Hypoxia-Inducible Factor Prolyl 4-Hydroxylase-2 Inhibition Protects Against Development of Atherosclerosis

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Objective—Small-molecule hypoxia-inducible factor prolyl 4-hydroxylase (HIF-P4H) inhibitors are being explored in clinical studies for the treatment of anemia. HIF-P4H-2 (also known as PHD2 or EglN1) inhibition improves glucose and lipid metabolism and protects against obesity and metabolic dysfunction. We studied here whether HIF-P4H-2 inhibition could also protect against atherosclerosis.

Approach and Results—Atherosclerosis development was studied in low-density lipoprotein (LDL) receptor–deficient mice treated with an oral HIF-P4H inhibitor, FG-4497, and in HIF-P4H-2-hypomorphic/C699Y-LDL receptor–mutant mice, all mice being fed a high-fat diet. FG-4497 administration to LDL receptor–deficient mice reduced the area of atherosclerotic plaques by ≈50% when compared with vehicle-treated controls and also reduced their weight gain, insulin resistance, liver and white adipose tissue (WAT) weights, adipocyte size, number of inflammation-associated WAT macrophage aggregates and the high-fat diet–induced increases in serum cholesterol levels. The levels of atherosclerosis-protecting circulating autoantibodies against copper-oxidized LDL were increased. The decrease in atherosclerotic plaque areas correlated with the reductions in weight, serum cholesterol levels, and WAT macrophage aggregates and the autoantibody increase. FG-4497 treatment stabilized HIF-1α and HIF-2α and altered the expression of glucose and lipid metabolism and inflammation-associated genes in liver and WAT. The HIF-P4H-2-hypomorphic/C699Y-LDL receptor–mutant mice likewise had a ≈50% reduction in atherosclerotic plaque areas, reduced WAT macrophage aggregate numbers, and increased autoantibodies against oxidized LDL, but did not have reduced serum cholesterol levels.

Conclusions—HIF-P4H-2 inhibition may be a novel strategy for protecting against the development of atherosclerosis. The mechanisms involve beneficial modulation of the serum lipid profile and innate immune system and reduced inflammation. (Arterioscler Thromb Vase Biol. 2016;36:608-617. DOI: 10.1161/ATVBAHA.115.307136.)

Key Words: atherosclerosis ■ cholesterol ■ hypoxia ■ immunology ■ inflammation

The hypoxia-inducible factor (HIF), an αβ dimer, and the HIF-prolyl 4-hydroxylases (HIF-P4Hs) play a central role in the hypoxia response. The HIF-P4Hs hydroxylate 2 prolyl residues in HIF-1α and HIF-2α, thus targeting the HIFα subunits for rapid proteosomal degradation. 1–3 There are 3 HIF-P4H isoenzymes, HIF-P4H-1 to 3 (also known as PHDs1–3 and EglN2, EglN1, and EglN3, respectively), and a fourth transmembrane HIF-P4H-4 that all require oxygen, Fe²⁺, 2-oxoglutarate, and ascorbate. 4 Thus, hydroxylation cannot proceed in hypoxia, in which HIFα escapes degradation and is translocated to the nucleus, where it dimerizes with HIFβ and initiates the transcription of HIF target genes. 1–3 All HIF-P4Hs regulate the stability of HIFα, but HIF-P4H-2 is the main isoenzyme. 1–3 Its knockout in mice is embryonic lethal, whereas the knockouts of HIF-P4Hs 1 and 3 are viable. 5 Broad-spectrum conditional HIF-P4H-2 knock-out results in massive erythrocytosis, dilated cardiomyopathy, hyperactive angiogenesis, and premature death, whereas cell-type specific and partial HIF-P4H-2 deficiency provide protection against cardiac ischemia. 1–3 HIFα can be stabilized pharmacologically under normoxia by inhibiting HIF-P4Hs with small-molecule compounds that are structural analogues of 2-oxoglutarate. 3

No data are available on the role of any HIF-P4H in atherosclerosis, but HIF has been reported to have both beneficial and pathological roles in this condition. 6,7 We have generated HIF-P4H-2 hypomorphic mice (Hif-p4h-2<sup>2/2<sup>5</sup></sup>) that express reduced amounts of HIF-P4H-2 mRNA in various tissues and show stabilization of HIF-1α and HIF-2α but do not develop polycythemia or have a reduced life span. 6,7 The Hif-p4h-2<sup>2/2</sup>

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mice are protected against obesity and have improved glucose tolerance, less white adipose tissue (WAT) macrophage aggregates, increased insulin sensitivity, and reduced serum cholesterol levels whether fed normal chow or a high-fat diet (HFD). This phenotype was copied in wild-type mice treated with an oral HIF-P4H inhibitor, FG-4497. Because high cholesterol levels and metabolic syndrome predispose to atherosclerosis, we set out to study whether HIF-P4H-2 inhibition could protect mice from its development.

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

Results
HIF-P4H Inhibition Reduced Weight Gain, Insulin resistance, Fat Accumulation, Adipose Tissue Inflammation, and Serum Cholesterol Levels in Low-Density Lipoprotein Receptor–Deficient Mice Fed a HFD
We administered a small-molecule 2-oxoglutarate HIF-P4H antagonist, FG-4497, for 13 weeks to Ldlr−/− mice fed a HFD for this same period. FG-4497 inhibits all HIF-P4Hs and stabilizes HIF-1α and HIF-2α. The weight gain of the FG-4497–treated Ldlr−/− mice was reduced when compared with that of the vehicle-treated Ldlr−/− mice (Figure 1A). The FG-4497–treated mice also had reduced weights of liver and gonadal WAT and their adipocyte sizes, and numbers of WAT macrophage aggregates were lowered (Figure 1B–1F). The homeostasis model assessment-insulin resistance scores were reduced in the FG-4497–treated mice (Figure 1G); this accounting especially for the reduced serum insulin levels (1170±420 pmol/L for vehicle-treated and 410±90 pmol/L for FG-4497–treated controls; P<0.004), suggesting that lower insulin levels in FG-4497–treated mice contributed to lower cholesterol levels.

