Dual-Tracer Autoradiography with Thallium-201 and Iodine-125-Metaiodobenzylguanidine in Experimental Myocardial Infarction of Rat

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Dual-isotope scintigraphic studies with ^{201}Tl and radioiodinated metaiodobenzylguanidine (MIBG) suggest that acute myocardial infarction causes extensive regional myocardial denervation beyond the infarcted area. We therefore investigated the histopathological and biochemical significance of the discrepancy between ^{201}Tl and ^{125}I-MIBG distribution determined by dual-tracer autoradiography in experimental myocardial infarction. **Methods:** Left coronary arteries of 12 male Wistar rats were ligated for 30 min followed by reperfusion. Dual-tracer autoradiography of infarcted heart sections was performed with ^{201}Tl and [^{125}I]MIBG 4 hr or 2 days after coronary reperfusion, followed by immunohistochemical staining with myoglobin monoclonal antibody to determine the area of myocardial infarction. Ultrastructural alterations and myocardial norepinephrine (NE) content in the region determined by dual-tracer autoradiography and myoglobin immunostaining were studied. **Results:** Thirty-minute coronary ligation with 4-hr reperfusion produced myocardial infarction associated with discrepant region in the peri-infarcted myocardium characterized by decreased [^{125}I]MIBG uptake and normal ^{201}Tl distribution (discrepant region), as determined by dual-tracer autoradiography. In the discrepant region, which disappeared after 2 days, the nerve terminals showed loss of granular cores, with normal structures between normal myocytes. The mean myocardial NE level in the discrepant region was significantly lower than that in the nonischemic region (255.2 ± 85.9 versus 549.5 ± 82.5 ng/mg). **Conclusion:** The uptake discrepancy of ^{201}Tl and [^{125}I]MIBG observed in the infarcted heart represents a transient functional denervation of the regional cardiac sympathetic nerve terminals in the noninfarcted myocardium.

Key Words: iodine-125-MIBG; thallium-201; dual-tracer autoradiography; myocardial infarction; adrenergic denervation

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Radioiodinated metaiodobenzylguanidine (MIBG) is thought to have the same uptake, storage and release mechanisms as norepinephrine (NE) in adrenergic nerve terminals (*1*). It is not, however, metabolized by catechol-*o*-methyl-transferase and monoamine oxidase and thus can be viewed as "nonmetabolized" NE (*2–4*). Radioiodinated MIBG imaging has proved to be a useful diagnostic tool, especially in the heart, which is richly innervated by adrenergic nerves, and has been used to assess adrenergic nerve activity in various cardiac diseases (*5–20*).

Clinical and experimental evidence suggest that cardiac sympathetic nervous function (as demonstrated by [^{123}I]MIBG myocardial scintigraphy) may be more sensitive to ischemic damage than the myocardium itself (as demonstrated by ^{201}Tl myocardial scintigraphy) (*21–27*). Alternatively, scintigraphy with [^{123}I]MIBG in acute myocardial infarction has shown more extensive regional myocardial denervation than that identified in the area of myocardial infarction by ^{201}Tl scintigraphy. Because of methodological limitations, few histopathological and biochemical studies have investigated the difference between radioiodinated MIBG and ^{201}Tl scintigraphic determinations of infarct size. Even with SPECT, it is difficult to identify a direct correspondence between regional scintigraphic distribution and histopathological or biochemical alterations. Autoradiography, in contrast, can clearly delineate the regional distribution of a tracer, permitting identification of direct correspondence between tracer distribution and histopathological and biochemical changes. We therefore investigated the histopathological and biochemical significance of discrepancies in tracer uptake in experimental myocardial infarction using dual-tracer autoradiography with [^{125}I]MIBG and ^{201}Tl.

MATERIALS AND METHODS

Pilot Study

To determine the presence of coronary reflow in the infarcted myocardium after reperfusion preceded by 30-min coronary occlusion in the rat, we performed a pilot study using methylene blue dye staining and electron microscopy.

