NADPH and Iron May Have an Important Role in Attenuated Mucosal Defense in *Helicobacter pylori* Infection?

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Abstract: Host intracellular iron has been recognized as an important cofactor in induction of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidative burst as antimicrobial defense mechanism. It is plausible that iron chelator directly inactivates NADPH oxidase by chelating the active site heme iron of flavocytochrome b558 thus blocking the transfer of electrons from NADPH to oxygen and its reduction to superoxide anion**.** Thus, altering the equilibrium of intracellular iron could influence the course of infection to the enhancement of the pathogen with regard to oxidative stress.

Keywords: *Helicobacter pylori*, NADPH, iron, free radicals.

INTRODUCTION

Helicobacter pylori (*H. pylori*) have evolved metabolic strategies to maintain a mild inflammation of gastric epithelium while limiting the extent of immune effectors activity. The bacteria induces infiltration of gastric mucosa by neutrophils, macrophages, and T and B lymphocytes. Chronic gastritis is a local immune response to *H. pylori* infection. In the early stage, chronic gastritis appears as an immune response of the gastric mucosa to *H. pylori* infection in the form of mononuclear cell infiltration (immunocompetent lymphocytes and immunoglobulin-secreting plasma cells) in antrum and corpus mucosa , with a predominance of inflammation in the antrum. However, this immune and inflammatory response cannot clear the infection, and leaves the host prone to complications resulting from chronic inflammation [1]. Migration of inflammatory cells to the injury site is followed by respiratory burst. Respiratory burst is characterised by massive production of reactive oxygen species (ROS) in an inflammatory environment and plays a key role in defence against environmental pathogens [2]. Under pathological conditions such as acute and chronic infection and inflammation, overproduction of ROS, highly reactive metabolites, will induce lethal damage to cellular integrity and survival, resulting in reversible or irreversible tissue injury [3].

Oxidative killing of engulfed bacteria requires nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in phagocytic cells. NADPH oxidase- generated $O_2^{\bullet-}$ is necessary for bacterial destruction. In cell membranes, NADPH oxidase becomes activated, and an electron transfer takes place from NADPH in cells to oxygen inside and outside cells. The oxygen molecules that receive an electron become superoxide radicals, which is rapidly converted to hydrogen peroxides (H_2O_2) by spontaneous dismutation or enzymatic dismutation by superoxide dismutase (SOD).Under stress conditions, $O_2^{\bullet-}$ acts as an oxidant of [4Fe–4S] cluster-containing enzymes and facilitates production of highly reactive hydroxyl radical (•OH) from H_2O_2 by making Fe^{2+} available for the Fenton reaction [4,5].The hydroxyl radical, has a high reactivity with a very short *in vivo* half-life of approx. 10−9 secunde, presented as a very dangerous radical. Thus when produced *in vivo* •OH reacts close to its site of formation [6]. However, ROS reaction with poorly liganded iron species can lead to the catalytic production of very dangerous hydroxyl radical which is a major cause of chronic inflammation [7].

THE ROLE OF IRON IN GENERATING ROS AS A COFACTOR IN FLAVOCYTOCHROME B558

Phagocytic leukocytes play major roles in the innate immune response to pathogens. An important component of this response is the ability of leukocytes to generate ROS *via* a membrane-associated NADPH oxidase (Nox) [1, 8]. In addition to its essential role in many processes in living organisms, host intracellular iron has been recognized as an important cofactor in inducing antimicrobial defense mechanisms: NADPH-dependent oxidative burst [1,9].

Nox proteins show structural homology to the cytochrome b_{558} of leukocytes [10]. This multi-component enzyme uses electrons derived from intracellular NADPH to generate superoxide anion, which subsequently dismutates to H_2O_2 and other ROS that are used for host defense against

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bacterial and fungal pathogens. However, the inappropriate or excessive action of Nox has been implicated in the pathogenesis of inflammatory tissue injury and several additional disease states [11].