FG-4497 Treatment Reduces Aortic Plaque Area in LDL Receptor–Deficient Mice
The areas of calcified plaques in the full-length aortas and at the aortic origin were reduced by ~50% in the FG-4497–treated HFD-fed Ldlr−/− mice when compared with the vehicle-treated controls (Figure 2A and 2B); no difference being found between the groups in the amount of plaque collagen (Figure 2C). There was a trend for a reduced total area of foam macrophages in the FG-4497 mouse plaques, and this difference disappeared when expressed in proportion to the reduced total plaque area (Figure 2D). The area of the necrotic cores of the FG-4497 mouse plaques was <30% of that in the controls (Figure 2E). The plaque sizes in the whole aorta and at the aortic origin correlated positively with body weight, serum cholesterol level, and the number of WAT macrophage aggregates suggesting that their reductions in the FG-4497–treated mice were protective against atherosclerosis development (Figure 3A–3F).

FG-4497 Treatment Stabilized HIF-1α and HIF-2α and Altered the Expression of Glucose and Lipid Metabolism Genes in the WAT and Liver of LDL Receptor–Deficient Mice
FG-4497 administration stabilized HIF-1α and HIF-2α in the liver (Figure IA in the online-only Data Supplement). The hepatic mRNA levels of Sterol regulatory element-binding protein (Srebp1c), its targets Acetyl-CoA carboxylase α (Accα), Fatty acid synthase (Fas), and Stearoyl-CoA desaturase-1 (Scd1) were reduced in the HFD-fed FG-4497–treated Ldlr−/− mice, whereas that of Insulin receptor substrate 2 (Irs2), a reported HIF-2α target, which downregulates Srebp1c, was increased (Figure IB in the online-only Data Supplement). The hepatic expression level of the key gene regulating cholesterol synthesis 3-OH-3-methylglutaryl-CoA reductase (Hmgcoar) and that of 3-OH-3-methylglutaryl-CoA synthase (Hmgcoas) was decreased in the FG-4497–treated Ldlr−/− mice to 68% and 62% of the vehicle-treated ones (P=0.06 and 0.08, respectively), whereas the mRNA for lipoprotein lipase (Lpl) was increased to 206% (P=0.07; Figure IB in the online-only Data Supplement). The hepatic level of the glucose-regulated Glut2 was reduced in the FG-4497–treated Ldlr−/− mice, and the expression of genes for glycolytic enzymes, established HIF-1α targets, was in general increased in the FG-4497–treated Ldlr−/− mice (Figure IB in the online-only Data Supplement). The WAT of the HFD-fed FG-4497–treated Ldlr−/− mice showed induction of HIF target glucose transport and glycolytic genes and Pyruvate dehydrogenase kinase 1 (Pdk1) and reduction in the expression of the inflammation marker Chemochine (C-C motif) ligand 2 (Ccl2) (Figure IC in the online-only Data Supplement), the level of CCL2 being also lower in pooled sera of the FG-4497–treated Ldlr−/− mice than in the vehicle-treated ones (2.44 versus 2.91 pg/mL). The WAT Cc12 mRNA levels correlated positively with the WAT macrophage aggregate number and the area...
of atherosclerotic plaques (Figure ID in the online-only Data Supplement).

**Pharmacological HIF-P4H Inhibition Reduced Hepatic Cholesterol Synthesis and Increased Levels of Circulating Atherosclerosis-Protecting Autoantibodies in LDL Receptor–Deficient Mice**

The levels of cholesterol, its precursors squalene, lanosterol, cholesterol, lathosterol, and desmosterol and the plant sterols, campesterol and sitosterol, which reflect cholesterol absorption efficiency, were determined in the liver and serum of the HFD-fed FG-4497 or vehicle-treated Ldlr−/− mice after 13 weeks. In agreement with the data in Figure 1H, the serum cholesterol level was reduced in the FG-4497–treated mice, but no decrease was seen in the liver total cholesterol level (Table II in the online-only Data Supplement). The level of squalene, the first measurable intermediate of cholesterol biosynthesis after acetyl-CoA, was reduced both in the liver and serum of the FG-4497–treated animals, and its hepatic levels correlated positively with serum insulin levels (r=0.578; P=0.038), whereas the level of the anti-inflammatory desmosterol and the plant sterol campesterol was increased in the liver but not in the serum (Table II in the online-only Data Supplement).

Natural autoantibodies to oxidized lipids are reported to be protective against atherosclerosis development. Lipid oxidation leads to proinflammatory products, such as malondialdehyde-acetaldehyde (MAA). Oxidation-specific epitopes for natural autoantibodies are generated in the LDL that accumulates in vascular walls. Because the FG-4497–treated Ldlr−/− mice had less WAT inflammation and were protected against atherosclerosis, we studied autoantibody

![Figure 1](https://example.com/figure1.png)

**Figure 1.** FG-4497 treatment reduced weight gain, white adipose tissue (WAT) inflammation, insulin resistance, and serum lipid levels in low-density lipoprotein (LDL) receptor–Deficient Mice. **A**, Weights of 3-month-old male Ldlr−/− mice treated with high-fat diet (HFD) and FG-4497 or HFD and vehicle for 13 wk. **B**, Liver weights at 13 wk. **C**, WAT weights at 13 wk. **D**, Cross-sectional area of WAT adipocytes at 13 wk. **E** and **F**, Number of WAT macrophage aggregates (*) (aggregates/field) analyzed using CD68 staining. Scale bar, 100 µm. **G**, Homeostasis model assessment-insulin resistance (HOMA-IR) scores at 13 wk. **H**, Serum total cholesterol (s-Chol), (I) high-density lipoprotein (HDL) cholesterol, and (J) LDL+very low-density lipoprotein (VLDL) cholesterol levels. **K**, HDL/LDL+VLDL cholesterol ratios, and (L) triglyceride levels at 0, 9, and 13 wk. n=7 per group.
binding to copper-oxidized LDL (CuOx-LDL) and MAA-modified LDL (MAA-LDL) in their sera. Total IgG and IgM levels and the oxidation-specific autoantibody levels increased similarly in the sera of the FG-4497–treated and vehicle-treated groups during HFD feeding (Figure 4A–4F). The only significant difference between the 2 groups was the continuous increase in CuOx-LDL IgG autoantibodies in the vehicle-treated groups during HFD feeding (Figure 4G and 4H).