Protocol. Fifteen male Wistar rats, weighing 200–250 g, fed ad libitum, were used. Each rat was anesthetized with sodium pentobarbital (40 mg/kg body weight) and ventilated 60 times per minute with a volume-cycled respirator. The left coronary artery was ligated using the technique described by Selye et al. (*28*) and modified by Deloche et al. (*29*). Briefly, a thoracotomy was performed to exteriorize the heart; the left coronary artery was then ligated; the heart was returned to its normal position; and the thorax was closed. We attempted to reduce the effects of pneumothorax by applying slight lateral pressure to the thorax immediately after the incision was closed. An electrocardiographic study, using four extremity leads and one midline precordial lead, was performed during the first 30 min of the procedure while animals were in the ventral position. The coronary artery ligature was released by traction on the exteriorized portion of the suture. Reperfusion was performed 30 min after ligation. The animals, divided into three groups of five animals each, were given 0.2 ml of a 1% solution of methylene blue dye through the femoral vein over 30 sec immediately, 30 min or 1 hr after reperfusion under anesthesia. Thirty seconds after injection of the dye, the rats were killed by rapid thoracotomy and excision of the heart. The hearts were cut perpendicular to the long axis of the left ventricle at the midventricular level, and the basal portions of the ventricles were photographed. Small tissue blocks were obtained from four regions of the apical portion of the 1-hr reperfused heart: anterior, lateral,

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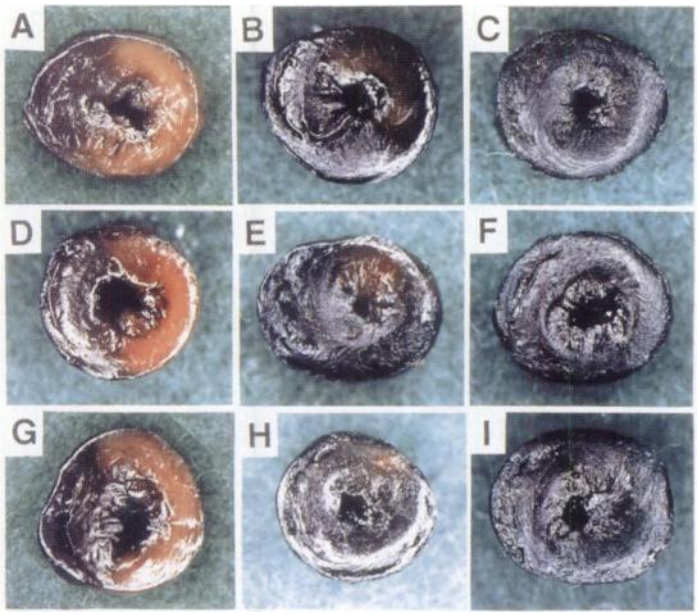

FIGURE 1. Representative transverse sections of left ventricles from nine different rats. (A,D,G) Transverse sections from rats with 60-sec reperfusion. The dark area is the region of myocardium perfused by methylene blue. The lighter area is an unstained region extending over a large area of the left ventricle. (B,E,H) Transverse sections from rats with 30-min reperfusion. Methylene blue-nonperfused myocardium was observed in a smaller area of the anterolateral wall of the left ventricle. (C,F,I) Transverse sections from rats with 1-hr reperfusion. The entire sectional area was well perfused with methylene blue.

posterior and septal walls of the left ventricle. The tissue blocks were immersed in a phosphate-buffered 3% glutaraldehyde solution (pH 7.4) for 3 hr at 4°C. The blocks were then postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead hydroxide and examined under the electron microscope.

Results. Methylene blue, when injected intravenously before excitation of the heart, stains all vessels that are perfused with arterial blood after the injection. Tissue receiving flow is identified by perfusion with methylene blue dye. Sixty seconds after reperfusion, a large area of the left ventricle (anterior, lateral and posterior walls) was not perfused with methylene blue, and 30 min after reperfusion, methylene blue-nonperfused myocardium was still observed in a smaller area of the anterolateral wall of the left ventricle (Fig. 1). One hour after reperfusion, the entire sectional area of the left ventricle was well perfused with methylene blue dye (Fig. 1), but the fine structures of myocardial cells from the anterior, lateral and posterior regions of the apical left ventricles showed irreversible ischemic changes, as previously described by Jennings et al. (*30–33*).

Experimental Protocol

Temporary Ligation of Left Coronary Artery. Twelve male Wistar rats, weighing 200–250 g, were used. The left coronary artery of each rat was ligated and reperfused as described in the pilot study.

