Moderate Hypoxia Potentiates Interleukin-1β Production in Activated Human Macrophages

Eduardo J. Folco, Galina K. Sukhova, Thibaut Quillard, Peter Libby

Rationale: Inflammation drives atherogenesis. Animal and human studies have implicated interleukin-1β (IL-1β) in this disease. Moderate hypoxia, a condition that prevails in the atherosclerotic plaque, may conspire with inflammation and contribute to the evolution and complications of atherosclerosis through mechanisms that remain incompletely understood.

Objective: This study investigated the links between hypoxia and inflammation by testing the hypothesis that moderate hypoxia modulates IL-1β production in activated human macrophages.

Methods and Results: Our results demonstrated that hypoxia enhances pro-IL-1β protein, but not mRNA, expression in lipopolysaccharide-stimulated human macrophages. We show that hypoxia limits the selective targeting of pro-IL-1β to autophagic degradation, thus prolonging its half-life and promoting its intracellular accumulation. Furthermore, hypoxia increased the expression of NLRP3, a limiting factor in NLRP3 inflammasome function, and augmented caspase-1 activation in lipopolysaccharide-primed macrophages. Consequently, hypoxic human macrophages secreted higher amounts of mature IL-1β than did normoxic macrophages after treatment with crystalline cholesterol, an endogenous danger signal that contributes to atherogenesis. In human atherosclerotic plaques, IL-1β localizes predominantly to macrophage-rich regions that express activated caspase-1 and the hypoxia markers hypoxia-inducible factor 1α and hexokinase-2, as assessed by immunohistochemical staining of carotid endarterectomy specimens.

Conclusions: These results indicate that hypoxia potentiates IL-1β expression in cultured human macrophages and in the context of atheromata, therefore unveiling a novel proinflammatory mechanism that may participate in atherogenesis.

Key Words: autophagy • hypoxia • inflammasomes • inflammation • interleukins

Inflammation plays a primordial role in all phases of atherogenesis, from the initial recruitment of innate and acquired immune cells to the arterial wall to later atherothrombotic events. Proinflammatory cytokines and chemokines secreted from these immune cells critically mediate the inflammatory response that operates in atherosclerosis. Among these cytokines, interleukin-1α (IL-1α) and IL-1β have received particular attention. Both cytokines signal through the IL-1 type I receptor. The balance of these 2 IL-1 isoforms and IL-1 receptor antagonist, a member of the IL-1 family that acts as an endogenous competitive inhibitor, modulates cytokine-receptor interactions and downstream signaling. IL-1α generally remains associated with the plasma membrane of the cell that produces it and therefore acts locally, whereas IL-1β can act at a distance after being proteolytically processed and secreted primarily by monocytes and macrophages. A diverse array of extracellular danger signals triggers the assembly of inflammasomes—large multiprotein platforms containing caspase-1 that process and activate the IL-1β precursor. In vitro and in vivo experiments have also implicated several noninflammasome proteases in the processing of pro-IL-1β, including the matrix metalloproteinases (MMPs) -2, -3, and -9; the sulfhydryl proteases cathepsin B and cathepsin L; and several neutrophil proteases.
and the association of circulating levels of IL-1β with risk factors of coronary artery disease, have indicated the participation of IL-1β in the development of atherosclerosis in human subjects. The ongoing Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), a large phase 3 clinical trial, tests directly the hypothesis that neutralization of IL-1β reduces the incidence of thrombotic events in patients after myocardial infarction who remain at high risk because of persistent inflammation despite standard-of-care treatment.

Clinical trial, tests directly the hypothesis that neutralization of IL-1β reduces the incidence of thrombotic events in patients after myocardial infarction who remain at high risk because of persistent inflammation despite standard-of-care treatment including intensive statin therapy.

