Isoproterenol-Induced Myocardial Fibrosis in Relation to Myocyte Necrosis


Treatment of rats with the $\beta$-adrenergic agonist isoproterenol results in cardiac hypertrophy, myocyte necrosis, and interstitial cell fibrosis. Our objectives in this study have been to examine whether hypertrophy and fibrosis occur in a compensatory and reparative response to myocyte loss or whether either process may be occurring independently of myocyte loss and thus be a reactive response to adrenergic hormone stimulation. We have examined this question by evaluating each of these responses in rats treated with different doses and forms of isoproterenol administration. Myocyte necrosis was evaluated using in vivo labeling with monoclonal antimyosin for identification of myocytes with permeable sarcolemma, which was indicative of irreversible injury. Myocardial fibrosis was evaluated by morphometric point counting of Gomori-stained tissue sections and by assessment of the stimulation of fibroblast proliferation by determination of increased levels of DNA synthesis. Stimulation of fibroblast DNA synthesis was determined from DNA specific radioactivities and radioautography after pulse labeling with $[^3H]$thymidine. The evidence provided by this study suggests that the degree and timing of myocardial hypertrophy does not follow the course of myocyte loss and, thus, appears to be either a response to altered cardiac loading or a reactive response to $\beta$-adrenergic hormone stimulation rather than a compensation for myocyte loss. Myocardial fibrosis, on the other hand, appears to be more closely related to myocyte necrosis with respect to collagen accumulation in the same areas of the heart, its dose-response relation to the amount of isoproterenol administered, and the timing of increased DNA synthesis, or fibroblast proliferation, after myocyte loss. (Circulation Research 1989;657–670)

A n increase in collagen content and concentration and an interstitial fibrosis have been observed in many instances of myocardial hypertrophy resulting from various forms of ventricular pressure overload.1–9 Myocyte loss has been suggested as a factor influencing the appearance of increased collagen concentration in a number of cases,5,10–12 but the evidence has not consistently supported myocyte loss as the primary stimulus for increased collagen since there are instances in which myocyte loss was not judged to be a factor in the development of fibrosis.5,8,13 In myocardial hypertrophy resulting from volume overload,2,8,14,15 thyroxine treatment, and exercise training,2,16 myocyte loss has not been observed, and an increase in collagen content occurred only to the extent that overall myocardial mass increased. Collagen concentration and the interstitial volume fraction remained equal to or less than that of controls.2 In focusing on the instances where collagen concentration and the interstitial volume fraction is increased above control levels, we have sought to determine if the initial rise in collagen concentration arises solely as a result of myocyte loss, or alternatively, if a reactive process leads to this result.17

In this connection, adrenergic hormones and their analogues also represent a potent stimulus for both myocardial hypertrophy18–23 and fibrosis.24,25 In most studies26–29 and a recent review,30 significant myocyte necrosis occurs with these agents, and thus, hypertrophy and fibrosis have been characterized as a compensatory response to myocyte loss.25 Although $\alpha$-adrenergic stimulation has been shown to induce hypertrophy in tissue culture,31–33 where myocyte loss is not a factor, it is unclear whether $\alpha$- and/or $\beta$-adrenergic stimulation in vivo might directly influence myocyte hypertrophy or fibrosis indepen-
dently of a reparative component due to myocyte loss.

A major difficulty encountered with regard to evaluation of this question is related to the sensitivity of commonly used methods for direct estimation of myocyte necrosis and fibrosis after treatment with either adrenergic hormones or pressure overload. Thus, it has not been possible to establish the temporal and spatial relation between the degree of fibrosis resulting from these interventions with the extent of myocyte necrosis that preceded it. In order to examine this relation more closely, we have used the technique of in vivo labeling of necrotic myocytes with monoclonal antimyosin for evaluation of myocyte necrosis. This determination has been combined with an estimation of increased fibroblast proliferation using both the incorporation of \([H]tymidine into myocardial DNA and estimation of the volume fraction of interstitial connective tissue by morphometric point counting. In this manner we have compared both the temporal and spatial relations between myocardial necrosis and fibrosis in isoproterenol-treated rats.

The evidence provided by this study would seem to suggest that the degree and timing of myocardial hypertrophy is not totally dependent upon myocyte loss, whereas the initial development of interstitial fibrosis is more closely coupled to injury. This conclusion is based on the levels of each response (myocyte necrosis, myocardial hypertrophy, and interstitial fibrosis) that are generated after various doses and forms of isoproterenol administration and their temporal sequence.