Dual-Tracer Autoradiography. The rats were divided into two groups of six animals each and were killed 4 hr or 2 days after reperfusion. One hour or 2 days after reperfusion, each rat received 40 μCi (15 kBq) of [^{125}I]MIBG dissolved in isotonic saline through the femoral vein under light anesthesia. Three hours after injection of MIBG, the animals were anesthetized lightly and infused with 1 mCi (37 MBq) ^{201}Tl. Fifteen minutes after the infusion of ^{201}Tl, the rats were again anesthetized with pentobarbital and killed by rapid thoracotomy and excision of the heart. The hearts were cut into two portions parallel to the atrioventricular groove. The basal portions were frozen at −80°C in liquid nitrogen. Using a cryostat, we cut 16-μm-thick sections from the cutting face at −30°C. Sections were placed on clean glass slides and air-dried for 20–30 min. The glass slides were mounted on cardboard and apposed to x-ray films for exposure. The first autoradiographic exposure was performed for 18 hr to reveal the distribution of ^{201}Tl. The second exposure was initiated 30 days later, after decay of ^{201}Tl activity; [^{125}I]MIBG imaging required 40 days for adequate image quality. Single-tracer autoradiography of each tracer performed under the same conditions confirmed that ^{201}Tl images were not visualized under the exposure conditions for imaging [^{125}I]MIBG uptake and that [^{125}I]MIBG images were not visualized under the exposure conditions for imaging ^{201}Tl uptake. The remaining ventricles were stored at −80°C until assayed for NE.

Myoglobin Immunohistochemistry. After autoradiographic processing, the tissue sections were prepared with 10% normal rabbit serum, washed three times in phosphate-buffered saline (PBS) and incubated with mouse monoclonal anti-myoglobin antibody for 30 min. After being washed three times in PBS, sections were incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulin. After repeated washing in PBS, slides were incubated with avidin and biotinylated horseradish peroxidase for 30 min. Diaminobenzidine tetrahydrochloride was used as a chromogen. After repeated PBS washes, slides were counterstained with diluted hematoxylin, dehydrated and sealed.

Electron Microscopic Examination. Small tissue blocks were obtained from eight regions of the apical portion of the heart: the epicardial and endocardial regions of the anterior, lateral, posterior and septal walls of the ventricle. These regions were matched to the regions determined by dual-tracer autoradiography and myoglobin immunostaining. The tissue blocks were immersed in a phosphate-buffered 3% glutaraldehyde solution (pH 7.4) for 3 hr at 4°C. They were then postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead hydroxide and examined under an electron microscope.

Determination of Myocardial NE Content. The remaining ventricles of the basal portion were dissected into three blocks of regions, as determined by dual-tracer autoradiography and myoglobin immunostaining: (a) a region showing myocardial infarction; (b) a region showing discrepant uptake of thallium and MIBG; and (c) a nonischemic region. These blocks were then weighed within the cooled chamber. Myocardial NE was extracted, and the content was determined by high-performance liquid chromatography using electrochemical detection.

Statistical Analysis

Results are expressed as mean ± s.d., and statistical analysis was performed using one-way analysis of variance (ANOVA) and Student's t-test; $p < 0.05$ was accepted as statistically significant.

RESULTS

Dual-Tracer Autoradiography and Myoglobin Immunostaining

Gross evidence of varying degrees of myocardial infarction was apparent in hearts from rats with 30-min coronary ligation. The area of necrosis lacked myoglobin and therefore was not stained by myoglobin immunostaining (Fig. 2). Myoglobin immunostaining clearly delineated the infarcted region, and the area identified by myoglobin immunostaining was identical to the infarcted region identified by ^{201}Tl autoradiography. Normal myocardial uptake of ^{201}Tl and [^{125}I]MIBG had almost disappeared from the infarcted region with clear delineation.

