Pharmacology of Smooth Muscle Cell Replication

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The suggestion that smooth muscle cell proliferation contributes to hypertension, atherosclerosis, and restenosis after angioplasty has led to a growing interest in the use of drugs to inhibit this process. This review summarizes pharmacological studies of smooth muscle cell proliferation in vitro and in vivo and identifies specific mediators of proliferation that are implicated by drugs binding with high affinity to enzymes or receptors. (Hypertension 1992;20:713-736)

KEY WORDS • atherosclerosis • growth substances • hypertension, essential • muscle, smooth, vascular

This review emphasizes agents that influence smooth muscle proliferation. Four overlapping categories of agents that could be discussed are growth factors, drugs with known pharmacological effects, endocrine hormones with growth regulatory properties, and intracellular mediators of replication. This review will concentrate on the last three categories. A great deal has already been written in other reviews about the growth factors, but the much longer list of possible targets discussed here has not been reviewed systematically. Moreover, a great deal is known about the pharmacology of some of the molecules in our review, so their therapeutic value in the treatment of conditions arising from smooth muscle replication is likely to be realized more quickly in a clinical sense than treatments based on the still very poorly understood pharmacology of growth factors.

Pathology of Smooth Muscle Proliferation

Smooth muscle proliferation has been implicated as playing a central role in atherosclerosis and hypertension. The evidence that smooth muscle cell proliferation occurs in these diseases is clear. As we will briefly review, however, evidence for a causal connection is not well established.

Smooth Muscle Replication in Hypertension

The argument for a role of smooth muscle cell replication in hypertension is an outgrowth of the structural hypothesis of hypertension proposed by Folkow. Folkow explained that the ability of a resistance artery to restrict blood flow depends on the thickness of the vessel wall as well as on the size of the vessel lumen, the responsiveness of the wall to vasoactive stimuli, and the level of stimulation by vasoconstrictors. Folkow's idea is based on the simple physical fact that a thicker vessel wall will act as an amplifier, so that an equal vasoconstrictor stimulus will produce greater narrowing of the lumen in a hypertensive vessel than in a normotensive vessel. Studies of essential and secondary hypertension have confirmed that hypertensive vessel walls are thickened. Indeed, there is evidence that such structural changes precede any elevation in blood pressure, and, at least for the spontaneously hypertensive rat, wall thickening may depend on the increased sympathetic innervation that develops in the spontaneously hypertensive rat vessel wall within the first month of life. A correlation exists between the development of sympathetic innervation and the development of arterial medial hypertrophy in normotensive and spontaneously hypertensive rats. Of course, wall thickening need not be correlated with smooth muscle replication. There is one report, however, that DNA synthesis precedes secondary hypertension, and most studies of hypertrophied vessel walls of hypertensive animals also show an increase in DNA content either as an increase in cell number (hyperplasia) or in DNA content of each cell (hyperploidy).

In summary, increase in smooth muscle mass is well established as a feature of hypertension, and the change in mass, at least in some cases, appears to be related to DNA synthesis. This DNA synthesis could contribute to the chronic nature of hypertension, because DNA, once formed, is a stable molecule that only decays after cell death. Because the increased DNA should provide an increased template for protein synthesis, hyperplasia or hyperploidy might be expected to maintain increased mass. Unfortunately, we lack evidence that increased mass can itself be a primary event in hypertension or that therapies directed at either mass change or replication will have any effect on blood pressure. Perhaps more convincing evidence will become available when we have more specific agents able to control smooth muscle cell hypertrophy or replication.

Smooth Muscle Replication in Atherosclerosis

The role of the smooth muscle cell in atherosclerosis can be summarized briefly: Atherosclerotic lesions occur in the intima at sites of smooth muscle proliferation.
The smooth muscle cells contribute to the lesion by synthesis of cell mass and extracellular matrix. Lipid accumulates within the smooth muscle cells, in the extracellular matrix elaborated by the smooth muscle cells, and in macrophages that accumulate in the plaque. Debate continues as to whether smooth muscle cell proliferation is the first event in lesion formation, but there is no doubt that all occlusive lesions depend on smooth muscle cells for much of their mass. Indeed, in sudden cardiac death, typical lesions consist largely of smooth muscle cells and their extracellular matrix.

As with hypertension, the coincidence of pathology is not clear evidence that an antiproliferative strategy will be effective in treating atherosclerosis. Recent cell kinetic studies show that the level of replication in the normal human plaque is very low, perhaps one or two divisions per year. It is possible that most of the replication is an early event, occurring during vascular development and therefore not of interest therapeutically. Alternatively, replication might occur episodically, perhaps as a result of inflammatory events. Knowing when such events might occur seems unlikely, so chronic therapy may be necessary to ensure suppression of replication at the critical times.

Smooth Muscle Replication After Angioplasty: Three-Wave Model

Like atherosclerosis itself, the lesion formed after angioplasty is a result of aberrant smooth muscle accumulation in the intima. Angioplasty is an important topic for this review for three reasons. First, in experimental models, the response to angioplasty occurs rapidly enough to allow various agonists or antagonists to be readily studied. Second, the high incidence of restenosis after angioplasty in humans makes this likely to be the first target for clinical intervention with inhibitors of smooth muscle proliferation. Third, animal studies of arterial injury have begun to provide data on the role of growth factors in the control of smooth muscle cell proliferation in vivo.

The animal model of arterial injury that bears the closest resemblance to angioplasty is balloon catheter de-endothelialization. The most thoroughly investigated system is balloon catheter injury to the rat common carotid artery. The first phase of the response is a dramatic increase in the replication rate of smooth muscle cells in the media injured by the balloon dilatation. This response is massive, involving as many as 50% of the smooth muscle cells in the wall, and probably represents replacement of cells that die in the media as a consequence of the injury. This repair response does not directly contribute to the accumulation of cells in the intima, but it is possible that this "first wave" is necessary to provide cells that ultimately form the intimal lesion. The first wave is completed within 4 days. In the rat, however, there are normally no intimal smooth muscle cells, so formation of the neointima requires migration of cells from the media into the intima, the "second wave" of lesion formation. Smooth muscle cells migrate and can be seen forming a neointima by 3–4 days after injury. Although a discussion of potential pharmacological inhibition of smooth muscle cell migration is beyond the scope of this review, it is possible that this may be a legitimate target for limiting restenosis after angioplasty. The utility of this approach clearly depends on the contribution of migration to the accumulation of smooth muscle cells in the human arterial intima, an issue that is as yet unresolved. Once these cells are in the intima, approximately 50% of the cells go on to replicate, forming the lesion. This "third wave" involves a much lower daily replication rate, as low as 1–5%.

In any attempt to design a therapeutic regimen, it is important to consider the probability of affecting each of the three waves and the role that they may play in the restenotic process. For example, it is unlikely that the first wave itself contributes to intimal thickening. Indeed, by inhibiting repair of the injured media, one might even increase the possibility that the "healed" vessel will have a narrowed lumen. On the other hand, without an adequate first wave, too few cells may be available to migrate, and neointimal formation may be retarded. Agents that affect the second wave (migration) or third wave (neointimal proliferation) would seem much more certain to have beneficial effects. However, we also need to be aware of a lack of data relating intimal mass to lumen size. In a recent study of the effects of hypertension on atherosclerotic narrowing, hypertension greatly accelerated formation of the neointima but had no effect on narrowing of the lumen.

In summary, the issues in restenosis are similar to those in hypertension. We know very little about why the lumen becomes narrow, although we know that the adverse effects of the lesion depend on the narrowing.

Growth Factors

Growth Factor Assays

As already mentioned, this review will not concentrate on the usual polypeptide growth factors, because these have been comprehensively reviewed elsewhere. Nonetheless, a brief overview is useful for completeness and because some of the agonists and antagonists discussed below may act by influencing growth factor activity.

It is important to understand how growth factors are usually defined. In theory, the assay system is quite simple: Cultured cells are arrested by being placed in medium deficient in growth factors. The suspected agonist is added, and growth is determined by measuring the rate of increase in cell number or by measuring incorporation of DNA-specific nucleotides such as thymidine or bromodeoxyuridine. However, this assay has several problems.

1. Definition of 'growth factor-deficient medium.' Cells in vivo exist in an extracellular medium very similar to lymph. Smooth muscle cells, however, grow quite well in lymph (Schwartz et al, unpublished data). Quiescence is usually achieved by use of an artificial medium such as a fully synthetic "serum-free" medium able to sustain nongrowing cells by simply allowing the cells to become quiescent by depleting undefined factors in the medium. Growth factors detected under these conditions may include molecules required for growth in vivo but not able to stimulate growth themselves.

2. Derivation of cells used for assays. Cells must be grown to do these assays, but cells in vivo replicate...
rarely. Thus, the factors detected by an assay may reflect selection of growth factor–dependent cells during the establishment of cells in culture. For example, smooth muscle cells obtained from rats and subcultured in plasma-derived serum, obtained without platelet release, do not require platelet-derived growth factor (PDGF) for growth in vitro, whereas cells initially obtained and passaged in whole blood serum do require PDGF.23

3. Effects of potent agonists other than growth factors. Because quiescence as defined in vitro is an arbitrary and artificial state, we should not be surprised if a number of molecules with functions other than the initiation of growth can facilitate replication in vitro. Referring back to the three waves discussed above, it would not be surprising if a large number of metabolically active hormones would alter the rate of cell growth in the third wave, i.e., when cells are already replicating. Similarly, when in vitro assays are considered, it may be important to distinguish factors that determine rates of cell growth from factors that can initiate growth from quiescence.

4. In vivo versus in vitro cell kinetics. The issue of replication rate is very confusing when we attempt to compare in vivo with in vitro phenomena. In vitro, “quiescence” usually implies a daily cell replication rate of 5% or even 10%. This compares with normal quiescent states for vascular smooth muscle of as little as 0.01%. Indeed, replicative responses of smooth muscle to hypertension or cholesterol feeding in vivo are usually less than the daily replication rate seen in quiescent cultured cells.24-26 In practice, this means that mitogenic agents with no apparent proliferative effect in vitro may be active in vivo at levels not measurable in the usual assays. Therefore, it would not be surprising to discover circumstances in which a specific inhibitor lowers replication rates in “quiescent” cultures, even though the addition of agonists at levels beyond those found in the basal medium has no further effect on replication. Indeed, Emmett and Harris-Hooker27 reported just such an inhibitory effect for saralasin under conditions in which angiotensin II did not appear to be mitogenic.

Effects of Growth Factors in the Three-Wave Model

Recently, the availability of large amounts of growth factors, usually as expression products of recombinant DNA, has led to initial understanding of the possible roles of several growth factors in the three waves of the in vivo angioplasty response described above in the rat carotid artery injury model. We will briefly summarize the results.

