Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6189–6193, July 1993
Pharmacology

Carvedilol, a cardiovascular drug, prevents vascular smooth muscle cell proliferation, migration, and neointimal formation following vascular injury

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Communicated by Stephen J. Benkovic, March 11, 1993 (received for review January 4, 1993)

ABSTRACT **Carvedilol is a cardiovascular drug currently used for the treatment of hypertension. Clinical studies have recently demonstrated efficacy in angina and congestive heart failure. Recently, carvedilol has been shown to attenuate oxygen free radical-initiated lipid peroxidation and to inhibit vascular smooth muscle mitogenesis induced by a wide variety of growth factors. These findings are of interest since smooth muscle proliferation and abnormal lipid metabolism are proposed to play an important role in the pathogenesis of atherosclerotic plaque formation and in development of stenotic lesions following vascular injury by balloon angioplasty and coronary artery bypass grafting. On the basis of these observations, the antiproliferative actions of carvedilol have been explored in detail. In human cultured pulmonary artery vascular smooth muscle cells, carvedilol (0.1–10 μM) produced a concentration-dependent inhibition of the mitogenesis stimulated by platelet-derived growth factor, epidermal growth factor, thrombin, and serum, with IC_{50} values ranging from 0.3 to 2.0 μM. Carvedilol also produced a concentration-dependent inhibition of vascular smooth muscle cell migration induced by platelet-derived growth factor, with an IC_{50} value of 3 μM. The extensive neointimal formation that occurs following balloon angioplasty of rat carotid arteries was markedly attenuated by carvedilol (1 mg/kg, i.p.; twice daily starting 3 days before angioplasty and continuing until 14 days after angioplasty). Quantitative image analysis demonstrated that carvedilol reduced the neointimal growth following angioplasty by 84% without altering either medial or adventitial cross-sectional areas. These observations indicate that carvedilol may also be effective in the treatment of pathological disorders principally associated with abnormal vascular smooth muscle growth, such as atherosclerosis and acute vascular wall injury induced by angioplasty or coronary artery bypass grafting.**

Abnormal vascular smooth muscle proliferation is associated with cardiovascular disorders such as atherosclerosis, hypertension, and most endovascular procedures. Abnormal vascular smooth muscle proliferation is a common complication of percutaneous transluminal coronary angioplasty (PTCA). The incidence of chronic restenosis resulting from vascular smooth muscle proliferation following PTCA has been reported to be as high as 40–45% within 3–6 months (1, 2). Several neurohumoral factors, including angiotensin II and norepinephrine, as well as growth factors, including platelet-derived growth factor (PDGF) and basic fibroblast growth factor (FGF), have been implicated in the development of vascular restenosis *in vivo* (2–10).

The high incidence of vascular reocclusion associated with PTCA has led to development of *in vivo* animal models of restenosis and the search for agents to prevent restenosis. Angiotensin II receptor antagonists, angiotensin-converting enzyme (ACE) inhibitors, α-adrenoceptor antagonists, and growth factor antibodies have generally produced only a modest (10–50%) reduction of vascular restenosis in such animal models (3, 5–7). Clinical studies with ACE inhibitors (which showed only a slight protective effect in animal models of restenosis) have failed to demonstrate significant efficacy in the prevention of angiographically defined restenosis in humans (11). This limited or insignificant protection against vascular restenosis afforded by agents with specific mechanisms of action most likely reflects the complex nature of the pathophysiology underlying vascular restenosis; a multiplicity of chemotactic and mitogenic factors are believed to be involved in this response to vascular wall injury, and it is unlikely that interfering with the actions of only one of these factors will prove to be beneficial.

Carvedilol is a newly developed cardiovascular drug with multiple actions. *In vitro* studies have demonstrated that this compound is a competitive β- and α_1-adrenoceptor antagonist (12–15). At higher concentrations, carvedilol is also a calcium channel antagonist and an antioxidant (16). The antihypertensive action of carvedilol is mediated primarily by decreasing total peripheral vascular resistance without causing the concomitant reflex changes in heart rate commonly associated with other antihypertensive agents (12–14). Carvedilol also markedly reduces infarct size in rat, canine, and porcine models of acute myocardial infarction (15), possibly as a consequence of its antioxidant action in attenuating oxygen free radical-initiated lipid peroxidation (16).

