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Hypoxia in Plaque Macrophages: A New Danger Signal for Interleukin-1β Activation?

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The recruitment of inflammatory cells to the arterial wall and their critical role in increasing plaque size and complexity is now dogma in the field of atherosclerosis [1]. Macrophages compose the majority of the inflammatory burden in plaques and incite many of the deleterious responses that exacerbate disease. Thus, the mechanisms by which macrophages are activated to secrete cytokines and other inflammatory mediators are of intense interest. The atherosclerotic milieu is replete with cellular stressors such as modified ApoB-containing lipoproteins (e.g. oxidized LDL) and reactive oxygen species (ROS) which can act as triggers of the inflammatory response. These Danger signals compose a repertoire of triggers of so-called “sterile inflammation” that characterizes atherosclerosis.

The prototypical mediator of sterile inflammation is the pro-inflammatory cytokine IL-1β[2]. Due to its potent downstream effects, the production of mature, biologically active IL-1β is tightly regulated at two distinct steps [3]. In the priming phase, activation of signaling pathways such as NF-κB by Toll-like receptors, other cytokines, or IL-1β itself, leads to the transcriptional induction of precursor IL-1β (pro-IL-1β). Generation of the mature form is then governed by activation of a complex of proteins known as the inflammasome. Although several distinct complexes with unique triggers have been described, the NLRP3 inflammasome composed of nucleotide-binding oligomerization domain (NOD)-like receptor family member (NLRP3), apoptosis-associated speck-like protein (ASC), and pro-Caspase-1 is the most relevant to metabolic diseases such as atherosclerosis. A variety of pathogen- or endogenously-derived danger signals (known as pathogen- or damage-associated molecular patterns, PAMPs and DAMPs) can activate the NLRP3 inflammasome, leading to proteolytic activation of Caspase-1 and consequent cleavage of pro-IL-1β to the mature form [3]. The process of inflammasome activation and its contribution to atherogenesis has been the focus of significant recent investigation in the field. Cholesterol crystals, which were previously thought to be inert byproducts of aberrant lipid metabolism in the atherosclerotic plaque, have now been shown to be potent inducers of the NLRP3 inflammasome and IL-1β secretion in macrophages, akin to other crystalline DAMPs [4-6].

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Even the pro-inflammatory action of oxidized LDL appears to occur in part through CD36-mediated uptake into lysosomes and conversion to cholesterol crystals [7].

Given the ability of IL-1β to further enhance the pro-inflammatory response and recruitment of immune cells, its critical role in exacerbating atherosclerotic progression has long been appreciated [8]. Thus, the discovery of endogenous stressors found in the atherosclerotic milieu with profound effects on the macrophage IL-1β response is of significant interest. In this regard, the role of one such cellular stressor, hypoxia, on inflammatory signaling and atherosclerosis is intriguing. The presence of hypoxia, which is observed in both human atheroma and animal models of atherosclerosis, is consequential particularly in advanced plaques with increased hypercellularity and lesion complexity [9, 10]. Local hypoxia is thought to arise from a combination of increased metabolic demand in macrophages and reduced oxygen supply stemming from increased diffusion distances across the complex lesion [11, 12]. The role of hypoxia as a trigger for numerous atherogenic cellular responses is supported by a large body of literature [13]. A universal feature of these responses appears to involve the stabilization and transcriptional activation of hypoxia-inducible factor-1α (HIF-1α) [14]. At lower oxygen levels, cells are known to produce mitochondrial ROS, leading to the stabilization of HIF-1α[15]. HIF-1α induces proteolytic, pro-angiogenic, pro-apoptotic, and other destabilizing factors that increase plaque complexity [12, 13]. Hypoxia also distinctly alters cellular metabolism, where a shift to anaerobic glycolysis occurs [13]. This affects cellular energy balance and the availability of metabolic intermediates required for optimal cellular function. An important outcome is the alteration of macrophage lipid homeostasis toward a foam cell formation phenotype [16]. Not surprisingly, hypoxia-induced cellular derangements in the plaque result in accelerated atherosclerosis in several pro-atherogenic animal models [17-19].

Despite the many parallels between hypoxic and pro-inflammatory effects on plaque progression, few direct links between hypoxia and pro-inflammatory signaling pathways (including IL-1β signaling) have been described [12, 13]. Hypoxic conditions can activate the NF-κB pathway through as yet undefined mechanisms [20]. Several reports have shown that hypoxia can induce the transcription of pro-inflammatory cytokines, especially IL-1β [21, 22]. Most recently, work by Tannahill, *et al* detailed a novel mechanism by which lipopolysaccharide (LPS) stimulates IL-1β transcription through HIF-1α, a process that depends on succinate and metabolic switching of macrophages to glycolysis [23]. Although the synergistic secretion of IL-1β upon exposure to both LPS and hypoxia was noted, the specific role of hypoxia in IL-1β transcription was not evaluated. An overview of the current understanding of the regulation of IL-1β production with links to hypoxia in atherosclerotic macrophages is shown in Figure 1A.

