HIF-1α and PFKFB3 Mediate a Tight Relationship Between Proinflammatory Activation and Anaerobic Metabolism in Atherosclerotic Macrophages

Ahmed Tawakol,* Parmanand Singh,* Marina Mojena, María Pimentel-Santillana, Hamed Emami, Megan MacNabb, James H.F. Rudd, Jagat Narula, José A. Enriquez, Paqui G. Través, María Fernández-Velasco, Ramón Bartrons, Paloma Martín-Sanz, Zahi A. Fayad, Alberto Tejedor, Lisardo Boscá

Objective—Although it is accepted that macrophage glycolysis is upregulated under hypoxic conditions, it is not known whether this is linked to a similar increase in macrophage proinflammatory activation and whether specific energy demands regulate cell viability in the atheromatous plaque.

Approach and Results—We studied the interplay between macrophage energy metabolism, polarization, and viability in the context of atherosclerosis. Cultured human and murine macrophages and an in vivo murine model of atherosclerosis were used to evaluate the mechanisms underlying metabolic and inflammatory activity of macrophages in the different atherosclerotic conditions analyzed. We observed that macrophage energetics and inflammatory activation are closely and linearly related, resulting in dynamic calibration of glycolysis to keep pace with inflammatory activity. In addition, we show that macrophage glycolysis and proinflammatory activation mainly depend on hypoxia-inducible factor and on its impact on glucose uptake, and on the expression of hexokinase II and ubiquitous 6-phosphofructo-2-kinase. As a consequence, hypoxia potentiates inflammation and glycolysis mainly via these pathways. Moreover, when macrophages’ ability to increase glycolysis through 6-phosphofructo-2-kinase is experimentally attenuated, cell viability is reduced if subjected to proinflammatory or hypoxic conditions, but unaffected under control conditions. In addition to this, granulocyte-macrophage colony-stimulating factor enhances anaerobic glycolysis while exerting a mild proinflammatory activation.

Conclusions—These findings, in human and murine cells and in an animal model, show that hypoxia potentiates macrophage glycolytic flux in concert with a proportional upregulation of proinflammatory activity, in a manner that is dependent on both hypoxia-inducible factor-1α and 6-phosphofructo-2-kinase. (Arterioscler Thromb Vasc Biol. 2015;35:1463-1471. DOI: 10.1161/ATVBAHA.115.305551.)

Key Words: anoxia ■ atherosclerosis ■ energy metabolism ■ macrophages ■ positron-emission tomography

A body of research extending over three quarters of a century shows that myeloid cells including macrophages are highly dependent on glycolysis for energy metabolism.1 Subsequently, several lines of evidence have linked macrophage proinflammatory activation with cellular energetics.2-4 However, the precise nature of this link remains poorly understood. It was recently shown that hypoxic macrophages upregulate the expression of glucose transporters, hexokinase II, and the ubiquitous form of 6-phosphofructo-2-kinase. (encoded by PFKFB3) to substantially increase the glycolytic flux.5 However, it remains unknown whether this increased glycolytic flux occurs to simply maintain constant energy delivery in an anaerobic environment or it parallels a similar increase in proinflammatory activation. Moreover, the relationship between macrophage proinflammatory activation and energetics is not well understood in the setting of an atherogenic milieu. Accordingly, in this series of investigations, we sought to investigate the relationship between 2 fundamental processes: energy metabolism and inflammatory activation of macrophages in an atherogenic environment.

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From the Cardiology Division, Massachusetts General Hospital and Harvard Medical School, Boston (A.T., P.S., H.E., M.M.); Cardiology Division, Weill Cornell Medical College, New York Presbyterian Hospital, NY (P.S.); Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), Madrid, Spain (M.M., M.P.-S., P.G.T., M.F.-V., P.M.-S., L.B.); Centro de Investigación en Red en Enfermedades Hepáticas y Digestivas (CIBERHED), Instituto de Salud Carlos III, Madrid, Spain (M.M., M.P.-S., P.G.T., M.F.-V., P.M.-S., L.B.); Hospital General Universitario Gregorio Marañón, Madrid, Spain (M.M., M.P.-S., P.G.T., M.F.-V., L.B.); Hospital General Universitario Gregorio Marañón, Madrid, Spain (M.M., M.P.-S., P.G.T., M.F.-V., L.B.); Centro Nacional de Investigaciones Cardiovasculares, Melchor Fernández Almagro, Madrid, Spain (J.A.E.); The Salk Institute for Biological Studies, La Jolla, CA (P.G.T.); Idiapaz, Hospital Universitario La Paz, Madrid, Spain (M.F.-V.); and Unitat de Bioquímica i Biologia Molecular, Departament de Ciències Fisiològiques II, Universitat de Barcelona, Barcelona, Spain (R.B.).

