The Role of Hypoxia-inducible Factor in Mycobacterium tuberculosis-infected Macrophages

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Tuberculosis is caused by Mycobacterium tuberculosis infection. During M. tuberculosis infection, there is a decrease in the partial pressure of oxygen in the granuloma microenvironment, which causes the hypoxia-inducible factor (HIF) to become stable. HIF functions as a transcription factor that regulates the expression of genes crucial for metabolic adaptation in hypoxic conditions. Recent research suggests that HIF plays a vital role in infectious and inflammatory conditions. Several studies have demonstrated that HIF signaling can enhance macrophages antimicrobial activity and bactericidal effect against M. tuberculosis, such as increasing macrophage autophagy, enhancing the effects of rifampicin, inhibiting p38 MAPK signaling, enhancing the regulation of effector antimicrobial pathways mediated by human β defensin 2 (hBD2) and vitamin D receptor (VDR), redirecting energy metabolism to glycolysis, and producing various cytokines. All these responses ultimately result in the inhibition of intracellular M. tuberculosis growth. HIF has therapeutic implications, potentially being a new candidate for host-directed therapy as a complement to existing antituberculosis drugs. Understanding the role of HIF in macrophages during M. tuberculosis infection and comprehending the host-pathogen relationship with M. tuberculosis is advantageous for developing future therapies.

Keywords: Mycobacterium tuberculosis, macrophages, hypoxia-inducible factor

Introduction

Tuberculosis, caused by Mycobacterium tuberculosis infection, is an airborne infectious disease, which remains a major health problem worldwide.¹,² The treatment of tuberculosis with antibiotics poses a significant challenge due to the increasing prevalence of drug-resistant strains. This necessitates prolonged treatment, resulting in heightened toxicity, the occurrence of side effects, and suboptimal treatment effectiveness. Therefore, the development of new strategies for tuberculosis therapy is imperative to complement existing antibiotic treatments.³-⁵ These strategies aim to enhance the host's immune response without introducing toxicity or fostering drug resistance.
The pathogenesis of tuberculosis is closely associated with the hypoxic microenvironment in the tissue, where tuberculous granulomas form under hypoxic conditions.\textsuperscript{6,7} In this pathological scenario, immune cells must adapt to hypoxia to maintain their function and integrity by stabilizing the hypoxia-inducible factor (HIF). HIF functions as a cellular hypoxia sensor and controls critical functions of immune cells necessary to protect the body from microbial pathogens.\textsuperscript{8,9}

In \textit{M. tuberculosis} infection, a decrease in the partial pressure of oxygen in the granuloma microenvironment leads to the stabilization of HIF-1 levels.\textsuperscript{10} Recent research has highlighted the crucial role of HIF-1 in infectious and inflammatory diseases, emphasizing its significance in improving the antimicrobial function of macrophages and suppressing pathogen growth.\textsuperscript{11-13} This pathway presents a potential target for host-directed therapy against tuberculosis.\textsuperscript{13} This article aims to delve further into the role of HIF in \textit{M. tuberculosis}-infected macrophages, drawing on existing research. It is anticipated that this article will contribute valuable information to serve as a foundation for developing host-directed therapy for tuberculosis.

\textbf{Mycobacterium tuberculosis}

\textit{M. tuberculosis} is an aerobic bacterium in the form of a granular bacillus, which does not have spores, and is not motile. The length and width of \textit{M. tuberculosis} are approximately 1-4 µm and 0.3-0.6 µm, respectively. \textit{M. tuberculosis} grows and reproduces well at a temperature of 37°C with an optimal pH range of 6.4-7.0.\textsuperscript{14} The replication time for \textit{M. tuberculosis} is around 20-36 hours, which is longer than other bacteria.\textsuperscript{15} This is due to the thick outer membrane of \textit{M. tuberculosis} composed of mycolic acid, which hinders nutrient absorption. However, this thick mycolic acid layer can enhance the bacterium's resistance to lysosomal enzymes.\textsuperscript{16,17} The main characteristic of \textit{M. tuberculosis} is the complexity of its cell wall structure, which is rich in polysaccharides and lipids, creating a barrier against various drugs and harmful compounds.\textsuperscript{15,16} On the outer layer, in addition to mycolic acid, there are other components such as lipoarabinomannan, mannoglycoprotein, and mannose-capped lipoarabinomannan (Man-LAM), which are crucial virulence factors for \textit{M. tuberculosis}. Meanwhile, the inner layer consists of components like arabinogalactan, phosphatidyl-myoinositol mannosides (PMI), and peptidoglycan.\textsuperscript{18,19}

