclooxygenase activity and the mitochondrial electron transport chain (68). However, the only system specialized in this function is the NADPHox complex (69).

The NADPHox is a mutimeric complex composed by membrane and cytosolic subunits. It has been suggested that its appearance accompanies the emergence of multicellular structures in order to ensure harmonious development, once its presence is observed in organisms as diverse as metazoans, plants and even in the colonial protozoan *Dictiostelium discoideum* (70, 71). The very first NADPHox characterized was the phagocytic NADPHox (69). This multienzymatic complex comprehends a cytochrome *b*558, comprising gp91^{phox} and p22^{phox}, and two dissociated cytosolic components, p47^{phox} and p67^{phox}. In addition, a low molecular weight G protein Rac participates in the assembly of the active

complex. NADPHox is inactive in resting neutrophils, but the proper
activation of these cells results in phosphorylation of NADPHox p47phox subunit mediated by protein kinase C (PKC) or other kinases such as p38 MAPK and Akt, among others (68). It is followed by the translocation of cytosolic subunits to the plasmalemma and association to the membrane-bound subunits, comprising the catalytic active NADPHox, which can then generate high amounts of O2- into extracellular or phagosomal milieu (69).

Most of the studies performed in order to address the role of ROS in neutrophil lifespan endow the classical view of ROS as deleterious, cytotoxic molecules, particularly those generated by NADPHox. The strongest evidence supporting this popular hypothesis is based on the study of neutrophils from chronic granulomatous disease (CGD) patients, which lack one or more functional NADPHox subunits resulting in complete inability in generating ROS that, in turn, determines a life-threatening condition with a very poor prognosis (72). Neutrophils in these patients display a prolonged lifespan, being less susceptible to both spontaneous and death receptor-induced apoptosis (73). Moreover, NADPHox inhibitors were shown to delay neutrophil spontaneous apoptosis while pro-oxidant molecules enhanced neutrophil apoptosis (73).

PICD is also a NADPHox derived-ROS phenomenon, as observed during intracellular killing by neutrophils of different microrganisms (32, 56-58). However, during this process, neutrophils generate high amounts of ROS into phagolysosome and it is quite reasonable to infer that the leakage of these oxidants to the cytosol would trigger catastrophic consequences, collapsing cell integrity through such an abrupt alteration of cellular redox potential (60).

There has been a growing number of evidence highlighting the underappreciated function of NADPHox derived-ROS in neutrophil homeostasis as a pro-survival second messenger. A number of pro-inflammatory molecules, such as LTB4, formylated peptides and IL-8 induce NADPHox activation in neutrophils (74, 75). Moreover, LPS triggers the NADPHox-dependent ROS generation as well as the activation of the pro-survival transcription factor NF-κB in human neutrophils in a ROS-dependent manner (76). It is also well accepted that many of the required signaling pathways involved in the inhibition of neutrophil apoptosis are redox-sensitive (67), and that the effector caspase-3 can be both activated or inhibited in the presence of ROS (77). However, this confuse scenario demands other strategies in order to elucidate the precise role of ROS in neutrophil biology.

Recently, our group characterized heme (ferriprotoporphyrin IX) as a potent pro-inflammatory molecule, activating neutrophil chemotaxis both *in vitro* and *in vitro*, inducing IL-8 mRNA synthesis, and triggering NADPHox-dependent ROS generation in a pathophysiological concentration range (78). Moreover, free heme was able also to prolong neutrophil longevity by delaying the onset of apoptosis. This phenomenon, which is NADPHox-dependent, demands the activation of critical redox-sensitive signaling pathways, such as PI3K/Akt system, ERK and NF-□B (34). Moreover, we observed that these pro-survival effects evoked by heme ultimately engaged an alteration on the expression profile of pro- and anti-apoptotic Bcl-2 family proteins, reducing the Bad/Bcl-XL ratio in a ROS-dependent fashion, which in turn impaired Bax insertion into the mitochondria, safeguarding mitochondrial □□*m*. (34, 79). These studies corroborate to the statement of free heme as a prototypical proinflammatory molecule, proposing a putative role of free heme in the acute and chronic inflammation that escorts hemolytic episodes (80), and unravel a novel modulatory role for ROS generated endogenously by NADPHox in the control of neutrophil homeostasis. To date, the precise molecular target of free heme action remains obscure. However, a growing bold of evidence has shown that free heme can activate Toll-like receptor 4 and chemotactic GPCRs (81, 82). Noteworthy is the fact that free heme is an amphiphilic molecule, able to intercalate in the plasma membrane, what suggests that this porphyrin can alter cell membrane dynamics, changing the organization and the disposition of membrane micro-domains such as lipid rafts and caveolae. It could, in turn, promote ligand-independent activation of those receptors, triggering the signaling cascades involved in neutrophil effects.

