Reversal of hypoxia in murine atherosclerosis prevents necrotic core expansion by enhancing efferocytosis

Reversal of Hypoxia in Murine Atherosclerosis Prevents Necrotic Core Expansion by Enhancing Efferocytosis

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Objective—Advanced murine and human plaques are hypoxic, but it remains unclear whether plaque hypoxia is causally related to atherogenesis. Here, we test the hypothesis that reversal of hypoxia in atherosclerotic plaques by breathing hyperoxic carbogen gas will prevent atherosclerosis.

Approach and Results—Low-density lipoprotein receptor–deficient mice ($LDLR^{-/-}$) were fed a Western-type diet, exposed to carbogen (95% O_2, 5% CO_2) or air, and the effect on plaque hypoxia, size, and phenotype was studied. First, the hypoxic marker pimonidazole was detected in murine $LDLR^{-/-}$ plaque macrophages from plaque initiation onwards. Second, the efficacy of breathing carbogen (90 minutes, single exposure) was studied. Compared with air, carbogen increased arterial blood pO_2 5-fold in $LDLR^{-/-}$ mice and reduced plaque hypoxia in advanced plaques of the aortic root (−32%) and arch (−84%). Finally, the effect of repeated carbogen exposure on progression of atherosclerosis was studied in $LDLR^{-/-}$ mice fed a Western-type diet for an initial 4 weeks, followed by 4 weeks of diet and carbogen or air (both 90 min/d). Carbogen reduced plaque hypoxia (−40%), necrotic core size (−37%), and $TUNEL^+$ (terminal uridine nick-end labeling positive) apoptotic cell content (−50%) and increased efferocytosis of apoptotic cells by cluster of differentiation $107b^+$ (CD107b, MAC3) macrophages (+36%) in advanced plaques of the aortic root. Plaque size, plasma cholesterol, hematopoiesis, and systemic inflammation were unchanged. In vitro, hypoxia hampered efferocytosis by bone marrow–derived macrophages, which was dependent on the receptor Mer tyrosine kinase.

Conclusions—Carbogen restored murine plaque oxygenation and prevented necrotic core expansion by enhancing efferocytosis, likely via Mer tyrosine kinase. Thus, plaque hypoxia is causally related to necrotic core expansion. **(*Arterioscler Thromb Vasc Biol*. 2014;34:2545-2553.)**

Key Words: atherosclerosis ■ carbogen ■ hypoxia ■ macrophages

In many pathophysiological conditions, reduced oxygen tension (hypoxia) is a known stimulus of inflammation, angiogenesis, and apoptosis.[1,2] Because the progression of atherosclerosis is driven by the same processes,[3–5] we recently investigated whether hypoxia was present in atherosclerosis. Plaque hypoxia was detected specifically in macrophages of advanced human atherosclerotic lesions using administration of the hypoxia-specific marker pimonidazole,[6] as well as in advanced rabbit and murine plaques.[7–10] However, whether plaque hypoxia is an innocent bystander effect of macrophage influx or a driving force behind plaque progression is still unknown.

Because the in vitro effects of hypoxia on macrophage function are proangiogenic, proinflammatory,[11] and antifibrotic,[12] we hypothesized that hypoxia in plaque macrophages is proatherogenic. Thus, reoxygenation would be expected to prevent plaque progression. An attractive strategy to achieve plaque reoxygenation may be the breathing of hyperoxic gas carbogen, composed of 95% O_2 and 5% CO_2, as this successfully alleviated tumor hypoxia.[13–15] Hyperoxic carbogen will

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Nonstandard Abbreviations and Acronyms	
BMDM	bone marrow–derived macrophage
CD36	cluster of differentiation 36
LDLR$^{-/-}$	low-density lipoprotein receptor–deficient
LRP1	low-density lipoprotein receptor–related protein 1
MerTK^{kd}	Mer tyrosine kinase domain deficient

increase oxygen dissolved in blood, thus pO_2, rather than enhancing already maximal hemoglobin saturation. In addition, the CO_2 component of carbogen contributes to increased oxygen supply: 5% CO_2 ensures a respiratory drive, prevents vasodilation associated with 100% O_2, and shifts the hemoglobin–O_2 dissociation curve toward facilitated oxygen delivery.[16] Thus, oxygen blood content, oxygen delivery, and oxygen diffusion are vastly improved and enhance tissue oxygenation. Therefore, we hypothesized that carbogen delivery will reverse hypoxia in murine atherosclerotic plaques and thus reverse hypoxia–associated detrimental effects on plaque inflammation and progression.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Murine Plaque Macrophages Are Hypoxic, Independent of Plaque Stage

Low-density lipoprotein receptor–deficient (LDLR$^{-/-}$) mice were fed a Western-type diet for 0 to 16 weeks to monitor the onset and distribution of plaque hypoxia with pimonidazole at different plaque stages in the aortic root (Figure 1A).

First, specificity of pimonidazole and its detection antibody was confirmed in mice injected with vehicle or pimonidazole (Figure IA in the online-only Data Supplement). Plaque hypoxia colocalized mainly with MAC3+ macrophages and foam cells shown by multispectral analysis of double immunohistochemistry (Figure 1C and 1D). Pimonidazole detection in cultured bone marrow–derived macrophages (BMDM) sharply increased below 1% O_2 (≈10 mm Hg; Figure IB and IC in the online-only Data Supplement), confirming previous data.[17] Also, macrophage lipid loading per se did not result in pimonidazole positivity (Figure ID in the online-only Data Supplement). Earlier studies have also shown that oxidative stress exposure does not affect pimonidazole detection,[6] overall suggesting that pimonidazole staining truly marks hypoxic macrophages.

