Chronic Hypoxia Activates the Akt and β-Catenin Pathways in Human Macrophages

Jun-o Deguchi, Hiroyuki Yamazaki, Elena Aikawa, Masanori Aikawa

Objective—Macrophage activation contributes importantly to the pathogenesis of inflammatory diseases including atherosclerosis. Macrophages exist chronically under moderate hypoxia (2% to 5% O2) in inflamed tissues such as atherosclerotic plaques. However, macrophage phenotypes in such environments remain incompletely understood. This study tested the hypothesis that chronic moderate hypoxia induces macrophage activation and explored the underlying mechanisms.

Methods and Results—We cultured primary human macrophages derived from peripheral blood monocytes in moderate hypoxia (2% O2 tension) or normoxia (21% O2) for 10 days. Moderate hypoxia did not affect macrophage differentiation assessed via expression levels of scavenger receptor A. Chronic moderate hypoxia, but not normoxia, activated Akt and inactivated GSK-3β, a negative effector of Akt, thus allowing nuclear translocation of β-catenin. 2% O2 tension increased accumulation of hypoxia-inducible factors 1α (HIF-1α) transiently at 3 to 5 days. Hypoxia induced mRNA expression of the β-catenin-associated genes: MMP-7, CD44, and c-Myc. RNAi of TCF7L2, a cofactor of β-catenin, suppressed MMP-7 expression induced by hypoxia. Inhibition of Akt phosphorylation with LY294002 abolished hypoxia-induced GSK-3β inactivation, β-catenin activation, and MMP-7 expression. Macrophages under hypoxia were more resistant for oxLDL-induced apoptosis. Moreover, phospho-Akt colocalized with MMP-7 and CD44 expression in macrophages of human atherosclerotic plaques.


Key Words: macrophages ■ hypoxia ■ inflammation ■ atherosclerosis ■ MMP

Macrophages contribute importantly to various inflammatory states, including atherosclerosis and its acute thrombotic complications.1–8 We reported that macrophage expression of matrix metalloproteinases (MMP) promotes matrix remodeling and atherosclerotic plaque instability.9–11 Clinical and preclinical studies including our own also established a concept for atherosclerotic plaque regression/stabilization through lipid lowering as an antiinflammatory therapy.1,9,12,13 However, despite effective lipid lowering, coronary events remain a significant clinical problem. Macrophages also promote activation of adipocytes and hepatocytes, leading to the metabolic syndrome, a global threat associated closely with cardiovascular risk.14–16 Therefore, macrophage activation may form an amplification loop, involving local and systemic inflammation, and may thus promote multiple disease processes. Further understanding of mechanisms that trigger macrophage activation should thus lead to more effective diagnostic and therapeutic strategies for various inflammatory diseases.5,13,17

In addition to its role in acute tissue damage attributable to ischemia (eg, stroke, myocardial infarction), accumulating evidence suggests that hypoxia participates in the development of various chronic medical conditions including atherosclerosis.18–20 In vitro studies thus far have used severe hypoxia, such as 0% to 1% O2 tension, to understand the response of cell function to hypoxic condition. Hypoxia stabilizes hypoxia-inducible factors-1α and -2α (HIF-1α and -2α) by altering the conformation of a ubiquitin ligase. HIF proteins accumulate as transcription factors, inducing numerous hypoxic-responsive genes.18 Severe hypoxia also influences molecules other than HIF in various cell types, including phosphatidylinositol 3-OH kinase (PI3K) activity.21–25 PI3K activation leads to production of PI-3,4,5-P3 and PI-3,4-P2, second messengers essential for the translocation of Akt to the plasma membrane where PDK-1 and PDK-2 phosphorylated and activated Akt. Akt regulates several signaling pathways and mediates cell survival in stress conditions.26 Activated Akt inhibits the catalytic activity of glycogen synthase kinase (GSK)-3β, which impedes cytoplasmic β-catenin degradation.27,28 Proinflammatory stimuli also activate Akt in macrophages.29,30

Despite the in vivo evidence suggesting that arterial cells are exposed to moderate hypoxia (approximately 2% to 5% O2...
HIF-1

used standard methods. See supplemental materials (available online at http://atvb.ahajournals.org) for detailed methods.

Methods

Primary human macrophages, separated from peripheral blood monocytes,38,39 were maintained with 5% human serum in a humidified incubator containing 21% O2, 5% CO2, and 74% N2 (normoxia), or in an air-tight culture chamber containing a hypoxic gas mixture (2% O2, 5% CO2, and 93% N2) kept in a humidified incubator. Real-time RT-PCR, Western blotting, Gel shift assay, and immunohistochemistry used standard methods. See supplemental materials (available online at http://atvb.ahajournals.org) for detailed methods.