The conflicting effects of ROS on neutrophil biology could be explained regarding the activation status of the cell. The results regarding PICD depict a situation in which neutrophils' mission is accomplished – once in the inflammatory site they got activated, ingested and killed the pathogen. However, when neutrophils are activated by a soluble inflammatory mediator, the NADPHox assembly and activation, which occurs few minutes after exposure to the stimulus, leads to a diffuse ROS generation, which in turn paves the way to other effector functions of activated neutrophils through the activation of the redox-sensitive signaling pathways previously discussed (Figure 2).

5. PERSPECTIVES

The lesson learned by analyzing the studies establishing a role of NADPHox activity and their by-products (ROS) in neutrophil biology is that there is no room for simplistic scrutiny. Although the supposed simplicity underlying neutrophil functions, the control of neutrophil spontaneous apoptosis is an exquisite and sophisticated process regulated by a still open-ended number of signaling routes. This way, *when, how much, why and where* comprise expressions that necessarily

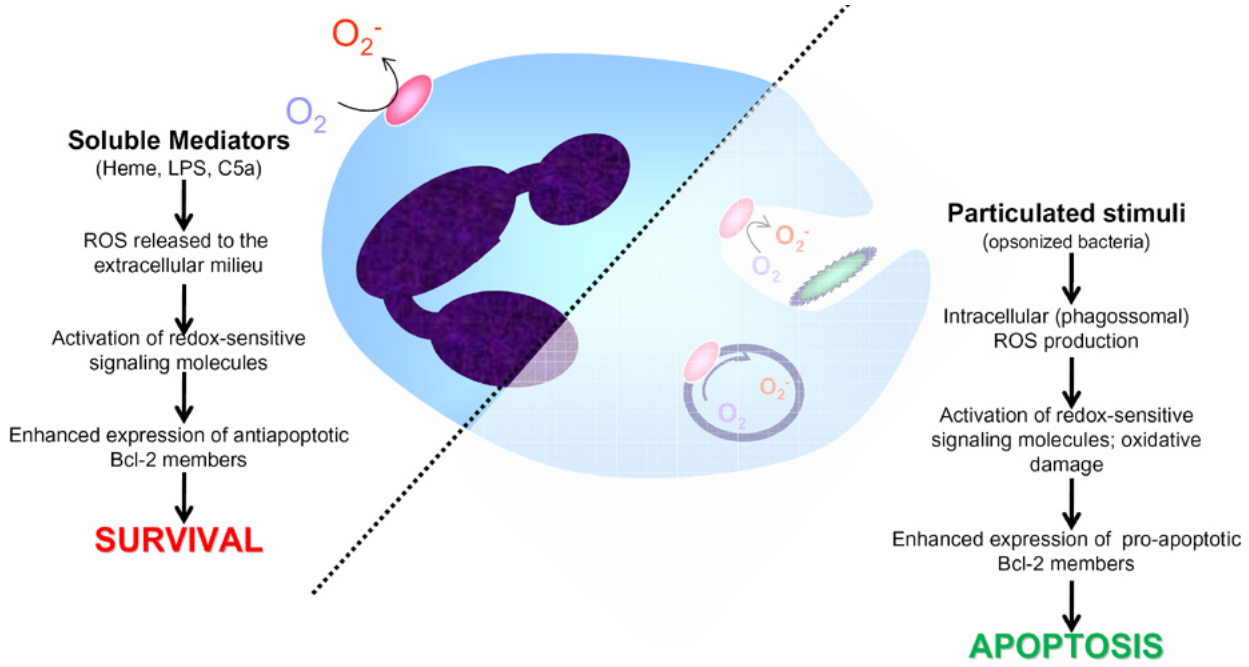

Figure 2. NADPH oxidase-generated ROS: a double-edge sword in neutrophil survival. The activation of neutrophils by soluble agonists leads to a potent, but diffuse production of ROS, which may leak to the intracellular milieu, activating redox-sensitive, anti-apoptotic signaling molecules with no major oxidative damage, and extends neutrophil survival. On the other hand, the activation of neutrophils by opsonized particles induces the concentrated organization of NADPHox, lining the membrane of the phagosome containing the engulfed particles. The concentrated intracellular generation of ROS shortens neutrophils' lifespan.

underlie the studies that seriously aim to elucidate this intricate puzzle. It is undoubtedly a challenging field of knowledge, and a better understanding of how these redox-sensitive signaling pathways are activated by ROS as well as defining the biological events coupled to such activation may lead to the development of new strategies for the treatment of chronic inflammatory disease.

6. ACKNOWLEDGEMENTS

We would like to express our gratitude to the researchers that have been devoting their career to bring a better understanding of neutrophil physiology. We are grateful to Dr. Andrea I. Doseff for the critical reading of the manuscript and valuable contributions. We also thank Dr. Iolanda M. Fierro and Heitor de Paula-Neto for helpful discussions. The authors are supported by grants from CNPq, CAPES, FAPERJ and SR-2/UERJ (Brazil).

