Cilazapril and Early Atherosclerotic Changes After Balloon Injury of Porcine Carotid Arteries

Jules Y.T. Lam, MD; Lucie Lacoste, MSc; and Martial G. Bourassa, MD

Background. Cilazapril, a converting enzyme inhibitor, has been shown to significantly decrease the extent of intimal smooth muscle cell proliferation after arterial injury in a normotensive rat model.

Methods and Results. To assess the influence of cilazapril on myofibrotic changes after balloon injury, control (n=7) or cilazapril-treated (n=9) (20 mg/kg b.i.d.) normal 3-month-old pigs underwent dilatation angioplasty of the right, and deendothelialization of the left common carotid artery. Carotid arteries isolated at 4 weeks were examined morphometrically with a computerized digitizer after in situ glutaraldehyde fixation. Both in the deendothelialized and dilated arteries, the surface area in square millimeters of the neointima and media were similar in the treated and control animals; therefore, the ratio of neointima to media was again not different. The neointimal changes included a combination of proliferative changes as well as organization of mural thrombus and were equally well distributed in the two groups of animals. At the time that the animals were killed, blood pressure (38.2±2.1 versus 56.8±4.8 mm Hg, p<0.003) and plasma angiotensin converting enzyme activity (5.6±4.1 versus 182.3±70.4, p<0.02) were lower in treated than in control pigs, but aortic media thrombogenicity (as assessed in an ex vivo perfusion flow chamber) and serum lipids were not different between groups.

Conclusions. In pigs, cilazapril did not alter the development of myofibrotic changes after mild or severe balloon injury despite plasma angiotensin converting enzyme activity inhibition. (Circulation 1992;85:1542–1547)

KEY WORDS • intimal hyperplasia • arterial wall injury • angiotensin converting enzyme inhibitors • restenosis

Experimental studies in various animal models have consistently shown that endothelial and arterial wall injury promote a sequence of events characterized by smooth muscle cell proliferation and migration into the intima that eventually lead to luminal stenosis and thrombosis; this has evolved into the concept of atherosclerosis being a response to injury. Proliferating smooth muscle cells in the intima with formation of extracellular matrix form the bulk of the fibrous lesions in response to arterial wall injury. In the clinical setting, the occurrence of restenosis after percutaneous transluminal coronary angioplasty appears to reflect the vascular response to balloon-induced injury. The factors that promote this proliferative response in vivo have not been defined, although a number of mitogens have been shown to stimulate smooth muscle cell proliferation in vitro. Factors or drugs that can retard this intimal proliferative response remain equally undefined.

Recently, it has been postulated that a local angiotensin system may participate in the regulation of the vascular smooth muscle cell response to arterial injury. This hypothesis derives from the observation that cilazapril, a converting enzyme inhibitor, can significantly decrease the extent of intimal smooth muscle proliferation after balloon injury in a normotensive rat model. However, in this rat model of mild arterial wall injury, other drugs such as heparin, the combination of cilazapril and heparin, and the vasodilator hydralazine have also been shown to be very effective in reducing neointimal hyperplasia. It is thus possible that the processes leading to intimal smooth muscle hyperplasia are complex, and it is not known whether these responses to drug therapy are animal species or animal model dependent or whether similar effects are obtained in response to more severe or deeper arterial injury, as is produced during clinical angioplasty. Therefore, the aim of this study was to assess the influence of cilazapril on the development of atherosclerotic changes after both mild and deep arterial wall injury in a porcine model.

Methods

Animals

The study involved normal normotensive, 2–3-month-old Yorkshire pigs. Nine pigs were treated with cilazapril 20 mg/kg p.o. twice daily for 5 weeks, and

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seven received no active drug and served as controls. One week into the study protocol, all the pigs were sedated with ketamine (150 mg i.m., Rogarsetic, Rogar/STB Inc., London, Ontario) and azaperone (80 mg i.m., Stresnil, Janssen Pharmaceutica, Mississauga, Ontario). The pigs were intubated, mechanically ventilated with room air by a Harvard respirator, and maintained anesthetized with 0.5% halothane (Fluthane, Ayerst Laboratories, New York, N.Y.). The electrocardiogram and intra-arterial pressure were continuously monitored throughout the procedure.

