Detection of Hypoxia by $^{[18F]}$EF5 in Atherosclerotic Plaques in Mice

Johanna M.U. Silvola, Antti Saraste, Sarita Forsback, V. Jukka O. Laine, Pekka Saukko, Suvi E. Heinonen, Seppo Ylӓ-Herttuala, Anne Roivainen, Juhani Knuuti

Objective—Atherosclerotic plaques with large lipid cores and inflammation contain regions of hypoxia. We examined the uptake of 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide ($^{[18F]}$EF5), a specific marker of hypoxia labeled for positron emission tomography, in mouse atherosclerotic plaques.

Methods and Results—Atherosclerotic mice of 2 different genetic backgrounds (low-density lipoprotein receptor$^{−/−}$apolipoprotein B$^{0/100}$ and insulin-like growth factor II/low-density lipoprotein receptor$^{−/−}$apolipoprotein B$^{100/100}$) were first fed a Western diet to induce development of plaques with variable phenotypes and then injected with $^{[18F]}$EF5. C57BL/6N mice served as controls. Aortas were dissected for biodistribution studies, autoradiography, histology, and immunohistochemistry. Uptake of $^{[18F]}$EF5 was significantly higher in the aortas of mice with large atherosclerotic plaques than in the C57BL/6N controls. Furthermore, autoradiography demonstrated, on average, 2.0-fold higher $^{[18F]}$EF5 uptake in atherosclerotic plaques than in the adjacent normal vessel wall. Hypoxia in plaques was verified by using an EF5 adduct-specific antibody and pimonidazole. The blood clearance of $^{[18F]}$EF5 was slow, with blood radioactivity remaining relatively high up to 180 minutes after injection.

Conclusion—Large atherosclerotic plaques in mice contained hypoxic areas and showed uptake of $^{[18F]}$EF5. Despite its slow blood clearance, the high uptake of $^{[18F]}$EF5 in plaques suggested that plaque hypoxia is a potential target for identifying high-risk plaques noninvasively.

Key Words: atherosclerosis ■ hypoxia ■ macrophages ■ positron emission tomography ■ $^{[18F]}$EF5

Atherosclerosis is a chronic inflammatory disease of the artery wall. The majority of myocardial infarctions result from sudden ruptures of atherosclerotic plaques. Atherosclerotic plaques that are vulnerable to rupture are characterized by extensive inflammation, neoangiogenesis, a thin fibrous cap, and a large lipid core. Circulating monocytes are recruited to plaques, where they differentiate into macrophages. The majority of macrophages transform into foam cells by accumulating lipids in their cytoplasm, thus forming the main component of the plaque lipid core. At this stage, plaques with a large necrotic core contain severely hypoxic areas. Hypoxia constitutes an important signal for plaque growth by inducing formation of lipid droplets in macrophages, increasing the secretion of inflammatory mediators, and increasing intraplaque angiogenesis. Therefore, we hypothesized that, if present, plaque hypoxia could offer a new biomarker of plaque growth and vulnerability.

$^{[18F]}$EF5 is a lipophilic compound and has better pharmacokinetic properties than other nitroimidazole compounds. Recently, $^{[18F]}$EF5 positron emission tomography imaging has been introduced as a promising tool for the detection of hypoxia in tumor tissues in patients and in experimental models.

In the present study, we aimed at investigating whether atherosclerotic plaques contain hypoxic regions as detected with $^{[18F]}$EF5 in atherosclerotic mouse models. In addition to $^{[18F]}$EF5 accumulation, we examined the detection of EF5 adducts by using the specific Cy-3-conjugated monoclonal antibody ELK3-51, and additionally, hypoxia was confirmed by using pimonidazole. We also compared the $^{[18F]}$EF5 uptake with the plaque histology and macrophage content.
Table 1. Basic Characteristics of Animals

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>n (F/M)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 – 9</td>
<td>6 (3/3)</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>8 – 13</td>
<td>6 (5/1)</td>
<td>39 ± 6</td>
</tr>
<tr>
<td>6 – 9</td>
<td>6 (0/6)</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>7 – 8</td>
<td>4 (3/1)</td>
<td>37 ± 9</td>
</tr>
</tbody>
</table>

*Investigated time point after [18F]EF5 injection. †F/M indicates female/male. ‡Mean ± SD.

