Evidence of Hypoxic Areas Within the Arterial Wall In Vivo

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Abstract—The anoxemia theory of atherosclerosis states that an imbalance between the demand and supply of oxygen in the arterial wall is a key factor for the development of atherosclerotic lesions. Direct in vitro and in situ measurements have shown that Po2 is decreased in the more deeply situated parts of the media, but the degree of hypoxia in vivo or the distribution of hypoxia along the arterial tree is not known. For this reason, we have developed a method for the detection of hypoxia in the arterial wall in vivo by using a hypoxia marker, 7-(4′-(2-nitroimidazol-1-yl)-butyl)-theophylline, that may be visualized by immunofluorescence. In the present study, we have used this method in rabbits with experimentally induced atherosclerosis. Our results indicate that zones of hypoxia occur at depth in the atherosclerotic plaque. The mechanism was probably an impaired oxygen diffusion capacity due to the thickness of the lesion, together with high oxygen consumption by the foam cells. Thus, we have for the first time demonstrated that hypoxia actually does exist in the arterial wall in vivo, lending support to the anoxemia theory of atherosclerosis.

Key Words: atherosclerosis ■ artery ■ hypoxia ■ hypoxia marker

The anoxemia theory of atherosclerosis1 highlights the fact that the more deeply situated parts of the arterial wall depend on diffusion to satisfy their need for oxygen and nutrients. When atherosclerotic lesions develop, the arterial wall thickness increases and diffusion capacity is impaired. At the same time, oxygen consumption is augmented,2,3 and an energy imbalance may occur. Local metabolic disturbances may be envisioned that may endanger regression or even result in progression of the atherosclerotic process, with the formation of a necrotic core.

In several studies in animals in vitro and in situ, a decreased oxygen concentration has been demonstrated in the arterial media, with a minimum Po2 of 0.3 to 0.7 kPa (20 to 50 mm Hg).4–7 So far, however, no data have been presented to verify that hypoxia is present in arterial tissue in the intact animal. At the same time, it is obvious that such data are crucial to validate the presented hypothesis.

In a recent article, we introduced a method for the assessment of hypoxia in arterial tissue in vitro.8 This method is based on the demonstration of a tissue-bound hypoxia marker, 7-(4′-(2-nitroimidazol-1-yl)-butyl)-theophylline (NITP), which was originally developed by Hodgkiss et al9 to detect hypoxia in tumors. The use of nitroimidazole derivatives (such as NITP) as hypoxia markers was introduced in the early 1980s,10 and applications in tumor research abound (eg, see References 11 through 14). The marker undergoes nitroreduction intracellularly (mainly by cytochrome P450 reductase)15 and reactive radicals are formed, which bind to cellular constituents in the absence of oxygen, hence, a marker of hypoxia. The main advantage with this method is that NITP may be administered in vivo, which makes it possible to assess hypoxia in arterial tissue in the intact animal.

In the present research, we have used this method to study arterial tissue in rabbits in vivo. Our results indicate that hypoxic zones do occur at certain locations in the arterial tree in the living animal. We believe that these results constitute a crucial piece of evidence in support of the anoxemia theory of atherosclerosis.

Methods

Medium, Chemicals, and Antibodies

Eagle’s minimum essential medium with Earle’s salts and 10 mmol/L NaHCO3 was used perioperatively, supplemented with 1% nonessential amino acids, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 60 mg/mL BSA (Serva Feinbiochimica). NITP was obtained from Lancaster Synthesis Ltd. This drug is poorly soluble in water, and the technique developed by Hodgkiss et al9 was used to prepare the solutions for intraperitoneal administration. Thus, NITP was first dissolved in dimethyl sulfoxide (Sigma Chemical Co) and then added to peanut oil (aflatoxin content <0.5 ppb; Apoteksbolaget) at 10% vol/vol. NITP was added at a final concentration of 50 (n=2) or 100 (n=4) μmol/mL to a 30-mL peanut oil mixture. All NITP solutions were prepared fresh before use.

