The present study examined the changes in reactivity to a variety of vasoconstrictor drugs of the rabbit carotid artery during development of an intimal thickening induced by injury with an inflated balloon catheter. The injured and the unoperated contralateral carotid arteries were studied at 2 and 6 weeks after the operation. To differentiate areas of the injured artery lined by modified smooth muscle cells from areas lined by regenerated endothelial cells, each rabbit was injected with Evans blue dye before sacrifice. Ring segments (3 mm length) from the control and injured arteries were mounted in organ baths to record the circumferential isometric force with a technique that ensured that all rings were set to equivalent initial resting conditions of passive transmural stretch. Compared with the controls, the experimental arteries had a significantly decreased maximum contraction (E_max) in response to KCl at both 2 and 6 weeks. The experimental arteries were also significantly less sensitive to the α₁-adrenoceptor agonist, methoxamine, at both 2 weeks (approximately sevenfold) and 6 weeks (fourfold), with a marked decrease in E_max at 2 weeks, which returned to control values at 6 weeks. There was no change in E_max to either serotonin or the thromboxane A₂ mimetic, U46619, in the experimental arteries at either time. There was, however, a small but significant increase in the sensitivity to both drugs. There was no difference in response to any of the constrictor agents between the white and blue regions of the experimental vessels. We propose that the selective increase in responsiveness to certain platelet-derived autacoids in the injured artery may be related to the changes in smooth muscle cell phenotype observed throughout the media and neointima and reported in our companion article. These modifications in arterial reactivity may contribute to the development of spasm in arteries with atherosclerotic lesions. (Arteriosclerosis 9:299-307, May/June 1989)
rabbit was injected with Evans blue dye (60 mg/kg body weight, Sigma Chemical Co., St. Louis, Missouri) in 0.9% saline via the marginal ear vein. Each rabbit was injected with heparin (500 units, i.v.) immediately before receiving an overdose of sodium pentobarbitone (40 to 60 mg/kg, i.v.). Both carotid arteries were carefully dissected out and were placed in Krebs' solution (21°C, pH 7.4), which was continuously bubbled with 95% O2/5% CO2. The composition of the Krebs' was: Na+ 144 mM, K+ 5.9 mM, Ca2+ 2.5 mM, Mg2+ 1.2 mM, HCO3− 25 mM, Cl− 128.7 mM, H2PO4− 1.2 mM, SO4 2− 1.2 mM, and glucose 11.0 mM.

The experimental and control carotid arteries were cut into ring segments 3 mm in length by using a double-bladed scalpel. The segments from experimental arteries were divided into “blue” rings (90% to 100% of the artery surface stained with Evans blue) and “white” rings (90% to 100% of the surface remained unstained). From each animal, two sets of artery rings were used, each set containing a control, a blue, and a white ring. Each ring segment was suspended on fine, stainless steel wire hooks (500 μm diameter) in a 25-ml jacketed organ bath containing Krebs' solution maintained at 37°C and continuously gassed with 95% O2/5% CO2 as described previously. One hook was suspended from a Grass FT03C transducer to monitor isometric circumferential force, which was then recorded on a single-channel, flat-bed recorder. The lower hook was fixed to a support leg attached to a micrometer. Six organ baths were used concurrently.

**Resting Tension/Internal Circumference Relationship**

Since these experiments aimed to compare the reactivity of arteries of different wall thickness and configuration (i.e., arteries with intimal thickening compared to control arteries), a procedure to set each ring segment at the same passive conditions before the construction of the concentration-response curves was followed. A modification of the method of Mulvany and Halpern was used to set each ring segment at an internal circumference and corresponding passive tension equivalent to 90% of the circumference the artery would have in situ when unstimulated (relaxed) and perfused with a transmural pressure of 100 mm Hg. The method used here has been described in detail elsewhere. Briefly, the two wires supporting the tissue were brought together and the micrometer reading was recorded. The vessels were then stretched by advancing the micrometer in steps at 1-minute intervals (see Figure 1A). At the end of each interval, the force developed (F) and micrometer reading were recorded. When the stretched artery is flat between the wires, the internal circumference of the vessel ring (Li) is given by the formula:

\[ L_i = (\pi + 2)d + 2f_i \]  

where \( d \) = the diameter of the wires and \( f_i \) = the separation of the wires after the \( i^{th} \) step (calculated from successive micrometer readings).