Human and experimental atheromata have hypoxic regions that result from high oxygen consumption and diffusion limitations in the plaque. Although severe hypoxia prevails in deep regions of atherosclerotic plaques, most cells within atheromata, including macrophages in the rupture-prone shoulder regions, experience chronic moderate hypoxia (2–5% O2). Chronic or intermittent hypoxia augment atherogenesis in mice. Several mechanisms may contribute to atheroma evolution and complications elicited by hypoxia. Low oxygen tension stimulates plaque angiogenesis, which may promote lesion growth, intraplaque hemorrhage, heme/iron-catalyzed oxidative stress, and recruitment of inflammatory cells. Hypoxia favors foam cell formation by promoting fatty acid synthesis and inhibiting fatty acid oxidation and cholesterol efflux in mononuclear phagocytes. Hypoxic cells shift their metabolism from mitochondrial respiration to anaerobic glycolysis, resulting in production of reactive oxygen species and reduced ATP availability—conditions that predispose to cell death and contribute to the formation of the plaque’s necrotic core. Hypoxia may contribute to the catabolism of the extracellular matrix, and therefore to lesion evolution, by inducing the expression of MMP-1, MMP-7, and MMP-9 and by increasing the activity of the lysosomal proteinases cathepsin S, cathepsin K, and cathepsin L, because of the pH drop that follows lactate production.

The studies described above have indicated that hypoxia may conspire with inflammation to promote the evolution and clinical complications of atherosclerosis, by mechanisms as yet incompletely understood. This study explored a possible intersection between hypoxia and inflammation by testing the hypothesis that moderate hypoxia potentiates IL-1β production in activated human macrophages. Our results demonstrate that cell exposure to hypoxia evokes distinct mechanisms that result in augmented release of secreted IL-1β. Hypoxia retards the autophagic degradation and therefore induces intracellular accumulation of pro-IL-1β and also augments NLRP3 expression and caspase-1 activity in human macrophages. Furthermore, we demonstrate that IL-1β colocalizes with markers of hypoxia and with activated caspase-1 in human carotid atherosclerotic lesions, uncovering 2 novel mechanisms by which hypoxia can aggravate this arterial disease by promoting inflammation.

### Methods

#### Reagents

Ultrapure *Escherichia coli* lipopolysaccharide and bafilomycin A1 were purchased from Invivogen (San Diego, CA), cholesterol and nigericin from Sigma (St Louis, MO), Z-YVAD-FMK from R&D Systems (Minneapolis, MN), and GM-6001 from Enzo Life Sciences (Farmingdale, NY). Cholesterol crystals were prepared as described.

#### Cell Culture

Human peripheral blood monocytes were isolated from freshly prepared leukocyte concentrates from healthy donors and differentiated to macrophages as described. Macrophages were preincubated for 24 hours in RPMI 1640 containing 5% human serum, under normoxic or hypoxic conditions, followed by the addition of the indicated reagents for various periods of time as described in each experiment. For culture in a hypoxic environment, experiments were conducted in Modular Incubation Chambers MIC-101 (Billups-Rothenberg, Del Mar, CA) filled with a gas mixture containing 2% O2, 5% CO2, and 95% N2.

#### Immunoblot

Whole-cell lysates from 25,000 cells were fractionated on 4% to 12% gradient SDS-PAGE gels (Life Technologies, Grand Island, NY) and transferred to polyvinylidene difluoride membranes. After blocking with 5% defatted milk and incubating with the appropriate antibodies, membranes were developed using a chemiluminescence reagent (Thermo Scientific, Waltham, MA). Anti-IL-1β was from Santa Cruz (Santa Cruz, CA), and anti-β-actin, anti-sequestosome 1 (SQSTM1)/p62, anti-cleaved caspase-1, anti-NLRP3, and anti-LC3B were from Cell Signaling (Danvers, MA). Densitometric analyses were performed using the National Institutes of Health Image J processing system.

#### Cytokine Assays

The concentrations of IL-1β, tumor necrosis factor-α, and IL-6 in culture supernatants were measured by ELISA, according to the manufacturer’s instructions. The IL-1β ELISA kit was purchased from Biologend (San Diego, CA), and the tumor necrosis factor-α and IL-6 kits were purchased from R&D Systems.

#### Metabolic Labeling and Pulse-Chase Experiments

Macrophages were primed for 3 hours with 5 ng/mL lipopolysaccharide in RPMI 1640 containing 5% human serum and radiolabeled with 0.4 μCi/mL of [35S] methionine and [3H] cystine (EXPRESS; Perkin Elmer, Waltham, MA) in the same medium for 4 hours. Cells were harvested (0 minutes) or washed 3× with PBS and incubated for various time intervals in complete medium supplemented with 1 mmol/L Met and 1 mmol/L Cys. After lysing cells with NP-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40, and a cocktail of protease inhibitors [Boston Bioproducts, Ashland, MA]) and centrifuging cell lysates at 10,000 rpm for 15 minutes, aliquots from the supernatants containing the same amount of protein were immunoprecipitated with IL-1β antibody, washed 3× with NP-40 lysis buffer, separated by SDS-PAGE, and analyzed by fluorography.

