Enhanced Superoxide Production in Experimental Venous Bypass Graft Intimal Hyperplasia Role of NAD(P)H Oxidase

Nick E.J. West, Tomasz J. Guzik, Edward Black, Keith M. Channon

Abstract—Vein graft intimal hyperplasia, due to smooth muscle cell (SMC) proliferation, remains a limiting factor in long-term vein graft patency. Increased superoxide production regulates SMC mitogenesis and contributes to reduced NO bioactivity in systemic models of vascular disease. We compared superoxide production in experimental venous bypass grafts with ungrafted veins and determined its enzymatic sources and cellular localization. Vascular superoxide production was measured in vein grafts and control jugular veins obtained from normocholesterolemic rabbits undergoing jugular vein–carotid artery interposition bypass grafting. Surgical isolation of the contralateral jugular vein, without bypass grafting, provided an additional control for the effects of surgical manipulation. Superoxide production was increased 3-fold in vein grafts compared with control veins. Systematic stimulation and inhibition of specific oxidases revealed that the major source of increased vein graft superoxide production was a membrane-associated NAD(P)H-dependent oxidase. Western blotting of vascular homogenates demonstrated corresponding increases in NAD(P)H oxidase p22phox (membrane-associated) and p67phox (cytosolic) subunits in vein grafts compared with jugular veins. There was marked intimal hyperplasia in vein grafts, and immunohistochemical staining of vessel cryosections revealed increased p22phox-expressing cells in vein grafts that were predominantly intimal SMCs. Superoxide generation is increased in experimental vein grafts compared with ungrafted veins. The principal source of increased superoxide generation in vein grafts is an NAD(P)H oxidase, expressed by intimal SMCs. These findings suggest a role for NAD(P)H oxidase–mediated superoxide production in the proliferative response to vascular injury in vein grafts. (Arterioscler Thromb Vasc Biol. 2001;21:189-194.)

Key Words: atherosclerosis ▪ vein grafts ▪ superoxide ▪ vascular smooth muscles

Vein grafts remain the mainstay of surgical treatment for peripheral and coronary artery disease but are prone to failure caused by accelerated atherosclerosis and occlusion, occurring at rates of ≥50% at 10 years after implantation. Vein grafts are characterized by intimal hyperplasia (resulting from vascular smooth muscle cell migration and proliferation), which develops as a consequence of early graft injury and is critical in the subsequent development of accelerated atherosclerosis.

See page 175

Increasing evidence suggests that superoxide production plays important roles in cardiovascular physiology and in the pathogenesis of vascular disease. Superoxide regulates redox-sensitive signaling pathways and acts as a direct vascular smooth muscle cell mitogen. Furthermore, superoxide modulates vessel remodeling by the activation of matrix metalloproteinases and influences vascular smooth muscle cell migration and apoptosis. Superoxide also exerts important proinflammatory and proliferative effects by scavenging vascular NO and producing peroxynitrite, a prooxidant species that can nitrosylate cellular proteins and lipids.

Potential sources of vascular superoxide production include NAD(P)H-dependent oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases, and NO synthases. NAD(P)H oxidase, a multisubunit enzyme originally characterized in neutrophils, is present in vascular smooth muscle cells and endothelial cells (see review). NAD(P)H oxidases are an important source of superoxide production in animal models of hypercholesterolemia and hypertension and in human blood vessels from patients with systemic atherosclerotic risk factors. In contrast to these systemic vascular disease states, the role of superoxide production in regulating the local response to vascular injury, such as vein graft intimal hyperplasia, remains unclear.

We sought to investigate the sources of superoxide production in experimental venous bypass grafts in normocho-
lesterolomic normotensive animals, a model characterized by smooth muscle cell proliferation.\textsuperscript{18} We have found that superoxide production is increased in vein grafts, mediated by an NAD(P)H oxidase. Increased NAD(P)H oxidase activity and increased levels of protein subunits in vein grafts are localized to intimal vascular smooth muscle cells.