Materials and Methods

A detailed description of the Materials and Methods is provided in the Supplemental Material, available online at http://atvb.ahajournals.org.

Animals

Atherosclerotic mice deficient for low-density lipoprotein receptor and synthesizing only apolipoprotein B100 (LDLR⁻ /⁻ ApoB100/100), as well as LDLR⁻ /⁻ ApoB100/100 mice overexpressing insulin-like growth factor II (IGF-II) in pancreatic β cells16 (IGF-II/LDLR⁻ /⁻ ApoB100/100) to induce diabetes, were fed a high-cholesterol Western-type diet (0.2% total cholesterol, TD 88137, Harlan Teklad) for 3 to 4 months, starting at the age of 4 to 8 months. Two different models were used to induce plaques with variable phenotypes. The IGF-II/LDLR⁻ /⁻ ApoB100/100 mice exhibited insulin resistance and hyperglycemia (type 2 diabetes), as well as hypercholesterolemia (from the LDLR⁻ /⁻ ApoB100/100 background) associated with a rapid development of atherosclerotic plaques. It was used in the present study as a model of the most inflamed types of atherosclerotic plaques.16 A subset of wild-type C57BL/6N mice received a regular chow diet and served as nonatherosclerotic controls. The experiments were approved by the local animal committee and carried out in compliance with the Finnish laws relating to the conduct of animal experimentation.

Synthesis of [18F]EF5

[18F]EF5 was synthesized from 2-(2-nitro-1H-imidazol-1-yl)-N-(2,3,3-trifluoroallyl)-acetamide, as described previously.7

Ex Vivo Biodistribution Studies

The biodistribution of intravenously administered [18F]EF5 at 90 minutes after injection was studied in 6 LDLR⁻ /⁻ ApoB100/100, 6 IGF-II/LDLR⁻ /⁻ ApoB100/100, and 6 C57BL/6N mice. The 90-minute accumulation time was based on the serum half-life of EF5 in mice (T½ = 40 minutes).17 Because the biological half-life of EF5 is relatively long, 4 additional LDLR⁻ /⁻ ApoB100/100 mice were studied at 180 minutes postinjection to explore the biodistribution of [18F]EF5 in more detail. The mice were anesthetized with isoflurane, injected with [18F]EF5 (11 MBq) via a tail vein, and euthanized 90 or 180 minutes after injection. The basic characteristics of the animals are shown in Table 1. The ascending aorta to the level of the diaphragm was dissected, and blood was rinsed with saline. The radioactivity in aorta, blood, fat, heart, kidney, liver, and muscle was measured using a gamma counter. The radioactivity concentration was expressed as a percentage of the injected activity per gram of tissue (%IA/g). In addition, a total of 12 atherosclerotic hearts (from 7 LDLR⁻ /⁻ ApoB100/100 and 5 IGF-II/LDLR⁻ /⁻ ApoB100/100 mice) were collected and preserved in formalin for further study.

Autoradiography

An autoradiography (ARG) analysis was performed to study the distribution of [18F]EF5 in the aortic tissue. Briefly, the aorta was frozen and cut in sequential longitudinal 8- and 20-μm cryosections. The sections were exposed to imaging plate, and after an exposure time of 4 hours, the imaging plates were scanned (Fuji Analyzer BAS 5000). After cogenration of the autoradiographs and images of hematoxylin/eosin-stained 20-μm sections, 18F radioactivities were measured in the following regions of interest: (1) plaque (excluding media), (2) adventitia (including adjacent fat), and (3) normal vessel wall (media remote from atherosclerotic lesions) and given as photostimulated luminescence units per mm². The background was subtracted from the image data, and the results from each mouse were normalized for injected activity and decay. An example of the ARG analysis is shown in Supplemental Figure I. As previously reported, the measurements by 2 independent observers are reproducible (coefficient of variation 4.5%).18