7. REFERENCES

1. M. A. Cassatela: Cytokines produced by polymorphonuclear neutrophils: Molecular and Biological Aspects. Chapman & Hall (1996)

2. I. M. Fierro, V. Nascimento-DaSilva, M. A. Arruda, M. S. Freitas, M. C. Plotkowski, F. Q. Cunha and C. Barja-Fidalgo: Induction of NOS in rat blood PMN *in vivo* and *in vitro*: modulation by tyrosine kinase and involvement in bactericidal activity. *J Leukoc Biol* 65, 508-514 (1999)

3. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch and A. Zychlinsky: Neutrophil extracellular traps kill bacteria. *Science* 303, 1532-1535 (2004)

4. T. A. Fuchs, U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann and A. Zychlinsky: Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 176, 231-241 (2007)

5. V. Witko-Sarsat, P. Rieu, B. Descamps-Latscha, P. Lesavre and L. Halbwachs-Mecarelli : Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest*. 80, 617-653 (2000)

6. T. Kato and S. Kitagawa: Regulation of neutrophil functions by proinflammatory cytokines. *Int J Hematol* 84, 205-209 (2006)

7. F. Sigurdsson, A. Khanna-Gupta, N. Lawson and N. Berliner: Control of late neutrophil-specific gene expression: insights into regulation of myeloid differentiation. *Semin Hematol* 34, 303-310 (1997)

8. R.J. Fahy, A.I. Doseff and M.D.Wewers: Spontaneous human monocyte apoptosis utilizes a caspase-3-dependent pathway that is blocked by endotoxin and is independent of caspase-1. *J Immunol.* 163, 1755-1762 (1999)

9. J. S. Savill, A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson and C. Haslett: Macrophage phagocytosis of

aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 83, 865-875 (1989)

10. S. L. Newman, J. E. Henson and P. M. Henson: Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages. *J Exp Med* 156, 430-442 (1982)

11. E. Metchnikoff: Lecture VII In: Lectures in the Comparative Pathology of Inflammation Delivered at the Pasteur Institute in 1891. F. A. Starling and E. H. Starling, translators. Dover, New York (1968)

12. R. A. Lockshin and Z. Zakeri: Apoptosis, autophagy, and more. *Int J Biochem Cell Biol* 36, 2405-2419 (2004)

13. C. Adrain, G. Brumatti and S. J. Martin: Apoptosomes: protease activation platforms to die from. *Trends Biochem Sci* 31, 243-247 (2006).