**Materials and Methods**

**Experimental Model**

Experimental studies were conducted in 173 male Sprague-Dawley rats (200–400 g) that were maintained on an ad libitum diet with free access to water. Isoproterenol hemisulfate (Sigma, St. Louis, Missouri) was prepared in isotonic phosphate-buffered saline (PBS) and administered by either of two methods: 1) daily subcutaneous injections of 50–5,000 \(\mu g/kg body wt\) in 0.2 ml solution or 2) continuous infusion of 1–22 mg/kg/day using minipumps (model 2002, Alza, Palo Alto, California) placed in the peritoneum. When minipumps were used, isoproterenol was dissolved in PBS containing 0.1\% sodium metabisulfite to prevent oxidation. Pumps were presoaked in sterile saline for 24 hours before implantation to ensure that the drug was being properly delivered. Control rats received 0.2 ml saline instead of isoproterenol.

In some cases, rats were pretreated with 5 mg/kg propranolol (\(\beta\)-adrenergic antagonist) 1 hour before isoproterenol administration. After 3 hours to 10 days of isoproterenol, animals were killed by \(CO_2\) asphyxiation or after methohexital (35–40 mg/kg i.p.) anesthetic. The thorax was opened, and the hearts were quickly excised, weighed, and further processed as described below.

**Evaluation of Myocyte Necrosis With Monoclonal Antimyosin**

Myocyte necrosis resulting from isoproterenol was assessed by in vivo labeling using monoclonal antimyosin. Necrotic myocytes that have developed sarcolemmal permeability allow antimyosin to enter the cell and bind myofibrillar myosin. Intact, uninjured cells exclude the antibody and remain unlabeled.

Both isoproterenol-treated and control rats were treated with 1 mg immunoglobulin G (IgG) fraction of monoclonal antibody CCM-52 intraperitoneally 24 hours before removal of heart. Animals killed less than 24 hours after isoproterenol treatment were pretreated with antimyosin. The antibody used in this study has been shown to be specific for cardiac myosin. Antibody administered intraperitoneally appears in the serum in less than an hour, reaches a maximal enzyme-linked immunosorbent assay serum titer by 3 hours, and remains at high serum titers for more than a week after a single injection (W.A. Clark, unpublished data).

After removal of the heart, 2-mm-thick midventricular cross sections were quickly frozen in isopentane cooled to the freezing point, \(-160^\circ C\), in liquid nitrogen. After cryostat sectioning of the heart, the sections were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, Pennsylvania) to localize myocardial cells that bind antimyosin monoclonal antibody. Immunofluorescent staining was evaluated with a microscope (Carl Zeiss, Thornwood, New York) equipped with epifluorescent optics optimized for FITC fluorescence.

**Morphometric Analysis**

A 2-mm-thick coronal section was taken from the equator of each heart and fixed in neutral buffered formalin. Formalin-fixed sections were dehydrated through a graded series of alcohol and xylene and embedded in paraffin. Five-micrometer paraffin sections were stained with hematoxylin and eosin for evaluation of histology and Gomori trichrome for distinguishing muscle and interstitial connective tissue.

Myocardial fibrosis was evaluated in each section of heart tissue using a morphometric point-counting procedure. Morphometry was conducted to evaluate the increase in the subendocardial volume fraction of fibrous tissue in the heart after isoproterenol treatment. To estimate interstitial volume fraction in the myocardium, cross sections of Gomori-stained tissue were viewed under a 441 point-counting grid at \(\times 100\) magnification. The fraction of grid points over muscle (red staining) and interstitial (blue staining) tissue was scored in each field. Four random fields, selected in each of the four quadrants of the ventricular cross section,
were counted in each heart in the zone lying within 1.4 mm of the ventricular endocardium. This counting protocol provided the greatest sensitivity in comparing fibrosis in different treatment groups since the greatest degree of fibrosis was confined to the subendocardium in all groups.