Histology

Following the ARG analysis, macrophages were identified on adjacent 8-μm sections using a rat anti-mouse Mac-3 antibody (BD Pharmingen, clone M3/84). The plaques were visually graded as noninflamed (none or occasional Mac-3 positive macrophages) or inflamed (groups of macrophages or abundant infiltration of macrophages)19 and compared with the corresponding [18F]EF5 uptake. To characterize atherosclerosis in more detail, formalin-fixed and paraffin-embedded hearts with the aortic root were transversely cut into 5-μm sections at the level of the coronary ostia, followed by staining with anti-mouse Mac-3 antibody and modified Movat pentachrome staining. The intima-to-media ratio was determined from the Movat-stained section, and the Mac-3 positive area of each plaque was calculated using the automated image analysis software (Image-Pro Plus 5.0, Media Cybernetics).

Verification of Plaque Hypoxia

Plaque hypoxia was verified through the detection of EF5 adducts, after injection of nonlabeled EF5 (kindly provided by C.J. Koch), using a specific monoclonal antibody ELK3-51 conjugated to the fluorescent dye Cy-3 in 4 additional (2 LDLR⁻ /⁻ ApoB100/100 and 2 IGF-II/LDLR⁻ /⁻ ApoB100/100) mice. Furthermore, plaque hypoxia was confirmed by the staining of tissue sections after injection of pimonidazole in 2 additional (1 LDLR⁻ /⁻ ApoB100/100 and 1 IGF-II/LDLR⁻ /⁻ ApoB100/100) mice, using a fluorescein isothiocyanate-conjugated anti-pimonidazole antibody.

Statistics

All results are expressed as mean ± SD. Nonpaired data comparisons between 2 groups were made using the t test, and comparisons between multiple groups were made using ANOVA with the Tukey correction. The paired t test was used for comparing paired data between 2 groups. All analyses were performed with SAS (version 9.1, SAS Institute, Inc., Cary, NC). Values of P < 0.05 were considered statistically significant.

Results

Characterization of Atherosclerosis

The aortas of the C57BL/6N control mice showed no atherosclerosis. The atherosclerotic LDLR⁻ /⁻ ApoB100/100 mice developed atherosclerotic plaques covering large areas of aorta and also in the abdominal aorta. Atherosclerotic plaques were also seen in IGF-II/LDLR⁻ /⁻ ApoB100/100 mice, but they were less extensive than in LDLR⁻ /⁻ ApoB100/100 mice. Representative Mac-3- and Movat-stained aortic root sections are shown in Figure 1. In all mice, the plaques were mostly of the fibroatheroma type with a well-defined fibrous cap (Figure 1). The plaques of LDLR⁻ /⁻ ApoB100/100 mice were larger and had typically larger necrotic cores than the fibrosis-rich plaques seen in IGF-II/LDLR⁻ /⁻ ApoB100/100 mice. Consistent with this, the intima-to-media ratio in the aortic root was significantly larger in LDLR⁻ /⁻ ApoB100/100 mice (3.6 ± 1.3) than IGF-II/LDLR⁻ /⁻ ApoB100/100 mice (0.9 ± 0.2, P < 0.001). The atherosclerotic plaques in both LDLR⁻ /⁻ ApoB100/100 and IGF-II/LDLR⁻ /⁻ ApoB100/100 mice were highly inflamed. The extent of Mac-3 positive areas in the plaques from the
0.35%IA/g) than in control C57BL/6N mice (1.10±0.23% IA/g, P=0.0002) at 90 minutes postinjection. In IGF-II/LDLR−/−ApoB100/100 mice, the uptake of [18F]EF5 (1.18±0.17%IA/g) was at the same level as in C57BL/6N mice (not significant). At 180 minutes postinjection, the blood uptake of [18F]EF5 was still 1.95±0.26%IA/g in LDLR−/−ApoB100/100 mice, revealing a slow blood clearance of [18F]EF5. The aorta-to-heart and aorta-to-blood ratios were comparable among LDLR−/−ApoB100/100, IGF-II/II-LDLR−/−ApoB100/100, and C57BL/6N mice at both 90 and 180 minutes after injection.

