Prolyl hydroxylase and collagen metabolism after experimental myocardial infarction

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JUDD, JOSEPH T., AND BERNARD C. WEXLER. Prolyl hydroxylase and collagen metabolism after experimental myocardial infarction. Am. J. Physiol. 228(1): 212-216. 1975.—Mature male Sprague-Dawley rats were subjected to an isoproterenol-induced myocardial infarction. Animals were sacrificed on a daily basis in order to assess the temporal changes in prolyl hydroxylase activity and collagen metabolism during the acute stages of myocardial necrosis and repair. Total myocardial hydroxyproline, as an index of collagen content, increased promptly and markedly, beginning on day 1, and remained elevated thereafter. The incorporation of [14C]-proline into definitive hydroxyproline of mature collagen was also increased. The activity of the enzyme prolyl hydroxylase, which regulates the rate of conversion of proline to hydroxyproline in collagen, was elevated by day 2, remained high through day 4, and then declined to a relatively constant but still slightly elevated level throughout the period of repair. It is believed that changes in these parameters of collagen metabolism reflect changes in myocardial fibroblastic cell and ground substance pertinent to fundamental aspects of repair of the injured myocardium.

hydroxyproline; connective tissue; necrosis; wound healing; isoproterenol

ADMINISTRATION OF THE POTENT beta-adrenergic stimulating drug isoproterenol to rats produces myocardial ischemia and necrosis, with biochemical and histopathological changes that simulate those accompanying naturally occurring myocardial infarction (4, 18-21). Changes in serum enzymes (18, 21) indicative of the severity of myocardial necrosis and repair. Total myocardial hydroxyproline, as an index of collagen content, increased promptly and markedly, beginning on day 1, and remained elevated thereafter. The incorporation of [14C]-proline into definitive hydroxyproline of mature collagen was also increased. The activity of the enzyme prolyl hydroxylase, which regulates the rate of conversion of proline to hydroxyproline in collagen, was elevated by day 2, remained high through day 4, and then declined to a relatively constant but still slightly elevated level throughout the period of repair. It is believed that changes in these parameters of collagen metabolism reflect changes in myocardial fibroblastic cell and ground substance pertinent to fundamental aspects of repair of the injured myocardium.

MATERIALS AND METHODS

Mature male Sprague-Dawley rats weighing 350-400 g were used in all experiments. Myocardial injury was induced by the subcutaneous injection of isoproterenol HCl, given in two doses, spaced 24 h apart, each containing 50 mg/100 g body wt. Control animals were injected with saline. The animals were killed by decapitation and blood was collected from the severed neck vessels. Hearts were removed as rapidly as possible, placed in saline while still beating, and then opened and washed further in saline to remove as much blood as possible. After blotting and weighing, the heart was either placed in acetone to dehydrate or quick frozen in a Dry Ice acetone bath to await enzymatic analysis or extraction of collagen (vide infra).

Prior to the determination of total myocardial hydroxyproline, the heart was extracted with acetone in a Soxhlet-type extraction apparatus for 24 h to remove lipid and water, dried, and weighed. The relatively lipid-free tissue was then hydrolyzed in 5 ml of 4 N HCl for 24 h at 98-99°C. Hydroxyproline was determined by the method of Prockop and Udenfriend (14).

The incorporation of [U-14C]proline into citrate-soluble and insoluble collagen during the repair phase after isoproterenol-induced necrosis was determined. Four and one-half days after the initial dose of isoproterenol, 16 control and 16 experimental animals were given 20 μCi of [U-14C]-proline dissolved in 1 ml of saline by intraperitoneal injection. At 6, 12, 24, and 36 h thereafter, four control and four experimental animals were killed by decapitation, blood was collected for counting, and the heart was removed, washed in saline, and placed in 20 vol of cold 0.2 M citrate buffer, pH 3.6. The tissue was minced with scissors and then homogenized with a Vir-Tis 45 homogenizer at high speed for a total time of 5 min (1 min of homogenization followed by 1 min of cooling in ice repeated 5 times). A few
drops of octyl alcohol were added as a preservative, and the sample was shaken at 4°C for 24 h. The residue, recovered by centrifugation at 10,000 g for 15 min, was resuspended and shaken as above a total of 3 times, and the supernatant from each extraction was pooled.

