**Enhanced Smooth Muscle Cell Coverage of Microvessels Exposed to Increased Hemodynamic Stresses In Vivo**


**Abstract**—During vascular remodeling in adult organisms, new capillary growth is often coupled with the adaptation of arterioles and venules, a process that requires the recruitment and differentiation of precursor cells into smooth muscle. We studied the in vivo adaptation of microvessels in the presence of elevated pressure and circumferential wall stress using a ligation strategy for mesenteric microvascular networks. Acute pressure increases of 42.6±18% and 17.1±3.3% were respectively elicited in the 25- to 30-μm-diameter venules and arterioles supplying the networks. Wall shear rates were not significantly changed; however, diameters were increased in >10-μm-diameter venules and >20-μm-diameter arterioles. Smooth muscle cell contractile phenotype was determined in all microvessels by observing the expression of smooth muscle myosin heavy chain (SM-MHC; a marker of fully differentiated smooth muscle) and smooth muscle α-actin (a marker for all smooth muscle, including immature smooth muscle of fibroblast/pericyte lineage). The ratio of SM-MHC positive vessel length to smooth muscle α-actin–positive vessel length increased >2-fold after 5 and 10 days of the ligation treatment. Smooth muscle proliferation was studied by bromodeoxyuridine incorporation, and the increase in SM-MHC–labeled microvessel length density was accompanied by no measurable change in proliferation of SM-MHC–labeled cells 5 and 10 days after ligation. These results indicate that after a period of 5 or 10 days, mesenteric microvessels <40 μm in diameter exposed to elevated pressure and wall strain exhibit an enhanced coverage of mature, fully differentiated smooth muscle cells. *(Circ Res, 2003;92:929-936.)*

**Key Words:** microcirculation ■ angiogenesis ■ smooth muscle differentiation ■ hemodynamic stress

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The complex process of microvascular remodeling, including the arterialization of capillaries and structural adaptation of existing arterioles, occurs in several normal physiological and pathological conditions, such as wound healing, myocardial collateralization, and adaptation to exercise. Within each of these physiological or pathological conditions, several angiogenic stimuli, including hypoxia, inflammation, and alterations in hemodynamic stresses, may exert significant effects. It is likely that a combination of mechanical factors and biochemical signals derived from tissue parenchyma produce integrated control of microvascular structure. Indeed, the extreme complexity of the in vivo angiogenic microenvironment has made it difficult to understand the role that each individual stimulus may play in angiogenesis. In the present study, we present an experimental design aimed at separating, to a degree, the effects of hemodynamic stress on microvascular remodeling from the influence of hypoxia, inflammation, and humoral factors.

It has been well established that in larger vessels such as the aorta and the carotid arteries, alterations in hemodynamic stresses trigger structural remodeling of the vessel wall through the synthesis or degradation of cellular and matrix material. Furthermore, the formation of new collateral pathways from small arterioles is hypothesized to occur in response to an increase in shear stress that triggers an inflammation-like cascade of events in which the recruitment of inflammatory cells accompanies vessel enlargement. In microvessels, defined here as those vessels <100 μm in diameter, studies have suggested that circumferential wall stress or fluid shear stress governs the recruitment and differentiation of smooth muscle cells. Likewise, it has been proposed that the transformation of capillaries into arterioles, a part of the microvessel remodeling process, is governed by elevated wall stress in microvascular networks. Others have suggested that microvascular networks exposed to a combination of elevated wall shear rate and circumferential wall strain exhibit an increased capillary density. Although each of these studies has provided correlative evidence that hemodynamic stresses may be important mediators of microvessel remodeling, a definitive link between hemodynamic stresses and microvessel adaptation, as established by in vivo experimental methods, remains elusive.

