Effects of L-749,329, an ET_A/ET_B Endothelin Receptor Antagonist, in a Porcine Coronary Artery Injury Model of Vascular Restenosis

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Background—Previous studies in animal models of angioplasty have suggested a role in neointimal hyperplasia for endothelins (ETs), potent vasoconstricting peptides that also exert growth-promoting effects. The present studies were undertaken to test the hypothesis that endothelin receptor blockade can reduce neointimal thickening in injured porcine coronary arteries.

Methods and Results—An ET_A/ET_B antagonist, L-749,329, was evaluated as an inhibitor of intimal thickening in a porcine balloon/stent model of coronary artery injury. L-749,329 competitively inhibited [125I]ET-1 binding to porcine ET_A (IC_{50} 0.3 nmol/L) or ET_B (IC_{50} 20 nmol/L) receptors and inhibited ET-1–stimulated signaling in cell culture. In anesthetized pigs, big ET-1–stimulated increases in systemic blood pressure were totally inhibited after intravenous infusion of L-749,329 (0.2 mg · kg^{-1} · h^{-1}). In vascular injury studies, pigs were treated with vehicle or L-749,329 (1 mg · kg^{-1} · h^{-1}) beginning 2 days before and continuing 28 days after experimental angioplasty. Left anterior descending, left circumflex, and/or right coronary arteries were injured by inflation of an angioplasty balloon wrapped with a coiled metallic stent. After 28 days, mean neointimal thickness in the L-749,329–treated group was reduced by 9.0% compared with vehicle-treated controls, but this effect was not statistically significant (P=0.13).

Conclusions—Blockade of endothelin receptors for 28 days with only a mixed ET_A/ET_B receptor antagonist is insufficient to substantially inhibit intimal hyperplasia after balloon/stent coronary artery injury in the pig, in contrast to results with a selective ET_A antagonist. The effects of selective or mixed ET_A/ET_B antagonists in diseased vessels remain to be determined in this model. (Circulation. 2001;103:1899-1905.)

Key Words: restenosis • endothelin • coronary disease • angioplasty

The endothelins constitute a family of endothelium-derived polypeptides that are among the most potent vasoconstrictors known.1,2 The major endothelin receptor subtypes (ET_A and ET_B) are expressed in vascular smooth muscle, where they mediate vasoconstriction,3 whereas the ET_B subtype on endothelial cells is believed to mediate vasorelaxation.4 These receptors have become targets for the development of agents to treat hypertension, myocardial ischemia, and congestive heart failure.5

In addition to their acute effects on vascular tone, endothelins are recognized to exert growth-promoting effects,6 a response associated with activation of the ET_A receptor.7 Endothelin receptors are elevated in atherosclerotic coronary arteries.8 Elevated plasma levels of immunoreactive ET-1 or its immediate precursor, big ET-1, have been detected in patients with angina pectoris9 and acute myocardial infarction.10 In addition, elevated endothelins have been observed acutely after cardiac catheterization11 or PTCA.12 The clinical results, together with in vitro data,13 have suggested that endothelins may be involved in atherosclerosis, vascular hypertrophy, and restenosis after angioplasty.14

Balloon denudation of rabbit carotid arteries was associated with prolonged elevation in immunoreactive ET-1 and induction of ET_B binding activity localized to the neointima.15,16 In rat carotid arteries, mRNAs for endothelin precursors, endothelin-converting enzyme-1, and both ET_A and ET_B receptors were induced by balloon injury.17 Moreover, infusion of ET-1 was found to potentiate carotid neointimal formation provoked by balloon angioplasty in rats.18,19 In inhibitor studies, long-term administration of antagonists with activity toward both ET_A and ET_B receptors markedly reduced neointimal formation in balloon-injured rat carotid arter-
ies,20,21 The ET₄-selective antagonist BQ-123 was ineffective in rat ²⁰ and rabbit ¹⁵ carotid artery studies, suggesting a predominant role for the ET₄ receptor in lesion development. In contrast to these findings, several ET₄-selective agents have been reported to inhibit neointimal thickening in rat and porcine vascular injury studies.²²-²⁵ Thus, results reported to date do not clearly indicate whether the ET₄ or ET₅ receptor subtype is of principal importance in the response to injury. In the present studies, we evaluated a potent, mixed ET₄/ET₅ endothelin receptor antagonist for its ability to inhibit intimal thickening in a porcine coronary artery model of vascular restenosis.²⁶

Methods

Endothelin Receptor Ligands

Porcine sequence ET₁ and big ET₁ (Peptide Institute), BQ-123 (Bachem), and sarafotoxin 56C (Peninsula) were prepared as recommended by their respective vendors. For PTCA studies, L-749,329 (racemic L-754,142²⁷) was prepared at 50 mg/mL in 0.9% saline. All agents were filter sterilized before administration.