Figure 2. FG-4497 treatment reduced the area of atherosclerotic plaques and their necrosis in low-density lipoprotein receptor–deficient mice. The mice were analyzed after feeding with high-fat diet and treatment with FG-4497 or vehicle for 13 wk. A. Aortic plaques analyzed by Movat staining. Scale bar, 500 µm. B. Plaques in a cross section of the aortic origin analyzed using Masson's trichrome staining and the ratio of collagen area:the whole plaque area. Scale bar, 500 µm. C. Collagen in the aortic plaques, analyzed using Masson's trichrome staining and the ratio of collagen area:the whole plaque area. Scale bar, 500 µm. D. Foam macrophages of the aortic plaques displayed as a cross-sectional area and as a proportion of the whole plaque area analyzed using CD68 immunostaining. Scale bar, 200 µm. E. Necrotic core area of the plaques analyzed using Movat staining. Scale bar, 500 µm. n=7 per group.

Genetic HIF-P4H-2 Inhibition Protects From Atherosclerosis

FG-4497 inhibits all 3 HIF-P4Hs with similar IC₅₀ values. To study whether the atherosclerosis-protective phenotype

Figure 3. Correlation of the area of atherosclerotic lesions in the FG-4497–treated low-density lipoprotein receptor–deficient mice to body weight and the levels of serum cholesterol and white adipose tissue (WAT) macrophages. Correlations between (A) body weight and the area of aortic plaques expressed as a percentage of the total aortic area. B. Body weight and the area of plaques in a cross section of the aortic origin. C. Serum cholesterol (s-Chol) level and the area of aortic plaques expressed as in A. D. Serum cholesterol level and the area of plaques in a cross section of the aortic origin. E, the numbers of WAT macrophage aggregates and the area of aortic plaques expressed as in A. F. Numbers of WAT macrophage aggregates and the area of plaques in a cross section of the aortic origin. n=7 per group.
was specifically caused by HIF-P4H-2 inhibition, we crossed our Hif-p4h-2<sup>−/−</sup> mice with Ldlr<sup>−/−</sup> mice, but obtained no live Hif-p4h-2<sup>−/−</sup>/Ldlr<sup>−/−</sup> pups, their death occurring at E9.5 (data not shown). We therefore crossed the Hif-p4h-2<sup>−/−</sup> mice with the Ldlr<sup>Hlb301</sup> mice, which have a missense mutation C699Y in LDL receptor, and obtained live double gene-modified pups. The Ldlr<sup>Hlb301</sup> mice were generated with chemical ethynitrosourea mutagenesis, and their genome has not been fully characterized for potential additional mutations. These Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice had similar, but not identical, reductions in their tissue Hif-p4h-2 mRNA levels to those of the parental Hif-p4h-2<sup>−/−</sup> mice (Figure IIA in the online-only Data Supplement). Similar to the parental line, HIF-1α, was stabilized in WAT of the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice, whereas in liver, opposite to the Hif-p4h-2<sup>−/−</sup> mice, HIF-1α but not HIF-2α was stabilized (Figure IIB in the online-only Data Supplement). There were no differences in the hepatic mRNA levels of Srebp1c, Acca, Fas, Scd1, and Irs2 between the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> and the Ldlr<sup>Hlb301</sup> mice (Figure IIC in the online-only Data Supplement). The Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice were lighter than the Ldlr<sup>Hlb301</sup> mice when fed normal chow, but this difference disappeared when fed HFD (Figure 5A). There was also no difference between the genotypes in WAT weight or adipocyte size, but the numbers of WAT macrophage aggregates were lower in the double gene-modified mice (Figure 5B and 5C). No difference was either seen in the homeostasis model assessment-insulin resistance scores (Figure IID in the online-only Data Supplement). Surprisingly, the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice, unlike the Hif-p4h-2<sup>−/−</sup> mice, did not have reduced serum cholesterol levels either on normal chow or on HFD (Figure 5D). The Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice were nevertheless protected against atherosclerosis development, as shown by the ≈50% reduction in the area of plaque over the total aortic area and the ≈40% reduction at the aortic origin (Figure 5E and 5F). The area of foam macrophages was >60% smaller in the plaques of the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice than that of the Ldlr<sup>Hlb301</sup> mice (Figure 5G), and the area of the necrotic core was likewise reduced by >60% (Figure 5H), whereas no difference was seen in the amount of plaque collagen (Figure IIE in the online-only Data Supplement). The number of WAT macrophage aggregates correlated positively with plaque sizes in the whole aorta and at the aortic origin, suggesting that also in this model reduced WAT inflammation associated with atherosclerosis protection (Figure 5I).

There was a trend for increases in the levels of serum IgM autoantibodies to CuOx-LDL and MAA-LDL before exposure to HFD in the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice relative to the Ldlr<sup>Hlb301</sup> mice; these increases being statistically significant since 9 weeks on HFD (Figure 6A). There was also a significant increase in serum IgG autoantibodies to CuOx-LDL and MAA-LDL at 9 weeks in the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice (Figure 6B). The amount of IgM CuOx-LDL and IgM MAA-LDL autoantibodies correlated negatively with plaque necrotic core area ($r = -0.452; P = 0.016$ and $r = -0.542; P = 0.003$, respectively), and the with plaque size at the aortic origin (Figure 6C). Our data suggest that the modulation of adipose tissue inflammation and innate immunity rather than changes in lipid metabolism may have played an important role in the protection against atherosclerosis in the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice.