When vessels are gently denuded of endothelium, the first wave is greatly decreased. This is consistent with the view expressed above that the first wave is a repair process dependent on injury to medial smooth muscle. However, when different growth factors were infused at the time of gentle injury, only basic fibroblast growth factor (bFGF) had a mitogenic effect comparable to the usual response to balloon injury. Transforming growth factor–β and PDGF BB were markedly less effective. Moreover, antibodies to bFGF greatly decreased the response seen after balloon injury. These data, combined with evidence that bFGF is present in the uninjured vessel wall, led Lindner et al28 to propose that bFGF acts as a wound hormone to control the first wave.

Similar studies by Clowes et al29 and Ross et al30 using infused PDGF and antibodies to PDGF, imply that its major role is stimulation of migration, i.e., the second wave. These observations help to resolve the paradoxical observation that antiplatelet antisera have no effect in abolishing first wave replication but retard the formation of the intimal lesion.18

Much less is known about controls in the third wave. Growth factor infusion studies have suggested that transforming growth factor–β and angiotensin II may be active at this stage.24,31

In this review, we summarize current knowledge concerning drugs that influence smooth muscle cell proliferation. Many of these agents interact with high affinity with receptors or enzymes and therefore implicate specific molecules in the positive or negative mediation of proliferation. With this in mind, we have grouped drugs in terms of their interactions with potential mediators. For each of these mediators, the following questions are considered: 1) Provenance. What are the possible in vivo sources of the mediator? Is it present in adequate concentration? Do vascular smooth muscle cells bear receptors that interact with it? 2) Effects of exogenous administration. Does exogenous administration of the proposed mediator by an appropriate route significantly influence proliferation? 3) Use of antagonists. Does administration of a suitable antagonist to the proposed mediator influence smooth muscle cell proliferation in the predicted way?

This review is therefore limited to a discussion of those potential mediators of smooth muscle cell proliferation that have an associated pharmacology. The second part of the review considers drugs that influence arterial smooth muscle cell proliferation but that do not implicate specific mediators.

Endogenous Agents That May Control Vascular Smooth Muscle Cell Proliferation

Adenosine

Adenosine is a nucleoside formed from 5'-adenosine monophosphate (AMP) by the action of 5'-nucleotidase.32 The concentration of adenosine in canine arterial blood plasma is 0.26 μM but rises substantially in some vascular beds after nerve stimulation.33 Cultured arterial smooth muscle cells possess both the A1 and A2 adenosine receptor subtypes.34-33 Occupation of the A1 receptor by adenosine inhibits adenylate cyclase activity and thus reduces the intracellular concentration of cyclic AMP, whereas occupation of the A2 receptor has the opposite effect.35 Jonzon et al36 correlated these effects of adenosine receptor stimulation with changes in the rate of DNA synthesis in rat aortic smooth muscle cells. Exposure of quiescent cells to human platelet PDGF resulted in an increase in the [3H]thymidine labeling index, which was significantly inhibited by adenosine at concentrations greater than 10 μM. Inhibition of PDGF-stimulated DNA synthesis was also observed with the adenosine analogues 5'-N-ethylcarboxamidoadenosine and 1-phenylisopropyladenosine. The latter was approximately equipotent with adenosine, which had 10% of the potency of 5'-N-ethylcarboxamidoadenosine. This adenosine analogue has
greater affinity for A2 receptors, suggesting that these may play a role in the control of vascular smooth muscle cell proliferation.

**Adrenocorticosteroid Hormones**

The biosynthesis and release of adrenocorticosteroid hormones from the adrenal cortex is controlled by corticotropin. The release of this hormone from the anterior pituitary gland is stimulated by corticotropin releasing factor, which is present in high concentration in neurosecretory cells in the paraventricular nucleus of the hypothalamus. The predominantly secreted adrenocorticosteroid hormone in humans is cortisol, or hydrocortisone, which is present in plasma at a total concentration of 0.14–0.55 μM under normal conditions.37

Järveläinen et al38 isolated smooth muscle cells from human fetal aorta and grew them in secondary culture. Addition of hydrocortisone at concentrations of 1 μM and greater significantly inhibited [3H]thymidine incorporation into DNA, and DNA content was significantly reduced at concentrations of 100 nM and greater. Berk et al39 found similar results using rat thoracic aortic smooth muscle cells growing in secondary culture. Addition of hydrocortisone (2 μM) significantly inhibited cell replication and the incorporation of [3H]thymidine into DNA. Inhibition of replication was also seen with the synthetic glucocorticoid dexamethasone (1 μM) but not with the sex hormones testosterone (2 μM) or progesterone (2 μM). Inhibition of cell growth by hydrocortisone was associated with a change in cell morphology, with the cells appearing larger, flatter, and more polygonal. The authors suggest that this morphological change could cause density-dependent inhibition of cell growth at lower cell densities, which is consistent with the growth profile of hydrocortisone-treated cells. The growth rate during the logarithmic phase was equal to that in control cultures, but the plateau phase was achieved at a lower cell density.

Longenecker et al40,41 also examined the effects of glucocorticoids on smooth muscle cell growth in vitro. Using bovine aortic smooth muscle cells, they found significant inhibition by dexamethasone at a concentration of 1 nM. This inhibition was counteracted by growing the cells on dishes coated with extracellular matrix; matrix elements can bind many polypeptide growth factors, and these may have overcome the inhibition by dexamethasone. Hydrocortisone (0.1 μM) and corticosterone (0.1 μM) also inhibited growth. Interestingly, the growth of primary cultures of bovine aortic smooth muscle cells was markedly more sensitive to dexamethasone than the growth of secondary cultures. In their 1984 study, Longenecker et al41 found that dexamethasone reduced both the growth rate and the final saturation density of smooth muscle cells, which contradicts the lack of effect on growth rate found by Berk et al.39 However, the earlier report from Longenecker et al40 showed a significant effect only on saturation density. Hauss et al42 examined the inhibitory effects of the synthetic glucocorticoid prednisolone on the proliferation of rat and porcine thoracic aortic smooth muscle cells in secondary culture. Significant inhibition of proliferation of porcine cells was observed at a concentration of 56 μM. Parenteral administration of prednisolone in rats by an unspecified route for 4 days (2 mg per rat per day) produced a slight inhibition of the growth of smooth muscle cells subsequently isolated from the aortas of these animals. The development of neointimal hyperplasia in the ear artery of rabbits in response to a crushing injury was significantly inhibited by intravenous treatment with prednisolone (0.2 mg per rabbit per day).43

Dexamethasone, at concentrations as low as 10 nM, was found by Hirosumi et al44 to inhibit the proliferation of rat thoracic smooth muscle cells growing in secondary culture. Inhibitory effects on smooth muscle cell migration were noted at concentrations greater than 100 pM. This study also investigated the effect of intramuscular administration of dexamethasone on the development of the neointima in response to investment of the rabbit carotid artery with a polyethylene cuff for 21 days. Intimal thickening was dose-dependently inhibited by dexamethasone, the lowest effective dose being 1 mg/kg/day. Presumably, this effect was the result of inhibition both of smooth muscle cell proliferation and of smooth muscle cell migration to the intima. Dexamethasone (0.05 mg/kg of body weight per day) has also been shown to inhibit intimal thickening in the balloon-injured rabbit common carotid artery45,46 and at 0.2 mg/kg of body weight per day in a rat vasculitis model.47 In this model, a thread soaked in bacterial lipopolysaccharide is laid along the adventitial surface of the femoral artery. Leukocytes adhere to the endothelium in the vicinity of the thread, and a neointima develops that is composed primarily of smooth muscle cells. Dexamethasone inhibits the accumulation of leukocytes in this system; it is possible that the effects of glucocorticoids in other inflammatory models of arterial injury, such as periarterial cuffing48 or arterial crushing,43 are related to inhibition of leukocyte function.

The main action of the glucocorticoids is induction of lipocortin, a protein that inhibits phospholipase A2.49 This enzyme is responsible for the liberation of arachidonic acid from cellular phospholipids, and its inhibition therefore reduces eicosanoid biosynthesis. The inhibitory effects of glucocorticoids on arterial smooth muscle cell proliferation thus may be the consequence of reduced availability of growth-stimulatory eicosanoids. The effects of eicosanoids on arterial smooth muscle cell growth are reviewed below.

If glucocorticoids were an important growth-inhibitory influence on smooth muscle cells in the arterial wall, one might expect that hypophysectomy would cause an increase in smooth muscle cell proliferation in response to vascular injury, because removal of the pituitary reduces circulating levels of hydrocortisone by approximately 90%.49 In fact, hypophysectomy significantly inhibits the development of intimal thickening in the balloon-catheterized rat aorta.49,50 Bettmann et al50 also found that hypophysectomy accelerated the regrowth rate of endothelium in balloon-denumed rat aorta and suggested that the inhibition of intimal thickening in hypophysectomized rats could be the consequence of suppression by the endothelium. However, Tiell et al51 did not observe any difference in endothelial regrowth between control and hypophysectomized animals, suggesting that the inhibition of neointimal development was not the indirect consequence of rapid recovery by endothelium. This leads to the conclusion that the pituitary is necessary for the elaboration of an essential cofactor for smooth muscle cell proliferation.
Bettmann et al were unable to reverse the inhibitory effect of hypophysectomy by coadministering thyroxin, hydrocortisone, deoxycorticosterone acetate, and growth hormone. However, the pituitary also produces effect of hypophysectomy by coadministering thyroxin, synthesis, secretion, and activity of a wide range of hormones and neurotransmitters in the body. Dissection of the pathways involved in hypophysectomy-induced suppression of smooth muscle cell proliferation will be a formidable task, but determination of the mitogenic activity for smooth muscle cells of plasma-derived serum prepared from normal and hypophysectomized animals may provide some useful clues. This approach was used by Stiles et al, who determined the effects on the growth of the BALB/c 3T3 murine fibroblast cell line of plasma prepared from hypophysectomized rats. This material had 5% of the potency of normal rat plasma in promoting DNA synthesis in cells briefly exposed to PDGF. The activity of hypophysectomized rat plasma was restored to normal by addition of somatomedin C.

**Angiotensin**

Angiotensin II is a vasoactive octapeptide formed from its inactive decapeptide precursor, angiotensin I, by the action of a dipeptidyl carboxypeptidase known as angiotensin converting enzyme (ACE). Angiotensin I is derived from the cleavage of angiotensinogen by renin. A reasonable case may be made for the presence in vascular tissue of all of the components required for the synthesis of angiotensin II. Canine aortic smooth muscle cells have been shown to synthesize renin in vitro, and mRNA has been detected in rat periarteric tissues for both renin and angiotensinogen. Rat aorta has been shown to be capable of generating angiotensin II from angiotensin I, suggesting that this vessel contains ACE activity. These authors suggested that this property is present in both endothelium and vascular smooth muscle, because conversion of angiotensin I occurred in aortic rings even after the removal of the endothelium by rubbing. Similar results were obtained in rabbit aorta by Saxe et al. An alternative explanation is that the endothelium of rat and rabbit aorta does not exhibit ACE activity, but these results certainly indicate that ACE activity is present in the arterial wall.