Evaluation of Myocardial DNA Synthesis

Stimulation of DNA synthesis in heart tissue was analyzed after 48 hours of isoproterenol treatment both by estimation of total incorporation of [3H]thymidine into DNA and by radioautography. [Methyl-3H]thymidine (TRK.686, specific activity>50 Ci/mmol, Amersham, Arlington Heights, Illinois) was administered by intraperitoneal injection (1 µCi/g) 4 hours before removal of heart. Thymidine incorporation into DNA was determined by a modification of the procedure described by Morkin and Ashford.1 Hearts were quickly excised, weighed, and placed in ice-cold saline. The hearts were divided into right ventricle and left (plus septum) ventricles, finely minced with scissors, and homogenized with a polytron (Brinkmann Instruments, Westbury, New York) in 2 ml ice-cold PBS. Two millimeters of cold 10% trichloracetic acid (TCA) was added to the homogenate to precipitate DNA and proteins. The tissue homogenates were centrifuged, washed three times with 4 ml cold 5% sodium acetate in 90% ethanol to remove unincorporated [3H]thymidine. After centrifugation, the supernatant was discarded, and the precipitates were resuspended in 2 ml of 5% TCA. DNA was extracted by heating the samples at 90° C for 30 minutes. After centrifugation, aliquots of the supernatant were taken for determination of tritium radioactivity by scintillation counting and determination of DNA content by the Burton method.38 DNA standards were prepared from calf thymus DNA, and the concentrations of the standard were determined by its absorbance at 260 nm. Stimulation of DNA synthesis was determined from the specific radioactivity (cpm/µg DNA) in each tissue sample.

Identification of cell types involved in DNA synthesis was achieved by radioautography. After hematoxylin and eosin staining of paraffin sections of [3H]thymidine-labeled heart tissue, sections were air dried from the aqueous phase. Slides were then coated at 45° C with Kodak NTB-3 nuclear tract emulsion diluted 1:1 with distilled water. After draining and drying, the coated slides were stored desiccated for 7 days at 4° C. The radioautographs were developed in half-strength Kodak D-11, fixed, washed, dehydrated, and mounted in permount.

Statistical Analysis

All quantitative data generated in this study were analyzed using the SPSS/PC+ statistical package. Data for all groups were compared simultaneously by analysis of variance. If a significant F value was obtained, pairwise comparisons were performed using the Scheffe method at specified levels of significance.

Results

Acute Myocyte Necrosis After a Single Dose of Isoproterenol

In vivo labeling with monoclonal anticardiac myosin provides a highly sensitive and specific means for characterizing injury resulting from treatment with isoproterenol. A single subcutaneous injection of isoproterenol results in the appearance of necrotic myocytes within hours of treatment. Using monoclonal antimyosin to assess the development of myocyte membrane permeability, we found antibody-labeled fluorescent myocytes within 3 hours (Figure 1A). The level of antimyosin staining appeared to be increased at 6–12 hours and maximal at 24 hours after isoproterenol treatment (Figure 1B and 1C). During this period, the affected myocytes were largely confined to the subendocardial region of the heart. Isoproterenol produced a pattern of scattered myocyte fluorescence involving isolated cells and groups of cells with no well-defined zone of injury (Figure 1B and 1C).

At 48 hours after administration of a single dose of isoproterenol, the number of fluorescent necrotic myocytes was considerably decreased over that observed at 24 hours (Figure 2), with evidence of extensive breakdown of the remaining labeled cells (Figure 2, inset). Hearts examined between 3 and 8 days after treatment had no evidence of fluorescent myocytes, and no myocyte labeling of any kind was ever observed in control rats that had been treated with monoclonal antibody without isoproterenol (Table 1).

These data were taken to suggest that after subcutaneous administration of a single dose of isoproterenol, there was a rapid appearance of necrotic myocytes in the subendocardium. The cell debris created by this process was then cleared by a healing process that had removed most traces of necrotic cells by 48 hours after treatment.

Myocyte Necrosis After Repeated Dosing With Isoproterenol

Repetitive treatment with isoproterenol results in down regulation of adrenergic receptors with considerable attenuation of the effect of subsequent dosing. Since antibody-labeled myocytes were not observed more than 3 days after injection of a single dose of isoproterenol, we examined whether repetitive daily dosing with 1,000 µg/kg isoproterenol for 8 days would reduce the level of necrosis observed 24 hours after the final treatment in comparison with that observed 24 hours after a single dose (Table 1). Substantial evidence of new necrosis was observed in rats treated according to this protocol (Figure 3). Unlike the pattern described after a single dose of isoproterenol in which labeling was confined to discrete cells, continued dosing pro-
duced a more diffuse staining pattern (Figure 3, inset). Both intracellular and some extracellular staining was observed, indicating a substantial leaking of myosin from injured myocytes. Thus, repeated dosing with isoproterenol appears to induce additional necrosis for at least 8 days in some rats.