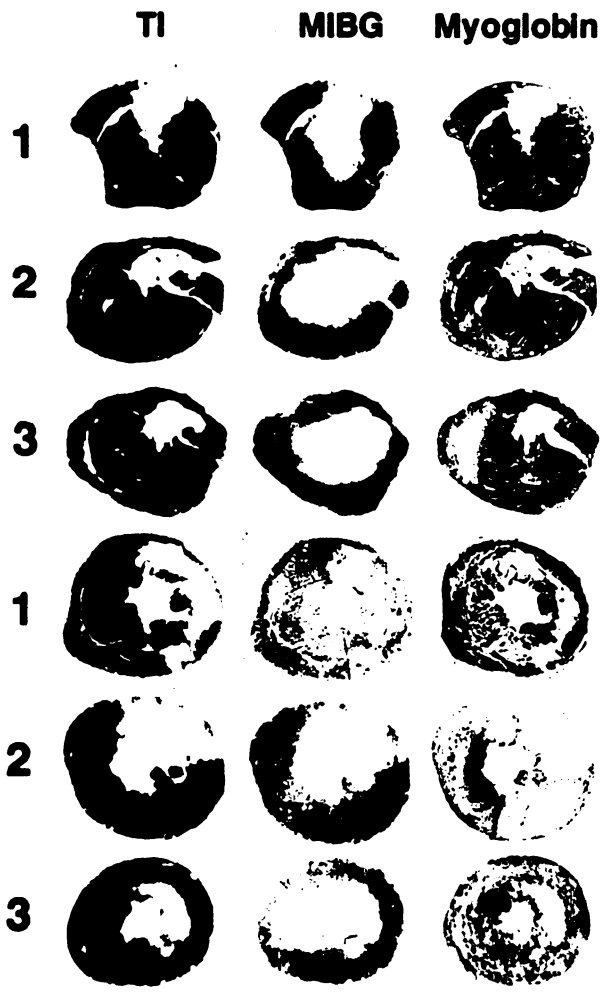

FIGURE 2. Representative dual-tracer autoradiograms with ^{201}Tl, [^{125}I]MIBG and myoglobin immunostaining of hearts from three different rats with 30-min coronary artery ligation. Myoglobin immunostaining and ^{201}Tl autoradiograms show identical defect regions representing myocardial infarction. Normal uptake of ^{201}Tl and [^{125}I]MIBG has almost disappeared from the infarcted region. (Upper panel) Four hours after reperfusion. (Lower panel) Two days after reperfusion.

Moreover, at 4 hr after reperfusion, decreased MIBG uptake was observed within a wider area of the infarcted region, extending all around the subendocardium with a clear demarcation. In addition, this discrepant region showed greater increased MIBG uptake than the infarcted region and disappeared after 2 days, when the areas in which uptake of the two tracers were identical. Two days after reperfusion, the noninfarcted myocardium showed a relative decrease of MIBG uptake in association with enlargement of the ventricular cavity.

Electron Microscopic Observations

In the nonischemic region of the rat heart with 4-hr reperfusion, adrenergic nerve terminal swelling, called varicosities, containing dense-cored vesicles, was observed in the proximity of cardiac muscle cells (Fig. 3A). In the region with uptake discrepancies, the nerve varicosities showed apparently normal structures with frequent loss of granular cores, and no degenerative changes were observed in the adjacent cardiac muscle cells (Fig. 3B).

In the infarcted region of the rat heart with 4-hr reperfusion, most nerve varicosities had fairly intact structures with loss of granular cores, but some showed degenerative changes; myocardial cells, however, showed marked degenerative changes

