Long-Term Effects of Nonselective Endothelin A and B Receptor Antagonism in Postinfarction Rat

Importance of Timing

Quang Trinh Nguyen, MSc; Peter Cernacek, MD, PhD; Martin G. Sirois, PhD; Angelino Calderone, PhD; Nathalie Lapointe, MSc; Duncan J. Stewart, MD; Jean L. Rouleau, MD

Background—Some controversy exists as to the effects of endothelin (ET) receptor antagonism on long-term post–myocardial infarction (MI) evolution, particularly as it relates to the timing of the intervention after MI (<24 hours versus 10 days).

Methods and Results—Sham rats and rats surviving an acute MI for >20 hours (n = 301) were assigned to treatment with saline or the nonselective ET_A and ET_B receptor antagonist LU 420627 (LU) started <24 hours (early) or 10 days (late) after MI and continued for 100 days. Long-term LU treatment led to increased mortality of rats with large MI, regardless of the timing of initiation of therapy. Early initiation of LU reduced survival from 61% to 16% (P < 0.001 versus untreated), and later initiation reduced survival to 36% (P = 0.012 versus untreated and P < 0.001 versus early initiation). Early initiation of LU led to scar thinning, further left ventricular (LV) dilatation, LV dysfunction, and an excessive rise in right ventricular systolic pressure. Later initiation of LU did not modify scar formation but resulted in LV dilatation and dysfunction compared with the untreated group. Cardiac fibrosis tended to increase in the LU-treated MI groups. LU in the sham group reduced cardiac endothelial constitutive nitric oxide synthase but did not modify the changes that occurred with a large MI.

Conclusions—The use of the nonselective ET_A and ET_B receptor antagonist LU results in reduced survival, ventricular dilatation, and dysfunction whether started early or late after MI. Early initiation of LU resulted in scar expansion and a particularly unfavorable outcome. (Circulation. 2001;104:2075-2081.)

Key Words: myocardial infarction • survival • endothelium-derived factors • remodeling
improvement in ventricular function regardless of how long after MI it was started (3 hours to 1 week). Nonselective ET-1 receptor blockade has also resulted in improved survival when started 7 to 10 days after MI. The earlier use (3 to 20 hours) of ET_{A} receptor antagonists after MI, however, resulted in greater ventricular dilatation and either no change or deterioration in ventricular function due to impaired scar healing and thus scar expansion. These results raise the possibility that the detrimental effects of the early use of ET-1 receptor antagonists after MI are limited to patients with small and moderate MI. The nonselective ET_{A} and ET_{B} receptor blockade would result in even more beneficial effects than those with bosentan, which nevertheless has more ETA than ETB selectivity.

The objectives of this study were thus to compare the effects of early (<24 hours) and late (10 days) initiation of the nonselective ET_{A} receptor antagonists and that the use of more nonselective ET_{A} and ET_{B} receptor blockade would result in even more beneficial effects than those with bosentan, which nevertheless has more ETA than ETB selectivity.

The nonselective ET_{A} and ET_{B} receptor antagonist LU was used because it antagonizes ET_{A} and ET_{B} receptors equally.

Methods

Animals
Male Wistar rats (Charles River, St-Constant, Quebec, Canada) weighing 200 to 250 g were chosen. The use and care of laboratory animals was according to the Canadian Council for Animal Care and was approved by the Animal Care Committee of the Montreal Heart Institute.

MI Operative Procedure
Anesthesia was induced with 2% halothane, and rats were intubated and mechanically ventilated on a small-rodent ventilator (Harvard Apparatus). During surgery, rats were maintained with 1% to 1.5% halothane in a mixture with O_{2} (100%).

MI was induced by ligation of the left anterior descending coronary artery, as previously described. Sham-operated rats had the 6-0 silk suture inserted but not tied.

There was a high perioperative mortality (36%) within 20 hours of surgery. The survivors were randomly divided into the different treatment groups so as to have approximately the same number of survivors in each group. Rats were classified according to MI size at the end of the study. Rats with large MI were defined as those having an LV scar of >45% of the ventricular cross-sectional circumference or a ratio of scar to body weight >0.2 mg/g. Rats with small and moderate MI were not considered further in this study.