The purpose of this study was to test the hypothesis that chronic alterations in hemodynamic stress elicit changes in microvessel remodeling through modifications in smooth muscle cell phenotype. To this end, we developed an experimental model in which ligations are strategically placed in...
the rat intestinal circulation to increase pressures and vessel diameters in a small region of the mesenteric microvasculature, without concomitant changes in blood flow to the surrounding tissue. Smooth muscle differentiation was examined in these networks by the expression of smooth muscle α-actin (SMA), a marker of mature and immature smooth muscle, and smooth muscle–myosin heavy chain (SM-MHC), a marker of mature smooth muscle only.13–15 Our results indicate that rat mesenteric microvessels <40 μm in diameter that are exposed chronically to elevated pressure and circumferential wall strain experience enhanced coverage by mature (SM-MHC–positive) smooth muscle cells.

Materials and Methods

Acute Treatments

All experiments were performed in accordance with the guidelines of the University of Virginia Animal Care and Use Committee. For the acute pressure, diameter, wall shear rate, and intestinal perfusion measurements, female Sprague-Dawley rats (258 ± 11.4 g) were anesthetized with an intramuscular injection of ketamine (80 mg/kg body weight), xylazine (0.08 mg/kg body weight), and atropine (0.08 mg/kg body weight). Pentobarbital (20 mg/kg body weight) in 0.9% saline was systemically injected intravenously 30 minutes after the initial anesthetic injection. The mesentry was exteriorized for intavital microscopy and continuously superfused with 37°C Ringer’s solution degassed with 5% CO2/95% N2. Mesenteric microvessels were observed using a saltwater immersion objective (Zeiss, 20× NA).

Acute Pressure Measurements

Pressure measurements were made in arterioles and venules that branched directly off arcuate vessels and supplied or drained microvessel networks in the mesentery windows of interest. The anatomical position of these networks and the vessels measured are depicted in Figure 1. A glass micropipette (2-μm tip) connected to a servo-nulling pressure measurement system (IPM, Inc)16 was inserted into the vessel, pressure was recorded for 3 minutes, the micropipette was removed, and ligations were placed on artery vein pairs according to Figure 1. After 10 minutes of ligation or sham treatment with the mesenteric tissue continuously superfused with degassed Ringers solution and the micropipette remaining in the superfusate, pressure was remeasured at the original position. Each set of before and after measurements was treated in a pairwise fashion during subsequent statistical analyses. The same technique was used for the sham experiments, but the ligatures were placed in the adipose tissue surrounding the vessels that would normally be ligated.

Acute Shear Rate and Diameter Measurements

Microvessel diameters were recorded in representative segments <40 μm in diameter, including intermediate arterioles, intermediate venules, feed arterioles, and feed venules in treated and sham mesentery microvessel networks. At least 20 segmental diameters were measured in each network before and at least 10 minutes after treatment. The diameters were grouped such that A3, A2, and A1 represent arterioles >30, 20 to 30, and 10 to 20 μm in diameter, respectively. In the case of venule segments, V3, V2, and V1 indicated the same diameter categories as arterioles. Shear rate values were obtained from centerline velocities and diameters measured in microvessels immediately branching off the arcuate vessels using a dual-slit velocity measurement system (Circusoft). Wall shear rates were calculated using an empirical relationship17,18 that accounts for the blood velocity profile in microvessels of similar diameter. Sham ligations for the velocity and diameter measurements were performed as described previously.

Intestinal Arteriole Diameter and Perfusion Measurements

See the expanded Materials and Methods section, available online at http://www.circresaha.org in the data supplement.

Chronic Treatments

The next phase of the study used sham (n=10) and treated (n=10) animals, which were anesthetized as in the acute experiments. Using sterile techniques, a longitudinal incision was made in the abdomen along the linea alba, and the mesentery and intestine were exposed and placed onto a specially designed sterile stage with a surface temperature maintained at 37°C. The stage was covered with gauze moistened with 0.9% saline at 37°C. Throughout the entire procedure, the tissue was irrigated with 0.9% saline at 37°C. Ligatures were placed in the artery/vein pairs as in the acute experiments. The ligature placement is shown in Figure 1. Nylon suture 10-0 (Ethicon, Inc) was placed in the locations indicated in Figure 1 for sham and treated groups; however, for sham treatments, ligatures were placed in the adipose tissue surrounding the vessels. After placement of the ligatures, the tissue was returned to the abdominal cavity. The incisions were sutured closed and the animals were allowed to recover.