In the current issue of Circulation Research, Folco *et al* suggest a novel link between hypoxia, inflammasome activation, and induction of the macrophage IL-1β response [24]. In order to gain a holistic understanding of IL-1β production under hypoxic conditions, Folco and colleagues interrogated the effect of moderate hypoxia in human macrophages and plaques at several levels of regulation, including transcription, pro-IL-1β processing, and inflammasome activation. First, they make the interesting observation that hypoxia synergistically elevates LPS-induced pro-IL-1β levels in a manner independent of

transcription. Follow-up pulse-chase experiments confirmed slower pro-IL-1β degradation under hypoxia, an observation that implicates either proteosomal or autophagic dysfunction. Several prior reports have suggested a complex role for autophagy in IL-1β production. Autophagy can both facilitate the degradation of pro-IL-1β [25] as well as dampen the ability of the NLRP3 inflammasome to convert pro-IL-1β to its active form [6, 26, 27]. Using the potent lysosomal (and by extension, autophagy) inhibitor Bafilomycin, Folco *et al* note that the profound difference in LPS-induced pro-IL-1β accumulation between hypoxic and normoxic conditions is abrogated. Buttressing their data with two markers of autophagy, p62 and LC3, they conclude that disruption of autophagic degradation is a prominent mechanism by which hypoxia increases pro-IL-1β levels.

At first pass, a hypoxia-mediated disruption of autophagic degradation is at odds with several previous reports demonstrating reduced oxygen levels in potent induction of autophagy, a process that is HIF-1α dependent [28]. In agreement with this literature, when Folco *et al* compare the rate of autophagic flux in their experimental model, the protein levels of two well-known targets of autophagic degradation (p62 and LC3) are indeed reduced under hypoxic conditions, suggesting increased autophagy. How could it be possible that autophagic degradation of pro-IL-1β can have slower kinetics with hypoxia while autophagy pathways are induced? Folco and colleagues suggest that selective autophagy might be the answer. The discriminatory capacity of cells to target specific proteins or organelles for autophagic degradation is known as selective autophagy and a repertoire of proteins have been described to date that mediate this process [29, 30]. A well-characterized mechanism for the selective targeting of proteins for autophagic degradation involves protein polyubiquitination, binding to the chaperone p62, and cargo delivery to autophagosomes via p62's LC3-binding domain [29, 30]. As evidence for such a process occurring in macrophages, Folco *et al* use immunofluorescence microscopy to show a loss of p62 co-localization with pro-IL-1β in hypoxic conditions. This observation would imply that with hypoxia, p62's interaction with competing proteins/organelles is favored, thus limiting p62 availability for selective pro-IL-1β degradation. At present, it is unknown whether pro-IL-1β undergoes polyubiquitination and the degree to which it interacts with and is cleared by a p62-dependent process, but this interesting possibility can be further evaluated *in vitro* (e.g. reconstitution experiments where the ubiquitin-binding domain of p62 is manipulated). It is noteworthy that polyubiquitination of the inflammasome complex and p62-dependent autophagic degradation has also been proposed as an alternative mechanism of limiting IL-1β production [27].

Folco and colleagues go on to implicate hypoxia at another level of IL-1β production, the NLRP3 inflammasome. They show that induction of IL-1β secretion by LPS is synergistically activated only upon incubation of macrophages with the known inflammasome trigger, cholesterol crystals, under hypoxic conditions. They support this finding by demonstrating selectivity for IL-1β (i.e. no changes were noted in TNFα and IL-6), concomitant elevation of cleaved (i.e. activated) caspase-1, an abrogation of this activation by caspase-1 inhibition, and a similar synergistic relationship between hypoxia and another potent inflammasome activator Nigericin. They suggest that at least part of the hypoxia-induced inflammasome hyperactivation is related to transcriptional increases in NLRP3, an essential component of the inflammasome complex. Finally, as a link to the *in*

vivo setting, they demonstrate that cholesterol crystals, IL-1β, activated caspase-1, and several markers of hypoxia such as HIF-1α are highly co-localized to the same macrophage-rich areas of human atherosclerotic plaques. Surprisingly, the IL-1β response of human macrophages to LPS and cholesterol crystals showed mild non-significant elevations, akin to LPS and hypoxia. Although this is distinctly different that the robust elevations of IL-1β when similar assays are conducted in murine macrophages [4, 6, 23], differences in experimental design as the authors suggest or inherent differences between human and murine macrophages might be an explanation. Also the precise mechanism by which hypoxia synergistically activates the inflammasome complex warrants further investigation. Is hypoxia-induced transcriptional upregulation of NLRP3 the predominant reason for enhanced inflammasome activity? Is hypoxia-induced NLRP3 transcription dependent on HIF-1α activation? An enticing alternative mechanism is hypoxia's role in more direct cytoplasmic activation of the inflammasome complex. Lower oxygen levels are a potent trigger for mitochondrial ROS [15] which in turn is one of the most potent triggers of the inflammasome complex [26]. Furthermore, cholesterol crystals among other crystalline DAMPs are thought to activate the inflammasome by disrupting the membrane integrity of lysosomes [4]. Thus, it would be interesting to evaluate whether hypoxia exacerbates crystalline-mediated lysosomal membrane integrity.