14. C. Ward, I. Dransfield, E. R. Chilvers, C. Haslett and A. G. Rossi: Pharmacological manipulation of granulocyte apoptosis: potential therapeutic targets. *Trends Pharmacol Sci* 20, 503-509 (1999)

15. R. C. Taylor, S. P. Cullen and S. J. Martin: Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* 9, 231 – 241 (2008)

16. F. Takizawa, S. Tsuji and S. Nagawa: Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. *FEBS Lett.* 397, 269–272 (1996)

17. S. Arur, U. E. Uche, K. Rezaul, M. Fong, V. Scranton, A. E. Cowan, W. Mohler and D. K. Han: Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev. Cell* 4, 587–598 (2003)

18. O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory: Macrophage recognition of ICAM-3 on apoptotic leukocytes. *J. Immunol.* 162, 6800–6810 (1999)

19. D. W. Gilroy, T. Lawrence, M. Perretti, A. G. Rossi: Inflammatory resolution: new opportunities for drug discovery. *Nat Rev Drug Discov* 3, 401-416. (2004)

20. C. N. Serhan and J. Savill: Resolution of inflammation: the beginning programs the end. *Nat Immunol* 6, 1191-1197 (2005)

21. D. R. Green: Apoptotic pathways: ten minutes to dead. *Cell* 121, 671-674 (2005)

22. C. Akgul and S. W. Edwards: Regulation of neutrophil apoptosis via death receptors. *Cell Mol Life Sci* 60, 2402-2408 (2003)

23. D. Scheel-Toellner, K. Wang, R. Craddock, P. R. Webb, H. M. McGettrick, L. K. Assi, N. Parkes, L. E. Clough, E. Gulbins, M. Salmon and J. M. Lord: Reactive oxygen species limit neutrophil life span by activating death receptor signaling. *Blood* 104:2557-2564 (2004)

24. B.M. Murphy, A. J. O'Neill, C. Adrain, R. W. Watson and S. J. Martin: The apoptosome pathway to caspase activation in primary human neutrophils exhibits dramatically reduced requirements for cytochrome c. *J Exp Med* 197, 625-632 (2003)

25. J. E. Chipuk, L. Bouchier-Hayes and D. R. Green: Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ* 13, 1396-1402 (2006)

26. D. D. Newmeyer and S. Fregusson-Miller: Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* 112, 481-490 (2003)

27. N. Borregaard and T. Herlin: Energy metabolism of human neutrophils during phagocytosis. *J Clin Invest* 70, 550-557 (1982)

28. N. A. Maianski, D. A. Roos and T. W. Kuijpers: Tumor necrosis factor alpha induces a caspase-independent death pathway in human neutrophils. *Blood* 101, 1987-1995 (2003)

29. N. A. Maianski, J. Geissler, S. M. Srinivasula, E. S. Alnemri, D. Roos and T. W. Kuijpers: Functional charachterization of neutrophil mitochondria in neutrophils: a role restricted to apoptosis. *Cell Death Diff* 11, 143-153 (2004)

30. G. Fossati, D. A. Moulding, D. G. Spiller, R. J. Moots, M. R. White and S. W. Edwards: The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. *J Immunol* 170, 1964-1972 (2003)

31. C. Akgul, D. A. Moulding and S. W. Edwards: Molecular control of neutrophil apoptosis. *FEBS Lett* 487, 318-322 (2001)

32. B. Zhang, J. Hirahashi, X. Cullere and T.N. Mayadas: Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation. *J Biol Chem* 278, 28443-28454 (2003)

33. A. S. Cowburn, J. Deighton, S. R. Walmsley and E. R. Chilvers: The survival effect of TNF-alpha in human neutrophils is mediated via NF-kappa B-dependent IL-8 release. *Eur J Immunol* 34, 1733-1743 (2004)