Immunohistochemistry and Specimen Observation

After 5 or 10 days, animals were anesthetized and euthanized, and mesentery windows from sham or treated groups were extracted, cleared of surrounding fat, whole-mounted on gelatin-coated slides, and fixed for 4 hours at 4°C in PBS (pH 7.4) containing 4% paraformaldehyde. After fixation and washing in PBS, specimens were incubated with 1:50 000 rabbit polyclonal anti–SM-MHC antibody19 in antibody buffer (PBS with 0.1% saponin, 3% BSA, and 1:20 dilution goat serum) for 12 hours at 4°C. Secondary antibody

Figure 1. Schematic illustration of the ligation scheme designed to locally elevate hemodynamic stress in mesenteric microvessels. Solid circles indicate points of ligation (venous and arterial). This figure also shows the networks observed during acute measurements of pressure, centerline velocity, and diameter at the onset of the ligations. Pressure and centerline velocity measurements were made in arterioles and venules supplying the circled networks. For scale, the actual width of one mesentery window is ~2 cm at the widest point and the length is ~3 cm.
incubations to reveal SM-MHC expression were performed for 1 hour at room temperature in 1:300 goat anti-rabbit CY2 conjugate (Jackson, West Grove, Pa) in antibody buffer. Finally, to reveal SMA expression, tissues were incubated in 1:500 CY3 conjugated mouse monoclonal anti-SMA (Clone IA4, Sigma) in antibody buffer for 1 hour at room temperature. Mesenteric windows from weight-matched untreated controls were harvested and stained in the same manner.

When immunostaining was completed, tissues were observed with a Nikon TE-300 inverted microscope with confocal accessories (Bio-Rad μ-Radiance). For each sample, the settings for gain and laser intensity were determined in a representative, non-immunolabeled field of view to exclude nonspecific background signal. The iris opening was fully opened for all images acquired for quantification purposes, allowing for image thicknesses of ~12 μm using a Nikon ×20 oil/water objective. These settings were then used for the entire sample to acquire the images for postimaging analysis.

To determine the SMA and SM-MHC length densities, 2 confocal images (displaying CY2 and CY3 labeling) were acquired for every field of view containing vascular segments within a given mesentery window. The area circumscribing the vascular segments was calculated from the total area of the fields of view containing vascular segments. The length of the vascular segments was determined by tracing over each SMA or SM-MHC–positive segment with a 1-pixel-wide line, thresholding, and computing the total line length (Scion, Inc) for both SMA and SM-MHC. The lengths were normalized to vascular area for a length density measurement. The vascular length densities were also used to calculate a SM-MHC/SMA length density ratio.

**SM-MHC Cell Proliferation**

For cell proliferation studies, in addition to untreated controls, sham and ligated mesenteries were labeled 5 and 10 days after treatment. Animals were prepared as described earlier for chronic treatment, but the harvesting process was modified to deliver a 2-hour pulse of bromodeoxyuridine (BRDU) at the end of the treatment period. Animals were anesthetized as they were for acute surgery. BRDU 10⁻⁵ mol/L in Ringer solution (37°C, degassed with 5% CO₂, 95% N₂) was continuously superfused to the abdominal cavity via the previously made abdominal incision. After the BRDU pulse, mesentery tissue was removed and extracted to a vinal cavity via the previously made abdominal incision. After the

**Acute Microvessel Pressure Measurements**

Pressure was measured before and 10 minutes after ligation or sham treatment in 25- to 30-μm-diameter arterioles and venules that branched directly off an arcuate vessel and supplied or drained a mesenteric microvessel network. Measurements were made in the same microvessel before and after placement of the ligations or sham treatments. After ligations, pressure significantly increased in these arterioles and venules by an average of 17.1±2.3% and 42.55±18%, respectively (Figure 2). No significant changes were observed in the sham experiments.