Pharmacological Interventions
Surviving rats were randomized 20 hours after surgery into 3 groups: (1) control (untreated), (2) LU started 20 hours after MI, and (3) LU started 10 days after MI (Figure 1). LU 100 mg · kg^{-1} · d^{-1} or vehicle (0.9% saline) was given by gavage twice daily during the first week. Thereafter, LU was given in drinking water, whereas the control group drank water without any additions. All rats were followed up for a total of 100 days (14 weeks) after MI. The body weight and water intake were measured weekly to ensure that the animals received the appropriate daily dose of LU throughout the study. To verify the plasma levels of LU achieved by this treatment, these were measured in randomly selected rats after 2 weeks of treatment by radio receptor assay as previously described. The levels found were 9.6 ± 1.9 μmol/L (n = 8), documenting an efficient blockade of both ET_{A} and ET_{B} receptors, the K_{i} of both ET_{A} and ET_{B} receptors to this compound being lower by >3 orders of magnitude. The LU compound is a nonselective ET_{A} and ET_{B} receptor antagonist with K_{i} values of 2 and 6 nmol/L, respectively. The half-life is 2 hours. The efficacy of ET_{B} receptor blockade was verified by evaluating the effects of injections of increasing doses of the ET_{B} receptor-selective agonist BQ3020, 0.01 to 1 nmol/kg (Sigma), on mean arterial pressure of anesthetized rats 2 weeks after sham operation (n = 13) or MI (n = 12). The BQ3020 was prepared in PBS at pH 7.4. Long-term LU treatment with 100 mg · kg^{-1} · d^{-1} markedly shifted the dose-response curve of BQ3020 to the right, indicating significant ET_{B} receptor blockade (results not shown).

Long-Term Hemodynamic Effects
Fourteen weeks after surgery, rats were anesthetized with halothane 2%, and the percentage was reduced to 0.5% to 0.8% 10 minutes before hemodynamic recordings. LV and right ventricular (RV) hemodynamics were then obtained as previously described, and thereafter, cardiac hypertrophy and/or LV remodeling was assessed. Rats that died after 72 hours after MI but before the hemodynamic study had morphological assessment of MI size but were not used for other measurements, except for survival. Those that died between 24 and 72 hours were assumed to have had a large MI. All rats that died after randomization are considered in the survival analyses.
Passive Pressure-Volume Relationship and Ventricular Remodeling

After the rats were killed by an injection of 20 mmol/L KCl, an LV passive pressure–volume relationship was assessed as previously described, and the heart was filled with saline to a pressure of 15 mm Hg, sealed, and fixed in its distended form in 10% formalin phosphate buffer for 24 hours. The heart was then cut halfway between the base and apex, and 2 slices were obtained 1 mm above and below the middle cut and stained with hematoxylin-phloxine safran for assessment of MI size, scar, and epicardial and endocardial characteristics, as previously described. Collagen was quantified by computer-assisted image analysis of samples from both cross sections cut into 8-μm-thick slices and stained with Sirius red F3BA as a 0.1% solution in saturated aqueous picric acid as previously described.

Assessment of Cardiac Hypertrophy

This group of rats was killed by exsanguination, and the heart was rapidly excised, washed in Krebs-Henseleit solution, and dissected into right and left atria, RV, LV (including septum), and the scarred area. The scarred area was pinned on a paper, and its surface was determined by planimetry. Each tissue was then weighed individually.

Assessment of Plasma and Tissue ET-1 and Plasma Angiotensin II

Plasma angiotensin II levels were measured as previously described. The plasma ET-1 concentrations and the ET-1 concentration of the lung, the RV, and the viable portion of the LV were determined by previously established methods with a detection limit of 0.12 pg/tube and an interassay coefficient of variation of 11%. The cardiac content of immunoreactive ET-1 is expressed in pg/mg tissue wt, and the pulmonary content is expressed in fg/mg tissue protein, to correct for lung congestion in rats with heart failure.

Myocardial ecNOS Protein Expression Analysis

Myocardial endothelial constitutive nitric oxide synthase (ecNOS) protein expression analysis was performed according to our previously reported method.

Drugs

The ET\textsubscript{a},ET\textsubscript{b} receptor antagonist LU was kindly provided by Dr. M. Kirchengast (Knoll AG, BASF Pharma, Ludwigshafen, Germany).

Statistical Analysis

All values were expressed as mean±SEM. Results were analyzed by 2-tailed Student’s t test for unpaired data and by ANOVA followed by Dunnett’s test when appropriate. Statistical significance was assumed at a value of P<0.05. Kaplan-Meier survival curves over the whole follow-up period were constructed and analyzed by the generalized Savage (Mantel-Cox) test.

