Chapter 8
Phagosomal and Lysosomal NO Synthesis

8.1 NO in Multivesicular Bodies, Phagosomes and Secondary Lysosomes

Engulfment of particles by endocytosis is one of the most ancient and evolutionarily conserved cellular processes in the eukaryotic cell (Chang 2009). Endocytosis starts with the recognition and binding of particles by cell-surface receptors; followed by budding of the cell membrane and the formation of an endosome which internalizes the bounded particles. Finally, the endosome undergoes fusion with lysosomes containing hydrolytic enzymes to degrade the engulfed cargo (Fang 2004).

Late endosomes often enclose intraluminal vesicles that are formed by the endosomal membrane growing inward (Fig. 8.1). These structures are also called multivesicular bodies (Loesch et al. 1997; O'Neill and Quah 2008). They may fuse with the plasma membrane and release their intraluminal vesicle content to the extracellular environment. The secreted vesicles contain bacterial antigen motifs and may function as signals for immune cells (Record et al. 2011). For instance, exosomes derived from bacterially infected macrophages carry bacterial coat components and stimulate bystander macrophages and neutrophils to secrete proinflammatory mediators and increase NO production (O'Neill and Quah 2008). Interestingly, the circulating exosomes of platelets also generate NO in septic shock, which evokes myocardial nitrosative injury (Azevedo et al. 2007). Platelet exosomes are also capable of inducing endothelial NO and peroxynitrite ($ONOO^-$) generation, thus evoking apoptosis and vascular damage (Gambim et al. 2007).

Phagocytosis is a special type of endocytosis: it is required for the engulfment of solid particles such as pathogens, xenobiotics, protein complexes and cell debris of necrotic or apoptotic cells (Fig. 8.1). The endosome formed in the phagocytosis process is termed a phagosome (Weissmann 1964; Hirsch 1965). In vertebrate-type phagocytosing cells such as macrophages, the association of NOS with phagosomes has been shown (Winberg et al. 2007). These cells employ NO synthesis as a pathogen killing mechanism, to evoke nitrosative damage of the engulfed microbes (Malawista et al. 1992). Accordingly, microbial antigens increase NOS-activity,

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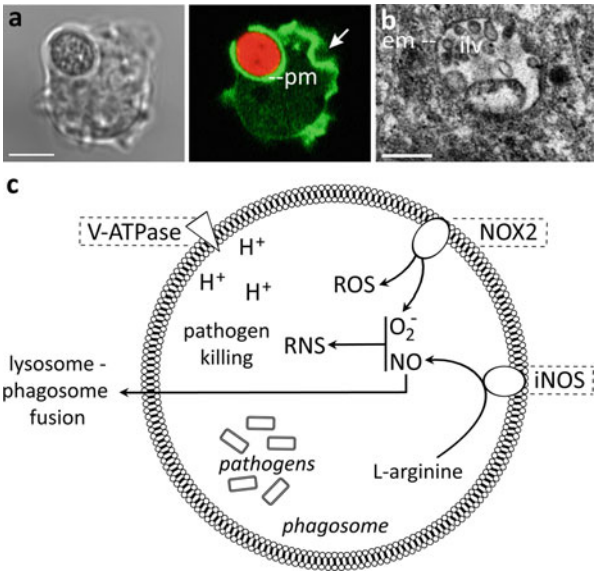

Fig. 8.1 Synthesis of NO in phagosomes. Example of phagocytosis: *Dictyostelium discoideum* amoeba engulfs a rhodamine-labelled yeast cell **a** The arrow shows a newly formed phagocytic cup. The phagosome membrane (*pm*) surrounds the particle. Green fluorescent protein is used to tag actin filaments. Phase contrast (on the left) and confocal image (on the right), scale bar 5 µm. Reprinted with permission (Schleicher and Jockusch 2008). Late endosomes may form multivesicular bodies: the endosome membrane (*em*) surrounds several intraluminal vesicles (*ilv*) which may be released from the cell and activate immune cells **b** Author's TEM image, scale bar 200 nm. The pathogen-containing phagosomes synthesize *NO* which forms *RNS* to kill *pathogens* or facilitate *lysosome-phagosome fusion* by affecting actin organization around the *phagosome*. The vesicular ATPase (*V-ATPase*) which generates an acidic environment in the *phagosome*, and the phagocyte oxidase (*NOX2*) which generates *ROS*, also ensures *pathogen killing* **c**

which is then involved in phagocytosis (Zagryazhskaya et al. 2010), pathogen killing and inflammation (Franchini et al. 1995; Nath and Powledge 1997; Sethi et al. 2001).