**Acute Diameter and Shear Rate Measurements**

Diameters were measured for all microvessels types before and after placement of the ligations. Figure 3 demonstrates that a significant increase in absolute diameter change was observed for the ligated group in comparison with the sham group for arterioles >20 μm and for all measured venules. Arterioles (A3) and venules (V3) >30 μm in diameter experienced an increase in diameter of 6.95±3.98% and 11.22±6.34%, respectively. Arterioles (A2) and venules (V2)
with diameters of 20 to 30 μm showed an increase in diameter of 11.76% and 14.62%, respectively. Finally, arterioles (A1) and venules (V1) in the 10- to 20-μm-diameter category demonstrated a 9.75% and a 23.38% diameter increase, respectively. Sham experiments showed virtually no change in vessel diameter (Figure 3) or wall shear rate (Figure 4) from preligation to postligation. No significant differences in wall shear rate were observed between the treated and sham groups (Figure 4).

**Regional Perfusion Measurements**

Blood perfusion was measured in ligated, collateral, and control regions of intestine using fluorescent microspheres. The ligated and collateral regions are depicted in Figure 1. Comparisons were made to flow in control regions of intestine, which were at least 20 cm from the treated areas. As seen in Figure 5A, there was no significant difference in perfusion between the 3 tested regions, indicating that no ischemia was present. These values are in agreement with previously reported values for perfusion in the resting rat intestine. To test whether local autoregulatory mechanisms were normalizing intestinal perfusion, the diameter of each primary artery/vein branch within the collateral and ligated zones was measured before and after the ligations were made.

**Smooth Muscle α-Actin/Smooth Muscle Myosin Heavy Chain Expression**

The chronic effects of the ligation strategy on microvascular remodeling were studied by observing the expression of smooth muscle contractile proteins in microvessel networks of treated and sham mesentery windows. At 5 and 10 days after placement of the ligations, the affected mesentery windows were whole-mount–immunolabeled for SMA and SM-MHC. SMA-labeled and SM-MHC–labeled mesenteric networks from 5-day ligated, 10-day ligated, and 10-day sham groups are shown in Figure 6. Note that the total density of SMA-positive microvessels is similar in all groups. These results are quantified in Figure 7, where it is apparent that no significant differences in SMA-positive microvessel length density were observed within and between groups (Figure 7A). SM-MHC–positive length density was increased significantly in ligated groups relative to sham groups at both 5 and 10 days and was unchanged over the time period between 5 and 10 days for both the sham and ligated groups (Figure 7B). Figure 7C shows that the 5- and 10-day length density ratios of SM-MHC–positive segments to SMA-positive segments were 0.44±0.05 and 0.57±0.18, respectively. For comparison, the sham-treated windows had a significantly lower SM-MHC to SMA length density ratio for both 5 and 10 days at 0.16±0.04 and 0.20±0.06, respectively. These results indicate that in the ligation-treated group, fully differentiated...
smooth muscle extends additionally along the microvascular network, terminating closer to the true capillaries.

### Smooth Muscle Cell Proliferation

SM-MHC–labeled vessels observed during the proliferation study ranged in diameter from 4.9 to 76.1 μm. SM-MHC–labeled cells demonstrated no proliferation response after 5 and 10 days for both sham and ligation treatments compared with the untreated controls, as seen in Figure 8. At days 5 and 10, the proliferation response of the SM-MHC–labeled cells was either equal to or slightly below that of the sham-treated group and was not significantly different from the untreated control group.

### Discussion

The purpose of this study was to determine whether chronic alterations in hemodynamic stresses influence microvascular adaptation through modifications in smooth muscle coverage of microvessels. An experimental design that allows separation of hemodynamic stresses from other angiogenic factors was implemented. Microvascular pressures and diameters were significantly increased in the targeted mesenteric networks immediately after placement of the ligations. After 5 and 10 days of ligation treatment, the total length of microvessel exhibiting SM-MHC expression was significantly increased over sham control, but no significant differences in smooth muscle cell proliferation were observed. These results indicate that increased pressure and wall strain elicit microvascular remodeling via the modulation of smooth muscle coverage in the microvessel network.