Plaque hypoxia may either arise from reduced oxygen supply to the growing plaque or from increased oxygen demand by inflammatory cells. A strong correlation between MAC3 content and pimonidazole positivity supports the latter explanation while plaque size was not a determinant for plaque hypoxia (Figure 1E). A slight reduction in macrophage content after 16 weeks was mirrored by a reduction in hypoxia (Figure 1A and 1D). Thus, murine plaque hypoxia is dictated by macrophage content, not by plaque thickness and oxygen supply. Pimonidazole was detected directly adjacent to the luminal arterial oxygen supply, in small fatty streaks after 4 weeks Western-type diet, while being absent in the media of diseased and nondiseased arteries (Figure 1A). Although pimonidazole is metabolized by living cells only, reflected by a significant univariate correlation of plaque necrosis and hypoxia, necrosis was not a significant predictor in multivariate regression (Figure 1A and 1E). In addition, tissue-resident macrophages in inflammation-rich fatty livers and lymphoid organs of Western-type diet–fed LDLR$^{-/-}$ mice were hypoxic (Figure IIA–IIC in the online-only Data Supplement). As even venous pO_2 is 4-fold higher than the 10 mm Hg detection threshold of pimonidazole,[17] arterial and venous white blood cells were negative for pimonidazole (Figure IID in the online-only Data Supplement), as expected.

In conclusion, tissue-resident and plaque macrophages are hypoxic. Plaque macrophages remain the only predictor of plaque hypoxia after multivariate regression, not plaque size or necrotic core content.

Carbogen Exposure Increased Arterial pO_2 and Alleviated Plaque Hypoxia

Carbogen significantly increased arterial pO_2 in awake LDLR$^{-/-}$ mice (Figure 2A). Simultaneously, blood lactate was drastically reduced, while pCO_2 was slightly elevated (Figure 2A), and pH, glucose, and electrolytes (data not shown) unchanged. Within minutes after cessation of carbogen breathing, all blood levels, except for lactate, returned to baseline (data not shown).

As carbogen successfully increased arterial pO_2, its effect on plaque hypoxia was studied. Single 90-minute carbogen exposure in LDLR$^{-/-}$ mice with advanced, hypoxic plaques led to a dramatic reduction of plaque hypoxia in the aortic arch and root compared with similarly sized plaques in mice breathing air (Figure 2B and 2C). Macrophage content, the main predictor of plaque hypoxia, was similar in both groups (Figure 2B and 2C). Likewise, repeated carbogen exposure was able to alleviate plaque hypoxia in LDLR$^{-/-}$ mice, which were fed a Western-type diet for an initial 4 weeks followed by 4 weeks of diet and carbogen or air exposure (both 90 min/d). Hypoxia decreased with 50% in the aortic arch and 42% in the root compared with control (Figure 2D and 2E) while macrophage content was not affected by carbogen gas (Figure 2D–2F). Moreover, carbogen was able to reoxygenate significantly liver and spleen (Figure IIA–IIC in the online-only Data Supplement).

To prevent oxygen toxicity possibly associated with long-term carbogen exposure, carbogen exposure was limited to 4 weeks. Chronic carbogen exposure did not affect plasma cholesterol, triglycerides, hemoglobin, or hematocrit, or erythropoiesis, or myelopoiesis as shown by flow cytometry analysis[18] of stem cells and myeloid progenitors in bone marrow. Also it did not impact monocyte, granulocyte, T cell, B cell, and dendritic cell numbers in blood, spleen, and lymph node (Figure III in the online-only Data Supplement).

Plaque Reoxygenation Improved Efferocytosis and Apoptosis In Vivo

Notably, plaque reoxygenation through daily carbogen exposure was able to prevent necrotic core expansion by 37% in advanced plaques of the aortic root compared with control