Results

Chronic Moderate Hypoxia Activates Akt

We first found chronic moderate hypoxia does not affect macrophage differentiation as gauged by expression of macrophage scavenger receptor class A (Supplemental Figure I). Primary human macrophages cultured under severe hypoxia (0% to 1% O2 tension) did not survive more than several days (data not shown). Several studies indicate that transient hypoxic shock induces Akt activation in various cell types. Exposing macrophages to 2% O2 tension for 10 days increased phosphorylated Akt (Ser473), indicating kinase activation of Akt, whereas expression levels of total Akt remained unchanged (Western blotting, Figure 1A, left). Akt activation also occurred in macrophages cultured under normoxia for the first 10 days and subsequently exposed to moderate hypoxia for 7 days. Notably, the amount of pAkt in these macrophages was similar to that in those exposed to moderate hypoxia continuously for 17 days (supplemental Figure II). We further examined the time-course of HIF-1α protein levels. HIF-1α levels in macrophages increased transiently at 3 and 5 days after exposure to 2% O2 tension, but returned to the levels similar to the cells cultured under normoxia by day 7 (Figure 2C, left). The protein levels of hypoxia-inducible factors, HIF-1α and -2α, did not increase under 2% O2 tension at day 10 (Figure 1B, bottom).

Hypoxia Inactivates GSK-3β and Permits β-Catenin to Translocate Into the Nuclei

GSK-3β, a downstream element of the Akt pathway, is constitutively active without phosphorylation.27 Active Akt phosphorylates Ser9 residue of GSK-3β, inactivating this molecule.28 Western blots from total cell lysates show that exposure of macrophages to 2% O2 tension for 10 days increased GSK-3β phosphorylation, indicating that GSK-3β became inactive, whereas the total amount of GSK-3β protein remained unchanged (Figure 2A).

GSK-3β induces β-catenin degradation and thus serves as a negative regulator for this pathway. Phosphorylated (inactivated) GSK-3β allows β-catenin to accumulate in the cytoplasm and, in turn, translocate into the nuclei, leading to the induction of β-catenin-associated genes.27 Under 2% O2 tension, β-catenin increased in the nuclear fraction while the total cell lysate contained a similar amount (Figure 2B). The nuclear fraction used in these experiments did not contain detectable levels of CD68, a membrane-bound protein, thus indicating little if any contamination of membranous components (supplemental Figure III). Immunofluorescence captured by confocal laser scanning microscopy further indicates that macrophages cultured under normoxia contained a greater amount of β-catenin near the cell membrane (Figure 2C, left), whereas in the cells cultured under 2% O2 tension β-catenin accumulated near the nuclei (Figure 2C, center), or translocated into the nuclei, a hallmark of activation of this signaling mechanism (Figure 2C, right).27

Chronic Moderate Hypoxia Induces MMP-7 Expression via the β-Catenin Pathway

Because nuclear-translocated β-catenin induces the transcription activity of a series of genes, we tested the hypothesis that...
Hypoxia induces β-catenin–associated molecules including MMP-7, CD44, and c-Myc in macrophages. Human primary macrophages chronically exposed to 2% O₂ tension increased mRNA expression of MMP-7 (mean 52.4 ± 11.3-fold relative to 21% O₂, 11 donors, Figure 3A). Hypoxia induced production of pro–MMP-7 protein in macrophages (Figure 3B). Chronic exposure of macrophages to 2% O₂ tension also increased mRNA expression of CD44 (mean 27.3 ± 13.5-fold, 6 donors) and c-Myc (mean 5.8 ± 1.7-fold, 6 donors, Figure 3C). We further examined whether chronic moderate hypoxia induces MMP-7 expression through the β-catenin pathway. Transcription factor 7 like 2 (TCF7L2), a cotranscription factor essential for binding β-catenin to its target DNAs, increased in the nuclear fraction induced by hypoxia (Figure 4A). Moreover, hypoxia induced binding of nuclear protein extracts to an oligonucleotide probe containing a consensus sequence for the TCF site as demonstrated by gel shift assay (Figure 4B). Introduction of siRNA oligonucleotides to TCF7L2 decreased mRNA expression of TCF7L2 in human macrophages (Figure 4C, left). TCF7L2 siRNA substantially suppressed MMP-7 induction (Figure 4C, right), indicating that the β-catenin pathway plays a dominant role in MMP-7 expression induced under the hypoxic milieu.