Sheep anti-theophylline antibody (theophylline-8-BSA) was purchased from Biogenes Inc, Bournemouth, England. Biotinylated rabbit anti-sheep IgG antibody, fluorescein avidin D, biotinylated goat anti-avidin D, and normal rabbit serum were all obtained from Vector Laboratories, Burlingame, Calif. Nonimmune sheep IgG to be used as the nonspecific control antibody was bought from Cedarlane Ltd, Hornby, Canada. Dry milk powder was obtained from Semper.
Chemicals for the modified Russel-Movat pentachrome staining were obtained as follows: alcian blue from Polysciences Inc; crocein scarlet, phosphotungstic acid, and safranin O from Sigma Chemical Co; and acid fuchsin from Histolab Products AB. Sections were mounted on glass slides (SuperFrost/Plus, Menzel-Glaser) with Vectashield as the mounting medium (Vector Laboratories).

**Animals**

At 2 to 3 months of age, experimental atherosclerosis was induced in 8 male New Zealand White rabbits (Lidköpings Kaniufarm, Målsöa, Sweden) through a combination of a cholesterol-enriched diet (1% cholesterol in standard rabbit chow fed ad libitum) and mechanical injury induced by use of an embolectomy catheter. After premedication with ketamine 7.5 mg/kg and xylazine 3.5 mg/kg IV initially, plus half that amount every 15 minutes thereafter. After perioperative preparations, the rabbit was exsanguinated. Approximately 3 minutes after the cessation of spontaneous breathing, continuous rinsing with oxygenated medium at 37°C was initiated, and the aortic arch and proximal half of the thoracic aorta were removed. The major part of the adventitia was dissected away, and the vessel was cut into 6 to 8 segments 4 to 5 mm long and fixed in 4% buffered formaldehyde, pH 7.0, for 1 to 2 days. Specimens were also taken from selected organs, segments 4 to 5 mm long and fixed in 4% buffered formaldehyde, pH 6.5 (n = 1) and 4.7±0.2 (n = 5) hours before the animals were killed. During the NITP injection, the rabbit was sedated with ketamine 7.5 mg/kg and xylazine 3 mg/kg IM. The animals tolerated the injections well and showed no adverse reactions during the experiment. Blood samples were taken at intervals for the determination of NITP concentrations in plasma, which was performed by high-performance liquid chromatography by Dr M. Stratford, Gray Laboratory of the Cancer Research Campaign, Mount Vernon Hospital, Northwood, UK. The aortic arch and the thoracic aorta were removed by a method that has been developed to ensure maximum tissue integrity. After premedication with ketamine 7.5 mg/kg and xylazine 3 mg/kg, anesthesia was induced by administration of ketamine 8.5 mg/kg IV and xylazine 3.5 mg/kg IV initially, plus half this amount every 15 minutes thereafter. After perioperative preparations, the skin was incised and the peritoneum opened. Before the thoracic cage was opened, (20 to 30 minutes after the induction of anesthesia), an overdose of pentobarbitonal sodium (∼50 mg/kg) was given, and the rabbit was exsanguinated. Approximately 3 minutes after the cessation of spontaneous breathing, continuous rinsing with oxygenated medium at 37°C was initiated, and the aortic arch and proximal half of the thoracic aorta were removed. The major part of the adventitia was dissected away, and the vessel was cut into 6 to 8 segments 4 to 5 mm long and fixed in 4% buffered formaldehyde, pH 7.0, for 1 to 2 days. Specimens were also taken from selected organs, ie, myocardium, liver, kidney, striated muscle, esophagus, and spleen. One of the atherosclerotic rabbits with prominent lesions and 1 nonmanipulated rabbit served as controls. These rabbits did not receive NITP, but arterial tissue was otherwise treated in the same way.

As another control experiment, atherosclerotic segments were obtained in the same way, but instead of fixation, the tissue was rinsed in tap water, the sections were mounted. Sections incubated in the absence of the primary antibody and sections in incubations where nonimmune IgG substituted for the specific primary antibody served as controls.