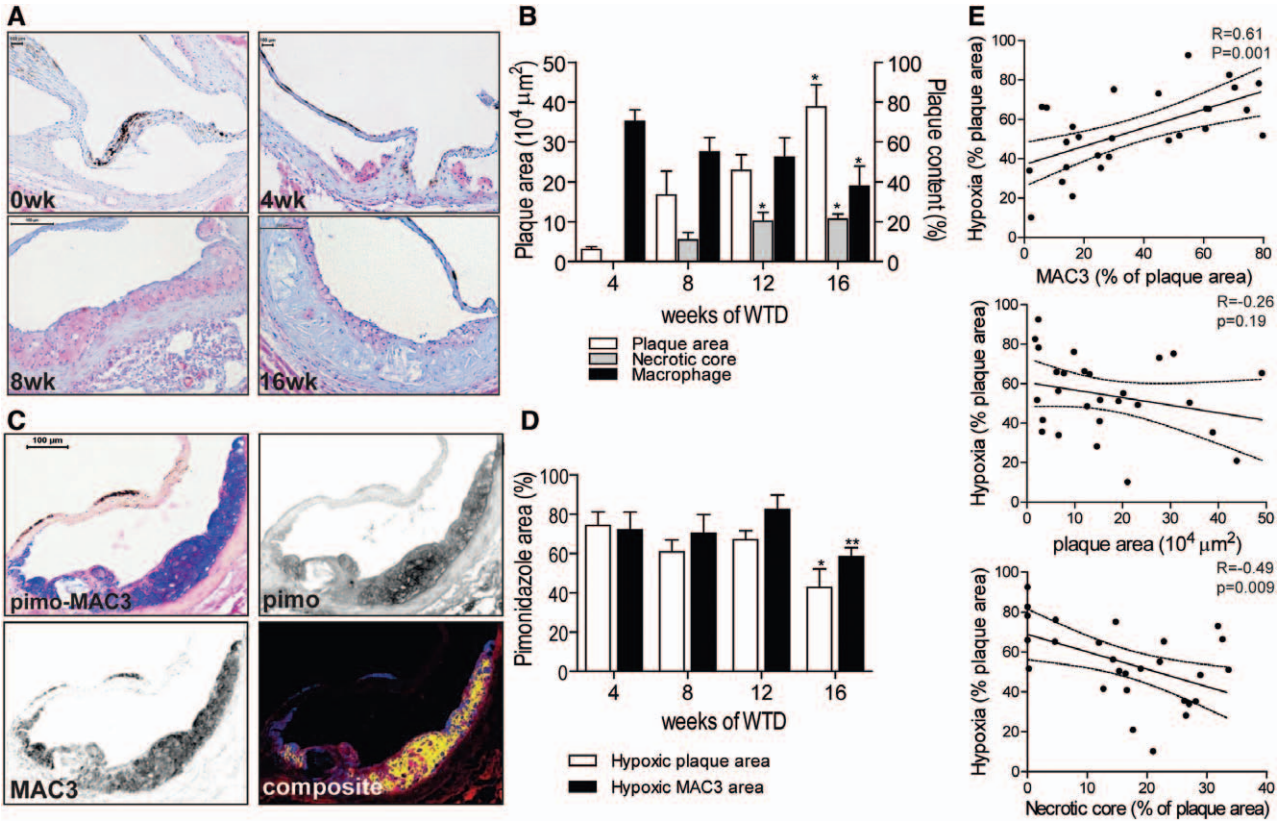

Figure 1. Murine plaque macrophages are hypoxic, independent of plaque stage. **A**, Representative pictures of pimonidazole-stained aortic root (red) from low-density lipoprotein receptor–deficient ($LDLR^{-/-}$) mice after 0, 4, 8, and 16 weeks of Western-type diet (WTD; n=5/group). **B**, Quantification of plaque area (left *y* axis), necrotic core (right *y* axis), and macrophage content (right *y* axis), **P*<0.05 vs 4 weeks. **C**, Illustration of principle multispectral analysis in aortic roots from $LDLR^{-/-}$ mice with advanced plaque, double-stained with pimonidazole (Pimo, red), and MAC3 (blue), respectively. **D**, Quantification of plaque hypoxia and hypoxic macrophages (MAC3, pimonidazole double positive) in $LDLR^{-/-}$aortic root (n=5/time point). *P*<0.05 4 vs 16 weeks, ***P*<0.01 12 vs 16 weeks. **E**, Scatter plots of plaque hypoxia with MAC3 (**top**), plaque area (**middle**), or necrotic core area (**bottom**) with univariate linear regression line and 95% confidence interval (dashed lines). Data from $LDLR^{-/-}$ mice fed WTD for 4, 8, 12, and 16 weeks (n=5 per time point+n=15 air breathing controls, n=35 in total).

while plaque size was not altered (Figure 3A). Necrotic core expansion is determined by the balance between apoptosis and the clearance of apoptotic cells by phagocytes, a process called efferocytosis. Efferocytosis was quantified in situ, as the ratio of macrophage-bound versus macrophage-free apoptotic cells in a terminal uridine nick-end labeling/MAC3 double immunohistochemistry.[19,20] Carbogen exposure reduced the density of apoptotic cells in the plaque in situ by 50% and improved efferocytosis of apoptotic cells by MAC3+ macrophages by ≈40% compared with air (Figure 3B and 3C). Increased efferocytosis was seen despite similar plaque macrophage content, size, and density (Figure 2E and 2F). Intraplaque proliferation, collagen content, T cell and total cell density, as well as tissue factor expression as a measure of thrombogenicity,[21] were unaffected by carbogen (Figure 3E–3H). In initial plaques of the aortic arch, carbogen did not affect plaque size or macrophage content (Figure IV in the online-only Data Supplement), despite successful reoxygenation (Figure 2D).

Neither Reactive Oxygen Species Formation Nor Macrophage Polarization Explains Hypoxic Repression of Efferocytosis

Generation of reactive oxygen species during carbogen-induced reoxygenation might be involved in apoptosis or efferocytosis. However, neither reactive oxygen species–mediated DNA damage, analyzed by 8-hydroxy-2′deoxy-guanosine immunohistochemistry, nor antioxidant capacity of the plasma was changed after chronic (Figure 4A) and single carbogen exposure (not shown). In addition, hypoxia did not alter macrophage reactive oxygen species activity in vitro (Figure 4A).

Apoptosis and efferocytosis were further studied in vitro. Chronic hypoxia (0.2% O_2) did not stimulate apoptosis in murine primary BMDM (Figure 4B) or J774 (data not shown) as single stimulus or augment apoptosis of additional endoplasmatic reticulum-stress stimuli such as 7-ketocholesterol or tunicamycin. In fact, cells even seemed to be protected from ER-stress–mediated apoptosis (Figure 4B). Efferocytosis of labeled, apoptotic Jurkat T cells by both the J774 murine macrophage cell line and BMDM was hampered in hypoxia (0.2% O_2) compared with 21% O_2 control (Figure 4C). Thus, hypoxia hampered efferocytosis in vitro.

