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The infectious hypoxia: occurrence and causes during *Shigella* infection

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Abstract

Hypoxia is defined as a tissue oxygenation status below physiological needs. During *Shigella* infection, an infectious hypoxia is induced within foci of infection. In this review, we discuss how *Shigella* physiology and virulence are modulated and how the main recruited immune cells, the neutrophils, adapt to this environment.

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Keywords: *Shigella*; Infectious hypoxia; Neutrophils

1. Introduction

*Shigella* are a Gram-negative, facultative anaerobic bacteria of the Enterobacteriaceae family and are the etiological agents of bacillary dysentery or shigellosis. Each year, 165 millions shigellosis cases are reported worldwide, leading to one million deaths, mainly in the developing world. Shigellosis is marked by fever and leads to hemorrhagic diarrhea; diagnosis relies on the detection of erythrocytes, polymorphonuclear neutrophils (neutrophils), and mucus in the stools.

*Shigella* encompasse four subgroups (*Shigella flexneri*, *Shigella sonnei*, *Shigella dysenteriae*, and *Shigella boydii*) (reviewed in Refs. [1] and [2]): *S. dysenteriae* includes 15 serotypes, *S. flexneri* 14 serotypes, *S. boydii* 20 serotypes, and *S. sonnei* a single serotype. The endemic form of the disease is caused essentially by *S. flexneri* 2a and *S. sonnei* [2].

*Shigella* colonize and invade the colon, preferentially targeting colonic crypts [3], and is associated with an acute inflammation [4]. The main steps of colonic infection by *Shigella* are the adhesion, invasion, intracellular replication, and cell-to-cell spreading. Each of these steps is likely to be regulated by environmental cues, including the oxygen tension, pH, temperature, and salts concentration. *Shigella* invasion of the host colonic epithelium is dependent of the Type Three Secretion Apparatus (T3SA). *Shigella* induce a controlled inflammatory response, which includes release of both inflammatory (IL6, IL-8, IL1β, TNFα and β) and anti-inflammatory cytokines (IL-10 and TGF-β) [15].

Within a few hours of *Shigella* invasion in the colonic mucosa, an important influx of inflammatory cells is observed, mainly driven by IL-8 released by infected epithelial cells. Neutrophils represent the predominant population of recruited cells, monocytes are also detected [5]. Lymphoid cells (mainly T cells) have also been observed within the rectal mucosa of infected patients [6]. The dissemination of *Shigella* within the colonic mucosa requires an efficient manipulation of the immune response (as reviewed elsewhere [7,8]). It has previously
been shown that *Shigella* induce monocyte [9], macrophage [10], and B cell [11] apoptosis and inhibits T cell migration [12].

Neutrophils are believed to play a key role in the *Shigella* clearance process through phagocytosis, intracellular killing, as well as the extracellular release of antimicrobial molecules. However, the respective survival of *Shigella* and neutrophils upon interaction remains controversial. It has been shown that Shigella induce neutrophil necrosis in a T3SA-dependent manner [13]. Conversely, it has been shown that neutrophils efficiently kill *Shigella* upon phagocytosis [14]. More recently, it has been shown in Zebrafish larvae infected with *Shigella* that neutrophils and macrophages engulf *Shigella*; although in this model, the impact of the microenvironment is not controlled and does not reflect the colonic lumen and mucosa low oxygen conditions [15].

Oxygen, among other environmental cues plays a critical role in host-pathogen interactions (also reviewed elsewhere [1]). Hypoxia induction during bacterial infection had been observed more than 50 years ago, although the cause remains elusive [2,16,17]. For technical reasons, the level of oxygen at bacterial, viral, or fungal infection sites could never been quantified *in situ* or in intact organs using non-disruptive techniques; the exception being the blood circulation compartment, which is the most accessible body compartment. However, the consequences of its modulation on pathogen physiology and virulence or potentially on immune response efficiency constitute a fundamental biological question.