The Nox system of stimulated phagocytic leukocytes (Nox2) catalyzes the one-electron reduction of oxygen to produce superoxide anion using NADPH as a substrate. When the phagocyte is activated through the action of soluble chemoattractants and chemokines, or phagocytic particles, the cytosolic components (Rac2, $p47^{phox}$ and $p67^{phox}$) of the oxidase are induced to assemble at the level of the membrane-associated flavocytochrome b_{558} (cyt b) the active enzyme. Cyt b has two subunits, $gp91^{phox}$ (Nox2) and $p22^{phox}$, as well as a NADPH binding site, two heme groups, and bound FAD [1, 8, 10]. The formation of this minimal complex enables the flow of electrons *via* a two-step mechanism, from NADPH to FAD (Step 1), then from FAD to the heme of cyt b, and finally to molecular oxygen (Step 2), whose one-electron reduction leads to the formation of superoxide anion [12,13]. Phagocytic cytochrome b_{558} is a unique heme-containing enzyme, which catalyzes one electron reduction of molecular oxygen to produce a superoxide anion with a six-coordinated heme iron [14]. The superoxide anion rapidly undergoes spontaneous dismutation (pH optimum of 4,8) to yield H_2O_2, which in turns supports the generation of an array of reactive species.

H. pylori disrupt NADPH oxidase targeting so that $O_2^{\bullet-}$ is released into the extracellular milieu and is not accumulated inside phagosome. The nascent *H. pylori* phagosomes acquire flavocytochrome b558, while $p47^{phox}$ and $p67^{phox}$ are not efficiently recruited on the phagosome membrane [15].

Iron chelation by desferrioxamine inhibited NADPH oxidase-dependent reactive oxygen species production in mice . It is plausible that iron chelator directly inactivates NADPH oxidase by chelating the active site heme iron thus blocking the transfer of electrons from NADPH to oxygen and its reduction to $O_2^{\bullet-}$. It was showed that chelation of host Fe *in vivo* exacerbates murine salmonellosis and that iron deprivation results in an inability to induce the NADPH oxidase – dependent production of ROS. Thus, altering the equilibrium of intracellular Fe influences the course of infection to the benefit of the pathogen [16].

In addition, a mechanism of rapid iron binding would be advantageous to prevent generation of oxygen radicals during fluxes of oxidative stress in inflammed mucosa. Oxidative damage to pathogenic bacteria constitutes a key part of the immune response of the host. To survive the effects of iron – mediated production ROS by host, *H. pylori* depends on significant role of *H pylori* neutrophil-activating protein (NapA). It is a member of Dps family, a group of ferritin-like-iron-binding proteins that are widespread in eubacteria and archaea These Dps family proteins binds iron and are implicated in oxidative stress resistance [17,18]. NapA is a powerful stimulant of ROS production by human neutrophils and monocytes [19]. This ROS production activate intracellular events including an increase in the cytosolic calcium ion concentration and phosphorylation of proteins, leading to assembly of the NADPH oxidase on the neutrophil plasma membrane [20,21]. Wang G *et al.* [22] demonstrated that *H. pylori* NapA has unique properties, with dual roles related to oxidative stress. NapA induce production of oxygen radicals from host cells and then protect the bacterial cells from iron-mediated oxidative DNA damage. The sequestration of intracellular free iron was proposed as a mechanism affording oxidative stress by NapA. Both roles contibute to the bacterium's long-term infection and pathogenesis.

Recently, it was found that NapA is able to prolong the lifespan of monocytes, the cell type which, together with neutrophils, mostly accumulate in the gastric mucosa of *H. pylori*-infected patients. NapA promotes the survival of Ficoll-purified neutrophils in a monocyte-dependent fashion. The mononuclear cell depletion of Ficoll-purified neutrophils completely abolished the pro-survival effect by NapA [23]. So, NapA exerts an essential contribution in triggering and maintaining inflammation.