In human smooth muscle cells derived from neonatal and juvenile aortas, growing in secondary culture in 10% fetal calf serum, exogenous angiotensin II significantly stimulates cell growth at concentrations of 100 nM and greater. However, this is insufficient evidence to conclude that angiotensin II is a smooth muscle cell mitogen. This study did not exclude the possibilities that angiotensin II was degraded in the culture medium to form uncharacterized mitogenic peptides or that it potentiated another mitogen present in fetal calf serum. Also, the mitogenic concentration of angiotensin II was at least 3,000 times higher than the concentration in human plasma. These objections were answered in part by Lyall et al in a study examining the effects of angiotensin II on the proliferation of rat mesenteric arterial smooth muscle cells growing in secondary culture in 10% fetal calf serum. Concentrations as low as 1 nM stimulated an increase in cell number, but this is still an order of magnitude higher than the human plasma concentration. Stimulation of cell replication was blocked by the angiotensin II structural analogue saralasin, which competes for the angiotensin II receptor. This suggests both that proteolytic degradation of angiotensin II is not involved in its mitogenic effect and that angiotensin II acts directly on the smooth muscle cell, rather than interacting with a serum component. The involvement of angiotensin II in the growth of arterial smooth muscle cells in vitro was highlighted by Emmett and Harris-Hooker. They found that saralasin significantly, dose-dependently, and reversibly inhibited smooth muscle cell proliferation in the presence of fetal calf serum.

Geisterfer et al were unable to detect any hypertrophic effect of angiotensin II at a concentration of 1 μM, using rat thoracic aortic smooth muscle cells growing in secondary culture either in 10% fetal calf serum or in a defined serum-free medium containing partially purified PDGF. However, at concentrations of 1 nM and greater, significant increases in the mean cellular protein content were observed, indicating hypertrophy. The hypertrophic effect was blocked by saralasin. The dose–response curve for the hypertrophic effect of angiotensin II was extremely flat, spanning five orders of magnitude between the lowest effective concentration and the maximally effective concentration. This may have been the consequence of breakdown of angiotensin II, which was added in fresh culture medium every 2–3 days; Lyall et al showed by radioimmunoassay that incubation for 3.5 hours in medium containing 10% fetal calf serum degraded 99.9% of exogenous angiotensin II.

Berk et al also found no effect of angiotensin II on the replication of rat thoracic aortic smooth muscle cells growing in secondary culture, although DNA synthesis increased significantly at concentrations of 100 nM and greater. This suggests that polyploid cells were being formed. Hypertrophic effects were observed at angiotensin II concentrations as low as 100 pM.

It is interesting to note that the effects of angiotensin II on smooth muscle cell proliferation seem to be related to exposure of the cells to serum. Angiotensin II appears to cause hyperplasia in smooth muscle cells maintained in growth-supporting concentrations of serum throughout the experiment. In contrast, in studies in which cells are rendered quiescent by incubation in medium containing little or no serum, hypertrophic effects are observed when angiotensin II is administered at the same time as reintroduction of serum. Hyperplastic effects of angiotensin II have not been observed under these conditions. A possible exception is the study by Hamada et al in which rat thoracic aortic smooth muscle cells in secondary culture were incubated in serum-free medium for 1 day before exposure to angiotensin II. The incorporation of [3H]thymidine into DNA was significantly stimulated at concentrations of 1 nM and greater. However, cell numbers were not determined, so it is not clear whether angiotensin II stimulated cellular replication or simply caused the development of polyploidy. Campbell-Boswell and Robertson examined the effects of angiotensin II in smooth muscle cells cultured in 1% fetal calf serum for 2 days before introduction of the experimental medium. Under these conditions, the stimulation of
cell replication by angiotensin II was approximately 75% of that found in cells maintained in 10% fetal calf serum throughout and exposed to the same concentration of angiotensin II. However, induction of quiescence by culturing the cells in 5% platelet-poor plasma-derived serum for 2 days did not inhibit the subsequent mitogenic effect of angiotensin II. Similar findings were reported by Scott-Burden et al who were able to detect a very weak mitogenic effect of 50 nM angiotensin II in rat vascular smooth muscle cells growing in 1% plasma-derived serum. This suggests that the hyperplastic response of smooth muscle cells is dependent on continuous exposure to serum and that, in the presence of low concentrations of serum, the mitogenic response gradually disappears. It is clear that cellular quiescence is not an issue, because quiescence induced by platelet-poor plasma-derived serum did not inhibit the mitogenic response to angiotensin II. It therefore appears that a non-platelet-derived factor or factors in serum is critical for the mitogenic response and that withdrawal of the factor for 2 or more days inhibits the response. Whether this factor operates by inducing a state of smooth muscle cell growth in which the cells are particularly sensitive to the mitogenic effects of angiotensin II or, alternatively, by inducing an intracellular signal necessary for the angiotensin II mitogenic signal to be fully transduced is open to question.

It is not clear what the cofactor for angiotensin II-induced mitogenesis might be. Campbell-Boswell and Robertson showed that removal of cationic substances from platelet-poor plasma-derived serum with carboxymethyl Sephadex abolished its ability to support the mitogenic effect of angiotensin II. During this procedure, the serum was also concentrated by ultrafiltration through a filter with a 10 kd cutoff. Therefore, the mitogenic cofactor is bound by carboxymethyl Sephadex or has a molecular weight less than 10 kd.

The mitogenic activity of angiotensin II under appropriate experimental conditions in vitro suggests that smooth muscle cells may respond similarly in vivo. This possibility was addressed in a study by Daemen et al in which angiotensin II was administered to rats by continuous subcutaneous infusion. The labeling index of smooth muscle cells in the carotid arterial media increased significantly both in unmanipulated vessels and in those subjected to balloon catheter injury. In the latter vessels, in which a neointima had been allowed to develop, angiotensin II infusion significantly increased the intimal smooth muscle cell labeling index also. However, it is not clear from this study whether there was an induction of smooth muscle cell proliferation by angiotensin II infusion, because the data do not rule out the possibility of an increase in cell ploidy.

The relative lack of potency of angiotensin I as a vasoconstrictive hormone has led to the development of synthetic ACE inhibitors as treatments for hypertension. Recently, interest has begun to focus on the use of ACE inhibitors to restrict the development of pathological changes in arteries. Kuriyama et al found that the ACE inhibitor captopril had no effect on the growth of the A7r5 embryonic rat thoracic aortic smooth muscle cell line in 10% fetal calf serum. This may reflect a lack of conversion of angiotensin I to angiotensin II under normal culture conditions. It may alternatively be the consequence of a restricted supply of angiotensin I, because Andre et al have shown that rat aortic smooth muscle cells are able to convert exogenous angiotensin I to angiotensin II.

The use of ACE inhibitors in studies of smooth muscle cell replication in vivo is associated with some serious difficulties of interpretation. In addition to catalysis of the conversion of angiotensin I to angiotensin II, ACE also cleaves a dipeptide from bradykinin and thus inactivates bradykinin. ACE inhibitors therefore reduce circulating levels of angiotensin II and increase circulating levels of bradykinin. Bradykinin is itself mitogenic for smooth muscle cells in vitro. Because angiotensin II causes release of aldosterone from the adrenal zona glomerulosa, ACE inhibitors may reduce circulating levels of this hormone. Also, angiotensin II causes increased sympathetic tone and increases vasopressin release through an effect on the central nervous system as well as facilitating the release of catecholamines from peripheral adrenergic nerve terminals.

Overturf et al administered the ACE inhibitor enalapril orally to normotensive and one-kidney, one clip hypertensive rabbits fed a normal or cholesterol-enriched diet. Drug treatment was associated with significant reductions in aortic weight and aortic cholesterol and triglyceride content in normally fed animals, both normotensive and hypertensive. These effects were not observed in the corresponding cholesterol-fed groups. Although one cannot attribute the effects of enalapril on aortic lipid content in normally fed animals directly to effects on smooth muscle cell proliferation, it is possible cautiously to conclude that ACE inhibition may have beneficial effects on arteries injured by hypertension. In contrast to the lack of effect of enalapril in rabbits rendered hyperlipidemic by dietary manipulation, beneficial effects have been obtained by ACE inhibition with captopril in other hypercholesterolemic animal models. These include the cholesterol-fed cynomolgus monkey and the Watanabe heritable hyperlipidemic rabbit. Powell et al investigated the effect of oral administration of the ACE inhibitor cilazapril to rats in which the left common carotid artery was injured with a balloon catheter. This procedure induces the replication of smooth muscle cells in the tunica media, accompanied by migration to and proliferation in the tunica intima. Cilazapril significantly inhibited the accumulation of smooth muscle cells in the intima and also decreased the proportion of the arterial circumference adjacent to neointima. Of course, this does not prove that cilazapril inhibits smooth muscle cell proliferation in vivo, because this effect could equally well be caused by inhibition of extracellular matrix accumulation or of smooth muscle cell migration. Indeed, the reduced circumferential extent of the neointima in cilazapril-treated animals supports the latter hypothesis. If cilazapril simply inhibited proliferation, smooth muscle cells would still be expected to be present in the intima, because nonproliferating smooth muscle cells do migrate to the intima in vivo. Because large portions of the arterial circumference were free of neointima, it is likely that cilazapril inhibits smooth muscle cell migration and may also inhibit smooth muscle cell proliferation. Another ACE inhibitor, benazepril, inhibits migration in this model but has no effect on smooth muscle.
Epinephrine and norepinephrine are synthesized ultimately from the amino acid phenylalanine. The major sites of synthesis are postganglionic sympathetic nerve fibers and the adrenal medulla. The plasma levels of epinephrine and norepinephrine in rats are 1.1 and 4.0 \( \mu \text{M} \), respectively; the values in normal human plasma are 0.2 and 2.7 \( \mu \text{M} \), respectively. Sympathetic agonists stimulate the growth of arterial smooth muscle cells in vitro. Blaas and Boissel\(^9\) showed that epinephrine dose-dependently stimulates the proliferation of rat thoracic aortic smooth muscle cells in secondary culture, the minimum effective concentration being 1 nM. In this study, norepinephrine and the nonselective \( \beta \)-adrenergic receptor agonist isoproterenol were also found to stimulate cellular proliferation. The role of \( \beta \)-adrenergic receptors in the stimulation of proliferation was confirmed by showing that the nonselective \( \beta \)-adrenergic receptor antagonist propranolol significantly inhibited the response to epinephrine. However, there was no effect of propranolol or of the nonselective \( \alpha \)-adrenergic receptor antagonist phenolamine, both at a concentration of 10 \( \mu \text{M} \), on the proliferation of control cultures growing in calf serum. Bell and Madri\(^8\) found that norepinephrine (1 \( \mu \text{M} \)) stimulates the proliferation of bovine aortic smooth muscle cells growing in secondary culture. Epinephrine and norepinephrine stimulate with equal potency the proliferation of rat thoracic aortic smooth muscle cells growing in fetal calf serum, but epinephrine is more efficacious.\(^8\) Peak effects were produced at a concentration of 1 \( \mu \text{M} \). The proliferation of the A7r5 smooth muscle cell line is stimulated by 10 \( \mu \text{M} \) norepinephrine and is inhibited by the \( \alpha \)-adrenergic receptor antagonist bunazocine and by the \( \beta \) and \( \beta \)-adrenergic receptor antagonist labetalol.\(^9\)