Experimental Procedure

Through an inguinal incision, the femoral artery was isolated and cannulated with an 8F balloon dilatation catheter (Meditech polyethylene balloon; size, 8-mm diameter by 3 cm long). A single intravenous bolus of heparin (100 units/kg) was then given, and the catheter was advanced under fluoroscopic control into the right common carotid arterial segment between the fifth and the fourth cervical vertebrae, in which severe or deep arterial injury was performed by inflating the balloon five times for 30 seconds each at 6 atm pressure (Meditech Pressure Manometer) with 1-minute intervals between inflations. This balloon dilatation procedure simulates the transluminal coronary angioplasty performed clinically in humans. Mild or superficial injury of the left common carotid artery was then performed using a 3F Fogarty balloon inflated with minimal distending pressure (<0.5 atm) to just below the size of the artery, and the balloon was moved up and down the artery 2–3 cm at one time between the fifth and fourth cervical vertebrae for four times to ensure complete endothelial removal. Thereafter, the catheter was removed, the femoral artery was ligated, and the incision was closed; the animals were allowed to recover and were fed a normal chow diet with their assigned drug treatment for an additional 4 weeks.

Tissue Analysis

At the end of the study, the animals were killed and the carotid arteries were perfused anterogradely with 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate (pH 7.25) buffer at physiological pressure to fix the arteries in situ. The nonperfused thoracic/abdominal aorta was removed together with the fixed carotid arteries, which were cleaned and prepared for analysis. Serial equally spaced ring sections of the injured carotid arteries between the fifth and the fourth cervical vertebrae were stained with hematoxylin–floxin–safran and Movat pentachrome stains, which define the boundaries between the neointima and the media. Morphometric analysis of the histological cross-sections of the dilated right and mildly injured left common carotid arteries was performed using a computerized digitizer to planimeter the surface area of the neointima and media. First, the histological cross-sections were projected onto white paper by using a Tri-simplex microprojector (Bausch and Lomb, Rochester, N.Y.) at 40× magnification. The luminal perimeter as well as the internal and outermost external elastic laminae were traced. The tracings were then transposed over a digitizing tablet interfaced to an IBM AT microcomputer loaded with a commercial software package (Sigman Scan) in which the outlines were retracted. The digitizing tablet was calibrated before each measurement. The cross-sectional area (in square millimeters) of the neointima was obtained by subtracting the luminal area from that bounded by the internal elastic lamina and the medial area by subtracting the area bounded by the internal elastic lamina from that of the external elastic lamina. Measurements were performed blinded to treatment assignment. Six different histological cross-sections were analyzed per carotid artery segment injured, and the mean value was calculated for the neointimal area, medial area, and the ratio of neointima to media.

Aortic Media Thrombogenicity

The thrombogenicity of the nonfixed aortic media harvested from the treated and control animals at the time the animals were killed was assessed in an ex vivo superfusion flow chamber by using femoral arterial blood from a normal untreated pig. The arterial blood was drawn into Plexiglas superfusion chambers by a peristaltic pump (model 7013, Masterflex) and recirculated into the animal through the femoral vein. The cylindrical superfusion Plexiglas chambers were designed to mimic the tubelike shape of the vascular system and contain a window permitting direct exposure of the aortic media to the flowing femoral arterial blood, which was kept constant by the peristaltic pump. The internal diameter of the chamber was 1.0 mm, with a flow rate set at 20 ml per minute; the theoretically calculated shear rate was 3,360 sec⁻¹. A 5-minute superfusion was performed with the chamber kept at 37°C in a water bath. The aortic media used in the superfusion chambers was prepared by lifting up and peeling off the intima together with a thin portion of the subjacent media after longitudinally opening the aorta. The exposed media overlying the adventitia was then divided into 35×15-mm segments to be placed inside the superfusion chambers.