34. M. A. Arruda, A. G. Rossi, M. S. de Freitas, C. Barja-Fidalgo and A. V. Graça-Souza: Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-κB. *J. Immunol*. 173, 2023-2030 (2004)

35. F, Colotta, F, Re N, Polentarutti S. Sozzani and A. Mantovani: Modulation of granulocyte survival and

programmed cell death by cytokines and bacterial products. *Blood* 80,2012-2020 (1992)

36. M.J. Hebert, T. Takano, H. Holthofer and H.R. Brady: Sequential morphologic events during apoptosis of human neutrophils. Modulation by lipoxygenase-derived eicosanoids. *J Immunol* 157, 3105-3115 (1996)

37. R. W. Watson, O. D. Rotstein, J. Parodo, R. Bitar, J. C. Marshall: The IL-1 beta-converting enzyme (caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1β. *J Immunol* 161, 957-962 (1998)

38. J.B. Klein, M.J. Rane, J.A. Scherzer, P.Y. Coxon, R. Kettritz, J.M. Mathiesen, A. Buridi and K.R. McLeish: Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J Immunol* 164, 4286-4291 (2000)

39. A. Lee, M.K. Whyte and C. Haslett: Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukoc Biol* 54, 283-288 (1993)

40. F.R. Sheppard, M.R. Kelher, E.E. Moore, N.J. McLaughlin, A. Banerjee and C.C. Silliman: Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *J Leukoc Biol* 78, 1025-42 (2005)

41. K. Ley: Integration of inflammatory signals by rolling neutrophils. *Immunol Rev* 186, 8-18 (2002)

42. C. Barja-Fidalgo, M. A. Arruda, R. Saldanha-da-Gama and M. S. Freitas: Signaling pathways involved in leukocyte adhesiveness and migration during inflammation: Potential targets for therapeutic interventions? *Curr. Med. Chem* 5, 59-69 (2006)

43. O. Dwir, G. S Kansas and R. Alon: Cytoplasmic anchorage of L-selectin controls leukocyte capture and rolling by increasing the mechanical stability of the selectin tether. *J Cell Biol* 155, 145-156 (2001)

44. J.J. Peschon, J.L. Slack, P. Reddy, K.L. Stocking, S.W. Sunnarborg, D.C. Lee, W.E. Russell, B.J. Castner, R.S. Johnson, J.N. Fitzner, R.W. Boyce, N. Nelson, C.J. Kozlosky, M.F. Wolfson, C.T. Rauch, D.P. Cerretti, R.J. Paxton, C.J. March and R.A. Black: An essential role for ectodomain shedding in mammalian development. *Science* 282, 1281-1284 (1998)

45. P. Dello Sbarba and E. Rovida: Transmodulation of cell surface regulatory molecules via ectodomain shedding. *J Biol Chem* 383, 69-83 (2002)

46. I. Dransfield, S. C. Stocks and C. Haslett. Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. *Blood* 85, 3264-3273. (1995)

47. A. Noguera, E. Sala, A. R. Pons, J. Iglesias, W. MacNee and A. G. Agustí: Expression of adhesion molecules during apoptosis of circulating neutrophils in COPD. *Chest* 125, 1837-1842 (2004)

48. J. Schymeinsky, A. Mocsai and B. Walzog: Neutrophil activation via beta2 integrins (CD11/CD18): molecular mechanisms and clinical implications. *Thromb Haemost* 98, 262-273 (2007)

49. T. N. Mayadas, X. Cullere: Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol* 26, 388-395 (2005)

50. Z. Li: The αMβ2 integrin and its role in neutrophil function. *Cell Res* 9:171-178 (1999)

51. E. Crockett-Torabi, B. Sulenbarger, C. W. Smith, J. C. Fantone: Activation of human neutrophils through L-selectin and Mac-1 molecules. *J Immunol*. 154, 2291-2302 (1995)

52. B. B. Whitlock, S. Gardai, V. Fadok, D. Bratton and P. M. Henson: Differential roles for α (M)β (2) integrin clustering or activation in the control of apoptosis via regulation of akt and ERK survival mechanisms. *J Cell Biol* 151, 1305–1320 (2000)

