were stimulated to adhere and assemble actin by FMLP. Furthermore, CD did not inhibit adherence, but did disrupt the microfilament organization in a fashion similar to that seen with human neutrophils.
25. G. S. Worthen *et al.*, *Am. Rev. Resp. Dis.* **136**, 19 (1987).
26. D. C. Lien *et al.*, *J. Appl. Physiol.* **62**, 1236 (1987); C. M. Doerschuk *et al.*, *ibid.* **63**, 1806 (1987).
27. J. C. Sandison, *Anat. Rec.* **54**, 105 (1932); A. A. Palmer, *Q. J. Exp. Physiol.* **44**, 149 (1957); P.-I. Branemark, *Intravascular Anatomy of Blood Cells in Man* (Karger, Basel, 1971), pp. 43–55; U. Bagge and P.-I. Branemark, *Adv. Microcirc.* **7**, 1 (1977).
28. U. Bagge and M. Braide, *Prog. Appl. Microcirc.* **7**, 43 (1985); R. L. Engler, G. W. Schmid-Schonbein, R. S. Pavelec, *Am. J. Pathol.* **111**, 98 (1983).
29. M. A. Lichtman, *J. Clin. Invest.* **52**, 350 (1973); ——— and J. M. Rowe, *Blood* **60**, 279 (1982); L. C. McKee and R. D. Collins, *Medicine* **53**, 463 (1974).
30. G. A. Zimmerman and T. M. McIntyre, *J. Clin. Invest.* **81**, 531 (1988).
31. R. J. Conant and R. Solecki, *Int. J. Solids Struct.* **19**, 105 (1983).
32. C. Haslett *et al.*, *Am. J. Pathol.* **119**, 101 (1985); L. A. Smedly *et al.*, *J. Clin. Invest.* **77**, 1233 (1986).
33. Adherence of the cell to a rigid substratum promotes spreading, which may increase the measured stiffness. Both spreading and the consequent increase in stiffness may be prevented by plating the cells on polyhydroxyethyl methacrylate [poly(HEMA)] [J. Folkman and A. Moscona, *Nature* **273**, 345 (1978); B. B. Daily, thesis, Washington University, St. Louis, MO (1985)]. Similarly, neutrophils allowed to spread on glass were stiffer than neutrophils on poly(HEMA). Although neutrophils on poly(HEMA)-coated glass change shape when stimulated, phase-contrast light microscopic studies revealed no tendency to spread. Accordingly, we believe that the mechanical properties of the cells reported here reflect the majority of cells in suspension, and thus of cells circulating in the bloodstream. For our measurements, cover slips were coated with 100 μl of a solution of poly(HEMA) (15 μg/ml) (Aldrich) in ethanol and allowed to dry for 3 hours at 37°C.
34. New Zealand White rabbits, weighing 2.6 to 3.5 kg, were anesthetized with xylazine (2 mg/kg) and ketamine (30 mg/kg) administered intramuscularly. Labeled cells were infused through 23-gauge needles inserted into the marginal ear vein. Anesthesia was maintained by intramuscular injections of ketamine (15 mg/kg) at hourly intervals.
35. These studies were carried out in part in the F. L. Bryant, Jr., Research Laboratory for the Study of Mechanisms of Lung Disease. Supported by grants HL-27353, HL-40784, HL-36577, GM-27160, and GM-38838 from the NIH and DMB-8610636 from the NSF. G.P.D. is supported in part by NIH Clinical Investigator Award HL-02039 and a Medical Research Council of Canada Research Fellowship. G.S.W. has been the recipient of an American Heart Association Clinician-Scientist Award with funds provided in part by the Colorado Heart Association and by an RJR-Nabisco Research Scholars Award. We thank P. M. Henson for critical reading of the manuscript, G. I. Zahalak for helpful discussion, B. McConnaughey for design and maintenance of the cell poker, and J. LaBreque for help with statistical analysis. MAb 60.3 directed against the CD11-CD18 complex was a gift of J. Harlan and P. Beatty.