#### RNA Isolation and Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed by Superscript II (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reaction was performed in a MyiQ Single-Color Real-Time PCR system using SYBR Green I (Bio-Rad, Hercules, CA). The mRNA levels of the genes tested were normalized to 18S as an internal control. The primer sequences were: 18S, 5′-ATGGGCTTCTTAGTGGTG-3′ and 5′-GAAAGCCACTTGCTCCCTA-3′; IL-β, 5′-AAAGCTTTGTGATGCTGTC-3′ and 5′-GGACATGGAGAACACCACTTG-3′; NLRP3, 5′-ACCTGGGGGTCATGATGTT-3′; and 5′-TGTCCTACATTTAATTTG-3′.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BMDC</td>
<td>bone marrow-derived macrophage</td>
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<tr>
<td>CC</td>
<td>cholesterol crystal</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor 1α</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>SQSTM1</td>
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Immunofluorescence
Cells were fixed with 4% paraformaldehyde for 20 minutes at 4°C, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum and 1% bovine serum albumin in PBS, and incubated with rabbit polyclonal anti-IL-1β (Santa Cruz) and mouse monoclonal anti-SQSTM1/p62 (Abcam, Cambridge, MA) antibodies, followed by incubation with goat antirabbit IgG-Alexa 535 and goat antimouse IgG-Alexa 488 (Invitrogen). After staining nuclei with DAPI (Invitrogen), slides were mounted with Dako fluorescent mounting medium (Dako, Carpinteria, CA). Images were acquired in an Olympus BX63 fluorescence microscope, processed with Fluoview imaging software (Olympus), and analyzed blindly for fluorescent label colocalization using Image-Pro Plus (Media Cybernetics, Rockville, MD).

Immunohistochemical Study
Surgical specimens of human carotid plaques from endarterectomies (n=8) and of nonatherosclerotic arteries from cardiac transplantation donors (n=3) were obtained through protocols approved by the Human Investigation Review Committee at Brigham and Women’s Hospital. For colocalization of hypoxia-inducible factor 1α (HIF-1α) with hexokinase-2, caspase-1, and IL-1β, we used serial 6-μm frozen sections. The sections were incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, followed by overnight incubation at 4°C with mouse monoclonal antibodies to CD68 (1:900; clone KP1; Dako) or HIF-1α (1:150; BD Biosciences, San Diego, CA), or rabbit polyclonal antibodies to cleaved caspase-1 (1:10; Cell Signaling), hexokinase-2 (1:20; Cell Signaling) or IL-1β (1:150; Santa Cruz). Subsequent processing was performed with the Universal Dako LSAB kit (peroxidase; Dako Cytomation). Nuclear HIF-1α staining was visualized with DAB (brown nuclei), and sections were counterstained with methyl green (ready-to-use; Dako). Staining performed with all other antibodies was visualized with 3-amino-9-ethyl carbazole (red cytoplasm; ready-to-use; Dako) and counterstained with Gill hematoxylin solution (Sigma). The specificity of the antibodies used was determined by omitting primary antibodies, which led to negligible staining (not shown). For oil red O staining, air-dried sections were rehydrated in PBS, fixed in 4% paraformaldehyde, briefly rinsed with 60% isopropanol, and stained with oil red O for 30 minutes. After 2 short rinses with 60% isopropanol, sections were washed with water and counterstained with hematoxylin.66 Cholesterol crystals were visualized by cross-polarized light microscopy in adjacent sections fixed with 10% formalin that were not treated with any organic solvent. Micrographs of stained sections were taken with a Nikon (DXM1200F) digital camera attached to a Nikon (OPTIPHOT-2) microscope.

