Regional Desensitization of β-Adrenergic Receptor Signaling in Swine With Chronic Hibernating Myocardium

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Abstract—Contractile reserve during submaximal β-adrenergic stimulation is attenuated in patients and swine with hibernating myocardium. We tested the hypothesis that this arises as a regional adaptive response in β-adrenergic adenylyl cyclase coupling. Pigs (n=8) were studied 3 months after instrumentation with a left anterior descending artery (LAD) stenosis when flow (LAD, 0.7±0.2 versus 1.2±0.1 mL/min per gram in normal remote; P<0.05) and wall thickening (LAD, 5.5±3.2% versus 40.0±5.5% in remote; P<0.05) were reduced in the absence of infarction. Whereas basal cAMP production was normal (LAD, 87±18 versus 91±19 pmol/mg per minute; P=NS), responses to isoproterenol were blunted (LAD, 83±6 versus 146±25 pmol/mg per minute in remote; P<0.05). β-receptor density and subtype were unchanged, but there was a reduction in the number of high-affinity binding sites (LAD, 40±4% versus 53±7% in normal remote; P<0.05). The Gia1/Gsα ratio increased (LAD, 1.8±0.3 versus 0.99±0.3 in remote myocardium; P<0.05), although GppNHp-stimulated cAMP production was equivocally reduced. Forskolin responses were unchanged and similar to shams. These data indicate regional attenuation of β-receptor adenylyl cyclase signaling in hibernating myocardium. This blunts the local contractile response to β-adrenergic stimulation and may serve to protect against a myocardial supply/demand imbalance when external determinants of myocardial workload increase during sympathetic activation. (Circ Res. 2005;97:789-795.)

Key Words: β-receptors ■ hibernating myocardium ■ chronic ischemic heart disease ■ adenylyl cyclase
and affinity, stimulated cAMP production, and changes in regional G-protein expression in swine. The results demonstrate regional attenuation of β-adrenergic signaling in hibernating myocardium that provides an intrinsic adaptation to limit the development of regional ischemia during increases in external workload.

Materials and Methods

Experimental procedures and protocols conformed to institutional guidelines for the care and use of animals in research. Hibernating myocardium was produced in pigs as previously described.7 Tissue for the present study was obtained from a series of animals used to assess in vivo myocardial norepinephrine uptake.8 End diastole was defined at the peak of the stimulation.15 Tissue from the center of the hibernating region (adjacent to the LAD) was compared with normal remote LV myocardial samples taken adjacent to the posterior descending artery. Four additional sham animals were used to exclude changes in remote myocardium. Light microscopy and point counting were performed to quantify connective tissue.7

Membrane Preparation

Flash frozen (−80°C) subendocardial samples were powdered in a stainless-steel mortar and pestle cooled with liquid nitrogen and homogenized using a polytron (setting, 4 to 5) in a buffer containing 50 mM/L Tris (pH 7.4), 20% sucrose, 2 mM/L EGTA, 10 μg/mL Leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, and 5 μg/mL phenylmethylsulfonyl fluoride. After homogenates were centrifuged (10 minutes; 2500g), we centrifuged the supernatant (20 minutes; 48 000g). Pellets were resuspended in 50 mM/L Tris or 30 mM/L HEPES (pH 7.4) and frozen at −80°C for subsequent use in radioligand-binding experiments, adenylyl cyclase assays, and Western blot analysis. Membrane protein concentrations were determined in triplicate and yields were similar in LAD and remote regions (9±0.5 mg/g versus 10±0.5 mg/g; P=NS).