The supernatant containing that fraction of collagen soluble in 0.2 M citrate buffer, pH 3.6 (citrate-soluble fraction), was dialyzed in a cellulose membrane for 48 h against several changes of distilled water. The retentate was heated in a double boiler for 30 min and then filtered through filter paper (Whatman No. 1) that had been washed with boiling water immediately prior to use. The precipitate was washed with hot water, and the washes were combined with the filtrate. The residue obtained from this fraction was very small (or absent) and was discarded. The soluble collagen was recovered by lyophilization and hydrolyzed with 4 N HCl for 24 h at 98–99°C.

The residue remaining after extraction of the heart with citrate buffer was suspended in distilled water and heated in a double boiler for 3 h to convert insoluble collagen to gelatin. Undissolved protein was removed by filtration, and the residue was washed with boiling water. The filtrate was lyophilized and hydrolyzed for the determination of labeled hydroxyproline. The specific activity of [14C]hydroxyproline in the citrate-soluble and insoluble collagen fractions separated by this procedure was determined by the method of Prockop et al. (15). The residue remaining after removal of the insoluble collagen was recovered from the filter as completely as possible, hydrolyzed, and analyzed for hydroxyproline. Hydroxyproline content was too low to be determined quantitatively.

Prolyl hydroxylase activity was determined by the method of Hutton et al. with a tritium-labeled substrate prepared from chick embryos (1). At autopsy, hearts were removed, placed in 10 vol (wt:vol) of 0.25 M sucrose, and frozen with Dry Ice. They were then stored at −20°C until analyzed. The tissues were homogenized with a Ten Broeck type of ground-glass tissue grinder at 4°C. Aliquots were taken for determination of hydroxyproline and for total protein (10). The remaining homogenate was centrifuged at 15,000 g for 10 min and the supernatant analyzed for protein. An aliquot of supernatant (enzyme) was incubated at 30°C with 0.1 M Tris-HCl buffer, pH 7.5, cofactors as described by Hutton et al. (1, 2), and with protocollagen substrate sufficient to give final counts in the tritiated water formed 3–6 times that of a sucrose (no enzyme) blank.

The effect of enzyme (protein level of the supernatant) and time of incubation on the velocity of reaction was determined (Fig. 1). A 30-min incubation period with 4–6 mg protein was established as standard conditions for the assay.

RESULTS

Total myocardial hydroxyproline changes after isoproterenol-induced myocardial infarction are presented in Table 2. Hydroxyproline in the collagen fractions is presented in Table 2. Hydroxyproline content of the collagen was determined quantitatively. The earliest statistically significant increase in hydroxyproline is seen on the 4th and 5th days after injury, with further increases occurring to days 10–12, reaching a final level of hydroxyproline approximately twice that of control (uninjured) hearts. The wet weight of the heart (Table 1) was elevated on days 1–4 and on day 6. Protein content of the heart was also elevated on days 1, 2, and 6.

The incorporation of [14C]proline into collagen hydroxyproline during the period from 4.5 to 6 days postinfarction, when myocardial repair is proceeding rapidly, was determined (Fig. 3). Hydroxyproline content of the collagen fractions is presented in Table 2. Hydroxyproline in the citrate-soluble collagen represented 6.2 and 8.1% of that of the total collagen in control and infarcted hearts, respect-
### TABLE 1. Heart weight and protein content after isoproterenol-induced myocardial infarction