**Methods**

**Animals and Venous Bypass Graft Surgery**

Male New Zealand White rabbits (2 to 2.5 kg) were maintained on a normal diet of rabbit chow with water ad libitum. Anesthesia for surgery was induced with the use of midazolam (Hynnovel, 2 to 2.5 mg/kg body wt, Roche Pharmaceuticals) and all grafts were patent at the time of harvest. Experimental vein grafts, Surgically manipulated jugular veins, and native jugular veins were harvested 28 days after surgery. Animals were anesthetized, and vessels were excised as previously described.\textsuperscript{18} Fresh vessel segments were immediately washed and placed in ice-cold Krebs-HEPES buffer (mmol/L: NaCl 99, KCl 4.7, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.9, CaCl\textsubscript{2} 1.9, NaHCO\textsubscript{3} 25, glucose 11.1, and sodium HEPES 20) for transfer to the laboratory for superoxide assays, or snap-frozen in Optimal Cutting Temperature medium (Tissue-Tek, Sakura) for cryosections.

**Vessel Harvesting and Analysis**

Experimental vein grafts, surgically isolated jugular veins, and native jugular veins were harvested 28 days after surgery. Animals were anesthetized, and vessels were excised as previously described.\textsuperscript{18} Fresh vessel segments were immediately washed and placed in ice-cold Krebs-HEPES buffer (mmol/L: NaCl 99, KCl 4.7, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.9, CaCl\textsubscript{2} 1.9, NaHCO\textsubscript{3} 25, glucose 11.1, and sodium HEPES 20) for transfer to the laboratory for superoxide assays, or they were snap-frozen in liquid nitrogen for homogenization for superoxide assays and Western blotting. Other segments were snap-frozen in Optimal Cutting Temperature medium (Tissue-Tek, Sakura) for cryosections.

**Vascular Superoxide Production**

Superoxide production was measured by lucigenin-enhanced chemiluminescence with the use of previously described methods.\textsuperscript{11,17,19} Intact vessel segments were equilibrated in Krebs-HEPES buffer gassed with 95\% O\textsubscript{2}/5\% CO\textsubscript{2} for 30 minutes at 37°C. Lucigenin-enhanced chemiluminescence was measured in 2 mL Krebs-HEPES buffer containing lucigenin (5 \(\mu\)mol/L or 250 \(\mu\)mol/L)\textsuperscript{19} with use of a Berthold FB12 single-tube luminometer, modified to maintain a sample temperature of 37°C. Chemiluminescence was measured continuously for 10 minutes after allowing dark adaptation and was expressed as relative light units (RLU) per minute per milligram vessel dry weight. Some vessels underwent mechanical endothelial denudation by gentle rolling with forceps over the endothelial surface. In other experiments, vessels were homogenized on ice in HEPES buffer containing 1 mmol/L EDTA and protease inhibitors. Tissue debris was pelleted at 800 \(\times\)g for 10 minutes, and chemiluminescence was measured as described above, by adding portions of lysate to 2 mL Krebs-HEPES buffer containing lucigenin (250 \(\mu\)mol/L). In some experiments, vascular homogenates were separated into soluble (cytosolic) and particulate (membrane-associated) fractions by ultracentrifugation at 100 000 \(\times\)g for 45 minutes. Protein concentrations were determined by the Bradford assay. Chemiluminescence was expressed as RLU per minute per microgram protein. We validated this assay by performing parallel experiments with 5 \(\mu\)mol/L and 250 \(\mu\)mol/L lucigenin and by comparing these results together and independently with superoxide production assessed by ferricytochrome C reduction\textsuperscript{17} Further preliminary experiments determined the optimal weight of vessel segment or the protein concentration of homogenate for these assays.

**Western Immunoblotting**

Portions of vascular homogenate, equalized for protein content, were boiled in loading buffer containing 0.1 mol/L dithiothreitol, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Subunits of the NAD(P)H oxidase were detected by using mouse monoclonal antibodies against p22\textsuperscript{phox} (mAb 44.1, a generous gift of Dr J. Burritt, Montana State University, Bozeman) and p67\textsuperscript{phox} (Transduction Laboratories). Blots were scanned and analyzed by NIH Image version 1.62.