THE ROLE OF PENTOSE PHOSPHATE PATHWAY AND GLUTATHIONE

To evade oxidative killing *H. pylori* prevents NADPH oxidase assembly at the phagosome, with release of $NADP^+$ and large amounts of superoxide anions into the extracellular milieu . The NADPH oxidase complex causes a massive increase in non- mitochondrial oxygen consumption due to one electron reduction of oxygen to generate superoxide anions and a strong stimulation of the pentose phosphate pathway (PPP). Generated superoxide anions are rapidly converted to hydrogen peroxide which reacts with free iron to produce hydroxyl radicals, the most toxic of all reactive oxygen species . The PPP is an important biochemical pathway for breakdown of glucose in the liver and fat cells. It consists of irreversible oxidative pentose pathway (OPP) and reversible nonoxidative pentose pathway (NOPP). The OPP is the major provider of reducing equivalents while the NOPP provides C3-C8 glycolyl units which serve as cellular assembly units and as reserve energy metabolites [24,25]. The key enzyme of OPP, glucose-6-phosphate dehydrogenase has main role in production of NADPH needed for reductive biosynthesis and maintenance of cellular redox state [26]. The activity of glucose-6-phosphate dehydrogenase regulates the level of glutathione, the most important antioxidant of gastric mucosa [27]. Exposure of glutathione to ROS oxidizes it and forms glutathione disulfide which is reduced to glutathione by glutathione reductase. The NADPH reducing equivalents utilized for this reaction are produced by glucose-6-phosphate dehydrogenase [25,28].

Availability of glutathione is primarily controlled by activity of the oxidative pentose pathway. *H. pylori* directly decrease cellular glutathione [29,30]. During acute infection in mice, increase in glucose-6-phosphate dehydrogenase activity and glutathione levels occurs immediately, whereas in chronic infection in humans is not changed. This study indicated that increase in OPP activity and concomitantly increased glutathione availability in mice act as effective first-line mucosal antioxidant defense while in humans during chronic infection these defense appears to be less effective [31,32]. It is proposed that the OPP and GSH are of great importance in host mucosal response to *H. pylori* infection and that differences in OPP and GSH activity are

related to different responses seen in acute and chronic infection in both humans and animals. Therefore, it is proposed that an increase in OPP activity and concomintantly increased GSH levels may prevent the early active chronic gastritis in mice. This is a characteristic of the human pathology to *H. pylori* infection [32].

Since gastric cells are not able to take up intact GSH directly from plasma, the maintenance of intracellular GSH level in these cells depends on its reduction and de novo synthesis as well. Therefore, the role of NADPH in glutathione metabolism in these cells is extremely important.

CONSEQUENCES FOR EXCESS OR TOO LITTLE IRON

" Labile" iron represents a key factor that determines the generation of the extremely reactive OH. which dictates the ultimate outcome of ROS-induced effects. The iron catalysed Haber-Weiss reaction, which relies on the Fenton reaction between ferrous iron and hydrogen peroxide, is the major mechanism for generating hydroxyl radicals in biological systems [33,34]. H_2O_2 may also interact with heme-iron on the active site of heme-containing proteins leading to formation of ferryl-heme forms and unstable free radicals [35,36]. Also, further oxidation may lead to liberation of heme-bound iron with obvious deleterious effects to the cell [37]. This may result in the peroxidation of adjacent lipids and lead to oxidative damage of DNA and other macromolecules. Both, severe iron overload and iron deficiency may be deleterious. Because iron is intimately involved in the production of energy and oxygen transport, iron deficiency is a serious problem causing cell damage by reduction of cell growth and proliferation, hypoxia and death. An excess of iron systemically and at the cellular level leads to deleterious effects including free radical-induced damage to cells, cellular components, tissues and organs [38]. Iron, apart from participating in H_2O_2 toxicity, may also act as mediator in H_2O_2-induced signaling.There are indications that iron acts as a second messenger in several signaling processes [39-42].

Limiting the availability of intracellular Fe is one way by which host can control the replication of intracellular pathogens. However, the labile equilibrium of Fe availability for both host and pathogen is illustrated by the following two extreme situations: 1) too little iron in patients suffering from severe anemia and 2) excess of iron in patients with either hereditary or dietary Fe overload are at higher risk of developing disease following infection with intracellular bacteria [43].The restriction of intracellular Fe can prevent the growth of the bacteria within the cell, while sufficient Fe must be available for the induction of antimicrobial mechanisms in host cells [16].