Development of Necrosis After Chronic Low-Dose Isoproterenol

A single injection of isoproterenol, even in doses as low as 125 μg/kg, produces myocyte necrosis. It was reasoned that with a bolus injection of isoproterenol, in which there was a marked increase in heart rate and a drop in systemic pressure, the hemodynamic effect on the myocardium might in itself generate enough subendocardial ischemia to generate myocyte necrosis. Thus, we examined whether isoproterenol delivery via osmotic minipumps, in which acute high levels were not experienced, might be accomplished without the generation of myocyte necrosis. Contrary to expectations, antibody-labeled necrotic myocytes were detected after 48 hours in rats receiving from 1 to 22 mg/kg/day isoproterenol (0.2–5 μg/min) via osmotic minipumps (Table 1 and Figure 4).

Inhibition of Necrosis With Propranolol

Pretreatment of rats with 5 mg/kg of the β-antagonist propranolol 1 hour before a single subcutaneous dose of 1 mg/kg isoproterenol substantially reduced the level of myocyte necrosis to near the detectable limit (Table 1).

Stimulation of Myocardial Hypertrophy: Dose-Response

In addition to the acute response of myocyte necrosis after isoproterenol, there was also an induction of myocardial hypertrophy and fibrosis. To compare the relation of these processes with myocyte necrosis, we evaluated myocardial hypertrophy and fibrosis after the same treatment protocols described above. Statistically significant (p<0.05) hypertrophy was observed within 2 days after treatment with 500 μg/kg/day s.c. isoproterenol (Table 2).
TABLE 1. Frequency of Isoproterenol-Induced Necrosis Evaluated by In Vivo Labeling With Monoclonal Anti-myosin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Myocyte labeling levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control:</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>Single ISO injection</td>
<td></td>
</tr>
<tr>
<td>(1 mg/kg s.c.) &lt;1 day</td>
<td>5 1 1 9</td>
</tr>
<tr>
<td>2 days</td>
<td>2 2 1</td>
</tr>
<tr>
<td>3–10 days</td>
<td>21 . . . .</td>
</tr>
<tr>
<td>PROP (5 mg s.c.) + ISO</td>
<td></td>
</tr>
<tr>
<td>(1 mg/kg s.c.) 1 day</td>
<td>2 1 1 1</td>
</tr>
<tr>
<td>Daily ISO injections</td>
<td></td>
</tr>
<tr>
<td>(1 mg/kg s.c.) 8 days</td>
<td></td>
</tr>
<tr>
<td>ISO infusion with minipump</td>
<td></td>
</tr>
<tr>
<td>(1–22 mg/kg/day i.p.)</td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td></td>
</tr>
</tbody>
</table>

ISO, isoproterenol; PROP, propranolol. The frequency and levels of immunofluorescent staining of cardiac myocytes with monoclonal anti-myosin were determined in rats treated with isoproterenol. Categories of fluorescent staining were as follows: 0, no detectable fluorescence in any cells; 1, fewer than five fluorescent positive cells per cross section; 2, a single group of five or more positive cells; 3, two to five clusters of positive cells; 4, more than five clusters of positive cells.

Stimulation of Myocardial Fibrosis: Dose-Response to Repeated Isoproterenol

Ten days of treatment with isoproterenol produced marked connective tissue accumulation in the subendocardium in both the left ventricle and septum. This effect is well illustrated in the photomicrograph shown in Figure 5A. Connective tissue accumulation in this rat treated for 10 days with isoproterenol (1,000 μg/kg) is clearly evident in the subendocardium, whereas there is essentially no increased connective tissue in the subepicardium. Cellular detail in the subendocardial region is shown in greater detail in Figure 5B. Isoproterenol appears to stimulate both an increase in the fibroblast cell population and the fibrous extracellular matrix. Figure 5C shows the same field as shown in Figure 5B with filters adjusted to show collagen fiber staining. Comparable areas of a control untreated heart is shown in Figure 5D and 5E.
To quantitate isoproterenol stimulation of regional fibroplasia in the heart, we used morphometric point counting. Subendocardial fibrosis averaged 8-10 times greater than control levels after treatment with 125-5,000 μg/kg isoproterenol for 10 days (Figure 6). Subepicardial fibrosis was not significantly greater than control values in any of

**TABLE 2. Myocardial Hypertrophy Induced by 2 Days of Treatment With Subcutaneous Isoproterenol**

<table>
<thead>
<tr>
<th>Isoproterenol</th>
<th>BW (g)</th>
<th>HW (g)</th>
<th>HW/BW (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>232.0±9.6</td>
<td>0.69±0.04</td>
<td>3.02±0.06</td>
</tr>
<tr>
<td>50 μg/kg</td>
<td>257.8±10.3</td>
<td>0.85±0.03</td>
<td>3.28±0.08</td>
</tr>
<tr>
<td>500 μg/kg</td>
<td>250.0±12.1</td>
<td>0.88±0.09</td>
<td>3.48±0.18*</td>
</tr>
<tr>
<td>1,000 μg/kg</td>
<td>265.7±14.8</td>
<td>0.96±0.05*</td>
<td>3.62±0.05†</td>
</tr>
<tr>
<td>2,500 μg/kg</td>
<td>243.0±7.5</td>
<td>0.90±0.01*</td>
<td>3.82±0.11†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. BW, body weight; HW, heart weight. Statistical significance was derived by pairwise comparison with control values by use of the Scheffe method after analysis of variance.