Akt Inhibition Abolishes GSK-3β Phosphorylation, β-Catenin Translocation, and MMP-7 Expression in Chronic Moderate Hypoxia

Akt phosphorylates and inactivates GSK-3β, a major inhibitor of the β-catenin pathway. Western blot analysis demonstrated that LY294002, a selective inhibitor of PI3-kinase, almost completely suppressed Akt activation, inhibited GSK-3β inactivation, and blocked nuclear accumulation of β-catenin induced by hypoxia in human primary macrophages (3 donors, Figure 5A). Moreover, inhibition of Akt suppressed hypoxia-induced MMP-7 mRNA expression (3 donors, Figure 5B). We further examined whether pharmacological inhibition of GSK-3β mim-
ics the effect of moderate hypoxia. GSK-3β inhibitor VIII increased β-catenin nuclear accumulation in macrophages cultured under normoxic condition (Figure 5C).

Chronic Moderate Hypoxia Reduces Macrophage Death Induced by Oxidized LDL

Preclinical evidence suggests that hypoxia activates the PI3-kinase/Akt pathway and promotes survival of various cell types.23,28,40,41 We thus examined whether macrophages under moderate hypoxia are more resistant for cell death. Oxidized LDL induced caspase activity in a concentration-dependent manner in human primary macrophages cultured under normoxic condition. The ratio of apoptotic macrophages cultured under hypoxia was significantly less compared to those under normoxic condition (Figure 5D).

pAkt, MMP-7, and CD44 Colocalize With Macrophages in Atherosclerotic Plaques

We performed double immunofluorescence to localize activated Akt, MMP-7, and CD44 in macrophages of human carotid endarterectomy specimens (Figure 6A). Intimal macrophages (CD68) contained pAkt and MMP-7 proteins and pAkt and CD44, respectively. Independent experiments on specimens from 12 patients produced similar results. Peptide absorption to the anti–MMP-7 antibody abolished MMP-7 signal in the lesion (Figure 6B). The data represent a subset of 3 cases that produced similar results. We further examined whether β-catenin activation occurs in human atherosclerotic plaques. Confocal laser scanning microscopy localized β-catenin in the nuclei of these macrophages (n=3, Figure 6C).

Discussion

Macrophage activation plays critical roles in the pathogenesis of various inflammatory conditions including atherosclerosis, metabolic syndrome, calcification, neurological disorders, and cancer.1–8,14–16 Accumulating evidence indicates that moderate hypoxia may participate in these disease processes.18–20,31–36 However, macrophage biology under hypoxia, particularly chronic moderate hypoxia as opposed to anoxia or severe hypoxia, remains obscure.42,43 The present study demonstrated that chronic moderate hypoxia promotes activation of the Akt and β-catenin axis in macrophages and expression of downstream gene products including MMP-7, CD44, and c-Myc,
MMP-7 plays central roles in inflammatory ailments.\textsuperscript{44} MMP-7 may not only participate in remodeling of inflamed arteries through cleavage of various extracellular matrices, but also promotes thrombogenicity by cleaving tissue factor pathway inhibitor.\textsuperscript{45} In cancer, MMP-7 likely figure critically not only in ECM degradation but also in the regulation of biochemical processes such as activation, degradation, and shedding of non-ECM proteins.\textsuperscript{46} MMP-7 may also serve as an important marker in human cancer progression. We recently demonstrated that Notch signaling promotes macrophage activation.\textsuperscript{39} Interestingly, recent evidence suggests that Notch signaling requires MMP-7.\textsuperscript{47} Accumulating evidence has further demonstrated that MMP-7 also functions in various other fundamental biological and pathological processes such as innate immunity and inflam-

**Figure 6.** Colocalization of pAkt (Ser473), MMP-7, and CD44, and nuclear translocation of $\beta$-catenin in macrophages of human carotid atherosclerotic plaques. A, Immunohistochemistry for macrophages in a human carotid atherosclerotic plaque. L and M denote the lumen and medial side, respectively. Rectangulars correspond to high power views in panels B and C further analyzed. Double immunofluorescence indicated that macrophages, as identified by CD68 antibody, stained positively for pAkt. pAkt also colocalized with MMP-7 and CD44. Analyses on carotid lesions from 12 patients yielded similar results. B, Peptide absorption abrogated immunoreaction of macrophages to MMP-7 antibody in 2 different cases. C, Confocal laser scanning microscopy detected $\beta$-catenin in the nuclei of human carotid atherosclerotic plaques. Analysis of 6 fields randomly chosen in each of the 3 patients yielded similar results. PI denotes propidium iodide for nuclear staining.
mation.44 MMP-7 also generates soluble tumor necrosis factor-α, a pluripotent proinflammatory cytokine. Additionally, we demonstrate in human macrophages that chronic moderate hypoxia induces mRNA expression of other β-catenin–related genes including CD44 and c-Myc, which also contribute to inflammation, tissue injury/repair, and atherogenesis.45–51