### HIF-P4H-2 Hypomorphic Mice Have Increased Levels of Circulating Atherosclerosis-Protecting Autoantibodies and Reduced Hepatic Cholesterol Synthesis

To study, whether the increased atherosclerosis-protecting autoantibody levels in the Ldlr<sup>Hlb301</sup>/Hif-p4h-2<sup>−/−</sup> mice were
because of HIF-P4H-2 deficiency or some noncharacterized mutation in the LdlrHlb301 mice, we determined autoantibody binding to modified LDL in the sera of the Hif-p4h-2gt/gt mice. The levels of CuOx-LDL and MAA-LDL IgG and IgM autoantibodies were higher in the Hif-p4h-2gt/gt than in the wild-type mice, whereas there was no difference in IgA levels (Figure 6D).

We also determined the levels of cholesterol and its precursors in the Hif-p4h-2gt/gt mice. There was no difference in total hepatic cholesterol levels between the Hif-p4h-2gt/gt and the wild-type mice, but the level of squalene was significantly reduced in the Hif-p4h-2gt/gt livers, as in FG-4497–treated mice, supporting reduced synthesis (Table III in the online-only Data Supplement). The hepatic desmosterol level was increased in the Hif-p4h-2gt/gt mice agreeing with data with the pharmacological inhibitor (Table III in the online-only Data Supplement). Serum cholesterol level was reduced in the Hif-p4h-2gt/gt mice (Table III in the online-only Data Supplement) similar to the FG-4497–treated Ldlr−/− mice (Figure 1H; Table II in the online-only Data Supplement). The 30% reduction in the serum squalene level in the Hif-p4h-2gt/gt mice was not statistically significant, but the serum level of the next measurable cholesterol synthesis intermediate, lanosterol, was significantly reduced (Table III in the online-only Data Supplement).

Having previously found that the hepatic mRNA levels for Srebp1c, Accα, and Fas were lower and that for Irs2 higher in the Hif-p4h-2gt/gt mice than in the wild-type littermates,9 we now observed a significant reduction in the hepatic Scd1 mRNA, while the reduction to 85% in Hmgr and the induction of Lpl to 181% did not reach significance (Figure 6E); however, all these changes being similar to those seen here for the HFD-fed FG-4497–treated Ldlr−/− mice (Figure 1B in the

**Figure 5.** Genetic hypoxia-inducible factor prolyl 4-hydroxylase (HIF-P4H)-2 inhibition protects high-fat diet (HFD)-fed LdlrHlb301 mice from atherosclerosis. A, Weight of male LdlrHlb301 and LdlrHlb301/Hif-p4h-2gt/gt mice on chow and after 4, 9, and 13 wk on HFD. B, WAT weight and WAT adipocyte size measured as a cross-sectional area at 13 wk. C, The number of macrophage aggregates (*) in the WAT of LdlrHlb301, Hif-p4h-2gt/gt, and LdlrHlb301 mice analyzed with hematoxylin-eosin staining. Scale bar, 200 µm. D, Serum cholesterol levels of LdlrHlb301 and LdlrHlb301/Hif-p4h-2gt/gt mice on chow and after 13 wk on HFD. E, Aortic plaques analyzed with Sudan IV staining and the measured ratio of plaque area:the whole aortic area. F, Plaques in a cross section of the aortic origin analyzed using hematoxylin-eosin staining and the measured plaque area. Scale bar, 500 µm. G, Foam macrophages of aortic plaques displayed as cross-sectional areas and as the ratio of this area:the whole plaque area analyzed using CD68 immunostaining. H, Areas of the necrotic core of the plaques measured using Movat staining. I, Correlation between the numbers of WAT macrophage aggregates and the area of plaques in a cross section of the aortic origin. n=14 per group.
Figure 6. Genetic hypoxia-inducible factor prolyl 4-hydroxylase (HIF-P4H)-2 inhibition is associated with increased levels of atherosclerosis-protecting autoantibodies and reduction of cholesterol synthesis regulating genes in liver. A, Serum total IgM, IgM bound to copper-oxidized low-density lipoprotein (CuOx-LDL) and IgM bound to malondialdehyde-acetaldehyde-modified LDL (MAA-LDL) in male Ldlr<sup>Ldlr<sub>H</sub></sup>*<sup>301</sup> and Ldlr<sup>Ldlr<sub>H</sub></sup>*<sup>301</sup>/Hif-p4h<sup>P4H-2</sup><sup>gt/gt</sup> mice at 0, 9, and 13 wk on HFD. B, Serum total IgG, IgG bound to CuOx-LDL, and IgG bound to MAA-LDL at 0, 9, and 13 wk. C, Correlation between the level of CuOx-LDL IgM at 13 wk and the area of plaques in a cross section of the aortic origin. n=14 per group. D, Natural autoantibodies in the serum of male Hif-P4H<sup>P4H-2</sup><sup>gt/gt</sup> (gt/gt) and wild-type mice (wt) at the age of 3 to 7 mo (n=7–8 per group). E, Quantitative polymerase chain reaction (qPCR) analyses of the mRNA levels of lipid metabolism genes in the liver of Hif-P4H<sup>P4H-2</sup><sup>gt/gt</sup> mice when compared with the wt (n=7–8 per group). The expression of each gene was studied relative to $\beta$-actin. F, qPCR analyses of the mRNA levels of Hif1a and Hif2a and lipid and glucose metabolism genes in the primary Hif-P4H<sup>P4H-2</sup><sup>gt</sup> hepatocytes followed by Hif1a or Hif2a siRNA transfection relative to control siRNA transfection (n=4 per group). Abca1 indicates ATP-binding cassette subfamily A member 1; Acac, acetyl-CoA carboxylase; ApoB, apolipoprotein B; Fas, fatty acid synthase; Scd1, stearoyl-CoA desaturase 1; Hmgcr and Hmgs, HMG-CoA reductase and synthase; Insig1 and Insig2, insulin-induced gene 1 and 2; Ldr, low density lipoprotein receptor; Lpl, lipoprotein lipase; Irs2, insulin receptor substrate 2; Pdk1, pyruvate dehydrogenase kinase 1; Pfkl, phosphofructokinase; and Srebp1c, sterol regulatory element-binding protein 1c.
online-only Data Supplement). The Hif-p4h−2/− mice, unlike the FG-4497−treated Ldlr−/− mice, show stabilization of only HIF-2α in their livers and no induction in the glycolytic HIF-1α targets.9

