Endothelin Receptor Antagonism Ameliorates Mast Cell Infiltration, Vascular Hypertrophy, and Epidermal Growth Factor Expression in Experimental Diabetes

Richard E. Gilbert, Jonathan R. Rumble, Zemin Cao, Alison J. Cox, Pauline van Eeden, Terri J. Allen, Darren J. Kelly, Mark E. Cooper

Abstract—Vascular hypertrophy, a feature of experimental and human diabetes, has been implicated in the pathogenesis of the microvascular and macrovascular complications of the disease. In the present study, we sought to examine the role of endogenous endothelin and its relation to vascular growth factors in the mediation of vascular hypertrophy in experimental diabetes and to examine the contribution of mast cells to this process. Vessel morphology, endothelin, growth factor gene expression, and matrix deposition were studied in the mesenteric arteries of control and streptozotocin-induced diabetic Sprague-Dawley rats treated with or without the dual endothelin-1 receptor antagonist bosentan (100 mg \( \cdot \) kg\(^{-1} \cdot \) d\(^{-1} \)) during a 3-week period. Compared with control animals, diabetic animals had significant increases in vessel weight, wall-to-lumen ratio, mast cell infiltration, extracellular matrix deposition, and gene expression of epidermal growth factor (EGF) and transforming growth factor-\( \beta_1 \). In diabetic, but not control, vessels, not only were EGF mRNA and endothelin present in endothelial cells, but also their expression was observed in adventitial mast cells. Immunoreactive endothelin was present in the media of mesenteric vessels of diabetic, but not control, animals. Bosentan treatment significantly reduced mesenteric weight, wall-to-lumen ratio, mast cell infiltration, extracellular matrix deposition, and EGF mRNA but did not prevent the overexpression of transforming growth factor-\( \beta_1 \) mRNA in diabetic rats. These findings suggest that endogenous endothelin and EGF may play a role in diabetes-induced vascular hypertrophy and that mast cells may be pathogenetically involved in this process. (Circ Res. 2000;86:158-165.)

Key Words: endothelin • mast cell • growth factor • vasculature • diabetes

Diabetes remains a major cause of vascular disease not only because of its microvascular complications but also because of its association with a more accelerated and diffuse form of atherosclerosis. Vascular hypertrophy, a characteristic feature of human and experimental diabetes, has been implicated in the pathogenesis of the vascular complications of this disease. The superior mesenteric vascular tree, which is a site of extensive study in other vascular disease states, such as hypertension and atherosclerosis, exhibits vascular hypertrophy and endothelial dysfunction with experimental diabetes. In these vessels, there is evidence of extracellular matrix (ECM) expansion, intimal proliferation, and media enlargement.

The role of locally active growth factors in the mediation of vascular remodeling has been the subject of intensive research. The results of recent studies suggest a significant interaction between transforming growth factor-\( \beta \) (TGF-\( \beta \)) and receptor tyrosine kinase (RTK)-activating growth factors in the mediation of the hypertrophic process in vascular tissue. Indeed, a combination of such growth factors may also be involved with the hypertrophic process at other sites, such as in diabetes-associated renal enlargement, for which both TGF-\( \beta \) and the RTK-activating growth factor epidermal growth factor (EGF) have been implicated. Although these growth factors may originate from resident cells, they may also derive from growth factor–rich infiltrating cells, such as the mast cell, which was recently implicated in angiogenesis and atherosclerosis.

Previous studies have indicated that although vascular hypertrophy in diabetes may involve glucose-dependent mechanisms, glucose-independent factors such as angiotensin II, bradykinin, and nitric oxide may also be important in its pathogenesis. However, the role of the vasoactive hormone endothelin (ET), which was recently implicated in angiogenesis and mediated vascular hypertrophy, has not been examined in the diabetic context.