In this review, we will define hypoxia in the context of *Shigella* infection, introducing the concept of the “Infectious Hypoxia” before describing how neutrophils and *Shigella* are physiologically adapted to low oxygen environments focusing on the pathogen metabolism and virulence modulation and the regulation of neutrophils survival and antimicrobial activity. In conclusion, we will discuss the potential causes and pathophysiological consequences of hypoxia induction during *Shigella* infection.

2. 1-Pathophysiological hypoxia(s)

A prerequisite for the characterization of a pathophysiological oxygen level in an infected tissue is the definition of the physiological oxygen level, named normoxia. The normoxic tissue oxygen level varies between organs, depending on their perfusion efficiency (input) and oxygen consumption, associated with their metabolic activity (output). As mentioned above, for technical reasons, the precise measure of physiological oxygen in most organs remains difficult and is still unknown. Most oxygen pressures (pO2 organ) reported in animal models were determined using a Clark electrode, which is a disruptive method [3,16,17].

In pathophysiological conditions, hypoxia is defined as a tissue oxygenation status that stands below normoxia and results from an inefficient oxygen supply or increased oxygen consumption. Accordingly, the oxygen level associated with hypoxia will vary between and within organs. Organ hypoxia induction may be associated with extrinsic or intrinsic factors. Extrinsic causes are associated with a reduced oxygen supply to an organ, perhaps due to a low red blood cell haemoglobin concentration (anemic hypoxia), a limited capillary perfusion (geographic hypoxia), a reduced local blood flow (ischemic hypoxia), insufficient cardiac activity (stagnant hypoxia), abnormal pulmonary function (hypo/hypoxic hypoxia) [4,18], or a reduced environmental oxygen pressure (at the sea level, pO2 atm = 160 mmHg; at 8848 m, pO2 atm = 53 mmHg), causing a drop in arterial pressure of oxygen associated with a lowered fraction of oxygen-saturated haemoglobin (oxygen saturation), defining the environmental hypoxia.

Intrinsic causes of hypoxia are characterized by a transient or permanent increase of the organ oxygen consumption. It might be physiological or pathophysiological. Physiological hypoxia induction can be illustrated by muscle contraction, which is associated with an increased oxygen consumption rate by myocytes, leading to a transient hypoxia induction. As a physiological response, the resulting and combining increase of the partial pressure of carbon dioxide (pCO2), pH decrease, and temperature increase leads to a local reduction of the haemoglobin affinity for oxygen: the so-called “Bohr effect” (first described by Dr. Christian Bohr in 1904). This physiological adaptation leads to an increased oxygen supply to the organ, counteracting the causes of hypoxia and restoring normoxic conditions. Pathophysiological hypoxia induction may be associated with the infiltration of “newcomers”, such as immune cells in an organ, associated with increased oxygen consumption. This phenomenon has been recently described in the context of sterile inflammation and was named ‘inflammatory hypoxia’. The recruitment of neutrophils was identified as the main cause of hypoxia induction, mainly due to the NADPH oxidase-dependent oxygen consumption, inhibited by Diphenyleneiodonium (DPI) [5–8,19–21].

In this review we will define a new type of hypoxia, named “Infectious Hypoxia”, associated with pathogen invasion, in particular bacterial pathogens. During infectious processes, the organ cell composition is dramatically modified; in this model, the “newcomers” are invading pathogens and recruited immune cells. The metabolic activity of the “newcomers” is hypothesized to modify the overall organ oxygen consumption. Hence, hypoxia can be associated with an increased consumption of the available oxygen by bacteria if they are able to consume oxygen as a final electron acceptor for their respiration (aerobic and facultative anaerobic bacteria). It may also be associated with immune cell respiration (mitochondria activity) or Reactive Oxygen Species (ROS) production (NADPH oxidase activity). Both “newcomers” oxygen consumption would represent potential intrinsic causes of hypoxia induction. Until now, no report has addressed this issue, although hypoxia has been described as a critical factor modulation neutrophil antimicrobial functions, notably through a limitation of the oxidative burst mediated by the NADPH oxidase, during *Staphylococcus aureus* infection [22]. The main immune cell population recruited at a site of bacterial infection are neutrophils; their role in the infectious hypoxia induction has not yet been characterized. It has to be
noticed that the alteration of the microcirculation by pathogen cell-surface components or secreted toxins (i.e. Shiga toxins targeting endothelial cells [9,10,23,24]), may also reduce the oxygen supply to the infected organ.