**Immunohistochemistry and Image Analysis**

Immunohistochemistry was performed by using mouse monoclonal antibodies in 6-\(\mu\)m vessel cryosections, as described.\textsuperscript{18} To investigate vascular smooth muscle cell phenotype/differentiation,\textsuperscript{20} the following were used: antibodies against smooth muscle \(\alpha\)-actin (Sigma), vimentin and desmin (both from Boehringer-Mannheim Biochemica), and smoothelin\textsuperscript{21} (Chemicon). Furthermore, staining was performed to identify the p22\textsuperscript{phox} subunit of the NAD(P)H oxidase (Dr J. Burritt, Montana State University, Bozeman), macrophages (rabbit RAM 11, Dako), and polymorphs (rabbit CD18, Serotec). Immune complexes were visualized by avidin-biotin complex formation and Vector red staining (Vector Laboratories). Intimal/mediastinal thickness (micrometers) and cell counts (number per micrometer squared) were assessed at 4 points around the circumference of vessel sections, with at least 2 sections per vessel used for analysis. Image analysis was performed with a Leica DRMBE microscope, Pixera PVC-100C image capture hardware, and Pixera Pro/Coriel Photo-Paint 8 software.

**Oxidative Fluorescent Microtopography**

In situ superoxide generation in vessel sections was evaluated by use of the oxidative fluorescent dye dihydroethidium. Tissue cryosections (30-\(\mu\)m thickness) were incubated with dihydroethidium, and fluorescence images were obtained with use of a Bio-Rad MRC 1024 scanning confocal microscope, as previously described.\textsuperscript{22} Images were captured by using identical microscope settings for all jugular vein and vein graft specimens.

**Statistical Analysis**

Data are expressed as mean±SEM. In all cases, \(n\) refers to numbers of animals. Statistical significance of differences was assessed by Student \(t\) tests, and a value of \(P<0.05\) was considered statistically significant.

**Results**

A total of 40 vessels from 28 animals were studied (17 native jugular veins, 6 surgically manipulated jugular veins, and 17 experimental vein grafts). All animals survived to 28 days, and all grafts were patent at the time of harvest.

**Superoxide Generation From Jugular Veins and Vein Grafts**

Basal superoxide production was determined by lucigenin-enhanced chemiluminescence from intact vessel rings (Figure 1). Specificity of the assay for superoxide was demonstrated by coinubcation with superoxide dismutase (SOD, 350 U/mL). Surgical manipulation of the jugular veins did not alter superoxide generation compared with that in native jugular veins. In contrast, superoxide production was significantly increased in vein grafts compared with jugular veins from the same animals (19.9±3.3 versus 8.4±2.3 RLU · \(s^{-1} \cdot mg^{-1}\), respectively, \(P<0.01; n=11\). Removal of endothelium from intact vein graft rings did not significantly alter superoxide production.
Sources of Superoxide Generation in Jugular Veins and Vein Grafts

To investigate the sources of superoxide production in experimental vein grafts, we determined superoxide production from intact vessel rings in response to a range of inhibitors of specific oxidase enzyme systems and in vascular homogenates in response to substrates for specific oxidases. Superoxide production from intact vessels was markedly reduced by diphenylene iodonium (DPI, 100 μmol/L), an inhibitor of flavin-containing oxidase enzymes. Incubation with N-methyl-L-arginine (1 mmol/L) resulted in a modest inhibition of superoxide generation, whereas oxypurinol, rotenone, and indomethacin did not alter superoxide production (Figure 2). Correspondingly, when substrates for specific oxidases were added to vascular homogenates, the greatest stimulation of superoxide production was generated by NADH and, to a lesser extent, by NADPH (Figure 3). Compared with jugular veins, vein grafts from the same animals showed a 3-fold increase in NADH-stimulated superoxide production (2.40 ± 0.5 versus 6.38 ± 0.8 RLU · s⁻¹ · mg⁻¹, respectively, *P < 0.005; n = 11). Similar results were obtained when identical experiments were performed with a lower concentration of lucigenin (5 μmol/L), although under these conditions, NADPH rather than NADH appeared to be the preferred substrate, perhaps because of the favorable artifactual interaction between lucigenin at this concentration and NADH⁵ (Figure I, which can be accessed online at http://atvb.ahajournals.org). Superoxide generation stimulated by NADH was inhibited by DPI in a manner similar to that observed under basal conditions (190 ± 3.5 [NADH] versus 33.0 ± 2.06 [NADH + DPI] RLU · s⁻¹ · mg⁻¹, *P < 0.005; n = 6). Other oxidase substrates produced either no increase in superoxide generation (xanthine and succinate) or only a modest increment (arachidonic acid), with no difference between vein grafts and jugular veins.