Recently, we have demonstrated that carvedilol is able to block mitogen-stimulated proliferation of cultured rat aortic vascular smooth muscle cells *in vitro* (17). The most striking observation from these studies is that carvedilol is able to block the proliferative actions of several pharmacologically unrelated mitogens, including thrombin, PDGF, epidermal growth factor (EGF), angiotensin II, and endothelin 1, with an IC_{50} of ≈1 μM. This is an action that is not shared by other β-adrenoceptor antagonists, such as labetalol, celiprolol, or sotalol. Because carvedilol inhibited the proliferative actions of multiple mitogenic stimuli, a clear advantage may exist over specific growth factor antagonists. We therefore hypothesized that carvedilol may demonstrate superior protective effects against vascular smooth muscle proliferation in blood vessels subjected to acute injury induced by balloon angioplasty. The present study demonstrates that carvedilol

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Abbreviations: PTCA, percutaneous transluminal coronary angioplasty; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; HPF, high-power field.

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produces potent inhibition of vascular smooth muscle cell proliferation, migration, and angioplasty-induced neointimal proliferation in the rat common carotid artery.

MATERIALS AND METHODS

Culture of Vascular Smooth Muscle Cells. Primary cultures of rat aortic vascular smooth muscle for use in the migration studies were prepared by an explant technique as described (18). Cryopreserved primary cultures of human pulmonary artery smooth muscle cells (passage 3) for use in the DNA synthesis studies were obtained from Clonetics (San Diego). Cells were grown in a modified MCDB 131 formulation containing 5% fetal bovine serum, EGF at 10 ng/ml, basic FGF at 2 ng/ml, 1 μM dexamethasone, gentamicin sulfate at 10 μg/ml, and amphotericin B at 10 ng/ml (Clonetics).

DNA Synthesis. Human vascular smooth muscle cells were plated into 24-well plates (Corning) (2×10^4 cells per cm^2; passage 6) and grown to confluence (3 days). Cells were then made quiescent (G_0) by substituting serum-containing medium with Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml) for 48 hr. Cells were replenished with fresh medium once between and after the 48-hr quiescent period. Carvedilol was added 15 min prior to the addition of a mitogen for an additional 24-hr incubation. DNA synthesis was assessed by measuring the radioactivity incorporation (4 hr) of [^{3}H]thymidine into the trichloroacetic acid-insoluble fraction.

Migration of Vascular Smooth Muscle. The procedure for assessing vascular smooth muscle cell migration has been described (19). Briefly, rat aortic vascular smooth muscle cells (passage 3) were suspended (1×10^6 cells per ml) in serum-free DMEM supplemented with 0.2% bovine serum albumin (Sigma). Migration assays were performed in modified Boyden chambers using Transwell (Costar) cell culture chambers with a polycarbonate (pore size, 8 μm) membrane. PDGF was dissolved in DMEM and placed in the lower compartment in the presence or absence of carvedilol. Vascular smooth muscle cells (5×10^5 cells) were then loaded in the upper compartment and incubated for 24 hr at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Nonmigrated cells on the upper surface were scraped away gently and washed three times with phosphate-buffered saline. Filters were fixed in methanol and stained with Giemsa. The number of vascular smooth muscle cells per ×100 high-power field (HPF) that had migrated to the lower surface of the filters was determined microscopically. Four HPFs were counted per filter. Experiments were performed either in duplicate or in triplicate.

Balloon Angioplasty of Rat Carotid Arteries. The animals used in this study were divided into two groups: (*i*) those used for hemodynamic studies and (*ii*) those used for histopathologic examination of the degree of neointimal proliferation following carotid artery balloon angioplasty. These two major groups were further subdivided into animals that were treated with carvedilol (1 mg/kg, i.p.; twice daily; ≈5 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) and those that served as controls (which received an equal volume of carvedilol vehicle). All animals were pretreated with either carvedilol or vehicle 3 days prior to commencing either the hemodynamic studies or carotid artery angioplasty (the latter group receiving carvedilol or vehicle for 14 days after surgery, at which time the animals were sacrificed for histological processing of the carotid arteries).