Results

Hypoxia Induces IL-1β Expression by Stabilizing Pro-IL-1β Protein in Human Macrophages
Treatment of human macrophages with lipopolysaccharide, a potent Toll-like receptor-4 agonist, induces the expression of many proinflammatory genes, including IL-1β. To test the effect of moderate hypoxia on lipopolysaccharide-induced pro-IL-1β expression, we exposed cells to 2% O2 for 24 hours, followed by treatment with lipopolysaccharide under the same hypoxic conditions for various periods of time. Compared with cells maintained under normal O2 tension, cells subjected to hypoxia accumulated substantial pro-IL-1β protein, as evaluated by immunoblot (Figure 1A and 1B), but contained similar levels of IL-1β mRNA after lipopolysaccharide stimulation (Figure 1C). Thus, hypoxia exerts strong post-transcriptional control of pro-IL-1β concentration.

The rates of translation and of proteolytic degradation determine the steady-state levels of intracellular proteins. Radwan et al77 have shown that tyrosine kinase 2 exerts translational control of IL-1β production in mouse bone marrow–derived macrophages (BMDMs). We did not find evidence supporting a role of hypoxia on tyrosine kinase 2 activation in human macrophages (not shown). We therefore hypothesized that hypoxia would reduce the degradative rate of pro-IL-1β and tested this conjecture in pulse-chase experiments. Radiolabeled pro-IL-1β decayed rapidly in normoxic macrophages (t1/2 of 2.74±1.25 hours), whereas exposure of cells to hypoxia notably stabilized pro-IL-1β (T1/2=6.79±0.75 hours; P<0.05 versus normoxia; Figure 2).

Paradoxically Reduced Autophagic Catabolism Contributes to Pro-IL-1β Protein Accumulation in Hypoxic Human Macrophages
Harris et al38 recently reported that autophagic degradation controls intracellular levels of pro-IL1β in immortalized mouse BMDMs. We therefore evaluated the involvement of...
autophagy in regulating pro-IL-1β concentration in primary cultures of human macrophages and investigated whether hypoxia affects pro-IL-1β targeting to the autophagic pathway. The autophagy inhibitor bafilomycin A1, a vacuolar H^+ ATPase inhibitor that prevents fusion of autophagosomes with lysosomes, strongly augmented accumulation of pro-IL-1β in normoxic cells, indicating that autophagy controls pro-IL-1β levels in human macrophages (Figure 3A, lanes 2 and 3, and Figure 3B). Hypoxic macrophages accumulated substantially higher amounts of pro-IL-1β after lipopolysaccharide stimulation than did normoxic macrophages (Figures 1 and 3A, lanes 2 and 5, and Figure 3B). Bafilomycin A1 mitigated this differential accumulation of pro-IL-1β in hypoxic cells compared with normoxic cells (Figure 3A, lanes 3 and 6, and Figure 3B), indicating that the lower concentrations of pro-IL-1β observed in normoxic cells stemmed from a higher rate of autophagic degradation. This observation is surprising, considering that moderate hypoxia generally augments autophagy,39 and it would therefore likely accelerate rather than retard the degradation of a substrate for autophagy such as pro-IL-1β. We affirmed that hypoxia indeed induced autophagy in our experiments by examining the steady-state levels of SQSTM1/p62 and LC3B, well-characterized substrates of the autophagic pathway, whose abundance correlates inversely with the extent of autophagy.40 Hypoxic cells contained lower concentrations of SQSTM1/p62 and of LC3B compared with normoxic cells (Figure 3A, lanes 1, 2, 4, and 5, and Figure 3C and 3D), and bafilomycin A1 caused SQSTM1/p62 and LC3B accumulation, confirming that hypoxia induced autophagy under conditions of our experiments (Figure 3A, 3C, and 3D). Pro-IL-1β colocalized to a higher extent with SQSTM1/p62, a cargo receptor that targets proteins to the autophagic machinery,40 in normoxic macrophages than it did in hypoxic macrophages, as assessed by immunostaining of cells treated with lipopolysaccharide and bafilomycin A1 and quantification of the fluorescent label colocalization (Figure 3E and 3F). Taken together, these results indicate that moderate hypoxia augments the accumulation of pro-IL-1β by limiting its selective targeting to the autophagic machinery.