The activation of macrophages by bacterial components (e.g. lipopolysaccharide, LPS) or inflammatory cytokines (e.g. interferon-γ, IFNγ) evokes an inflammatory or M1 phenotype acquisition (Benoit et al. 2008). This M1-type polarization of macrophages increases the expression of iNOS, leads to the enrichment of iNOS in the phagosome membrane and evokes a NO-burst (Winston et al. 1999). Vesicular iNOS is derived from a cytosolic iNOS isoform, by a post-translational protein modification, which increases the membrane association of the molecule (Vodovotz et al. 1995). In resting macrophages iNOS is associated with non-lysosomal vesicles, which undergo fusion with phagosomes thus translocate iNOS to the phagosomal membrane upon activation and phagocytosis (Vodovotz et al. 1995).

Phagosomes of M1 macrophages also contain NADPH-dependent phagocyte oxidase which produces superoxide (O_2^-) and hydrogen peroxide (H_2O_2) thus increases ROS generation within the phagosome (Fig. 8.1) (Winberg et al. 2007). With the

activation of phagosomal NO synthesis the phagocyte oxidase activity can also be increased (Brennan et al. 2004) and the generated O_2^- forms $ONOO^-$ with NO. Under the acidotic pH of the phagosome NO also gives nitrous acid (HNO) and other reactive nitrogen species (RNS), which evoke nitrosative damage of the engulfed pathogens (Jordao et al. 2008; Ehrt and Schnappinger 2009). Phagosome NO synthesis also restricts the iron-availability of the engulfed cells thus limiting their survival (von Bargen et al. 2011). Phagosomal NO synthesis not only ensures the elimination of the pathogenic cells, but also helps F-actin assembly around the phagosomes, which facilitates phagosome-lysosome fusion (Winberg et al. 2007) (Fig. 8.1).

Certain intracellular pathogens, such as *Mycobacterium tuberculosis*, *Leishmania donovani* and *Rhodococcus equi*, have evolved defensive mechanisms, by which they arrest the fusion of lysosomes with the phagosome and thus avoid degradation by lysosomal enzymes (Winberg et al. 2007; von Bargen et al. 2011). Various cell surface molecules of the engulfed pathogens mediate the inhibition of the lysosome-phagosome fusion: e.g. lipophosphoglycans or trehalose dimycolate (Jordao et al. 2008; Ehrt and Schnappinger 2009). However, when macrophages are being activated by IFNγ or LPS, their NO burst overshadows these defense mechanisms and lysosomes fuse properly with the phagosomes (Winberg et al. 2007).

Phagocytosing immune cells of invertebrates also synthesize NO, and they may respond with increased NO synthesis to various microbial products (Nieto-Fernandez et al. 1999; Beck et al. 2001). Rhizopoda, the most ancient phagocytosing eukaryotes show NOS-like activity (Rojas-Hernandez et al. 2007) and also display reductive NO synthesis (Risgaard-Petersen et al. 2006). These eukaryotes utilize phagocytosis to engulf unicellular organisms, thus they may be considered the archetypes of phagocytosing immune cells. To date, whether a NO burst occurs during their phagocytosis however, has not been established. Destruction of engulfed pathogens by cytotoxic effects of NO is therefore an attribute of multicellular eukaryotes, and this mechanism is conserved in the evolution of the innate immune system (Tauber 2003; Fang 2004).

8.2 Lysosomes of Granulocytes are Sources of NO

Granulocytes constitutively express iNOS, eNOS, and nNOS and display calmodulin-dependent L-arginine/L-citrulline conversion (Maruo et al. 1999; Cedergren et al. 2003; Heijnen et al. 2006; Saini et al. 2006; Saluja et al. 2010; Saluja et al. 2011). Although NOS is also distributed in the cytoplasm and associated with the nucleus (Heijnen et al. 2006; Saluja et al. 2010; Saluja et al. 2011), electron microscopic analysis has revealed that granulocyte-specific lysosomes, the so-called eosinophil and neutrophil granules are the most important NOS-containing organelles in granulocytes (Fig. 8.2).