<table>
<thead>
<tr>
<th>Days after Isoproterenol</th>
<th>Wet Weight, g</th>
<th>Protein, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.393 ± 0.057</td>
<td>322.7 ± 13.2</td>
</tr>
<tr>
<td>1</td>
<td>1.707 ± 0.062</td>
<td>432.6 ± 30.9</td>
</tr>
<tr>
<td>2</td>
<td>1.846 ± 0.111</td>
<td>440.8 ± 33.5</td>
</tr>
<tr>
<td>3</td>
<td>1.722 ± 0.044†</td>
<td>351.6 ± 09.8</td>
</tr>
<tr>
<td>4</td>
<td>1.733 ± 0.074†</td>
<td>343.0 ± 19.2</td>
</tr>
<tr>
<td>5</td>
<td>1.555 ± 0.049</td>
<td>316.6 ± 17.3</td>
</tr>
<tr>
<td>6</td>
<td>1.645 ± 0.065*</td>
<td>410.6 ± 08.9†</td>
</tr>
<tr>
<td>7</td>
<td>1.569 ± 0.052</td>
<td>267.0 ± 08.0</td>
</tr>
<tr>
<td>8</td>
<td>1.432 ± 0.040</td>
<td>302.2 ± 29.0</td>
</tr>
<tr>
<td>9</td>
<td>1.450 ± 0.063</td>
<td>311.8 ± 31.9</td>
</tr>
<tr>
<td>10</td>
<td>1.447 ± 0.056</td>
<td>281.8 ± 19.8</td>
</tr>
<tr>
<td>12</td>
<td>1.447 ± 0.039</td>
<td>322.0 ± 16.5</td>
</tr>
<tr>
<td>14</td>
<td>1.436 ± 0.052</td>
<td>332.4 ± 18.6</td>
</tr>
<tr>
<td>16</td>
<td>1.431 ± 0.036</td>
<td>278.8 ± 09.5</td>
</tr>
<tr>
<td>18</td>
<td>1.564 ± 0.076</td>
<td>278.2 ± 17.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. Controls, n = 15; experimental, n = 5 per group. Significant difference from controls as calculated from a t test: * P < 0.05; † P < 0.01; ‡ P < 0.005.

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### DISCUSSION

The early stages of isoproterenol-induced myocardial injury are characterized by many of the same biochemical and
histological changes observed after other experimental procedures or infarction resulting in ischemic myocardial injury and necrosis. Within a few hours of established ischemia there is marked cardiac edema with the accumulation of glycoproteins, presumably of serum origin (5). Within 24 h after injury, there is an increase in myocardial mucopolysaccharides, mainly hyaluronic acid (3,6), which persists up to the 4th day postinfarction. Early cellular infiltration of the injured myocardium is observed by 24 h, with the appearance of what appear to be young mesenchymal cells. By the 2nd to 3rd day there is an intense cellular infiltration of the myocardium, consisting principally of leukocytes and lymphocytes. In addition, fibroblasts are readily observed at this time, scattered throughout the intermuscular spaces (ecmwcative areas) and also clustered about necrotic muscle fibers. By the 6th day, foci of cellular reaction persist in the heart muscle in conjunction with areas of extensive scar tissue formation, but without the generalized cellular reaction seen earlier. Histologically, repair appears to be virtually complete as early as 1 wk postinfarction (5).