Similar findings were reported by Nakaki et al., who investigated the effects of catecholamines on the restimulation of DNA synthesis in quiescent cloned rat aortic smooth muscle cells in serum-free medium. In subconfluent cultures, norepinephrine stimulated DNA synthesis at concentrations at and greater than 100 \( \mu \text{M} \). In confluent cultures, the stimulatory effect was seen only at concentrations greater than 100 nM. These effects were inhibited by the nonselective \( \alpha \)-adrenergic receptor antagonist phentolamine and by the \( \alpha \)-adrenergic receptor antagonist prazosin. The \( \alpha \)-adrenergic receptor agonist phenylephrine also stimulated DNA synthesis, but with lower potency than norepinephrine. Norepinephrine was found to inhibit the restimulation of DNA synthesis in cloned cells of high passage number, an effect apparently caused by \( \beta \)-adrenergic receptor stimulation, because it was blocked by the selective antagonist butoxamine. This compound also augmented the stimulatory effects of norepinephrine on DNA synthesis in cells of lower passage number. These results led the authors to conclude that stimulation of \( \alpha \)-adrenergic receptors stimulates arterial smooth muscle cell growth but that stimulation of \( \beta \)-adrenergic receptors has an inhibitory effect. This runs counter to the conclusion of Blaas and Boissel\(^8\) that isoproterenol stimulates smooth muscle cell proliferation, but it is possible that this discrepancy is related to the rather different assay conditions used in the two studies. At any rate, we may conclude that stimulation of the \( \alpha \)-adrenergic receptor induces or augments the proliferation of smooth muscle cells in vitro.

This hypothesis also appears to hold true in vivo. Epinephrine stimulates DNA synthesis in the cells of the aortic tunica media in rabbits fed a cholesterol-enriched diet;\(^2\) presumably, these are smooth muscle cells. Bevan\(^3\) found that sympathetic denervation of the ear artery by removal of the superior cervical ganglion in young rabbits causes a significant reduction in smooth muscle cell DNA synthesis. Chemical sympathectomy with 6-hydroxydopamine causes a reduction in the number of smooth muscle cells in rabbit aortic tunica media.\(^4\) In rats sympathectomized by a combination of immunological and chemical means, there is a significant reduction in the number of medial smooth muscle cell layers in large and small mesenteric arteries, but not in the superior mesenteric artery.\(^5\) Medial cross-sectional area was also reduced in large mesenteric arteries. Administration of the \( \alpha \)-adrenergic receptor agonist methoxamine to chickens results in the formation of thoracic aortic intimal accumulations of smooth muscle cells.\(^9\) Another \( \alpha \)-adrenergic receptor agonist, phentolamine, does not stimulate DNA synthesis in the thoracic aorta of normal rats.\(^6\) These disparate results may be related to differences in aortic morphology between chickens and rats; in chickens, there are occasional smooth muscle cells in the subendothelial intima,\(^6\) but these are not found in normal rats.\(^9\) Perhaps in the uninjured vessel increased \( \alpha \)-adrenergic receptor stimulation by exogenous agonist provokes the prolifer-
Eicosanoids

The eicosanoids are a family of products derived from the oxidation of fatty acids and include the prostaglandins, prostacyclins, thromboxanes, and leukotrienes.

An appreciable amount of evidence now suggests that arterial smooth muscle cells can synthesize eicosanoids. Huttner et al.100 showed that guinea pig aortic smooth muscle cells secrete prostaglandins of the E and I series into the culture medium when supplied with appropriate fatty acid precursors. Cornwell et al.101 were able to attribute this to de novo synthesis of prostaglandins rather than release from preformed stores by showing that the prostaglandin endoperoxide synthetase (cyclooxygenase) inhibitor indomethacin reduced the concentrations of prostaglandin (PG) E and PGF, in conditioned medium. PGE, PGF, and PGF, are synthesized by porcine aortic smooth muscle cells supplied with exogenous arachidonic acid, but there is no secretion of thromboxane A,102 Using bovine pulmonary arterial smooth muscle cells, Menconi and colleagues103 detected synthesis of PGF, and PGD, which was augmented by exogenous arachidonic acid. There was no detectable synthesis of PGE or thromboxane A,. However, punch samples of bovine pulmonary arterial tunica media synthesized PGE, PGF, and thromboxane A,. PGF, synthesis was not determined. Larrue et al.104 also compared the prostaglandin synthetic activity of rabbit aortic smooth muscle cells in intact artery and in culture. PGE, PGF, and PGF, were all synthesized in response to addition of arachidonic acid. The major product, both ex vivo and in vitro, was PGF,.

Thromboxane A, production by rat thoracic aortic smooth muscle cells was documented by Ishimuta et al.105,106 Thromboxane A, was also synthesized by excised thoracic aorta, but it is not possible to attribute this exclusively to smooth muscle cells, because endothelial cells and fibroblasts can also synthesize thromboxane A,.103

Canine and human coronary arteries synthesize leukotriene (LT) C, LTD,, and LTE, when incubated in vivo with arachidonic acid and the calcium ionophore A23187.107 Cultured rabbit aortic smooth muscle cells have been shown to generate lipoxygenase products from exogenous arachidonic acid.108 Human umbilical arterial smooth muscle cells generate PGE, PGF, PGF, 12-hydroxyptadecatrienoic acid, and 11-hydroxy-eicosatetraenoic acid from exogenous arachidonate in vitro.108 PGE, is the major metabolite. All of these are cyclooxygenase products; no evidence was found for processing of exogenous arachidonic acid via the lipoxygenase pathway.

Prostaglandin synthesis by smooth muscle cells has been shown to be stimulated by a variety of factors. Interleukin-1 stimulates prostaglandin synthesis by smooth muscle cells growing in culture.109-111 PDGF dose-dependently stimulates PGF synthesis by porcine aortic smooth muscle cells in culture, at concentrations of 100 pg/ml and higher. Similar effects, but at higher PDGF concentrations, were found in porcine aortic rings denuded of endothelium.112 The same authors also found that PDGF-stimulated PGF, synthesis was markedly lower in human atherosclerotic femoral and carotid arteries than in ostensibly normal vessels. Morphological classification of human atherosclerotic lesions showed that both basal and PDGF-stimulated PGF, production were reduced in fatty streaks, intimal thickenings, fibrous plaques, and complicated lesions. In lesions classified as lipid deposits and fatty dots, basal production of PGF, was not significantly reduced, but the response to PDGF was blunted.

A number of groups have found stimulatory effects of vasoactive agents on smooth muscle cell prostaglandin production. Vasopressin increases PGF, production by the A10 smooth muscle cell line, an effect augmented by epidermal growth factor.113 Other vasoactive agents that augment PGF, production include bradykinin,114 angiotensins I and II but not angiotensin III,103 and serotonin.114

The ability of prostanoids to modulate the proliferation of arterial smooth muscle cells in vitro was first demonstrated by Huttner et al.100 The growth of guinea pig aortic smooth muscle cells was dose-dependently inhibited by PGE, and PGE, the minimum effective concentrations being 2 and 20 µM, respectively.

Pietilä et al.115 confirmed and extended these results. They incubated secondary cultures of rabbit aortic smooth muscle cells with 10 µM PGE, PGE, PGF, or PGF,. Significant inhibition of the incorporation of [3H]thymidine into DNA was observed with all these prostaglandins; the maximum inhibitory effect was a 40% reduction in incorporation produced by PGE,.

Although several groups subsequently have reported the inhibitory actions of E-series prostaglandins on arterial smooth muscle cell proliferation in vitro, it has become clear that study conditions are critical determinants of the observed effect. In the studies of Huttner et al.,100 Pietilä et al.,115 Sjölund et al.,116 Smith et al.,117 and Orekhov et al.,118 E-series prostaglandins inhibited the proliferation of arterial smooth muscle cells growing continuously in serum. Loesberg et al.119 rendered human aortic smooth muscle cells quiescent by culturing them for 3 days in 0.5% human serum. Cell growth was restored by addition of 1% serum supplemented with PDGF, and PGE, was added at various time intervals later. Addition of PGE, (25 nM) between 0 and 12 hours after restimulation inhibited [3H]thymidine incorporation into DNA, but addition 14–18 hours after restimulation was without effect. Nilsson and Olsson120 used a similar protocol to examine the effects of PGE, on rat thoracic aortic smooth muscle cells. Restimulation of quiescent cells by addition of PDGF was inhibited by PGE, (140 nM), but only if it was given within 6 hours of restimulation. Using the A10 embryonic rat thoracic aortic smooth muscle cell line, Owen121 found that a minimal restimulation of quiescent cells by addition of insulin was significantly and dose-depen-
dently augmented by PGE\(_1\). The half-maximal effect was seen at a concentration of 4 \(\mu M\). No such stimulatory effect was observed in quiescent cells that were restimulated with 10% fetal calf serum or with PDGF plus insulin. Addition of PGE\(_1\) to cultures growing normally in fetal calf serum, PDGF, or insulin significantly and dose-dependently inhibited DNA synthesis, the half-maximal effect being produced by a concentration of 20 \(\mu M\). It therefore appears that the inhibitory effect of PGE\(_1\) is produced either by administration to proliferating cells or by administration to quiescent cells within a few hours of restimulation. There is a crucial difference, however, in sensitivity between these two protocols. The concentration of PGE\(_1\) required to inhibit the proliferation of growing cells was on average 100 times greater than that required to inhibit the restimulation of quiescent cells.