In response to infection, mammals sequester iron into iron-binding proteins (e.g. ferritin, lactoferrin, siderocalin) and locations less accessible to most invading microbes (cytoplasm of macrophages and hepatocytes) [44]. The inflammation alters iron metabolism by decreasing the iron content of plasma (hypoferremia of inflammation). The anemia of inflammation develops if inflammation is sufficiently severe or chronic, it differs from iron deficiency anemia by the presence of stainable iron in bone marrow macrophages, and by elevated serum ferritin—both indicative of adequate iron stores. The molecular basis of these changes was only recently elucidated and centers on the regulation of hepcidin by inflammation [45].

The peptide hormone hepcidin has recently emerged as a master regulator of iron regulation at the physiological level. The high iron stores and/or inflammation are able to increase hepcidin. Increased hepcidin causes internalisation of ferroportin (cellular iron exporter), with decrease in efflux of Fe(II) from peripheral cells, causing IL-6 mediated hypoferraemia. The efflux of Fe(II) from major iron-transporting tissues (duodenal enterocytes, iron-recycling macrophages and iron-storing hepatocytes) into plasma is reduced. Iron accumulates in their cytoplasmic ferritin. Prolonged hypoferraemia limits the iron supply for hemoglobin synthesis and erythropoiesis causing of anemia [46,47]. However, hepcidin is overexpressed in inflammatory disease and is marker of early inflamation [7]. In patients with inflammation, an abundance of hepcidin should lead to poor uptake of dietary iron from the gastrointestinal tract, iron sequestration in macrophages, little iron recycling to the erythron for red-cell production, and microcytic anemia. This pathophysiology is termed inflammatory block [48].

During *H. pylori* infection, the bacterium enhances gastric lactoferrin, which captures iron from transferrin. However, lactoferrin has tough competition in bacterial iron-binding molecules, siderophores [49]. This lactoferrin bound iron can be utilized by *H. pylori*, subsequently depleting host extracellular iron, leading to hypoferremia. *Helicobacter pylori* gastric infection has emerged as a new cause of refractory iron deficiency anemia but the pathogenetic mechanism of anemia is still unknown [50].

Phagocytic NADPH oxidase is impaired in iron-deficient patients with anemia and restored to normal levels after iron supplementation [51]. Excess iron may increase oxidative stress and play a role in vascular inflammation and atherosclerosis. It was showed that desferrioxamine inhibits LPS-induced, NADPH oxidase–mediated oxidative stress and, hence, NF-_B activation and adhesion molecule expression in a murine model of local inflammation.

Iron chelation may be helpful in treating atherosclerotic vascular diseases by ameliorating oxidative stress and inflammation [40].

It has been clearly demonstrated the critical role of host deprivated iron in preventing production of hydroxyl radicals *via* Fe- catalyzed Haber-Weiss reaction and generation of superoxide anion, the precursors of several types of ROS such as hydrogen peroxide, hydroxyl radical and hypochlorous acid (HOCl).

During *Helicobacter pylori* infection Fe is required by the host cell and the bacterium as well. The biological mechanism by which *H. pylori* induces the alternation in the iron stores is not fully understood but it seems to involve several pathways including gastrointestinal blood loss, decrease in the absorption of dietary iron and enhanced uptake of the iron by the bacterium [52-54]. *Helicobacter pylori*