Previously, collagen formation, estimated by increased myocardial hydroxyproline and by histopathological techniques, was demonstrated to occur as early as 3 days after injury, with substantial increases in myocardial hydroxyproline occurring by the 5th-7th days (5). In the present investigation, determination of changes in myocardial hydroxyproline indicates that healing of the injured myocardium proceeds rapidly from the 3rd-10th or 12th days postinfarction, with little change thereafter up to 18 days. This increase in myocardial hydroxyproline represents in part the synthesis of mature collagen, e.g., scar tissue, as demonstrated by the isotopic-incorporation data. Jackson and Bentley (3) have shown that collagen in developing connective tissue exists in a continuous spectrum of aggregates of varying strengths of cross-linkages, those with lower cross-linkage representing the more recently formed molecules. Further, these aggregates may be extracted with salt solutions of various concentrations and thus of concomitantly various abilities to disaggregate and dissolve the collagen. In view of the small amount of soluble collagen in the heart, and in order to work with individual heart specimens rather than pooled hearts, we elected to separate only the fraction soluble in 0.2 M citrate buffer, pH 3.6, from the insoluble collagen (extracted after gelatinization), assuming that the citrate-soluble fraction represents all the most recently synthesized collagen and that the insoluble fraction contains mature collagen as found in scar tissue and in normal fibrous tissues or structures. There was little change in the incorporation of isotope in normal hearts during a 36-h period in either the citrate-soluble or insoluble fractions. However, there was a four- to fivefold increase in specific activity of hydroxyproline in the citrate fraction from infarcted hearts in a comparable 36-h period from 4 to 6.5 days after injury, indicating rapid synthesis of collagen in the repairing heart. There was no consistent pattern of change in the specific activity of this fraction, however, due possibly to a high rate of turnover of the labeled collagen as the newly synthesized molecules are either broken down or converted into mature insoluble forms. There was a 24% increase in the hydroxyproline of insoluble collagen from infarcted hearts, together with a progressive increase in labeling of the hydroxyproline between 4.5 and 6.5 days after injury. Although the biochemical techniques used do not separate collagen of mature scar tissue from that normally present in the fibrous connective tissue of the heart, we believe that these data demonstrate de novo scar tissue formation during the healing phase of experimental myocardial infarction, rather than any effect on preexistent fibrous tissue of the heart. However, some effect on the collagen of existing myocardial fibrous tissue (such as remodeling induced by changes in mechanical stress) in the repairing heart cannot be ruled out.

Myocardial prolyl hydroxylase activity was greatly elevated on the 2nd through 4th days after injury, reaching a level 7-10 times that of normal control hearts by day 3, and then by day 5 declining to levels 1.5-2 times that of controls, where it remained for the final period of this investigation. This rapid increase and decline in prolyl hydroxylase during the first 3 days after myocardial injury closely parallel the inflammatory changes in the heart and are consistent with the results reported by other workers showing that prolyl hydroxylase activity is elevated in inflammatory conditions (12). However, in view of the early appearance of connective tissue cells in the injured myocardium (5), discussed above, we feel that the changes in enzyme activity observed up to the 4th day after injury reflect activation of connective tissue synthesizing cells, with a subsequent decline in activity as much of the early, extensive inflammatory reaction in the heart is resolved without correspondingly extensive and irreversible muscle damage. The level of enzyme activity then remains slightly elevated as repair proceeds in foci of necrotic muscle. Furthermore, the in vitro enzyme activity does not necessarily represent active enzyme in the heart, but rather simply the presence of cells having the potential to synthesize collagen. Continuation of elevated levels of enzyme in the heart as repair proceeds is consistent with observations of Mussini et al. (11) that prolyl hydroxylase activity increases markedly at the site of a wound. Failure of the level of enzyme to decline as the hydroxyproline level in the heart plateaus may be due to continuing irritation and repair in the muscle induced by contraction in the presence of abnormal scar tissue. Histologically, fibroblasts persist in such foci throughout healing and thereafter. However, experimentally induced cardiac and skeletal muscle hypertrophy is associated with an activation of prolyl hydroxylase (9,16), indicating that, at least in part, the elevated enzyme activity observed in the present work may be due to a similar effect. This may be especially pertinent to the observed elevation in enzyme activity after healing appears complete.

These findings suggest that the appearance of mesenchymal cells, and later adult fibroblasts, and perhaps white blood cell elements bear a pertinent relationship to our earlier findings of definitive alterations in ground substance (5-8) and to our present findings of definitive changes in collagen metabolism related to healing in injured myocardial tissue.

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REFERENCES