Platelet thrombus deposition on the aortic media was quantitated using ¹¹¹In-labeled autologous platelets injected into the normal untreated animal 18 hours before using its femoral arterial blood for the superfusion experiment. Platelets were labeled according to previously described techniques. The radioactivity of the aortic media in the superfusion chambers as well as the radioactivity from weighed samples of blood were determined from a gamma well counter after correction for radionuclide decay. The quantitative platelet deposition (×10⁴) on the arterial segments were then calculated with the circulating blood platelet count obtained from a Coulter counter and the ¹¹¹In activity on the aortic media and in blood, using the equation

\[
\text{number of deposited platelets} = \frac{\text{¹¹¹In cpm in arterial media} \times n \text{ platelets/ml blood}}{\text{¹¹¹In cpm/ml blood}}
\]

Physiological and Biochemical Parameters

Heart rate and blood pressure were measured before and after the study, as well as serum lipid levels. Plasma angiotensin converting enzyme activity was determined by measuring the release of hippuric acid from the
substrate Hip-His-Leu. In four separate animals, plasma angiotensin converting enzyme activity was measured before and at half-hour, 1-hour, and at 1-hour intervals thereafter for 11 hours after a single oral dosing of 20 mg/kg to document drug absorption and the duration of its enzyme inhibition.

**Data Analysis**

Results are expressed as mean±SEM unless otherwise stated. Differences between group means were assessed by Student's t test. Values were considered significant at p<0.05.

**Results**

Cilazapril was well absorbed by the pigs. After a single oral dosing of 20 mg/kg, there was an over 95% inhibition of plasma angiotensin converting enzyme activity within a half hour of drug ingestion from 499±30.3 to 21.8±5.2 nmol/l/sec. This activity continued to decline to attain a minimum activity at about 4-6 hours after dosing (2.2±1.2–1.3±0.8 nmol/l/sec, respectively). This inhibition of the angiotensin converting enzyme activity persisted throughout the 11-hour period tested and was 2.5±1.4 nmol/l/sec 11 hours after drug ingestion. Therefore, the twice-daily dosing regimen used assured continuous suppression of the enzyme activity over a 24-hour period.

At the end of the study, that is, after 5 weeks of treatment with cilazapril, plasma angiotensin converting enzyme activity was significantly lower in the treated animals than in the controls, with an inclusion in excess of 96% of the plasma enzyme activity of control animals (Figure 1).

Mean femoral arterial blood pressure was similar in the treated and control animals before the study (62.4±3.5 versus 61.8±4.0 mm Hg, respectively; p=NS). However, at the end of the study, mean arterial pressure was significantly lower in the treated animals (38.2±2.1 mm Hg) relative to the control animals (56.8±4.8 mm Hg, p=0.003). Heart rate was similar in the treated and control animals before (85.9±4.6 versus 78.9±4.2 beats per minute, p=NS) and at the end of the study (70.7±5.8 versus 74.8±6.6 beats per minute, respectively; p=NS).

Four weeks after both the superficial or mild and deep or severe arterial injury, extensive neointimal thickening characterized by a combination of fibrocellular proliferation, extracellular matrix accumulation, and organization of mural thrombus (Figures 2A, 2B, and 2C) was observed at the site of vessel injury. After the superficial injury, the surface area of the neointima and the media were similar (p=NS) in the treated and control animals; therefore, the ratio of neointima to media was not different (Figure 3). Changes occurring after 4 weeks in the deeply injured arteries were again similar (p=NS) in the treated and control animals (Figure 4). Although the neointimal changes in the control animals were somewhat less than in the treated animals, they were not statistically significant.

These neointimal changes were not associated with any changes in serum total cholesterol before (2.2±0.2 versus 2.6±0.2 mmol/l, p=NS) and after (2.3±0.1 versus 2.3±0.2 mmol/l, p=NS) the study in the treated and control animals, respectively. Foam cell accumulation was not evident in the area of neointimal changes; likewise, there were no associated changes in the aortic media thrombogenicity of the treated relative to the control animals, as measured by the extent of 111In-labeled platelet deposition (18.1±1.8×10⁶ versus 15.4±2.8×10⁶, respectively; p=NS) in the superfusion flow chamber under controlled blood flow conditions.