*These authors contributed equally to this article.

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1463
Inflammation plays an important role in a wide range of diseases. Atherosclerosis is a chronic inflammatory condition accounting for the largest share of mortality in the developed world and is one of the most devastating of inflammatory conditions. Consequently, there is a critical need for greater understanding of the biological mechanisms underlying atherosclerotic inflammation and its complications, including plaque rupture and intraplaque hemorrhage. Atherosclerosis is characterized by macrophage-predominated inflammation existing in the context of proinflammatory cytokines, oxidized low-density lipoproteins (oxLDL), and hypoxia within the arterial wall. Despite mounting evidence that hypoxia promotes inflammation in cancer, obesity, and other inflammatory diseases, in atherosclerosis, the mechanism and precise contribution of hypoxia to other plaque constituents (eg, cytokines and oxLDL) on the proinflammatory milieu remains to be established and a recent study questioned whether proinflammatory stimuli present in atheroma upregulate macrophage glucose metabolism at all.

We studied macrophage responses to a range of proinflammatory stimuli relevant to atherosclerosis and observed that proinflammatory stimuli substantially increased both the rate of glycolysis (flux) and proinflammatory activation (eg, tumor necrosis factor (TNF)-α production). Moreover, we found that glycolytic flux and proinflammatory activation show a significant correlation over a range of physiological inflammatory stimuli and across normoxic and hypoxic conditions. We also noted that hypoxia substantially potentiates the effect of cytokines on both glycolytic flux and proinflammatory activation (in similar proportions). We then demonstrated that blocking hypoxia sensing or enzymes responsible for high glycolytic flux substantially reduced both the proinflammatory activation and the glycolytic flux of macrophages. Moreover, inhibition of glycolysis after PFKFB3 expression has a profound effect on murine and human macrophage viability, leading to enhanced necrotic and apoptotic death under proinflammatory conditions. Subsequently, we confirmed the in vivo relevance of our findings in an animal model.

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

Results
Hypoxia Potentiates Macrophage Proinflammatory Activation and Glycolysis
In atherosclerosis, as with other inflamed tissues, the microenvironment plays a key role in determining the activation state of macrophages. Cytokines are an important constituent of the inflammatory milieu influencing macrophage commitment along a classical (M1 proinflammatory) or alternative (M2 anti-inflammatory) polarization. To establish a firm basis for the series of experiments in this study, we sought to confirm the known effects of various M1 versus M2 activators on elicited peritoneal murine macrophage polarization. In addition, we evaluated the relative action of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that is relevant to the atheroma and a potent macrophage regulator, which served as a physiological classical stimulus given its ubiquitous, endogenous expression in rodents and humans. Despite the relative importance of GM-CSF to macrophage biology in vivo, the metabolic impact of GM-CSF on macrophage metabolism is not well understood. Accordingly, we examined macrophages after exposure to cytokine (classical stimuli: TNF-α, interleukin (IL)-1β, interferon-γ), lipopolysaccharide (innate stimulus), IL4/IL13 (alternative stimulus), and GM-CSF. Using complementary techniques to evaluate the polarization (Figure 1A–1D), we observed as expected that M1 markers increased after classic or innate activation (with lipopolysaccharide and cytokine), whereas M2 markers increased after stimulation with IL4/IL13. Furthermore, we observed that GM-CSF exerts a M1-like polarization, resulting in mild increases in NO and TNF-α accumulation, (Figure 1B and 1C), well-accepted signatures of the M1 activation. Furthermore, under either classic or innate stimuli, we observed a shift in the gene expression of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB), from the liver type-PFK2 (PFKFB1), which has a low net kinase activity, to the more active inducible form PFKFB3 (Figure 1D). This response was further exacerbated under hypoxic conditions, as suggested by the increased hypoxia-inducible factor (HIF)-1α levels (Figure 1E and 1F). It is well established that PFKFB3 has greater kinase than bisphosphatase activity and thus favors the rise in fructose-2,6-bisphosphate (Fru-2,6-P2), a potent regulator of upper glycolysis that is required for high glycolytic rates.