3. Infectious hypoxia during pathogen infection

Hypoxia induction has been reported upon tissue infection by a wide range of pathogens. Most studies were performed in animal models (mouse, rat, rabbit, guinea pig) systemically injected with hypoxia reporters, nitroimidazole derivatives [11,25], such as pimonidazole (i.e. Hydroxyprobe) [12,26] or 2-nitroimidazole (i.e. EF5) [13,27]. These reporters specifically accumulate in hypoxic cells and can subsequently be immune-detected. It is important to note that hypoxia detection using this approach allow the distinction between normoxic and hypoxic conditions but do not allow correlations between the cell oxygenation level and the fluorescence intensity. More importantly, this approach is not a quantitative method, meaning that the cell oxygenation level cannot be determined; its use remains limited to the observation of a pathophysiological decrease of the local oxygen availability (hypoxia detection).

The oxygen metabolism is altered in experimental bacteremia. Intravascular injection of Escherichia coli results in 90% reduction of the hepatic partial pressure of oxygen; as compared to the control, the systemic oxygenation remains stable [14,28]. Tuberculous granulomas are highly hypoxic in mouse and human lungs infected with Mycobacterium tuberculosis. M. tuberculosis mouse lung infection is associated with a massive recruitment of CD4+ T cells, CD8+ T cells, DEC205+ dendritic cells, and F4/80+ macrophages [15,29]. The hypoxic environment of the granuloma was shown to be associated with M. tuberculosis latency [30].

Melican and colleagues performed the first direct oxygen measurement during bacterial infection, using a modified Clark-type microelectrode in a rat extracorporal kidney model infection of E. coli (pyelonephritis model). Strikingly, the kidney oxygen level decreased from 40 mmHg (5% O2) to 5 mmHg (0.6% O2) within 4 h post-infection. The rapid local oxygen consumption and clotting resulted in a localized ischemia [31]. More recently, the oxygen level was quantified in extracorporal mouse ceca infected by Salmonella typhimurium (enterocolitis model) using oxygen-sensitive foil and ratiometric luminescence imaging. Jennewein and colleagues showed that the gut tissue oxygen level decreased from 78 Torr (11% O2) to 16 Torr (2% O2). The authors showed that the local oxygen depletion impaired the phagocyte NADPH oxidase (PHOX) and the nitric oxide synthase activities, enhancing S. typhimurium intracellular replication and survival within macrophages [32]. These pioneering quantification studies of tissue oxygenation during bacterial infection confirmed the infectious hypoxia concept. However, the causes of infectious hypoxia were not identified, and the use of an extracorporal organ model might lead to extra-oxygenation of the organs exposed to atmospheric oxygen, resulting in biased quantitative measurements.

The evolution of partial pressure of the colonic mucosa during Shigella infection has not yet been determined. Shigella specifically target the colon, which is physiologically poorly perfused and oxygenated.