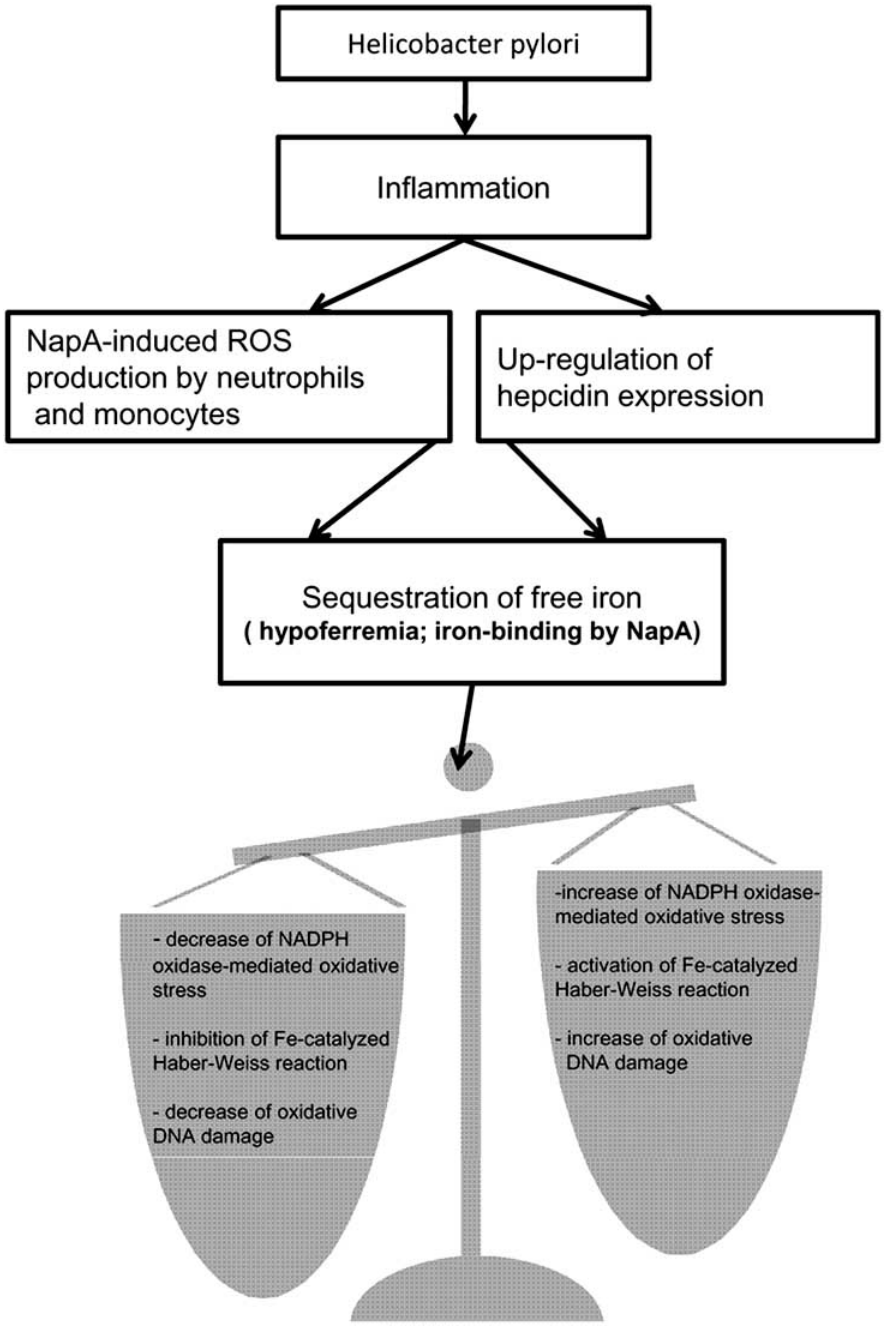

Fig. (1). NADPH oxidase –mediated oxidative stress in *H. pylori* infection. *Helicobacter pylori*-induced inflammation cause ROS production by inflammation cells and increase of hepcidin, leading to sequestration of iron. All these reactions may be involved in modulating NADPH oxidase-mediated oxidative stress, Fe-catalyzed Haber-Weiss reaction and oxidative DNA damage which are important components of mucosal defence.

infection is known to be a cause of iron deficiency anemia (IDA) that is unresponsive to iron supplements. *H. pylori* bind iron to a specific receptor by iron-repressible outer membrane proteins (IROMPs) under conditions of restricted iron. Recently, Lee *et al*. [55] showed that specific *H. pylori* strains associated with IDA demonstrated an advantage in iron acquisition due to a higher expression of IROMPs in the iron-depleted state. These results may explain in part why some patients with *H. pylori* infection are more prone to develop clinical IDA under restricted iron conditions in the host [55]. The meta-analysis of observational studies suggested an association between *H. pylori* and IDA. In randomized controlled trials, eradication of *H. pylori* can improve hemoglobin and serum ferritin levels but not significantly [56].