\textbf{Macrophages and phagocytosis}

Alveolar macrophages, the frontline defenders of the innate immune response, recognize \textit{M. tuberculosis} components through various receptors, such as mannos receptors, toll-like receptors, and scavenger receptors. The binding between receptors and pathogen ligands activates intracellular signaling, leading to the rearrangement of the actin cytoskeleton, and the formation of pseudopods that surround the bacteria, which result in the formation of a phagocytic cup. Eventually, the bacteria are internalized into the phagosome.\textsuperscript{20,21} Macrophage cells form the phagosome lumen and migrate towards the site of infection, which in turn causes an increase in oxygen (O\textsubscript{2}) consumption because the cell requires ample O\textsubscript{2} to generate energy in the process of oxidative phosphorylation and to activate enzyme complexes involved in phagosome formation.\textsuperscript{22} This is known as the oxygen burst, leading to relative hypoxia in macrophage cells due to increased oxygen needs while oxygen flow remains constant. Macrophage oxygen burst produces reactive molecules that oxidize microbes, damaging proteins and DNA in microbial cells. Hypoxia at the cellular level triggers the activation of HIF transcription factors and will maintain the presence of HIF as long as the infection continues.\textsuperscript{23} Although the active lifespan of HIF protein lasts only a few days and will eventually be degraded, other HIF proteins will continue to be produced, ensuring their presence remains stable within the cell.\textsuperscript{22,24}

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex present in the macrophage membrane oxidizes NADPH to NADP\textsuperscript{+}, producing superoxide (O\textsubscript{2}\textsuperscript{-}). Superoxide acts as an antibacterial agent, although it cannot penetrate bacterial cell walls. Superoxide kills bacteria through non-enzymatic or enzymatic dismutation (assisted by superoxide dismutase enzyme), which generates hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Myeloperoxidase (MPO) reacts with H\textsubscript{2}O\textsubscript{2} and chloride ions, producing hypochlorous acid (HOCl), a potent antimicrobial compound.\textsuperscript{25,26} Unlike superoxide, H\textsubscript{2}O\textsubscript{2} can penetrate cell membranes and kill various microorganisms by forming intracellular OH. H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2} depend on the presence of oxygen.\textsuperscript{21,27} Reactive oxygen species (ROS) interaction with other cellular mediators, such as nitric oxide (NO) and hypochlorous acid, which produces hydroxyl (OH), can enhance ROS cytotoxicity. ROS can directly attack pathogens inside the phagosome.
HIF

HIF is a transcription factor that plays a role in adapting to hypoxic conditions. Changes in oxygen levels trigger the body's response to adapt to such conditions by expressing regulatory genes that maintain body homeostasis in a fluctuating environment. HIF regulates the transcription of genes involved in various processes, such as angiogenesis (e.g., vascular endothelial growth factor; VEGF), glycolysis (e.g., glucose transporter-1; Glut-1), and HIF autoregulation (prolyl hydroxylase (PHD). HIF is a basic helix-loop-helix (bHLH) composed of subunit-α and β that bind to DNA, forming a subunit dimerization. The HIF complex is modulated due to its sensitivity to oxygen.

Under normoxic conditions, HIF subunit-α is hydroxylated on prolyl residues by PHD enzymes. HIF subunit-α is then recognized and ubiquitinated by the Von Hippel Lindau (VHL) protein complex, leading to its degradation by the proteasome. Another factor inhibiting HIF transcription is the factor inhibiting HIF-1 (FIH). FIH hydroxylates asparagine residues of HIF subunit-α, preventing HIF subunit-α from interacting with the transcription coactivator p300. Conversely, under low oxygen levels (hypoxia), PHD enzyme activity decreases, preventing the hydroxylation of HIF subunit-α. This leads to the stabilization and accumulation of HIF subunit-α in the cytoplasm. HIF subunit-α, along with HIF subunit-β, forms a transcription complex that translocates to the nucleus, where this complex binds to the hypoxic response element (HRE) on the target gene promoter (Figure 1).