A subset of neutrophil granules (the so-called azurophilic granules) and the eosinophil granules also contain heme-peroxidases (EC 1.11.1.7): myeloperoxidase (MPO) and eosinophil peroxidase (EPO), respectively. A product of MPO is

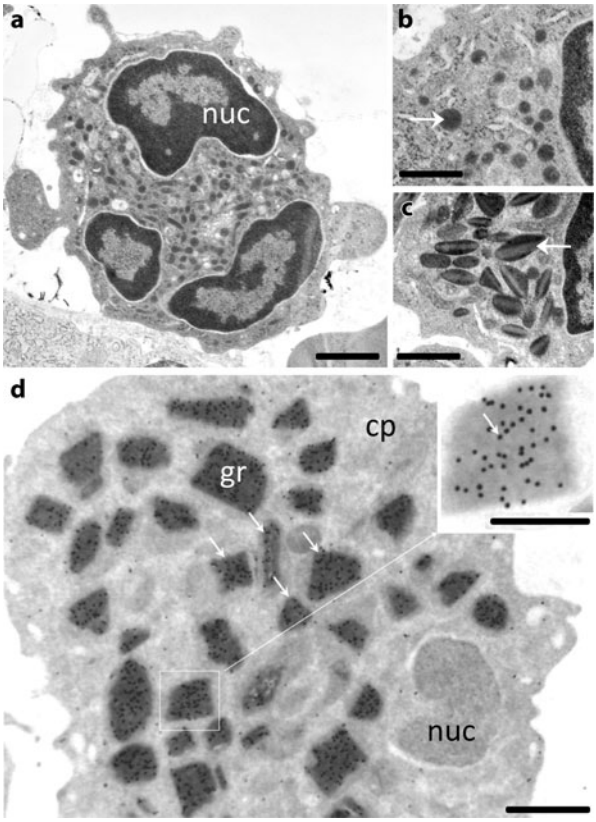

Fig. 8.2 Leukocyte granules contain NOS. Leukocyte granules are lysosome-like vesicles containing various proteins implicated in host defense, hydrolytic enzymes, and plasma membrane components, receptors of complements, chemoattractants and NOS. Some of the granules undergo fusion with the phagosomes and they are involved in the killing and degradation of microorganisms. Granule contents may also be released into the extracellular space, where they play distinct roles in inflammation. TEM images showing a neutrophil granulocyte of mouse **a** neutrophil granules **b** and eosinophil granules **c** form mouse granulocytes. *nuc* – nucleus, *white arrow* points to granules; scale bar 650 nm (a), 300 nm (b, c); Author's images. TEM images showing colloidal gold-labeling of NOS (*white arrows*) in the granules of a human eosinophil granulocyte **d** (Saluja et al. 2010). *cp* – cytoplasm, *gr* – eosinophil granule, *nuc* – nucleus, arrows label NOS signal (colloidal gold); scale bar 200 nm, in insert 500 nm. (Source: With courtesy of Dr. Madhu Dikshit)

hypochlorous acid (HOCl), which is an effective pathogen killing substance, while EPO generates hypobromite, another oxidizing agent which ensures defense against helminths and bacteria (Fang 2004). Both MPO and EPO are responsible for generating RNS from degradation products of NO, such as NO_2^- and peroxynitrous acid (HONOO). In activated granulocytes, NO degrades to NO_2^- or combines with O_2^- to $ONOO^-$, which then forms HOONO (Pryor and Squadrito 1995) (Fig. 8.3). MPO can convert NO_2^- and HOONO to other RNS, such as nitryl chloride (NO_2Cl) and