Adenylyl Cyclase Activity

Adenylyl cyclase activity was determined using a modification of the method by Salomon et al.16 Assays were performed in 100 μL of solution containing 15 mM/L HEPES (pH 7.4), 5 mM/L MgCl2, 0.4 mM/L ATP, 5 mM/L creatine phosphate, 0.5 mM/L EGTA, 50 mM/L NaCl, 1 mM/L cAMP, 60 μM/L creatine phosphokinase, 1 mM/L 3-iso-butyl-2-methoxanthine, and 2 μCi [32P]ATP. Reactions were initiated by adding 100 μg of membrane protein and incubated at 37°C for 20 minutes. The reaction was terminated by adding 100 μL of stopping solution (2% sodium dodecyl sulfate, 40 mM/L ATP, and 1.4 mM/L cAMP). We added [3H] (5000 counts per minute) to each reaction for estimation of column recovery. The [32P]/cAMP produced was separated from [32P]ATP by sequential Dowex and alumina chromatography. Column recovery was 85% to 95%. Adenylyl cyclase activity was determined under basal conditions and after stimulation with isoproterenol (10−4 mol/L) in the presence of 0.1 mM/L GTP, forskolin (10−4 mol/L), and GppNHp (10−4 mol/L). In preliminary experiments, we performed dose-response curves with each of these agents to determine the concentration eliciting maximal adenylyl cyclase cAMP production for use in subsequent studies. Data represent total cAMP production. Baseline cAMP production before stimulation was not different from the basal state (P>0.05, ANOVA).

Western Blotting

Regional expression of Gxs proteins (25 μg/lane) and G-protein receptor kinase (GRK) (40 μg/lane) was determined on membrane preparations as previously described.17 Paired experimental and normal samples were run simultaneously to minimize variability. Equal loading and transfer were confirmed in preliminary experiments (Coomassie blue and Ponceau S staining). Nitrocellulose membranes were incubated with antibodies (Santa Cruz Biotechnology Inc) to Gxs proteins (Gαs [SC-823], 1:1000; Gβγ [39-CI], 1:1000 dilution) and GRK-2 and GRK-5 (GRK-2 [SC-562] and GRK-5 [SC-565]; each at 1:500 dilution) overnight at 4°C. They were subsequently incubated for 1 hour with 0.1% TBS containing horseradish peroxidase–conjugated anti-rabbit IgG at 1:10 000 dilutions, visualized with the Supersignal West Peco chemiluminescent substrate (Pierce, Ill), and quantified with a laser densitometer (Bio-Rad Inc).

Radioligand Binding Experiments

β-Adrenergic receptors were quantified using 125I-iodocyanopindolol (5 to 450 mM/L) in saturation isotherm experiments conducted in membrane preparations as previously described.18 In brief, 100 μg of membrane protein prepared as described above was suspended in binding buffer containing 250 mM/L HEPES, 20 mM/L MgCl2, and 0.2% BSA. The membranes were incubated with increasing concentrations of 125I-iodocyanopindolol for 1 hour at 37°C in a water bath. Nonspecific binding was defined as binding in the presence of 10 μM/L propranolol. All reactions were performed in triplicate and specific binding was >80%. The reactions were terminated by rapid filtration using a Brandel M-29R cell harvester (Gaithersburg, Md) through Whatman GF/C filters followed by 4 washes with 4 mL of wash buffer (20 mM/L Tris-HCl [pH 7.4], 2 mM/L MgCl2). The filters were transferred to polystyrene tubes and counted in a gamma counter for 1 minute. The antagonist binding data were analyzed using the Graph Pad Prism program. The proportion of receptors in high-affinity and low-affinity states were determined in competition-binding experiments by incubating 100 mM/L 125I-Iodocyanopindolol with sixteen increasing concentrations of isoproterenol (10−11 to 10−4 mol/L) in the absence of guanine nucleotides. The binding assays were performed in the buffer described above with 3-hour incubation at 30°C. All reactions were performed in duplicate, with nonspecific binding assessed in the presence of 0.1 mM/L isoproterenol. The displacement curves were fit to 1-site or 2-site binding models using the Graph Pad Prism 4 curve-fitting program. A 1-way ANOVA compared 2- and 1-site fits. Finally, proportions of β1 and β2 receptors were determined in competition-binding experiments using 16 different concentrations of CGP20712A (10−12 to 10−4 mol/L), a selective β2 antagonist.

Statistical Analysis

Data are reported as the mean±SEM. After an ANOVA, post hoc analysis was performed with paired 2-tailed t tests. Unpaired t tests were used for sham comparisons. A value of P<0.05 was significant.

Results

All pigs were in good health at the time of study and 2–3.5-triphenyl tetrazolium chloride staining showed no evidence of acute or healed infarction. Angiography demonstrated total occlusion of the LAD, which filled by collaterals arising from both left and right coronary arteries. There was anterior hypokinesia consistent with viable dysfunctional myocardium. Although point counting demonstrated an in-
crease in interstitial connective tissue typical of hibernating myocardium (7.3±1.4% versus 3.6±0.5% in remote regions; P<0.05), >90% of both regions was comprised of myocytes.