Because anti-inflammatory M2c macrophages are thought to be more efficient in phagocytosis/efferocytosis,[22] the effect of hypoxia on polarization was studied. Hypoxia reduced M2 gene expression (interleukin 10 and mannose receptor), whereas M1 genes (interleukin 6 and inducible nitric oxide synthase) were strongly upregulated (Figure 4D). In vivo, unlike inducible nitric oxide synthase, mannose receptor+

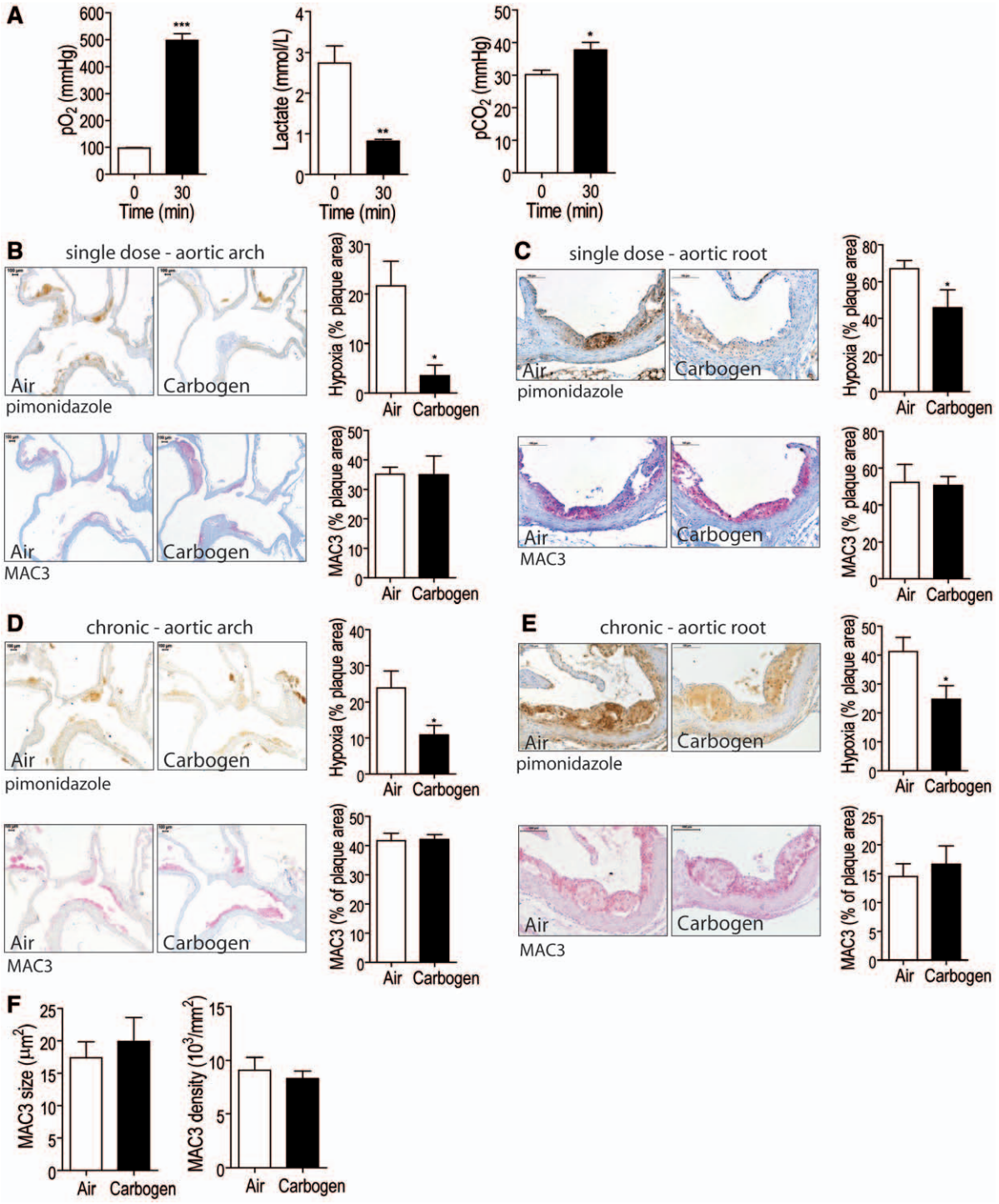

Figure 2. Carbogen increased arterial pO_2 and alleviated plaque hypoxia. **A**, Arterial pO_2 (**left**), lactate concentration (**middle**), and pCO_2 (**right**) before and after 30 minutes of carbogen exposure in low-density lipoprotein receptor–deficient ($LDLR^{-/-}$) mice on chow, ***P<0.001, **P<0.01 vs before exposure. **B**, Representative pictures and quantification of pimonidazole (brown) and MAC3 (red) immunohistochemistry in aortic arch and branches of $LDLR^{-/-}$ mice exposed to single dose of air or carbogen. **C**, Representative pictures and quantification of plaque hypoxia and macrophages in the aortic root on single dose of air or carbogen. **D**, Representative pictures and quantification of plaque hypoxia and macrophages in the aortic arch and (**E**) root of $LDLR^{-/-}$ mice after chronic carbogen or air treatment. *P<0.05 vs air. **F**, Quantification of macrophage size (left graph) and density (right graph) on air or carbogen treatment.

macrophages were hardly present in the plaque; therefore, adventitial mannose receptor+ cells were quantified. A trend toward less M1 in the plaque and more M2 expression in the adventitia of carbogen-treated mice was found (Figure 4E and 4F). In addition, plasma cytokine levels were essentially not changed, with a trend toward less interleukin 6 in the carbogen-treated group (Figure 4G).

