16. The time scale of a single fluctuation (for example, single bond dissociation) is probably of the order of 10^{-5} to 10^{-6} second (15). Thus, it is likely that many thousands of events will have occurred within the first 1/30 second. This makes it plausible that the system is approximately in a steady state for the first few seconds.
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Reductions in Arterial Diameter Produced by Chronic Decreases in Blood Flow Are Endothelium-Dependent

B. Lowell Langille and Frank O'Donnell

A 70 percent reduction in the rate of blood flow through the common carotid artery in rabbits caused a 21 percent decrease in the diameter of this artery within 2 weeks. The smooth muscle relaxant papaverine did not attenuate the response; therefore, such reductions in diameter probably reflect a structural modification of the arterial wall rather than sustained contraction of smooth muscle. This arterial response to reduced blood flow was abolished when the endothelium was removed from the vessels. It appears that the endothelium is essential for the compensatory arterial response to long-term changes in luminal blood flow rates.

The inner diameters of mammalian arteries are correlated with luminal blood flow rates throughout development (*1*). This dependence of arterial size on blood flow is not totally preprogrammed in vascular development, however, because experimental interventions that alter blood flow cause corresponding changes in arterial diameter (*2, 3*). Thus, local hemodynamic forces are important in determining vascular structure. This responsiveness to local blood flow persists into adulthood (*4*) and may play a role in arterial occlusive disease. For example, flow reductions due to partial occlusion may be exacerbated by constrictor responses to the reduced flow. On the other hand, early increases in flow through collateral vessels after arterial occlusion may be enhanced by a dilatory response to the increased flow.

Flow-induced changes in arterial size are mediated locally because they are restricted to the sites at which flow is altered, they are not abolished by arterial denervation, and they are not dependent on coincident changes in arterial pressure (*2, 5*); however, little else is known about the mechanisms underlying this important response. Changes in arterial size, whether contractile or structural, must be effected by vascular smooth muscle cells, since these are the only cells in the media of adult arteries, but it is unlikely that these cells directly sense changes in blood flow rates through the vessel lumen. Such direct sensitivity to flow would imply that the cells detect changes in shear stress; however, physiological shear stresses cause extremely little bulk deformation of the arterial wall. For example, 100 dynes/cm_2, a very high shear stress in vivo (*6*), will cause less than 1 percent bulk deformation (shear strain) of the arterial wall tissue (*7*). Therefore, it is unlikely that cells deep in the vessel wall can detect changes in blood flow.

Instead, we and others (*5, 8*) have reasoned that the endothelium, which is in direct contact with luminal blood, may detect changes in flow and mediate responses of the smooth muscle. The endothelium may release vasoactive substances (*9*) that adjust smooth muscle tone and vessel wall growth modulators (*10*) that initiate structural modifications of the vessel wall. There is evidence that the release of some of these agents is dependent on shear stress (*9*). In the study reported here, we altered blood flow rates through the one common carotid artery of rabbits and determined the influence of endothelial denudation on inner arterial diameter.

Adult male New Zealand White rabbits were anesthetized intramuscularly with ketamine (22 mg/kg) and xylazine (2.2 mg/kg) and the left external carotid artery was exposed and ligated at its origin. Therefore, flow through the left common carotid artery was reduced to internal carotid artery flow. In seven of these rabbits, electromagnetic flowmeters were implanted on either the left (four rabbits) or right (three rabbits) common carotid artery 1 week before surgery, and blood flows were recorded until 1 week after surgery. Ligation of the left external carotid caused a 70 percent reduction in blood flow, from 26.5 ± 3.00 to 8.0 ± 0.70 ml/min (means ± standard errors), and no significant change in flow through the contralateral common carotid.