28 December 1988; accepted 15 May 1989

Inhibitors of Angiotensin-Converting Enzyme Prevent Myointimal Proliferation After Vascular Injury

JERRY S. POWELL,* JEAN-PAUL CLOZEL, RITA K. M. MÜLLER, HERBERT KUHN, FRIDOLIN HEFTI, MARKUS HOSANG, HANS R. BAUMGARTNER

The role of a local angiotensin system in the vascular response to arterial injury was investigated by administering the angiotensin-converting enzyme (CE) inhibitor cilazapril to normotensive rats in which the left carotid artery was subjected to endothelial denudation and injury by balloon catheterization. In control animals, by 14 days after balloon injury, the processes of smooth muscle cell (SMC) proliferation, migration of SMCs from the media to the intima, and synthesis of extracellular matrix produced marked thickening of the intima, with reduction of the cross-sectional area of the lumen. However, in animals that received continuous treatment with the CE inhibitor, neointima formation was decreased (by about 80 percent), and lumen integrity was preserved. Thus, the angiotensin-converting enzyme may participate in modulating the proliferative response of the vascular wall after arterial injury, and inhibition of this enzyme may have therapeutic applications to prevent the proliferative lesions that occur after coronary angioplasty and vascular surgery.

SMOOTH MUSCLE CELL (SMC) proliferation in the intima of muscular arteries and formation of extracellular matrix are major processes that lead to vascular stenosis in arteriosclerosis, after vascular surgery, and after coronary angioplasty (*1*). Although these processes occur in the normal physiology of the vascular wall during development and in response to vascular injury, in excess they may cause significant morbidity (*2*). After endothelial denudation and injury to the arterial wall, the proliferative response in the intima (Fig. 1, A and B) is the result of normally quiescent SMCs migrating from the media to the intima, proliferating both in the media and in the intima (*3*), and producing extracellular matrix.

Several organs contain local angiotensin systems. Both the production of angiotensin II (AII) and its interaction with specific AII receptors occur in these tissues, apparently independently of the plasma renin-angiotensin system (*4*). Angiotensin-converting enzyme (CE) is a membrane-bound enzyme present in the walls of large vessels (both arteries and veins) (*5*), and medial SMCs have high numbers of specific AII receptors (*6*). In addition, treatment of hypertensive animals with inhibitors of CE reduces the medial hypertrophy of muscular arteries that results from chronic hypertension (*7*). We postulated that a local angiotensin system may participate in regulation of the vascular response to arterial injury and investigated the role of endogenous AII production by examining the effects of the specific long-acting CE inhibitor cilazapril (*8*) in an animal model of the proliferative response to arterial injury. This inhibitor was selected because it lowered blood pressure over a 24-hour period and reduced the medial hypertrophy of hypertensive rats (*8*).

Endothelial denudation and vascular injury were achieved in the left common carotid artery of 4-month-old male normotensive rats (*9*) (400 to 500 g) as described (*3*). A balloon catheter (2 French Fogarty, Edwards Laboratories, Santa Anna, California) was passed through the external carotid into the aorta; the balloon was inflated with sufficient water to distend the common carotid and was then pulled back to the external carotid. This procedure was repeated three times and then the catheter was removed. Complete denudation of the endothelium was achieved throughout the common carotid, with some injury to medial SMCs, as assessed by morphological examination of random controls 24 hours after the procedure. The rate of intimal thickening in the rat carotid in response to balloon injury slows considerably after 14 days (*10*). Therefore, at 14 days the animals were anesthetized and perfusion-fixed as described (*11*), with the modification that 2.5% glutaraldehyde and 90-mmHg perfusion pressure were used. Carotid arteries were isolated from adherent tissue and embedded in Epon 812. Semithin sections (1 μm) were stained with toluidine blue and basic fuchsin and processed for morphometric evaluation (*3*). Sections from the middle fifth of the carotid were analyzed, with the right carotid serving as a control for the balloon-catheterized left carotid. Rats were selected randomly to be given either placebo or cilazapril (10 mg/kg per day, mixed with normal food). Animals were coded so that operation and analysis

Pharmaceutical Research Department, F. Hoffmann–La Roche Ltd., Grenzacherstrasse 124, CH-4002 Basel, Switzerland.