To maintain the complexity of the response to hypoxia, there are three variations of the HIF subunit-α: HIF-1α, HIF-2α, and HIF-3α. HIF-1α and HIF-2α have similar structures and undergo similar proteolysis mechanisms, but the expression of HIF-2α is much more limited than that of HIF-1α. Several studies explain that there is an increase in the levels of HIF-1α and HIF-2α in hypoxia-induced macrophages, and both transcription factors can translocate to the nucleus. Additionally, it has been found that the expression of HIF-2α lasts longer and persists with re-oxygenation compared to HIF-1α. This indicates that HIF-1α and HIF-2α are transcription factors that collaborate in regulating macrophage function through the activation of a series of different genes in hypoxic conditions.

The role of HIF in M. tuberculosis-infected macrophages

There has been extensive research on HIF-1α to comprehend the host's relationship with M. tuberculosis pathogens as a pharmacological target for managing chronic inflammation, boosting immune responses, and addressing drug-resistant infectious diseases. HIF-1α can regulate various gene expressions crucial for the anti-infective function of M. tuberculosis-infected macrophages. This involves enhancing macrophage autophagy, boosting rifampicin's effectiveness, and transitioning energy metabolism from oxidative phosphorylation to glycolysis. This metabolic shift is achieved by upregulating the expression of glycolytic enzymes to ensure a sustained adenosine triphosphate (ATP) supply under oxygen-limited conditions. Additionally, the HIF response in macrophages also influences expression of antimicrobial peptides (defensin, cathelicidin), production of granule proteases (cathepsin G, neutrophil elastase), NO induction, various cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-6, IL-1, IL-12), and enhances the regulation of antimicrobial effector pathways mediated by human β defensin 2 (hBD-2) and the vitamin D receptor (VDR). All of these responses ultimately result in the inhibition of intracellular M. tuberculosis growth. HIF-1α deficiency in macrophages leads to disrupted macrophage metabolic adaptation to hypoxia, causing decreased macrophage migration and a reduced ability to kill bacteria. Furthermore, in the absence of HIF-1α, the anti-infective effects of interferon (IFN)-γ are compromised, leading to inadequate production of anti-infective factors like prostaglandin E2 (PGE2), NO, cytokines, and inflammatory response factors.
HIF activation enhances autophagy in *M. tuberculosis*-infected macrophages

Recent research indicates that the activation of HIF-1 can enhance macrophage cell autophagy. This action suggests the involvement of the HIF-1 signaling cascade in macrophages. There is an increase in HIF-1 expression in macrophage cells (U937) infected with *M. tuberculosis* H37Rv when treated with CoCl2, a chemical anoxia reagent that inhibits HIF-1 hydroxylation. The elevation of HIF-1 levels is suspected to result from various mechanisms. Initially, it could result from the initiation of toll-like receptor (TLR) mechanisms, counterbalanced by the negative regulatory mechanisms of TLR signaling.

Another potential explanation is the triggering of the cellular hypoxia response, leading to the activation of genes regulated by HIF-1, which encompasses essential genes related to anti-infection cytokines.

Several markers can be used as indicators of autophagic activity, such as LC3. LC3 is a protein known to monitor autophagic activity. The presence of the autophagic molecular marker LC3B was observed in lung tuberculosis tissue in contrast to the normal lung tissue adjacent to lung cancer. Mammalian cells possess three isoforms of LC3, namely LC3A, LC3B, and LC3C. In the autophagy process, LC3B is converted from a soluble form (16kDa) to a lipidated form (14kDa). Lipidated LC3B is widely used as an autophagy marker. The expression of lipidated LC3B was observed in *M. tuberculosis*-infected macrophages, whether or not CoCl2 was administered. However, in *M. tuberculosis*-infected macrophages treated with 3-(5’-Hydroxymethyl-2’-furyl)-1-benzylindazole (YC-1), a HIF-1 inhibitor, there was minimal LC3B lipidation, while those treated with CoCl2 showed a clearer increase in lipidated LC3B. This indicates an increase in U937 macrophage cell autophagy, concurrent with HIF-1 signaling activation.