Under resting conditions, heart rate averaged 94±7 bpm, LV systolic pressure averaged 147±7 mm Hg, and LV end-diastolic pressure averaged 25±2 mm Hg. Measurements of resting flow and wall thickening confirmed the presence of hibernating myocardium. In the subendocardium (Figure 1A), flow was reduced at rest (LAD, 0.75±0.14 versus 1.19±0.14 mL/min per gram in normal remote myocardium; P<0.05). Like the hypokinesis demonstrated on ventriculography, LAD wall thickening was also significantly reduced (Figure 1B; LAD, 5.5±3.2% versus 40.0±5.5% in remote; P<0.05). Following adenosine, subendocardial flow in the hibernating region was critically impaired (0.7±0.2 mL/min per gram) and unable to increase above resting values, whereas flow in the remote region increased 4 times (4.7±0.8 mL/min per gram; P<0.05, LAD versus remote). Thus, subendocardial samples had physiological features of hibernating myocardium in the absence of infarction.

Adenylyl Cyclase Activity
Figure 2 summarizes results of the adenylyl cyclase activity assays. Total cAMP accumulation was similar under basal conditions (LAD, 87±18 versus 91±19 pmol/mg per minute in remote). Stimulation with isoproterenol (10−4 mol/L) resulted in an attenuated cAMP accumulation in hibernating myocardium (LAD, 83±6 versus 146±25 pmol/mg per minute in remote; P<0.05). Stimulation of adenylyl cyclase with GppNHP (10−4 mol/L) was also attenuated in hibernating myocardium, but the difference was of borderline significance (LAD, 202±37 versus 277±64 pmol/mg per minute in remote; P=0.11). Forskolin-stimulated responses (10−4 mol/L) were not different (LAD, 276±39 versus 299±43 pmol/mg per minute in remote; P=NS). Thus, there was a regional attenuation of β-mediated stimulation of adenylyl cyclase and normal stimulation with forskolin in chronic hibernating myocardium.

In sham instrumented pigs, basal (LAD, 103±19 versus 97±22 pmol/mg per minute in remote; P=NS), isoproterenol-stimulated (LAD, 156±14 versus 152±18 pmol/mg per minute in remote; P=NS), and forskolin-stimulated (LAD, 348±31 versus 335±28 pmol/mg per minute in remote; P=NS) CAMP were similar. Remote myocardial values in shams were not significantly different from remote myocardial regions. Basal measurements before stimulation were not significantly different (P>0.05, ANOVA).

G-Protein and GRK Immunoblotting
Analysis of Ga protein levels (Figure 3) revealed an increase in Gia levels in hibernating myocardium (LAD, 11.2±2.2 versus 7.2±1.2 densitometric units in remote; P<0.05). There was a trend toward reduced membrane-associated levels of Gsα, which did not reach statistical significance (LAD, 7.7±1.2 versus 12.2±3.3 densitometric units in remote; P=0.15). There were no regional differences in Gia or Gsα proteins in sham animals. Thus, the Gia/Gsα ratio in hibernating regions was significantly increased (LAD, 1.84±0.3 versus 0.9±0.3 in remote myocardium; P<0.005). GRK-2 protein levels were similar in remote and hibernating tissue, whereas GRK-5 protein showed a trend toward increasing in hibernating myocardium (LAD, 7.8±1.2 versus 6.8±1.1 densitometric units in remote; P=0.15).

Radioligand Binding
The Table summarizes results of radioligand-binding experiments. Scatchard analyses r2 values were 0.96±0.01. There was no difference in total β-receptor–binding sites using 125I-iodocyanopindolol in hibernating versus remote myocardium. Displacement experiments using 125I-iodocyanopindolol to assess changes in high- and low-affinity receptors (Figure 4) consistently fit best to a 2-site model (ANOVA, P<0.05) and were shifted to the right in hibernating myocardium. This
reflected a reduction in the number of high-affinity binding sites in hibernating myocardium (40 ± 4% versus 53 ± 7%; *P* < 0.05). Competition-binding experiments done in the presence of increasing concentrations of CGP20712A (Figure 5), a selective β1 antagonist, did not reveal any differences in the proportion of β1 and β2 receptor subtypes (Table).