Results

Survival

Of the 472 rats that had surgery, 171 died within 20 hours of surgery and were excluded from the study. The other 301 rats were randomized to control (n=54), early LU (n=162), and late LU (n=85). During the 14-week treatment period, survival of the sham-operated control (n=10) and LU (n=15) groups was excellent (100%), with none of the rats dying. Survival in the control large-MI group was 61%. Early

<table>
<thead>
<tr>
<th>TABLE 1. Cardiac Hemodynamics</th>
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<tr>
<td>HR, bpm</td>
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<tr>
<td>Sham control (n=10)</td>
</tr>
<tr>
<td>Sham + LU (n=15)</td>
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<tr>
<td>Large MI control (n=23)</td>
</tr>
<tr>
<td>Large MI + LU early (n=22)</td>
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<tr>
<td>Large MI + LU late (n=26)</td>
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HR indicates heart rate; MAP, mean arterial pressure. Values are mean±SEM.

*P<0.05 vs sham; †P<0.05 vs control.
post large MI had the worst survival (26%, $P<0.05$ versus control large MI, 72%), and survival in the late LU-treated group was intermediate (48%, $P<0.05$ versus control large MI).

**Hemodynamic Effects of LU Treatment**

In the sham group, LU treatment had only mild effects on the hemodynamic parameters measured (Table 1). LU caused a slight decrease in LV systolic pressure (LVSP) and maximum rate of LV pressure rise (+dP/dt).

Control rats with a large MI had a decrease in LVSP, an increase in LV end-diastolic pressure (LVEDP), and a decrease in all measured indices of contractility and relaxation compared with their sham-operated counterparts. This was accompanied by an increase in RV systolic (RVSP) and end-diastolic (RVEDP) pressures. Early LU-treated rats with large MI had a similar decrease in LVSP and in mean arterial pressure. They had a greater increase, however, in LVEDP, RVSP, and RVEDP. They also had a greater decrease in LV +dP/dt when corrected for LVSP, indicating further LV dysfunction compared with control large MI. The late LU large-MI group had hemodynamic changes between those of the control and early LU large-MI groups.

Control rats with a large MI studied 2 weeks after MI ($n=6$) had significant LV dysfunction and pulmonary hypertension. Rats receiving LU early and euthanized 2 weeks after MI ($n=6$) had more marked LV dysfunction, LVSP being $72 \pm 2$ versus $83 \pm 3$ mm Hg for control MI, $P<0.05$, LVEDP $24 \pm 1$ versus $20 \pm 1$ mm Hg, $P<0.05$, LV +dP/dt $2277 \pm 40$ versus $3087 \pm 101$ mm Hg/s, $P<0.05$, and RVSP $48 \pm 1$ versus $37 \pm 2$ mm Hg, $P<0.05$, for control MI.

**Passive LV Pressure-Volume Relationship**

LU treatment did not modify the pressure-volume relationship of sham-operated hearts. A rightward shift of the pressure-volume relationship was found in the control large MI group compared with sham. Early treatment with LU in the large-MI group shifted the curve rightward ($P<0.01$) compared with the control large-MI hearts (Figure 3A). Late treatment with LU also tended to shift the curve rightward, but this shift was not as marked and was significant only when heart volume was corrected for body weight (Figure 3B).

**Cardiac Remodeling and Morphological Studies**

The sham-MI LU-treated group had a slight increase in ratios of lung weight to body weight. Ratios of LV weight to body

<table>
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<th>TABLE 2. Morphology of Heart</th>
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<tr>
<td>BW, g</td>
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<tr>
<td>Sham (n=4)</td>
</tr>
<tr>
<td>Sham + LU (n=9)</td>
</tr>
<tr>
<td>Large MI (n=11)</td>
</tr>
<tr>
<td>Large MI + LU early (n=10)</td>
</tr>
<tr>
<td>Large MI + LU late (n=12)</td>
</tr>
</tbody>
</table>

BW indicates body weight; wt, weight. Values are mean±SEM.

* $P<0.05$ vs sham; † $P<0.05$ vs control.
weight were similar in all 5 groups, whereas the ratio of RV weight to body weight increased similarly in all 3 large MI groups. Ratios of lung weight to body weight followed a similar pattern, the 3 large-MI groups having an increase compared with the control sham-operated group (Table 2).