[18F]EF5 Uptake in Atherosclerotic Plaques

The ARG analysis revealed significantly higher [18F]EF5 uptakes in the plaques than in the normal vessel wall in all the atherosclerotic mice. In total, 1505 regions of interest (523 in plaques, 555 in normal vessel wall, and 427 in adventitia) were analyzed from 16 atherosclerotic and 6 control mice. At 90 minutes after injection, the plaque–to–normal vessel wall ratio was 2.2±0.2 (P=0.0001 plaque versus wall) in LDLR−/−ApoB100/100 mice and 1.9±0.2 (P=0.0004 plaque versus wall) in IGF-II/LDLR−/−ApoB100/100 mice. At 180 minutes after injection, the plaque–to–normal vessel wall ratio was 1.9±0.2 (P=0.01 plaque versus wall) in LDLR−/−ApoB100/100 mice. Importantly, [18F]EF5 uptake in the normal vessel wall was similar between the atherosclerotic strains and control mice (Table 3). The uptake of [18F]EF5 in plaques did not show clear predilection sites, and there were no differences between calcified and noncalcified regions. In addition to plaques, the adventitia immediately adjacent to aorta showed [18F]EF5 uptake in both atherosclerotic mouse strains and controls. The average plaque-to-adventitia ratio was 1.0±0.2 (n=16; not significant) and the normal vessel wall-to-adventitia ratio did not differ significantly between atherosclerotic mice.

**Table 2. Ex Vivo Biodistribution of [18F]EF5 at 90 and 180 Minutes After Tracer Injection**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>LDLR−/−ApoB100/100 (90 Minutes, n=6)</th>
<th>IGF-II/LDLR−/−ApoB100/100 (90 Minutes, n=6)</th>
<th>C57BL/6N (90 Minutes, n=6)</th>
<th>LDLR−/−ApoB100/100 (180 Minutes, n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>1.88±0.35*</td>
<td>1.18±0.17</td>
<td>1.10±0.23</td>
<td>1.19±0.19</td>
</tr>
<tr>
<td>Blood</td>
<td>2.97±0.22*</td>
<td>1.84±0.48</td>
<td>1.88±0.19</td>
<td>1.95±0.26</td>
</tr>
<tr>
<td>Fat</td>
<td>0.76±0.21*</td>
<td>0.43±0.06</td>
<td>0.41±0.08</td>
<td>0.39±0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>2.53±0.58</td>
<td>1.54±0.64</td>
<td>1.85±0.30</td>
<td>1.67±0.58</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.18±1.69</td>
<td>12.04±0.95</td>
<td>5.16±0.75</td>
<td>5.05±1.02</td>
</tr>
<tr>
<td>Liver</td>
<td>7.47±5.69</td>
<td>8.08±4.27</td>
<td>4.60±1.74</td>
<td>3.77±0.62</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.48±0.17</td>
<td>1.31±0.41</td>
<td>1.59±0.17</td>
<td>1.57±0.25</td>
</tr>
</tbody>
</table>

Results are expressed as %IA/g (mean±SD). *Statistically significant difference in comparison with control mice (C57BL/6N).
In this article, we report for the first time that hypoxia in atherosclerotic models. Figure 2 shows the EF5 adducts and subsequent staining with anti-pimonidazole antibody in both fluorescent microscopy after injection of pimonidazole and antibody. A positive signal was also seen in plaques by adventitia and wall areas were not stained with ELK3-51 layers of plaques was confirmed by fluorescent microscopy of nonlabeled EF5 in both atherosclerotic models. The presence of hypoxia-specific EF5 adducts in the deep hypoxic areas have been detected in advanced human atherosclerotic lesions and in rabbit models of atherosclerosis. Until recently, it has been controversial whether mouse atherosclerotic plaques are hypoxic. The threshold for hypoxia depends on the oxygen diffusion distance, which is governed by plaque thickness. In mice, the plaque thickness is usually smaller than the depth at which severe hypoxia is detected in rabbit and human plaques (100 to 250 μm). However, it was recently shown that the plaques in the carotid artery in LDLR−/− mice are more hypoxic (4.4 to 55.2% of plaque hypoxia) compared with the plaques in the carotid arteries of symptomatic patients (0.4 to 23.5%) as detected by pimonidazole. In a tumor model, the 2 hypoxia markers, namely pimonidazole and EF5, were found to colocalize. In the present study, we verified hypoxia in LDLR−/− ApoB100/100 and IGF-II/LDLR−/− ApoB100/100 mouse aortic plaques by means of injecting EF5 adduct specific antibody and pimonidazole.