### Experimental Model

This ligation strategy is a modified form of those used previously to study the adaptation of larger collateral arteries feeding the intestine. In these previous studies, elevations in wall shear rate or blood flow were reproduced consistently by strategically placing ligations in arteries in the rat mesentery. With these models, changes in hemodynamics elicit a medial thickening in mesenteric vessels ranging from ≈300 to 800 μm in diameter and changes in the expression of PDGF-A in the medial and intimal portions of the affected large arteries in the mesentery. The goal of the experimental model presented here was to reproducibly alter pressure and wall strain in microvessel networks and study the subsequent changes in microvessel wall investment by differentiated smooth muscle cells.

The present ligation approach redirects blood supply such that the unoccluded arcuate vessels surrounding the mesentery window of interest must supply and drain blood from the intestinal regions affected by the ligations. Based on the data from the perfusion experiments (Figure 5), the arcuate arteries surrounding the window of interest, along with smaller collateral connections in the intestinal wall, adequately supply these affected areas of the intestine. The increased intestinal area of perfusion for the unoccluded vessels results in an increase in the number of capillary resistance units fed by the unoccluded arteries on either side of the window of interest. The addition of new parallel resistances (capillary units) serves to decrease the total input resistance experienced by those arteries, resulting in an elevated flow rate in the arcuate arteries around the mesentery window of interest.

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**Figure 6.** Confocal microscopic images of SMA (A, D, and G) and SM-MHC (B, E, and H) expression in an untreated mesentery window (A, B, and C) and mesentery windows exposed to ligation treatment for 5 (D, E, and F) and 10 days (G, H, and I). C, F, and I are overlays of the preceding SMA and SM-MHC images. SMA expression is red, whereas SM-MHC expression is shown in green. Segments colored red are positive only for SMA (white arrows), whereas yellow regions indicate areas where both SMA and SM-MHC expression occurs. Bar=50 μm.
As seen in Figure 2A, placing these ligations also causes arteriolar input pressure to the microvascular networks in the mesenteric connective tissue windows to increase. For these pressures to increase, the arcuate artery that carries blood from the mesenteric artery to the arteriolar inputs of the mesenteric networks must dilate. We believe that there are 2 possible explanations for why this occurs. First, we know that the ligations force blood flow in the arcuate arteries to increase. This flow increase will, in turn, cause an increase in wall shear stress that could trigger a shear dependent dilation. Moreover, other investigators have shown that when these arcuate vessels are exposed to high flow, they remodel their structure such that luminal diameter is increased.22,23 Second, placing the ligations around the vessel could directly produce a conducted vasodilation response that is transferred upstream to the arcuate vessels between the mesenteric artery and the arteriolar inputs to the microvascular networks being studied. The venous pressure increase shown in Figure 2B occurs because arteriolar pressure increases (Figure 2A) and total network resistance decreases (a consequence of the diameter changes reported in Figure 3). Based on simple hemodynamic principles, increasing input pressure and lowering resistance dictates that venous pressure must also increase.

The model presented here creates changes in microvascular pressure and circumferential wall strain in such a way as to allow for the separation of their effects from other confounding angiogenic factors such as ischemia, humoral factors, and inflammation. As shown by the intestinal perfusion and diameter measurements in Figure 5, the ligatures did not cause ischemia or local autoregulation in the intestine. Thus, it was unlikely that hypoxia induced growth factors or metabolites diffused from the intestine into the mesenteric window of interest. The possible influence of circulating humoral factors and inflammation was addressed by the sham animals, which underwent practically identical surgeries yet exhibited no increase in SM-MHC length density at either 5 or 10 days. Even so, it is important to point out that even the sham-operated animals do not control for potential changes in nerve activity and hypoxia that may occur directly at the site of ligation. We believe that such effects were probably negligible or nonexistent, because these ligation sites were a considerable distance (≈2 cm) from the mesenteric windows being studied. Moreover, these potentially limiting factors have not adversely affected other studies that have examined vascular remodeling in the mesenteric arcuate vessels after ligation.22,23

