Built for the kill. Studies on the neutrophil NADPH oxidase

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Publication date
2004

Chapter 7

Summary and Discussion

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The most abundant cell in the human immune system is the neutrophil, which is specialized in the destruction of invading microorganisms such as bacteria and fungi. To fulfill this task successfully the neutrophil is equipped with features that are unmatched by other cells in the immune system. First, neutrophils have the capacity to migrate to a site of infection at a very high pace, guided by their ability to sense chemokines and bacterial products. Second, as professional phagocytes, neutrophils can ingest large numbers of microorganisms. Finally, the neutrophil is heavily armed with anti-microbial enzymes, which will be launched at phagocytosed microbes, resulting in the degradation of these pathogens.

One of the most important anti-microbial enzymes in the neutrophil is the leukocyte NADPH oxidase[1]. This enzyme converts oxygen into superoxide, using NADPH as electron donor[2]. Superoxide forms the basic compound from which other, more agressive reactive oxygen species (ROS) are derived[1]. ROS, some of which are highly toxic, have a central role in the killing of ingested microbes. The NADPH oxidase consists of a membrane-bound flavocytochrome, cytochrome b_{558}, a heterodimer formed by the glycoprotein gp91^{phox} (*phox*: *ph*agocyte *ox*idase) and p22^{phox}[1]. Gp91^{phox} is the catalytic subunit of the NADPH oxidase, bearing two hemes, one FAD molecule and a binding place for NADPH[3]. The primary function of p22^{phox} seems to be the stabilisation of gp91^{phox} in the membrane, because without p22^{phox}, gp91^{phox} is not expressed[4]. For the activation of cytochrome b_{558}, the interaction with three cytosolic proteins, p47^{phox}, p67^{phox} and Rac2, is necessary[5-7]. These proteins, together with a fourth protein, p40^{phox}, translocate to the membrane and bind to cytochrome b_{558} upon activation. This complex of proteins forms the active NADPH oxidase.

The importance of a functional NADPH oxidase for the anti-microbial response of neutrophils is best illustrated in patients suffering from chronic granulomatous disease (CGD)[8]. This inherited immunodeficiency syndrome is characterized by recurrent life-threatening bacterial and fungal infections and is the consequence of a defect in the production of superoxide by the NADPH oxidase. A mutation in any of the five essential NADPH oxidase components, gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox} and Rac2, can lead to CGD[9]. Until now, no mutations have been described in p40^{phox} of CGD patients, raising questions about the importance of this protein for the activity of the NADPH oxidase.

Besides the generation of ROS, the NADPH oxidase has another very important function in the killing of phagocytosed pathogens. The NADPH oxidase

transfers electrons from NADPH to oxygen over the phagosomal membrane, creating a membrane potential over this membrane. To compensate for the negative charge in the phagosome, H^+ ion is transported into the phagosome. However, this is not the only positively charged ion that is transported into the phagosome; there is also substantial charge compensation by influx of K^+ ions. The influx of K^+ ions proved to be essential for the liberation of anti-microbial proteases from the negatively charged matrix of granules fused with the phagosome[10]. Without a functional NADPH oxidase, the liberation of the proteases from the matrix does not occur, and subsequent degradation of phagocytosed microbes by these enzymes is then prevented. The onset of two distinctive means of microbial degradation, the oxidative and the proteolytic cascades, are thus regulated both by the actions of the NADPH oxidase.

In this thesis, several aspects of the NADPH oxidase were investigated. In **Chapter II** an attempt was made to identify the FAD-binding residues in $gp91^{phox}$. Although a small number of mutations have been identified in CGD patients that are situated in proposed FAD-binding regions in $gp91^{phox}$ and lead to loss of FAD binding, the number of mutations is too low to predict the FAD-binding residues with any certainty. To circumvent this problem, potential FAD-binding residues were mutated and the mutant $gp91^{phox}$ proteins were expressed in cells containing all NADPH oxidase components except $gp91^{phox}$. First, the C-terminal part of $gp91^{phox}$ was aligned with two related FAD-containing enzymes, ferredoxin reductase and nitrate reductase. On the basis of these alignments, two models of the C-terminus of $gp91^{phox}$ were built, which were used to identify potential FAD-binding residues. Several of these residues were subsequently mutated by a novel site-directed mutagenesis method. Using this method, an amino-acid residue can be mutated into all possible amino-acid residues in a single PCR reaction. Two selected residues were submitted to this procedure, which resulted in 26 new mutations in $gp91^{phox}$. The mutants were then expressed in K562 cells that expressed all essential NADPH oxidase components except $gp91^{phox}$, thereby generating cells that were, in case of transfection with a functional $gp91^{phox}$ protein, able to produce superoxide.