53. C. Rubel, S. Gomez, G. C. Fernandez, M. A. Isturiz, J. Caamano and M. S. Palermo: Fibrinogen-CD11b/CD18 interaction activates the NF-κB pathway and delays apoptosis in human neutrophils. *Eur J Immunol* 33, 1429-1438 (2003)

54. R. F. Guo, L. Sun, H. Gao, K. X. Shi, D. Rittirsch, V. J. Sarma, F. S. Zetoune and P. A. Ward: *In vivo* regulation of neutrophil apoptosis by C5a during sepsis. *J Leukoc Biol* 80, 1575-1583 (2006)

55. A. L. Coelho, M. S. De Freitas, A. Mariano-Oliveira, D. C. Rapozo, L. F. Pinto, S. Niewiarowski, R. B. Zingali, C. Marcinkiewicz and C. Barja-Fidalgo: RGD- and MLD-disintegrins, jarastatin and EC3, activate integrin-mediated signaling modulating the human neutrophils chemotaxis, apoptosis and IL-8 gene expression. *Exp Cell Res* 292, 371-384 (2004)

56. M. Aleman, P. Schierloh, S.S. de la Barrera, R.M. Musella, M.A. Saab, M. Baldini, E. Abbate and M.C. Sasiain: Mycobacterium tuberculosis triggers apoptosis in peripheral neutrophils involving toll-like receptor 2 and p38 mitogen protein kinase in tuberculosis patients. *Infect Immun* 72, 5150-5158 (2004)

57. H.O. Song, M.H. Shin, M.H. Ahn, D.Y. Min, Y.S. Kim and J.S. Ryu: Trichomonas vaginalis: Reactive oxygen species mediates caspase-3 dependent apoptosis of human neutrophils. *Exp Parasitol* 118, 59-65 (2007)

58. S. Sim, S.J. Park, T.S. Yong, K.I. Im and M.H. Shin: Involvement of β (2)-integrin in ROS-mediated neutrophil

apoptosis induced by Entamoeba histolytica. *Microbes Infect* 9, 1368-1375 (2007)

59. L. Lindbom and J. Werr. Integrin-dependent neutrophil migration in extravascular tissue. *Semin Immunol* 14, 115-121 (2002)

60. Y. Taooka, J. Chen, T. Yednock and D. Sheppard: The integrin alpha9beta1 mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. *J Cell Biol* 145, 413-420 (1999)

61. J. Werr, X. Xie, P. Hedqvist, E. Ruoslahti and L. Lindbom: Beta1 integrins are critically involved in neutrophil locomotion in extravascular tissue *in vivo*. *J Exp Med* 187, 2091-2096 (1998)

62. B. D. Cox, M. Natarajan, M. R. Stettner and C. L. Gladson: New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. *J Cell Biochem,* 99, 35-52 (2006)

63. S. K. Mitra, D. A. Hanson, D. D.Schlaepfer: Focal adhesion kinase: in command and control of cell motility.Nat Rev Mol Cell Biol 6, 56-68 (2005)

64. K. Umanskiy, C. Robinson, C. Cave, M. A. Williams, A. B. Lentsch, J. Cuschieri and J. S. Solomkin: NADPH oxidase activation in fibronectin adherent human neutrophils: A potential role for beta1 integrin ligation. *Surgery*. 134, 378-383 (2003)

65. E. A. Ross, M. R. Douglas, S. H. Wong, E. J. Ross, S. J. Curnow, G. B. Nash, E. Rainger, D. Scheel-Toellner, J. M. Lord, M. Salmon and C. D. Buckley: Interaction between integrin alpha9beta1 and vascular cell adhesion molecule-1 (VCAM-1) inhibits neutrophil apoptosis. *Blood* 107:1178-1183 (2006)

66. K. Raza, C. E. Buckley, M. Salmon and C. D. Buckley: Treating very early rheumatoid arthritis. *Best Pract Res Clin Rheumatol* 20, 849-863 (2006)