In the present study, we sought to determine whether endogenous ET and its interactions with growth factors may mediate the trophic vascular changes in experimental diabetes. In addition, we sought to examine the role of mast cells as a potential source of growth factors that may contribute to this hypertrophic process.
Materials and Methods

Animals
Eighty male Sprague-Dawley rats (age 8 weeks, weight 200 to 250 g) were randomized to receive streptozotocin (STZ) at a dose of 45 mg/kg (diabetic) or to receive citrate buffer alone (control). Animals were then further randomized to either no treatment or the ET\_A antagonist bosentan (Hoffman-La Roche) at a dose of 100 mg/kg via daily gavage. Animals were sacrificed at 3 weeks after STZ injection. In each group of 20 animals (control, diabetic, bosentan-treated control, and bosentan-treated diabetic rats), 12 were used for histological studies and 8 were used for Northern blot analysis. In the latter group, animals were sacrificed through decapitation, and the superior mesenteric arterial tree was isolated as previously described.\(^{14}\)

Northern Blot Analysis
Mesenteric arteries stored at \(-80^\circ\)C were homogenized (Ultra-Turrax, Janke and Kunkel), and total RNA was isolated according to the acid guanidinium thiocyanate–phenol–chloroform extraction method.\(^{16}\) RNAs extracted from each of 8 animals per group were linearized, and antisense riboprobes were generated. In situ hybridization was performed with use of the indirect avidin-biotin complex method.\(^{17}\) The cDNAs coding for TGF-\(\beta\), rat TGF-\(\beta\)-smooth muscle actin–immunostained sections, and a PCR product for prepro-ET-1 were cloned into pBluescript KS\(^+\) (Stratagene). The cDNA coding for murine prepro-EGF (gift of Dr P Fuller, Melbourne, Australia) and rat TGF-\(\beta\)-\(\beta\) (gift of Dr Qian, NIH, Bethesda, Md).

In Situ Hybridization
The cDNAs coding for TGF-\(\beta\) and a PCR product for prepro-ET-1 were cloned into pBluescript KS\(^+\) (Stratagene). The cDNA coding for EGF was cloned into pGEM 3Z (Promega). cDNAs were then linearized, and antisense riboprobes were generated. In situ hybridization was performed as previously described.\(^{17}\)

Histochemistry and Immunohistochemistry
Histological studies of vascular architecture were performed in a subset of animals (12 per group) as previously described.\(^{7}\) Sections were stained with either hematoxylin and eosin or Mason’s trichrome\(^{18}\) for examination of the ECM. Mast cells were visualized as previously described.\(^{14}\) Sections were stained with either hematoxylin and eosin or Masson’s trichrome18 for examination of the ECM. Mast cells were visualized with use of both 0.1% toluidine blue (Sigma Chemical Co) \(^{19}\) and immunostaining for tryptase.\(^{15}\) Immunostaining for ET and smooth muscle cells was also performed with a polyclonal rabbit and anti-human ET antibody (AMRAD Biomedical) or a polyclonal \(\alpha\)-smooth muscle actin antibody (Biogenes). Immunohistochemistry was performed with use of the indirect avidin-biotin complex method as previously described.\(^{5}\)

Quantification of Histopathology
The proportion of vessel wall occupied by the media was assessed through the use of \(\alpha\)-smooth muscle actin–immunostained sections, and the ratio of the wall to the lumen was quantified through the use of a video-imaging system as previously described.\(^{20,21}\) Quantification of ECM matrix was performed on trichrome-stained sections with the use of a semiquantitative scale (adapted from O’Brien et al\(^{22}\)) and through calculation of the proportion of area occupied by matrix, vessel wall media, and immunoreactive ET with computer-assisted image analysis as previously described.\(^{23,24}\) Mast cells were quantified in toluidine blue–stained sections (6 per group), in which the number of positively stained cells were counted by an observer who was masked to the study group of origin.

Statistics
All values are given as mean ± SEM unless otherwise specified. Data were analyzed with ANOVA. Comparisons between group mean values were performed with Fisher’s least significant difference method. Semiquantitative data derived from the histological scoring of trichrome-stained sections and mast cell numbers were analyzed with use of the Kruskal-Wallis test. \(P<0.05\) was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Animal Data
All animals that were injected with STZ developed diabetes as defined as a plasma glucose level of >15 mmol/L. Diabetes was associated with reduced weight gain that was not influenced by bosentan treatment (Table 1). Plasma glucose levels were similar in diabetic animals that did and did not receive bosentan (Table 1). Systolic blood pressure

![Figure 1](http://circres.ahajournals.org)  
Figure 1. Mesenteric vessel weight (top) and wall/lumen area ratio (bottom). Data are expressed as mean ± SEM. *\(P<0.01\) vs control. †\(P<0.01\) vs diabetes.
was similar in control and diabetic rats that did and did not receive bosentan (Table 1). Food intake was increased in diabetic animals compared with control animals but was not influenced by bosentan treatment (Table 1). Mesenteric vessel weight was greater in untreated diabetic rats than in control animals and was attenuated by bosentan (Figure 1).