**Smooth Muscle Investment of Microvessels and Hemodynamic Stress**

We have shown previously that the expression of SMA and SM-MHC can be used to monitor the differentiation state of microvascular smooth muscle cells in vivo.9,15,24 SMA, which is expressed at the earliest stages of smooth muscle differentiation, is used as a marker of immature and mature smooth muscle. SM-MHC, which is expressed at the latest stages of smooth muscle differentiation, is used as a marker of mature smooth muscle.13–15,25,26 Figure 7B illustrates that total SM-MHC length density is increased after 5 and 10 days of
ligation treatment. Moreover, in Figure 7C we show that, compared with the sham groups, where only 15% to 20% of SMA-positive vessel length consists of mature smooth muscle, 45% to 50% of SMA-positive vessel length is fully mature in the ligation-treated networks at both 5 and 10 days after treatment.

We have observed here and in other studies that the differentiation of smooth muscle cells from SMA only to SMA-expressing and SM-MHC–expressing phenotypes occurs in a continuous proximal to distal pattern along the developing vessels13 and that SM-MHC–positive vessels always express SMA. Based on these observations, the expansion of the SM-MHC–positive cell population cannot be attributed to the addition of new vascular SM-MHC–positive segments that lack SMA expression. Therefore, SMA length density is always greater than SM-MHC length density, and the addition of new vascular segments is always reflected, at first, as an increase in SMA length density. Thus, over the entire population of cells with smooth muscle lineage, as defined by the SMA label, a significant number of immature smooth muscle cells differentiate toward a fully mature smooth muscle phenotype after 5 and 10 days of exposure to the ligation treatment. Given these patterns of smooth muscle differentiation and the result that the ratio of SM-MHC length density to SMA length density increases, we assert that immature smooth muscle cells on preexisting microvessels receive a spatially controlled and localized differentiation signal that causes subsequent SM-MHC expression. It is important to note that the increase in SM-MHC coverage could also be the result of proliferation or recruitment of cells of smooth muscle lineage, ultimately destined to become SM-MHC–expressing cells. However, it is unlikely that the source of new SM-MHC–labeled cells could be from proliferation of existing SM-MHC–labeled cells, because, in comparison with sham treatments, there is no significant difference in smooth muscle cell proliferation 5 or 10 days after placement of the ligations (Figure 8).

Increased pressure in the microvessels serves to increase diameter and wall strain (leading to an increase in circumferential wall stress), which we hypothesize to be a localized mechanical stimulus that triggers the differentiation of immature smooth muscle. Although mean shear rates were unchanged with ligation, individual vessels may have experienced transient wall shear stress changes that could also play a role in the observed remodeling response. In vivo, there is evidence suggesting that smooth muscle phenotype is sensitive to elevated pressure or wall strain. Hypertensive rats exhibit pressure increases of ∼10 mm Hg in precapillary arterioles within the cremaster27 and intestinal28 microvasculature, and it has been shown that, with hypertension, SM-MHC B expression is increased in precapillary arteriole smooth muscle cells.29 Additionally, our findings agree with in vitro evidence30 that 10% cyclic strain can elevate the expression of the SM1 and SM2 isoforms of SM-MHC in cultured smooth muscle cells. In our study, SM-MHC expression was enhanced in the presence of strains that, from our diameter data, we estimated to be 9.7% and 13.2% for arterioles and venules, respectively. It is also possible that increased wall shear stress or circumferential wall stress triggered a local inflammation-like cascade of events in the vessel wall. In other studies, such inflammatory responses, triggered by hemodynamic stimuli, have been shown to mediate vascular remodeling.2 Alternatively, increased pressure may lead to an increase in transmural fluid flux, creating an elevation in transmural shear stress imposed directly on the vascular smooth muscle cells.31 However, to our knowledge, there is no experimental link between shear stress and SM-MHC expression in microvascular smooth muscle cells.