The rabbits were killed by barbiturate overdose 2 weeks after surgery and a methacrylate casting compound (Batson's No. 17) was immediated infused into the carotids via the thoracic aorta under physiological pressure (100 mmHg) and allowed to set for 30 to 60 minutes. The rigid casts, with arterial tissue, then were removed and immersed in a fixative. The next day the tissue was removed from the cast and cross-sec-

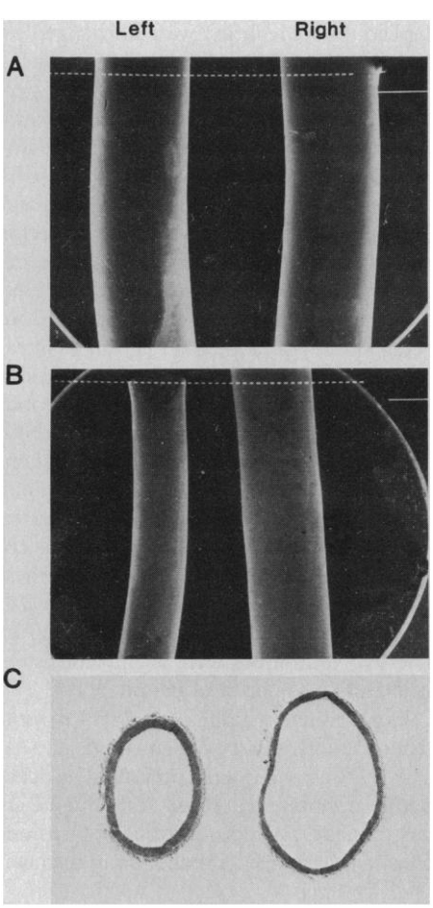

Fig. 1. (A) Scanning electron micrograph of methacrylate casts of the right and left common carotid arteries of a normal rabbit. (B) Micrograph of carotid casts 2 weeks after left carotid flow was reduced. Markers are 100 μm. (C) Histological cross sections of carotid arteries 2 weeks after left carotid flow was reduced.

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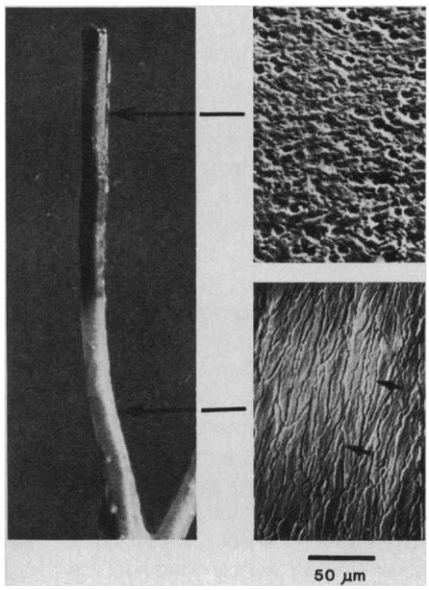

Fig. 2. Techniques used to identify deendothelialized segments of arteries. On the left is a common carotid artery that has been denuded (upper half) of endothelium. Vessel is darkened where Evans blue has stained the denuded segment. On the right are high-power scanning electron micrographs of casts of denuded (top) and endothelialized (bottom) vessels. Shorter arrows indicate impressions of cell boundaries on cast of undenuded vessel.

tioned for histological examination. Measurements of the internal diameters of both common carotid arteries were made from the casts using a stereomicroscope with an eyepiece graticule. The diameter of the left common carotid artery was 79 ± 3 percent of the diameter of the right, whereas no difference was seen in control animals (Fig. 1, A and B). The same finding was observed when the casting procedure was replaced by simple perfusion fixation of the arteries under physiological pressures, and mean diameters were measured from histological cross sections (Fig. 1C). Results were essentially the same when the comparisons were made of external arterial diameters, which differed from internal diameters by less than 10 percent. Therefore, diameter reduction was due to reduced vessel size rather than a proliferation of wall tissue.

To assess the role of the endothelium in this response, we used two techniques to remove this cell lining from segments of both carotid arteries before ligation of the left external carotid. A 2F Fogarty balloon catheter was inserted retrogradely into one lingual artery and advanced to the midregion of the left common carotid. The balloon on the catheter tip was inflated until moderate resistance to withdrawal was felt and the catheter was withdrawn 3 cm, abrading the endothelial surface (*11*). The balloon was deflated and procedure repeated twice. The catheter was then removed and the insertion hole in the lingual artery was sutured in a nonocclusive manner. The other carotid artery was deendothelialized in the same way. The left external carotid was ligated immediately after denudation.