To establish a causality between HIFα stabilization and the observed phenotype, we extracted primary hepatocytes from the Hif-p4h−2/− mice and knocked down Hif1α or Hif2α by siRNA transfections in them. Two independent siRNAs for Hif1α and Hif2α resulted in >85% and 60% to 70% down-regulation of the corresponding mRNA levels, respectively, and interestingly, downregulation of one isoform resulted in upregulation of the other (Figure 6F). The downregulation of either Hif1α or Hif2α resulted in downregulation of Irs2 and upregulation of Srebp1c, Acca, Fas, and Scd1 mRNA levels. Downregulation of Hif1α in these Hif-p4h−2/− hepatocytes devoid of HIF-1α stabilization did not alter the mRNA levels of the glycolytic genes Pfk1 and Pdk1, as expected, whereas downregulation of Hif2α increased their levels, most likely because of simultaneous Hif1α mRNA upregulation (Figure 6F). The mRNA for Hmgcr was upregulated when either Hif1α or Hif2α was downregulated indicating that it is regulated by both HIFαs.

Discussion

Hypercholesterolemia and dyslipidemia are regarded as the key risk factors for the development of atherosclerosis, and therefore the treatments concentrate on lowering the elevated cholesterol levels in risk subjects. HIF has been suggested to have both detrimental and beneficial roles in atherosclerosis.6,9 Most human studies show that increased HIF-1α expression correlates with more advanced atherosclerotic plaques.19 On the other hand, overexpression of HIF-1α in mouse lymphocytes attenuated inflammation and reduced aortic lesions.6 Epidemic studies point to an association between altitude-induced hypoxia and protection against ischemic heart disease and suggest that HIF stabilization may be involved in this protection.20 HIF-P4H-2 inhibition leads to improved lipid and glucose metabolism, reduced serum cholesterol levels, and protection from obesity and metabolic dysfunction.9,21,22 We present evidence here that pharmacological and genetic HIF-P4H-2 inhibition also protects from atherosclerosis development.

Cholesterol levels are regulated in multiple ways. We show here that HIF-P4H-2 inhibition driven HIF-1α and HIF-2α stabilization resulted in reduced serum cholesterol levels via several mechanisms (Figure III in the online-only Data Supplement). First, HIF-P4H-2 inhibition reduced insulin resistance and its levels in serum. Because insulin increases the activity of 3-OH-3-methylglutaryl-CoA reductase, the lower insulin levels after HIF-P4H-2 inhibition likely reduced its catalytic activity, which accounted for the lower squalene transport to the liver,9 which is known to downregulate Srebp1c by competing with binding of the FOXO transcription factor to the Irs2 promoter insulin-responsive elements.23 It has also been reported that Srebp1c is a direct target of insulin, and therefore the lower insulin levels after HIF-P4H-2 inhibition may have contributed to the lower Srebp1c mRNA levels also independently from Irs2 increase.23 Moreover, it has been shown that both HIF-1α and HIF-2α can directly downregulate Srebp1c,11 which agrees with our data from the primary hepatocytes. SREBPs are key transcriptional regulators of hepatic lipid synthesis, SREBP1c being primarily, but not exclusively, responsible for fatty acid and SREBP2 for cholesterol synthesis.25 Therefore, the reduced mRNA levels of hepatic Hmgcr in the FG-4497−treated Ldlr−/− and to a smaller extent in the Hif-p4h−2/− mice were likely because of their reduced Srebp1c levels because no reduction in Srebp2 mRNA was detected. Furthermore, the hepatic expression levels of the Srebp1c downstream targets Fas, Acca, and Scd1 were reduced in the FG-4497−treated Ldlr−/− and Hif-p4h−2/− mice probably accounting for decreased fatty acid synthesis and de novo lipogenesis, as found earlier in the Hif-p4h−2/− liver,9 and explaining the reduced liver weights seen here. Third, the overall improved glucose tolerance after global HIF-P4H-2 inhibition, which manifested in the FG-4497−treated Ldlr−/− mice as a reduction in the glucose-regulated hepatic Glut2 mRNA level, likely reduced glucose uptake by the liver and diminished the levels of acetyl-CoA, the building block for cholesterol synthesis, as reported earlier for the Hif-p4h−2/− mice.9 The observed increased hepatic Lpl mRNA levels in the FG-4497−treated Ldlr−/− and the Hif-p4h−2/− mice, and the increased levels of campesterol in the former, were most likely methods to compensate for the reduced endogenous cholesterol synthesis in liver. In summary, these findings suggest that hepatic HIF-2α stabilization and reduced insulin resistance, seen in the FG-4497−treated Ldlr−/− and Hif-p4h−2/− mice, but not in the Ldlr−/− mice, were responsible for the lower serum cholesterol levels.