After transfection, the different $gp91^{phox}$ mutants were first tested for expression of cytochrome b_{558} by flow cytometric analysis. Surprisingly, all mutants were expressed, which is in sharp contrast to what is found in CGD patients, where most amino-acid substitutions in $gp91^{phox}$ lead to loss of cytochrome b_{558} expression[11]. However, $gp91^{phox}$ mutants that had been identified

in CGD patients, and which showed lowered expression of cytochrome b_{558} in the neutrophils of these patients, were also expressed at lower levels in K562 cells. Similar results were obtained when gp91^{phox} mutants were expressed in other cell lines, i.e. mutants that were expressed at lower levels in CGD neutrophils also showed a lower level of expression in these cell lines[12;13]. Although we cannot exclude that there will be subtle differences in expression of gp91^{phox} mutants, the prerequisites for cytochrome b_{558} expression seem to be similar in neutrophils and the cell lines used in this study.

Several of the newly generated mutants, although expressed at levels comparable to wild-type gp91^{phox}, displayed no detectable enzymatic activity. These non-functional gp91^{phox} mutants were then expressed in a granulocytic cell line, PLB-985. In this particular cell line, the expression of gp91^{phox} has been knocked out by gene targeting, and the expression of cytochrome b_{558}, after complementation with mutants of gp91phox, was higher than the expression in K562 cells. Mutant cytochrome b_{558} was purified from these cells and attempts were made to determine the FAD-binding capacity of these mutants. Unfortunately, we were not able to measure FAD binding in wild-type cytochrome b_{558}. Therefore, the FAD-binding properties of our newly generated mutants are still elusive. Future experiments with the purified recombinant C-terminal part of gp91^{phox} bearing the different mutations will perhaps provide more insight in their FAD-binding properties.

Chapter III describes the identification of a new mutation in glucose-6-phosphate dehydrogenase (G6PD), the enzyme that catalyzes the conversion of glucose-6-phosphate into 6-phosphogluconolactone. This reaction is accompanied by the generation of NADPH through the reduction of NADP. The conversion of glucose-6-phosphate into 6-phosphogluconolactone by G6PD is one of the reactions of the hexose monophosphate (HMP) pathway, the only source of NADPH in cells that lack mitochondria, such as red blood cells (RBC). Although granulocytes have mitochondria, there are indications that these mitochondria are less functional than those in other blood cells[14]. Normally, G6PD deficiency only becomes manifest in RBC, since these cells are long-living and do not synthesize new proteins, and most G6PD mutations lead to reduced stability of the protein[15]. However, in rare cases, when the activity and/or stability of the mutant G6PD protein is very low, the amount of NADPH that can be generated in the granulocytes is so low that the activity of the NADPH oxidase is affected[16]. This can give rise to CGD-like symptoms, i.e. an increased susceptibility to bacterial

and fungal infections. We identified two patients from two unrelated families, suffering from hemolytic anemia and recurrent infections, with the same new variant of the G6PD protein. In this variant, which was named G6PD Amsterdam, the leucine at position 61 is deleted. Indeed, G6PD activity as well as G6PD protein levels were undetectable in the RBC and in the granulocytes of both patients. Moreover, the granulocytes of both patients displayed a reduced capacity to produce superoxide, which is most likely the cause of their susceptibility to infections. Recombinant G6PD Amsterdam displayed reduced protein stability as well as reduced activity compared to the wild-type enzyme. However, the effects were not as dramatic as expected, and therefore the low levels of G6PD Amsterdam expression in the patient's cells were suspected to be the consequence of instability of the mRNA encoding this enzyme. The expression of the mRNA encoding G6PD Amsterdam was investigated in a heterozygous individual, the mother of one of the patients. G6PD Amsterdam mRNA was undetectable in the mother's blood cells, despite the presence of the mutation in one of her X chromosomes, proving the instability of this mRNA. The instability of the G6PD Amsterdam mRNA very likely contributes to the manifestation of G6PD deficiency in the granulocytes, because in other cases of G6PD deficiency that lead to granulocyte dysfunction the enzymatic properties of the G6PD enzyme were much more affected than in G6PD Amsterdam. It is generally believed that G6PD deficiency does not easily affect granulocytes, because these cells are short-living, i.e. G6PD variants with a reduced half-life will not be completely lost before the granulocytes are already undergoing apoptosis. However, in case of the G6PD Amsterdam mutation, the unstable mRNA is likely to be the cause of a very limited amount of synthesized G6PD protein in the granulocytes, which leads to a deficiency in the NADPH supply.