**Discussion**

There are several new and important findings from our study. First, there is a regional attenuation of β-adrenergic–mediated cAMP production in subendocardial samples from hibernating myocardium that occurs in the absence of infarction. This was not attributable to alterations in adenyl cyclase activity because direct stimulation with forskolin was not affected. Second, whereas there was no alteration in total β-receptor density in vitro, competition-binding curves demonstrated a reduction in β-receptor affinity. This was associated with a regional reduction in the number of high-affinity binding sites and increase in Gαi2 proteins. Collectively, our results support the notion that an adaptive downregulation of β-adrenergic responsiveness contributes to attenuating increases in regional metabolic demand during increases in external workload from sympathetic activation.

**Effects of Reversible Ischemia on β-Adrenergic Signaling**

The effects of ischemia on β-receptor signaling have been studied in a variety of models of reversible transmural...
ischemia leading to stunned myocardium. Although the specific alterations depend on the model used, as well as the duration of ischemia, most of the available data have demonstrated no change or evidence of accentuated β-receptor adenylyl cyclase coupling. For example, Sato et al found wall thickening after isoproterenol increased more in stunned myocardium than in control, a finding potentially consistent with β-adrenergic hypersensitivity following ischemia.11 Whereas this was associated with a small increase in β-receptor density, isoproterenol-stimulated cAMP production was unchanged. In chronically stunned myocardium, in vivo β-adrenergic stimulation normalized regional function, although effects on β-adrenergic adenylyl cyclase production were not reported.10 In a model using multiple brief episodes of ischemia, Boraso et al found no changes in cAMP, β-receptor density, affinity, or G-protein levels.19 Likewise, prolonged moderate ischemia or short-term hibernation in swine was associated with contractile reserve to dobutamine and unchanged β-receptor levels, with no evidence of denervation hypersensitivity.12 Thus, following reversible ischemia, the functional response to transient β-adrenergic stimulation is either normal or accentuated and biochemical evidence of alterations in cellular β-receptor signaling is limited.

Hammond et al studied β-receptor signaling in pigs subjected to repetitive ischemia from an ameroid constrictor placed around the left circumflex artery.20 Although demand-induced ischemia was produced in their model, the physiological significance of the stenosis was not sufficient to cause chronic reductions in resting flow or function. Thus, these investigators were able to evaluate the effects of repetitive ischemia on β-adrenergic adenylyl cyclase coupling independently of the progression to stunned or hibernating myocardium. Like the present study, they reported a regional reduction in β-receptor affinity with a shift to a low-affinity state in dysfunctional myocardium. Although these changes were accompanied by a reduction in β-receptor number, there were no effects on stimulated adenylyl cyclase activity. These paradoxical findings were explained by a reciprocal reduction in Gsα and an increase in Gsβ compensating for the alteration in β-receptor number and affinity.

Our results in hibernating myocardium differ from those reported in myocardium subjected to reversible ischemia with or without stunning in several respects. We found that cAMP production in response to isoproterenol stimulation was regionally reduced in chronic hibernating myocardium. This supports previous in vivo functional observations in this model demonstrating an attenuated contractile response to β-adrenergic stimulation with epinephrine when assessed using regional wall motion by ventriculography,7 regional wall-thickening measurements from echocardiography,8 and subendocardial segment length shortening.9,14 In each circumstance, β-adrenergic stimulation improved but did not normalize myocardial function in hibernating myocardium. Radioligand-binding data in the presence of 125I-cyanopindolol demonstrated that Bmax (total receptor binding sites) was similar in remote and hibernating regions, indicating that total β-adrenergic receptor density was normal. Competition binding revealed a loss of high-affinity receptor binding sites in hibernating myocardium, which at least partly explained the reduced ability of β-adrenergic stimulation to increase cAMP in response to isoproterenol. The mechanism for the loss of high-affinity sites is most likely an uncoupling of the receptor from the G-protein. While speculative, phosphorylation of the receptor by protein kinases, including G-protein coupled receptor kinases, could cause uncoupling of the receptor–G-protein complex.21–23 Although the increase in GRK-5 levels was not significant, and GRK-2 protein levels were unchanged, it is plausible that their activities are significantly enhanced in hibernating myocardium and will require further studies.