Atherosclerosis is not only because of high cholesterol levels. Immunology plays a central role in its development and progression and increased titers of autoantibodies to Ox-LDL are associated with atherosclerosis protection.5,16,26 These autoantibodies, part of innate immunity and mainly secreted by the B-1 cells, recognize the inflammation-induced modified LDL particles and can prevent the excessive accumulation of cell debris and modified LDL in the artery wall.6,26 The Ldlr−/−/Hif-p4h−2/− mice, which had reduced numbers of WAT macrophage aggregates and increased autoantibody levels against CuOx-LDL and MAA-LDL, but no reduced serum cholesterol levels, were also protected against the development of atherosclerosis. The FG-4497−treated Ldlr−/− mice also had higher levels of autoantibodies against CuOx-LDL at 13 weeks than did the vehicle-treated Ldlr−/− mice and reduced numbers of WAT macrophage aggregates. The increased levels of autoantibodies correlated negatively with plaque size at the aortic origin, and the reduced WAT macrophages correlated positively with plaque size both in the whole aorta and at the aortic origin, suggesting that changes in innate immunity and WAT inflammation may have played a role in the protection of these mice from atherosclerosis development (Figure III in the online-only Data Supplement). The plaques at the aortic origins of the FG-4497−treated HFD-fed Ldlr−/− and Ldlr−/−/Hif-p4h−2/− mice...
mice had smaller necrotic cores, which can have at least partially accounted for their increased autoantibody levels. Importantly, the increased levels of autoantibodies were also seen in the Hif-p4h-2−/− mice verifying their specificity for the HIF-P4H-2 inhibition and bringing up the possibility of therapeutic use of HIF-P4H-2 inhibition to increase autoantibodies for host homeostasis mediation for the treatment of other chronic inflammatory diseases in addition to atherosclerosis.

HIFs become stabilized during inflammatory processes, and hypoxia and inflammation are intimately linked. Genetic and pharmacological HIF-P4H inhibition and the following HIF stabilization have been associated with protection from some inflammatory conditions, such as the inflammatory bowel disease including ulcerative colitis and Crohn disease. The protection against colitis included HIF-1α-mediated induction of FoxP3 and increase in abundance and function of regulatory T cell (Tregs). Because Tregs, and the suppressive cytokines produced by them, have been implicated in atherosclerosis protection, pharmacological HIF-P4H-2 inhibition may further diminish vascular inflammation by inducing them. More recently, HIF-1α-driven optimization of carbohydrate metabolism, also acquired with a nonspecific 2-oxoglutarate inhibitor analogue DMOG, during an inflammatory acute lung injury, provided lung protection and dampened inflammation. The data available suggest that HIFα stabilization via HIF-P4H inhibition can modulate immunologic responses, making them potential therapeutic targets in the treatment of chronic inflammatory diseases. Our data provide evidence for the pharmacological use of HIF-P4H-2 inhibition for the prevention of atherosclerosis development, such therapy combining reductions in serum cholesterol levels with modulation of innate immunity and protection against inflammation.

Acknowledgments

FG-4497, a frequently used experimental tool for studying HIF-P4H inhibition in cells and in vivo, was developed and synthesized by Drs. Lee Flippin and Michael Arend at FibroGen Inc. We thank T. Aatsinki, L. Kaipiainen, E. Lehtimäki, S. Rannikko, M. Siurua, and R. Tauriainen for excellent technical assistance.

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Disclosures

K.I. Kivirikko is a scientific founder and consultant of FibroGen Inc., which develops hypoxia-inducible factor-prolyl 4-hydroxylases inhibitors as potential therapeutics. K.I. Kivirikko and J. Myllyharju own equity in this company, and the company has sponsored research in the laboratory headed by K.I. Kivirikko and currently supports research in that headed by J. Myllyharju. G. Walkinshaw is a senior cell biology director at FibroGen Inc.

References

20. Ezzati M, Horwitz ME, Friedman AB, Roach R, Clark T, Murray CJ, Hoivnag B, Altitude, life expectancy and mortality from...
Epidemiological studies have pointed to an association between altitude-induced hypoxia and protection against ischemic heart disease and suggest that hypoxia-inducible factor (HIF) stabilization may be involved in this protection. HIF can be stabilized pharmacologically under normoxia by inhibiting HIF prolyl 4-hydroxylases. HIF prolyl 4-hydroxylase-2 inhibition has been shown to lead to improved lipid and glucose metabolism and protection from obesity and metabolic dysfunction. Our research here show that activation of the hypoxia response via pharmacological or genetic HIF prolyl 4-hydroxylase-2 inhibition prevents atherosclerosis development in mice. The mechanism involves reduction in serum cholesterol levels and adipose tissue inflammation and increases in circulating atherosclerosis-protecting autoantibodies. Small-molecule HIF prolyl 4-hydroxylase inhibitors, such as those that are currently in clinical trials for the treatment of anemia, may therefore be potential drugs for the treatment of atherosclerosis.
Hypoxia-Inducible Factor Prolyl 4-Hydroxylase-2 Inhibition Protects Against Development of Atherosclerosis
Lea Rahtu-Korpela, Jenni Määttä, Elitsa Y. Dimova, Sohvi Hörkkö, Helena Gylling, Gail Walkinshaw, Jukka Hakkola, Kari I. Kivirikko, Johanna Myllyharju, Raisa Serpi and Peppi Koivunen

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http://atvb.ahajournals.org/content/suppl/2016/02/04/ATVBAHA.115.307136.DC1

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SUPPLEMENTAL MATERIAL

Online-only Tables I-III

Online-only Figure Legends and Figures I-III
## Online-only Table I Sequences of the qPCR Primers.

<table>
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<th>Gene</th>
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<td>Hif-p4h-2</td>
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<td>Cel2</td>
<td>CCTGCTGTTACAGTGG</td>
<td>ATGCGATCATCAGGGTC</td>
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Online-only Table II  Cholesterol Synthesis Metabolites in the Liver and Serum of FG-4497-Treated and Vehicle-Treated Ldlr−/− Mice.

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<th>Metabolite</th>
<th>Liver</th>
<th>Serum</th>
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<tr>
<td></td>
<td>Vehicle (ng/mg liver/g body weight)</td>
<td>FG-4497</td>
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<tr>
<td>Cholesterol</td>
<td>18.0 x 10³ ± 1.8 x 10³</td>
<td>19.0 x 10³ ± 2.3 x 10³</td>
</tr>
<tr>
<td>Squalene</td>
<td>179.9 ± 20.2</td>
<td>111.6 ± 19.9</td>
</tr>
<tr>
<td>Lanosterol</td>
<td>2.7 ± 0.3</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Desmosterol</td>
<td>11.6 ± 0.5</td>
<td>16.0 ± 1.4</td>
</tr>
<tr>
<td>Cholestenol</td>
<td>3.7 ± 0.5</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>Lathosterol</td>
<td>27.0 ± 4.1</td>
<td>31.5 ± 5.7</td>
</tr>
<tr>
<td>Campesterol</td>
<td>2.5 ± 0.2</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

The hepatic values are shown per mg liver weight further corrected for the differences in the body weight, all values shown being means ± SEM.