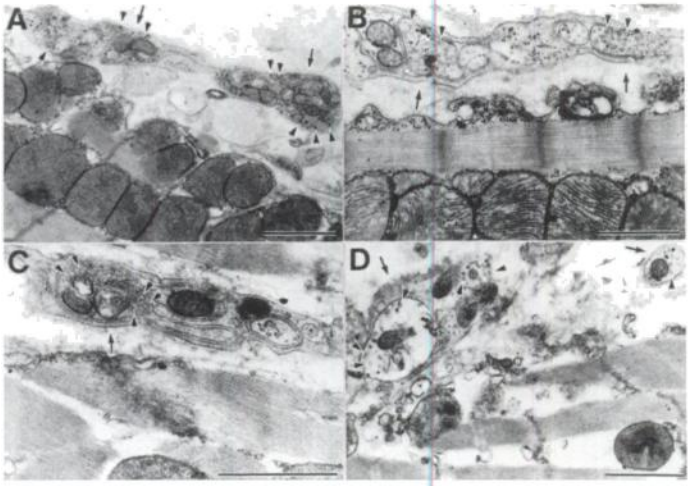

FIGURE 3. (A) Electron micrograph of nonischemic myocardium from rat with 30-min coronary ligation and 4-hr reperfusion. Adrenergic nerve terminal swellings (varicosities) were observed in the proximity of a cardiac muscle cell (arrows). Many synaptic vesicles containing granular cores were seen in the varicosities (arrowheads). (B) Electron micrograph of an adrenergic nerve in the region with uptake discrepancy in a rat with 30-min coronary ligation and 4-hr reperfusion. Varicosity showed a fairly intact structure (arrow). Synaptic vesicles in the varicosity showed loss of granular cores (arrowheads). Adjacent myocardial cell showed no degenerative changes. (C) Electron micrograph of infarcted myocardium from a rat with 30-min coronary ligation and 4-hr reperfusion. Varicosity had an apparently normal structure. Synaptic vesicles in the varicosity showed loss of granular cores (arrowheads). Degenerative changes were observed in the adjacent cardiac muscle cells. (D) Electron micrograph of infarcted myocardium from rat with 30-min coronary ligation and 2-day reperfusion. Varicosities showed degenerative changes with swelling (arrows). Synaptic vesicles in the varicosities showed loss of granular cores (arrowheads). Adjacent myocardial cell showed marked degenerative changes. (A–D) Scale bar = 1 μm.

(Fig. 3C). Two days after reperfusion, degenerative changes of the nerve varicosities became apparent between the necrotic myocardial cells in the infarcted region (Fig. 3D). These varicosities showed a loss of granular cores. In the nonischemic region, nerve varicosities with normal structures frequently showed a loss of granular cores.

Myocardial Norepinephrine Content

The NE content decreased significantly in the discrepant regions compared with that in the nonischemic area after 4-hr reperfusion (Table 1). The NE content was significantly lower in the infarcted than the nonischemic region after 4 hr and 2 days. Four hours after reperfusion, the NE content was lower in the infarcted than the discrepant region, but the difference was not significant. Two days after reperfusion, the nonischemic region showed a significant decrease in myocardial NE content

TABLE 1
Myocardial Norepinephrine Content

Time	Nonischemic region	Discrepant region	Infarcted region
4 hr after reperfusion	549.5 ± 82.5	255.2 ± 85.9*	196.5 ± 49.6*
2 d after reperfusion	389.2 ± 84.6	DA	117.5 ± 31.2†

*$p < 0.01$ versus nonischemic region, one-way ANOVA.
†$p < 0.01$ versus nonischemic region, Student's t-test.
Data are expressed as mean value ± s.d.
Nonischemic region = myocardial region showing normal uptake of ^{201}Tl and [^{125}I]MIBG; discrepant region = myocardial region showing decreased MIBG uptake with normal thallium distribution; infarcted region = myocardial region where normal uptake of ^{201}Tl and [^{125}I]MIBG and myoglobin immunoreactivity are absent; DA = disappeared.

compared with that in the nonischemic region at 4 hr after reperfusion.

DISCUSSION

Pilot Study

When administered 1 hr after reperfusion preceded by 30-min coronary occlusion, methylene blue dye perfused the region where the fine structure of the myocardial cell showed irreversible ischemic changes. Therefore, we administered [^{125}I]MIBG in rats 1 hr after reperfusion preceded by 30-min coronary occlusion in the experimental protocol. The reperfusion model used in the present study has proven useful for the investigation of myocardial behavior of radiopharmaceutical tracers in ischemic myocardium.

Dual-Tracer Autoradiography and Myoglobin Immunostaining

Dual-tracer autoradiography clearly delineated regional distribution of tracers and allowed us to investigate closely the histopathological and biochemical changes in the corresponding region. The discrepant region was located mainly in the anterior subendocardial region adjacent to the infarcted area, but it also extended to the subendocardium around the left ventricular cavity. The region with discrepant ^{201}Tl and [^{125}I]MIBG uptake extended to the subendocardium, which was wider than the area with interruption of coronary perfusion. Alternatively, the distribution of the discrepant region showed coronary perfusion-adrenergic innervation mismatch. Relative sensitivity of subendocardial region to ischemic damage may result in subendocardial denervation of adrenergic nerve terminals, but the mechanisms responsible for this mismatch are not clear from the present study. These findings, however, have not been described in previous reports and seem to have significant implications for interpreting the myocardial behavior of MIBG in this early phase of myocardial infarction. Further study is required to unravel this mechanism.

Because myocardial uptake of ^{201}Tl depends on both myocardial viability and coronary perfusion, the region with discrepant ^{201}Tl and [^{125}I]MIBG uptake may represent viable myocardium with transient functional denervation of sympathetic nerve terminals caused by ischemic damage. Thus, the denervated region at 4 hr after reperfusion, identified by [^{125}I]MIBG, was considered to be reduced at 2 days after reperfusion and became identical to the region in which ^{201}Tl uptake and myoglobin immunoreactivity were absent.