**Histochemistry**

To visualize the structural correlates of the observed immunofluorescence, parallel sections were stained with the Russel-Movat pentachrome method in some cases. In our experience, optimal staining was obtained after some modifications of the protocol by using a higher concentration (2%) of alcian blue and a considerably lower concentration of the alcoholic safranin solution (0.1%).

**Microscopy**

Bright-field or fluorescence microscopy was performed on an Axioshot or an Axiocert microscope. For fluorescence, filter set 16 (BP 485/20, FF 510, LP 520; Carl Zeiss) was used. Photomicrographs of the outlines of the serial sections were obtained on the same microscope with the use of the differential interference contrast setup. Images for publication were registered with a color video camera (Sony 3CCD, DXC-930P, Sony Co) in integrating mode and KS400 image analysis software (Carl Zeiss).

**Statistics**

SDs of the mean were used as the measure of dispersion unless stated otherwise.

**Results**

**NITP in Plasma**

To the best of our knowledge, NITP had not been used in rabbits before, which means that no pharmacokinetic data were available on suitable doses and modes of administration. Also, for practical reasons, it was not feasible to perform extensive methodological experiments. Thus, in the present experiments, we were guided by protocols that have been used in tumor-bearing mice in which hypoxia was assessed by flow cytometry.

The plasma levels of NITP rose to a maximum between 11.3 and 98.1 μmol/L within 11/2 hours and then decreased, with a half-life of ∼1 to 3 hours (Figure 1). The maximum concentrations tended to be lower and the half-life longer than those described in the mouse (≈100 μmol/L and 11/2 hour, respectively). The NITP exposure, calculated as area under the curve (AUC), was 230±157 (range, 29 to 415) μmol·h⁻¹·L⁻¹ in the rabbits. In the tumor-bearing mice, the AUC was 154 μmol·h⁻¹·L⁻¹.
For comparison, in the in vitro system that we used to test NITP, the concentration was 250 μmol/L during the 3 hours of incubation, which would correspond to an AUC of 750 μmol · h⁻¹ · L⁻¹.9 In the in vitro control experiment that was performed in the present study, the NITP exposure corresponded to an AUC of 190 to 750 μmol · h⁻¹ · L⁻¹.

Immunofluorescence in Arterial Tissue

Rabbits With Experimental Atherosclerosis

Sections (216) from ≈12 levels in each of 6 NITP-treated rabbits were studied. In ≈2/3 of these, only minor lesions or no lesions were seen. Approximately another 2/3 had prominent lesions, whereas the remainder were in between. As summarized in the Table, the overwhelming majority of lesions exceeding a thickness of 400 to 500 μm exhibited distinct zones of immunofluorescence. In Figure 2, these zones are shown at low magnification together with a routine, stained adjacent section. At higher magnifications (Figures 3 and 4), the fluorescence appears to be mainly localized intracellularly in groups of foam cells situated in the interior parts of the lesions. The zones of immunofluorescence were 200 to 300 μm wide and situated at a distance of 200 to 300 μm from the endothelial surface. In Figure 5, a diagrammatic 3-dimensional reconstruction of 1 serially sectioned segment is depicted. The fluorescence occupies the center of the lesion like a wedge.

In many sections, fluorescence was also noted in the luminal endothelium (Figure 6) and in the endothelium of the vasa vasorum. In control incubations with nonspecific antibodies, this reaction was reproduced in the endothelium, whereas no fluorescence was observed in the foam cells (Figure 7).