**Discussion**

Injury to blood vessels appears to produce a stereotypical response leading to marked intimal thickening, a process that is believed to reflect one of the early stages in the development of atherosclerosis. This process appears to develop independently of the type of vessel wall injury (immunological or mechanical) and has been demonstrated in a number of animal models, including the rat, rabbit, swine, and in humans after coronary angioplasty and saphenous vein aortocoronary bypass surgery. In the present study using normolipemic swine, significant neointimal thickening is observed at 4 weeks after balloon injury, confirming previous reports. However, the data presented also show that these neointimal changes were not modified by pretreatment with the angiotensin converting enzyme inhibitor cilazapril. The extent of neointimal thickening was unchanged after either mild or severe balloon injury despite adequate drug absorption as shown by the almost complete inhibition of the plasma angiotensin converting enzyme activity and the significant depression of mean arterial pressure.

The twice-daily regimen of cilazapril used in this study was selected to provide continuous suppression of angiotensin converting enzyme and to achieve a lowering of blood pressure response throughout the day. At the end of the study, plasma angiotensin converting enzyme activity was indeed markedly depressed, as was the physiological parameter of blood pressure. The absence of a beneficial effect of cilazapril on the development of neointimal hyperplasia in this study differs from the observations previously reported by Powell et al in rats. Although it is possible that a significant lowering of blood pressure may contribute to the observed decrease in neointimal changes after cilazapril or hydralazine treatment in the rat model, it is less likely that such a mechanism was operative in this study in which a significant 39% fall in blood pressure produced...
no significant change in neointimal thickening. Similar to the Powell study, however, which has shown the need for continuous administration of cilazapril during the response of the vascular wall, cilazapril was started 7 days before vessel wall injury and then continued for the 4 weeks of the study. In Powell's and Chobanian's studies, a large difference in atherosclerosis development in favor of drug therapy was observed. This was a very significant difference ($p<0.0001$) with the 22 animals evaluated. However, in our study involving 16 animals, the extent of neointima formation as well as the ratio of neointima to media tended to be higher in the treated group than in the control group for both the mild and severe injury. Because of this potentially adverse effect of drug therapy, it becomes very unlikely that a beneficial treatment effect (even at the level of $p<0.05$) could be demonstrated even if the number of animals was increased; thus, it is unlikely that this negative study was due to a type II error. In addition, a similar dose of cilazapril was used, which is almost 100 times the equivalent dose that might be used in the clinical setting. This high dose of cilazapril used in the

**Figure 2.** Photomicrographic sections showing various components of the myofibrotic response after balloon arterial injury in pigs, using hematoxylin–phloxine–safran stain. Panel A: Extensive neointimal myofibrotic changes (I) can be seen bounded by the lumen (L) on one side and by the internal elastic lamina (arrow) on the other side. Part of the media (M) is also seen. Panel B: Representative section showing reorganization of a mural thrombus (T) superimposed on extensive neointimal myofibrotic changes and obstructing the lumen. Panel C: Higher power of the neointimal changes showing fibrocellular proliferation and extracellular matrix accumulation.

**Figure 3.** Plots show neointimal thickening 4 weeks after superficial or mild arterial injury by balloon deendothelialization at $<0.05$ atm pressure. The planimeted surface area (in square millimeters) of the neointima (1.16±0.2 vs. 0.92±0.34) and media (2.18±0.15 vs. 2.0±0.22) were similar in the treated and control animals, respectively; therefore, the ratio of neointima to media was not different. In uninjured areas of the artery, no intimal proliferation was observed.

**Figure 4.** Plots show neointimal thickening 4 weeks after severe or deep arterial injury by balloon angioplasty at 6 atm pressure. The planimeted surface area (in square millimeters) of the neointima (0.82±0.27 vs. 0.34±0.16) and media (1.98±0.15 vs. 2.15±0.32) were again similar in the treated and control animals, respectively. The ratio of neointima to media was also not different. In uninjured areas of the artery, no intimal proliferation was observed.
rat and the pig model may be of very little physiological significance for the present ongoing trials in humans, in whom the dose used is relatively much lower.