In this study, the ex vivo biodistribution results demonstrated a significantly higher aorta uptake of [18F]EF5 in those LDLR−/− ApoB100/100 mice that showed extensive and large atherosclerotic plaques than in the control mice. Our observation that aortic [18F]EF5 uptake of diabetic IGF-II/LDLR−/− ApoB100/100 mice was comparable to that of control mice is likely explained by the small size of plaques, as verified by the calculation of intima-to-media ratio in the aortic root of IGF-II/LDLR−/− ApoB100/100 mice. Thus, our results suggest that hypoxia is consistently present in advanced and large atherosclerotic plaques in atherosclerotic mice.

ARG showed much higher [18F]EF5 uptake in atherosclerotic plaques than in normal vessel wall in all atherosclerotic mice. However, the slow blood clearance suggests that [18F]EF5 is strongly bound to the plasma proteins, which lengthens its half-life in the circulation. The circulatory half-life of EF5 has been reported to be approximately 40 minutes in mice. Uptake of [18F]EF5 in adventitia observed in this study was nonspecific, probably because of the presence of blood in the adventitial microvessels and tissue processing. This is suggested by a deficiency of EF5 adducts in the immunofluorescence staining. The slow blood clearance and the high adventitial uptake may limit the value of [18F]EF5 for in vivo imaging of atherosclerosis, but this remains to be tested in other models and in humans.

Hypoxia may have an important role in the progression of atherosclerotic lesions by promoting lipid accumulation, inflammation, and angiogenesis. It has been reported earlier that the hypoxia observed in advanced atherosclerotic plaques is directly correlated to the extent of macrophages and

Discussion

In this article, we report for the first time that hypoxia in atherosclerotic plaques of atherosclerotic mice can be detected with [18F]EF5 by using ex vivo methods. The uptake of [18F]EF5 was higher in the aortas of atherosclerotic LDLR−/− ApoB100/100 mice with large plaques than in C57BL/6N control mice. ARG showed consistent, focally increased uptake of [18F]EF5 in atherosclerotic plaques, as compared with normal vessel wall of the same animals. The uptake of [18F]EF5 appeared to be unrelated to the degree of plaque inflammation confirmed by anti-Mac-3 antibody immunohistochemical staining. The blood concentrations of [18F]EF5 remained quite high, potentially limiting the feasibility of [18F]EF5 for noninvasive imaging of atherosclerotic plaques with [18F]EF5 in this model.

Hypoxic areas have been detected in advanced human atherosclerotic lesions and in rabbit models of atherosclerosis. Until recently, it has been controversial whether mouse atherosclerotic plaques are hypoxic. The threshold for hypoxia depends on the oxygen diffusion distance, which is governed by plaque thickness. In mice, the plaque thickness is usually smaller than the depth at which severe hypoxia is detected in rabbit and human plaques (100 to 250 μm). However, it was recently shown that the plaques in the carotid artery in LDLR−/− mice are more hypoxic (4.4 to 55.2% of plaque hypoxia) compared with the plaques in the carotid arteries of symptomatic patients (0.4 to 23.5%) as detected by pimonidazole. In a tumor model, the 2 hypoxia markers, namely pimonidazole and EF5, were found to colocalize. In the present study, we verified hypoxia in LDLR−/− ApoB100/100 and IGF-II/LDLR−/− ApoB100/100 mouse aortic plaques by means of injecting EF5 adduct specific antibody and pimonidazole.