*p<0.05.
| p<0.01.

**TABLE 3. Myocardial Hypertrophy Induced by 10 Days of Treatment With Subcutaneous Isoproterenol**

<table>
<thead>
<tr>
<th>Isoproterenol</th>
<th>BW (g)</th>
<th>HW (g)</th>
<th>HW/BW (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>349.0±2.41</td>
<td>1.19±0.03</td>
<td>3.42±0.07</td>
</tr>
<tr>
<td>125 μg/kg</td>
<td>354.4±7.2</td>
<td>1.61±0.07*</td>
<td>4.54±0.12*</td>
</tr>
<tr>
<td>250 μg/kg</td>
<td>355.8±4.7</td>
<td>1.65±0.03*</td>
<td>4.65±0.06*</td>
</tr>
<tr>
<td>500 μg/kg</td>
<td>361.6±7.8</td>
<td>1.64±0.05*</td>
<td>4.56±0.15*</td>
</tr>
<tr>
<td>1,000 μg/kg</td>
<td>332.0±5.8</td>
<td>1.42±0.05</td>
<td>4.23±0.13*</td>
</tr>
<tr>
<td>2,500 μg/kg</td>
<td>353.2±8.6</td>
<td>1.71±0.06*</td>
<td>4.85±0.21*</td>
</tr>
<tr>
<td>5,000 μg/kg</td>
<td>344.0±4.0</td>
<td>1.74±0.03*</td>
<td>5.05±0.15*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Statistical significance was derived by pairwise comparison with control values by use of the Scheffe method after analysis of variance.

*p<0.05.
| p<0.01.
FIGURE 5. Photomicrographs of sections of rat heart. Panel A: Gomori-stained rat heart (left ventricle removed) treated for 10 days with 1 mg/kg/day isoproterenol and photographed under a 546 nm interference filter (green). Interstitial cells in the subendocardium appear light while muscle tissue appears dark. Magnification, ×17. Panel B: Cellular detail in subendocardium under 546 nm filter. Magnification, ×175. Panel C: Same field as panel B and photographed under Wratten 1A filter to show enhanced collagen fiber contrast staining. Magnification, ×175. Panel D: Gomori-stained untreated control rat heart under 546 nm filter. Magnification, ×175. Panel E: Gomori-stained untreated control rat heart under Wratten 1A filter. Magnification, ×175.

the isoproterenol treated groups, as is shown in Figure 5A (morphometric data not shown). An increase in the degree of subendocardial fibrosis was noted when the doses of isoproterenol were increased from 125 to 5,000 μg/kg (Figure 6).

Effect of Single Versus Repetitive Isoproterenol on the Degree of Fibrosis

To determine whether the amount of subendocardial fibrosis that developed with the initial treatment with isoproterenol was enhanced by repetitive doses, we compared the degree of fibrosis that resulted after either single or multiple doses at different concentrations. The results shown in Figure 7 clearly show that there is a marked cumulative effect regarding the fibrous tissue formation that is produced by isoproterenol. A single subcutaneous injection of 1,000 μg/kg generated about 5.5% subendocardial fibrosis in rats that were examined 10 days after treatment. This is approximately twice
FIGURE 6. Bar graph showing effect of dose of isoproterenol (ISO) on subendocardial interstitial volume fraction after 10 days. BW, body weight. Rats were treated with 10 daily injections of the doses of isoproterenol indicated. After staining of ventricular cross sections of the heart with Gomori stain, the degree of fibrosis was estimated by point counting. Statistical difference from control group is indicated by *p<0.01 and #p<0.05. The number of cases included for each data point is the same as given in Table 3, with the exception that in the control group, n=8.