*P < 0.05 and ***P < 0.001.
Online-only Table III Cholesterol Synthesis Metabolites in the Liver and Serum of the *Hif-p4h-2*^{gt/gt} and Wild-Type Mice.

<table>
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<tr>
<th>Geometric Isomer</th>
<th>Liver (ng/mg liver/g body weight)</th>
<th>Serum (µg/100ml)</th>
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<tr>
<td></td>
<td>WT</td>
<td>Hif-p4h-2^{gt/gt}</td>
<td><em>P</em></td>
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<tr>
<td>Cholesterol</td>
<td>60.3 ± 4.1</td>
<td>65.1 ± 2.7</td>
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<tr>
<td>Squalene</td>
<td>615.6 ± 71.4</td>
<td>402.2 ± 42.6</td>
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<tr>
<td>Lanosterol</td>
<td>43.9 ± 7.2</td>
<td>49.8 ± 4.5</td>
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<td>Desmosterol</td>
<td>44.5 ± 4.3</td>
<td>60.0 ± 5.4</td>
<td>0.05*</td>
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<tr>
<td>Cholestenol</td>
<td>9.8 ± 0.7</td>
<td>10.3 ± 0.9</td>
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<td>Lathosterol</td>
<td>40.1 ± 4.6</td>
<td>54.8 ± 8.7</td>
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<td>Campesterol</td>
<td>1402.4 ± 162.9</td>
<td>1057.7 ± 139.1</td>
<td>0.14</td>
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<tr>
<td>Sitosterol</td>
<td>303.2 ± 22.6</td>
<td>271.4 ± 22.3</td>
<td>0.36</td>
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</table>

Values shown are means ± SEM. *P ≤ 0.05
**Online-only Figure I.** Analysis of HIFα Levels in the Liver and HIF Target, Lipid Metabolism and Inflammatory Genes in the Liver and WAT of LDLR-Deficient Mice Treated with FG-4497. **A,** Western blot analysis showing stabilization of HIF-1α and HIF-2α proteins in the liver. β-actin was used as a loading control. **B, C** qPCR analyses show altered mRNA levels in the liver and WAT between the FG-4497-treated and vehicle-treated male mice fed HFD for 13 weeks. The mice were 3 months old at the onset of HFD. N = 8 per group. Glut1, Glut2 and Glut4 stand for glucose transporters 1, 2 and 4; Hk1, hexokinase-1; Pfkl, phosphofructokinase; Pgk1; phosphoglycerate kinase-1; Gapdh, glyceraldehyde phosphate dehydrogenase; Eno1, enolase 1; Ldha, lactate dehydrogenase a; Pdk1 and Pdk4, pyruvate dehydrogenase kinases 1 and 4; Lep, leptin; and Adipoq, adiponectin.
dehydrogenase kinases 1 and 4; Pparα and Pparγ, peroxisome proliferator-activated receptors α and γ; Pnpla2, patatin-like phospholipase domain containing 2; Accα, acetyl-CoA carboxylase α; Adipoq, C1Q and collagen domain containing adiponectin; Lpl, lipoprotein lipase; Acba1, ATP-binding cassette sub-family A member 1; Ccl2, chemokine (C-C motif) ligand 2; Tp1, terminal protein 1; Srebp1c and Srebp2, sterol regulatory element-binding protein 1c and 2; Fas, fatty acid synthase; Scd1, stearoyl-CoA desaturase 1; Hmgcr and Hmgcs, HMG-CoA reductase and synthase; Insig1 and Insig2, insulin-induced gene 1 and 2; Irs1 and Irs2, insulin receptor substrates 1 and 2; and ApoB, apolipoprotein B. D, Correlation of the WAT Ccl2 mRNA levels with WAT macrophage aggregate numbers and the area of aortic plaques.
Online-only Figure II. Genetic HIF-P4H-2 Inhibition Protects From Atherosclerosis Despite No Reduction in Insulin Resistance and No Hepatic HIF-2α Stabilization. A, qPCR analysis displaying the expression levels of Hif-p4h-2 mRNA in the liver, WAT and skeletal muscle (SKM) of male and Ldlr<sup>Lhb301/Lhb301</sup>/Hif-p4h-2<sup>gt/gt</sup> mice relative to Ldlr<sup>Lhb301</sup> mice. B, Western blot analysis of HIF-1α and HIF-2α proteins in the WAT and liver. β-actin staining was used as a loading control. C, qPCR analysis displaying the mRNA expression levels of lipid metabolism genes in the liver of male Ldlr<sup>Lhb301/Lhb301</sup>/Hif-p4h-2<sup>gt/gt</sup> mice relative to Ldlr<sup>Lhb301</sup> mice. D, HOMA-IR scores of male Ldlr<sup>Lhb301/Lhb301</sup> and Ldlr<sup>Lhb301/Lhb301</sup>/Hif-p4h-2<sup>gt/gt</sup> mice fed chow. E, Collagen in plaques at 13 weeks. N=14 per group. All data are means ± SEM, ***P<0.001. WAT stands for white adipose tissue; Acca, acetyl-CoA carboxylase α; Srebp1c, sterol regulatory element-binding protein 1c; Fas, fatty acid synthase; Scd1, stearoyl-CoA desaturase 1; and Irs2, insulin receptor substrate 2.
FG-4497-treatment inhibits HIF-P4H-2, and also HIF-P4Hs 1 and 3, resulting in the stabilization of HIFαs and changes in gene expression. The mechanisms involved in the atherosclerosis protection include reduced insulin resistance, modifications in hepatic metabolism and reduction of cholesterol synthesis leading to lower serum cholesterol, LDL cholesterol and triglyceride levels, attenuation of WAT inflammation and increased levels of circulating autoantibodies against oxidized plasma lipids. Genetic knockdown of HIF-P4H-2 phenocopied the reduction in hepatic cholesterol synthesis and WAT inflammation and increases in autoantibodies.
HIF Prolyl 4-Hydroxylase-2 Inhibition Protects Against Development of Atherosclerosis