Role of Altered G-Protein Expression on Adenylyl Cyclase in Hibernating Myocardium

The differences in β-adrenergic signaling caused by chronic ischemia and hibernating myocardium lead to opposing effects of these states on G-protein expression. We found reciprocal alterations in the levels of Ga proteins in hibernating myocardium with the inhibitory subunit Giα2 increasing and the stimulatory subunit Gsα decreasing. These are the exact opposite of the changes reported in myocardium with normal resting function subjected to chronic repetitive ischemia by Hammond et al.20 Coupled with the differences in β-adrenergic–mediated cAMP production, the results support the notion that hibernating myocardium reflects a fundamentally different phase of the chronic adaptive response to repetitive ischemia.

The functional importance of alterations in G-protein levels in hibernating myocardium is unclear because relative protein changes were greater than the reduction in GppNHp-stimulated adenylyl cyclase activity. This could reflect the possibility that G-protein changes have no functional impact because of the molar excess of G-proteins as compared with receptors and adenylyl cyclase molecules.24 Alternatively, it could reflect differential activation of the individual G-proteins or compartmentalization of a pool of G-proteins that, along with receptors and adenylyl cyclase molecules, colocalize in microdomains of the cell membrane lined by caveolar proteins.25 Thus, the stoichiometric excess of G-proteins in the whole cell might not represent the pool of G-proteins available to the receptors in caveolar microdomains. Finally, it is conceivable that there is an effect on the dose-response relation to GppNHp that could be brought out at submaximal concentrations, which we did not study. Further studies will be required to determine the functional impact of chronic alterations in G-protein expression because these changes may impact on other adaptive responses in hibernating myocardium.

Relation of Postsynaptic Reductions in β-Adrenergic Receptor Signaling to Cellular Hypertrophy and Reductions in Presynaptic Norepinephrine Uptake

Although regional, our findings in hibernating myocardium are reminiscent of those reported in models of heart failure, where there are global reductions in β-receptor signaling in the presence of systemic neurohormonal activation and reduced presynaptic norepinephrine uptake.26 In some heart
failure models, these changes are accompanied by global reductions in the expression of the sarcoplasmic reticulum calcium-uptake proteins, phospholamban, and the sarcoplasmic reticulum Ca\(^{2+}\) ATPase, which is seen regionally in the absence of neurohormonal activation and heart failure in hibernating myocardium.\(^{17}\) Whereas global cellular hypertrophy occurs in the failing heart, hibernating myocardium is associated with regional hypertrophy secondary to low but chronic rates of apoptosis.\(^{27}\) A number of laboratories have demonstrated altered \(\beta\)-adrenergic adenylyl cyclase coupling in models of cellular hypertrophy that are not associated with decompensation or neurohormonal activation.\(^{28}\) Thus, it is conceivable that the alterations in \(\beta\)-adrenergic affinity and attenuated cAMP production reflect a common response of the hypertrophied cardiac myocyte.

Although the presence of normal \(\beta\)-adrenergic signaling in remote regions from the same heart excludes systemic neurohormonal activation as a mechanism in the present study, compartmental increases in norepinephrine are an alternative mechanism leading to the regional downregulation in \(\beta\)-receptor signaling. We have previously demonstrated that regional norepinephrine uptake is moderately reduced in hibernating myocardium, as assessed by the uptake of \(^{125}\)I-meta-iodobenzylguanidine ex vivo\(^{15}\) and imaging \(^{11}\)C-hydroxyephedrine uptake using positron emission tomography in vivo.\(^{29}\) These regional changes may reflect a loss of sympathetic nerves or a functional impairment in norepinephrine uptake. If reduced norepinephrine uptake was indicative of partial sympathetic denervation, it would be predicted to result in accentuated functional responses and increased \(\beta\)-adrenergic–mediated cAMP production. Although such effects have been seen several weeks after surgical sympathetic denervation in animals,\(^{30}\) the blunted cAMP production and functional responses during \(\beta\)-adrenergic stimulation argue against this mechanism in hibernating myocardium.