The successive values of \( f_i \) were used to calculate the successive values of \( L_i \), while the values of \( F_i \) were used to calculate the wall tension \( T_i \) (circumferential wall force per unit length) with the following formula:

\[ T_i = \frac{F_i}{2g} \]  

where \( g = \) ring segment length (=3 mm). The series of \( T_i \) and \( L_i \) values derived for each artery ring were then fitted by an exponential equation:

\[ T_i = A_0e^{B L_i} \]  

where \( A \) and \( B \) are constants for that particular artery ring. The constant \( B \) gives a measure of the slope of the exponential over the entire curve and, hence, an estimate of the stiffness of the artery ring.
The wall tension/internal circumference exponential curves give the dimensions of each artery ring under normalized conditions. The internal circumference ($L_{100}$), corresponding to the point on the fitted exponential for which the effective transmural pressure was 100 mm Hg, was determined for each ring (see Figure 1B). This value of $L_{100}$ gives an estimate of the internal circumference the vessel would have in situ when unstimulated and under a transmural pressure of 100 mm Hg. Based on the Laplace equation:

$$P = \frac{2\pi T}{L}$$

which relates the effective transmural pressure, $P$, to the internal circumference, $L$, and corresponding wall tension, $T$, a computer-fitting technique determines the point on the exponential line that corresponds to a transmural pressure of 100 mm Hg and then gives the estimate of $L_{100}$. Since previous studies in this laboratory have found that maximum force development for artery rings occurs when they are initially stretched to 90% of $L_{100}$, the computer calculates 90% of $L_{100}$ and then displays the micrometer setting necessary to place the artery ring at 0.9 $L_{100}$.

The ring segments were left to equilibrate in the organ baths for 30 to 60 minutes after being set at 0.9 $L_{100}$. Then for each animal, cumulative (0.5 log unit) concentration–response curves were obtained to serotonin in one set of artery rings, while full curves were constructed to a thromboxane A₂-mimetic, U46619, in the second set of ring segments. In each case after the maximum contractions had been achieved, the ability of the arteries to relax was tested by adding the vasodilators acetylcholine and glyceryl trinitrate. The organ baths were then rinsed 90% of $L_{100}$ and then displays the micrometer setting necessary to place the artery ring at 0.9 $L_{100}$.

Comparison of the plotted passive wall tension/internal circumference exponential constant ($\alpha$) revealed no significant change in passive arterial wall stiffness between 2 and 6 weeks. While the average diameter of the blue rings was 6 weeks. While the average diameter of the blue rings was

Effect of Evans Blue on Vascular Reactivity

Segments of unoperated carotid arteries were incubated for 1 hour in either Krebs' solution or a solution of Evans blue of a concentration approximate to that which would be present when injected into the intact animal (i.e., 0.3 mg/ml in Krebs' solution) before constructing full concentration–response curves to either serotonin or U46619, as described above.

Statistical Analysis

The concentration–response curves to serotonin, U46619, and methoxamine were analyzed as increase in force from resting and were fitted to a logistic equation to determine maximum response ($E_{\text{max}}$) and the concentration of drug that elicited 50% of the maximum contraction ($E_{50}$). The $E_{\text{max}}$ and $E_{50}$ values were then analyzed similarly. The data were tested for homogeneity of variances using Cochran's C test. In the experiments with serotonin, the groups of $E_{50}$ values were found to have heterogeneous variances and thus the square root transformation was used on the data before further analysis. The data were then analyzed using a split-plot design analysis of variance to test the significance of the effect of area (control, blue, or white) and the effect of time after endothelial denudation (2 or 6 weeks). The Student-Newman-Keuls multiple comparisons test was then used to compare the significance of differences between mean values for different areas. The $E_{\text{max}}$ values derived in response to KC (30 mM) were analyzed similarly.

The $E_{\text{max}}$ and $E_{50}$ values derived from artery rings incubated with and without Evans blue before organ bath study were compared using a paired t test.