Left common carotid artery balloon angioplasty was performed under aseptic conditions in anesthetized (sodium pentobarbital, 65 mg/kg, i.p.) male Sprague–Dawley rats (380–420 g) that had been pretreated for 3 days with either carvedilol or vehicle. After an anterior midline incision was made, the left common carotid artery was identified and cleared of adherent tissue to allow a Diffenbach clip to be placed around it without causing crush injury to the vagus or the associated superior cervical ganglion and sympathetic cord. After temporary cessation of carotid artery blood flow, a 2-F Fogarty arterial embolectomy catheter (Baxter Healthcare, Santa Ana, CA) was inserted into the lumen of the left external carotid artery. Once in position, the hemostat was removed and the catheter was guided a fixed distance (5 cm) down the external carotid and left common carotid artery to a point such that the tip of the catheter was adjacent to the aortic arch. Once in position, the balloon was inflated with fluid sufficient to generate slight resistance with the vessel wall when the catheter was withdrawn (20). With the balloon inflated, the catheter was then withdrawn at a constant rate (≈2 cm/sec) back to a point proximal to the site of insertion in the external carotid artery. This procedure was performed a total of three times; then the catheter was removed, the external carotid was ligated, and the wound was closed. Animals were housed in Plexiglas cages in pairs under a 12-hr light/dark cycle with access to standard laboratory chow and drinking water ad libitum.

Common carotid arteries were removed from rats 14 days after they had undergone balloon angioplasty for use in histopathologic studies. The extent of neointima formation evident on day 14 was quantified histologically. Vessels were perfusion fixed under constant pressure (95–100 mmHg) *in situ* immediately after a sodium pentobarbital overdose. The entire length of the common carotid artery, extending from the aortic arch to the carotid artery bifurcation (≈5 cm long), was removed for histological processing. Arterial cross-sections (8 μm) were cut from paraffin blocks containing the middle third of these arteries and were processed for hematoxylin and eosin staining. The cross-sectional areas of the blood vessel layers (intima, media, and adventitia) were quantified by using a Bioscan Optimus (Edmonds, WA) cell imaging system.

All experiments were performed specifically in accordance with the guidelines of the Animal Care and Use Committee, SmithKline Beecham Pharmaceuticals and the American Association of Laboratory Animal Care.

Materials. Human EGF and PDGF A/B were purchased from Boehringer Mannheim, [^{3}H]thymidine (specific activity, 110 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear, and human thrombin was purchased from Sigma. Carvedilol was kindly provided by Boehringer Mannheim. Solutions of carvedilol were prepared for injection immediately before each administration by dissolving 5 mg of compound in a vehicle that consisted of 0.3 ml of acid/ethanol (equal volumes of 100% ethanol and 1 M HCl) in 4.7 ml of sterile distilled water. All other chemicals used were reagent grade from commercial sources and were used without further purification.

Statistics. Values are expressed as means ± SEM and n represents the number of animals or separate experiments studied in a particular group. Statistical comparisons were made by one-way analysis of variance with a P value < 0.05 accepted as significant.

RESULTS

Inhibition of Human Vascular Smooth Muscle Mitogenesis. Carvedilol (0.1–10 μM) produced a concentration-dependent inhibition of mitogenesis stimulated by PDGF (1 nM), EGF (1 nM), thrombin (0.1 unit/ml), and fetal bovine serum (1%) in cultured human pulmonary artery smooth muscle cells. The IC_{50} values for carvedilol against mitogenesis stimulated by the growth factors and serum were between 0.3 and 2 μM (Fig. 1). This effect was fully reversible, as cells regained full