It appeared that this nonspecific immunofluorescence was a suitable marker for the vasa vasorum. Although not specifically studied, vasa vasorum were regularly observed in the

### Specific Immunofluorescence in Lesions of Varying Thickness

<table>
<thead>
<tr>
<th>Size of Lesion</th>
<th>Specific Immunofluorescence</th>
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<tbody>
<tr>
<td>Minimal or no lesion</td>
<td>Absent</td>
</tr>
<tr>
<td>Lesion &lt;400 to 500 μm</td>
<td>171 (230)</td>
</tr>
<tr>
<td>Lesion &gt;400 to 500 μm</td>
<td>166 (246)</td>
</tr>
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Rabbits with experimental atherosclerosis were given hypoxia marker intraperitoneally. Bound marker in sections from aortic tissue was demonstrated by immunofluorescence. In this table, the number of sections with detectable marker is listed as a function of the thickness of the lesion. Average AUC (μmol · h⁻¹ · L⁻¹) is given in parentheses. Specific fluorescence is clearly associated with the thickness of the lesions. There is no difference in the AUC between sections with and without fluorescence. Please note that the number of observations exceeds the total number of sections, 216, because sections with lesions of different thickness were included in >1 size category.

![Image](https://example.com/image.png)

**Figure 2.** Two adjacent cross sections of the thoracic aorta from a rabbit with experimental atherosclerosis. The rabbit was given the hypoxia marker NITP intraperitoneally 4.5 hours before sacrifice. One section was stained with Russel-Movat’s pentachrome stain (left). In the other section (right), bound NITP was detected by FITC immunofluorescence. Apple-green fluorescence is seen at several locations. The areas with the most intense fluorescence, easily discernible at this low magnification, are indicated with arrows. Below, the whole circumference of the section is given a diagrammatic representation. The internal elastic lamina has been stretched out along the abscissa with the plaque above and the media below. The part of the plaque that showed fluorescence is depicted in white, whereas the rest of the plaque is green. The filled gray areas correspond to those zones that had intense fluorescence. The figures show that

![Image](https://example.com/image.png)

**Figure 3.** Cross section of the aortic arch (left) and the thoracic aorta (right) from a rabbit with experimental atherosclerosis. The rabbit was given the hypoxia marker NITP intraperitoneally 4.5 hours before sacrifice, and bound marker was detected by FITC immunofluorescence. The luminal surface (arrows), internal elastic lamina (lei), and adventitia (adv) are indicated. Where the plaque is thick (left), a zone of specific yellow-green fluorescence was observed in the abluminal part (blue dashed line), indicative of in vivo hypoxia. Fluorescence is especially intense in a group of centrally located foam cells. Where the plaque is thinner (upper right) or almost absent (lower right), no immunofluorescence was seen. In the background nonspecific yellow-brown autofluorescence is shown. AUC in this rabbit was 284 μmol · h⁻¹ · L⁻¹. Composite picture from 8 to 20 visual fields. Bars=100 μm.

NITP-associated immunofluorescence, indicative of tissue hypoxia, is present in the deep portions of the atherosclerotic plaque. It should be noted that at some locations in these deep portions, areas were observed where no fluorescence was obvious (gray outlines), p, m, and a denote plaque, media, and adventitia, respectively. AUC in this rabbit was 387 μmol · h⁻¹ · L⁻¹. Composite pictures from 25 to 30 visual fields. Bar=1000 μm.
adventitia and in the outermost portion of the media. It seemed that the distance from the zones of specific immuno-
fluorescence to the vasa vasorum was similar to the distance
to the main lumen (Figure 7). Fluorescence was sometimes
noted in the adventitia (Figure 3); this reaction was also
reproduced by staining with nonspecific primary antibodies
(Figure 7).

**Controls**

No immunofluorescence was seen in aortic sections obtained
from animals that had not received NITP, 1 rabbit with experimental atherosclerosis and 1 nonmanipulated rabbit
(Figure 8). Furthermore, no immunofluorescence was seen in
control sections when the primary antibody was omitted
(Figure 8).

**In Vitro Incubations**

NITP exposure was lower during the in vivo experiments
than during the incubations performed to test the hypoxia
marker in vitro. For this reason, aortic segments were
incubated at reduced NITP concentrations under hypoxic
conditions that had been previously shown to induce binding.
As shown in Figure 9, the intensity of the immunofluores-
cence was reduced, but clearly detectable, at an NITP
exposure (190 μmol · h⁻¹ · L⁻¹) within the same range as the
one used in vivo (230±157 μmol · h⁻¹ · L⁻¹).