Our results support a fundamentally different postsynaptic response than that seen in the denervated heart. The most likely explanation is that alterations in \(^{125}\)I-meta-iodobenzylguanidine and \(^{11}\)C-hydroxyephedrine reflect chronic functional abnormalities in the presynaptic norepinephrine uptake mechanism rather than denervation. In this circumstance, locally increased norepinephrine overflow during sympathetic activation would be predicted to lead to a regional downregulation in \(\beta\)-adrenergic receptor function rather than \(\beta\)-adrenergic hypersensitivity. A similar mechanism has been hypothesized to contribute to chamber-specific \(\beta\)-receptor downregulation in heart failure.\(^{31}\) A functional abnormality is also supported by studies evaluating the response to sympathetic nerve stimulation in vivo. Like postsynaptic \(\beta\)-stimulation with epinephrine, stellate ganglion stimulation and eliciting presynaptic norepinephrine release using tyramine result in blunted rather than absent or hypersensitive contractile responses in hibernating myocardium.\(^{14}\) Teleologically, this could serve to uncouple local myocyte demand from increases in external workload during sympathetic activation to allow a regional balance between reduced oxygen delivery and reduced demand to be maintained during submaximal stress.

Limitations

To avoid potential effects of the stimulation on \(\beta\)-receptor function, we did not evaluate contractile responses in the same animals. Individual correlation between cAMP production and in vivo functional responses was not possible. Nevertheless, our previous work in this well-characterized model demonstrates consistently attenuated contractile responses to epinephrine infusion in a number of studies.\(^{7–9,14}\) More detailed in vivo studies assessing the functional consequences of changes in \(\beta\)-receptor adenylyl cyclase coupling (eg, using higher doses and other agonists stimulating adenylyl cyclase activity) will require experiments where the effects of blunted \(\beta\)-receptor function can be dissociated from ischemia. These challenging studies will necessitate experiments stimulating adenylyl cyclase through different pathways before and immediately after coronary revascularization of chronically instrumented animals. Finally, although the in vitro assays primarily reflect a cardiomyocyte population by volumetric fraction, there are small differences in connective tissue as well as membranes contributed by endothelium and vascular smooth muscle. The similarity of regional forskolin responses in hibernating and sham animals suggests that such tissue heterogeneity was not the cause of regional reductions in isoproterenol responses in hibernating myocardium.

Clinical Implications

Our data support the notion that the lack of contractile reserve during \(\beta\)-adrenergic stimulation of viable dysfunctional myocardium can arise as the result of an intrinsic attenuation of \(\beta\)-adrenergic signaling. This may be of considerable consequence in patients with ischemic cardiomyopathy where areas of normal, infarcted, and hibernating myocardium coexist within the same heart. The resultant heterogeneity in myocardial \(\beta\)-adrenergic function could produce substantial inhomogeneity in myocardial repolarization, leading to a dynamic substrate factor for the development of ventricular tachycardia and fibrillation and sudden death during sympathetic activation. Indeed, patients with hibernating myocardium have a high short-term mortality\(^{32}\) that is paralleled by the high rate of sudden cardiac death caused by ventricular tachycardia and fibrillation in swine with hibernating myocardium.\(^{33}\)

Finally, although further clinical correlation is required, our findings could explain the higher sensitivity of nuclear imaging as compared with assessing contractile reserve with \(\beta\)-adrenergic stimulation to identify hibernating myocardium. This appears to be particularly problematic in the patient subgroup in whom resting flow is regionally reduced versus those in whom flow is normal.\(^{4,5}\) Thus, in patients with reduced resting flow, imaging viability with approaches that can ascertain the extent of infarction and fibrosis may be superior to imaging function during \(\beta\)-adrenergic stimulation, which may be intrinsically reduced.

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10. Iyer and Canty /H9252


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In an article by Iyer and Canty (*Circ Res.* 2005;97:789–795), the wall thickening data cited in the article was published erroneously. In both the Abstract and text of the article, the data is incorrectly cited as 5.5±3.2%, whereas it should have been cited as 15.5±3.2%. The corrected text is shown below. The publisher regrets this error.

Page 789, Abstract, line 5: “. . . thickening (LAD, 15.5±3.2% versus 40.0±5.5% in remote; P<0.05). . .”

Page 791, left column, line 13: “. . . reduced (Figure 1B; LAD, 15.5±3.2% versus 40.0±5.5% in remote; P<0.05).”