Next, it was investigated whether M2 polarization before hypoxia could rescue hypoxia-mediated repression of efferocytosis. Under normoxic conditions, M2c polarization

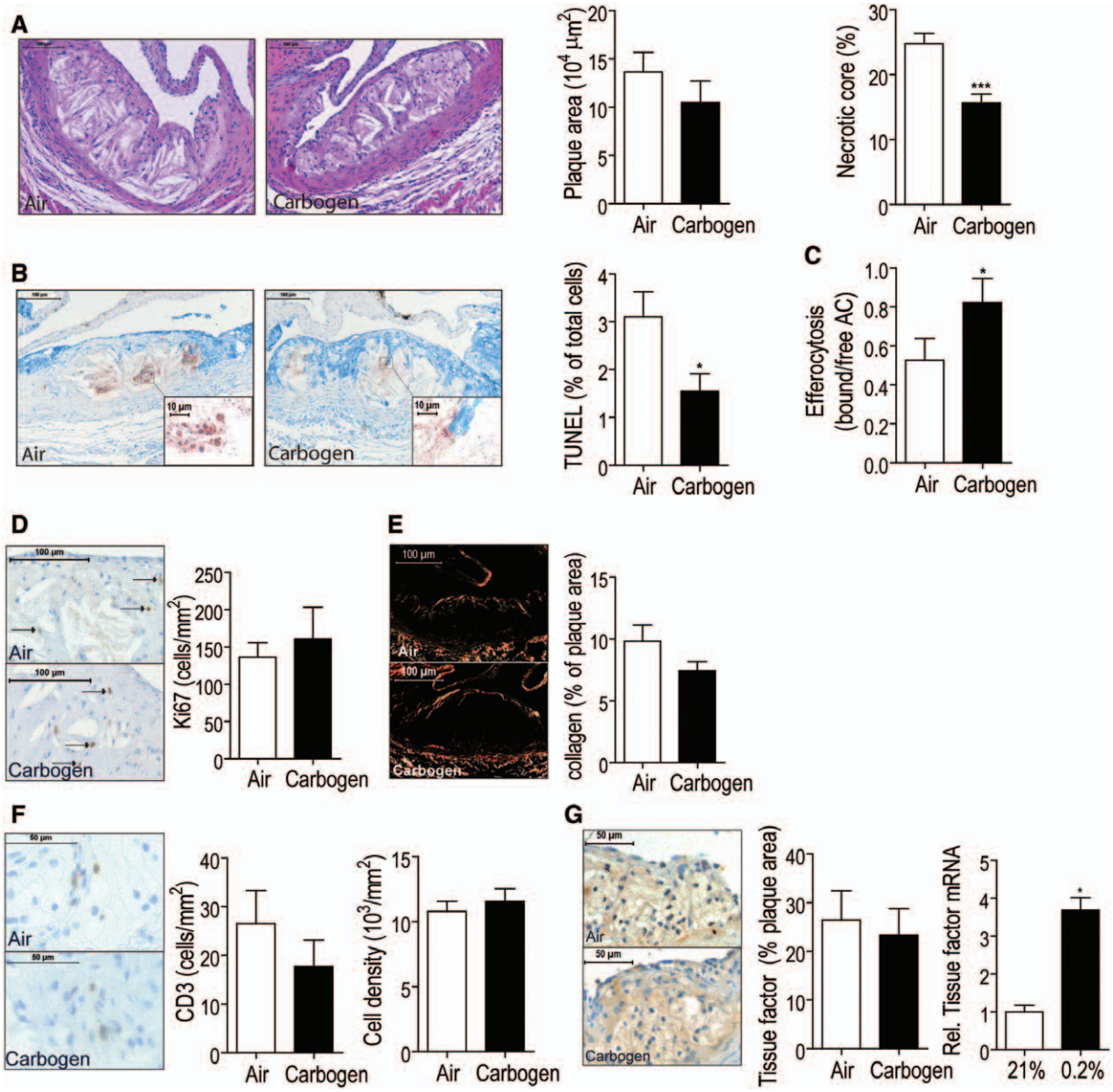

Figure 3. Plaque reoxygenation prevented necrotic core expansion and stimulated efferocytosis in vivo. **A**, Hematoxylin and eosin staining and quantifications of aortic root plaque size (left graph) and necrotic core content (right graph) of male low-density lipoprotein receptor–deficient (LDLR$^{-/-}$) mice treated with air or carbogen and fed a Western-type diet for 8 weeks. ***P<0.001 vs air. **B**, Terminal uridine nick-end labeling (TUNEL) and MAC3 double immunohistochemistry and weak counterstain with hematoxylin (1:50) of LDLR$^{-/-}$ aortic roots treated with air or carbogen. Insets show free TUNEL+ nuclei (brick red) in air-treated group, while TUNEL+ nuclei were associated with macrophages (blue) in carbogen-treated group. Graphs represent quantification of apoptosis and (**C**) efferocytosis (ratio MAC3-bound/free TUNEL+cells), *P<0.05 vs air. **D**, Quantification of proliferation (Ki67) expression, (**E**) collagen content, (**F**) T-cell density and total cell density, as well as (**G**) tissue factor expression, as a measure for thrombogenicity, in aortic roots of LDLR$^{-/-}$ mice after chronic air or carbogen exposure (left graph) and in bone marrow–derived macrophages exposed to 21% or 0.2% O_2 (right graph).

enhanced macrophage efferocytosis capacity compared with unpolarized BMDM, and, unexpectedly, so did M1 polarization (Figure 4H). However, polarization to M2c or M1 was not able to restore the repression of efferocytosis by hypoxia, suggesting that macrophage polarization is not involved in oxygen-dependent regulation of efferocytosis.