Observations Under Atherogenic Conditions
Next, we evaluated the actions of key atherosclerotic constituents (oxLDL, LDL, and hypoxia) on murine macrophage proinflammatory activation and glycolysis. We found that oxLDL and GM-CSF (but not LDL) each resulted in proinflammatory activation of murine macrophages (Figure 2A). Notably, hypoxia on its own did not increase proinflammatory stimulation. However, in cells exposed to GM-CSF or oxLDL, hypoxia substantially potentiated proinflammatory activation (Figure 2A, right). The effects on glycolysis of these atherosclerotic constituents were similar to those on TNF-α production: oxLDL, GM-CSF, and cytokine each resulted in substantial increases in Fru-2,6-P2 concentration (Figure 2B). Moreover, although hypoxia alone caused a minimal increase in glycolysis, hypoxia markedly potentiated the glycolytic flux in the presence of proatherogenic mediators (cytokines and oxLDL; Figure 2B, right; Figure 2C). Furthermore, while hypoxia alone was not associated with an increase in glucose consumption in nonactivated macrophages, hypoxia substantially potentiated the effect of cytokine and GM-CSF (Figure 2D). These results agree with the expression levels of genes encoding the glucose transporter Glut-1 and the genes encoding hexokinase.
II and PFKFB3, the enzyme responsible for the high-throughput synthesis of Fru-2,6-P₂. We observed a similar action of hypoxia on the expression of those genes: hypoxia had a minor impact on its own, but substantially potentiated the actions of cytokine and GM-CSF (Figure 2E).

Close Inter-Relationship Between Glycolysis and Proinflammatory Activation. Role of TNF-α
Because we observed that both macrophage proinflammatory activation and energetics increase in the presence of atherosclerotic mediators, we sought to define the strength of the inter-relationship between these 2 processes. We observed a strong linear correlation ($R=0.97$; $P<0.001$) between glycolytic flux (as Fru-2,6-P₂ levels) and proinflammatory activity (measured as TNF-α production) under both hypoxic and normoxic conditions (Figure 3A). Hence, we demonstrate that macrophage glycolysis keeps pace with the proinflammatory activated state of the cell regardless of oxygen tension. Moreover, because GM-CSF promotes TNF-α synthesis, we
sought to determine its contribution to the glycolytic phenotype. As Figure 3B shows, the presence of neutralizing antibodies against TNF-α in the culture medium significantly attenuated the expression of PFKFB3, the rise in Fru-2,6-P₂ levels and the release of lactate, providing a link for TNF-α between the inflammatory and glycolytic profiles.

**PFKFB3 Regulates Both Glycolytic Metabolism and Inflammation**

Given the strong linear association between macrophage glycolysis and inflammatory activity in murine cells, we next sought to evaluate whether (1) a high glycolytic rate was necessary for inflammatory activation and (2) this relationship was maintained in human macrophages. To investigate this, human macrophages were separately treated with 2 inhibitors of PFKFB3: (1) a human small interfering RNA targeting PFKFB3 and (2) (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) (3PO), a selective inhibitor of PFKFB3. In the first experiment, we observed that silencing PFKFB3 effectively attenuated PFKFB3 without affecting the constitutively expressed PFKFB1 or hexokinase II (Figure 4A), and thus did not affect basal glycolysis (Figure 4B). However, we observed that inhibition of PFKFB3 activity under stimulatory conditions results in a significant reduction in glycolytic flux (measured as Fru-2,6-P₂ and lactate release; Figure 4A), and thus did not affect basal glycolysis (Figure 4B). However, we observed that inhibition of PFKFB3 activity under stimulatory conditions results in a significant reduction in glycolytic flux (measured as Fru-2,6-P₂ and lactate release; Figure 4A), and thus did not affect basal glycolysis (Figure 4B).

Hereafter, we tested the hypothesis that high-flux glycolysis is required to maintain macrophage viability under conditions of proinflammatory stimulation. In this experiment, we observed that blocking PFKFB3 did not reduce the viability of resting macrophages; however, under conditions of proinflammatory stimulation, the viability of activated macrophages decreased when PFKFB3 was inhibited (Figure 4D). Taken together, these observations provide evidence that the ability to upregulate PFKFB3 activity is necessary to maintain the viability of activated (but not resting) macrophages.