Inhibitory effects on arterial smooth muscle cell proliferation have also been noted with PGA\(_1\),\(^{117}\) PGA\(_2\),\(^{117}\) FGB,\(^{117}\) PGD\(_2\),\(^{117,118,120}\) PGE\(_1\),\(^{100,111,117,119-120}\) and PGJ\(_2\).\(^{117}\) Effects with F-series prostaglandins have been contradictory. Pietila et al\(^{116}\) found significant inhibitory actions with PGF\(_{2\alpha}\) (10 \(\mu M\)) and PGF\(_{2\beta}\) (10 \(\mu M\)), but Cornwell et al\(^{110}\) found significant stimulation. No effect was found with PGF\(_2\gamma\) (0.6 \(\mu M\)) by Nilsson and Olsson\(^{120}\) or at unspecified doses by Smith et al\(^{117}\) and Loesberg et al.\(^{119}\)

Prostacyclin (PGI\(_2\)) in the form of its relatively stable analogue carbacyclin, has been found to inhibit the incorporation of \([^3H]\)thymidine into DNA of human aortic intimal smooth muscle cells growing in primary culture, at micromolar concentrations.\(^{118,122,123}\) Similar effects were noted with authentic prostacyclin by Morisaki et al.\(^{124}\) using rabbit aortic smooth muscle cells growing in secondary culture. Prostacyclin was without effect, at concentrations up to 100 \(\mu M\), on the restimulation of growth in quiescent bovine aortic smooth muscle cells.\(^{125}\)

Ishimitsu et al\(^{105,106}\) have shown that a stable thromboxane A\(_2\) analogue, 9,11-epithio-11,12-methano-thromboxane A\(_2\), dose-dependently stimulates the incorporation of \([^3H]\)thymidine into DNA of rat thoracic aortic smooth muscle cells, with a maximal effect at a concentration of 10 \(\mu M\). This concentration also significantly prolonged the doubling time. Similar results were achieved by Akopov et al,\(^{125}\) using the stable thromboxane A\(_2\) analogue U46619 at a concentration of 0.7 \(\mu M\) to stimulate the proliferation of human aortic intimal smooth muscle cells. However, the thromboxane synthetase inhibitors sodium 5-(3-pyridinylmethyl)benzo-furan-2-carboxylate and dazoxiben both failed to inhibit the proliferation of canine arterial smooth muscle cells growing in fetal calf serum.\(^{126}\)

LTB\(_4\) was initially found to inhibit rabbit aortic smooth muscle cell growth in culture, the half-maximal effect being produced at a concentration of 28 \(\mu M\).\(^{117}\) Later, Palmberg et al\(^{127,128}\) showed that LTB\(_4\), LTC\(_4\), and LTD\(_4\) all significantly stimulate proliferation in rat thoracic aortic smooth muscle cells growing in primary culture, with maximal effects being produced at a concentration of 10 nM. LTD\(_4\) produced the biologically inactive isomer of LTD\(_4\), (5S,12S)-dihydroxy-(6,8,10,14)-eicosatetraenoic acid. LTB\(_4\), LTC\(_4\), and LTD\(_4\) stimulated DNA synthesis in smooth muscle cells in serum-free medium at concentrations as low as 10 fM. These cells cannot be characterized uniformly as quiescent, because 15% of them incorporated \([^3H]\)thymidine into DNA in the absence of leukotrienes. The capacity of leukotrienes to propel quiescent cells into the cell cycle remains undetermined. The growth stimulatory effect of LTB\(_4\), but not of LTC\(_4\), was inhibited by indomethacin and aspirin, suggesting that this effect is modulated by a cyclooxygenase product.\(^{127}\) Brinkman et al\(^{108}\) investigated the effects of inhibitors of lipoxigenase on the proliferation of human umbilical arterial smooth muscle cells in vitro. Both nordihydroguaiaretic acid and caffeic acid inhibited proliferation, suggesting an involvement of lipoygenase products. There was no evidence in these cells of metabolism of exogenous arachidonic acid via the lipoxigenase pathway, leading the authors to suggest that lipoxigenase metabolism of endogenous fatty acids other than arachidonate may play a role in the control of smooth muscle cell proliferation.

Investigation of the effects of drugs that interfere with endogenous eicosanoid synthesis is a potentially useful way of obtaining information regarding the role of this process in the modulation of smooth muscle cell proliferation. The major mechanism of action of the glucocorticoids is inhibition of phospholipase A\(_2\).\(^{46}\) It is possible, at least in the rat model, that glucocorticoids may also interfere with the release or activity of histamine and serotonin.\(^{129}\) Dexamethasone and cortisone also reduce levels of cyclooxygenase mRNA in cultured vascular smooth muscle cells, thereby reducing its activity.\(^{130}\) The effects of steroids on smooth muscle cell proliferation are discussed above. It is possible that their growth-inhibitory actions are the consequence of the impaired biosynthesis of growth-stimulatory eicosanoids such as thromboxane A\(_2\),\(^{105,106}\) LTB\(_4\), LTC\(_4\), LTD\(_4\),\(^{127,128}\) and perhaps PGF\(_{2\alpha}\).\(^{101}\) Two synthetic inhibitors of phospholipase A\(_2\), \(\alpha\)-bromophenacylbromide and mepracine, have been shown to inhibit the proliferation of human umbilical arterial smooth muscle cells in vitro.\(^{108}\) Hauss et al\(^{42}\) found that aspirin inhibited the proliferation of porcine aortic smooth muscle cells in secondary culture. The proliferation of aortic smooth muscle cells isolated from rats dosed with aspirin was also inhibited. However, Lindblad et al\(^{126}\) found no effect of aspirin, at concentrations up to 560 \(\mu M\), on the growth of canine carotid arterial smooth muscle cells in fetal calf serum. Another cyclooxygenase inhibitor, ibuprofen, inhibited proliferation of these cells at a concentration of 240 \(\mu M\). Indomethacin has been found either not to affect smooth muscle cell proliferation in vitro\(^{104}\) or to augment it.\(^{124,125}\) The effects of in vivo administration of cyclooxygenase inhibitors are discussed in detail in the section dealing with antplatelet drugs, because these agents are potent inhibitors of platelet aggregation.

In light of the generally negative findings with cyclooxygenase inhibitors on smooth muscle cell proliferation in vitro, it is tempting to speculate that any growth-stimulatory eicosanoid or eicosanoids inhibited by glucocorticoid treatment are lipoygenase products.

Cicletanine is a diuretic that stimulates the synthesis of prostacyclin in rat thoracic aortic smooth muscle cells, both from endogenous and exogenous arachidonic acid.\(^{123}\) Cicletanine inhibits the restimulation of quies-
cent rat mesenteric arterial smooth muscle cells in response to serum or PDGF.\textsuperscript{134}

**Heparinoids**

Heparin is a heterogeneous group of glycosaminoglycans and is particularly abundant in lung, liver, and intestinal mucosa. It is found intracellularly in mast cells, which accumulate in most organs around small blood vessels.\textsuperscript{135} Heparan sulfate is a structurally related compound that is present in extracellular matrix and at cell surfaces and that has been shown to be present in the aorta.\textsuperscript{136,137}

Hoover et al\textsuperscript{138} showed that heparin dose-dependently inhibits the proliferation of rat aortic smooth muscle cells growing in 20% fetal calf serum, with 50% inhibition of growth occurring at a concentration of approximately 10 $\mu$g/ml. This corresponds to 0.67 $\mu$M, assuming a molecular weight of 15 kd. There was no difference in growth-inhibitory activity between anticoagulant and nonanticoagulant heparin fractions. Because the latter species has low affinity for antithrombin III, it is unlikely that the growth-inhibitory properties of heparin are related to inactivation of the proteolytic properties of thrombin. Reilly et al\textsuperscript{139} went on to show that the inhibition of bovine aortic smooth muscle cell proliferation is maximized by preincubation of quiescent cells with heparin for 48 hours before reinduction of serum.

The effects of heparin on smooth muscle cell growth have also been investigated in animal models of arterial injury. Clowes and Karnovsky\textsuperscript{140} administered heparin by intravenous infusion to rats in which the common carotid artery was injured by air drying. Infusion began 24 hours after injury. There was no difference in neointimal thickening between control and heparin-treated rats 10 days after injury. During the next 4 days, there was considerable expansion of the intima that was inhibited by heparin, with the result that at 14 days after injury the neointimas of injured carotid arteries were significantly smaller in the heparin-treated group. This difference was attributed to inhibition of smooth muscle cell proliferation. Similar results were reported by Guyton et al,\textsuperscript{141} who also showed a dose-related inhibitory action of heparin on neointimal thickening. This effect was observed with both anticoagulant and non-anticoagulant heparin fractions. Reduction of neointimal thickening was also observed in the balloon-injured rabbit aorta after continuous intravenous infusion of heparin.\textsuperscript{142} Local release of heparin from a gel enclosed in a periadventitial cuff around the rat common carotid artery significantly inhibits balloon catheter-induced neointimal thickening in this vessel.\textsuperscript{143} Intravenous administration of heparin to rats for 14 days significantly inhibits the increase in the DNA content of the common carotid artery caused by balloon catheter injury.\textsuperscript{144,145} Further direct evidence of an effect of heparin on arterial smooth muscle cell proliferation in vivo was obtained by Majesky et al,\textsuperscript{146} who measured the [%H]thymidine labeling index of smooth muscle cells in the rat common carotid artery after balloon injury. Intravenous infusion of heparin significantly reduced the labeling index measured 33 hours after injury. Delay of commencement of heparin administration until 18 hours after injury did not prevent the inhibitory effect, but delay until 27 hours after injury abolished the inhibition.

This is rather surprising in view of the finding by Clowes and Karnovsky\textsuperscript{140} that heparin inhibits neointimal thickening in the air-drying model of arterial injury through a mechanism that becomes manifest 10 days after injury. These results suggest that heparin inhibits both medial smooth muscle cell proliferation and neointimal expansion. This latter effect could be the result of inhibition of neointimal smooth muscle cell proliferation, of synthesis of extracellular matrix in the neointima, or of migration of smooth muscle cells from the media to the intima. There is experimental support for all of these alternatives.\textsuperscript{144,145} The mechanism by which heparin inhibits smooth muscle cell proliferation has not been established, although several hypotheses may be considered. In smooth muscle cells growing in vitro, heparin binds via specific, high-affinity receptors\textsuperscript{147,148} and prevents progression through the G, phase of the cell cycle.\textsuperscript{149} The specific suppression by heparin of the elevation of the levels of the mRNAs for c-myb and 2F1 may constitute a mechanism by which the antiproliferative effect is exerted. Another possible mechanism involves inhibition of intracellular Ca\textsuperscript{2+} mobilization through an effect on the inositol 1,4,5-trisphosphate receptor.\textsuperscript{150-151} A third alternative mechanism of antiproliferative action involves the capacity of heparin to bind to specific structural domains in the bFGF molecule.\textsuperscript{152,153} Although this mechanism may not be important for the inhibition by heparin of smooth muscle cells growing in serum, which contains little bFGF, it could play a role in vivo. Lindner et al\textsuperscript{154} have suggested that, after balloon catheter injury to the rat common carotid artery, bFGF is released from wounded smooth muscle cells and stimulates replication among their neighbors. Exogenous heparin could compete for bFGF with binding sites in the extracellular matrix. The growth factor would therefore partition into the systemic circulation, where clearance mechanisms would supervene. Lastly, heparin has been shown to disrupt the deposition of thrombospondin in the extracellular matrix of smooth muscle cells.\textsuperscript{155,156} Thrombospondin appears to be functionally essential for smooth muscle cell proliferation in vitro.\textsuperscript{157}

Unfortunately, the hypothesis that endogenous heparinoids play a role in the control of arterial smooth muscle cell replication cannot be tested. There are no agents currently available that can reduce or remove the influence of these molecules, and this makes it difficult to evaluate their biological significance. It has been suggested that a smooth muscle cell growth-inhibitory heparinoid is released from endothelial cells on exposure to a platelet-derived endoglycosidase.\textsuperscript{158-160} Synthetic inhibitors of this enzyme may help to elucidate the role of heparinoids in smooth muscle cell growth.