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Hypoxia may have an important role in the progression of atherosclerotic lesions by promoting lipid accumulation, inflammation, and angiogenesis. It has been reported earlier that the hypoxia observed in advanced atherosclerotic plaques is directly correlated to the extent of macrophages and
neovascularization.\textsuperscript{5} Consistent with previous studies, we found that plaques were rich in macrophages (up to 60\% of plaque area). In this study, the uptake of [\textsuperscript{18}F]EF5 was at the same level in the inflamed and noninflamed plaques as detected by Mac-3 staining. However, it is important to notice that the content of macrophages in plaques is heterogeneous, and macrophages can express different antigens.\textsuperscript{22} Expression of Mac-3 antigen is upregulated during differentiation of monocytes into activated macrophages, which can be detected by anti-Mac-3 antibody immunohistochemical staining in the cap area and in the shoulder regions of the plaques. It has been previously shown that in advanced human carotid atherosclerotic plaques, the cap shows mild or no hypoxia, whereas the shoulder segment of the plaque is not hypoxic, irrespective of the infiltration of macrophages. However, the hypoxia is present in the center of an advanced plaque and colocalizes with CD68-positive macrophages.\textsuperscript{5} This may also explain the difference in the [\textsuperscript{18}F]EF5 uptake and Mac-3 positive macrophages in our study.

The ARG analysis of very small structures is challenging, and variation in the results is expected. We compensated for this by analyzing several regions in serial sections, thus adding the effect of multiple measurements. The variability of the ARG method has been previously tested by 2 independent observers, and the coefficient of variation was always <10\%, which is considered acceptable.\textsuperscript{18} In conclusion, our study provides evidence that hypoxia in advanced atherosclerotic plaques can be a potential target for identifying high-risk plaques. Our findings encourage pursuing additional studies with hypoxia-specific tracers for detecting high-risk atherosclerotic plaques by noninvasive imaging.

Acknowledgments

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Disclosures

None.

References

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Supplement Material

Materials and Methods

Animals

Atherosclerotic mice deficient of low-density lipoprotein receptor and synthesizing only apolipoprotein B100 (LDLR\(^{-/-}\)ApoB\(^{100/100}\) in C57BL/6\(\times\)129/SvJae background, The Jackson Laboratory, Bar Harbor, ME, strain #003000) as well as LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice over-expressing insulin-like growth factor-II (IGF-II)\(^1\) in pancreatic beta cells (IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\), A. I. Virtanen Institute, University of Kuopio, Finland) were fed with a high-cholesterol Western-type diet (TD 88137, Harlan Teklad, with 42% of calories coming from fat and 0.2% from cholesterol, without sodium cholate) for 3–4 months, starting at the age of 4–8 months. The IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice exhibited insulin resistance and hyperglycemia (type 2 diabetes), and hypercholesterolemia (from the LDLR\(^{-/-}\)ApoB\(^{100/100}\) background) associated with a rapid development of atherosclerotic plaques. It was used in the present study as a model of the most inflamed types of atherosclerotic plaques.\(^1\) A subset of wild-type C57BL/6N mice were fed with a regular diet and served as non-atherosclerotic controls. The experiments were approved by the Lab-Animal Care & Use Committee of the State Provincial Office of Southern Finland and carried out in compliance with the Finnish laws relating to the conduct of animal experimentation.

Synthesis of \([^{18}\text{F}]\text{EF5}\)

\([^{18}\text{F}]\text{EF5}\) was synthesized from 2-(2-nitro-\(^1\)H-imidazol-1-yl)-N-(2,3,3-trifluoroallyl)-acetamide using \([^{18}\text{F}]\text{F}_2\) as the labeling reagent, as described previously\(^2\). The specific radioactivity of \([^{18}\text{F}]\text{EF5}\), decay-corrected to the end of synthesis, was always >3.7 GBq/\(\mu\)mol and the radiochemical purity was >98.5% throughout the study.
Ex vivo Biodistribution Studies