**HIF-1α Also Regulates Both Glycolytic Metabolism and Inflammation**

Because we observed that hypoxia potentiates macrophage proinflammatory activation, we hypothesized that interruption of hypoxia-sensing might attenuate inflammation in an atherosclerotic environment. Indeed, within the context of cancer, it has recently been shown that hypoxia-induced overexpression of HIF-1α results in metabolic adaptation (a shift to glycolysis from Krebs cycle and oxidative phosphorylation) which in turn facilitates cell survival. We sought to evaluate whether a similar relationship exists in atherosclerotic macrophages. We observed that silencing HIF-1α substantially reduced glycolytic flux (as Fru-2,6-P₂ and lactate; Figure 5A and 5B) across oxygen tensions and under various proinflammatory conditions. In parallel, blocking HIF-1α substantially reduced proinflammatory activation of macrophages (as TNF-α production; Figure 5C). Furthermore, we observed that inhibition of either PFKFB3 or HIF-1α resulted in proportionate reductions in both glycolysis (as Fru-2,6-P₂) and proinflammatory activation (R=0.68, P=0.007; Figure 5D). In addition to this, we evaluated the role of hypoxia on the inducibility of PFKFB3 in response to GM-CSF. As Figure 5E shows, macrophages transfected with a plasmid carrying the HRE sequence of the human pfkfb3 promoter (29 nucleotides, from −1297 to −1269) linked to a luciferase reporter exhibited an increase not only in activity because of hypoxia but also by the action of GM-CSF, a response that was enhanced under hypoxic conditions. Collectively, these observations suggest that both upregulation of upper glycolysis and proinflammatory activation are dependent on HIF-1α across oxygen tensions, highlighting the cross talk between hypoxic and nonhypoxic signaling pathways in the regulation of metabolic adaptation in response to atherosclerotic stimuli. Indeed, silencing of HIF-1α provoked a loss in viability with increased rates of apoptosis and necrosis compared to controls (Figure 5F). This was especially evident in the presence of oxLDL or under hypoxic conditions. This finding reaffirms that an intact HIF-1α system is required for macrophage survival in an atherosclerotic microenvironment and that survival of activated macrophages is also dependent on high glycolytic flux, through upregulation of PFKFB3. In addition to this, O₂ consumption decreased in M1 macrophages. Moreover, under

Figure 3. Close inter-relationship between murine macrophage proinflammatory activation (TNF-α) and Fru-2,6-P₂ concentration as indicator of glycolysis. A. Correlation between the accumulation of TNF-α in the culture medium and intracellular Fru-2,6-P₂ concentrations under the experimental conditions described in Figures 1 and 2. B. Neutralizing goat antimouse TNF-α Abs (10 μg/mL) in the culture medium prevent 6-phosphofructo-2-kinase (PFKFB3) induction, the rise in Fru-2,6-P₂ concentration and the release of lactate in macrophages treated for 24 hours with granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/mL) under normoxic and hypoxic (1% O₂) conditions. Goat IgG (10 μg/mL) was used as control immunoglobulin. Results show a representative blot and the mean±SD of three experiments. **P<0.01 vs the same IgG condition.
low glucose availability, forcing respiration with FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) resulted in cell death of M1 cells, but not in resting or M2 counterparts (Figure 6B), but enhanced nonmitochondrial O₂ consumption in agreement with ROS production, in particular, in the presence of oxLDL (Figure 6C and 6D).

Inhibition of PFKFB3 and HIF-1α Attenuates Glycolytic Flux in ApoE−/− Mice

We then sought to evaluate the impact of selective inhibition of PFKFB3 and HIF-1α on glycolytic flux in atherosclerotic mice (ApoE−/− mice fed a high-fat diet). Glycolysis in the arterial wall was assessed using ^18F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomographic imaging before and after impairing PFKFB3 (using 3PO or PFKFB3 small interfering RNA). Silencing hypoxia-inducible factor (HIF)-1α decreases human macrovascular energetics, proinflammatory activation, and viability. Human macrophages were maintained under normoxia or hypoxia and silenced with siRNA for HIF-1α, and then activated with human macrophage colony-stimulating factor (GM-CSF) or cytokine (CK; 20 ng/mL each). A, Effect of small interfering RNA (siRNA) for HIF-1α on the levels of Fru-2,6-P₂ and lactate release after stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) or cytokine (CK; 20 ng/mL each). B, Interrelationship between TNF-α and Fru-2,6-P₂ levels under the different experimental conditions described in Figures 4 and 5 (including normoxia, hypoxia, low-density lipoprotein [LDL] and oxidized LDL [oxLDL] and silencing PFKFB3-2 kinase [PFKFB3] and HIF-1α), and inhibition of PFKFB3 (3PO; 10 μmol/L). C, Time-course of tumor necrosis factor (TNF)-α accumulation under hypoxia and after silencing HIF-1α. D, Interrelationship between TNF-α and Fru-2,6-P₂ levels under the different experimental conditions described in Figures 4 and 5 (including normoxia, hypoxia, low-density lipoprotein [LDL] and oxidized LDL [oxLDL] and silencing PFKFB3-2 kinase [PFKFB3] and HIF-1α), and inhibition of PFKFB3 (3PO; 10 μmol/L). E, After transfection of macrophages with a luciferase reporter plasmid containing the HRE domain of the pfkfb3 gene promoter, or the mutated sequence lacking HRE binding (pHREwt), and inhibition of PFKFB3 (3PO; 10 μmol/L). F, After activation for 60 hours with GM-CSF or CK, the percentage of apoptotic/necrotic cells was determined. Results show the mean+SD (n=3; A, B, and D) or a representative experiment out of 3 (C). **P<0.01 vs the same condition with scramble RNA (A and B), vs the basal condition under normoxia (E) or vs the corresponding LDL condition (F). **P<0.01 vs the same condition under normoxia (A and B).
after removal of the arterial tissues, we measured the tissue quantities of PFKFB3 and Fru-2,6-P2 and found that their tissue levels mirrored the measurements of FDG uptake across the tested conditions (Figure 7B and 7C). Similarly, the tissue measurements of proinflammatory mediators TNF-α and CCL2 were similarly affected by the inhibitors (Figure 7D). Also, silencing or inhibition of PFKFB3 resulted in an enhancement of active caspase 3 in the plaque (Figure 7E), confirming the loss in cell viability under these conditions. Interestingly, all animals were alive and stable after treatment courses. Given the tenacious relationship between glycolysis and inflammation, these in vivo observations raise the possibility that antagonism of HIF-1α or modulation of glycolysis may provide a pharmacological approach to alleviate the inflammatory burden in atherosclerotic disease.