Alternatively, a polyethylene catheter (PE 50) was inserted retrogradely into the lingual artery and advanced until the tip of the catheter was 1 cm inside the common carotid artery. A ligature around the carotid secured the catheter tip and stopped blood flow past this point. The vessel then was clamped 3 cm farther upstream and a smaller bore catheter (PE 10) was then advanced through the larger catheter to the upstream clamp. Physiological saline was infused through this smaller tubing and vented through the larger, to flush the blood from the isolated arterial segment. The segment was then flushed for 60 seconds with 2 percent Triton X-100 dissolved in physiological saline and reflushed with a rapid flow of saline. Triton X-100 disrupts the endothelial cell membrane (*12*); this level of exposure was found to be the minimum necessary to cause complete loss of the endothelium. After these procedures were completed, the catheters were removed and the left external carotid artery was ligated. No damage to deeper wall structures was detected when sections of the denuded carotid arteries were examined histologically. Furthermore, dose-response curves for topically applied norepinephrine were unchanged by endothelial denudation (*13*).

Sham denudation procedures were performed in two animals. Saline rather than Triton X-100 was flushed through the isolated arterial segments.

After 2 weeks, the rabbits were killed and data on vessel diameters were collected as for rabbits without endothelial denudation, except that the following procedure was used to confirm denudation. One hour before being killed, the rabbits were given an intravenous injection of Evans blue; immediately after death, but before casting, the carotids were flushed with 0.25 percent $AgNO_3$ (*14*). Evans blue binds to albumin and stains the vessel wherever the loss of endothelium allows this plasma protein to penetrate. $AgNO_3$ precipitates at the endothelial cell junctions and leaves an impression of these junctions in the casts of the vessel (*14*) wherever the endothelium is intact (Fig. 2). The two techniques were totally consistent at identifying regions of denudation.

Flow reduction alone caused a 21 percent reduction in left carotid diameter (Fig. 3A). This response was totally prevented by deendothelialization, but those segments of artery outside the deendothelialized regions consistently showed a reduction in diameter

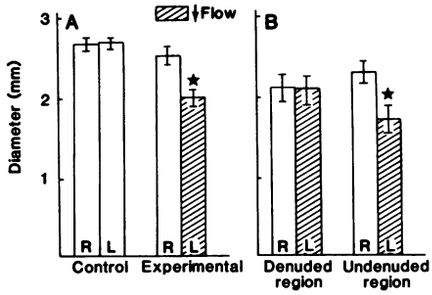

Fig. 3. (A) Mean diameter of right (R) and left (L) common carotid arteries in normal animals (control) and 2 weeks after reduction of blood flow in the left carotid artery (experimental). (B) Mean carotid artery diameters 2 weeks after endothelium was removed from segments of both carotid arteries and flow to the left carotid was reduced. At left are diameters measured in regions denuded of endothelium. At right are diameters taken from endothelialized regions of the same vessels. Data are means ± standard errors ($n = 6$ per group). Asterisks indicate that left carotid artery diameter was significantly different from right ($P < 0.05$, paired sample t-test).

similar to that of undenuded vessels (Fig. 3B).

The most probable explanation for these results is that the endothelium initiates arterial responses to alterations in blood flow rate. Although deendothelialization introduces factors other than the loss of endothelial cell function, we know of no such factor that would prevent narrowing in the vessel experiencing flow reduction. For example, an obvious consequence of endothelial denudation is platelet adherence and activation, but this normally produces arterial constriction, rather than preventing it. In any event, platelet adherence falls to minimal levels in well under the 2-week duration of our experiments (*15*). A second effect of denudation is proliferation of wall tissue, but again this would lead to a reduction in luminal diameter (*16*). We did observe minimal intimal thickening (<30 μm) that encroached insignificantly on the 2-mm diameter of these carotid arteries. This observation is consistent with the finding of Reidy and Silver (*17*) that deendothelialization alone does not stimulate such proliferation. They suggested that intimal proliferation may be secondary to the injury of medial smooth muscle cells that is caused by many denudation procedures. In our experiments the Triton X-100 technique and extreme care to avoid overdistending the vessels when using the balloon catheter reduced the possibility of such injury.