The biodistribution of intravenously (i.v.) administered $[^{18}\text{F}]\text{EF5}$ at 90 min after injection was studied in 6 LDLR$^{-/}$ApoB$^{100/100}$, 6 IGF-II/LDLR$^{-/}$ApoB$^{100/100}$, and 6 C57BL/6N mice. The 90-min accumulation time was based on the serum half-life of EF5 in mice ($T_{1/2}=40 \text{ min}$). Since the biological half-life of EF5 is relatively long, 4 additional LDLR$^{-/}$ApoB$^{100/100}$ mice were studied at 180 min post-injection to explore the biodistribution of $[^{18}\text{F}]\text{EF5}$ in more detail. The mice were anaesthetized with isoflurane and injected with $[^{18}\text{F}]\text{EF5}$ ($11 \pm 2 \text{ MBq in } 150 \mu\text{l of saline}$) via a tail vein, and sacrificed 90 min or 180 min after injection. The basic characteristics of the animals are shown in Table 1. Blood samples were drawn under deep isoflurane anaesthesia by cardiac puncture before cervical dislocation and subsequent dissection of aorta, fat, heart, kidney, liver and muscle. The ascending aorta to the level of diaphragm was dissected and blood was rinsed with saline. The radioactivity in aorta, blood and other tissue samples was measured in a well-type gamma counter (Triathler 3”, Hidex, Turku, Finland), cross-calibrated with a dose calibrator (VDC-202, Veenstra Instruments, Joure, The Netherlands). Background counts were subtracted, and the radioactivity decay was corrected to the time of injection. The dose remaining in the tail was also compensated. The radioactivity concentration was expressed as a percentage of the injected activity per gram of tissue ($\%\text{IA}/\text{g}$). A total of 13 atherosclerotic hearts (from 7 LDLR$^{-/}$ApoB$^{100/100}$ and 5 IGF-II/LDLR$^{-/}$ApoB$^{100/100}$ mice) were collected and preserved in formalin for further studies.

Autoradiography

An autoradiography (ARG) analysis of aorta was performed in order to study the distribution of $[^{18}\text{F}]\text{EF5}$ in the aortic tissues of each investigated mouse. The aorta was frozen, and sequential longitudinal 8 and 20 $\mu\text{m}$ cryosections were cut, apposed to an imaging plate (Fuji Imaging Plate...
BAS-TR2025) and after an exposure time of 4 h, the imaging plates were scanned (Fuji Analyzer BAS 5000; internal resolution 25 μm). The 20 μm sections were stained with hematoxylin and eosin (HE) and examined for morphology under a light microscope. After a careful co-registration of the autoradiographs and HE images, three types of regions of interest (ROIs) were defined. The ROIs were analyzed from (1) plaque, (2) adventitia, and (3) normal vessel wall for count densities (photostimulated luminescence per unit area, PSL/mm²) using Tina 2.1 software (Raytest Isopemessgeräte, GmbH, Straubenhardt, Germany). The background area count densities were subtracted from the image data and the results of each mouse were normalized for injected dose and decay. An example of ARG analysis is shown in Supplemental Figure I. The variability of the autoradiography method has been previously tested by two independent observers⁴.

**Histology**

Following the ARG analysis, macrophages were identified on adjacent 8 μm cross-sections using a rat anti-mouse Mac-3 antibody (BD Pharmigen, Clone M3/84, 1:5000) as we have described previously⁴. The degree of inflammation in the plaques was semi-quantitatively assessed by the first author together with an experienced pathologist (J.L.). In the semi-quantitative analysis of Mac-3 stained areas, the plaques were visually graded as non-inflamed (= none or occasional Mac-3 positive macrophages) or inflamed (groups of macrophages or abundant infiltration of macrophages)⁵. An ARG analysis of the [¹⁸F]EF5 uptake was made from Mac-3 antibody stained 8 μm sections and the macrophages in the plaques were defined as belonging to one or the other category. In order to characterize atherosclerosis in more detail, formalin-fixed and paraffin-embedded hearts with the aortic root from seven LDLR⁺/⁻ApoB⁴₀₀/⁴₀₀ and five IGF-II/LDLR⁺/⁻ApoB⁴₀₀/⁴₀₀ mice were transversely cut into 5 μm sections at the level of the coronary ostia, followed by staining with anti-mouse Mac-3 antibody and modified Movat’s pentachrome staining.
The intima-to-media ratio was determined from the Movat stained section, and the Mac-3 positive area of each plaque was calculated using the automated image analysis software (Image-Pro Plus 5.0, Media Cybernetics, USA).