**Histology and Immunohistochemistry**

Histomorphometric analysis revealed a significant increase in the media wall/lumen ratio (Figure 1) in diabetic rats compared with control animals. Treatment with bosentan significantly reduced the media wall/lumen ratio. Trichrome-stained sections demonstrated expansion of collagenous ECM in mesenteric vessels of diabetic animals compared with that of control animals (Figure 2). With the use of both semiquantitative scoring and computerized image analysis, more ECM was observed in vessels from diabetic than in vessels from control rats (Table 2). Bosentan treatment significantly reduced the extent of ECM expansion in vessels from diabetic rats to levels observed in control rats (Table 2).

Numerous toluidine blue–stained and tryptase-positive mast cells were present in the adventitia of diabetic rat vessels (Figure 3). In contrast, few mast cells were detected in control or bosentan-treated diabetic animals (median mast cells/section [range]: control 1 [0–3], diabetic 10 [4–17], and bosentan-treated diabetic 3 [0–11]; $P<0.01$ control versus diabetic, $P<0.05$ diabetic versus bosentan-treated diabetic).

In control animals, ET was detected only in the vessel intima. In contrast, abundant immunoreactive ET was noted in vascular smooth muscle cells of the media of both diabetic and bosentan-treated diabetic animals (Figure 4). When corrected for wall area, there was no difference in the extent

![Figure 2. Representative photomicrographs of mesenteric arteries from control (A), bosentan-treated control (B), diabetic (C), and bosentan-treated diabetic (D) rats (Masson’s trichrome stain, magnification ×240).](image)

**TABLE 2. Extracellular Matrix in Mesenteric Vessels Assessed by Semiquantitative Scoring and Image Analysis**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + Bosentan</th>
<th>Diabetes</th>
<th>Diabetes + Bosentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix (score)</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>2.5 ± 0.2*</td>
<td>1.4 ± 0.2†</td>
</tr>
<tr>
<td>Matrix (% area)</td>
<td>8.8 ± 3.2</td>
<td>13.5 ± 3.0</td>
<td>39.1 ± 3.2*</td>
<td>24.2 ± 5.1‡</td>
</tr>
</tbody>
</table>

$n=6$ for all groups.

* $P<0.01$ vs control.

† $P<0.01$ vs diabetes.

‡ $P<0.05$ vs diabetes.
of vessel ET between diabetic and bosentan-treated diabetic rats. In diabetic rats, immunoreactive ET was also detected in mast cells but not in mast cells from control or bosentan-treated animals (Figure 4).

Northern Blot Analysis
EGF increased 2- to 3-fold in mesenteric vessels of diabetic rats and was significantly reduced in diabetic animals treated with bosentan (Figures 5 and 6). TGF-β1 mRNA was also overexpressed in diabetic animals but was unchanged by bosentan treatment (Figures 5 and 6). No effect on 18S rRNA expression was noted in the vessels of either bosentan-treated or untreated diabetic rats.

In Situ Hybridization
Emulsion-dipped, hematoxylin and eosin–counterstained sections of vessels from diabetic rats revealed abundant EGF mRNA in the intima, as well as in the adventitia, where transcript was present in a granular pattern within large, toluidine blue–staining mast cells (Figures 3B and 7). Both intimal and mast cell EGF mRNA was less abundant in bosentan-treated diabetic rats. TGF-β1 mRNA was present in the adventitia and intima of mesenteric vessels from diabetic animals and was unchanged by bosentan treatment (data not shown). Only sparse hybridization for TGF-β and EGF mRNA was noted in untreated control and bosentan-treated control animals.

ET-1 mRNA was detected in the endothelial cells of mesenteric arteries and was particularly abundant in the vessels from untreated animals with only minimal transcript detected in the vessel media (Figure 8).

No hybridization was observed in sections incubated with sense riboprobes for TGF-β1, EGF, or ET-1.

Discussion
The present study demonstrates several findings in relation to mesenteric vascular hypertrophy in experimental diabetes. First, not only was vascular hypertrophy in diabetic animals associated with increased vessel wall ET, but also its amelioration was demonstrated through the administration of the ETA/B receptor antagonist bosentan, suggesting a role for endogenous ET in this pathological process. Second, both EGF and ET were expressed by mast cells in addition to cells of the vessel wall, implicating infiltrating as well as resident cells in the development of diabetes-associated vascular hypertrophy. Third, although diabetes was associated with the overexpression of both TGF-β and EGF, blockade of the ET receptors reduced only EGF mRNA, suggesting that the normalization of all cytokines may not be necessary to achieve a reduction in vascular hypertrophy.