It is interesting to note that in another model of atherosclerosis involving the rabbit, captopril has also not been shown to influence the development of aortic atherosclerosis, although it had a beneficial effect on atherosclerosis development in Watanabe rabbits. Thus, it is not clear whether the responses observed in the rats or the Watanabe rabbits are animal species dependent, or even animal model dependent. It is possible that the inhibition of swine plasma angiotensin converting enzyme as documented in this study did not reflect inhibition of the local tissue renin–angiotensin system. Recent data suggest that plasma angiotensin converting enzyme activity does reflect quite closely the total transformation of angiotensin I to angiotensin II, and because of the 5-week prolonged and continuous drug administration in this study and the significant fall in blood pressure, it is likely that at least some inhibition of tissue renin–angiotensin system has occurred. In addition, other important differences may exist between the rats, rabbits, and swine regarding the content of the converting enzyme in different tissues, the kinetics of the converting enzyme reaction, and the degree of penetration or binding of the drug to the converting enzyme.

Although intimal thickening represents a vascular response to vessel wall injury occurring in humans and in numerous animal models, the exact mechanisms involved have not been identified. Powell et al have raised the possibility that the renin–angiotensin system may be involved in this process. However, the responses to heparin and hydralazine in the same model suggest that other mediators may also be involved. Other studies suggest that local thrombin, platelets, and monocytes may also play a role. Therefore, intimal thickening and restenosis after angioplasty is believed to be a multifactorial process involving not only the vessel wall and its interaction with circulating blood elements but also its interaction with growth factors that influence cellular proliferation. The actual role and relative importance of these various factors in humans and in animal models must be defined, and different mechanisms may assume increasing importance in each of the various conditions. Many of these factors may even mimic or amplify the effects of each other. It is unclear whether the renin–angiotensin system can influence or modulate the platelet–vessel wall interaction or the effects of other growth factors in vivo. In swine, angioplasty as performed in this study causes acute platelet thrombus deposition, which may contribute to intimal thickening both by thrombus reorganization and by the release of mitogenic platelet-derived growth factor (PDGF) and other growth factors from the platelet-rich thrombus stimulating intimal hyperplasia. In a rabbit model, Liu et al have shown that teipid, which can inhibit PDGF-induced cellular proliferation in vitro, can also prevent the development of restenosis in vivo. Besides the release of PDGF, platelets may influence vascular smooth muscle cell growth by the release of other mitogens such as serotonin, transforming growth factor B, epidermal growth factor, and release of growth-inhibiting heparin from the endothelial cells. However, a platelet-mediated process may not be the only mechanism involved in the swine model, and it would have been interesting to assess the influence of heparin or hirudin, a direct thrombin inhibitor, alone and in combination with cilazapril, to appreciate the relative importance of these mechanisms in this animal model. It is of interest that in rabbits, hirudin has recently been shown to decrease restenosis. The loss of endogenous growth inhibitors as well as the release of mitogenic factors from injured and dying endothelial cells may also contribute to smooth muscle growth.

Restenosis after angioplasty and intravascular stenting is a major problem limiting the expanded use of these procedures. However, an ideal animal model for the study of human atherosclerosis and the phenomenon of restenosis after angioplasty does not exist at present. Rabbits, dogs, or rats have been used previously. None of these animals develop atherosclerosis naturally, and in the atherosclerotic rabbit model, foam cell lesions predominate in association with a serum cholesterol level in excess of 1,000 mg/dl. Although the intimal changes observed in the rabbits may reflect mainly a response to mild injury, it is unclear whether more severe injury as occurs in humans will result in a similar or more aggressive response. In this study, pigs were used: These animals develop atherosclerosis spontaneously without cholesterol feeding, and the similarity of their platelet coagulation system to that of humans may provide a closer approximation of the platelet coagulation system interactions with the vessel wall at the site of injury. Both mild and severe injury can be readily performed, although when used at 2–3 months of age, atherosclerotic lesions were not present.

Conclusions

Both mild and severe carotid arterial injury in pigs promote the development of intimal thickening that is not affected by pretreatment with the converting enzyme inhibitor cilazapril. The clinical significance of these findings is unknown. However, these data and methods may provide some useful insights not only into the relevance of animal models of injury to human arterial disease caused by vessel wall injury, such as by balloon angioplasty, but also into the mechanisms of drug effects in current clinical trials using cilazapril in the prevention of coronary arterial restenosis in humans.

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