Lea Rahtu-Korpela, Jenni Määttä, Elitsa Y. Dimova, Sohvi Hörkkö, Helena Gylling, Gail Walkinshaw, Jukka Hakkola, Kari I. Kivirikko, Johanna Myllyharju, Raisa Serpi, Peppi Koivunen

Materials and Methods

Animal Experiments

Generation of the Hif-p4h-2−/− mice has been described.1 Ldlr−/− and LdlrHlb301 mice2 were obtained from the Jackson Laboratory and LdlrHlb301/Hif-p4h-2−/− mice were generated by mating the Hif-p4h-2−/− mice with the LdlrHlb301 mice. All the mice were in the C57BL/6 background. All experiments were performed according to protocols approved by the Provincial State Office of Southern Finland. The mice were fed either a standard rodent diet or HFD (18% and 42% kcal fat, respectively). FG-4497 was dissolved in 0.5% NaCMC (Spectrum) and 0.1% Polysorbate 80 (Fluka), and the solvent alone was also used as a vehicle control. FG-4497, 60 mg/kg, or vehicle were administered orally three times a week.

Histological Analyses

Five-μm sections of formaldehyde-fixed paraffin-embedded tissue samples were stained with hematoxylin-eosin (HE) and viewed and photographed with a Leica DM LB2 microscope and Leica DFC 320 camera. The area of adipocytes was quantified from HE-stained sections as described.3 Macrophage aggregates were analyzed using an anti-CD68 antibody (ab955, Abcam) and the EnVision Detection System (Dako). The number of macrophage aggregates was calculated from 5-8 fields/sample.

The extent of atherosclerosis was analyzed using en face lipid staining of the whole aorta and the aortic origin with Sudan IV, as described.4 The hearts were sectioned to localize the aortic origin, and its plaque area was quantified in three consecutive sections of the largest plaques using the Photoshop software. The amount of collagen and the areas of necrotic core and foam macrophages in the plaques
were determined in sections adjacent to the largest plaques with Masson’s trichrome, Movat’s pentachrome and an anti-CD68 antibody (ab955, Abcam) staining, respectively, and quantified as above.

**Quantitative Real-Time PCR (qPCR)**

Total RNA was isolated from the tissues with an EZNA total RNA kit II (OMEGA Bio-tek) or the TriPure isolation reagent (Roche Applied Science) and reverse-transcribed with an iScript cDNA synthesis kit (Bio-Rad). qPCR was performed with iTaq SYBR Green Supermix and ROX (Bio-Rad) in a Touch Thermal Cycler and a CFX96 Real-Time System (Bio-Rad). The primers used are presented in Table I of the online-only Data Supplement.

**Isolation and Transfection of Mouse Primary Hepatocytes with siRNA**

Primary mouse hepatocytes were isolated from 10-week-old *Hif-p4h-2gt/gt* males by collagenase perfusion and cultured as described previously. Fetal calf serum (10 %) was present during the initial 5 h after which the cultures were incubated in serum-free William's E medium containing 2.0 g/l D-glucose, 5 mg/l insulin, 5 mg/l transferrin and 5 μg/l sodium selenate (Sigma-Aldrich). After 24 h primary mouse hepatocytes were transfected with X-tremeGENE siRNA transfection reagent (Sigma Aldrich) using 150 pmol per well in six-well plates of siRNAs targeting *Hif1α* or *Hif2α* (Sigma Aldrich). As a scrambled control GFP siRNA (Sigma Aldrich) was used. After an initial 6 h incubation period the transfection medium was replaced by fresh serum-free medium and 48 h post-transfection cells were collected for total RNA isolation.

**Analysis of Circulating Natural Antibodies**

The modified lipoprotein antigens CuOx-LDL and MAA-LDL were prepared and used in chemiluminescence immunoassays to determine autoantibody binding as described previously. The total serum immunoglobulin concentrations were determined by chemiluminescent immunoassay.
**Determination of Serum Lipids and Serum and Liver Non-cholesterol Sterols and Squalene**

Total serum cholesterol, HDL cholesterol and triglyceride levels were determined by an enzymatic method (Roche Diagnostics), and the Friedewald equation\(^9\) was used to calculate approximate LDL+VLDL cholesterol levels. Serum and liver cholesterol, squalene, lanosterol, cholestenol, lathosterol, desmosterol, campesterol and sitosterol were quantified by capillary gas-liquid chromatography (GLC, Agilent 6890N Network GC System, Agilent Technologies) using a 50 m non-polar Ultra 2 capillary column (5 % phenyl-methyl siloxane, Agilent Technologies) with 5α-cholestanate as an internal standard.\(^10\)

**Determination of Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) Scores and Serum CCL2 Levels**

HOMA-IR scores were calculated from the glucose and insulin concentrations determined from blood with a glucometer and its serum fraction with a Rat/Mouse Insulin ELISA kit (EZRMI-13K, Millipore), respectively. Multi-Analyte ELISArray Kit (MEM-005A, Qiagen) was used to determine the serum CCL2 levels.

**Western Blotting**

NE-PER extraction reagents (Thermo Scientific) were used to prepare nuclear fractions. Samples of 30-100 μg were resolved by SDS-PAGE, blotted and probed with HIF-1α (NB100-479, Novus), HIF-2α (ab199, Abcam) and β-actin (NB600-501, Novus) antibodies.

**Statistical Analyses**

Student’s two-tailed \(t\)-test was used for comparisons between two groups. Areas under the curve (AUC) were calculated by the method of summary measures. Pearson’s correlation coefficient was calculated to compare linear dependences between two variables. All data are shown as means ± SEM.
References