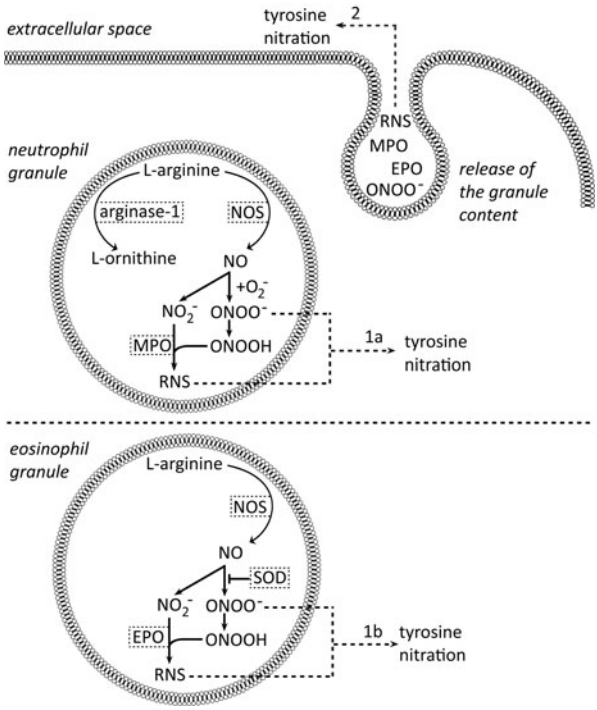

Fig. 8.3 Synthesis of *NO* in leukocyte granules. In the leukocyte granules, *NOS* synthesizes *NO* from *L-arginine*. In *neutrophil granules*, *arginase-1* competes for the substrate with *NOS*, and may thereby limit *NO* levels. Peroxidases (*MPO*, *EPO*) produce reactive nitrogen species (*RNS*) from derivatives of *NO* (NO_2^-, $ONOO^-$, *ONOOH*). Antioxidant enzymes, e.g. *SOD*, may limit the generation of *RNS*. The *RNS* evoke *tyrosine nitration* in the cell (*1a*, *1b*) or in the *extracellular space* (*2*)

nitrogen dioxide (NO_2) (Floris et al. 1993; Eiserich et al. 1998; But et al. 2004). Similarly, EPO also metabolizes NO_2^- to RNS in eosinophil granulocytes (Wu et al. 1999; Takemoto et al. 2007b). Activated human neutrophil granulocytes show increased MPO activity along with their elevated NO production and both NO and NO_2^- are capable of increasing MPO activity (Sethi et al. 2001; But et al. 2004).

Nitrotyrosine is abundant in granules containing both iNOS and peroxidases (Heijnen et al. 2006), suggesting that close vicinity of NO synthesis and peroxidase activity results in tyrosine nitration. In accordance with this scenario, upregulation of iNOS increases the level of 3-nitrotyrosine in eosinophil granulocytes (Duguet et al. 2001) and increased iNOS expression and tyrosine nitration occurs at inflammatory sites infiltrated by neutrophil or eosinophil granulocytes (Wu et al. 1999; Iijima et al. 2001). The lack of iNOS or inhibition of NOS abolishes the generation of intracellular RNS in granulocytes (Numata et al. 1998; Iijima et al. 2001; Koarai et al. 2002). EPO-deficiency also diminishes tyrosine nitration in eosinophil granulocytes in response to allergen challenge in mice, showing that peroxidase activity is required for protein nitration (Duguet et al. 2001). Although $ONOO^-$ evokes tyrosine nitration by itself, MPO and EPO generated RNS play the leading role in nitration of tyrosine residues in granulocytes (Eiserich et al. 1998; But et al. 2004).

8.3 Effects of Protein Nitration Evoked by Granulocytes

The generation of RNS and consequent protein nitration may provide an additional microbial killing mechanism in granulocytes (Malawista et al. 1992; Malawista et al. 1996; Gutierrez-Correa et al. 2000). For instance, tyrosine nitration by resident eosinophil granulocytes of the gastric mucosa (Takemoto et al. 2007b) is involved in defense against pathogens, such as *Helicobacter pylori* (Kuwahara et al. 2000). However, tyrosine nitration by tumor-infiltrating neutrophil granulocytes may also evoke genotoxic damage and contribute to the burden of genetic abnormalities associated with tumor progression (Sandhu et al. 2000). In various inflammatory disorders such as asthma, atopic dermatitis and allergic reactions, granulocyte-evoked tyrosine nitration also accounts for tissue damage and remodeling (Maruo et al. 1999; Kubo et al. 2005; Prado et al. 2006). Production of NO in granulocytes and consequent protein nitration is therefore considered as a cytotoxic, often harmful and inflammation provoking mechanism.

However, tyrosine nitration of chemoattractant molecules, such as interleukin-8 and monocyte chemotactic protein-1 impairs their ability to increase granulocyte chemotactic activity (Sato et al. 2000c; Sato et al. 2000b). Eosinophil granulocytes also display diminished chemotaxis in response to tyrosine nitrated eotaxin, interleukin-5 and RANTES (normal T cell expressed and secreted) (Sato et al. 1999; Sato et al. 2000a). Tyrosine nitration of immunoglobulin-G impairs its ability to induce inflammatory granulocyte activation (Uesugi et al. 2000). Tyrosine nitration of chemotactic factors therefore diminishes granulocyte recruitment to inflammatory sites. Moreover, tyrosine nitration also inhibits granulocyte adherence to endothelial cells, therefore NO may limit the endothelial injury evoked by activated granulocytes (Banick et al. 1997; Su et al. 1998). The activation of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) also decreases granulocyte rolling and adhesion by a mechanism dependent on NO production (Napimoga et al. 2008). Apart from tyrosine-nitrated proteins, other nitrated organic compounds, such as unsaturated fatty acids also exert an anti-inflammatory profile by attenuation of neutrophil degranulation, O_2^- generation and integrin expression (Coles et al. 2002).