Discussion

The findings of this study highlight the basis for and importance of macrophage metabolic adaptation within atherosclerosis. We show that hypoxia potentiates macrophage glycolytic flux along with a proportional upregulation of proinflammatory activity (in a manner that is dependent on both HIF-1α and PFKFB3). Moreover, we show that antagonizing either HIF-1α or PFKFB3 activities simultaneously impairs both glycolysis and proinflammatory activation in vitro, and we provide translational evidence of this phenomenon in vivo. Furthermore, we show that impairing either HIF-1α or PFKFB3 in the setting of proinflammatory stimulation results in loss of monocyte viability after activation of caspase cascades. The potential impact of reduced monocyte/macrophage viability in atherosclerotic plaques is unclear. Macrophage proliferation within plaque has been described, and reduced viability of such cells, as well perhaps of other macrophages, may indeed be beneficial. However, there is also the possibility of detrimental effects of enhancing cell death with possible postapoptotic necrosis if effecrosis is deficient. Thus,
additional work is needed to better understand the implications of such modulation of macrophage viability within the atheroma. The observation that hypoxia increases glycolysis is a phenomenon referred to, as the Warburg Effect and appears to be involved in macrophage physiology at different points of their maturation and function. One proposed explanation for Warburg observation is that hypoxia obligates a shift in metabolism, from the tricarboxylic acid cycle to glycolysis simply to compensate for loss of aerobic metabolism. However, our observations provide an alternate explanation for the Warburg Effect, at least as far as it relates to atherosclerotic macrophages, namely that (1) hypoxia also increases the proinflammatory activation of macrophages and (2) the glycolytic rate is increased in a manner that keeps pace with this increased inflammatory and proatherogenic activity, namely because of the contribution of oxLDL to the enhanced glucose consumption and ROS production. Collectively, these observations give rise to a paradigm wherein hypoxia can act to exacerbate a cycle of inflammation (Figure 8). Within this paradigm, cytokines trigger upregulation of HIF-1α, which is stabilized under hypoxic conditions. After transcriptional induction of PFKFB3 leads to increased proinflammatory activation and increased glycolysis. The activated macrophages then produce additional cytokines to perpetuate this vicious inflammatory cycle. Our data have potentially important implications for the treatment of atherosclerotic inflammation. We found that selective inhibition (or silencing) of PFKFB3, HIF-1α, or both results in a substantial reduction in proinflammatory activation. Along these lines, PFKFB3 has been recently shown to play a regulatory role in proliferation and angiogenesis. Accordingly, drugs that target PFKFB3 or hypoxia sensing, which are also currently in development as cancer therapeutics, should be further studied in the context of atherosclerosis. Moreover, studies should assess whether targeting PFKFB3 or reducing hypoxia sensing within plaques will decrease atherosclerotic inflammation and its clinical consequences. Indeed, in a previous work using macrophages deficient for HIF-1α in the myeloid lineage, we were surprised by the observation that glycolysis remained increased and PFKFB3 was also overexpressed, perhaps because of the fact that HIF-2α assumed part of the transcriptional control caused by HIF-1α. Therefore, the rationale for the use of PFKFB3 inhibitors offers many advantages because this enzyme is only expressed under hypoxic and proinflammatory conditions. The study findings also have important implications for imaging of atherosclerosis. Although the bulk of previous evidence suggests that proinflammatory stimulation is associated with increased glycolysis, recent study suggested that hypoxia and not proinflammatory activation augments macrophage glucose metabolism. That report proposed that imaging measures of glucose metabolism (eg, with labeled 2-deoxyglucose) may provide an indirectly assessment tissue hypoxia in vivo. However, the findings of the current study suggest that measures of glycolysis are not well suited for assessing hypoxia. Specifically, we observe, across oxygen tensions, that glycolytic flux remains tightly related to the state of macrophage proinflammatory activity, (eg, TNF-α production; Figure 3), thus supporting the use of FDG to image inflammatory cell activity within atheromatous plaques. On the contrary, we found that oxygen tension per se is not a good predictor of the degree of inflammation because proinflammatory activity varies markedly within both normoxic and hypoxic conditions, depending on the availability of other proinflammatory factors, such as oxLDL and cytokines (Figure 2A). Accordingly, hypoxia-directed probes (such as FMISO [18F-fluoromisonidazole]), while likely colocalize to sites of inflammation, may not be so useful for reporting on the level of proinflammatory activation that exists in those lesions. The central importance of inflammation to atherosclerosis warrants pursuit of a deeper understanding of the interconnection between hypoxia, glycolysis, and inflammatory activation within an atheroma. This study demonstrates that a surprisingly tight interconnection exists between macrophage glycolysis and proinflammatory activity, that the link requires HIF-1α and transcriptional induction of PFKFB3, and that inhibition of either under proinflammatory conditions results in a reduction in inflammatory activity. Hence, modulation of macrophage metabolic adaptation may provide an opportunity to develop novel treatments against atherosclerotic inflammation.