*To whom correspondence should be addressed.

were performed without knowledge of which treatment individual animals received.

The effect of cilazapril on blood pressure reaches a plateau by 1 week (*8*). Therefore, we began treatment with the drug 6 days before balloon injury so as to achieve a maximal effect as measured by the physiological parameter of lower blood pressure. In normotensive rats a slight but significant decrease in blood pressure from 109 ± 4.9 to 86 ± 4.8 mmHg (mean ± SEM, n = 12) occurred as measured by tail cuff (*12*). The dose of cilazapril used for these studies was selected because it was the lowest dose that had sustained effects on blood pressure over a 24-hour period in hypertensive rats. When administered 6 days before injury and continuously until the animals were killed 14 days after injury, the CE inhibitor suppressed the myointimal proliferative response to balloon catheterization (Table 1, group A). Both the amount of neointima and the extent of internal elastic lamina (IEL) covered by neointima were reduced significantly. Primarily as a consequence of decreased neointima formation, the lumen remained almost as patent as the normal artery. The decreased amount of neointima appeared to reflect a reduction in all components of the intimal thickening, with fewer SMCs and less matrix formation (Fig. 1C).

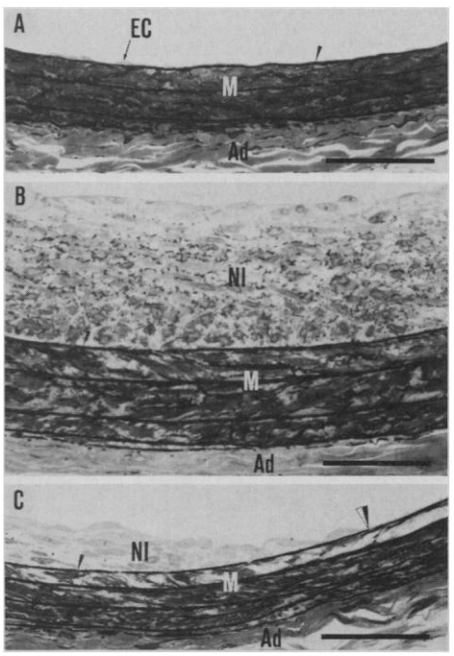

Fig. 1. The vascular response to injury as depicted by light micrographs of semithin (1 μm) cross sections of rat carotid arteries. (**A**) Carotid artery of an uncatheterized normal rat. (**B**) Carotid artery of a placebo-treated rat 14 days after balloon catheterization. (**C**) Carotid artery of a rat treated with cilazapril (10 mg/kg per day per os, continuously from 6 days before to 14 days after balloon injury), shown at 14 days after balloon catheterization. The arrowheads mark the IEL. Abbreviations: Ad, adventitia; EC, endothelial cells; M, media; and NI, neointima. Bars, 50 μm. The lumen is at the top in each section. These sections are representative of the findings summarized in Table 1. The intimal thickening (or NI) in response to injury is the result of the new accumulation of proliferating SMC and connective tissue. In the media, the SMCs near the IEL show evidence of damage after balloon injury (B and C). In the rat treated with cilazapril the coverage of the IEL by neointima formation is much reduced, and there is IEL denuded of endothelial cells but not covered by neointima [half-shaded arrowhead in (C)].

To assess whether inhibition of CE activity was required continuously, or only during or immediately after injury, for suppression of the proliferative response, we tested several additional groups of rats (Table 1, groups B through E). Both neointima formation and the extent of IEL covered by neointima were reduced when the CE inhibitor was administered continuously, either from 1 hour before balloon injury (group D) or from 2 days after injury (group E). However, neither neointima formation nor the extent of IEL coverage was reduced by a single administration of the CE inhibitor 1 hour before balloon injury (group C) or by administration from 6 days before to 2 days after injury (group B). These data suggest that for maximal suppression of neointima formation, the CE inhibitor must be administered continuously during the response of the vascular wall. The apparent advantage of prior administration of the inhibitor may reflect the time required to achieve complete inhibition of CE activity.