The differences in the severity of disease between one of the patients and his two brothers, who both display the same mutation and have comparable levels of G6PD activity in their RBC and superoxide-producing capacity of their granulocytes, are intriguing. One of the brothers does not suffer from either hemolytic anemia or susceptibility to infections, while the other does suffer from mild hemolytic episodes. It will be interesting to investigate the involvement of other genes that may modulate the severity of the hemolytic anemia and/or the susceptibility to infections.

In **Chapter IV** the translocation of Rac2 and $p67^{phox}$ was studied in living cells by means of fluorescence recovery after photobleaching (FRAP) experiments

of GFP-tagged versions of these proteins. First, the expression and functionality of the GFP-tagged $p67^{phox}$ and Rac2 was verified. The constructs were expressed in a granulocytic cell line, PLB-985 and in a $gp91^{phox}$-deficient derivative of this cell line. The localization of p67GFP and GFP-Rac2 was then studied in these cell lines during phagocytosis of serum-treated zymosan (STZ). Like in X-CGD neutrophils, we found that the retention of p67GFP and GFP-Rac2 at the phagosomal membrane was dependent on the expression of cytochrome b_{558}. The turn-over of p67GFP and GFP-Rac2 at the phagosomal membrane determined by FRAP experiments, turned out to be very high. By this approach the rapid exchange of $p67^{phox}$ and Rac2 at the phagosomal membrane with free, cytosolic $p67^{phox}$ and Rac2 was shown, which strongly suggests that the cytosolic components of the NADPH oxidase and cytochrome b_{558} do not form a stable complex, but interact with $gp91^{phox}$ and then dissociate rapidly. Although the involvement of several signal transduction pathways was excluded by the use of specific inhibitors of these pathways, we were not able to identify the mechanism that drives this continuous translocation.

Many different protein-protein and protein-lipid interactions are essential for translocation of $p47^{phox}$, $p67^{phox}$ and Rac2 to and association with cytochrome b_{558}. For instance, $p47^{phox}$ has been described to associate with $p22^{phox}$ but also with phospoinositides residing in the phagosomal membrane[17;18]. $P67^{phox}$ interacts with Rac2, with $gp91^{phox}$, but also with $p47^{phox}$ [19-21]. Rac2 can insert its prenylated tail in the membrane, which could be the basis of the translocation of this protein, but it also interacts with $gp91^{phox}$, an interaction that is essential for retention of this protein at the phagosomal membrane[22]. Although the association/dissociation of the cytosolic proteins and cytochrome b_{558} could be regulated by different means, the most likely mechanism for the constant turn-over seems to be the cycling of Rac2 between the GTP- and GDP-bound state. The GDP- and GTP-bound forms of Rac2 are likely to have different affinities for cytochrome b_{558}, thereby regulating their association with the phagosomal membrane. Rac2 is transiently associated with the phagosome if cytochrome b_{558} is absent, proving that the interaction of Rac2 with the cytochrome is essential for continued Rac2 localization on the phagosome. Therefore, changes in the affinity of Rac2 for the cytochrome, by changes in the GTP- or GDP-bound state of Rac2, might regulate the association/dissocation of Rac2 with the phagosomal membrane. Furthermore, Rac2 is the only cytosolic protein that is able to interact with cytochrome b_{558} in the absence of $p47^{phox}$ and $p67^{phox}$, while it is essential for the correct localization of

these proteins at the phagosomal membrane[23;24]. By regulating the affinity of Rac2 for cytochrome b_{558} it might be possible to regulate the association/dissociation of $p47^{phox}$ and $p67^{phox}$ with cytochrome b_{558}. In the past, several attempts have been made to unravel the importance of changes in the GTP-or GDP-bound state of Rac2 for NADPH oxidase activity by the use of dominant negative or constitutively active forms of the Rac GTPase[25]. However, conflicting results were obtained in these studies, leaving the issue of GTP/GDP cycling of Rac2 in NADPH oxidase activity unsolved.