3.1. Physiological oxygenation of the colonic compartments

The colonic luminal compartment is almost anoxic, due to the intense metabolic activity of the large, resident bacterial population, the microbiota (10^10–10^12 cfu/mL feces). In this compartment, the limited amount of oxygen favors anaerobic bacteria and outnumbers aerobic bacteria [33]. The partial pressure of oxygen within the colonic mucosa is estimated around 24 mmHg (3% O2), although for technical reasons, no direct measurement of intact colon in situ with non-disruptive method could yet be achieved in humans. Importantly, the epithelial lineage of the mouse colon has been shown to be physiologically hypoxic, promoting the stabilization and the activation of the transcriptional regulator HIF-1α, described as a key factor for the maintenance of the epithelial barrier function [19]. A thick layer of mucus (between 100 μm and 700 μm in humans [34,35]) protects the colonic tissue from the microbiota. We previously demonstrated that oxygen diffuses through the mucus layer, following Fick's law, establishing an oxygen gradient between the oxygenated colonic mucosa and the quasi-anoxic luminal compartment [36].

When reaching and invading the colonic mucosa, neutrophils are the main immune cells recruited during the shigellosis. They are believed to play a central role in Shigella clearance in vivo. As mentioned above, neutrophils evolve, like Shigella, under various oxygen conditions from their site of production (bone marrow) to the inflamed or infected organ. In the last part of this review, we will describe the neutrophil adaptation to low oxygen conditions during its journey within the human body.

3.2. Physiological adaptation of neutrophils to hypoxic conditions from the bone marrow, to the blood circulation to infected sites

Neutrophils evolve in various oxygenated environments during their lifecycle. They are produced in the bone marrow during haematopoiesis, circulate in the blood and transmigrate in perfused organs during inflammatory or infectious processes. Otherwise, senescent neutrophils are cleared equally in the bone marrow, the spleen, and the liver [37].

Haematopoiesis occurs in the bone marrow, resulting in the production and maturation of circulating blood cells, including neutrophils through the differentiation of Haematopoietic Stem Cells (HSC) (Fig. 1). Mature neutrophils mainly remain stored in the bone marrow (85%), while a limited amount circulates in the blood plasma fraction (15%); this proportion might significantly increase through their rapid mobilization during inflammation or infection, mediated by G-CSF. The bone marrow has long been known to be a hypoxic environment [38], although the precise oxygen tension in the human

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bone marrow remains unknown. The partial pressure of oxygen (pO2) in the mouse bone marrow was recently shown to be heterogeneous, but globally below 32 mmHg (<4% O2); the peri-sinusoidal region seems to be the most hypoxic region (<10 mmHg, <1.2% O2) [39]. However, the intrinsic cause of the bone marrow low oxygen tension remains unclear, considering that this compartment is highly perfused by a dense microcirculation network.

The overall blood partial pressure of oxygen is elevated: the arterial pO2 equals 75–100 mmHg, and the venous pO2 equals 30–50 mmHg [40]. However, the oxygen solubility coefficient is low (0.0031 mL/mmHg of oxygen/dL of blood), and the oxygen is mainly transported by red blood cells combined to haemoglobin. The remaining oxygen fraction dissolved in the blood plasma is estimated around 2% of the total transported oxygen [18]; as a consequence, the plasmatic pO2 equals 0.75–1 mmHg (0.10–0.13% O2) in the arteries and 0.3–0.5 mmHg in the veins (0.04–0.07% O2). As a conclusion, leukocytes, including neutrophils, evolve in the blood plasma fraction under quasi-anoxic conditions, although no experimental data based on direct measurements in situ are yet available. Neutrophils will only face higher partial pressure of oxygen when transmigrating to perfused organs during inflammation or infectious processes. Based on previous reports stating that inflammatory and infectious sites are hypoxic, the exposure of neutrophils to oxygen is hypothesized to be transient.

Neutrophils are well adapted to hypoxic conditions. Neutrophil survival is increased under hypoxic or anoxic conditions, in a HIF-1α-dependent manner [41]. We recently demonstrated that the combination of anoxia and glucose supplementation preserve neutrophil viability and function in vitro [42]. Additionally, it has been shown that HIF-1α was important for neutrophils’ bactericidal functions [43,44].
Adversely, it was shown that hypoxia was a negative factor for bacterial clearance in the lower respiratory tract [45].