Another marker that is used to monitor autophagic activity is p62/sequestosome 1 (SQSTM1). During the formation of autophagosomes, the autophagic junction protein p62/SQSTM1 selectively transports substrate proteins to autophagosomes, ultimately leading to its degradation by lysosomes. Therefore, a reduction in p62 levels during autophagy indicates functional autophagy. p62 expression in *M. tuberculosis*-infected macrophages which were treated with YC-1 was not altered. Conversely, the administration of CoCl2 resulted in a notable decrease in p62 in *M. tuberculosis*-infected macrophages. Therefore, the increase in LC3B lipidation and the decrease in p62 in *M. tuberculosis*-infected cells treated with CoCl2 indicate that HIF-1 activation can significantly enhance functional autophagy in killing intracellular bacteria.

HIF activation enhances the bactericidal effect of rifampicin

Rifampicin is a first-line anti-tuberculosis drug that specifically inhibits the action of bacterial RNA polymerase, preventing the transcription process and leading to bacterial death. Therefore, rifampicin significantly influences the effectiveness of eliminating *M. tuberculosis* bacteria. There was a study exploring the impact of HIF-1 activation on the intracellular proliferation of *M. tuberculosis* H37Rv and the bactericidal properties of rifampicin in U937 macrophage cells. The research results indicate that HIF-1 activation can suppress the proliferation of *M. tuberculosis* H37Rv and enhance the anti-tuberculosis effect of rifampicin on macrophages. In conclusion, these results propose that targeting HIF-1 activation holds promise in enhancing the anti-tuberculosis capabilities of macrophages and the bactericidal effects of rifampicin, a crucial anti-tuberculosis drug.

HIF activation inhibits the p38 mitogen activation protein kinase (MAPK) pathway

During *M. tuberculosis* infection, p38 MAPK regulation is activated either directly through secreted factors and bacterial cell wall components or indirectly through the liberation of pro-inflammatory cytokines, such as TNF or IL-1, from host cells that are activated. Therefore, the p38 MAPK signaling pathway orchestrates various inflammatory, stress, and necrotic cell death responses in host cells that have been infected with *M. tuberculosis*. Increased p38 MAPK becomes a pathogen target to enhance its virulence and survival through replication. The initiation of the p38 MAPK pathway in response to *M. tuberculosis* infection results in the buildup of PGE2, cyclooxygenase II, and the liberation of cyclic AMP. This, in turn, culminates in the excretion of matrix metalloproteinase (MMP)-1, secretion known to play a crucial role in *M. tuberculosis* pathogenesis and tissue damage. Thus, p38 MAPK activation serves as a key signaling player in *M. tuberculosis* pathogenesis. A study indicates that inhibiting p38 MAPK in *M. tuberculosis*-infected C57BL/6 mice can reduce the inflammatory...
response, granuloma formation, lung pathology, and enhance antibiotic activity. This effect is marked by a significant reduction in mycobacterial counts in the lungs compared to antibiotic treatment alone. Therefore, inhibiting p38 MAPK in M. tuberculosis-infected tissues can create an immunologically protective microenvironment, potentially serving as a suitable treatment for M. tuberculosis infection. In a recent study, THP-1 macrophage cells were stimulated with M. tuberculosis extract and treated with or without molidustat. Molidustat is a compound that prevents the degradation of HIF-1α by inhibiting PHD, resulting in the stabilization of HIF-1α. This stabilization is triggered by M. tuberculosis antigen signaling through TLR, causing HIF-1α to translocate into the nucleus. Molidustat treatment resulted in a decrease in the regulation of p38 MAPK in M. tuberculosis-infected macrophages. These results indicate that stabilizing HIF with molidustat inhibits the p38 MAPK signaling pathway. This suggests that HIF-1-induced p38 MAPK inhibition may be utilized as an additional complementary therapy targeted at the host in addition to anti-mycobacterial drugs.