**Serotonin**

Serotonin (5-hydroxytryptamine) is found in platelets, and also in the mast cells of rodents and cattle, but not in those of humans.\textsuperscript{161} Serotonin initiates DNA synthesis in quiescent bovine aortic smooth muscle cells, with a maximal effect at a concentration of 1 $\mu$M or 100 $\mu$M.\textsuperscript{162} Serotonin (100 nM) also stimulates DNA synthesis in continuously growing rat thoracic aortic smooth muscle cells.\textsuperscript{72} Similar effects were noted at this concentration by Bell and Madri\textsuperscript{160} using bovine aortic smooth muscle cells. To put these concentrations in
The evidence for mitogenic effects of thrombin on vascular smooth muscle cells is controversial. Ishida and Tanaka grew rabbit aortic smooth muscle cells in 10% fetal calf serum, then added thrombin at the same time as complete serum withdrawal. Concentrations up to 10 nM produced no effect, and cell numbers actually decreased over a period of 7 days when exposed to a concentration of 100 nM. Significant mitogenic effects of 10 nM thrombin were observed in neonatal rat vascular smooth muscle cells by Huang and Ives and in bovine aortic smooth muscle cells by Graham and Alexander. Berk et al. failed to detect any effect of α-thrombin on the proliferation of quiescent rat vascular smooth muscle cells at concentrations up to 100 nM, despite marked increases in protein synthesis, intracellular calcium ion concentration, and intracellular pH. These results suggest that the proteolytic activity of thrombin may be an important determinant of its activation of signal transduction mechanisms in vascular smooth muscle cells. The wide spectrum of proteolytic activity of thrombin (reviewed by Fenton) encourages the speculation that a proteolytically modified polypeptide or protein may be the proximal agonist in smooth muscle cell cultures treated with α-thrombin. This hypothesis is supported by the studies of Walz et al. who found that the contraction of strips of rabbit aorta to different preparations of thrombin was absolutely dependent on proteolytic activity, and also by the discovery that the thrombin receptor on platelets is activated by proteolytic modification of the receptor protein. However, bovine aortic smooth muscle cells have been shown to respond mitogenically to nanomolar concentrations of both α-thrombin and diisopropylfluorophosphate-conjugated, enzymatically inactive α-thrombin. Hirudin, a leech polypeptide that specifically inhibits thrombin, blocked its mitogenic effect. The authors suggest that hirudin interacts with a part of the thrombin molecule, distinct from its active proteolytic site, which is responsible for conferring mitogenic activity. Another possibility is that there is more than one type of thrombin receptor, with proteolytic activation being required by some but not by others.

Hirudin and other inhibitors of thrombin such as 3,4-dihydro-3-benzyl-6-chloromethylcoumarin could be used to elucidate the role of thrombin in the control of vascular smooth muscle cell proliferation in vivo. Warfarin is a congener ofbishydroxycoumarin that interferes with the hepatic synthesis of the vitamin K-dependent clotting factors VII, IX, X, and II, prothrombin. It therefore produces a hypoprothrombinemia and reduces the capacity of the blood to generate thrombin. Oral administration of warfarin to rats subjected to air-drying injury to the common carotid artery significantly reduces the neointimal thickening measured 4 weeks after injury.

Somatostatin

Somatostatin is a tetradecapeptide hormone that inhibits the release of growth hormone from the pituitary. It is found in the hypothalamus and in ganglia of the sympathetic nervous system. Nanomolar concentrations of angiopeptin, an octapeptide analogue of somatostatin, inhibit DNA synthesis in explants of rat common carotid artery, suggesting that this material has an effect on vascular smooth muscle cell proliferation that is not mediated by growth hormone. Subcutaneous administration of angiopeptin inhibits DNA synthesis and neointimal thickening in the rat common carotid artery after air-drying injury, but this property is not shared by some other analogues of somatostatin that also inhibit the release of growth hormone. Angiopeptin also inhibits DNA synthesis and neointimal thickening in balloon-injured rabbit arteries.

Thrombin

Thrombin (factor IIa) is a serine protease that catalyzes the conversion of fibrinogen to fibrin and activates factors V, VIII, and XIII. It is derived from prothrombin (factor II) by the coordinated actions of factor V, factor Xa, phospholipid, and calcium ions and is therefore formed at sites of vascular injury. Its physiological inhibitors include antithrombin III, α2-macroglobulin, and α1-antitrypsin, but thrombin can be deposited on subendothelial extracellular matrix in such a way that it is protected from inhibition by antithrombin III. The approximate maximum concentration of thrombin achieved during blood coagulation is 140 nM.
Drugs That Modify Vascular Smooth Muscle Cell Proliferation

Antiplatelet Drugs

A wide variety of agents has been used in humans to inhibit platelet aggregability. Inhibition of cyclooxygenase results in reduced platelet aggregation. The effects of inhibitors of this enzyme on the proliferation of vascular smooth muscle cells in vitro are discussed in the section dealing with eicosanoids.

Cyclooxygenase inhibitors have been used in a number of in vivo models of smooth muscle cell proliferation. In the cuffed rabbit carotid artery, indomethacin treatment had no significant effect on intimal thickening.44 Neither aspirin nor flurbiprofen, another cyclooxygenase inhibitor, influenced the formation of a neointima in the rat carotid artery subjected to air-drying injury.182 August and Tilson182 also found aspirin to be without effect in this model. Aspirin did not influence the degree of intimal thickening occurring in canine carotid arteries after endarterectomy.164 Aspirin was without effect on smooth muscle cell proliferation in the injured rabbit ear artery.165 However, Völker and Faber166 found that dietary administration of aspirin to rats resulted in a significant reduction in balloon catheter-induced neointimal thickening in the common carotid artery. This treatment also reduced the thickness of the media.

Dipyridamole has a number of activities, most of which appear to be related to inhibition of cyclic AMP phosphodiesterase.187 In addition to its effects on platelet aggregation, it is a vasodilator and it inhibits the uptake of adenosine into erythrocytes.188 It has also been suggested that it decreases the release of PDGF from human platelets during blood clotting because it inhibits the mitogenic effects of serum on human skin fibroblasts.189 It is also an antioxidant.190 Dipyridamole inhibits the proliferation of canine carotid arterial smooth muscle cells in vitro, at a concentration of 20 μM, which is 10 times greater than the plasma concentration achieved in clinical use.126 No effect was seen at lower concentrations. Morisaki et al190 found that 1 μM dipyridamole significantly increased the proliferation rate of guinea pig aortic smooth muscle cells growing in serum. Oral administration of dipyridamole to baboons injured by continuous infusion of homocystine resulted in a significant reduction in intimal lesion score.191 This score was derived by counting layers of intimal smooth muscle cells in cross sections of the abdominal aorta and the iliac and femoral arteries. Administration of dipyridamole to cholesterol-fed dogs with autologous vein-to-artery grafts significantly reduced the intimal thickening measured 6 weeks later.192 There was no effect on the plasma concentrations of the major metabolites of thromboxane A2 and prostacyclin. Dipyridamole treatment significantly reduced the accumulation of smooth muscle cells in the intimas of rabbit ear arteries subjected to repeated mechanical injury, as did the structurally related compound AH-P719.185 In contrast to these positive findings, Koster et al193 found that dipyridamole significantly increased the number of smooth muscle cells in the aortic intima of cholesterol-fed rabbits.

A number of studies have investigated the effects of a combination of dipyridamole with aspirin on the development of vascular lesions. In a nonhuman vein graft model, this therapy reduced the intimal cross-sectional area measured 16 weeks after surgery.104 Hagen et al195 measured the degree of luminal narrowing in the region of Gore-Tex grafts inserted in the abdominal aortas of nonhuman primates. Aspirin/dipyridamole therapy reduced the degree of luminal narrowing in the aorta distal to the graft. However, the vessels were not fixed in situ under pressure, so it is not clear whether drug treatment affected postmortem aortic contraction or intimal hyperplasia. In a rabbit balloon injury model, treatment with aspirin and dipyridamole caused an increase in the intima/media ratio in the thoracic aorta 14 days after injury.196 Similar effects were noted by Murday et al197 in a rabbit vein graft model. Radic et al198 measured the rate of smooth muscle cell DNA synthesis, intimal nuclei per unit vessel circumference, and intima to intima-plus-media ratio in rabbits with balloon injury to the abdominal aorta. Combination therapy with aspirin and dipyridamole had no significant effect on any of these indexes. This therapy was also ineffective in a study reported by Rodgers et al199 in which stents were implanted in the left anterior descending coronary arteries of hypercholesterolemic pigs.

Ticlopidine, another inhibitor of platelet function, had no effect on the growth of rat aortic smooth muscle cells in serum.44 There is one preliminary report of inhibition by ticlopidine of neointimal thickening in a rabbit balloon injury model,200 but this compound was ineffective in the repeated mechanical injury model used on rabbit ear arteries by Ingerman-Wojenski and Silver.165

U-53,059, a thiazole that inhibits platelet aggregation, reduces the extent of the pulmonary arterial lesions caused in dogs by infection with Dirofilaria immitis.201 The authors suggest that this is due to reduced myointimal proliferation. However, the same group found no effect of U-53,059 on neointimal thickening in the balloon-injured rabbit aorta.202

It must be concluded from these studies that the case for an antiproliferative effect of antiplatelet therapy is not proved.

Calcium Antagonists

The calcium antagonists are a group of compounds that share the property of inhibiting the flux of Ca2+ ions into cells through a specific voltage-sensitive membrane channel, called the L-type channel. This channel is relatively impermeable to Ca2+ ions when the membrane is normally polarized but opens when a sufficient degree of depolarization is reached. In arterial smooth muscle cells, this influx of Ca2+ ions and subsequent elevation of the concentration of cytoplasmic-free Ca2+ is a critical element in the contractile process.