FIGURE 7. Bar graph showing evaluation of repetitive dosing and concentration of isoproterenol (I) on the degree of interstitial fibrosis in the subendocardium. Rats were treated with 125 or 1,000 μg/kg body wt s.c. isoproterenol that was repeated daily for the times indicated. Data is also included for a group treated with 5 mg/kg/day propranolol (Pp) 1 hour before receiving 1 mg/kg/day isoproterenol. Interstitial fibrosis was evaluated in the subendocardium by point counting. *Statistical difference from control group, p<0.01. The number of cases for each group are 8, 4, 6, 5, 7, and 6 according to their order of appearance on graph.

FIGURE 8. Bar graph showing the effect of dose of isoproterenol on the stimulation of myocardial DNA synthesis. Rats were treated with daily subcutaneous (sc) doses of isoproterenol. On the second day of treatment, the animals were pulse-labeled with [3H]thymidine for 4 hours. The specific radioactivity of DNA cpm/μg is given. The number of cases in each group are n=4 for control, n=6 for sham Alza pump, n=5-7 in the daily subcutaneous groups, and n=2-3 in minipump groups.

the control level of connective tissue. Repeating this treatment for 2 days doubles the amount of fibrous tissue produced, even in rats examined 24 hours after the second treatment rather than at 10 days. Considerably greater fibrosis is observed in rats receiving 10 daily doses of isoproterenol. However, rats that received 125 μg/kg isoproterenol given in 10 daily doses had four times the level of fibrosis as compared with rats receiving 1,000 μg/kg in one dose. Thus, prolonged stimulation with lower doses of isoproterenol produces greater fibrosis than a single higher dose. Development of subendocardial fibrosis could be blocked by pretreating the rats with 5 mg of the β-antagonist propranolol 1 hour before administration of each daily subcutaneous dose of 1,000 μg/kg isoproterenol.

Myocardial DNA Synthesis

To better characterize the relation between myocyte necrosis and stimulation of fibrosis during the period immediately after isoproterenol treatment, we measured the direct stimulation of DNA synthesis in the heart. The advantage of using DNA synthesis for estimation of stimulation of myocardial fibrosis is twofold. First, the difference in DNA synthesis rates in comparison with controls is greater than the difference in percent fibrosis measured by morphometry. Secondly, peak stimulation of DNA synthesis occurs within 48 hours of treatment. Thus, the timing of the peak activation of DNA synthesis can be more closely compared with the onset of treatment than the development of fibrosis, and the temporal relation between necrosis and fibrosis can be more accurately determined than that achieved using morphometry alone.

Rats that were treated with 50-5,000 μg/kg/day isoproterenol for 2 days were pulse-labeled with [3H]thymidine for 4 hours before sacrifice. DNA was extracted from the left ventricular, septal, and right ventricular samples of each heart, and the specific radioactivity (cpm/μg DNA) was determined in each sample. As was observed in the development of fibrosis (Figure 6), stimulation of myocardial DNA synthesis appeared to be related to the dose of isoproterenol (Figure 8). The incor-
poration of \(^{3}H\)thymidine into DNA of the left ventricle and septum was increased more than 10 times control values in rats receiving more than 1,000 \(\mu\)g/kg isoproterenol (Figure 8). Stimulation of DNA synthesis was also observed in the right ventricle after treatment with 1,000 \(\mu\)g/kg isoproterenol (mean+SEM, 233±61 cpm/\(\mu\)g; \(n=6; p<0.01\)) versus control right ventricle although levels were slightly lower than those observed in the left ventricle.

Administration of isoproterenol with minipumps, which presumably prevented the appearance of acute high serum levels of isoproterenol, did not prevent the appearance of myocyte necrosis, as shown above (Figure 4) nor did it prevent the stimulation of myocardial DNA synthesis (Figure 8). Although the same dose of 1 mg/kg/day isoproterenol produced a considerably higher stimulation of DNA synthesis when given by a single injection than by minipump, higher levels of isoproterenol delivered by minipump produced the same maximal stimulation of DNA synthesis (Figure 8). A proportionality was also observed between the level of necrosis and DNA synthesis in rats given isoproterenol by minipump; two animals with the lowest necrosis score in Table 1 received the lowest dose, 1 mg/kg/day, via the minipump.

Radioautographic Analysis

To further evaluate the regional differences in the stimulation of DNA synthesis in the myocardium, as well as to determine whether this response was limited to the fibroblast population, we conducted radioautography on paraffin sections of \(^{3}H\)thymidine-labeled hearts. Virtually all nuclear labeling in both control and isoproterenol-treated hearts was limited to the nonmyocyte cells. A gradient of labeling frequency was observed progressing from the subendocardium to subepicardium in treated animals (Figure 9). Elevated labeling indexes were also noted in the right ventricle (Figure 9).