Receptor Binding Assays

Levels of L-749,329 in pig plasma were estimated by radioceptor assays with rat aortic smooth muscle cell (SMC) cultures²⁸ in which only the ET₄ receptor subtype is detectable. Confluent cultures in 24-well culture dishes were washed with 0.5 mL of Waymouth’s medium containing 0.1% bovine serum albumin (BSA), 20 mmol/L N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], pH 7.3 (WBH). Binding incubations (0.3 mL of WBH plus 10 μM heparin) contained 50 pmol/L [¹²⁵I]ET-1 (DuPont-NEN) plus 10% (vol/vol) test plasma or 10% control plasma spiked with known concentrations of L-749,329. Incubation, washing, harvesting of bound [¹²⁵I]ET-1, and data analysis were performed as described previously.²⁸ A similar procedure was used for assays of endothelin receptor antagonist potency in pig SMC and LLC-PK1 cells,²⁹ except that binding incubations contained 0.01% BSA and no plasma or heparin. Specific binding was defined as total binding minus binding occurring in the presence of 1 μmol/L ET-1.

Measurement of Inositol Phosphate Production

Confluent cultures of pig coronary artery SMCs in 24-well dishes were labeled for 48 hours at 37°C with 3 μCi of [³²P]myo-inositol (Amersham; 18.3 Ci/mmol) in inositol-free DMEM (Irvine), 0.5% fetal bovine serum. Cells were washed with DMEM containing 1 mg/mL myo-inositol, 10 mmol/L LiCl, and 0.1% BSA and stimulated with ET-1 in the presence or absence of L-749,329 for 60 minutes at 37°C. [³²P]Inositol phosphates were measured as described previously.³⁰

Instrumentation

All studies were performed according to procedures approved by the Merck-West Point Institutional Animal Care and Use Committee. Juvenile male or female Yorkshire pigs (weight ~30 kg) were catheterized for compound infusion and blood sampling as described previously.²⁸ When required for blood pressure monitoring, a vascular access port was placed in the common carotid artery and secured subcutaneously in the right lateral cervical area. Arterial pressure in animals under isoflurane anesthesia was measured by DTX pressure transducer systems with ECG monitoring. Animals were allowed to recover in their home cages and were observed until stable.

Angioplasty Studies

Animals were premedicated with 650 mg of aspirin (24 hours before angioplasty), 30 mg of nifedipine (2 hours before), and 10 000 U of heparin (immediately before). After 2 days of pretreatment with vehicle (0.9% saline) or L-749,329 (50 mg/mL; 1 mg·kg⁻¹·h⁻¹), animals were reanesthetized and subjected to angioplasty of the left anterior descending, left circumflex, and/or right coronary arteries under fluoroscopic imaging.²⁸ The balloon was inflated to 8 atm for 15 seconds to deploy the coiled, tantalum wire stent, resulting in a 1.2- to 1.4-fold ratio of balloon diameter to initial vessel diameter. After intravenous administration of 1 g of cefoxitin, animals were allowed to recover in their home cages and were maintained for 28 days after angioplasty with constant intravenous infusion of vehicle or test compound. Jugular catheters were flushed aseptically with saline 3 times per week and locked with 50% glucose/heparin. Blood samples were drawn weekly for measurement of plasma drug levels. Ampicillin (500 mg PO) was given daily.

Analysis of Neointimal Thickening

Animals were euthanatized with pentobarbital (60 mg/kg IV) on day 28 after angioplasty. Formalin-fixed coronary arterial segments containing the expanded stent were excised and processed for 5-µm cross-sectioning and hematoxylin/eosin and elastin-van Gieson staining as described previously.²⁶,²⁸ Sections from each vessel were scored for degree of injury and neointimal thickness using the section showing the most severe injury. The measurements for each vessel were averaged to produce a single data point. Luminal areas and areas circumscribed by internal and external elastic laminae were measured at the site of injury. Preangioplasty lumen size was estimated by measuring the uninvolved luminal area ~5 mm distal to the site of balloon/stent deployment.