By the use of actin-depolymerizing agents we showed a role for the actin cytoskeleton in the initial translocation of $p67^{phox}$ and Rac2 to the plasma membrane. Binding of serum-treated zymosan (STZ) to the cell surface rapidly induces translocation of these proteins to the site of attachment. In cells that were treated with an actin-depolymerizing agent, the translocation of $p67^{phox}$ and Rac2, after binding of STZ to the cell surface, was absent. However, disruption of the actin cytoskeleton after the translocation had been initiated did not alter the localization or the FRAP characteristics of $p67^{phox}$ and Rac2, restricting the role of the actin cytoskeleton to the initial translocation of $p67^{phox}$ and Rac2.

In **Chapter V** a novel method was developed to detect hydrogen peroxide, encountered by bacteria inside the neutrophil. In this method, bacteria or other particles such as zymosan, are labelled with the fluorescent dye dihydrorhodamine-1,2,3 (DHR) which is converted to the fluorescent dye rhodamine-1,2,3 by hydrogen peroxide in a peroxidase-dependent reaction. By this DHR labelling, hydrogen peroxide encountered by the bacteria results in the generation of fluorescent rhodamine-1,2,3, which can be measured by flow cytometry. This method was then used to investigate possible differences in hydrogen-peroxide-inducing capacity of different *Salmonella typhimurium* strains. Indeed, differences were found between wild-type *Salmonella* and an attenuated strain, a so-called Spi-2 mutant, which is unable to prevent the activation of the NADPH oxidase[26]. Another strain, in which the gene encoding the Sspj protein was deleted, also showed increased fluorescence after infection of neutrophils. The characteristics of this last mutant were further investigated, and this particular strain proved to be more sensitive to hydrogen peroxide than the wild-type strain. No differences were found between the wild-type strain and the Sspj mutant in their ability to prevent the activation of the NADPH oxidase on the membrane of the *Salmonella*-containing vacuole. The Sspj mutant is attenuated *in vitro* and *in vivo*, and the increased susceptibility to hydrogen peroxide is a likely explanation for its

decreased virulence, which is restored in CGD mice[27]. Furthermore, the detection of fluorescence in this hydrogen-peroxide-sensitive but otherwise wild-type strain, shows that *Salmonella* encounters ROS after infection of human neutrophils, although this is not detected in the wild-type strain under the conditions used.

This phenomenon was further explored in **Chapter VI** in which wild-type and an LPS mutant (rough) strain of *Salmonella typhimurium* were used to identify the role of TLRs in the activation of the NADPH oxidase. Again, the bacteria were labelled with DHR, but the infection rate was now increased, which resulted in detectable fluorescence for the wild-type strain. The rough strain, however, was less effective in inducing DHR fluorescence, suggesting a role for LPS in the activation of the NADPH oxidase. Since TLR4, the receptor for LPS, is expressed on human neutrophils, the importance of signalling via this receptor for the activation of the NADPH oxidase after *Salmonella* infection was further investigated. First, the various TLRs were found to be located on the cell surface and in the different neutrophil granules. A TLR4-specific cell-permeable peptide, which is able to inhibit LPS-mediated signalling via this receptor, was then used to determine the importance of TLR4 signalling for the activation of the NADPH oxidase after infection of neutrophils with *Salmonella*. Indeed, the DHR fluorescence in the wild-type *Salmonella*-infected neutrophils was inhibited by the action of this inhibitory peptide, showing the importance of TLR4 signalling in the activation of the NADPH oxidase. A TLR4-specific blocking monoclonal antibody was used to show that LPS-mediated signalling via TLR4 does not take place at the cell surface. Thus LPS-mediated TLR4 signalling in this system occurs intracellularly.

Our data identify a role for TLR4 in the intracellular detection of pathogens, which is coupled to activation of the NADPH oxidase in neutrophils. Since other TLRs are localized similarly in neutrophils it is very likely that these TLRs are also able to "sense" intracellular pathogens and activate the NADPH oxidase. It remains to be determined whether neutrophils belong to one of the few cell types that are able to couple the detection of intracellular pathogens to the activation of microbicidal systems or that other cell types[28;29] that express TLRs have a similar capacity to activate microbicidal systems. If so, the recently identified homologues of $gp91^{phox}$, the Nox family[30], which have been suggested to be involved in anti-microbial defense, are interesting candidates as targets of TLR signalling.

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