Next, we determined the specific activity of NADH-stimulated superoxide production in subcellular fractions of homogenates prepared from experimental vein grafts and native jugular veins by ultracentrifugation into soluble (cytosolic) and particulate (membrane) fractions; almost all of the NAD(P)H oxidase activity was localized to the particulate fraction (1.27 ± 0.73 [basal cytosolic] versus 89.0 ± 25.4 [membrane] RLU · s⁻¹ · μg⁻¹ and 3.85 ± 0.58 [cytosolic + NADH] versus 466 ± 108 [membrane + NADH] RLU · s⁻¹ · μg⁻¹, P < 0.05 in each case; n = 3).

Taken together, the stimulation of superoxide generation by NADH and NADPH, inhibition by DPI, and localization to the cell membrane suggest that an NAD(P)H oxidase accounts for increased superoxide production in experimental vein grafts.

Presence of NAD(P)H Oxidase Components in Vein Grafts

We sought to detect protein subunits of the NAD(P)H oxidase enzyme by Western blotting of vascular homogenates (Figure 4). Relative to the α-actin internal control, p22phox (membrane-bound) and p67phox (cytosolic) subunits were present in increased amounts in vein grafts compared with jugular veins when blot intensities were assessed by computer-assisted analysis, in similar proportion (3-fold increase) to the observed difference in superoxide production between the vessel types (band intensities were as follows: for p22phox, 67.11 ± 29.0 [jugular vein] versus 179 ± 30.3
[vein graft] arbitrary units, \( P < 0.05 \); for p67phox, 27.4±10.2 [jugular vein] versus 135±16.2 [vein graft] arbitrary units, \( P < 0.005 \); n=4 jugular veins and 5 vein grafts). Furthermore, superoxide generation from vascular homogenates was directly correlated with the relative amounts of p22phox or p67phox protein present in individual vessels (n=9 vessels; for p22phox, \( r^2 = 0.50, P < 0.05 \); for p67phox, \( r^2 = 0.78, P < 0.005 \)), suggesting that the increase in superoxide generation in vein grafts is associated with a corresponding increase in the NAD(P)H oxidase enzyme protein subunits.

**Cell Types Associated With NAD(P)H Oxidase Activity**

We used immunohistochemistry with computerized image analysis to investigate the number, location, and types of cells expressing the NAD(P)H oxidase p22phox subunit in jugular veins and vein grafts. Mature vein grafts showed marked intimal hyperplasia, with positive staining for smooth muscle \( \alpha \)-actin (Figure 5; intimal thickness 3.0±0.8 [jugular vein] versus 37±1.8 [vein graft] \( \mu m, P < 0.005 \)). There was also a modest increase in medial thickness in vein grafts, but the intima-to-media ratio in these vessels was nevertheless greatly increased (intima-to-media ratio 0.6±0.6 [jugular vein] versus 3.9±0.3 [vein graft], \( P < 0.0005 \)). Cells staining positively for p22phox were present in greatly increased numbers in vein grafts (127±9 [jugular vein] versus 364±38 [vein graft] cells/mm\(^2\), \( P < 0.005 \)). These cells were localized predominantly within the vein graft intima and media, were stained positively for smooth muscle \( \alpha \)-actin, and were particularly prominent in the superficial (subendothelial) layer of the intima (Figure 5). We also immunostained for smoothelin, a cytoskeletal marker of fully differentiated (contractile) smooth muscle cells, to investigate the phenotype of the medial and intimal smooth muscle cells in jugular veins and vein grafts. In jugular veins, smoothelin immunostaining was colocalized with medial \( \alpha \)-actin staining. However, in vein grafts, the media and the deeper layers of the hyperplastic intima stained for smoothelin, whereas the more superficial \( \alpha \)-actin–positive smooth muscle cells in the intima did not stain for smoothelin, suggesting that these smooth muscle cells are of a less differentiated (noncontractile) phenotype (Figure 5). Staining for other cytoskeletal markers of cellular differentiation confirmed that vimentin stained almost all cells, including the endothelium, whereas desmin,

whose expression indicates increasing smooth muscle cell differentiation, showed a staining pattern similar to that seen with smoothelin. These findings are summarized in Table I (which can be accessed online at http://atvb.ahajournals.org).