All values reported are estimates of the mean±SE. For neointimal thickness, injury score, and luminal areas, estimates were calculated by maximum likelihood methodology and a linear mixed-effects model with a random effect for each pig. Separate analyses were performed on data from the L-749,329–treated and control pigs. Neointimal thickness was fit on the logarithmic scale; all other variables were fit with the scale on which they were measured. Comparisons of injury score and external elastic lamina areas for the 2 treatment groups were made by a maximum likelihood ratio test; probability values for these comparisons were 2-sided. Comparisons of the treatment means that were adjusted for injury score (neointimal thickness and luminal areas) were made with linear regression and the jackknife procedure³⁰ to estimate standard errors. Specifically, after we checked for parallelism, log(neointimal thickness) or log(luminal area) was regressed on injury score for the injured vessels of antagonist-treated and control pigs. Regression lines with equal slopes were fit for the treated and control pigs, allowing the difference between treatment and control to be measured by the difference in intercepts. By this method, the comparisons were on a “per pig” basis. Probability values reported for these comparisons are 1-sided.

Results

Potency of L-749,329 Toward Porcine ET₁ Receptors

L-749,329 is the racemic form of the nonpeptide endothelin receptor antagonist L-754,142, which exhibits potent activity toward both the ET₁ (Kᵢ = 0.062 nmol/L for the cloned human receptor) and ET₂ (Kᵢ = 2.25 nmol/L) subtypes.²⁷ The ability of L-749,329 to interact with porcine endothelin receptors was confirmed in binding studies with porcine coronary artery SMCs and LLC-PK1 porcine kidney epithelial cells (Figure 1). In the presence of 50 nmol/L [¹²¹I]ET-1 radioligand, L-749,329 blocked ET₁ binding with an IC₅₀ of ~0.3 nmol/L. [¹²¹I]ET-1 binding to porcine SMCs was totally inhibited by the ET₁-selective antagonist BQ-123 (IC₅₀ ~1 nmol/L) but not appreciably by the ET₂-selective ligand sarafotoxin 6-C, indicating that the ET₁ receptor subtype is expressed predominantly in these cells (Figure 1A). In cultures of LLC-PK1 cells, which principally express the ET₂ receptor.
Inhibition of ET-1 Signaling by L-749,329

Pig coronary artery SMCs prelabeled with [3H]myo-inositol were stimulated with ET-1 in the presence or absence of L-749,329. Binding of [125I]ET-1 to intact pig coronary artery SMCs (A) or LLC-PK1 cells (B) was measured in presence of competing ligands. Specific binding is expressed as percentage of control specific binding (cpm bound without competing ligand minus cpm bound in presence of 1 μmol/L ET-1). Results shown (mean±SD, n=3 determinations) are representative of 3 experiments. ●, L-749,329; ▽, BQ-123; ▼, sarafotoxin 6C (S6C).

Dose-Finding Experiments

Pressor responses to ET-1 or its biosynthetic precursor, big ET-1, were measured in anesthetized animals before and after a series of intravenous infusions of L-749,329. ET-1 (0.25 to 0.5 nmol/kg) produced rapid and prolonged increases in mean arterial pressure (MAP); at lower doses of ET-1 (0.1 to 0.2 nmol/kg), a transient depressor phase of response was noted 1 to 3 minutes after ET-1 administration (not shown). Depressor responses to ET-1 have been linked to ETB-mediated vasodilation in other species. Bolus doses of L-749,329 (1 to 10 mg/kg) were associated with decreases in both the magnitude and duration of MAP increases stimulated by ET-1. In separate studies, doses of ET-1 (0.25 nmol/kg) and big ET-1 (0.5 nmol/kg) that yielded similar maximum MAP responses in the pig (30 to 40 mm Hg) were determined empirically (Figure 3, “Control”). Consistent with previous reports, big ET-1 produced an increase in MAP that was slower in onset and relatively prolonged compared with that stimulated by ET-1. In the experiment shown, constant intravenous infusion of L-749,329 at 1.0 mg · kg⁻¹ · h⁻¹ for 24 hours produced 78% inhibition of the pressor response to ET-1 and total inhibition of the response to big ET-1.
ET-1 (Figure 3). This degree of inhibition of the response to ET-1 was not exceeded even when L-749,329 infusion was increased to 5 mg · kg⁻¹ · h⁻¹, whereas rates lower than 0.2 mg · kg⁻¹ · h⁻¹ were associated with diminished inhibition of both the ET-1 and big ET-1 responses. The rates of infusion associated with maximal inhibition of ET-1 and big ET-1 pressor responses gave rise to plasma levels of L-749,329 ≥340 nmol/L. On the basis of these results, a dose of 1 mg · kg⁻¹ · h⁻¹ was chosen for the PTCA studies, with a target plasma L-749,329 concentration of ≥400 nmol/L.