**Immunofluorescence in Other Tissues**

Strong immunofluorescence was noted in the mucosa of the
esophagus (Figure 10). Immunofluorescence was also noted
in the pericentral regions of the liver lobules (Figure 10).
Weak fluorescence was observed in some of the collecting
tubuli of the kidney. No immunofluorescence was noted in
the myocardium, spleen, or skeletal muscle.

**Discussion**

A method for the demonstration of arterial wall hypoxia by
using the hypoxia marker NITP was recently described from
our laboratory. The testing was carried out in vitro, and it
was proposed that the method could also be used in vivo. In
the present study, we have applied this technique in rabbits
with experimental atherosclerosis.
In the rabbit aorta, distinct NITP-associated immunofluorescence was observed in atherosclerotic lesions that were >400 to 500 μm thick (Figures 2, 3, and 7), and the fluorescence was reproduced in consecutive serial sections (Figure 5). The fluorescence was localized intracellularly to foam cells (fc) deep within the plaque. Nonspecific fluorescence (right) is obvious in the luminal endothelium (arrows) and in the endothelium of the vasa vasorum (vv) but is notably absent among foam cells. p, m, and a denote plaque, media, and adventitia, respectively. The luminal border is indicated by arrows and dashed line. AUC in this rabbit was 387 μmol · h⁻¹ · L⁻¹. Composite pictures from 8 to 10 visual fields. Bar=500 μm.

When a rabbit aorta with experimental atherosclerosis was incubated under hypoxic conditions in vitro, foam cells in the atherosclerotic lesions accumulated NITP.⁸ In those experiments, smooth muscle cells bound NITP at a Po₂<2 to 3 mm Hg, whereas foam cells seemed to bind the hypoxia marker at a somewhat higher Po₂ but still in a clearly hypoxia-dependent manner. Thus, the most obvious interpretation of our present findings is that the arterial tissue in the zones of NITP immunofluorescence had suffered from local hypoxia in vivo. From the in vitro data, the Po₂ had most likely been ~30 to 40 Pa (2 to 3 mm Hg) in the periphery of these zones. It should also be pointed out that the NITP exposure in vivo was considerably lower than that in the in vitro “calibration” experiments (AUCs of 230±157 and 750 μmol · h⁻¹ · L⁻¹, respectively). Still, control incubations indicated that hypoxia-dependent immunofluorescence was already clearly detectable at an NITP exposure of 190 μmol · h⁻¹ · L⁻¹, similar to that used in vivo (Figure 9). It has been suggested that the binding of NITP is proportional to the square root of the concentration,²¹ which is also in agreement with estimations from published in vitro data, indicating that a reduction of the NITP concentration by a factor 5 could lead to a decrease in hypoxic fluorescence by 50%.⁹ Thus, our present data might have underestimated rather than exaggerated the true zones of hypoxia in vivo. Furthermore, it is interesting that classic data by Jurjus and Weiss⁵ based on polarographic measurements in vitro, agree well with our observations. They found a Po₂ of 0 Pa in the atherosclerotic aortic arch in rabbits at a depth of 300 μm from the lumen when the total arterial wall thickness exceeded 880 μm, which is similar to the observations in the present study.
Alternative Mechanisms of NITP Binding

Although hypoxia markers have been shown to bind to cellular constituents in various tissues in a clearly hypoxia-dependent manner, binding to apparently normoxic tissue has also been described. This points to the possibility that other, oxygen-independent mechanisms may exist and confound the interpretation. Thus, nitroreduction might be channeled by the 2-electron transfer enzyme detoxicating diaphorase [(NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2], thus bypassing the oxygen-dependent neutralization of the reactive radicals that cause binding. This mechanism has been proposed to explain the apparently oxygen-independent NITP binding to the esophageal mucosa in mice, and in fact, the presence of this enzyme has been demonstrated there. However, others failed to show an effect on NITP binding through inhibition of the enzyme. In addition, in the same article, binding in the esophagus seemed, in fact, to be oxygen dependent, at least in vitro. Furthermore, it has been argued that the detoxicating diaphorase reaction is a very poor producer of reactive radicals in comparison with the reaction mediated by cytochrome P450. Hypoxia markers have also been used to demonstrate hypoxic areas in the liver, but the results have been suspected to merely reflect local differences in nitroreductase activity. However, by using retrograde perfusion of murine liver, Arteel et al have demonstrated that NITP binding in the liver was clearly hypoxia dependent. Thus, whereas some controversy remains, most data favor the interpretation of NITP binding as a true marker of tissue hypoxia. That our own findings do reflect tissue hypoxia is supported by the fact that the NITP binding in vivo reproduced the clearly hypoxia-dependent binding in vitro within the same type of cells and tissue. Also, if PO2-independent mechanisms were important, one would also have expected some immunoreaction in cells closer to the vessel lumen in presumably better-oxygenated areas.