Burke et al recently reported that acute severe hypoxia (0.5% O2 tension) increases MMP-7 mRNA expression.52 However, several in vivo studies have found an oxygen tension of 2% to 5% O2 in the arterial wall.31–36 Bjornheden et al demonstrated that more severely hypoxic zones occur only in the deep portion of atherosclerotic plaques, and recently, Sluimer et al used pimonidazole (Hypoxyprobe-1) to localize a sign of hypoxia (<1% O2) in macrophages of the center of human atherosclerotic plaques, but not in a shoulder segment,53 suggesting that most cells within the plaques undergo exposure to moderate rather than severe hypoxia.55 Particularly, activated macrophages in the shoulder region that contribute to so-called plaque instability likely experience chronic exposure to moderate hypoxia. A recent study demonstrated induction of various macrophage genes after transient exposure to hypoxia (1% O2, 16 hours).42 Notably, primary human macrophages in the present study did not survive more than several days under severe hypoxia (0% to 1% O2) in vitro. Severe hypoxia regulates gene expression, including angiogenic and atherogenic genes through HIF proteins.19 The present study showed no substantial changes in HIF-1α or HIF-2α protein expression after 7- or 10-day exposure to 2% O2 tension, whereas HIF-1α induction occurred transiently at day 3 or 5. Therefore, the role of HIFs in the effects of chronic moderate hypoxia in macrophages remains unclear. Addressing this question requires further investigations.

Severe hypoxia (0% to 1% O2 tension) activates the PI3K-Akt pathway for cell survival. Akt targets GSK-3, mammalian target of rapamycin, insulin receptor substrate-1, and cyclin-dependent kinase inhibitors p21CIP/WAF1 and p27KIP1, indicating that it regulates protein synthesis, glycogen metabolism, and cell proliferation.26 Several reports demonstrated that the Akt pathway participates in cell activation.54,55 GSK-3β exists in cytoplasm as a kinase-active form and subjects β-catenin to degradation. After phosphorylation of GSK-3β, β-catenin accumulates and translocates into the nuclei, resulting in increased transcriptional activity of β-catenin-regulated genes.27,56 Miller et al reported that minimally modified LDL, an early and physiologically relevant form of oxidatively-modified LDL found in the arterial wall, activates Akt in macrophages.30 Proliferation and survival of macrophages may play a critical role in the pathogenesis of vascular inflammation.38,57,58 In this study, macrophages exposed to chronic moderate hypoxia were more resistant to oxidized LDL–induced apoptosis. Akt activated by glucose-oxidized LDL may participate in macrophage proliferation.29 The present study demonstrates that Akt activation in human monocyte-derived macrophages by chronic moderate hypoxia causes inactivation of GSK-3β and nuclear accumulation of β-catenin. Collectively, these studies indicate that Akt plays a pivotal role in the proinflammatory phenotype of macrophages, providing a new mechanism in macrophage biology, whereas some studies have indicated that GSK-3β plays a proinflammatory role in part by activating NF-κB.59 Despite evidence for the role of Akt in β-catenin accumulation and MMP-7 induction in the present study, modification of the Wnt pathway could also mediate these responses,60 a subject for future study.

The present study demonstrates that chronic moderate hypoxia promotes activation of the Akt and β-catenin pathways and expression of molecules associated with inflammatory diseases such as atherosclerosis and its acute complications, the metabolic syndrome, and cancer.44–47,49–51 These results suggest that induction of macrophage products in chronically hypoxic tissues such as atherosclerotic plaques and adipose tissue may further amplify the inflammatory milieu. Our observations expand the mechanistic understanding of macrophage activation and provide new insight into the pathogenesis of chronic inflammatory diseases.

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Disclosures

None.

References

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Primary human macrophages cultured in normoxia (21% O$_2$) or moderate hypoxia (2% O$_2$) for 10 days (Day 1 thru Day 11) produced similar levels of mRNA encoding macrophage scavenger receptor class A (SR-A), detected by real-time RT-PCR. Each bar at Day 11 indicates a mean fold-increase ± S.E.M. relative to Day 1 control in 3 different donors.
Akt activation also occurred in macrophages cultured under normoxia for the first 10 days and subsequently exposed to moderate hypoxia for 7 days (gray bar). The amount of pAkt in these macrophages was similar to that in those exposed to moderate hypoxia continuously for 17 days (black bar).
Supplemental Figure III

The nuclear fraction was not contaminated with membrane fraction, as determined by Western blotting with an antibody against CD68.