67. J. L. Martindale and N. J. Holbrook: Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol* 192, 1-15 (2002)

68. H. Kamata and H. Hirata: Redox regulation of cellular signaling. *Cell Signal* 11, 1-14 (1999)

69. S. J. Chanock, J. el Benna, R. M. Smith and B. M. Babior: The respiratory burst oxidase. *J Biol Chem* 269, 24519-24522 (1994)

70. H. Lalucque and P. Silar: NADPH oxidase: an enzyme for multicellularity? *Trends Microbiol* 11, 9-12 (2003)

71. B. Lardy, M. Bof, L. Aubry, M.H. Paclet, F. Morel, M. Satre and G. Klein: NADPH oxidase homologs are required for normal cell differentiation and morphogenesis in Dictyostelium discoideum. *Biochim Biophys Acta* 1744, 199-212 (2005)

72. P.G. Heyworth, A.R. Cross and J.T. Curnutte: Chronic granulomatous disease. *Curr Opin Immunol* 15, 578-584 (2003)

73. Y. Kasahara, K. Iwai, A. Yachie, K. Ohta, A. Konno, H. Seki, T. Miyawaki and N. Taniguchi: Involvement of reactive oxygen intermediates in spontaneous and CD95 (Fas/APO-1)-mediated apoptosis of neutrophils. *Blood* 89, 1748-1753 (1997)

74. B. Dewald and M. Baggiolini: Activation of NADPH oxidase in human neutrophils. Synergism between fMLP and the neutrophil products PAF and LTB4. *Biochem Biophys Res Commun* 128, 297-304 (1985)

75. S. A. Jones, M. Wolf, S. Qin, C. R. Mackay and M. Baggiolini: Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2. *Proc Natl Acad Sci U S A* 93, 6682-6686 (1996)

76. K. Asehnoune, D. Strassheim, S. Mitra, J. Y. Kim and E. Abraham: Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. *J Immunol* 172, 2522-2529 (2004)

77. R. W. Watson: Redox regulation of neutrophil apoptosis. *Antioxid Redox Signal* 4, 97-104 (2002)

78. A. V. Graça-Souza, M. A. Arruda, M. S. de Freitas, C. Barja-Fidalgo and P. L. Oliveira: Neutrophil activation by heme: implications for inflammatory processes. Blood 99, 4160-4165 (2002)

79. M. A. Arruda, P. Barcellos-de-Souza, A. L. Sampaio, A. G. Rossi, A. V. Graça-Souza and C. Barja-Fidalgo: NADPH oxidase-derived ROS: key modulators of heme-induced mitochondrial stability in human neutrophils. *Exp Cell Res*. 312, 3939-3948 (2006)

80. M. A. Arruda, A. V. Graça-Souza and C. Barja-Fidalgo: Heme and innate immunity: New insights for an old molecule. *Mem Inst Oswaldo Cruz* 100, 799-803 (2005)

81. R. T. Figueiredo, P. L. Fernandez, D. S. Mourao-Sa, B. N. Porto, F. F. Dutra, L. S. Alves, M. F. Oliveira, P. L. Oliveira, A. V. Graça-Souza and M. T. Bozza: Characterization of heme as activator of Toll-like receptor 4. *J Biol Chem* 282, 20221-20229 (2007)

82. B. N. Porto, L. S. Alves, P. L. Fernández, T. P. Dutra, R. T. Figueiredo, A. V. Graça-Souza and M. T. Bozza: Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. *J Biol Chem* 282, 24430-24436 (2007)

Key Words: Neutrophil, Apoptosis, Mitochondria, Signaling Transduction, NADPH oxidase, Reactive Oxygen Species, Cell Activation, Inflammation, Heme, Chronic Diseases, Review

Send correspondence to: Christina Barja-Fidalgo, Departamento de Farmacologia, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Av. 28 de setembro 87 fds, Vila Isabel, Rio de Janeiro, 20551-030, RJ, Brazil, Tel: 55 21 2587-6398, Fax: 55 21 2587-6808, E-mail: barja-fidalgo@uerj.br

http://www.bioscience.org/current/vol14.htm