Discussion

Administration of the \(\beta\)-adrenergic agonist, isoproterenol, results in cardiac hypertrophy, myocyte necrosis, fibroblast proliferation, and connective tissue accumulation. Our objective in this study has been to compare the temporal, spatial, and dose-response relation of the levels of necrosis, fibrosis, and hypertrophy to determine whether fibrosis and hypertrophy occur in reparation of injury and as a consequence of myocyte loss. Alternatively, if the level of either of these responses was independent of myocyte loss, it could be suggestive of a reactive response to adrenergic hormone stimulation.

To characterize the onset, timing, and extent of myocardial necrosis after isoproterenol treatment, we have used the technique of in vivo labeling with monoclonal antimyosin as a sensitive and specific probe for the development of myocyte necrosis.\(^{24}\) Monoclonal antimyosin is specific for myocytes with permeable sarcolemma and remains active in the circulation for more than a week. Rather than being the sole result of a passive entry of the probe into the injured cell, the antibody is concentrated by binding to myofibrillar myosin. Thus, antibody labeling provides a clear objective means to detect necrotic cells down to a level of a single myocyte per cross section. This technique may also serve to rule out myocyte macromolecular permeability in cells that might appear abnormal by histological criteria and yet remain unlabeled with antimyosin.

Using this approach, we have characterized the development of myocyte necrosis after isoproterenol treatment with a greater degree of sensitivity and specificity than has heretofore been possible. Our study confirms earlier reports that myocyte permeability develops within a very short period after isoproterenol administration.\(^{24,29}\) The level of labeling rises to a maximum between 12–24 hours after treatment and then declines to control levels by 72 hours. The rapid rise and fall of myocyte labeling suggests that necrosis was produced during a single short-lived period.

Rona et al\(^{24}\) using doses of isoproterenol considerably greater than those used here (85 mg/kg), reported a rapid appearance of grossly apparent necrosis within 8 hours of treatment, peak development by 48 hours, and regression and disappearance of necrotic muscle by 72 hours. The significance of the present study lies in the ability to detect necrosis even earlier and with considerably greater sensitivity. By identifying each new wave of necrosis after repeated injections or minipump dosing, we have been able to closely explore the temporal and spatial relation between myocyte necrosis, subsequent fibrogenesis, and myocardial hypertrophy.

The amount of subendocardial fibrosis present after 10 days of isoproterenol appears to be dose dependent. The full extent of fibrosis is not apparent by morphometric point counting, however, until several days after treatment. Using incorporation of \(^{3}H\)thymidine into myocardial DNA as a probe for stimulation of fibroblast proliferation, we were able to assess the level of this response as early as 24 hours after isoproterenol treatment. Maximal stimulation of DNA synthesis occurs approximately 48 hours after a single dose of isoproterenol.\(^{41}\) Stimulation of DNA synthesis at 48 hours also follows an approximate dose-response relation for the doses of isoproterenol we used.

Given that neither this study nor previous reports have been able to totally dissociate a fibrotic response from the occurrence of myocyte necrosis,\(^{25}\) it is still possible to qualitatively compare the dose-response, timing, and response to single, pulse, and chronic administration of different levels of isoproterenol to evaluate how closely the necrosis and fibrosis responses are related. The findings appear to indicate that there are at least three correlations between the devel-
FIGURE 9. Mosaic radioautograph showing the gradient of nuclear labeling in a section of the septum from the subendocardium to right ventricle in a rat treated with 1,000 µg/kg isoproterenol for 48 hours. The rat was pulse-labeled with [3H]thymidine for 4 hours before removal of heart, and paraffin sections were prepared for radioautography. LVL: Left ventricular lumen; RVL: Right ventricular lumen. Magnification, ×175.
opment of myocyte necrosis and myocardial fibrosis after isoproterenol administration.

First, myocyte necrosis and fibrosis both develop in the same myocardial regions. Maximal myocyte necrosis and myocardial fibrosis are both confined to the subendocardium and midwall.\(^{24,25}\) No significant scarring developed in the subepicardium, and necrotic myocytes were not observed in this region of the heart.

Second, the appearance of necrosis and fibrosis are closely related in time. Necrosis reaches a maximum at 12–24 hours after a single dose of isoproterenol. Stimulation of fibroblast DNA synthesis reaches a maximum at 48 hours.\(^{41}\)

Third, there is a similarity in the dose-response relation of both necrosis and fibrosis. Significant necrosis and fibrosis were both observed in hearts treated with 125 \(\mu\)g/kg/day s.c. isoproterenol. Increasing the doses or repetition of isoproterenol treatment resulted in increased levels of observed necrosis, greater stimulation of DNA synthesis, and increased myocardial fibrosis. If only a single dose of isoproterenol is given, the degree of subendocardial fibrosis observed 10 days later is only slightly greater than in control hearts. Repeated treatments produce additional myocyte necrosis and considerably greater levels of subendocardial fibrosis.