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Disclosures

None.

References

Significance

The present data show a connection between macrophage viability, activation and oxygen availability that impacts macrophage function in an atherosclerotic environment. This tight interconnection between macrophage glycolysis and proinflammatory activity, requiring HIF-1α and transcripitional induction of 6-phosphofructo-2-kinase, is susceptible to be modulated by pharmacological inhibitors of 6-phosphofructo-2-kinase (and hypoxia-inducible factor-1α). Therefore, selective inhibition of glycolytic activity may result in a loss of macrophage function and a potential stabilization of atherosclerotic lesions, with relevance in the pharmacological management of atherogenesis.
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FIGURE LEGENDS

Fig. S1. Inhibition/silencing of PFKFB3 attenuates glycolytic flux. Human macrophages were maintained under normoxia or hypoxia, and transfected for 18h with a specific pool of siRNA to silence PFKFB3 or inactive RNA control (scRNA) and then activated with human GM-CSF and CK. In another experiment, cells were treated with LDL or oxLDL (50 μg/ml) and then activated with GM-CSF or CK in the absence or presence of the PFKFB3 inhibitor 3PO. (A) Lactate accumulation in the culture medium was determined in cells treated with siRNA (5 nM) specific for FKFB3, and (B) the PFKFB3 inhibitor 3PO (10 μM). Results show the mean±SD of three experiments. *P<0.05; **P<0.01 vs. the same condition in cells treated with scRNA (A) or vehicle (B).
Materials and Methods

Chemicals. Reagents were from Sigma-Aldrich (St Louis, MO), Roche (Basel, CH), Invitrogen (Carlsbad, CA) or Merck/Millipore (Darmstadt, FRG). LPS was from InvivoGen (San Diego, CA). Human and murine cytokines were from PeproTech (Rocky Hill, NJ) or Gentaur (Kampenhout, Belgium). Commercial antibodies were from Santa Cruz Biotech (Santa Cruz, CA), Cell Signaling (Danvers, MA), Abcam (Cambridge, UK), R&D Systems (Minneapolis, MN), Sigma or PeproTech. siRNA were from Ambion/InvivoGen, OriGene (Rockville, MD) or Sigma-Aldrich. Serum and media were from Lonza (Walkersville, MD). Native human LDLs were purified to homogeneity by ultracentrifugation (1.063-1.21 g/dl; 1.066 g cholesterol/dl, and were maintained at 10 mg of protein/ml at -80ºC under N₂ atmosphere).

Treatment of animals and preparation of peritoneal macrophages. C57BL/6 mice were housed and bred in our pathogen-free facility. Experimental procedures for macrophage isolation were approved by the Committee for Research Ethics of our Institute (Madrid) in accordance with Spanish and European guidelines (see Animal Study Section). Animals were used aged 8 to 12-weeks as follows: Mice were i.p. injected 2.5 ml of 3% (weight/vol.) of thioglycollate broth, and after four days elicited peritoneal macrophages were prepared from anesthetized mice (4-6 animals per experiment) injected i.p. 10 ml of sterile RPMI 1640 medium at RT. After 10 min, the peritoneal fluid was aspirated avoiding hemorrhage and kept at 4ºC to prevent cell adhesion to the plastic. The cells were centrifuged at 200g for 10 min at 4ºC and resuspended and centrifuged twice with 25 ml of ice-cold PBS. An aliquot of the cell suspension was used to determine the cell density and the enrichment in the F4/80⁺ population by flow cytometry. Cells were seeded at 60-75% confluence in RPMI 1640 medium supplemented with 10% of heat inactivated FCS and antibiotics. After incubation for 3h at 37ºC in a 5% CO₂ atmosphere, the remaining non-adherent cells were removed by extensive washing with PBS. Experiments were carried out in phenol-red free RPMI 1640 medium and 1% of heat-inactivated FCS plus antibiotics.