Drugs

The drugs used were serotonin (5-hydroxytryptamine creatinine sulphate), acetylcholine bromide (both from Sigma), U46619 [(1,5,5)-hydroxy-11a,9a-(epoxymethano)-prosta-5Z,13E-dienoic acid; The Upjohn Company, Kalamazoo, Michigan], methoxamine (Burroughs Wellcome, Beckingham, Kent, UK), and glyceryl trinitrate (David Bull Laboratories, Melbourne, Victoria). All drugs except glyceryl trinitrate were made up in distilled water each day before use. Glyceryl trinitrate was made up in ethanol.

Light and Electron Microscopy

The artery segments were sliced into smaller ring segments (1 mm in length), rinsed thoroughly in 0.1 M phosphate buffer (pH 7.3), and then postfixed for 1 to 3 hours in 2.5% osmium tetroxide in phosphate buffer. The tissue was then thoroughly rinsed in phosphate buffer, dehydrated through graded solutions of acetone, and embedded in araldite/epon. Thick sections (0.5 to 1.0 μm) were cut and stained with methylene blue. Thin sections of selected blocks were cut on a Huxley ultramicrotome, mounted on copper mesh grids, stained with uranyl acetate and lead citrate, and then viewed at 60kV in a Philips 400 electron microscope.

Results

Passive Properties of Control and Experimental Arteries

A representative trace from the initial passive stretch–force relationship for a control artery is shown in Figure 1A. Comparison of the plotted passive wall tension/internal circumference relationships (see Figure 1B) and comparison of the values of $\beta$ (Table 1), the exponential constant, revealed no significant change in passive arterial wall stiffness in blue areas compared to the control arteries. However, the average $\beta$ value for white rings was greater than that of the control arteries ($p<0.01$), indicating that the passive arterial wall stiffness was slightly greater in these areas of the experimental vessels. There was no significant change in arterial wall stiffness between 2 and 6 weeks. While the average diameter of the blue rings was
Table 1. Summary of Resting Vessel Parameters

<table>
<thead>
<tr>
<th>Area and time after endothelial denudation</th>
<th>Exponential constant B ($\times 10^4$)</th>
<th>Internal diameter at L$_{100}$ (mm)</th>
<th>Pressure at 0.9 L$_{100}$ (mm Hg)</th>
<th>Isometric force at 0.9 L$_{100}$ (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.43±0.14</td>
<td>2.53±0.10</td>
<td>72.6±0.4</td>
<td>6.25±0.22</td>
</tr>
<tr>
<td>Blue</td>
<td>5.82±0.38</td>
<td>2.60±0.14†</td>
<td>70.5±1.1</td>
<td>6.08±0.39</td>
</tr>
<tr>
<td>White</td>
<td>6.97±0.64*</td>
<td>2.34±0.15</td>
<td>69.0±0.7</td>
<td>5.30±0.43</td>
</tr>
<tr>
<td>Six weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.70±0.32</td>
<td>2.48±0.08</td>
<td>72.0±1.1</td>
<td>5.84±0.29</td>
</tr>
<tr>
<td>Blue</td>
<td>5.44±0.34</td>
<td>2.78±0.21†</td>
<td>71.0±0.9</td>
<td>5.60±0.53</td>
</tr>
<tr>
<td>White</td>
<td>6.44±0.30*</td>
<td>2.36±0.11</td>
<td>69.8±1.3</td>
<td>5.53±0.32</td>
</tr>
</tbody>
</table>

Values are the means±SEM. There were 14 animals in each group.

*Significantly different from the control value and from the value in blue areas (p<0.01) as shown by a split-plot analysis. †Significantly different from the value in white areas (p<0.01) but not significantly different from the control value as shown by split-plot analysis.

Figure 2. Concentration-response relations for serotonin (−log M) in control and in white and blue areas of the experimental arteries at 2 and 6 weeks after endothelial denudation. Each point represents the mean of nine ring segments. The vertical error bars represent the SEM of the final points in the curves. The horizontal error bars are the SEM of the EC$_{50}$ values.