Angioplasty Studies

Seven pigs each were assigned to receive vehicle or L-749,329 infusion beginning 2 days before PTCA. Balloon/stent angioplasty was performed on a total of 21 vessels in the vehicle group and 20 vessels in the L-749,329–treated group. In the antagonist-treated animals, mean plasma levels of L-749,329 were 1300±540 nmol/L on the day of angioplasty and were maintained between 1210 and 1670 nmol/L throughout the 28-day study period. These levels are well above those determined independently to be associated with maximal blockade of pressor responses to endothelins. At day 28 after angioplasty, animals were euthanatized, and hearts were removed for histomorphometric analysis. Elastin-van Gieson–stained sections from each injured vessel were examined by light microscopy for determination of injury score and neointimal thickness (Figure 4). Mean injury scores did not differ between the 2 groups (P=0.76; Table).

Treatment with L-749,329 was associated with a 9.0% decrease in mean neointimal thickness when adjusted for injury score, but this effect was not statistically significant (P=0.13) (Figure 5). Mean vessel luminal area at the site of angioplasty (adjusted for injury score) was increased by 18% in the L-749,329–treated animals compared with the vehicle group; again, this effect was not statistically significant (P=0.06). When the net change in luminal area was calculated for each vessel (luminal area at the site of injury minus the preinjury luminal area approximated as the uninvolved luminal area measured 5 mm distal to the site of angioplasty), the increase associated with L-749,329 treatment dropped to 7.0% (P=0.36). Thus, the small reduction in neointimal thickness (9.0%) associated with L-749,329 treatment was accompanied by a small increase in luminal area (7.0%). The mean areas circumscribed by the internal elastic laminae in the 2 treatment groups were very similar (Table), consistent with the approximately reciprocating changes in luminal area and neointimal thickness noted above. Mean areas circumscribed by the external elastic lamina in the 2 groups also were similar.

Discussion

Endothelin receptor antagonists have been used to probe the involvement of endothelins in postangioplasty neointimal hyperplasia. Studies in animal models of vascular injury have suggested a role for endothelins in neointimal formation but have produced conflicting results with respect to the endo-
L-749,329 (0.2 mg · kg$^{-1}$·h$^{-1}$) were totally abolished after intravenous infusion of big ET-1, demonstrating increased in systemic blood pressure in response to big ET-1. In anesthetized pigs, L-749,329 competitively inhibited ET A receptors on cultured porcine cells. In conscious pigs, chronic intravenous infusion beginning 2 days before balloon/stent injury and continuing until animals were killed, plasma levels of the antagonist in the L-749,329–treated group were considerably in excess of levels associated with maximal pressor blockade, as well as the IC$50$ values determined in vitro. Mean neointimal thickness in the L-749,329–treated group was reduced by 9.0% compared to vehicle-treated controls, but this effect was not statistically significant ($P=0.13$).