In the present study, experimental diabetes was associated with increased weight and an increase in media/lumen ratio in mesenteric arteries. Although it might be postulated that such hypertrophy reflects diabetes-induced hyperphagia, bosentan decreased mesenteric vascular hypertrophy without influencing food intake or glycemic control. Furthermore, previous studies have shown that both ACE inhibition and the inhibitor of advanced glycation, aminoguanidine, also attenuate mesenteric vascular hypertrophy without reducing food intake, indicating that attributes of the diabetic state other than food intake underlie the pathogenesis of hypertrophy.

Several growth factors have been implicated in the hypertrophy of vascular and other tissues. In diabetes, previous studies have documented significant increases in the expres-
sion of TGF-β and EGF in association with renal hypertrophy. As in the kidney, functioning receptors for TGF-β, EGFR and ET (ET A and ET B) have all been documented in vascular tissue. EGF is a potent pro-proliferative growth factor for a wide variety of cell types, inducing activation of the cell cycle and increasing protein synthesis. Its mitogenic action is curtailed in the presence of TGF-β, which through the prevention of DNA synthesis and cell division, blocks cell cycle progression at G1/S transition and converts EGF-induced hyperplasia into hypertrophy. In contrast to their differing effects on cell cycling, the combination of EGF and TGF-β may be synergistic in stimulation of ECM synthesis in vascular smooth muscle cells. Indeed, in the diabetic vessels, matrix expansion was associated with the overexpression of both growth factors. However, in the present study, ET receptor blockade reduced EGF, but not TGF-β, gene expression, yet bosentan still reduced diabetes-associated vascular hypertrophy. These findings suggest that the normalization of all cytokines may not be mandatory for a therapeutic intervention to achieve an effect on vessel structure.
The cell-specific origins of growth factors within the vessel represent an important aspect of vascular biology. EGF has been shown to be expressed by vascular endothelial cells, and in the present study, its expression at this site was increased in association with diabetes. In addition to its presence in the intima, EGF transcript was found in abundance in mast cells in vessels from diabetic rats. Although mast cells are traditionally known for their role in allergic IgE-mediated reactions, there also is a non–immune-related mast cell phenotype that predominates in connective tissue rather than at mucosal surfaces. These nonimmune mast cells participate in cell migration, differentiation, and the dissolution and synthesis of ECM. Consistent with these actions, mast cells are associated with angiogenesis, atherosclerosis, and tissue fibrosis. In addition to heparin and tryptase, mast cells also are a rich source of various growth factors, including basic fibroblast growth factor and vascular endothelial growth factor. The present report identifies mast cell infiltration in association with diabetic vascular hypertrophy and shows that in addition to ET, mast cells expressed abundant EGF mRNA, thereby extending the repertoire of cytokine production of these cells and further implicating them in the mediation of tissue injury.

In the present study, immunoreactive ET was detected in both the endothelium and media of diabetic vessels. Indeed, recent in vivo studies by other groups have also noted the presence of immunoreactive ET in the media in various pathological settings, including coronary artery disease, hypertension, and transplant coronary artery disease. This contrasts normal vessels where ET is restricted to the vascular endothelium. However, in the present study, although ET peptide was detected in smooth muscle cells of vessels from diabetic rats, mRNA for prepro-ET was confined to the endothelial cell layer. These findings suggest that the presence of immunoreactive ET within medial smooth muscle cells may have arisen as a consequence of the well described abluminal secretion of ET by vascular endothelial cells.

In addition to the observation of increased vessel wall ET, a pharmacological approach that involves the blockade of ET receptors was used to demonstrate the role of ET in vascular hypertrophy in diabetes. The amelioration of the pathological changes in this model with ET receptor antagonism suggests a role for ET in this process, similar to that described in other experimental settings, including norepinephrine-induced ventricular hypertrophy, vascular hypertrophy with deoxycorticosterone acetate-salt hypertension, and angiotensin II infusion.