Preparation of human monocyte/macrophages. PBMCs were isolated from buffy-coats of blood from healthy donors (under their written informed consent, through the blood bank Cruz Roja, Blood Bank, Madrid, Spain) by centrifugation on Ficoll-Hypaque Plus (GE Biotech., UK) following the manufacturers’ protocol. The CD14-enriched fraction was collected after binding to MACS-hCD14-magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were differentiated into macrophages with human CSF-1 (20 ng/ml, PeproTech) for 5 days in RPMI 1640 supplemented with antibiotics and 10% FCS. After this period cells were kept for 48h in medium lacking CSF-1 and treated with the indicated stimuli. Purity of all cultures was verified by CD14⁺ staining using a flow cytometer; on average >95% of the detached cells were highly positive for this surface marker.

Transfection of macrophages and luciferase reporter assays. Cells (12-well dishes) were transfected with 600 ng/ml of the wild-type (pHREWt-PFKFB3.Luc) or the HRE mutated 29-nt region (pHREmut-PFKFB3.Luc), from -1297 to -1269, corresponding to the HRE domain of the human pfkfb3 gene promoter cloned in a pGL2-basic vector containing the c-fos minimal promoter (for a description see ²), using Lipofectamine 2000 (Invitrogen) and following the supplier's protocol. A plasmid encoding β-galactosidase was co-transfected (60 ng/ml) to normalize for the efficiency of transfection. Basically, macrophages were transfected in DMEM medium for 4h and the medium was replaced by RPMI 1640 for 12h. After this period, cells were submitted to normoxia or hypoxia (4h) and activated for 6h in the absence or presence of human GM-CSF. Cell extracts were prepared to determine luciferase and β-galactosidase activities (Promega Biotech. Spain). Transfections were performed in triplicate and expressed as the ratio of luciferase to β-galactosidase activities measured in the same cell lysate.
Flow cytometry. Cells were harvested at the indicated times and washed in cold PBS. After centrifugation at 4°C for 5 min and 200g, cells were stained with anti-F4/80 and anti-CD14 mAbs, or with propidium iodide (PI) or annexin-V and analyzed as previously described in a flow cytometer using a FC 500 Becton Dickinson FACScan flow cytometer (Mountain View, CA) with a CXP Software (Beckman Coulter).

PFKFB3 and HIF-1α silencing in macrophages. Cells were transfected overnight with a mixture of Lipofectamine and a pool of at least 3 different Silencer-select predesigned siRNAs (5 nM), following the instructions of the supplier. Controls with the corresponding scrambled (negative) RNAs (scRNA) were used to ensure the specificity of the silencing.

Incubation of macrophages under normoxia and hypoxia. Cell cultures were maintained under normoxia or with 2% to 0.5% O₂. Unless otherwise indicated hypoxia was considered 1% O₂. Media were maintained under these conditions to avoid fluctuations in the O₂ saturation and macrophage treatments were accomplished under these normoxic/hypoxic conditions. Hypoxia and normoxia experiments were run in parallel.

Metabolite assays. NO release was determined spectrophotometrically by the accumulation of nitrite and nitrate in the medium (phenol red-free). Nitrate was reduced to nitrite, and the latter was quantified with Griess reagent by adding 1 mM sulfanilic acid and 100 mM HCl (final concentration) in. Lactate was determined enzymatically in the culture medium using a previously described protocol. Fru-2,6-P₂ was extracted from cells (24-well plates) after homogenization in 100 μl of a fresh solution of 50 mM NaOH at 80°C, transferred to eppendorf tubes and heated at 80°C for 10 min. The metabolite was measured by the activation of the pyrophosphate-dependent 6-phosphofructo-1-kinase.

TNF-α and CCL2 measurements. The accumulation of the cytokines in the culture medium or in tissues was measured per triplicate using commercial kits (PeproTech for TNF-α and Sigma for CCL2), following the indications of the suppliers.

Measurement of oxygen consumption and ROS production. O₂ consumption was determined in a Seahorse device (Seahorse Bioscience) and analyzed using the XF Cell MitoStress test assay. ROS production was measured by the oxidation of DCFH as described.

Preparation of modified LDL. Native LDL (100 µg/ml) were incubated for 24h at RT with sterile copper sulfate (3 µM, final concentration). Oxidized LDL (oxLDL) were re-isolated by centrifugation at 125,000g and oxidation was evaluated as previously described.