Hypoxia Enhances IL-1β Secretion Induced by Lipopolysaccharide and Cholesterol Crystals in Human Macrophages

Atheromata contain cholesterol crystals (CCs).41 Recent work has shown that CCs elicit release of mature IL-1β from mononuclear phagocytes by activating the NLRP3 inflammasome, providing a link between cholesterol metabolism and inflammation.5,34 To examine the effect of hypoxia on IL-1β secretion, we exposed human macrophages to hypoxia for 24 hours, primed them with lipopolysaccharide for 2 hours, and incubated them for 6 hours in the presence or absence of CCs. Compared with their normoxic controls, hypoxic cells secreted 3- to 4-fold higher amounts of IL-1β in response to CCs, as assessed by ELISA of cell-conditioned media (Figure 4A). The response depended on CC concentration, reaching maximal values at 2 mg/mL (Figure 4B). Hypoxia did not augment secretion of IL-6 (Figure 4C) or tumor necrosis factor-α under the same conditions (Figure 4D). Metabolic labeling of macrophages treated as described above with [35S] methionine and [35S] cysteine, followed by IL-1β immunoprecipitation from cell-conditioned media, verified that hypoxic cells synthesized de novo and secreted a ≈17 kDa polypeptide that corresponds to cleaved, mature IL-1β, which was not detectable under normoxic conditions (Figure 4E). The caspase-1 inhibitor Z-YVAD-FMK, but not the pan-MMP inhibitor GM-6001, limited hypoxia-induced IL-1β secretion, indicating that caspase-1 participates in this process (Figure 4F).

Hypoxia Augments NLRP3 Induction and Inflammasome Activation in Lipopolysaccharide-Primed Human Macrophages

Efflux of intracellular K^+ is necessary and sufficient for NLRP3 inflammasome activation and caspase-1-dependent IL-1β secretion in lipopolysaccharide-primed mouse BMDMs.42 To test the effect of hypoxia on caspase-1 activation in human macrophages, we treated lipopolysaccharide-primed cells with the K^+/H^+ ionophore nigericin, a potent inflammasome activator.43 Cells exposed to hypoxia exhibited stronger caspase-1 activation than normoxic cells under these conditions, as assessed by the secretion of p20, a cleavage fragment of caspase-1 characteristic of the active enzyme (Figure 5A).
Nuclear factor κB–dependent regulation of NLRP3 expression controls inflammasome activation and caspase-1 activity in mouse BMDMs. Exposure of human macrophages to low oxygen tension augmented lipopolysaccharide-induced NLRP3 mRNA expression, compared with cells maintained under normal oxygen tension (Figure 5B). Concordantly, hypoxic lipopolysaccharide-treated cells exhibited higher levels of NLRP3 protein than normoxic lipopolysaccharide-treated cells (Figure 5C, lanes 2 and 4, and Figure 5D). Macrophages exposed to hypoxia but not treated with lipopolysaccharide also contained increased NLRP3 levels compared with their normoxic controls (Figure 5C, lanes 1 and 3, and Figure 5D). The addition of bafilomycin A1 did not alter NLRP3 levels, indicating that autophagy does not regulate NLRP3 protein concentration under these conditions (results not shown). Collectively, these results indicate that hypoxia regulates NLRP3 inflammasome activation at both NLRP3 mRNA and protein concentrations in human macrophages.

IL-1β Localizes in Macrophage-Rich and Potentially Hypoxic Areas of Atherosclerotic Lesions

We recently reported the abundant expression of proteins involved in glucose utilization in macrophage-rich areas of human plaques characterized as hypoxic. Examination of IL-1β localization in atheroma revealed that this cytokine also localizes predominantly in macrophage-rich regions of plaques that express the hypoxia markers HIF-1α and hexokinase-2 and activated caspase-1. Normal arteries did not contain detectable levels of hypoxia-regulated proteins, IL-1β, or activated caspase-1 (Figure 6A). These macrophage-rich hypoxic regions of plaques are lipid-rich, as assessed by their staining with oil red O, and contain crystalline cholesterol, visualized by its birefringence under cross-polarized light microscopy (Figure 6B). These results indicate that the hypoxia-induced proinflammatory mechanisms described above may operate in atherosclerotic lesions.