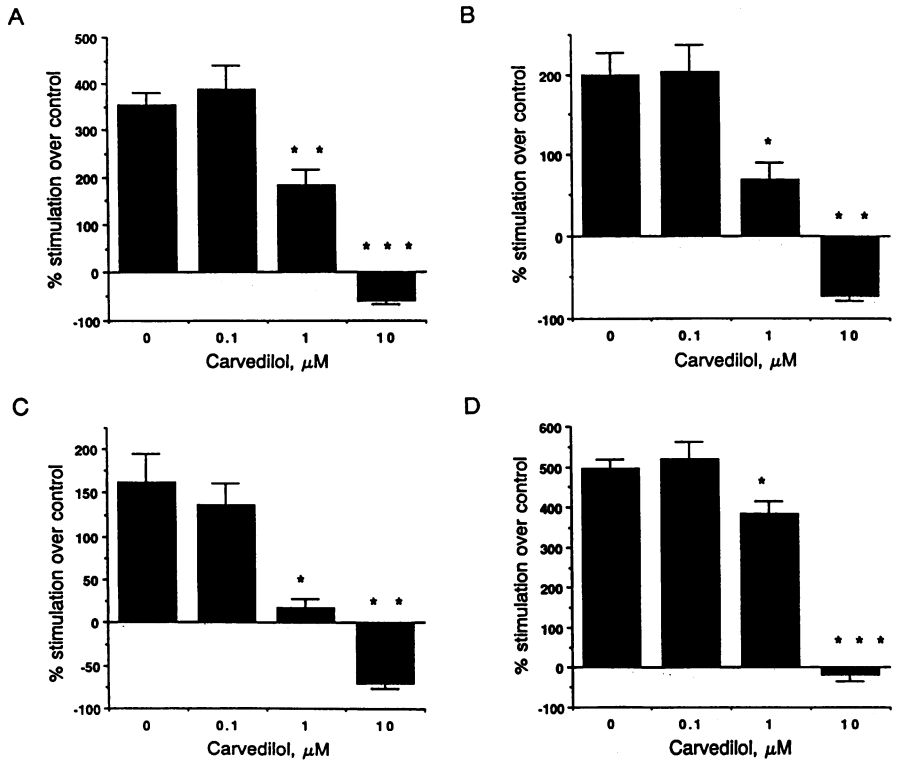

FIG. 1. Carvedilol produces concentration-dependent inhibition of PDGF- (1 nM) (*A*), EGF- (1 nM) (*B*), thrombin- (0.1 unit/ml) (*C*), and fetal bovine serum- (1%) (*D*) stimulated [^{3}H]thymidine incorporation in human pulmonary artery smooth muscle cell. Results are expressed as means ± SEM from four separate experiments, with triplicate determinations in each experiment. Basal [^{3}H]thymidine incorporation was 3129 ± 249 dpm. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

responsiveness to growth stimulation if carvedilol was washed out of the medium after a 24-hr incubation.

Inhibition of Vascular Smooth Muscle Cell Migration. PDGF produced concentration-dependent increases in the migration of rat vascular smooth muscle cells with a maximal effect obtained at a concentration of 1 nM (Fig. 2 *Inset*). When carvedilol was placed in the lower chamber with PDGF (1 nM), the migration response was inhibited significantly in a concentration-dependent manner with an IC_{50} value for carvedilol of 3 μM (Fig. 2). Preliminary data further suggest that when smooth muscle cells are incubated with carvedilol over prolonged periods, the IC_{50} of the inhibitory capacity of carvedilol is reduced 10-fold to ≈0.3 μM.

Inhibition of Neointima Formation by Carvedilol in Rats Following Balloon Angioplasty of the Common Carotid Artery.

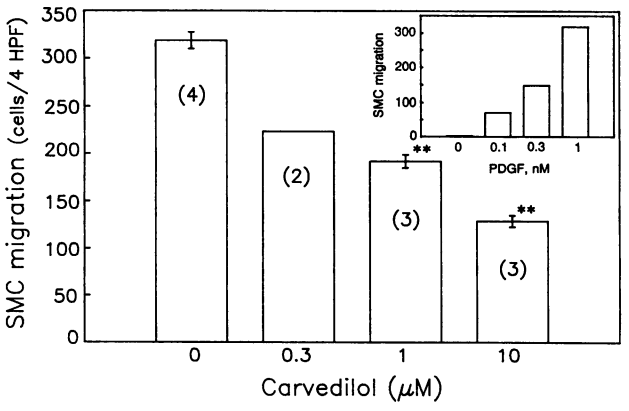

FIG. 2. Carvedilol inhibits migration of vascular smooth muscle cells (SMC) stimulated by PDGF. The lower compartment contained PDGF (1 nM) and the indicated concentrations of carvedilol. Rat aortic vascular smooth muscle cells (5×10^5 cells per 0.2 ml) were incubated for 24 hr in the upper compartment. At the end of the incubation period, the migration response was determined. Results are expressed as means ± SEM from two to four separate experiments, with duplicate determinations in each experiment. (*Inset*) Concentration–response determination for PDGF-stimulated rat aortic smooth muscle migration (SMC per 4 HPF) ($n = 2$).