HIF activation induces an increase in the regulation of VDR and hBD-2

VDR is a receptor that regulates gene expression for immune function, is involved in cytokine production, and influences vitamin D status as well as susceptibility to tuberculosis. VDR has a direct effect on immune function by diminishing the effects of complement factor C5a (chemotactic protein) and enhancing macrophage activity. The possible mechanism through VDR that may prevent or limit M. tuberculosis infection involves increasing the binding of bioactive vitamin D (1,25-dihydroxycholecalciferol) to VDR. Through this mechanism, calcitriol stimulates the expression of various natural antimicrobial peptides, notably cathelicidin LL 37 and β-defensin, while inhibiting matrix metalloproteinase enzymes responsible for breaking down the extracellular matrix in the lungs. hBD-2, an antimicrobial peptide with cationic properties, plays a role in immunity against M. tuberculosis, as blood monocytes transfused with hBD-2 have been reported to control the growth of M. tuberculosis better than native monocytes. The modulation of macrophage immune responses to the M. tuberculosis pathogen can be achieved through the stabilization of HIF using the PHD inhibitor molidustat. Molidustat stabilizes HIF-1α, which in turn induces the expression of VDR and hBD-2, resulting in the inhibition of M. tuberculosis proliferation within the cell. Molidustat significantly increases the expression of VDR and hBD-2 compared to the untreated control. The heightened expression of VDR and hBD-2 is crucial for determining the antimicrobial pathway in human macrophages.

These immunological effects influence the intracellular growth of M. tuberculosis, leading to a decreased growth of aggressive M. tuberculosis within human macrophages. The quantity of viable bacteria is notably reduced in macrophages treated with molidustat, and this effect is dose-dependent. The growth inhibition caused by molidustat closely corresponds to the growth hindrance induced by hypoxia. Therefore, HIF presents itself as a new and captivating prospect for direct intervention against infectious diseases instigated by intracellular tuberculosis bacteria. It acts as a supplementary element to current antibiotic treatments by boosting the antimicrobial function of macrophages. Therefore, the increase in HIF-1α can induce the expression of VDR and hBD-2, determining the response to the antimicrobial pathway in macrophages for the elimination of M. tuberculosis bacteria.

HIF activation modifies macrophage metabolism by enhancing glycolysis and inducing metabolic reprogramming of the citric acid cycle

HIF-1α is a transcription factor that presents in all myeloid cells, including macrophages. HIF-1α controls various genes involved in regulating immune function, cell division, and metabolism. Modifications to energy metabolism orchestrated by HIF-1α in macrophages disrupt the replication of M. tuberculosis. Firstly, elevated intracellular levels of HIF-1α stimulate glycolysis, mirroring the Warburg effect observed in tumors. The increased consumption of glucose in glycolysis results in heightened lactate formation. Some reports suggest that lactate monomers can activate macrophages, thereby enhancing their ability to eliminate intracellular M. tuberculosis. Secondly, elevated HIF-1α levels lead to a metabolic alteration in the citric acid cycle, influencing the generation of essential metabolites such as succinate and itaconic acid. Succinate can stabilize HIF-1α itself and acts as an inflammatory signal. Meanwhile, itaconic acid, which is generated through the decarboxylation of the cis-aconitate intermediate in the tricarboxylic acid cycle, can directly inhibit M. tuberculosis
growth. Mechanically, this inhibition occurs through the suppression of bacterial isocitrate lyase, a key enzyme in the glyoxylate shunt responsible for bacterial growth. In mice, heightened regulation of HIF-1α and glucose metabolism has been demonstrated to be crucial for macrophage migration activity, contributing to bactericidal activity dependent on HIF-1α. Therefore, the elevation of HIF can modify macrophage metabolism, resulting in various metabolite products that can inhibit the growth of *M. tuberculosis*. Although numerous studies have elucidated various roles of HIF in macrophage cells infected with *M. tuberculosis* bacteria, further research is still needed to delve deeper into these roles and explore other factors involved in enhancing immune function mediated by HIF.

**Conclusion**

The infection of *M. tuberculosis* causes the HIF in macrophages to stabilize as the cells experience a hypoxic condition. HIF regulates the expression of essential genes for metabolic adaptation during hypoxia. HIF signaling can enhance the antimicrobial activity and bactericidal effect of macrophages against *M. tuberculosis*. This includes increasing macrophage autophagy, enhancing the effects of rifampicin, inhibiting p38 MAPK signaling, upregulating the regulation of effector antimicrobial pathways mediated by hBD-2 and VDR, and redirecting energy metabolism to glycolysis. All of these responses result in the inhibition of intracellular *M. tuberculosis* growth. Knowledge of the function of HIF in macrophages during *M. tuberculosis* infection is beneficial for the development of future therapies.

**Authors Contribution**

NF contributes to the creation of initial written material, FCI serves as an editorial contributor, and MS contributes as an idea provider.

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