To eliminate the possibility that the inhibitor itself contributed directly to the observed suppression of neointima formation, we tested cilazapril and its active metabolite cilazaprilate for effects on cellular proliferation in vitro. Proliferation of human umbilical cord vein SMCs cultured in medium containing 10% serum was unaffected by either compound at concentrations up to 100 μg/ml (the in vivo concentration of the drug in the rats used in these studies being 0.03 μg/ml).

Two additional groups of experiments were performed to analyze potential mechanisms for the observed inhibition of myointimal proliferation. First, we tested another CE inhibitor, captopril (*13*), which is chemically different from cilazapril. Similar results were found when captopril (100 mg/kg per day) mixed with normal food was administered from 6 days before to 14 days after balloon injury. The cross-sectional area of neointima expressed as a percentage of media was significantly reduced compared to placebo-treated controls [42 ± 11% (mean ± SEM, n = 14) compared to 111 ± 10% (n = 13) ($P < 0.001$)], as was the coverage of IEL [51 ± 12% compared to 95 ± 3% ($P < 0.001$)]. In a second experiment we tested the calcium-channel antagonist vera-

Table 1. Formation of neointima after vascular injury by balloon catheterization. Cross-sectional area of neointima was measured by semithin sections of the middle fifth of the left carotid artery that were prepared 14 days after catheterization. Noninjured normal arteries had no neointima formation. Groups of treated animals underwent catheterization and balloon injury on the same days as placebo controls. Treatment groups were administered cilazapril (10 mg/kg of body weight per day) mixed with food, or by gavage for single doses. Treatment schedules were as follows: group A, from 6 days before to 14 days after balloon catheterization; group B, from 6 days before to 2 days after injury; group C, a single oral dose (10 mg/kg) 1 hour before balloon injury; group D, the single dose 1 hour before injury and then daily mixed with food to 14 days after balloon catheterization; group E, from 2 to 14 days after balloon injury. Cross-sectional surface areas of the neointima, media, and lumen and the length of the IEL were measured by using a computerized digitizer (Morphometry-System, DIASYS) with an IBM AT03 (Datalab, Thoerigen, Switzerland). Means ± SEM are given. The fourth column is the ratio of the cross-sectional area of the newly formed intima (neointima) to the cross-sectional area of the media of the injured carotid artery. Statistical significance was assessed by Student's two-tailed t test.

Group	n	Neointima area (10^3 μm^2)	Neointima/ media (%)	Coverage of IEL by neointima (%)
A				
Placebo	11	109 ± 15	101 ± 13	93 ± 5
Treated	11	20 ± 5***	23 ± 6***	35 ± 9 ***
B				
Placebo	9	100 ± 17	111 ± 13	100 ± 0
Treated	11	105 ± 13†	121 ± 13†	96 ± 4†
C				
Placebo	13	91 ± 12	122 ± 14	97 ± 3
Treated	14	83 ± 10†	94 ± 11†	93 ± 7†
D				
Placebo	14	115 ± 8	130 ± 9	99 ± 0.5
Treated	17	46 ± 7***	54 ± 8***	78 ± 9*
E				
Placebo	13	91 ± 12	122 ± 14	97 ± 3
Treated	14	43 ± 7**	53 ± 9***	71 ± 9*

*$P \leq 0.05$. **$P \leq 0.01$. ***$P \leq 0.001$. †Not significant.

pamil (*14*) at a concentration of 100 mg/kg per day [a dose that lowered arterial pressure by 29 ± 3 mmHg (mean ± SEM, $n = 10$)] by gavage from 6 days before to 14 days after balloon injury. At this dose no effect on myointimal proliferation was seen in the verapamil-treated rats [the cross-sectional area of neointima expressed as a percentage of media being 129 ± 18% ($n = 6$) in treated rats compared to 127 ± 13% ($n = 10$) in placebo-treated controls].