Since bolus subcutaneous injection results in acute high serum levels of isoproterenol, it was presumed that a low dose of isoproterenol infused over a prolonged period might produce myocardial fibrosis without the generation of significant necrosis. This did not appear to be the case. Myocyte necrosis and significant stimulation of DNA synthesis occurred even when we administered 1 mg/kg i.p. isoproterenol over 24 hours with an osmotic minipump. However, the levels of both necrosis and DNA synthesis were less than that observed when the same dose was given by daily subcutaneous injection.

The conclusion that initial fibrosis after isoproterenol treatment is predominantly related to repa-
ration of myocyte loss is based in part on the comparison of dose-response levels of each pro-
cess. While a quantitative estimation of the degree of fibrosis is relatively direct, estimation of the amount of myocardial necrosis resulting from isoproterenol is somewhat indirect, even when monoclonal antimyosin labeling is used. Fibrosis is a cumulative permanent response that may be evaluated in a number of ways at various periods after an intervention. Monoclonal labeling of necrotic myocytes, on the other hand, is a transient, time-varying process. Necrotic myocytes are probably labeled with antimyosin within a few minutes after development of membrane permeability. However, reparative processes immediately begin to break down the necrotic cell and remove it from the heart. Chronic or repeated dosing protocols stimulate new necrosis and myocyte labeling while previously labeled cells are being degraded. Thus, monoclonal antibody labeling can only identify the necrotic population during the period after development of membrane permeability and before removal from the heart. Direct quantification of the number of volume fraction of antibody-labeled myocytes at any specific time would most likely provide a significant underestimate of the true fraction of myocyte loss. A quantitative estimate of total myocyte loss would probably require direct morphometric counting procedures as has been used by Anversa et al.\(^{42}\)

Myocardial necrosis and fibrosis appear to be related processes that have not been observed independently of each other. The question of whether myocardial hypertrophy after isoproterenol treatment is a response related to myocyte loss\(^{25}\) or a reactive process related to \(\beta\)-adrenergic hormone stimulation\(^{20}\) has also been addressed in this study. Myocardial mass also increases significantly after isoproterenol treatment. In contrast to necrosis and fibrosis, however, myocardial hypertrophy appears to be less associated with the dose of isoproterenol over the ranges we evaluated than either myocyte necrosis or fibrosis. In our study, myocardial hyper-
trophy showed a graded response to increasing doses of isoproterenol after 2 days. However, unlike the levels of fibrosis, maximal stimulation of hyper-
trophy was observed over the range of 125–5,000 \(\mu\)g/kg/day after 10 days. It should be noted that both Collins et al\(^{25}\) and Alderman and Harrison\(^{19}\) found that longer periods were required for development of maximal hypertrophy. However, their studies evaluated lower levels of isoproterenol than we studied. Stanton et al,\(^{18}\) using doses equivalent to those used in our study, found both a dose- and time-dependent relation with regard to the degree of developed myocardial hypertrophy. Both Stanton et al\(^{18}\) and Collins et al\(^{25}\) have suggested that hypertrophy develops in compensation for myocyte loss after isoproterenol administration. However, in neither of these studies was the level of myocyte necrosis directly determined.

The results of this study show that isoproterenol produces myocyte necrosis, even when applied in low doses and with chronic infusion to avoid transient high levels. This necrosis is followed by increased fibrosis and hypertrophy. The fibrotic tissue response is graded and closely parallels necrosis. Hypertrophy also developed in a measured response related to isoproterenol dosage within 2 days of treatment but reached maximal levels at all doses with 10 daily repetitive treatments. Thus, hypertrophy may derive either from increased myocardial loading, which is a direct result of adrenergic hormone stimulation of the heart, or as a compensation for myocyte loss. Thus, at this point, it is not possible to totally rule out myocyte loss as a basic factor in any instance of increased myocardial fibrosis or increased collagen concentration (in contrast to increased collagen content, which is proportional to the level of increased hypertrophy). Application of monoclonal antimyosin for detection of
myocyte necrosis provides considerably greater sensitivity and, when applied in these instances, may provide significant new insight into both the causes and direct effects of myocyte loss in myocardial hypertrophy.

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