Neutrophils are primarily glycolytic; the level of the oxidative phosphorylation is negligible (Seahorse measurements) [46]; these bioenergetics properties are specific to neutrophils, as compared to other lymphocytes, monocytes, or platelets [47]. Consistently, neutrophils contain few mitochondria, which are also relatively small with poorly defined cristae and inner membrane definition. Their role appears to be restricted to apoptosis induction [48-50]. Mitochondria participate additionally to the neutrophil activation [51], chemotaxis, phagocytosis, and respiratory burst activation [52].

In conclusion, neutrophils are well adapted to the low oxygen tension encountered in the colonic mucosa during Shigella infection, which should promote their survival. However, to date, the level of oxygenation of the colonic mucosa during Shigella infection is not known. In addition, the oxygen-dependent modulation of neutrophil antimicrobial activity against Shigella or other bacterial remains elusive.

3.3. Hypoxia induction during Shigella invasion

Prior to reaching the epithelial surface, Shigella need to adapt to various oxygen levels from the lumen, the mucus layer, the colonic mucosa, and the colonic crypts [3]. This physiological adaptation is made possible by the ability of Shigella to use oxygen, but also other electron acceptors, such as nitrate (facultative anaerobe), for aerobic and anaerobic respirations. Oxygen limitation has been shown to modulate pathogenic bacterial adhesion, invasion, and intracellular adaptation (reviewed in Ref. [53]). In addition to oxygen level sensing, other environmental cues, such as the pH, salt concentration, and temperature, should be considered to better characterize the whole infectious process [2]. We and others demonstrated that Shigella sense anaerobic conditions in a Fumarate nitrate reductase regulator (FNR)-dependent manner [36,54,55]. In particular, we demonstrated that S. flexneri T3SA function was blocked in the absence of oxygen, through the FNR-dependent repression of Spa32 and Spa33 expression [36]. Vergara-Irigaray and colleagues confirmed these results by RNA-seq analysis and further showed that a wide range of Shigella flexneri virulence genes located in the virulence plasmid pathogenicity island were repressed under anaerobic conditions, including T3SA major components (MxiAC-DEGJLM) and essential T3SA effectors (IpaA, IpaB, IpaC, IpaD), although confirmatory studies are required to correlate these transcriptional regulations to the modulation of the production of the main virulence factors (ie. IpaB, IpaC) [55]. The ArcA/B two-component transcriptional regulatory system is also important for the adaptation of Shigella flexneri to
anaerobic conditions, particularly for the regulation of iron acquisition genes expression in these conditions [54,56].

However, to date no report describes the evolution of the colonic mucosa’s partial pressure of oxygen upon Shigella invasion, replication, and dissemination and how Shigella adapts to the putative oxygen tension variations. We hypothesized that an infectious hypoxia was induced during Shigella infection. To address this question, young guinea pigs were injected intracardiacly with a hypoxia reporter (EF5) prior to intrarectal infection with Shigella flexneri 5a (M90T). In non-infected animals, consistent with previous reports [19], the epithelial layer was hypoxic (Fig. 2A), probably due to the limited oxygen diffusion in the colonic mucosa from capillaries (<100 μm) and corresponding to a physiological hypoxia. Upon Shigella infection, massive numbers of neutrophils were recruited to the site of infection (Fig. 2B), and an intense hypoxic signal was observed within the infected colonic mucosa, demonstrating that an infectious hypoxia was induced by Shigella infection (Fig. 2B). It seemed that the hypoxia marker co-localized with Shigella foci of infection, although no correlation was confirmed at this stage. This preliminary study does not address the potential causes of infectious hypoxia induction.