The relationship between ET and the cytokines TGF-β and EGF is complex. ET-1 stimulates TGF-β synthesis, and in turn TGF-β may augment ET expression as part of a positive feedback cycle. In the present study, diabetes was associated with increased TGF-β mRNA in mesenteric arteries. However, this overexpression was not reduced by the blockade of ET receptors. These findings suggest that factors apart from ET may underlie the overexpression of TGF-β in the diabetic state. Indeed, several other mechanisms have been implicated in the upregulation of TGF-β in association with diabetes, including glucose-mediated overproduction of protein kinase C, advanced glycation end-products, activation of the renin-angiotensin system, and cell stretch associated with hyperperfusion. For EGF, the nature of the interaction with ET is less well understood. In previous experiments, ET-1 has been shown to potentiate the mitogenic action of EGF in smooth muscle cell culture, whereas EGF has been found to inhibit ET release. In the present study, ET receptor blockade diminished vascular EGF expression, sug-
suggesting that ET may act as a stimulus for EGF expression in the diabetic milieu.

In summary, vascular hypertrophy in diabetes may be mediated in part via increased and aberrant tissue expression of the vasoactive peptide ET and the RTK-activating growth factor EGF. The findings that the inhibition of ET action ameliorated EGF expression, mast cell infiltration, and the structural changes associated with diabetes suggest that this class of agent may offer a new therapeutic approach to the treatment of vascular disease in diabetes.

Acknowledgments

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Methods

Animals

Eighty male Sprague Dawley rats aged 8 weeks, weighing between 200 and 250 grams were randomized to receive streptozotocin (STZ) at a dose of 45mg/kg (diabetic) or citrate buffer alone (control). Animals were then further randomized to receive either no treatment or the \( \text{ET}_{A/B} \) antagonist bosentan (Hofmann-LaRoche, Basel, Switzerland) at a dose of 100 mg/kg by daily gavage. This yielded four groups, each of twenty animals: control, diabetic, bosentan-treated controls and bosentan-treated diabetic. Rats were given \textit{ad libitum} access to water and standard chow containing 20% protein (Clark, King & Co, Melbourne, Australia). Only STZ-treated animals with plasma glucose levels > 15 mmol/l were considered diabetic and included in the study. Animals were sacrificed at 3 weeks following STZ injection. Immediately prior to sacrifice, rats were weighed and systolic blood pressure was determined by tail cuff plethysmography [16]. In each group of 20 animals, 12 were used for histological studies and eight were used for Northern analysis. In the latter group, animals were sacrificed by decapitation after which the mesenteric vessels were removed and stripped of surrounding fat, connective tissue and veins to yield the superior mesenteric arterial tree as previously described [14]. The vessels were weighed, snap frozen in liquid nitrogen and subsequently stored at -80°C. Blood collected for determination of plasma glucose by the glucose oxidase technique in all animals [17].
Northern Analysis

Mesenteric arteries stored at -80°C were homogenized (Ultra-Turrax, Janke and Kunkel, Staufen, Germany) and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method [18]. RNA extracted from each animal was run as separate lanes in all experiments and Northern analysis performed as previously described [5]. In brief, RNA containing nylon filters were hybridized with a 400 base pair Eco RI/Hind III cDNA coding for murine preproEGF (gift of Dr P Fuller, Melbourne, Australia) and a 985 bp cDNA probe coding for rat TGF-β1 (gift of Dr Qian, NIH, Bethesda, MD). Intensity of hybridization was quantified using a phosphorescent imager (Fujix BAS-3000, Fuji Photo Film Co., Ltd, Japan) and all results were corrected for differences in RNA loading and transfer by rehybridization with an oligonucleotide probe for 18S rRNA. Results were expressed as the ratio of image intensity of TGF-β1 or EGF to 18S relative to control vessels which were arbitrarily assigned a value of 1.

In situ hybridization

The cDNA coding for TGF-β1 was cloned into pBluescript KS+ (Stratagene, LaJolla, CA), linearized with XbaI and the cDNA coding for EGF was cloned into pGEM 3Z (Promega, Madison, WI, USA) and linearized with Hind III. In situ hybridization was performed using a cDNA encoding rat perproendothelin-1 obtained by PCR using the following oligonucleotides: sense, 5′-CCGCCCCTGCTATGGAATCTC-3′ and anti-sense, 5′-GATGCCAGTGCTGCTACCAA-3′. PCR products were cloned into pBluescript KS+ (Stratagene), then sequenced and confirmed as identical to that previously reported [19]. The cDNA was then linearized with Hind III and an anti-sense
riboprobe was then generated using T3 RNA polymerase. Anti-sense riboprobes for both TGF-β1 and EGF were generated using T7 RNA polymerase and in situ hybridization was performed as previously described [20]. was performed as previously described [5]. After hybridization, slides were washed, incubated with RNase A (150 μg/ml), dehydrated in graded ethanol, air dried and exposed to X-Ray film (BIO MAX MR, Eastman-Kodak, Rochester, NY) for 1-3 days. Slides were then dipped in emulsion (K5, Ilford, Mobberley, UK), stored in a light-free box with desiccant at room temperature for 2 to 3 weeks, immersed in developer (D19, Eastman-Kodak), fixed (Hypam, Ilford) and stained with hematoxylin and eosin. Sections hybridized with sense probe for EGF and TGF-β1 were used as controls for non-specific binding.