Carvedilol treatment had no effect on the increases in body weight over the 17-day treatment period [380 ± 6 g ($n = 9$) and 377 ± 6 g ($n = 10$) in carvedilol- and vehicle-treated groups 3 days before surgery and 425 ± 9 g and 416 ± 7 g 14 days after balloon angioplasty, respectively]. Rats treated with carvedilol (1 mg/kg, i.p.; twice daily for 3 days) had significantly lower resting mean arterial blood pressures and heart rates (102 ± 5 mmHg and 305 ± 9 beats per min, respectively; $n = 6$) than those recorded in the vehicle-treated group (125 ± 3 mmHg and 360 ± 21 beats per min, respectively; $n = 7$).

Balloon angioplasty of the left common carotid artery produced marked intimal thickening in vehicle-treated rats (Fig. 3*A*), resulting in a highly significant, 20-fold increase in the intima/media ratio (Figs. 3*D* and 4). The contralateral right common carotid arteries that were not subjected to the angioplasty procedure were normal in both carvedilol- and vehicle-treated rats (Fig. 3*A*); i.e., no differences were observed between the intimal, medial, and adventitial areas, and these vessels had identical intima/media ratios. The extent of neointimal formation in the carotid arteries subjected to angioplasty was profoundly attenuated by carvedilol treatment, which caused an 84% decrease in intimal cross-sectional area (Figs. 3*A* and 4), and a comparable 81% decrease in the intima/media ratio (Figs. 3*A* and 4). Carvedilol treatment did not alter either medial or adventitial cross-sectional area (Fig. 3 *B* and *C*). Thus, carvedilol treatment afforded marked and highly significant protection from the myointimal proliferation and migration that results from vascular wall injury following balloon angioplasty.

DISCUSSION

The present study demonstrates that carvedilol affords profound protection (i.e., 84% reduction in intimal cross-sectional area) against balloon angioplasty-induced neointimal smooth muscle proliferation, migration, and vascular stenosis in the rat common carotid artery model. In addition, carvedilol significantly inhibits vascular smooth muscle cell migration *in vitro* and inhibits human vascular smooth muscle mitogenesis mediated by a wide variety of different mitogens,