Preparation of macrophage extracts. Cell cultures (6 cm dishes) were washed twice with ice-cold PBS and homogenized in 0.2 ml of buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM β-mercaptoethanol and 0.1 mM PMSF and a protease/phosphatase inhibitor cocktail (Sigma). The extracts were vortexed for 30 min at 4°C and centrifuged for 15 min at 13,000g. The supernatants were stored at -20°C. Proteins levels were determined using the Bio-Rad detergent-compatible protein reagent (Richmond, CA). All steps were carried out at 4°C.

Western blot analysis. Samples of cell extracts containing equal amounts of protein (30 µg per lane) were boiled in 250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and size-separated in 10-15% SDS-PAGE. The gels were blotted onto a PVDF membrane (GE Healthcare, UK) and processed as recommended by the supplier of the antibodies against the murine or human
antigens: phospho-STAT-5(Y694), STAT-5, phospho-ERK1/2, ERK1/2, NOS-2, COX-2, HK-II, PFKFB3, PFKFB1, MMP-9, HO-1, Arg-1, HIF-1α, active caspase 3 and β-actin. The blots were developed by ECL protocol (GE Healthcare) and different exposition times were performed for each blot with a charged coupling device camera in a luminescent image analyzer (Molecular Imager, BioRad) to ensure the linearity of the band intensities. Bands were normalized for the content of β-actin.

RNA isolation and RT-PCR analysis. 1 μg of total RNA, extracted with Trizol Reagent (Invitrogen) according to the manufacturer’s instructions, was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit for RT-PCR following the indications of the manufacturer (Roche). Real-time PCR was conducted with SYBR Green on a MyiQ Real-Time PCR System (Bio-Rad) using the SYBR Green method. PCR thermocycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15s, and 60°C for 1 min. All samples were analyzed for 36B4 expression in parallel. Each sample was run in duplicate and was normalized to 36B4. The replicates were then averaged, and fold induction (FI) was determined in a ΔΔCt based fold-change calculations. Primer sequences are available on request.

Caspase 3 activity assay. Tissue extracts (atheromatous caps) were prepared by homogenization in buffer A supplemented with 2 μg/ml TLCK, 5 mM NaF, 1 mM NaVO₄, 10 mM Na₂MoO₄ and 0.5% Nonidet P-40. After centrifugation of the cell lysate the activity of caspase 3 was determined with the fluorogenic substrate N-acetyl-DEVD-7-amino-4-trifluoromethylcoumarin (Merck/Millipore). The linearity of caspase assays was determined over a 30 min reaction period, and was expressed as percentage vs. the activity measured in animals fed a high fat diet.

Animal Model Study.
Animal care and experimental procedures were performed according to the Directive 2010/63/EU of the European Parliament, and the studies were approved by the Institutional Committee on bioethics (authorization 28079-37A to the Instituto de Investigaciones Biomédicas, CISC-UAM).

PFKFB3 and HIF-1α in vivo silencing. A mixture of at least 3 different Silencer-select predesigned siRNAs for PFKFB3 and HIF-1α were obtained from different sources (Ambion/InvivoGen, OriGene or Sigma-Aldrich). The transfection mixture was prepared using Invivofectamine 2.0 (InvivoGen) and was administered i.p. at 5 mg/kg per dose, following the instructions of the supplier. Administration of the corresponding scrambled (negative) RNAs (scRNA) was used to ensure the specificity of the silencing.

Atherogenesis in ApoE deficient mice and FDG-PET Image Analysis. 30 male ApoE deficient mice aged 3-4 Mo were fed a high-fat/high cholesterol diet for three weeks and, after anesthesia with isoflurane, FDG (1 mCi/kg; 0.2 ml) was administered i.p. and the ¹⁸F emission was analyzed in a microCT-microSPECT-microPET (INVEON) system. Images were analyzed and quantified, the first axial slice representing the descending aorta (the first PET/CT slice clear of the aortic arch). The measured maximal standardized uptake values (SUVmax) of the descending aorta for five consecutive slices in intervals of 3 mm was averaged to obtain a mean SUVmax. Measured background SUVs from the paraspinal muscles was used to obtain a corrected TBR. Interobserver variability using this technique was 9%, which is comparable to measured values in similar work by other investigators. When PFKFB3 and HIF-1α were silenced in intact animals, the siRNAs were administered at days 3, 7, 10, 14 and 17 after high-fat/high-cholesterol administration. 3PO was resuspended in Solutol and administered i.p. (50 mg/kg; 0.2 ml) at the same time periods. Animals were processed for biochemical analyses at the end of the experiment.
Data analysis

The data shown are the means ± SD of three to five experiments. Statistical significance was estimated with Student's $t$ test for unpaired observations or ANOVA followed by the Bonferroni test when appropriate. Differences with values of $P<0.05$ were considered statistically significant.
References