Various immunomodulatory factors are capable of inducing iNOS gene transcription in granulocytes and increased iNOS activity is proportional with tyrosine nitration under certain pathological conditions (Pautz et al. 2010). However, studies with iNOS deficient mice have pointed out that tyrosine nitration is not completely abolished by the lack of iNOS (Kenyon et al. 2002), since other NOS isoforms may also be sources of NO and cell types other than granulocytes also contribute to tyrosine nitration in inflamed tissues (Maarsingh et al. 2009). Collectively, inflammatory activation of granulocytes evokes NO production, and NO is converted to RNS by peroxidases, leading to tyrosine nitration. Protein nitration evokes nitrosative damage in the inflammatory environment; however, nitration of various biomolecules inhibits granulocyte-mediated inflammation.

Apart from the effects of NO-derived RNS, NO also acts through the cGMP/PKG pathway in granulocytes (Wyatt et al. 1993). The NO/cGMP/PKG signaling induces degranulation, the release of inflammation-modulating substances (Wyatt et al.

1993). Moreover, NO is also required for granulocyte chemotaxis and metalloproteinase secretion (Iijima et al. 2001; DiScipio et al. 2006). Under oxidative stress, NO generation also helps the survival of neutrophil granulocytes and contributes to sustained inflammation (Riazantseva et al. 2010). However, it has not been established whether the lysosomal NOS-pool would be the source of NO in these events.

8.4 Arginase-1 Reduces NO Synthesis in Neutrophil Granulocytes

Availability of L-arginine is a key determinant of NO biosynthesis. Neutrophil granulocytes constitutively express arginase-1, which hydrolyzes L-arginine to L-ornithine and urea (Munder et al. 2005; Munder et al. 2006). In neutrophil granulocytes, arginase-1 is confined to gelatinase containing granules, which are also sites of NO synthesis. Within the granules, arginase-1 consumes L-arginine, and thus reduces NO generation by NOS (Jacobsen et al. 2007) (Fig. 8.3). Competition of the two enzymes for the same substrate therefore, determines the level of NO production in the neutrophil granules. It has also been shown that L-ornithine and N_{ω}-hydroxy-L-arginine, an intermediate product of NO biosynthesis inhibit arginase-1, and L-ornithine also reduces uptake of L-arginine in NOS-containing cells (Maarsingh et al. 2009). Interplay between arginase-1 and NOS therefore, may ensure balanced NO production in the neutrophil granules. However, increased consumption of L-arginine by arginase-1 also increases tyrosine nitration (Takemoto et al. 2007a) since reduced availability of L-arginine increases O_2^- generation by the iNOS reductase domain (Xia et al. 1998), leading to production of $ONOO^-$ in the leukocytes (Maarsingh et al. 2009).

Because eosinophil granulocytes do not express arginase-1, the regulation of NOS activity through L-arginine levels is specific to neutrophil granulocytes (Luckner-Minden et al. 2010). Why eosinophil granulocytes are able to evoke higher levels of tyrosine nitration than neutrophil granulocytes (Takemoto et al. 2007b) may be due to the lack of regulation of NOS catalytic activity. In eosinophil granulocytes, catalase and superoxide dismutase (SOD) may counteract the RNS generation, without affecting NO synthesis (Takemoto et al. 2007b).

8.5 Chapter Summary

NO in the endosomes	• Endosome-derived multivesicular bodies may emit exosomes, which contain RNS and cause nitrosative damage in tissues; or activate NO synthesis in immune cells • In phagosomes NO and RNS are pathogen killing agents. Inflammatory stimuli increase iNOS transcription and the iNOS protein is targeted from the cytosol to the phagosome membrane
NO in the lysosomes	• Lysosomes of granulocytes produce NO which leads to protein nitrosylation, affects pathogen killing and inflammation. Substrate restriction may limit NO synthesis within the lysosomes

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