Discussion

The current study reveals that moderate hypoxia acts by 2 different and heretofore unrecognized mechanisms to augment IL-1β production in lipopolysaccharide-stimulated human macrophages: it limits the selective targeting of pro-IL-1β to autophagic degradation and increases NLRP3 expression and caspase-1 activation. Tanahill et al recently reported that hypoxia (1% O2) enhances IL-1β protein secretion in lipopolysaccharide-stimulated mouse macrophages and attributed, but did not conclusively demonstrate, this effect to transcriptional induction of the IL-1β gene mediated by a HIF-1α–binding site in the IL-1β promoter. The reasons for the discrepancy between our work and that by Tanahill et al remain unclear, but the different experimental approaches used in these 2 studies offer the simplest explanation: the present study used primary human macrophages subjected to moderate hypoxia (2% O2) and stimulated with lipopolysaccharide for 1 to 8 hours, versus mouse BMDMs exposed to severe hypoxia (1% O2) and treated with lipopolysaccharide for 24 hours in the study by Tanahill et al. In human macrophages, lipopolysaccharide stimulation requires a second signal (eg, CCs) to elicit secretion of IL-1β, whereas lipopolysaccharide suffices to induce IL-1β secretion in mouse BMDMs, thus...
highlighting the differences between the experimental conditions used in these 2 studies.

Autophagy, a pathway that involves the lysosomal degradation of damaged organelles and cytoplasmic components, may play a protective role in atherosclerosis. Limitation of supply of essential nutrients, including O₂, induces autophagy. In nutrient-rich conditions, autophagy operates at basal levels as a highly selective process that participates in the quality control of cellular organelles and in the regulation of the abundance of many cytoplasmic proteins, including pro-IL-1β.38, 48, 49 Our results show that hypoxia prolongs pro-IL-1β’s half-life despite activating autophagy, indicating decreased targeting of pro-IL-1β to autophagic degradation under low O₂ tension. A plausible explanation for this finding is that the half-life of pro-IL-1β likely depends on the intracellular availability of SQSTM1/p62, a cargo receptor that interacts with selected polyubiquitinated proteins and targets them to the autophagic machinery. Hypoxic cells have reduced levels of SQSTM1/p62 (Figure 3) and most likely a further reduced availability of this protein because of its diversion to mitophagy and other processes needed to maintain homeostasis under low O₂ tension. Thus, in contrast to the anti-inflammatory nature of normal autophagy, hypoxia elicits a selective deficit in autophagy that results in augmented pro-IL-1β accumulation and therefore increased inflammatory potential in activated human macrophages. Such defective autophagy contributes critically to certain inflammatory diseases such as Crohn colitis51 and chronic granulomatous disease.52

The induction of mature IL-1β secretion by CCs in human monocytes and mouse macrophages depends on caspase-1.5 We show that the caspase-1 inhibitor Z-YVAD-FMK, but not the MMP inhibitor GM-6001, limits hypoxia-induced IL-1β secretion.

Figure 4. Hypoxia potentiates interleukin-1β (IL-1β) secretion induced by lipopolysaccharide (LPS) and cholesterol crystals (CCs) in human macrophages. Cells were primed with 5 ng/mL LPS for 2 h and subsequently were stimulated with CCs (2 mg/mL in A, C, D, E, and F, or various concentrations in B) for 6 h. ELISA determined the concentration of the indicated cytokines in cell supernatants. A, IL-1β (n=11 donors; ⁠*P<0.001 vs LPS plus CCs in normoxia). B, IL-1β (n=3 donors). C, IL-6 (n=8 donors). D, Tumor necrosis factor-α (n=8 donors). Data are expressed in mean±SD. In C and D, data are relative to the values of treatment with LPS plus CCs in normoxic cells, defined as 100%. E, Macrophages were primed with 5 ng/mL LPS for 3 h and subsequently were radiolabeled with [35S]Met and [35S]Cys in the presence or absence of CCs for 4 h. IL-1β immunoprecipitates from culture supernatants were analyzed by SDS-PAGE and fluorography. F, Cells were treated as in A in the presence or absence of the indicated protease inhibitors. ELISA determined IL-1β concentration in cell supernatants. Data are expressed in mean±SD (n=4 donors) relative to the values in normoxic cells stimulated with LPS plus CCs, defined as 100%.
secretion in lipopolysaccharide-primed human macrophages treated with CCs, indicating the participation of caspase-1 in this process. The effect of hypoxia likely stems from its effect on NLRP3 expression, a limiting factor for caspase-1 activation triggered by the NLRP3 inflammasome.44 Of note, lipopolysaccharide-primed macrophages respond to various