Hypoxia Limits Efferocytosis via MerTK

Efferocytosis is a receptor-mediated process. Therefore, we studied whether hypoxia affects expression of known efferocytosis receptors. Expression of the efferocytosis receptor Mer tyrosine kinase (MerTK) and cluster of differentiation 36 (CD36) was reduced in hypoxic BMDM, whereas that of scavenger receptor A was unchanged. In contrast, low-density lipoprotein receptor–related protein 1 (LRP1) was upregulated (Figure 5A). As efferocytosis capacity was decreased on hypoxia and LRP1 was previously not involved in efferocytosis,[23] LRP1 is likely not involved.

Compromized function of MerTK was seen to underlie the impaired efferocytosis during murine atherogenesis in vivo.[20,24] As expected, disrupted MerTK signaling in MerTK kinase domain–deficient (MerTK^{kd}) BMDM led to repressed

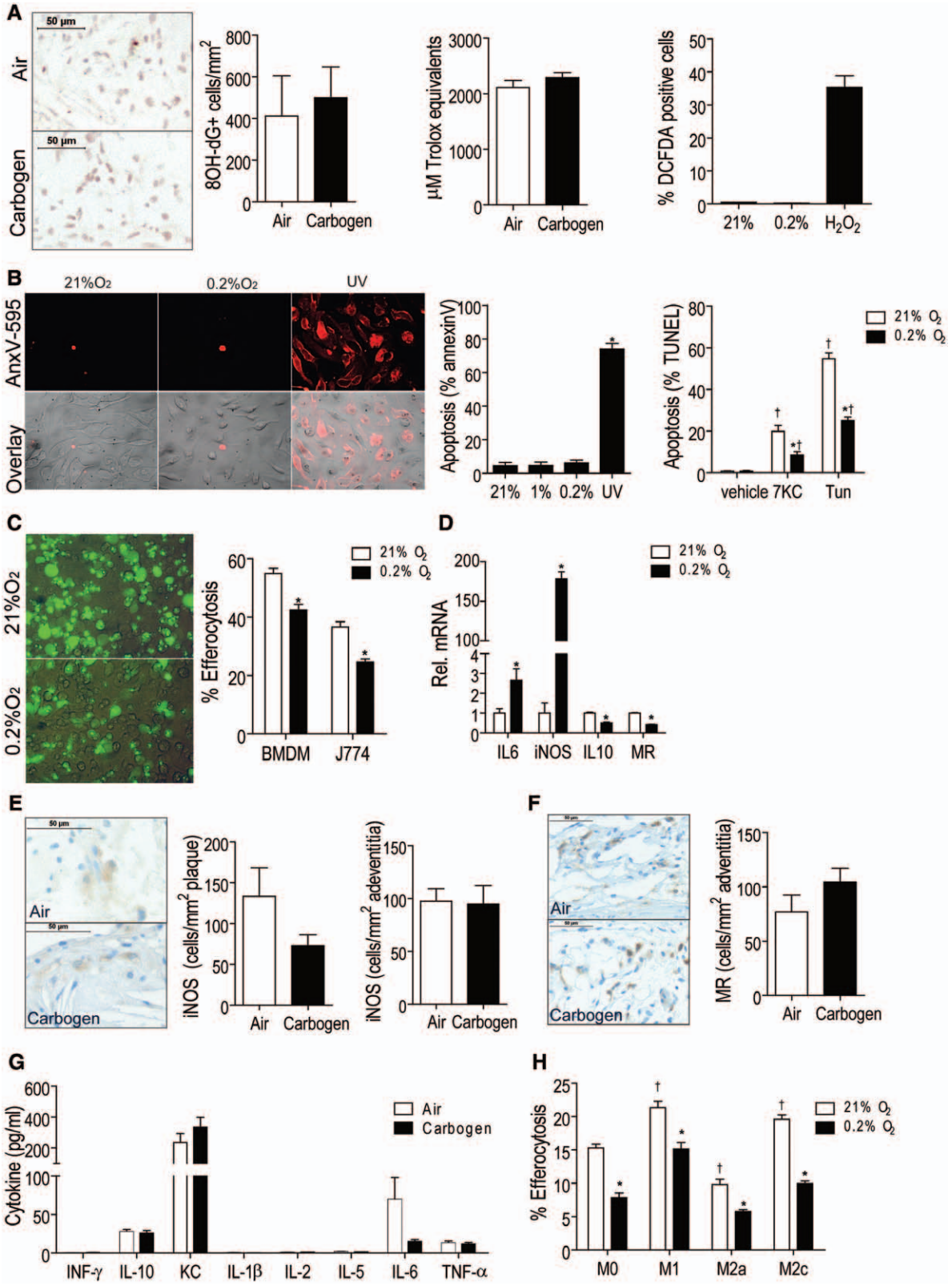

Figure 4. Neither reactive oxygen species (ROS) formation nor macrophage polarization explains hypoxic repression of efferocytosis. **A**, Representative images and quantifications of ROS-mediated DNA damage (8-hydroxy-2′deoxy-guanosine [8OH-dG]) in aortic roots of carbogen or air-treated mice (n=15/group, **left**), low-density lipoprotein receptor–deficient plasma antioxidant capacity relative to the standard trolox, a water-soluble vitamin E analog (trolox equivalents, middle graph), and 2′,7′-dichlorofluorescin diacetate (DCFDA) incorporation in bone marrow–derived macrophages (BMDM) exposed to 21%, 0.2% O_2 (right graph) for 24 hours. **B**, Representative images and quantifications of percentage of annexin A5 (red) apoptotic BMDM exposed to 21% O_2, 0.2% O_2 or ultraviolet (UV)-irradiated (left graph). Quantification of apoptosis (% terminal uridine nick-end labeling) in 7 ketocholesterol and tunicamycin-treated BMDM in 21% O_2 or 0.2% O_2. *P<0.05 vs normoxia, †P<0.05 vs normoxia vehicle. **C**, Representative images of calcein-acetoxymethyl–labeled apoptotic Jurkat T cells (Continued)