As discussed in our previous article, binding to foam cells might reflect still another alternative mechanism: ie, local hypoxia could upregulate detoxicating diaphorase synthesis, leading to an increased nitroreductase activity and the production of reactive intermediates, which could bind to NITP. However, others have shown that overexpression of detoxicating diaphorase by 3 orders of magnitude had very little influence on NITP binding. The observed immunofluorescence in apparently normoxic endothelial cells may seem paradoxical and in fact, has been described before. Occasionally, diffuse fluorescence was also noted in the adventitia (Figure 3). Most likely, however, these reactions do not reflect NITP binding, because they were reproduced in incubations with nonimmune primary antibodies (Figure 7). We also confirmed hypoxia marker binding to the esophageal epithelium and pericentral areas in the liver (Figure 10).

Levels of Hypoxia

Regarding the degree of hypoxia to which the immunodetectable NITP corresponded in our experiments, the estimates were derived from comparisons with our previous in vitro incubations, in which the same type of tissue had been used. Our conclusion was that bound marker corresponded to a PO2 of ~30 to 40 Pa (2 to 3 mm Hg). In cell lines, half-maximal binding of NITP derivatives is said to occur mostly at oxygen concentrations of 0.1% to 0.5%, probably corresponding to a detection level of 0.5% to 1% O2. This is somewhat lower than our estimate. However, in the only true tissue “calibration” of NITP (in this case, misonidazole), binding at 140 Pa (10 mm Hg) has been mentioned as the practical limit of detection. In that study, PO2 was measured directly with microelectrodes, and tissue binding was assessed by autoradiography. At 140 Pa (corresponding to 10 mm Hg), PO2 binding was 5 times the background level, whereas at 70 Pa (5 mm Hg), binding was as high as 9 times the background level. Although these data were obtained in tumor spheroids, it is possible that tissue binding of the hypoxia marker may in fact be more sensitive than previously suggested. One basic fact that it is also highlighted in the quoted study is that binding requires living cells. This means that, though hypoxic, most cells within the foam cell accumulations did not seem to have undergone necrosis.

“The anoxemia theory of atherosclerosis” states that hypoxia is a key factor for the development of atherosclerotic lesions. However, so far no data have been provided on the actual presence of hypoxia in the arterial wall in vivo. In the present study, we have demonstrated that hypoxic zones do occur within atherosclerotic plaques in rabbits when the lesions exceed certain dimensions and at a depth that is readily reached in humans. It seems likely that the hypoxia in these areas reflects an impaired diffusion capacity due to the thickness of the plaque, together with an increased demand in the metabolically active foam cells.

Acknowledgments

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Figure 10. Sections from liver (top) and esophagus (bottom) from rabbits that had been given the hypoxia marker NITP intraperitoneally 6.5 hours before sacrifice. Bound NITP was detected by FITC immunofluorescence. In the liver, immunofluorescence is obvious in the superficial pericentral areas in the hepatic lobule. Immunofluorescence is shown pericentrally but not in the midzone of the hepatic lobule. Immunofluorescence is obvious in the superficial esophageal mucosa. AUC in this rabbit was 109 μmol·h·L−1. Bars=250 μm.
Arterial Wall Hypoxia In Vivo

References