It could be argued that the reduction of [^{125}I]MIBG uptake after coronary artery ligation is a result of ligation of the nerve bundles accompanying the vessels rather than a result of ischemic change. During ligation of the coronary artery, adrenergic nerves are also ligated, and the resulting trauma to the cardiac nerves produces Wallerian degeneration. Wallerian degeneration, however, was not observed in the early phase (e.g., at 4 hr) after ligation in a previous study (*34*). Holmgren et al. (*35*) reported that 5 hr of ligation around the nerve bundles and vein only, avoiding the coronary artery, produced no reduction of catecholamine fluorescence in the rat heart. Moreover, they observed that adrenergic nerve fibers ran along the coronary arteries and were distributed in the perfusion areas of the associated coronary arteries. The discrepant region produced in the present study showed more extensive regional myocardial denervation than that identified in the area of myocardial infarction by ^{201}Tl autoradiography. Thus, it is unlikely that sympathetic axotomy is a factor contributing to the reduction of [^{125}I]MIBG uptake in the present study.

Ultrastructural Alterations of Adrenergic Nerve Terminals

In an electron microscopic investigation, the axonal ultrastructure was more resistant to ischemic damage than were the myocytes, as found in a previous study (*36*). Degenerative changes of fine structures occurred gradually in the axons but with a certain delay compared with that in the adjacent myocardial cells. These processes have been previously described and discussed in detail for ischemic myocardium (*37*), but ultrastructural alterations of the nerve terminals in the peri-infarcted (discrepant) region determined by dual-tracer autoradiography have not been investigated. We observed a disappearance of granular cores in the intact varicosities between nondegenerated myocytes in the discrepant region 4 hr after coronary reperfusion. Because the disappearance of granular cores in the intact varicosities represents functional denervation, the function of cardiac sympathetic nerve terminals seemed more sensitive to ischemic damage than the myocardium itself. These findings were observed in the discrepant region at 4 hr after coronary reperfusion, whereas the degenerative changes of sympathetic nerve terminals were observed only in the infarcted region. Alternatively, ischemic changes in the sympathetic nerve terminals were less extensive in the discrepant than in the infarcted region, which may have been responsible for the increased MIBG uptake and NE content in the discrepant region compared with that in the infarcted region.

Myocardial Norepinephrine Content

A marked reduction in NE content was observed in the infarcted region. Acute myocardial infarction leads to the increase of sympathetic nervous system activity, resulting in an increase of NE release and a decrease of NE reuptake in the adrenergic nerve terminals of the heart. Norepinephrine is released from ischemic tissue into the circulation (*38,39*), and infarcted tissue shows a marked decrease in NE content (*40–42*). Subsequent progressive reduction of NE was observed in the infarcted region 2 days after coronary reperfusion, when the adrenergic nerve terminals were completely destroyed. The noninfarcted region also showed a significant but smaller reduction of NE content 2 days after coronary reperfusion, reflecting the relative decreased activity of MIBG uptake as observed in the corresponding region determined by autoradiography. These findings may have reflected the presence of congestive heart failure. The autoradiogram showed an enlarged left ventricular cavity, indicating congestive heart failure. Previous studies have shown a similar reduction of NE content in heart muscle in congestive heart failure (*43–51*).

The discrepant region in the vicinity of the infarcted myocardium also showed a significant reduction of myocardial NE content, indicating increased NE release and decreased NE reuptake in the adrenergic nerve terminals. Nishimura et al. (*26*) also observed a transient reduction of myocardial NE content in the peri-infarcted region in experimental myocardial infarction in dogs. The present study revealed that the close regional relation between myocardial NE content and MIBG uptake activity of the sympathetic nerves and showed that the decreased NE content in the discrepant region was consistent with the autoradiographic and morphological findings showing functional denervation of regional sympathetic nerve terminals.

CONCLUSION

The present study demonstrated a discrepancy characterized by decreased MIBG uptake and normal thallium distribution in experimental acute myocardial infarction of rat heart, using dual-tracer autoradiography, and revealed the histopathological and biochemical features of this discrepant region. Our results

indicate that the discrepancy observed in the infarcted heart represents a transient functional denervation of the regional cardiac sympathetic nerve terminals of the noninfarcted myocardium.

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