**Verification of Plaque Hypoxia**

Plaque hypoxia was verified through the detection of EF5 adducts, after injection of non-labeled EF5 (kindly provided by C. J. Koch), using a specific monoclonal antibody ELK3-51 conjugated to the fluorescent dye Cy-3 (University of Pennsylvania, Philadelphia, USA) in 4 additional (2 LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> and 2 IGF-II/LDLR<sup>−/−</sup>ApoB<sup>100/100</sup>) mice. A high dose of non-labeled EF5 (10mM in 0.9% saline, 0.01 ml/g body weight) was i.v. administered to non-anaesthetized mice 3 h before the mice were sacrificed. Frozen 8 µm aorta sections were then processed for immunofluorescence staining as described previously<sup>6,7</sup>. Briefly, the sections were fixed in 4% paraformaldehyde for 1 h and rinsed twice in PBS with 0.3% polyoxyethylenesorbitanmonolaurate (Tween20). The sections were blocked for 1 h with 3% bovine serum albumin (BSA) in PBS with Tween20, and rinsed with in PBS with 0.3% Tween20. Then, the sections were stained for overnight in +4°C with 75 µg/ml Cy-3 conjugated ELK3-51 monoclonal antibody. After rinsing three times in PBS with 0.3% Tween20, the sections were stored in PBS until image acquisition. After imaging on a fluorescent microscopy, the sections were counterstained with HE. Aorta sections from mice not injected with EF5 were also stained and used as negative controls in order to assess the non-specific binding of the antibody.

Furthermore, plaque hypoxia was confirmed by the staining of tissue sections after i.v. injection of 50 mM pimonidazole (Hypoxyprobe™-1, Natural Pharmacia International, Inc, Burlington, USA) in 0.9% saline in two additional (1 LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> and 1 IGF-II/LDLR<sup>−/−</sup>ApoB<sup>100/100</sup>) mice. Pimonidazole (0.06 ml/g body weight) was injected 1 h before sacrifice,
followed by aorta preparation, formalin-fixing and embedding in paraffin. Serial 4 µm cross sections of aorta were cut and stained using the Hypoxyprobe™-1 kit (Natural Pharmacia International). The sections were rinsed twice (0.05 mol/l Tris-HCl with 0.05% Tween20), blocked for 10 min with 3% BSA (in 0.05 mol/l Tris-HCl with 0.05% Tween20) and rinsed again. Then, the sections were stained overnight in +4°C with fluorescein isothiocyanate-conjugated anti-pimonidazole (1:50, Hypoxyprobe™-1), rinsed three times and imaged. Adjacent sections were stained with HE. Aorta sections from mice not injected with pimonidazole were also stained and used as negative controls in order to assess the non-specific binding of the antibody.

**Statistics**

All results are expressed as mean ± SD values. Non-paired data comparisons between two groups were made using t-test and between multiple groups using Anova with Tukey’s correction. Paired t-test was used for comparing paired data between two groups. All analyses were performed with SAS (version 9.1, SAS Institute, Inc., Cary, North Carolina). Values of $P$ less than 0.05 were considered statistically significant.
Supplemental Figure I. An example of the ARG analysis of an aortic arch section.

(A) HE stain of an aortic arch section. (B) Patch image made from the HE stained section (A) superimposed with grey/black $^{18}$F-autoradiograph (not shown). (C) Image (B) in rainbow colors for an analysis of ROIs: R1 and R2 = plaque, R3 = adventitia, and R4 = normal vessel wall. Scale bar 500µm.
References