An overview of the role of hypoxia in atherosclerotic macrophage IL-1β production as proposed by Folco and colleagues is shown in Figure 1B. Taken together, these data support the notion that hypoxia imparts similar effects as other DAMPs and relevant inflammasome activators such as cholesterol crystals and implicates hypoxia as a previously unrecognized danger signal in atherosclerosis. In order to control the indiscriminate activation of IL-1β, nature has placed multiple regulatory steps in the processing of this potent pro-inflammatory cytokine. Hypoxia now adds a fascinating twist to the ever-increasing complexity of IL-1β regulation, the mastery of which will expand our understanding of clinically important chronic inflammatory conditions such as atherosclerosis.

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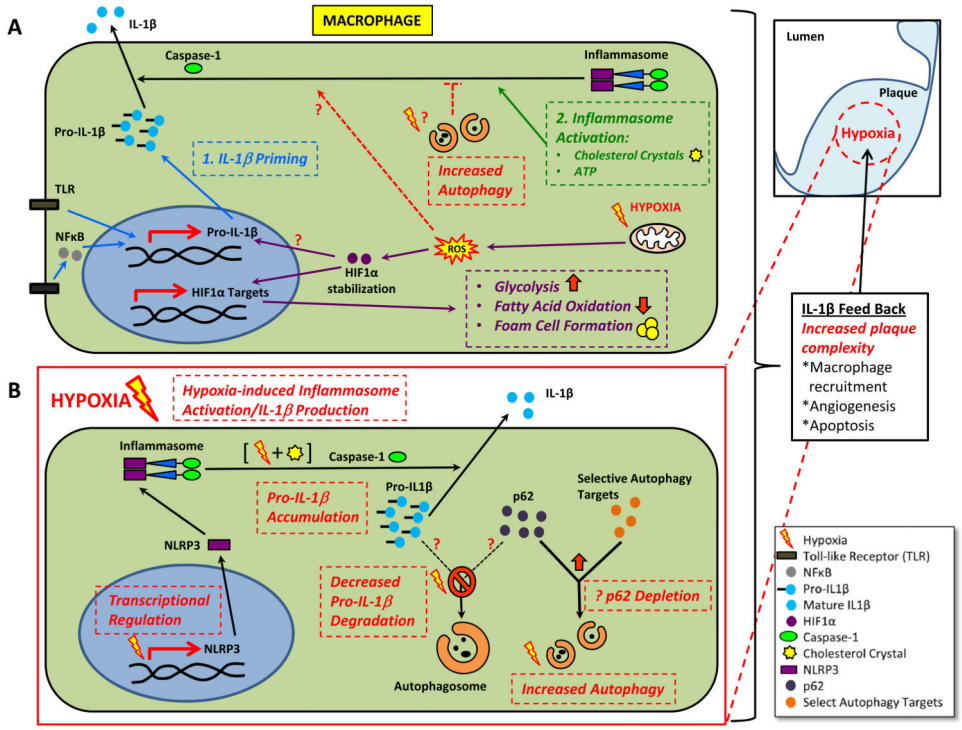

Figure 1. Current and Proposed View of IL-1β Regulation in the Context of Hypoxia in Atherosclerotic Macrophages

(A) IL-1β is generated by a 2-step process: 1) priming where pro-IL-1β can be transcriptionally induced by TLR ligands or cytokines, and 2) inflammasome activation where DAMPs such as cholesterol crystals can trigger inflammasome formation, caspase-1 activation, and cleavage of pro-IL-1β to mature/secretable IL-1β. Autophagy is known to be inhibitory to inflammasome activation. The relevant previously described roles of hypoxia are also depicted. Hypoxia can activate autophagy with unknown significance on inflammasome inhibition. Hypoxia can also induce mitochondrial ROS leading to HIF-1α stabilization with potential upregulation of pro-IL-1β transcripts. More characterized downstream effects of the hypoxia/HIF-1α axis are a metabolic shift to glycolysis and alterations in fatty acid metabolism favoring foam cell formation.

(B) The effects of hypoxia at various levels of IL-1β processing as proposed by Folco *et al* is depicted. Hypoxia can selectively prevent the degradation of pro-IL-1β by limiting its interaction with the p62 chaperone through as yet unclear mechanisms. A possibility is hypoxia-mediated induction of other p62-dependent autophagic degradation processes, limiting p62 availability. Hypoxia can lead to transcriptional induction of NLRP3, a key component of the inflammasome. The combination of hypoxia and known DAMPs such as cholesterol crystals can synergistically activate the inflammasome complex, caspase-1 activation, and cleavage of pro-IL-1β to mature/secretable IL-1β. It is unclear if this activation is solely dependent on increased NLRP3 levels or is further regulated in the cytoplasm.