In addition, 701 school-age children in Mexico City were screened to evaluate the effect of *Helicobacter pylori* eradication and iron supplementation on the iron nutritional

status in children with iron deficiency. This study showed that eradication of *H. pylori* plus iron supplementation increases pool of functional iron and increases the storage of iron in children with iron deficiency [57]. Above mentioned data further underline the contribution of *H. pylori* induced reduction of host iron to levels which have impact on oxidative burst. A dual roles of *H. pylori* NapA protein and pathophysiology of inflammatory block confirm the intricate nature of iron role in mucosal defence in *H. pylori* infection (Fig. **1**).

THERAPEUTIC GOAL TO TREAT OXYRADICAL OVERLOAD IN *HELICOBACTER PYLORI* INFECTION

The host immune response to the infection is largely ineffective as the bacterium persists and the inflammation continues for decades [23]. For the resolution of infl ammation, the activated neutrophils have to be safely removed. Neutrophil apoptosis has been suggested as a possible target for the control of neutrophil-mediated tissue injury. Exogenously added superoxide dismutase induces neutrophil apoptosis, and hydrogen peroxide has been suggested to be a possible major mediator of ROS-induced neutrophil apoptosis in a caspase-dependent manner. If the drug can be delivered efficiently to the inflammatory site, SOD may be useful as an inhibitory mediator of neutrophil-mediated inflammation [58].

Inhibitors of excessive $O_2^{\cdot-}$ generation have been indicated to be more effective antioxidants than radical scavengers, because $O_2^{\cdot-}$ anion is one of the precursors of several types of ROS. It is demonstrated that benzyl isothiocyanate is a potent inhibitor of leukocytic NADPH oxidase, generating a great amount of $O_2^{\cdot-}$ in oxidative burst. Benzyl isothiocyanate probably modifies the electron transport system of cytochrome b_{558} and has a potential to prevent inflammation-related carcinogenesis including skin cancer [59]. Lack of NADPH oxidase activity results in severe inflammation and reduced bacteria load in murine *Helicobacter infection* [60]. Also, individuals with lower neutrophile oxidative burst activity are more prone to *H. pylori* infection [61].

We speculate that these attenuated oxidative burst might favour *H. pylori* persistence, while at the same time promoting host tissue damage. Interesting piece of evidence has been provided by Keenan *et al.* [62]. They suggest that Nox2-based oxidase is likely to have a protective role by dampening inflammation and that the release of granule constituents from neutrophils contributes to tissue damage. However, Allen *et al.* [63] disagreed with this hypothesis. They suggested that *H. pylori* may cause aberrant activation of Nox 2-based oxidase, resulting in accelerated oxidative damage to the gastric mucosa. Thus, there is no doubt that neutrophils play central role in H. pylori –induced gastric damage. As hyperactivity of NADPH oxidase leads to tissue injury their modulation may have significant impacts on regenerative medicine and tissue engineering [64].

CONCLUSION

H. pylori infection is a condition of oxidative stress [65,66]. Host-iron availability has a crucial role in the host-pathogen relationship. In response to infection, host sequester iron into iron-binding proteins. The inflammation alters iron metabolism by decreasing the iron content of plasma (hypoferremia of inflammation) [44]. During *Helicobacter pylori* infection, deprivation of host iron could affect NADPH-dependent oxidative burst [52]. The infection leads to an imbalance in the oxidative pentose pathway and glutahione synthesis in the host. This PPP up-regulation is result of an increased need for antioxidant activity to offset the oxidant assault caused by the infection. The regulation of GSH availability through the up-regulation of OPP activity in *H. pylori* infected humans may provide a novel approach to preventing or reducing the mucosal damage occurring during infection [32]. Affected availability of iron and NADPH could have an important role in attenuated oxidative burst in *H. pylori* infection by favoring local tissue damage which is essential for *H. pylori* survival in gastric mucosa.

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