Figure 4 Continued. (green fluorescent) ingested by BMDM (phase contrast overlay with fluorescence) in 21% O_2 and 0.2% O_2. Quantification shows percentage of calcein+BMDM or J774 macrophages having ingested labeled, apoptotic Jurkat T cells. *P<0.05. **D**, M1 (interleukin 6 [IL6]; inducible nitric oxide synthase [iNOS]) and M2 (IL10; mannose receptor [MR]) gene expression of BMDM exposed to 21% or 0.2% O_2. *P<0.05. **E**, iNOS in plaque and adventitia and (**F**) MR expression in the adventitia of aortic roots after chronic carbogen compared with air. **G**, Plasma cytokine expression in carbogen and air-treated mice. **H**, Efferocytosis of apoptotic Jurkats by normoxic or hypoxic unpolarized (M0) BMDM or polarized toward an M1, M2a, or M2c phenotype. *P<0.05 vs normoxia of same polarization state, †P<0.05 vs normoxic M0. IL indicates interleukin; INF, interferon; KC, keratinocyte-derived cytokine; and TNF, tumor necrosis factor.

efferocytosis in normoxia (Figure 5B). Importantly, although hypoxia significantly reduced the efferocytosis capacity of wild-type (WT) BMDM, hypoxia was not able to repress efferocytosis in MerTK^{kd} BMDM any further (Figure 5B). This effect was specifically mediated by the MerTK^{kd} because expression of other efferocytosis receptor expression in MerTK^{kd} BMDM was unaltered, except for a reduction in CD36 mRNA (Figure 5C). However, as CD36 knockout did not affect efferocytosis (Figure 5D), as reported previously[23]; this suggests that hypoxic repression of efferocytosis in WT BMDM is no longer effective in MerTK^{kd} BMDM. Hypoxia-dependent downregulation of MerTK in WT is thus likely responsible for repression of efferocytosis in a hypoxic milieu, such as the atherosclerotic plaque.

Discussion

This study revealed that reversal of plaque hypoxia can inhibit the progression of atherosclerotic plaques to a vulnerable phenotype, by reducing the development of the plaque-destabilizing necrotic core. Also, it was shown that murine plaque macrophages are hypoxic from the initiation of atherosclerosis on, using the hypoxia-specific marker pimonidazole. Importantly, we revealed that hyperoxic carbogen gas not only achieved reoxygenation of atherosclerotic plaques, liver, and spleen in LDLR$^{-/-}$ mice but also prevented apoptotic cell accumulation in the plaque.

Apoptosis induction in vitro was not augmented by hypoxia, suggesting that reoxygenation restored the known deficiency in apoptotic cell clearance in atherosclerosis.[19] Mechanistically, hypoxia-dependent transcriptional downregulation of MerTK and subsequent reduction of apoptotic cell clearance by MerTK are the likely underlying cause for hampered efferocytosis by hypoxic macrophages. Thus, this study is the first to show a causal role of hypoxia in plaque destabilization, specifically in efferocytosis.

In addition, plaque thrombogenicity was studied using tissue factor expression, a key trigger of the extrinsic coagulation system causally linked to plaque thrombosis.[21] Interestingly, hypoxia enhanced macrophage expression of tissue factor, supporting the prior correlation of human plaque hypoxia to intraplaque hemorrhage.[6] Nevertheless, carbogen did not prevent the hypoxia-induced expression of plaque tissue factor.

The oxygen debt in atherosclerosis is thought to arise from the sizeable oxygen-consuming metabolism of inflammatory cells, as even macrophages of LDLR$^{-/-}$ fatty streaks directly adjacent to arterial oxygen supply, within the 100 to 200 μm oxygen diffusion distance, were hypoxic. This was already suggested by our previous work, showing that human plaque macrophages were hypoxic, despite the close proximity—within 40 μm—of arterial blood oxygen supply.[6] Moreover, hypoxia was also detected in most tissue-resident leukocytes of lymphoid organs in LDLR$^{-/-}$ mice. Not surprisingly, as hypoxia was found in many, if not all tissues with an extensive inflammatory influx, such as wound healing, obesity, rheumatoid arthritis, and tumors.[25–28] Because pimonidazole only forms adducts in low oxygen concentrations (<10 mm Hg oxygen),[17] this indicates that hypoxia in plaque macrophage arises from a high oxygen demand exceeding oxygen supply. Activated macrophages, in need of ATP for protein production and migration, show 9-fold enhancement of their oxygen consumption.[29] Oxygen usage of activated macrophages would then compared with the notoriously high O_2 consumption by cardiac myocytes[30] which were also positive for pimonidazole and sensitive to carbogen-mediated reoxygenation (data not shown). Therefore, it is highly likely that on activation, macrophages rapidly become hypoxic, even within the oxygen-diffusion limit.