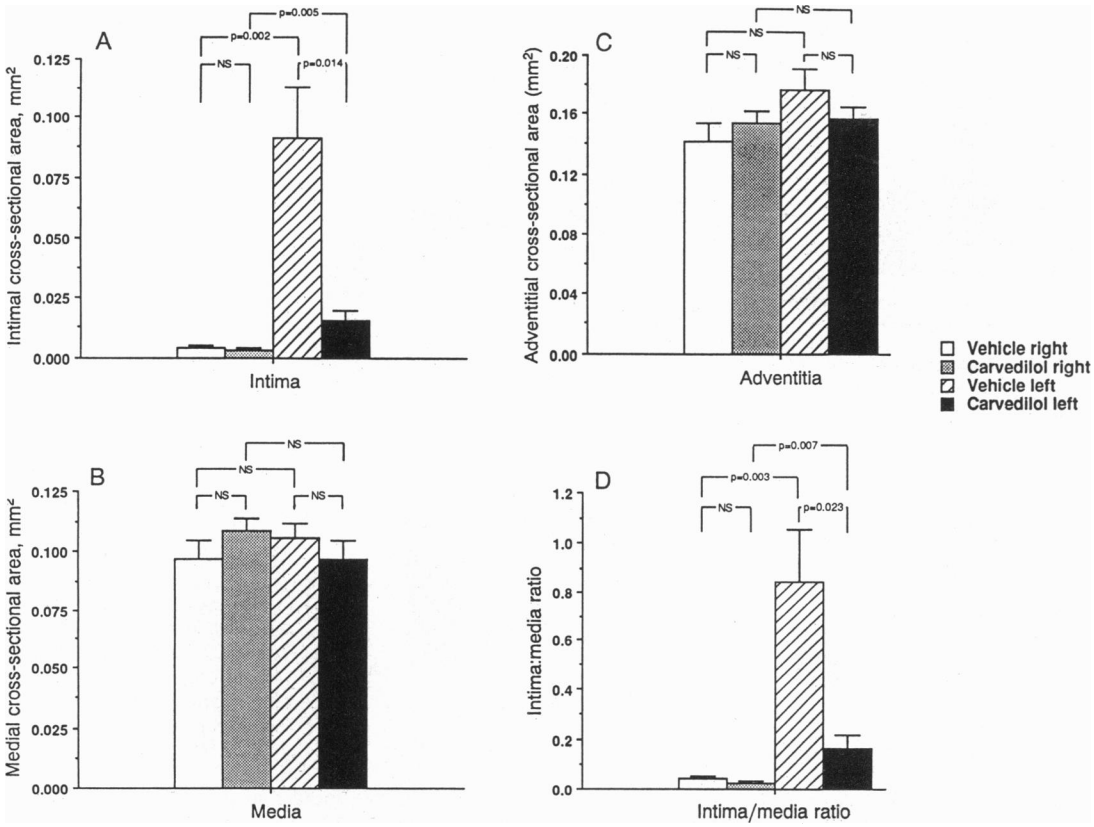

FIG. 3. Cross-sectional areas (mm^2) of tunica intima (*A*), tunica media (*B*), and tunica adventia (*C*) of left common carotid arteries removed 14 days after left common carotid artery angioplasty from rats that received either vehicle or carvedilol (1 mg/kg, twice daily). (*D*) Extent of myointimal proliferation expressed as the ratio of cross-sectional areas of the tunica intima to the tunica media. Contralateral right common carotid arteries from both the vehicle-treated and carvedilol-treated animals are also shown, demonstrating that intimal proliferation in response to angioplasty was limited to the left common carotid artery and also that carvedilol did not alter the morphometry of normal, unballooned contralateral vessels. NS, not significant.

which likely accounts for the pronounced protection of vascular restenosis after balloon angioplasty *in vivo*.

The protective mechanism of carvedilol is not the result of blockade of calcium channels or angiotensin II receptors, both of which have been implicated in the stenosis that results from balloon angioplasty, inasmuch as hemodynamic experiments demonstrated that this dosing regimen of carvedilol did not produce significant effects on calcium channels or angiotensin II receptors (data not shown). Furthermore, the calcium channel blocker nifedipine, in an animal model similar to that used here, produced <40% protection in the rabbit femoral artery after angioplasty (21). Although β-adrenoceptor blockade cannot be ruled out as a mechanism by which carvedilol protects against the vascular smooth muscle response to angioplasty, there is no evidence to suggest that these receptors are capable of mediating smooth muscle mitogenesis. In contrast, however, evidence does exist suggesting that α_1-adrenoceptor activation by circulating norepinephrine may be involved in luminal stenosis following angioplasty. However, the α_1-adrenoceptor antagonist prazosin (1 mg/kg, p.o.) produces only 16% inhibition of the vascular smooth muscle proliferation observed after rat carotid artery angioplasty (5). Furthermore, it is not likely that the hypotensive actions of carvedilol contribute to the observed antiproliferative actions inasmuch as other antihypertensive agents lack the marked effects on vascular restenosis observed with carvedilol. In addition, continuous administration of the angiotensin II receptor antagonist losartan over a similar time period produces a reduction in systemic blood pressure similar to that observed with carvedilol in this rat model but produced only a 48% reduction in neointimal proliferation (7). Similarly, equal hypotensive doses of several other antihypertensive agents, such as minoxidil or hydralazine, have failed to produce significant protection against vascular restenosis (22).