We do not know how endothelium might couple arterial responses to changes in blood flow. Although endothelium can release vasoactive substances (*9*), the response seen

after 2 weeks of flow reduction may involve responses other than adjustments of smooth muscle tone. For example, there may be a structural reorganization of the vessel wall. To assess this possibility, we ligated left external carotid arteries in an additional four rabbits. After 4 weeks the rabbits were reanesthetized, both carotids were exposed, and the smooth muscle relaxant papaverine (3.25 mg/ml) was applied topically to each vessel. Both dilated slightly, but the diameter reduction of the left carotid relative to the right was not reversed or even reduced by this dose of papaverine, even though it abolished the maximal constrictor response to norepinephrine (*13*). We infer that long-term, flow-induced reductions in arterial diameter are due to a structural modification of the vessel wall, although it remains possible that changes in smooth muscle tone contribute to the early phase of the response.

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Ras-Transformed Cells: Altered Levels of Phosphatidylinositol-4,5-bisphosphate and Catabolites

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Steady-state cellular levels of phosphatidylinositol-4,5-bisphosphate (PIP_2), 1,2-diacylglycerol (DAG), and inositol phosphates have been measured in two different fibroblast cell lines (NIH 3T3 and NRK cells) before and after transformation with three different *ras* genes. At high cell density the ratio of DAG to PIP_2 was 2.5- to 3-fold higher in the *ras*-transformed cells than in their untransformed counterparts. The sum of the water-soluble breakdown products of the polyphosphoinositides, inositol-1,4-bisphosphate and inositol-1,4,5-trisphosphate, was also elevated in *ras*-transformed NRK cells compared with nontransformed NRK cells. These findings suggest that the *ras* (p21) protein may act by affecting these levels, possibly as a regulatory element in the PIP_2 breakdown pathway.

INTERMEDIATES IN THE PHOSPHATIDYLINOSITOL turnover cycle apparently act as second messengers to mediate diverse responses in cells. Phospholipase C hydrolysis of phosphatidylinositol (PI) and its phosphorylated derivatives, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2), is stimulated by hormones, neurotransmitters, serum, and purified growth factors (*1*). Recent work from several laboratories has indicated that PIP_2 is the primary substrate for this breakdown (*2*). The breakdown products of PIP_2—diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3)—appear to act as intracellular regulators. Diacylglycerol activates a Ca^{2+}- and phospholipid-dependent protein kinase (kinase C) and can also act as a substrate for arachidonic acid release for production of prostaglandins (*3*); IP_3 stimulates Ca^{2+} release from intracellular vesicles into the cytosol (*4*). Both the DAG-activated kinase C and the IP_3-stimulated Ca^{2+} release cause activation of a plasma membrane Na^+-H^+ exchanger, which elevates cytoplasmic Na^+ and *p*H (*5*). Recent evidence has suggested that generation of these signals through stimulation of PI turnover is a critical event in growth factor stimulation of cell proliferation (*6*).

The transforming proteins encoded by the *ras* genes of the Kirsten and Harvey sarcoma viruses are homologous 21-kD proteins. Mutated versions of cellular homologs of *ras* have been implicated in the etiology of many human tumors of diverse origin (*7*). The ras protein is located in the plasma membrane and possesses GTP-binding capacity and low guanosine triphosphatase (GTPase) activity (*8*). Analogies between ras, transducin, and the proteins that regulate adenylate cyclase suggest a role for *ras* in transmembrane signal transduction (*9*), but its actual function in mammalian cells remains to be elucidated. In this study, levels of intermediates in the PI turnover cycle were measured quantitatively in NIH 3T3 and NRK cells and in these cells transformed by H, N, and K alleles of *ras* (see legend to Fig. 1). The results indicate that alterations in the steady-state levels of these membrane components is characteristic of *ras* transformation, suggesting that the ras protein may be involved in the regulation of PIP_2 breakdown.

PIP_2 and DAG levels in normal and *ras*-transformed cells were determined by growing cells in [^{3}H]glycerol. Cellular lipids were extracted in acidic chloroform-methanol and analyzed by thin-layer chromatography (TLC). Radioactivity remaining at the origin on the TLC plates developed in benzene–ethyl acetate (60:40) solvent (diacylglycerol plate) was used as a measure of total cellular [^{3}H]glycerol–containing phospholipid. PIP_2 and DAG were expressed as a percentage of this total, and a ratio of DAG to PIP_2 was obtained for each cell type. Figure 1 shows the results of experiments with cells at high density (0.8×10^7 to 1.0×10^7 cells per 100-mm plate). All three types of *ras*-transformed cells had signifi-

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