Scar weight was lower in the early LU large-MI group, but when adjusted for body weight, this was no longer significant. The early LU large-MI group had evidence of scar expansion and thinning, their scars having the lowest ratio of scar weight to surface and the greatest scar surface.

Long-term treatment with LU did not modify LV collagen volume density in the sham-operated group. The control large-MI group had an increase in LV collagen volume density, but the increase in the early and late LU large-MI groups was greater, reaching statistical significance in the late LU group (Figure 4).

Rats euthanized 2 weeks after MI had similar MI sizes as assessed by scar weight, 0.14±0.01 versus 0.12±0.01 mg, P = NS, for LU and control, respectively. LU-treated rats with a large MI, however, had greater RV wt/body wt, 1.44±0.11 versus 1.01±0.09 mg/g, P < 0.05, and greater lung wt/body wt, 11±1 versus 8.1±0.6 mg/g, P < 0.05, than control large MI.

Assessment of Plasma and Tissue ET-1 Levels and Plasma Angiotensin II

Plasma angiotensin II increased similarly in all 3 MI groups. Plasma ET-1 but not cardiac or pulmonary ET-1 increased in the sham LU group compared with their control counterparts. Control rats with a large MI had a similar increase in plasma ET-1 but also had a significant increase in cardiac and pulmonary ET-1 compared with the 2 sham groups. The 2 LU-treated groups had the greatest increase in plasma ET-1, but the increases in cardiac and pulmonary ET-1 concentrations were similar to those of the control large-MI group (Table 3).

Assessment of Cardiac ecNOS Protein

The hearts from control sham-operated rats were used to evaluate the basal tissue expression of ecNOS and served as control (100%) (Figure 5). Treatment of sham rats with LU reduced the normal basal level of ecNOS protein in heart (to 20.1%) to levels similar to those of the non-MI region of hearts with a large MI. The scarred areas had the lowest ecNOS expression (0% to 1.6%, P < 0.05). LU treatment did not modify ecNOS protein expression pattern in the hearts with a large MI.

Discussion

This study demonstrates that after large MI, long-term nonselective ET\(_A\) and ET\(_B\) receptor antagonism with LU leads to increased mortality, excessive LV dilatation and fibrosis, and further LV dysfunction. Early initiation (<24 hours) of LU after MI is particularly detrimental, leading to impaired scar healing, further ventricular dilatation and dysfunction, and reduced survival compared with later (10 days) post-MI initiation of this treatment. LU also reduces cardiac ecNOS in the sham-operated but not the large-MI group. This study thus indicates that nonselective ET\(_A\) and ET\(_B\) receptor antagonism with LU is detrimental after large MI, particularly when started early after MI.

The decrease in post-MI survival rate with LU contrasts with previous reports of improved survival with selective ET\(_A\), or nonselective ET\(_A\) and ET\(_B\) receptor antagonists. In the present study, post-MI survival with the nonselective ET\(_A\) and ET\(_B\) receptor antagonist LU was particularly poor when it was started early (<24 hours) after MI. In that group, there was evidence of poor scar healing and stretching (scar expansion), which contributed to further ventricular dilatation. LV dysfunction, evidence of RV hypertension, and reduced survival were already present 2 weeks after MI.

Although the survival of the group in which LU was started later (10 days) was better than that of the group in which LU was started early, post-MI survival with LU is still poorer than with sham treatment and compared with the late LU group. The decrease in post-MI survival rate with LU contrasts with previous reports of improved survival with selective ET\(_A\), or nonselective ET\(_A\) and ET\(_B\) receptor antagonists. In the present study, post-MI survival with the nonselective ET\(_A\) and ET\(_B\) receptor antagonist LU was particularly poor when it was started early (<24 hours) after MI. In that group, there was evidence of poor scar healing and stretching (scar expansion), which contributed to further ventricular dilatation. LV dysfunction, evidence of RV hypertension, and reduced survival were already present 2 weeks after MI.

Although the survival of the group in which LU was started later (10 days) was better than that of the group in which LU was started early, post-MI survival with LU is still poorer than with sham treatment and compared with the late LU group.

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**Figure 4.** Effects of large MI and LU therapy on LV collagen volume density. *P < 0.05 vs sham; #P < 0.05 vs large-MI control.