Chemotactic migration of medial smooth muscle cells into the intima is an important first step in the pathogenesis of neointima formation following balloon angioplasty. PDGF is believed to be a key substance for promoting smooth muscle cell migration and proliferation (9, 23). In the present study, carvedilol inhibited smooth muscle cell migration induced by PDGF with an IC_{50} value comparable with the potencies observed for inhibiting smooth muscle proliferation and antioxidant activity. The ability of carvedilol to inhibit myointimal formation *in vivo* may in part be related to direct inhibition of the physical migration of vascular smooth muscle from the tunica media into the tunica intima and also in part through antioxidant activity of carvedilol, which may inhibit the recruitment of macrophages and monocytes to the injury site. Since oxidized low density lipoprotein is also chemotactic in vascular smooth muscle, and carvedilol inhibits the oxidation of low density lipoprotein (24), this could be an additional mechanism contributing to the marked inhibition of neointimal formation *in vivo*.

While the precise molecular events leading to the antiproliferative and antimigratory actions of carvedilol await further elucidation, the present study clearly demonstrates that carvedilol affords pronounced protection in an animal model of neointimal formation and stenosis following angioplasty. The degree of protection produced by carvedilol is only matched by the recent report of an experimental c-*myb* antisense oligonucleotide (25). This was achieved only via the

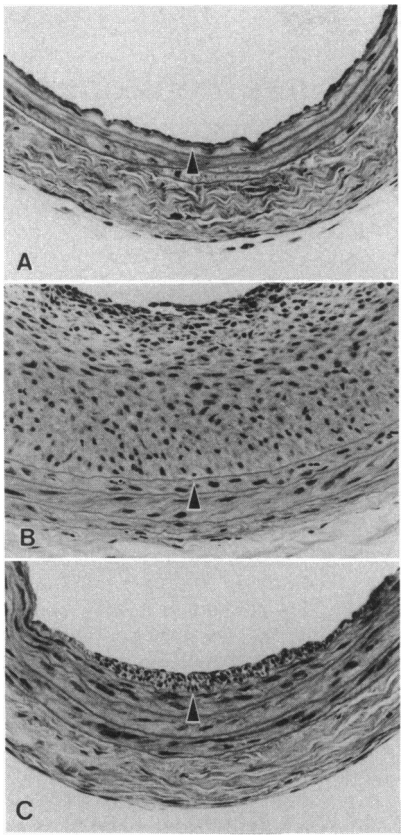

FIG. 4. Representative hematoxylin and eosin-stained cross-sections of contralateral right common carotid artery (no angioplasty) from vehicle-treated rats (*A*), left common carotid artery (angioplasty) from vehicle-treated rats (*B*), and left common carotid artery (angioplasty) from carvedilol-treated rats (*C*). Arteries were removed 14 days after angioplasty. Balloon angioplasty caused extensive neointimal proliferation. Arrowhead indicates internal elastic lamina. (×150.)

direct deposition of antisense construct onto the injured vascular surface in contrast to the present study in which the antistenotic effects were achieved by systemic administration, which is more amenable to conventional medical practice. The extent of carvedilol-induced protection (84%) using a total daily dose of 2 mg/kg far exceeds that reported for other compounds in several animal models.

It is now recognized that numerous pharmacologically unrelated chemotactic factors and mitogens are involved in the control of vascular smooth muscle growth. The implication of such observations is that, for successful management of abnormal smooth muscle growth, efficacious compounds may need to interfere with a common biochemical pathway shared by such factors (i.e., inhibition at some point distal to a specific cell-surface receptor). It might prove to be insufficient simply to inhibit a single growth factor, such as PDGF or thrombin. Thus, antibodies to either PDGF or FGF, widely believed to be among the more important mitogenic factors involved in vascular smooth muscle proliferation, have been reported to afford only modest (≈40%) protection in this animal model of neointimal formation (9, 10).

The present studies demonstrate that carvedilol inhibits vascular smooth muscle proliferation and migration *in vitro*, which apparently leads to a pronounced protection of myointimal proliferation *in vivo* following vascular injury by balloon angioplasty. Furthermore, previous observations that carvedilol attenuates oxygen free radical-initiated lipid peroxidation present additional implications for the treatment of atherosclerosis. Collectively, the vascular protective effect of carvedilol in this model of vascular injury-induced restenosis suggests that carvedilol may represent a therapeutic modality to prevent restenosis of coronary vessels following PTCA and other invasive vascular procedures.

We wish to thank Mildred Ezekiel, Patrick McKenna, Roseanna Mirabile, and Tom Covatta for their expert technical help.

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