Our results using a mixed ET A/ET B endothelin receptor antagonist differ from previous findings obtained with an ET A–selective agent in the same model system. Several factors may contribute to this difference. Although the present results suggest that blockade of both ET A and ET B receptors is not an effective means of reducing neointimal thickening in pig coronary artery, the value of ET A versus ET B selectivity remains unresolved. It is possible, for example, that preservation of vasodilatation mediated by ET B might be protective after vascular injury in the pig, such that ET B blockade might exacerbate luminal loss. Another difference between the 2 studies is route of administration (intravenous and oral), which may have produced a distribution of inhibitor that favored efficacy in the prior studies. In addition, different methods of statistical analysis were applied in the 2 studies. Finally, the mean injury scores in the present study (2.07 and 2.13 for the vehicle and L-749,329–treated groups, respectively) were uniformly higher than those measured in the previous study (ranging from 1.73 to 1.94); measurements for both studies were made by the same core investigators at the Mayo Clinic. Thus, the absence of significant efficacy in the present study may stem from our relatively higher degree of vessel injury. This idea is supported by the observation in the previous study that the intermediate dose of ET A–selective agent was ineffective against a mean injury score of 1.94, whereas a lower dose was effective against a lower mean injury score of 1.73. Similarly, the ET A–selective antagonist LU 135252 recently was reported to significantly reduce the neointimal:medial ratio in a porcine balloon-only injury model; balloon expansion alone typically imparts an injury no more severe than medial dissection (ie, equivalent to injury score 2), whereas stent deployment can rupture the...
external elastic lamina as well (injury score 3). The mean injury scores >2 in the present study reflect the greater incidence of external elastic lamina disruption.

Several limitations to the present studies should be noted. The animals used likely were normolipidemic, and the vessels targeted were not previously injured. Consequently, we cannot infer the effects of L-749,329 in atherosclerotic vessels. However, all of the prior studies mentioned above likewise were conducted in nondiseased arteries, and effects of endothelin antagonists still were manifest. Second, treatment with endothelin receptor antagonists is recognized to increase endothelin receptor levels in culture and increase circulating endothelin levels in vivo. Because we did not measure endothelin levels or monitor pressor inhibition throughout the 30-day study, it might be argued that compensatory changes in endothelin or receptor expression rendered L-749,329 less effective. This argument is countered by the observation that mean L-749,329 plasma levels were maintained at >1000 nmol/L, ie, >50-fold excess over the IC₅₀ for ET-1 binding. Third, we cannot exclude the possibility that adequate concentrations of L-749,329 did not reach sites critical for reducing neointimal hyperplasia, although the inhibitor clearly had access to sites involved in endothelin pressor responses.

A variety of studies have documented the activities of endotheinals and their receptors in normal and injured porcine coronary arteries. Endothelin-stimulated contraction of isolated strips of pig coronary arteries is composed of both ETA antagonist-sensitive and -insensitive components, suggesting that both ETA and ETB receptors can mediate constriction of these vessels. Addition of ETB receptors may be heterogeneous in pig coronary artery. Net release of ET-1 from pig coronary arteries has been balanced angiotensin receptor antagonists in a porcine coronary artery model of external elastic lamina disruption.

**References**


distinct signaling pathways in porcine kidney epithelial LLC-PK₁ cells.

30. Huckle WR, Hawes BE, Conn PM. Protein kinase C-mediated sequestration
of gonadotropin-releasing hormone receptors is associated with uncoupling

thalin have less vasoconstrictor activity in vitro but a potent pressor effect

endothelin-1 binding following balloon angioplasty of pig coronary
arteries: effect of the ETA receptor antagonist, LU 135252. *Cardiovasc

33. Yu JCM, Davenport AP. Regulation of endothelin receptor expression in

34. Haynes WG, Ferro CJ, O’Kane KPI, et al. Systemic endothelin receptor
blockade decreases peripheral vascular resistance and blood pressure in

35. Fukuroda T, Nishikibe M, Ohta Y, et al. Analysis of responses to endo-
thelins in isolated porcine blood vessels by using a novel endothelin

36. Pernow J, Modin A. Endothelial regulation of coronary vascular tone in
vitro: contribution of endothelial receptor subtypes and nitric oxide. *Eur

37. Harrison VJ, Randriantsosa A, Schoeffter P. Heterogeneity of endothelin-
sarafotoxin receptors mediating contraction of pig coronary artery. *Br J

38. Tonnessen T, Naess PA, Kirkeboen KA, et al. Release of endothelin from

(ET) and enhanced ET₄ receptor-mediated coronary vasoconstriction
after coronary thrombosis and thrombolysis in pigs. *J Cardiovasc

40. Katwa LC, Campbell SE, Tanner MA, et al. The upregulation of endo-
thelin and its receptors in porcine coronary arteries in a double balloon
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