**Table 3.** Plasma and Tissue Neurohormonal Concentrations After MI

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Tissue-ET-1</th>
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<tbody>
<tr>
<td>Ang II, pg/mL</td>
<td>ET-1, pg/mL</td>
</tr>
<tr>
<td>Sham</td>
<td>270±75 (n=10)</td>
</tr>
<tr>
<td>Sham + LU</td>
<td>305±61 (n=14)</td>
</tr>
<tr>
<td>Large MI</td>
<td>1157±218* (n=16)</td>
</tr>
<tr>
<td>Large MI + LU early</td>
<td>989±128* (n=16)</td>
</tr>
<tr>
<td>Large MI + LU late</td>
<td>1180±204* (n=15)</td>
</tr>
</tbody>
</table>

Ang II indicates angiotensin II. Data are mean±SD.

*P < 0.05 vs sham; †P < 0.05 vs control.
was started earlier, it was nevertheless worse than that of the untreated large-MI group. This was an unexpected result, because previous studies of selective ET<sub>A</sub> and nonselective ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists demonstrated improved, or at worst unaffected, survival when started later after MI. Previous studies of selective ETA and nonselective ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists demonstrated improved, or at worst unaffected, survival when started later after MI. Although differences in the protocol used (length of follow-up, MI size, drug administration protocol, etc) may account for some of the differences in survival between this and other studies, it is also possible that characteristics particular to LU, specifically its more balanced antagonism of ET<sub>A</sub> and ET<sub>B</sub> receptors, may account for some of the difference between the present and previous studies. The ET<sub>B</sub> receptor is important for ET-1 clearance and can stimulate NO release, an effect that has been demonstrated to be important in many situations. Bosentan, although frequently considered a nonselective ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist, is actually 40 times more selective for the ET<sub>A</sub> receptor than the ET<sub>B</sub> receptor.

The nonselective ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist LU resulted in further LV dysfunction in rats with large MI whether started early or late after MI. Hemodynamic abnormalities were already present 2 weeks after MI in the early LU rats, suggesting that abnormalities began early after MI in this group. These findings probably result from adverse ventricular remodeling that resulted in ventricular dilatation without corresponding hypertrophy of the LV, thus increasing wall stress. It could also result in part from the loss of the positive inotropic effects of ET-1. In addition, early LU rats had a greater increase in RVSP and in right atrial pressure (results not shown). These results contrast with studies of selective ET<sub>A</sub> receptor antagonists and bosentan, which demonstrated a preferential vasodilatory effect on the pulmonary vasculature and again suggest significant differences between this and other ET receptor antagonists, at least as it pertains to large MI.

Rats treated early after large MI with LU had impaired scar healing and scar expansion, resulting in excessive ventricular dilatation. Excessive ventricular dilatation after MI could in turn explain excessive LV dysfunction and worse survival. In this study, we extend results from selective ET<sub>A</sub> receptor antagonists to a nonselective ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist, LU. Presumably these findings result from the loss of the proinflammatory and profibrotic effects of ET-1, the expression of which is greatly enhanced in the scar. Rats treated later (10 days) after MI with LU did not have impaired scar healing but nevertheless had more ventricular dilatation 100 days after MI. These results differ from those of Fraccarollo et al with bosentan started 3 hours after MI and differ from all other studies evaluating ET receptor antagonists started later after MI, either selective ET<sub>A</sub> or nonselective ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists. Taken together, these results suggest that significant differences exist between LU and most other ET-1 receptor blockers and that care should be exercised in administration of LU after MI, particularly when scar healing and stabilization are not complete.

Nonselective receptor antagonism with LU resulted in increased cardiac fibrosis. This result differs from that of previous studies with both selective ET<sub>A</sub> and nonselective ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists, in which cardiac fibrosis decreased significantly despite varying effects on ventricular dilatation. This difference suggests that significant redundancy in the cardiac fibrotic process exists and that in our study, excessive heart failure in the LU groups led to activation of other local or systemic neurohumoral systems that initiate the development of extracellular matrix remodeling and fibrosis.

We documented a decrease in cardiac ecNOS in sham-operated LU-treated rats. This effect of nonselective ET receptor antagonism has not been described previously. Although not specifically evaluated in this study, an early decrease in cardiac ecNOS in the LU MI rats, if it involved the endothelium, could help explain the detrimental effects of LU.

Acknowledgments
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