In situ production of superoxide, visualized by oxidative fluorescent microtopography with the use of dihydroethidium (DHE), revealed superoxide production in cells in the vein graft intima and in the media, corresponding to areas of p22phox immunostaining (Figure II, which can be accessed online at http://atvb.ahajournals.org). In contrast, DHE staining in jugular veins was very weak and hardly visible when viewed with the same confocal scanning parameters. The specificity of DHE staining in vein grafts for superoxide production was confirmed by a striking reduction in signal after preincubation of vessel sections with either SOD or SOD coupled with polyethylene glycol. Furthermore, preincubation of sections with DPI to inhibit NAD(P)H oxidase activity also markedly attenuated DHE staining (Figure II; see above). Because inflammatory cells are a source of abundant NAD(P)H oxidase, we sought to identify macrophages and polymorphs in jugular veins and vein grafts by immunostaining with RAM 11 and CD18 antibodies, respectively. There were no differences between veins and vein grafts in RAM 11 staining (12±12 [jugular vein] versus 20±6 [vein graft] cells/mm\(^2\), \( P = NS \)) or in CD18 staining...
(31±15 [jugular vein] versus 64±17 [vein graft] cells/mm², P=NS). These observations suggest that the cells mediating increased expression and activity of NAD(P)H oxidase in vein grafts are principally smooth muscle cells in the media and intima, in particular, a population of undifferentiated smooth muscle cells in the superficial (subendothelial) region of the intima.

Discussion
We have used the rabbit jugular-carotid interposition bypass graft to investigate vascular superoxide production in vein grafts, a model of vascular injury characterized by smooth muscle cell intimal hyperplasia. Superoxide production is 3-fold higher in vein grafts compared with ungrafted jugular veins, and this increase is mediated by NAD(P)H oxidase. Furthermore, undifferentiated intimal smooth muscle cells expressing the p22phox subunit of NAD(P)H oxidase appear to be an important source of increased vein graft superoxide production.

Increased superoxide production is a feature of systemic vascular disease states in animal models and humans and may promote atherogenesis by reducing NO bioactivity. Recent evidence also suggests a direct role for superoxide production, specifically, NAD(P)H oxidase activity, in regulating vascular smooth muscle cell proliferation. NAD(P)H oxidase mediates thrombin-stimulated smooth muscle cell mitogenesis, and overexpression of NAD(P)H oxidase homologues leads to a transformed phenotype. Our data now identify locally increased NAD(P)H oxidase activity and protein levels in an in vivo model of vascular smooth muscle cell proliferation, without additional systemic factors such as hypercholesterolemia or angiotensin II–induced hypertension. These observations suggest that increased NAD(P)H oxidase–mediated superoxide production may be an important aspect of the local proliferative response to vascular injury and not just a systemic feature of atherosclerotic vascular disease states. In particular, we find that undifferentiated (smoothelin-negative) smooth muscle cells are associated with NAD(P)H oxidase expression and activity in vein grafts. This suggests that superoxide production from NAD(P)H oxidase may be an important modulator of smooth muscle cell phenotype in models of vascular injury. NAD(P)H oxidase has been previously identified in cultured vascular smooth muscle and endothelial cells. The enzyme is a multisubunit complex with 2 membrane-associated subunits (p22phox and gp91phox) and 3 cytosolic subunits (p40phox, p47phox, and p67phox), regulated by Rac G proteins. The p22phox membrane–associated subunit is absolutely required for enzymatic activity, and its expression is increased by angiotensin II in vivo and in vitro. The p67phox cytosolic subunit is also regulated by angiotensin II, and its immunodepletion in fibroblasts leads to a decrease in superoxide production. We demonstrated increased levels of both these subunits in vein grafts, in proportion to the increase in superoxide generation, suggesting that both of these subunits are directly involved in NAD(P)H oxidase enzyme activity. However, novel homologues of the gp91phox subunit, such as Mox-1, have recently been described in a variety of cell subtypes, including vascular smooth muscle cells, and other studies in knockout mice suggest redundancy among the NAD(P)H oxidase subunits. Future work is required to assess the importance of homologous NAD(P)H oxidase subunits in smooth muscle cell superoxide production in vivo and to identify how expression and activity of the NAD(P)H oxidase components are regulated in response to vascular injury.