The present observations, taken together with data showing CE activity (*5*), angiotensinogen mRNA (*15*), and AII receptors in the vascular wall (*6*), support the hypothesis that there is a local angiotensin system that has a role in the myointimal proliferative response of the vascular wall to injury.

The processes that lead to formation of the neointima are complex and poorly understood, but initially quiescent SMCs must be activated either directly by injury or indirectly by factors released early during the response, such as interferon-γ from lymphocytes (*16*) or platelet-derived growth factor (PDGF) (*1, 17*). Blocking the activation of SMCs, however, is unlikely to be the principal mechanism for the suppression of neointima formation, as administration of cilazapril only during the initial phase of the vascular response was ineffective.

It is probable that CE inhibitors suppress the vascular response to injury by interrupting the conversion of angiotensin I (AI) to the active AII, and thus preventing modulation of SMC proliferation and matrix protein synthesis by AII. Inhibition of CE also increases bradykinin levels, since CE is one of the enzymes that metabolize bradykinin (*4*). Although unlikely, the possibility that bradykinin mediates the suppression of the vascular response to injury cannot be excluded. Bradykinin, however, is a mitogenic agent (*18*), and therefore it might be expected that increased bradykinin concentrations would augment the proliferative response. The observation that the *mas* proto-oncogene product is an angiotensin receptor with mitogenic activity (*19*) raises the possibility that the proliferative response to vascular injury is mediated through these receptors on SMCs and requires continued stimulation by AII.

It has been reported that AII neither stimulated the proliferation of cultured rat aortic SMCs nor augmented cell proliferation induced by PDGF (*20*). However, AII did increase SMC protein content by 20% after 4 days, and this effect was blocked by saralasin, an antagonist of AII. In contrast, others have found that AII stimulated the proliferation of human vascular SMCs cultured in serum-depleted medium (*21*). It is possible that AII is effective only when added at specific phases of the cell cycle, as has been shown for the AII-augmented proliferation of NIH 3T3 cells stimulated by epidermal growth factor (*22*).

Infusion of heparin suppresses neointima formation in the rat balloon model used for our study (*23*). The heparin administration must be started by 24 hours after injury and is equally effective whether administered continuously for 21 days or for 3 days (*10*). However, in contrast to our results with CE inhibitors, the maximal suppression of neointima formation achieved with heparin has been generally about 50% (*23*). It remains to be tested whether CE inhibition can act synergistically with heparin to prevent the proliferative response.