In summary, we believe that this is the first in vivo study that has isolated, to a reasonable degree, the effect of circumferential wall strain on microvessel remodeling from potential confounding stimuli, including hypoxia, and inflammation. The network effect of increases in pressure or strain is an enhanced coverage of microvessels by fully differentiated smooth muscle cells.

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References


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Expanded Materials and Methods Section

To characterize possible changes in flow or vessel diameter in vessels supplying collateral and ligated zones of the intestinal tissue (see Figure 1) directly above and adjacent to the window of interest (labeled “window of interest” in Figure 1), we performed microsphere flow measurements and direct microscopic observations. Intestinal segments within the ligated zones (Figure 1) from each animal were observed with reflected light microscopy using a Zeiss ACM microscope and a low magnification objective (Leitz 10x / 0.22 NA). Pre-and post-ligation (at least ten minutes after placement of the ligatures) diameters of each intestinal arteriole branching directly off of the intestinal artery/vein segments (segments of arteries and venules running parallel to the long axis of the intestine), and between the outermost ligation point and the edge of the window of interest, were determined offline using digitized images of the video recordings.

After ligations were in place and the intestinal artery/vein segments had been observed, the arterial blood perfusion of the ligated, collateral, and control (a distance of at least 10 mesentery windows, or about 20 cm, on either side of the outermost ligations) regions of intestine were measured using a fluorescent microsphere perfusion approach. Briefly, red fluorescent microspheres (10 μm diameter spheres, Molecular Probes, Eugene, OR) were injected (0.2 mL, 3.6 * 10⁶ microspheres/mL) through a left-carotid artery cannula, advanced to the aortic arch, and followed by a 0.25-mL flush of physiologically isotonic saline. Simultaneously, blood was collected from the femoral artery and the collection continued for 60 seconds after microspheres were injected for a total collection time of 90 seconds. After euthanasia with an overdose of pentobarbitol, the intestinal tissue (~ 0.4 gm) in the ligated, collateral, and control regions was removed, weighed and
digested for 48 hours in 7 mL of 2M ethanolic KOH containing a known quantity of yellow-green microspheres (10 µm diameter, Molecular Probes, Eugene, Or.) to verify that there was insignificant loss of microspheres during tissue processing. The samples were then rinsed in 1% Triton X 100 and 10 µM PBS and dissolved in 2-Ethoxy Ethyl Acetate for 24 hours. A PTI M-2004 fluorimeter (Photon Technology Inc.) was used for all fluorescence measurements. To test the functionality of the fluorimeter, a standard curve was generated with known dilutions of microspheres prior to each measurement. A reference yellow-green sample, containing the same quantity of spheres added to each tissue sample, was measured and this measurement was later used as a factor to estimate the loss of red fluorescence due to processing for all of the tissue samples. Based on the fluorescence measurements, the tissue blood perfusion (Q, mL x min⁻¹ x gm⁻¹) was determined using the relationship:

\[
Q = \frac{f_{TR} \left( \frac{f_{TYG}}{f_{RYG}} \right)}{f_{BR} \left( \frac{f_{BYG}}{f_{RYG}} \right)} \left( \frac{R}{M} \right)
\]

Where R is the quantity of blood extracted from the femoral artery (mL x min⁻¹), \( f_{BR} \) is the red fluorescence of the blood sample, \( f_{BYG} \) is the yellow-green fluorescence of the blood sample, \( f_{TR} \) is the red fluorescence of the tissue sample, \( f_{TYG} \) is the yellow-green fluorescence of the tissue sample, M is the mass of the tissue sample, and \( f_{RYG} \) is the fluorescence of the yellow-green reference sample.