Our findings provide potential mechanisms to explain previous observations in experimental vein grafts. Antioxidants or free-radical scavengers reduce vein graft intimal hyperplasia, suggesting that superoxide is an important mediator of this process. Furthermore, the inhibition of ACE or the blockade of angiotensin II (type I) receptors also reduces intimal hyperplasia in vein grafts. Because NAD(P)H oxidase activity and p22phox expression are regulated by angiotensin II, our findings raise the possibility that NAD(P)H oxidase–dependent superoxide production may mediate the proliferative effects of angiotensin II in vein graft intimal hyperplasia and in other vascular injury states.

In addition to providing insights into the mechanisms of intimal hyperplasia in vivo, our observations in experimental vein grafts have direct clinical relevance. Vein graft disease continues to limit the clinical success of peripheral and coronary artery bypass graft surgery. Our findings suggest that strategies aimed at reducing superoxide production or blocking the upregulation of NAD(P)H oxidase may have therapeutic potential in reducing vein graft intimal hyperplasia and may provide a rationale for further clinical studies using antioxidants or agents that modulate angiotensin II in patients undergoing venous bypass graft surgery.

In conclusion, we have identified the NAD(P)H oxidase enzyme as the major source of increased superoxide production in experimental vein graft intimal hyperplasia in normo-cholesterolemic animals. Furthermore, NAD(P)H oxidase activity and protein subunits are localized to undifferentiated intimal smooth muscle cells, suggesting a role for NAD(P)H oxidase–mediated superoxide signaling in the proliferative response to vascular injury in vivo.

Acknowledgments
This work was supported by the British Heart Foundation (PG 98040). N.E.J.W. is a British Heart Foundation Junior Research Fellow. T.J.G. is a Crescendium Est Polonia Foundation International Fellow.

References
7. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the...


Enhanced Superoxide Production in Experimental Venous Bypass Graft Intimal Hyperplasia: Role of NAD(P)H Oxidase
Nick E. J. West, Tomasz J. Guzik, Edward Black and Keith M. Channon

doi: 10.1161/01.ATV.21.2.189
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/21/2/189

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2001/01/18/21.2.189.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Figure I. Comparison of NADH/NADPH-stimulated superoxide production determined from vascular homogenates using 250 µM or 5 µM lucigenin. Superoxide generation was maximal when samples were stimulated with NADH at high concentrations of lucigenin or NADPH at lower concentrations. Superoxide production correlated well between lucigenin concentrations, except at basal levels, where generated signals were very low (basal $r^2=0.41$, $p=0.065$; NADH $r^2=0.59$, $p<0.05$; NADPH $r^2=0.76$, $p<0.005$; xanthine $r^2=0.74$, $p<0.005$). Note differing scales on left and right axes, reflecting differing magnitudes of signal obtained.
**Figure II.** Dihydroethidium staining of vein grafts to detect in situ superoxide generation.

Tissue cryosections of vein graft were incubated with dihydroethidium at 37°C, either alone ('VG'), in the presence of 100 µmol/L diphenylene iodonium ('VG +DPI'), 300 U/ml superoxide dismutase ('VG +SOD') or 300 U/ml superoxide dismutase conjugated with polyethylene glycol ('VG +PEG-SOD'). Sections were then imaged by scanning confocal microscopy using identical scanning parameters in each case. The luminal surface is indicated by white arrows.
<table>
<thead>
<tr>
<th></th>
<th>JV</th>
<th>VG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Media</td>
<td>Media</td>
</tr>
<tr>
<td></td>
<td>Deeper layers</td>
<td>Subendothelial layers</td>
</tr>
<tr>
<td>Alpha-actin</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Vimentin</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Smoothelin</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Desmin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p22phox</td>
<td>-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Table I. Immunostaining for markers of smooth muscle cell differentiation.

All intimal smooth muscle cells stained positive for alpha-actin and vimentin.

Smoothelin and desmin, indicating enhanced differentiation, stained only cells in the deeper intima, in contrast to the p22phox positive staining of the subendothelial intimal smooth muscle cells. +++ indicates staining of all cells, ++ most cells, + some cells and +/- occasional cells. – indicates no staining.