At 2 and 6 weeks after endothelial denudation, there was no difference in the average maximum contractile force (E$_{max}$) developed in response to either serotonin or U46619 between the experimental and the control arteries (Figures 2 and 3 and Table 2), even though some rings from the experimental arteries developed greater tension than their corresponding controls. There was also no difference in reactivity (EC$_{50}$ values and E$_{max}$ values) between the white areas (lined by regenerated endothelium) and the blue areas (lined by modified smooth muscle cells) in response to either U46619 or serotonin. However, as indicated by the shift in EC$_{50}$ values, the experimental arteries had an enhanced (1.8- to 1.9-fold in blue and white areas, respectively) sensitivity to serotonin at 2 weeks, which was more pronounced at 6 weeks (2.2- to 3.5-fold in...
blue and white areas, respectively; see Table 2). Similarly, there was a small increase in sensitivity to U46619 (1.1- to 1.9-fold) at both 2 and 6 weeks (Figure 3; Table 2).

Three experimental artery rings (two at 2 weeks; one at 6 weeks) developed no active force to any of the agonists used here. Therefore, these rings were considered to be extreme outliers and were excluded from the statistical analysis. Histological examination revealed that these artery rings contained areas of considerable disruption of the medial smooth muscle cells.

At 2 weeks after endothelial denudation, the experimental arteries developed markedly less contractile force to methoxamine than did the control arteries (Figure 4, Table 2). The EC50 values indicate that at 2 weeks the experimental arteries were also significantly less sensitive (6.4- to 6.9-fold in blue and white areas, respectively) to methoxamine than were the control arteries (p<0.01). At 6 weeks, the average maximum contraction of the injured arteries to methoxamine had increased compared to that at 2 weeks (p<0.05); these values were now similar to that of the controls. Although there was a trend toward control values (Table 2), at 6 weeks the experimental vessels were still significantly less sensitive (2.5- to 4.2-fold in white and blue areas, respectively) to methoxamine than were the controls (p<0.01). There were no significant differences in the Emax or EC50 values in response to methoxamine between blue and white areas of the denuded arteries.

At both times after endothelial denudation, the experimental arteries developed significantly less tension (Emax) in response to KCl than did the control vessels (Figure 5; Table 2). There was no difference in Emax between the white and blue areas. Since full contraction curves to KCl were not obtained, we have not calculated the EC50 values. However, in the dose range used (5 to 30 mM), the curves for the experimental arteries to KCl did not appear to be displaced compared to the controls (Figure 5).

When the artery rings were examined histologically, there was no clear correlation between the maximum tension developed and the thickness of the intima or the thickness of the media. Despite the obvious medial damage observed in some poorly contracting rings, some other artery segments that had areas with very few cells remaining in the media were still able to contract to a dimension close to or greater than the control vessels.

To determine whether uptake of Evans blue dye into the artery wall affected arterial reactivity, some control artery rings were incubated in Evans blue (0.3 mg/ml in Krebs' solution) for 1 hour before organ bath study. Representative traces from one of three experiments are shown in Figure 6. Although the artery segments stained intensely blue, there were no differences between the EC50 and Emax values [mean±SE, 6.914±0.165 (−log10 M) and 4.96±0.41 g, respectively, for three experiments] in response to serotonin, compared to those obtained in control artery rings that were incubated for 1 hour in Krebs' solution [6.960±0.029 (−log10 M) and 5.48±0.62 g, respectively].

Discussion

In the current study, we examined the reactivity of the rabbit carotid artery after injury induced with a balloon catheter to four vasoconstrictor agents. Our major finding was that, although the experimental artery was markedly less reactive (in both sensitivity and range) to the α1-adrenoceptor agonist methoxamine particularly at 2 weeks after injury, there was no change in the maximum responses to serotonin or U46619. In fact, the injured arteries showed a small increase in sensitivity to both serotonin and U46619, particularly at 6 weeks after endothelial denudation. These results are consistent with the study of Consigny et al.,21 who examined vascular reactivity of the rabbit iliac artery after injury with a balloon catheter. Immediately after injury, these researchers found that the arteries were unable to contract in response to either noradrenaline or KCl. However, 4 weeks later, the sensitivity to serotonin had returned to control levels while there was still a sixfold decrease in the sensitivity to noradrenaline. In contrast to our results, Makhoul et al.22 noted a selective increase in sensitivity to noradrenaline but not to serotonin or histamine after balloon catheter injury to the aorta. These workers suggested that this effect was mediated through α1-adrenoceptors. The reasons for the discrepancies between their results and ours are unknown, although they may arise through differences in preparations or as a result of differences in the extent of the initial injury as a result of studying different vessels.