Figure 5. Hypoxia augments NLRP3 expression and caspase activity in human macrophages. A, Cells were primed with 5 ng/mL lipopolysaccharide (LPS) for 2 h and subsequently were stimulated with 10 μmol/L nigericin for 3 h. Cell supernatants were fractionated by SDS–PAGE and immunoblotted with antibodies to cleaved caspase-1. B, Cells were stimulated with 5 ng/mL LPS for 4 h, followed by RNA extraction and determination of NLRP3 mRNA levels by reverse transcription–quantitative polymerase chain reaction. mRNA levels of 18S served as an internal control for adjustment between samples. Data are expressed in mean±SD (n=4 donors). C, Cells were incubated with 5 ng/mL LPS for 7 h. Whole-cell lysates were fractionated by SDS–PAGE and immunoblotted with antibodies to NLRP3 (top) and β-actin (bottom). D, Densitometric analyses of the bands corresponding to NLRP3 in C. β-Actin served as an internal control for adjustment between samples. Data are expressed in mean±SD (n=6 donors) relative to the values in normoxic cells stimulated with LPS, defined as 100%.

Figure 6. Interleukin-1β (IL-1β) colocalizes with hypoxia markers and cleaved caspase-1 in macrophage-rich regions of human atherosclerotic lesions that contain cholesterol crystals. A, Representative immunohistochemical staining of serial sections of human atherosclerotic carotid lesions (n=8) for CD68 (macrophages), IL–1β, hexokinase-2 (HK-2), hypoxia-inducible factor 1α (HIF-1α), and cleaved caspase-1. B, Representative immunohistochemical staining of serial sections of human carotid artery atherosclerotic lesions (n=5) for CD68 (macrophages), HK-2, and oil red O (ORO). Cholesterol crystals were visualized by ORO staining and cross-polarized light microscopy. The original magnification is indicated on each image.
well-characterized activators of the NLRP3 inflammasome to vastly different extents under the experimental conditions used here. Indeed, IL-1β release induced by nigericin exceeds the secretion levels elicited by CCs or ATP by 1 order of magnitude. Consequently, our immunoblots detected secreted p20, a product of caspase-1 activation, on cell stimulation with nigericin but not with CCs or ATP. Collectively, our data indicate that levels of hypoxia highly relevant to atherosclerosis potentiate the generation of mature IL-1β in human macrophages, both in vitro and in the context of atheromata. These findings provide new evidence for the intersection of hypoxia and inflammation and disclose novel mechanisms by which low oxygen tension can aggravate atherosclerosis.

Acknowledgments

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Disclosures

None.

References

Caspase-1, a component of the inflammasome, activates pro-IL-1β for degradation.


What New Information Does This Article Contribute?

• This work further discovered a selective and unexpected defect in autophagy induced by moderate hypoxia as a contributor to a build-up of pro-IL-1β, a substrate for the inflammasome.

What Is Known?

• Many preclinical studies support a role for the primordial proinflamma-tory cytokine, interleukin-1 (IL-1), as a promoter of atherogenesis.

• Like many proteins critical in biological control, IL-1 has multiple levels of regulation including a requirement for limited proteolysis of a pre-cursor to produce its active form.

• Caspase-1, a component of the inflammasome, activates pro-IL-1β to the mature cytokine in response to certain cytokines and danger signals including crystalline cholesterol, a component of atherosclerotic plaques.

• This study identifies moderate hypoxia, a condition that prevails within atherosclerotic plaques, as a novel activator of the inflammasome, hence contributing to the generation of active IL-1β.

Novelty and Significance

These results enhance our understanding of the molecular mechanisms of inflammation that operate in the local environment of the atherosclerotic plaque. They also have direct clinical relevance, because strategies that target IL-1β signaling can reduce biomarkers of cardiovascular risk. A large-scale clinical end point trial currently underway will evaluate the hypothesis that neutralization of IL-1β can improve outcomes in patients with established coronary artery disease.


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