REFERENCES AND NOTES

1. R. Ross, *N. Engl. J. Med.* **314**, 488 (1986).
2. W. McBride *et al.*, *ibid.* **318**, 1734 (1988).
3. H. R. Baumgartner and A. Studer, *Pathol. Microbiol.* **29**, 393 (1966); A. W. Clowes, M. A. Reidy, M. M. Clowes, *Lab. Invest.* **49**, 208 (1983); A. W. Clowes and S. M. Schwartz, *Circ. Res.* **56**, 139 (1985).
4. D. J. Campbell, *J. Clin. Invest.* **79**, 1 (1987); V. J. Dzau, *Circulation* 77 (suppl. 1), 1 (1988); A. Pellicer *et al.*, *Science* **240**, 1660 (1988).
5. Y. Nakamura *et al.*, *J. Hypertens.* **6**, 105 (1988).
6. J. Penit, M. Faure, S. Jard, *Am. J. Physiol.* **244**, E72 (1983).
7. G. K. Owens and S. M. Schwartz, *Circ. Res.* **51**, 280 (1982); G. K. Owens, *ibid.* **56**, 525 (1985).
8. C. H. Hassall, A. Kröhn, C. J. Moody, W. A. Thomas, *FEBS Lett.* **147**, 175 (1982); I. L. Natoff *et al.*, *J. Cardiovasc. Pharmacol.* **7**, 569 (1985); J.-P. Clozel and F. Hefti, *ibid.* **10**, 350 (1987); J.-P. Clozel *et al.*, *J. Hypertens.*, in press.
9. Rats (rat-outbred Roche) were specific-pathogen free and from the Institute of Biomedical Research, Füllinsdorf, Switzerland.
10. A. W. Clowes and M. M. Clowes, *Lab. Invest.* **52**, 611 (1985).
11. C. C. Haudenschild and S. M. Schwartz, *ibid.* **41**, 407 (1979).
12. M. Gerold and H. Tschirky, *Arzneim. Forsch.* **18**, 1289 (1969).
13. M. A. Ondetti *et al.*, *Science* **196**, 441 (1977).
14. A. Fleckenstein, *Annu. Rev. Pharmacol. Toxicol.* **17**, 149 (1977).
15. L. A. Cassis *et al.*, *Hypertension* **11**, 591 (1988).
16. G. K. Hanson, L. Jonasson, J. Holm, M. M. Clowes, A. W. Clowes, *Circ. Res.* **63**, 712 (1988).
17. L. N. Walker *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7311 (1986).
18. N. E. Owen and M. L. Villereal, *Cell* **32**, 379 (1983).
19. T. R. Jackson, L. A. C. Blair, J. Marshall, M. Goedert, M. R. Hanley, *Nature* **335**, 437 (1988).
20. A. A. T. Geisterfer, M. J. Peach, G. K. Owens, *Circ. Res.* **62**, 749 (1988).
21. M. Campbell-Boswell and A. L. Robertson, *Exp. Mol. Pathol.* **35**, 265 (1981).
22. J. Norman *et al.*, *Am. J. Physiol.* **253**, F299 (1987).
23. A. W. Clowes and M. J. Karnovsky, *Nature* **265**, 625 (1977); J. R. Guyton, R. D. Rosenberg, A. W. Clowes, M. J. Karnovsky, *Circ. Res.* **46**, 625 (1980); R. L. Hoover, R. Rosenberg, W. Haering, M. J. Karnovsky, *ibid.* **47**, 578 (1980).

24. We thank M. M. Clowes and A. W. Clowes for helpful discussions; M. Broussé, C. Hungerbühler, M. Reichlin, F. Schodjai, S. Stäger, and P. Wyss for technical help; and D. Brütsch and J. Lobsiger for preparation of the manuscript.

3 January 1989; accepted 23 May 1989

The Periaqueductal Gray Matter Mediates Opiate-Induced Immunosuppression

RICHARD J. WEBER AND AGU PERT

The periaqueductal gray matter of the mesencephalon (PAG) subserves a variety of diverse autonomic functions and also appears to be a site for opiate action in the induction of immunosuppression. Microinjections of morphine into the PAG, but not into other opiate receptor–containing neuroanatomical sites, result in a rapid suppression of natural killer (NK) cell activity. The NK cell suppression can be blocked by prior peripheral administration of the opiate antagonist naltrexone. These findings demonstrate that certain central actions of opiates that produce changes in NK cell function are mediated through opiate receptors in the PAG and identify a brain region involved in opiate regulation of immune function.

OPIATES EXERT PROFOUND EFFECTS on immune function in vivo (*1*). Opiate addicts have increased susceptibility to infections (*2*), an effect related to deficits in immune function (*3*). Certain opiate agonists suppress antibody production (*4*), produce changes in the ability of leukocytes to respond to mitogenic stimuli (*5*), and decrease cytotoxic activity of natural killer (NK) cells (*6*). These immunosuppressive effects of opiates result in decreased survival in tumor-bearing animals (*7*) and increased susceptibility to bacterial and fungal infections (*8*) as well as to murine retroviral infections (*9*). Some in vivo effects of opiates on immune function are mediated indirectly through the central nervous system (CNS). For example, Shavit *et al.* (*10*) have shown that injections of morphine into the lateral cerebral ventricle suppress NK cell activity in rats. Conversely, peripheral administration of *N*-methyl morphine,

R. J. Weber, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.

A. Pert, Biological Psychiatry Branch, National Institute of Mental Health, Bethesda, MA 20892.