Two factors could be involved in the changes in reactivity of the vessels to the four vasoconstrictor agents in our experiments. First, in our previous study, we found...
extensive damage to the medial smooth muscle cells immediately after balloon catheter injury. As a result, in some areas there was a decreased number of intact smooth muscle cells at 2 and 6 weeks. This finding may, in part, explain the decreased reactivity to both methoxamine and KCl. However, the injured arteries were still able to develop tension equivalent to that of the controls in response to serotonin and U46619, with enhanced sensitivity to these agents. This suggests that the vessels were still able to contract maximally to certain stimuli, and that the changes in reactivity observed at 2 and 6 weeks were not entirely due to the initial injury. The decreased reactivity to methoxamine, particularly at 2 weeks, is unlikely to be due to a change in adrenergic uptake mechanisms since methoxamine has a low affinity for such pathways. The changes in smooth muscle phenotype that we observed in cells in the neointima and media between 2 and 6 weeks may be important here. At 2 weeks, the smooth muscle cells in the neointima and the remaining cells in the underlying media had a decreased volume...
ergonovine,24 and serotonin,6,25,29 but not to α-adrenergic agonists. In animal models, this enhanced reactivity appears to be related to the development of the atherosclerotic lesion, rather than to the presence of hypercholesterolemia per se.6 Likewise, in miniature pigs, coronary artery spasm can be induced specifically in areas with intimal thickening, an effect which is not further enhanced by elevated cholesterol levels.11 Numerous recent studies using stereology,27 specific antibody staining techniques,28,29,30 and cell culture31 have demonstrated that smooth muscle cells either adjacent to or within atherosclerotic plaques in humans or experimental animals express an altered phenotype.

The mechanisms responsible for smooth muscle cells of an altered phenotype having differing sensitivities to specific agonists are currently unknown but are under investigation. Studies of smooth muscle cells in primary culture would suggest that the muscle cells in the neointima 2 weeks after denudation would proliferate almost immediately in response to mitogens,32 synthesize increased amounts of extracellular matrix,33 have an increased number of receptors for lipoprotein,34 and have a decreased Na⁺/K⁺ pump activity.36 The changes in reactivity that we observed in this study may be the result of selective alterations in receptor numbers or in receptor-coupling mechanisms, which occur as a result of phenotypic modulation after endothelial denudation. A preliminary report suggests that the enhancement in sensitivity of atherosclerotic arteries to serotonin is due to an increased density of serotonergic receptors.36 In addition, while the cells of the neointima are in a modified phenotype, they may be able to alter the reactivity of the artery via release of endogenous vasoconstrictor substances. For example, the intimal smooth muscle cells may produce platelet-derived growth factor (PDGF),37 which has been recently shown to have potent vasoconstrictor activity.38 PDGF may act to selectively increase the availability of intracellular Ca²⁺ in surrounding smooth
muscle cells in response to other vasoconstrictors such as serotonin and U46619. Regenerated endothelial cells may also play a role in the altered reactivity of the vessel, releasing substances such as endothelium-derived relaxant factor, or the newly discovered vasoconstrictor peptide, endothelin.

In conclusion, we have shown that there are selective changes in the reactivity of the rabbit carotid artery to vasoconstrictor agents after balloon catheter-induced injury. The mechanisms involved in the marked decrease in reactivity to methoxamine and the concomitant increase in sensitivity to serotonin and U46619 remain unresolved, although there appears to be some correlation with changes in smooth muscle phenotype. Since changes in smooth muscle phenotype are also observed in atherosclerotic arteries, the data suggest an explanation of why spasm is a feature of these vessels. A clinically significant feature of advanced human atheroma is endothelial denudation followed by thrombus deposition. The enhanced responsiveness of smooth muscle cells in areas adjacent to, or even within, atheromatous plaques to platelet-derived vasoconstrictors released from the thrombi may result in an exaggerated arterial contraction. This hypercontraction, or spasm, may act together with the amplifying effect of the lumen encroachment of atherosclerotic lesions to produce severe focal narrowing, and even occlusion, of the arterial lumen. Such a scenario may explain the observation that spasm occurs in only specific areas of atherosclerotic arteries and in only a proportion of patients with coronary artery disease. The recent results of Lam et al. showing correlation between the degree of platelet deposition and the extent of localized vasoconstriction after angioplasty support the sequence of events that we suggest.

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