The modulation of tissue oxygenation during Shigella infection suggests that the interaction of the pathogen with recruited immune cells may occur first under normoxic conditions during the early steps of the mucosa invasion, which is consistent with the oxygen-dependent activation of Shigella T3SA [36], then within an hypoxic environment during the course of the infection (Fig. 3). In this context, Shigella and immune cells will have to adapt to this changing environment. This statement will have to be taken into account for in vitro models of Shigella interaction with host cells.

4. Conclusion, perspectives

Shigella and neutrophils are well adapted to low oxygen conditions, which are encountered during their journey within the human body until they reach the infectious site. Oxygen is required for the activation of Shigella virulence, through the T3SA activation [36], and neutrophils viability is increased under low oxygen environments [41,42]. Here we demonstrate...
that during the *Shigella* infectious process, an infectious hypoxia is induced and seems to be localized within infectious foci. This observation raises several key questions regarding the causes of this infectious hypoxia induction and the adaptation of *Shigella*'s physiology and virulence, but also the efficiency of neutrophil antimicrobial activity within this context.

First, considering *Shigella* needs oxygen to activate T3SA, it is envisaged that within the hypoxic foci, *Shigella* are no longer able to invade adjacent host cells or use this system to spread from cell-to-cell, as it was described by Campbell-Valois and colleagues [57]. The oxygen-dependent modulation of the production and secretion of the main cell-to-cell dissemination factor, IcsA [58] has not yet been characterized. Second, the neutrophils' antimicrobial functions (phagocytosis, intracellular killing, antimicrobial molecule secretion, ROS production) have not been characterized extensively under low oxygen conditions. NADPH oxidase-dependent ROS production occurring extracellularly and within the phagosome requires molecular oxygen [59]. Their production is hypothesized to be limited within hypoxic foci. It has been recently described that *Salmonella* impair NADPH oxidase function to increase its survival [32].

In conclusion, it still remains elusive whether 1) *Shigella* or neutrophils oxygen consumption is responsible of the infectious hypoxia and 2) whether *Shigella* or the immune response, encompassing neutrophils antimicrobial activities, benefit from the induction of hypoxia within infectious foci. Further studies will be required to answer these key questions and to better understand the importance of the oxygen modulation within the colonic mucosa during shigellosis and to improve therapeutic approaches.

5. Material and methods

5.1. Guinea pig model of *S. flexneri* intrarectal infection

Young guinea pigs (Hartley, <150 g) were anesthetized (Imalgene 100 mg/kg and Rompun 10 mg/kg), and 150 μL of a 10 mM EF5 solution (nitroimidazole, hypoxia marker, University of Pennsylvania School of Medicine) was injected intracardiely one hour before infection. When indicated, guinea pigs were subsequently infected intraretally with $10^9$ CFU exponentially grown *Shigella flexneri* 5a (M90T) pGFP as described previously [60]. Infection occurred during 8 h before animals were sacrificed and infected colons collected (n = 5) and fixed overnight in a 4% paraformaldehyde solution at 4 °C. For immunofluorescence staining, infected guinea pig colon samples were washed in PBS, incubated at 4 °C in PBS containing 15% sucrose for 8 h, followed by an overnight incubation in PBS with 30% sucrose. Samples were frozen in OCT (Sakura) on dry ice. 30 μm sections were obtained using a cryostat CM-3050 (Leica).

5.2. Immunofluorescence and imaging

Samples were immunolabeled overnight with in a ready-to-use solution containing the α-FE5-Cy3 conjugated antibody (supplied by the University of Pennsylvania School of Medicine) supplemented with 0.1% Triton X-100 and 1% Bovine Serum Albumin (Sigma—Aldrich) and 1:1000 MUB40-Alexa405 (1 mg/mL) (derivative from the MUB70 peptide [61], to be published elsewhere) for neutrophil labeling. Samples were washed three times in PBS prior mounting in Prolong Gold Antifade (ThermoFisher).

Sample imaging was performed using a Cell Voyager 1000 (CV1000, Yokagawa electric). Image analysis was performed using the Fiji software [62].

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