Once hypoxic, macrophages will primarily rely on glycolysis as main energy supply, as suggested by a hypoxia-dependent increase in glucose uptake.[31] Together, this may explain prior results of plaques with low glucose and ATP content, and high lactate levels.[32] Increased glycolytic activity has been linked with M1 polarization of macrophages.[33] Hypoxic macrophages presented with a proinflammatory M1 phenotype in vitro, although reoxygenation in vivo failed to switch polarization. Nevertheless, tumor-associated macrophages located in hypoxic tumor regions express an M2-like profile, and both M1 and M2 marker expression was found in hypoxic adipose tissue macrophages.[34,35] Hence, macrophage subtype distinction might not be as clear in vivo, and despite hypoxia, the

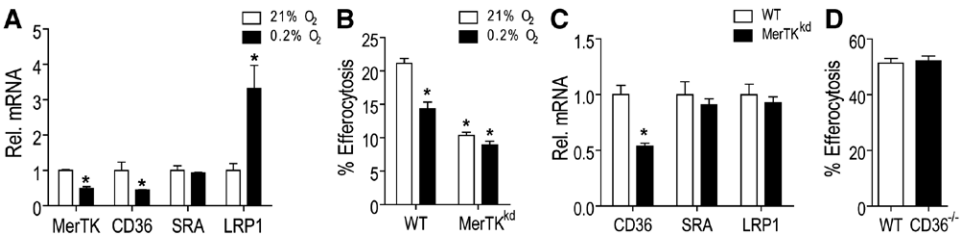

Figure 5. Hypoxia limits efferocytosis via Mer tyrosine kinase (MerTK). **A**, Efferocytosis receptor gene expression (scavenger receptor A [SRA]; low-density lipoprotein receptor–related protein 1 [LRP1]) in normoxia or 0.2% O_2. *P<0.05 vs normoxia of same gene. **B**, Efferocytosis in wild-type and Mer tyrosine kinase domain–deficient (MerTK^{kd}) bone marrow–derived macrophages (BMDM) exposed to 21% or 0.2% O_2. *P<0.05 vs wild-type normoxia. **C**, Efferocytosis receptor gene expression in WT and MerTK^{kd} BMDMs. *P<0.05 vs WT of same gene. **D**, Efferocytosis of apoptotic Jurkats by WT and CD36$^{-/-}$ BMDM.

microenvironment seems to influence macrophage polarization/function also in the plaque. M2c macrophages are the most superior efferocytic macrophage subtype in humans.[22] However, neither M1 nor M2 polarization could protect from hypoxia-mediated reduction in efferocytosis. Mechanistically, low oxygen tension downregulated MerTK and CD36 expression while upregulating LRP1. Both CD36 and LRP were not involved in efferocytosis in vitro as shown by us and others.[23] Hypoxic upregulation of LRP1 expression may be efferocytosis independent because LRP1 has a myriad of other functions related to atherosclerosis.[36] Only MerTK^{kd} mimicked the reduced efferocytosis seen in hypoxic macrophages. In fact, MerTK^{kd} has been shown to aggravate atherosclerosis in mice and worsen lupus-like autoimmunity,[20,24,37] suggesting a role of MerTK in chronic inflammatory diseases.

Although MerTK^{kd} bone marrow accelerated both atherosclerotic plaque size, as well as necrotic core size,[24] carbogen treatment only affected the latter. This may be explained by the potentially partial inhibition of MerTK expression, the intermittent nature of the carbogen treatment, and treatment onset after initiation of atherosclerosis. Although MerTK^{kd}-transplanted LDLR$^{-/-}$ recipient mice presented with a continuous and complete absence of MerTK activity before plaque initiation,[24] normoxic macrophages in vitro show a 50% increase in MerTK expression compared with hypoxic macrophages. Treatment of MerTK^{kd} mice with carbogen gas could provide definite proof of the MerTK-mediated carbogen effect.

This study is limited by the systemic nature of carbogen exposure. Therefore, many aspects of peripheral processes instrumental in atherogenesis were examined. Carbogen did not affect erythropoiesis, myelopoiesis, thrombogenicity, or plasma cholesterol level. Also, blood pressure and heart rate were unaffected.[38,39] Although unlikely, we cannot fully exclude an indirect effect of carbogen on atherogenesis.

Our results unveil an exciting new process to exploit for therapeutic strategies for atherosclerosis. In mice, carbogen gas was able to stabilize the atherosclerotic plaque; if translatable to the human situation, this may potentially prevent plaque ruptures and thrombosis. Carbogen and hyperbaric oxygen therapy were safely and successfully applied in cancer and diabetic foot patients, respectively.[40,41] Although in humans with sleep apnea, maintained oxygen supply through continuous positive airway pressure therapy already proved effective in reversing subclinical atherosclerosis,[42] effectiveness in existing human atherosclerosis remains unclear. In addition, currently developed tools for imaging of hypoxia[13,43] can be used to monitor the efficacy of carbogen to alter human plaque hypoxia and stability.

In conclusion, carbogen-mediated reoxygenation supports the role of plaque hypoxia as a driver of plaque instability through dysfunctional MerTK-mediated clearance of apoptotic cells and subsequent necrotic core expansion. Nevertheless, translation of carbogen therapy to human atherosclerosis will need to be confirmed using noninvasive imaging of plaque hypoxia and stability.

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Disclosures

None.

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Significance

This study is the first to show a functional link between hypoxia and atherosclerosis. This functional contribution of hypoxia uncloses hitherto overlooked targets in atherosclerosis treatment but also risk assessment and imaging of plaques. By identifying a mechanistic contribution of Mer tyrosine kinase in hypoxic macrophages, we established an additional point of attack in atherosclerosis management. Importantly, our study opens up a new field within atherosclerosis research because hypoxia might present a previously disregarded contributor to the plaque milieu, which should be considered in future mechanistic studies.