Many studies have focused on the influence of calcium antagonists on arterial smooth muscle cell proliferation in vitro, and these are summarized in Table 1. This table is subdivided into four groups of compounds, according to the scheme drawn up by the World Health Organization.215 It is interesting to note that, in general, the concentrations of calcium antagonists required to inhibit the onset of proliferation in restimulated quiescent cells are considerably lower than those required to inhibit growing cultures. For instance, the median con-
centration of dihydropyridine (Type II) calcium antagonists required to inhibit proliferation in arterial smooth muscle cells growing in serum is 100 μM; the median concentration in quiescent cultures is 1–2 μM. In other cell types, there is a relation between growth state and the ability to bind dihydropyridine calcium antagonists to L-type calcium channels. In the nonfusing muscle cell line BC3H1, specific binding of [[H]lsradipine was undetectable in cultures growing in 20% fetal calf serum. On reduction of the serum concentration to 0.5%, specific binding of [[H]lsradipine began to increase, reaching a peak 6 days after serum with-

| Table 1. Effects of Calcium Antagonists on Smooth Muscle Cell Proliferation In Vitro |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Type            | Compound        | Concentration (μM) | Measurement     | Stimulus        | Effect         | Cell origin     | Growth status  | Reference |
| I               | Verapamil       | 0.33*             | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 203        |
|                 | Verapamil       | 1                | DNA synthesis   | Serum           | Decrease       | Rabbit aorta    | Growing        | 204        |
|                 | Verapamil       | 5†               | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Verapamil       | 5†               | DNA synthesis   | EGF             | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Verapamil       | 10†              | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Verapamil       | 10               | DNA synthesis   | Serum           | No effect      | Human artery    | Primary        | 206        |
|                 | Verapamil       | 10               | Cell number     | Serum           | No effect      | Rabbit aorta    | Growing        | 209        |
|                 | Verapamil       | 10               | DNA synthesis   | PDGF            | Decrease       | Porcine aorta   | Quiescent      | 208        |
|                 | Verapamil       | 50               | DNA synthesis   | Serum           | Decrease       | Rabbit aorta    | Growing        | 210        |
|                 | Verapamil       | 100              | Cell number     | Serum           | No effect      | Rat aorta       | Growing        | 211        |
|                 | Verapamil       | 100†             | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 211        |
|                 | Sim 6080        | 5†               | Cell number     | Serum           | Decrease       | Rat aorta       | Quiescent      | 212        |
| II              | Nifedipine      | 0.1†             | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 211        |
|                 | Nifedipine      | 0.1*             | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 213        |
|                 | Nifedipine      | 1†               | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 211        |
|                 | Nifedipine      | 1-2*             | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 213        |
|                 | Nifedipine      | 2                | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Primary        | 213        |
|                 | Nifedipine      | 3.3†             | Cell number     | Serum           | Decrease       | Rat aorta       | Quiescent      | 134        |
|                 | Nifedipine      | 3.3†             | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 134        |
|                 | Nifedipine      | 3.3†             | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Growing        | 134        |
|                 | Nifedipine      | 5.4*             | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 203        |
|                 | Nifedipine      | 10               | Cell number     | Serum           | Decrease       | Rat aorta       | Growing        | 134        |
|                 | Nifedipine      | 10               | DNA synthesis   | PDGF            | Decrease       | Porcine aorta   | Quiescent      | 208        |
|                 | Nifedipine      | 10               | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Primary        | 211        |
|                 | Nifedipine      | 100              | Cell number     | Serum           | No effect      | Rat aorta       | Growing        | 210        |
|                 | Nifedipine      | 100              | Cell number     | Serum           | Decrease       | A7r5           | Growing        | 69         |
|                 | Nisoldipine     | 1-2*             | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 213        |
|                 | Nisoldipine     | 1-2*             | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 213        |
|                 | Nisoldipine     | 2                | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Primary        | 213        |
|                 | Nicardipine     | 1†               | DNA synthesis   | EGF             | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Nicardipine     | 5†               | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Nicardipine     | 5†               | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Nimodipine      | 1†               | Cell number     | Serum           | Decrease       | Rabbit aorta    | Quiescent      | 207        |
|                 | Nivardipine     | 100              | Cell number     | Serum           | No effect      | Rat aorta       | Growing        | 210        |
| III             | Diltiazem       | 10               | DNA synthesis   | Serum           | No effect      | Human artery    | Primary        | 123        |
|                 | Diltiazem       | 100              | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Growing        | 214        |
|                 | Diltiazem       | 100†             | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 211        |
|                 | Diltiazem       | 100              | Cell number     | Serum           | No effect      | Rat aorta       | Growing        | 210        |
|                 | Diltiazem       | 0.47*            | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 203        |
|                 | Diltiazem       | 1†               | DNA synthesis   | EGF             | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Diltiazem       | 5†               | DNA synthesis   | PDGF            | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Diltiazem       | 5†               | DNA synthesis   | Serum           | Decrease       | Rat aorta       | Quiescent      | 205        |
|                 | Diltiazem       | 100†             | Cell number     | Serum           | Decrease       | Rat artery      | Growing        | 134        |
| IV              | Flunarizine     | 2†               | Cell number     | Serum           | Decrease       | Rabbit aorta    | Quiescent      | 207        |

PDGF, platelet-derived growth factor; EGF, epidermal growth factor.
*IC50,
†Minimum effective concentration.
calcium channels from proliferating cells. If this were that Ca\(^{2+}\) influx via L-type channels is critical for proliferating cultures. The corollary of this argument is a loss of L-type maintained proliferation in growing cultures. Because high concentrations of calcium antagonists block other types of ion channel, the effects reported in growing cultures may not be related to specific blockade of calcium influx via L-type channels.

The hypothesis that influx of Ca\(^{2+}\) via L-type channels is necessary for arterial smooth muscle cells to achieve the transition from quiescence to active growth has been addressed in vivo. Administration of nifedipine orally to balloon-catheterized rats inhibited aortic smooth muscle cell proliferation, measured 48 hours after injury, if the compound was given between 8 and 20 hours after injury. Administration more than 30 hours after injury was ineffective, suggesting that smooth muscle cell proliferation is susceptible to the inhibitory influence of reduced Ca\(^{2+}\) ion influx via L-type channels only during a critical period 8–20 hours after balloon injury. These data suggest that nifedipine inhibits a process occurring during the activation of quiescent cells by balloon injury. Whether this is a direct effect or a consequence of the blood pressure–lowering effect of nifedipine is unknown.

In the same paper, Jackson et al. investigated the effects of nifedipine on balloon catheter-induced smooth muscle cell proliferation in the rabbit abdominal aorta. A dosage protocol that resulted in inhibition of aortic smooth muscle cell proliferation 2 days after balloon injury was ineffective if delayed by 7 days, proliferation being measured in this case 9 days after injury. This also indicates that nifedipine inhibits arterial smooth muscle cell proliferation at an early stage and presumably does not block at a point in the cell cycle that is traversed with every round of cell division.

The influx of calcium into smooth muscle cells can also be blocked by lanthanum, which displaces Ca\(^{2+}\) ions from specific binding sites on the cell membrane, and by the potassium channel opener minoxidil, which hyperpolarizes the cell membrane. Both of these agents inhibit smooth muscle cell proliferation in the rat thoracic aorta after balloon catheter injury.

Many studies have been carried out to investigate the antiatherogenic properties of calcium antagonists, usually in cholesterol-fed animals. These have been summarized previously. The results of these studies also suggest that calcium antagonists interfere with an early event in the development of experimental arterial lesions, an idea supported by Overturf. This argument has consequences for the use of calcium antagonists to treat human arterial disease. Administration of these drugs to individuals in whom smooth muscle cell proliferation is already an active process would be expected to be ineffective. Indeed, studies of their utility in percutaneous transluminal coronary angioplasty have yielded negative results. Strong positive support for the hypothesis that calcium antagonists inhibit the activation of quiescent smooth muscle cells comes from the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT). In this study, patients with angiographically proven mild coronary artery disease were randomly assigned to treatment for 3 years with either placebo or nifedipine. When angiograms were measured at the end of the treatment period, nifedipine therapy had no effect on the rate of progression of the lesions that were present at the beginning of the trial. However, the number of new lesions per patient was significantly lower. It therefore seems likely that calcium antagonists should show activity in other clinical settings in which quiescent arterial smooth muscle cells are stimulated to proliferate, such as organ transplantation or coronary bypass grafting. Calcium antagonists have shown beneficial effects in experimental models of vein bypass grafting.

An important question arising from the antiproliferative actions of calcium antagonists concerns the nature of the agonist or agonists stimulating Ca\(^{2+}\) ion influx via L-type channels, as this occurs only in response to membrane depolarization. What are the endogenous agents capable of promoting Ca\(^{2+}\) influx via these channels?

Thromboxane A\(_2\) has been shown to promote calcium influx into rabbit coronary artery smooth muscle cells by this route. Contraction of intact strips of this artery, evoked by application of nanomolar concentrations of the stable thromboxane A\(_2\) analogue 9,11-epithio-11,12-dimethano-thromboxane A\(_2\), was inhibited by approximately 50% by nanomolar concentrations of nifedipine. LTD\(_4\) produces contractions of strips of guinea pig basilar artery denuded of endothelium. The phasic component of these contractions is inhibited by submicromolar concentrations of nifedipine.

Endothelin is a 21-amino acid polypeptide that dose-dependently stimulates DNA synthesis in quiescent rat aortic smooth muscle cells, in the absence of serum. The peak effect is produced at a concentration of 1 nM and is completely blocked by treatment with 1 \(\mu\)M nifedipine. PDGF stimulates calcium influx into aortic smooth muscle cells via L-type channels. Nifedipine and verapamil both inhibit PDGF-stimulated DNA synthesis in aortic smooth muscle cells in vitro. \(\alpha\)-Adrenergic receptor agonists also stimulate calcium influx into arterial smooth muscle cells via L-type channels. The increase in intracellular Ca\(^{2+}\) concentration caused by application of norepinephrine to rat aortic smooth muscle cells is inhibited by verapamil and diltiazem.

Oxidized low density lipoprotein causes a slight contraction of strips of rabbit femoral artery, which is markedly potentiated by norepinephrine, phenylephrine, and serotonin. Diltiazem (10 \(\mu\)M) inhibits these contractions.

Therefore, thrombotic A\(_2\), LTD\(_4\), endothelin, PDGF, catecholamines, and oxidized lipoproteins are all capable of inducing calcium influx via L-type channels and are also potential mitogens in vivo. However, it is important to consider the likely provenance of each of these contractions.
these molecules, especially in the light of the report by Fingerle et al\textsuperscript{233} that arterial smooth muscle cell replication in the balloon-catheterized rat carotid artery is not affected by abolition of platelet adherence to the injured vessel. This suggests that the molecule or molecules mediating proliferation are derived from the plasma, arterial wall, or leukocytes adhering to the injured wall.

**Cyclosporin A**

Cyclosporin A is an immunosuppressive agent used to forestall tissue rejection after organ transplantation. It acts by inhibiting both the proliferation of T lymphocytes and their secretion of cytokines. In the balloon-injured rat common carotid artery, cyclosporin A treatment resulted in a reduction in neointimal volume.\textsuperscript{234} Because the drug had no effect on the proliferation of smooth muscle cells in vitro, the authors conclude that the inhibition of neointimal thickening was mediated by an effect on the immune system that resulted in impaired smooth muscle cell proliferation. Another possibility is that cyclosporin A treatment inhibits the migration of smooth muscle cells from the media to the intima. However, Ferns et al\textsuperscript{235} were able to detect an inhibitory effect of cyclosporin A on the growth of rabbit aortic smooth muscle cells in serum. This antiproliferative effect was accompanied by vacuolation of the cells, indicating the possibility of a mild toxicity. Cyclosporin A had no effect on the proliferation of smooth muscle cells in the rabbit common carotid artery after atraumatic removal of the endothelium.

**Trapidil**

Trapidil is a triazolopyrimidine that was originally developed as a coronary vasodilator and that has subsequently been found to lower elevated serum levels of low density lipoprotein and very low density lipoprotein in humans.\textsuperscript{236} It inhibits \(^{3}H\)thymidine incorporation into human thoracic aortic intimal smooth muscle cells growing in 10\% fetal calf serum, at concentrations at and greater than 5 \(\mu M\).\textsuperscript{236} The peak concentration achieved in rabbit plasma after subcutaneous administration is approximately 40 \(\mu M\)\textsuperscript{237}.