**Histochemistry and immunohistochemistry**

Histological studies of vascular architecture were performed in a subset of animals (n = 12/group). Animals in this subgroup were anesthetized with pentobarbital sodium (Nembutal, Bomac Laboratories, Asquith, Australia) and vessels were perfused at arterial pressure with 2.5% glutaraldehyde (n = 6/group) or 10% neutral buffered formalin (n = 6/group) via an intra-aortic cannula. Tissues were then prepared as previously described [7]. In brief, mesenteric vessels were placed in ice cold phosphate buffer where fat, connective tissue and veins were removed by blunt dissection. The resultant vessel preparation was then embedded in paraffin.

Sections were then either histochemically or immunohistochemically stained. Histochemical staining comprised the use of either haematoxylin and eosin,
Masson's trichrome [21] to examine extracellular matrix or the immersion of sections in 0.1% toluidine blue (Sigma) in 0.1N HCl for one minute at room temperature to visualize mast cells [22]. Immunostaining for tryptase was also performed as a confirmatory test for mast cell identification [13] using a monoclonal anti-human tryptase antibody mAb 1222 (Chemicon, Temecula, CA). Immunohistochemistry was performed using the indirect avidin-biotin complex method as previously described [5]. To examine for the presence of endothelin sections were incubated with a polyclonal rabbit and anti-human endothelin antibody (AMRAD Biomedical, Melbourne Australia). Smooth muscle was immunostained using a polyclonal α-smooth muscle actin antibody (Biogenes, San Ramon, CA).

**Quantification of Histopathology**

The proportion of vessel wall occupied by the media was demonstrated by the presence of α-smooth muscle actin containing cells and quantified using a video-imaging system (Video Pro 32, Leading Edge, Bedford Park, South Australia, Australia), connected to a light microscope (Zeiss, Oberkochen, Germany) with photographic attachment (Axiophot, Zeiss, Oberkochen, Germany) microscope. Using this device, the media (areas stained with the anti-actin antibody) and corresponding lumenal areas were determined in a median of 20 (range 10-35) vessels per animal and expressed as wall/lumen ratio, as previously described in other models of vascular hypertrophy [23, 24].

Quantitation of extracellular matrix was performed on trichrome-stained sections using two methods. Firstly, sections (n = 6/group) were examined by two
independent observers, blind to the disease status of the animals, using a semi-quantitative scale (adapted from [25]) wherein sections were graded as 0 = absent staining, 1+ = little staining (<20% tissue stained), 2+ = moderate staining (20-50% tissue stained) or 3+ = abundant staining (>50% tissue stained). In addition, the proportion area occupied by matrix, vessel wall media and immunoreactive endothelin was quantified using computer-assisted image analysis as previously described [26, 27]. In brief, the color ranges for matrix (blue on trichrome stained sections), media smooth muscle (red on trichrome stained sections) and endothelin (brown on immunoperoxidase labeled sections) were selected and image analysis was performed using a chromogen-separating technique [27]. Tissue section images from three non-overlapping, randomly selected fields were examined by light microscopy (Olympus BX-50, Olympus Optical, Tokyo, Japan) and digitized using a high resolution camera (Fujix HC-2000, Fujifilm, Tokyo, Japan). All images were obtained using a 20X objective lens. Digitized images were then captured on a Power Macintosh G3 computer (Apple Computer Inc., Cupertino, CA) equipped with an in-built graphic board and evaluated using analytical software (Adobe Photoshop 5.0, Adobe Systems, San Jose, CA).

Mast cells were quantified in toluidine blue-stained sections (n = 6/group) where the number of positively stained cells were counted by an observer masked to the study group of origin.