Oral administration of trapidil at a dose of 6 mg/kg/day to rats with air-drying injury to the common carotid artery significantly reduced the intima-to-media ratio measured at 14 days.\textsuperscript{238} Tiell et al\textsuperscript{239} administered trapidil orally to rats, starting 3 days before balloon catheter injury to the aorta. The daily dose was 180 or 360 mg/kg, the oral \(L_{D50}\) in rats is 235 mg/kg (\textit{Merck Index}, 1983). These treatment schedules both resulted in significant decreases in intimal thickening in the abdominal aorta, measured 14 days after balloon injury. The authors attribute this effect to inhibition of myointimal hyperplasia. One must question, however, the validity of a study in which very high doses of a vasodilator are given before balloon catheter injury. It is quite possible that reduced vascular tone in the aortas of trapidil-treated rats resulted in diminished balloon injury, and this could account for the differences in intimal thickening seen 2 weeks later.

Liu et al\textsuperscript{237} investigated the effects of trapidil in a rabbit model of postangioplasty restenosis. Animals were fed cholesterol and were subjected to focal balloon injury to the iliac arteries so that stenoses developed. These were dilated with an angioplasty balloon, and the degree of restenosis was determined 4 weeks later. Treated rabbits received subcutaneous trapidil, 60 mg/kg/day, beginning 2 days before balloon dilatation. There were significant reductions in both intimal and medial thicknesses in trapidil-treated animals when compared with controls. The authors tentatively attribute these beneficial effects to inhibition of smooth muscle cell proliferation. Determinations of serum cholesterol concentrations were not made; because trapidil has cholesterol-lowering properties, it is possible that the observed effects were related to differences in serum cholesterol concentration.

**Drugs That Interfere With Intracellular Mitogenic Signal Transduction Pathways**

**Calmodulin**

Calmodulin is a protein that mediates many of the intracellular effects of calcium ions. The complex between calmodulin and \(Ca^{2+}\) is disrupted by drugs such as W-7 and trifluoperazine. W-7 inhibits induction of DNA synthesis in quiescent rat aortic smooth muscle cells restimulated with serum\textsuperscript{213} or with epidermal growth factor or PDGF.\textsuperscript{205} Trifluoperazine also inhibited restimulation by these mediators.\textsuperscript{205}

**Cyclic Nucleotides**

Nitric oxide is believed to be an endogenous vasodilator generated by the endothelium. It causes an increase in cyclic guanosine monophosphate (GMP) concentration within smooth muscle cells by inhibiting cyclic GMP phosphodiesterase. Vasodilators that generate nitric oxide inhibit the proliferation of smooth muscle cells in vitro. Garg and Hassid\textsuperscript{240} showed that sodium nitroprusside, \(S-nitroso-N\)-acetlypenicillamine, and isosorbide dinitrate inhibit DNA synthesis and growth in rat aortic smooth muscle cells. Kariya et al\textsuperscript{241} obtained similar results with sodium nitroprusside using rabbit aortic smooth muscle cells.

Forskolin stimulates cyclic AMP formation by adenylate cyclase and inhibits the restimulation of proliferation by serum in quiescent rat cerebral microvascular smooth muscle cells.\textsuperscript{242} It also inhibits the growth of human aortic smooth muscle cells in serum.\textsuperscript{118} As already noted,\textsuperscript{109} angiopeptin, a somatostatin analogue, has recently been studied as a smooth muscle growth inhibitor. Although this approach was based on the notion that somatostatin inhibits release of pituitary hormones thought to be important in smooth muscle proliferation, this class of compounds has more general effects on protein secretion. These effects are believed to be due to \(G_{i}\) protein-induced decreases in cyclic AMP.\textsuperscript{243--246} The possibility that this pathway also directly mediates an inhibition of smooth muscle replication has not been explored.

**HMGCoA Reductase**

3-Hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase catalyzes the formation of mevalonic acid, a precursor in the biosynthesis of cholesterol. Several synthetic inhibitors of HMGCoA reductase have been developed as hypolipidemic agents, and some have been
investigated for possible effects on vascular smooth muscle cell proliferation.

Compactin significantly inhibited DNA synthesis in quiescent primate aortic smooth muscle cells restimulated with serum. This effect was reversed by addition of mevalonic acid to the cells but not by addition of cholesterol, suggesting that some other derivative of mevalonic acid is required for DNA synthesis in vascular smooth muscle cells. Mevinolin significantly inhibited DNA synthesis in quiescent bovine aortic smooth muscle cells restimulated with serum. It should be noted that products of mevalonic acid are incorporated into a number of cell proteins, including nuclear lamins.

In cholesterol-fed rabbits, mevinolin administration significantly reduced the neointimal cross-sectional area.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Category</th>
<th>Concentration</th>
<th>Measurement</th>
<th>Stimulus</th>
<th>Effect</th>
<th>Cell origin</th>
<th>Growth status</th>
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<td>268</td>
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</table>

PDGF, platelet-derived growth factor; NR, not reported.

*Cells were isolated from animals dosed orally, 70 mg/kg of body weight per day for 20 days.

†Minimum effective concentration.

‡Cells were isolated from animals dosed intraperitoneally, 0.5 mg/kg of body weight per day for 8 weeks.

§IC₅₀.
after air-drying injury to the femoral artery.\textsuperscript{231} Possibly, the reduced concentration of serum cholesterol in the mevinolin-treated group was responsible for this effect. However, in both control and treated groups, the serum cholesterol concentration was greatly elevated above normal, and it is conceivable that a direct effect on cellular proliferation contributed to the reduced neointimal thickening.

### Membrane Ion Transport

In many cell types, there is a close link between mitogenesis and elevation of cytoplasmic pH via a membrane Na\textsuperscript{+}/H\textsuperscript{+} exchange mechanism. Amiloride and its derivative, ethylisopropylamiloride, inhibit the Na\textsuperscript{+}/H\textsuperscript{+} exchange mechanism and prevent alkalinization of the cytoplasm. Bobik et al.\textsuperscript{252,253} have shown that ethylisopropylamiloride inhibits the proliferation both of quiescent rat aortic smooth muscle cells restimulated with serum and of cells growing exponentially in serum. Amiloride had similar effects but was less potent.

Monensin also inhibits cytoplasmic alkalinization by catalyzing the expulsion of sodium ions through the N\textsuperscript{+}/H\textsuperscript{+} exchange mechanism. It inhibits the proliferation of quiescent rat aortic smooth muscle cells incubated with either PDGF or serum, with an IC\textsubscript{50} of 5 μM.\textsuperscript{254}

Inhibition of the membrane Na\textsuperscript{+},K\textsuperscript{+}-ATPase with ouabain inhibits the proliferation of rat aortic smooth muscle cells growing in serum\textsuperscript{252} and depresses further the low rate of DNA synthesis in quiescent rabbit aortic smooth muscle cells.\textsuperscript{255}

### Microtubules

Colchicine causes disassembly of microtubules in cultured rat aortic smooth muscle cells and stimulates the proliferation of these cells in serum-free medium.\textsuperscript{256} When quiescent cells were exposed to PDGF or to serum, colchicine inhibited DNA synthesis. The authors suggest that disassembly of microtubules is an obligatory step in the pathway leading to DNA synthesis and that colchicine mimics this. Because colchicine inhibits endocytosis, the inhibitory effects on growth factor-stimulated growth may have been the consequence of decreased growth factor/receptor complex internalization.

### Protein Kinase C

The role of protein kinase C in vascular smooth muscle cell mitogenesis is not clear. It has been investigated both by using tumor-promoting phorbol esters that activate it and by using synthetic inhibitors, but the results are equivocal. Owen\textsuperscript{257} found that 12-\textit{O}-tetradecanoylphorbol-13-acetate (TPA) stimulated DNA synthesis in quiescent cultures of the A7r5 smooth muscle cell line. In quiescent cultures exposed to epidermal growth factor, TPA significantly inhibited DNA synthesis. In quiescent neonatal rat vascular smooth muscle cells, TPA did not stimulate DNA synthesis, but it did inhibit the mitogenic effect of α-thrombin.\textsuperscript{175} TPA also inhibited DNA synthesis in quiescent rabbit aortic smooth muscle cells restimulated with serum.\textsuperscript{258,259} The inhibitory effects of TPA in all of these reports were obtained with concentrations of approximately 5 nM. Similar concentrations significantly stimulated the proliferation of quiescent bovine smooth muscle cells isolated from carotid artery\textsuperscript{260} and pulmonary artery,\textsuperscript{261} and 100 nM TPA stimulated DNA synthesis in quiescent rat aortic smooth muscle cells.\textsuperscript{262–264}

The published results obtained with synthetic inhibitors of protein kinase C show a clearer picture. H-7 inhibited the restimulation of quiescent rat aortic smooth muscle cells by both PDGF and epidermal growth factor.\textsuperscript{262} Staurosporine was effective in quiescent rabbit aortic smooth muscle cells restimulated with serum,\textsuperscript{265} and K252a had similar effects in bovine carotid arterial smooth muscle cells.\textsuperscript{260}

### Tyrosine Kinase

Proliferation induced by epidermal growth factor in rat aortic smooth muscle cells was inhibited by the tyrosine kinase inhibitor genistein, with an IC\textsubscript{50} of 85 μM.\textsuperscript{266} Various tyrosine kinase inhibitors of the tyrphostin type reversibly inhibit PDGF-stimulated growth of rabbit arterial smooth muscle cells, with IC\textsubscript{50} values as low as 40 nM.\textsuperscript{267}

### Miscellaneous Agents

The effects of a variety of agents that cannot easily be categorized are summarized in Table 2 (in vitro studies) and Table 3 (in vivo studies).

#### Summary

The first point to be made in summary is that the potential list of compounds is more extensive than the usual limited focus on polypeptide growth factors. A number of fairly well understood mediators seem to be as active as growth factors in stimulating and inhibiting smooth muscle proliferation; these pathways merit further study. The issues are the usual ones for a pharmacological study. Mechanisms are most easily explored when we have well-defined antagonists as well as agonists. For many of the mediators discussed above, there is insufficient evidence to reach firm
conclusions because of a lack of specific antagonists. This is a problem particularly for molecules posited to have an inhibitory function such as steroids, heparinoids, or somatostatin. In other cases, tools are available and the experiments are feasible (angiotension, thrombin, eicosanoids, serotonin).

Second, the recent successful studies with growth factors and growth factor antagonists open a new frontier. The in vivo studies suggest that these molecules may control specific parts of the proliferative response; for example, bFGF controls the first wave of DNA synthesis as a repair response to cell death, whereas PDGF seems to play a role in migration into the intima. The greatest value of in vitro studies may be to reveal how these mediators exert their effects on cells and thus allow us to identify pathways that may be blocked either with new drugs or with existing drugs that affect relevant pathways.

Third, we must finish with a note of caution. Although the evidence that smooth muscle proliferation occurs is compelling in atherosclerosis, hypertension, and restenosis, the relation of smooth muscle proliferation to the final clinical event, i.e., vascular narrowing or obstruction, is not firmly established. Perhaps the surest benefit of being able to control smooth muscle growth will be to determine the extent to which this pathological response is etiologic in the clinical manifestation of vascular diseases.

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