Injury and Repair in Arterial Tissue in the Rabbit

ANALYSIS OF DNA, RNA, HYDROXYPROLINE, AND LACTATE DEHYDROGENASE IN EXPERIMENTAL ARTERIOSCLEROSIS

By Seppo Lindy, Heikki Turto, Jouni Uitto, Pekka Helin, and Ib Lorenzen

ABSTRACT

The aortic wall in rabbits was injured by pulling an inflated balloon catheter through the aorta. This was followed by an increase of the RNA-DNA ratio in the aortic wall 3 days after the injury. At the same time, the cathodic lactate dehydrogenase (LDH) isoenzymes composed of M subunits increased. The changes in LDH may reflect tissue hypoxia at the early phase after the injury. At 6 days procollagen proline hydroxylase activity was increased, suggesting increased capacity for collagen formation in the injured aorta. Two weeks after injury the total content of hydroxyproline and DNA were increased, indicating connective tissue proliferation and hyperplasia in the injured aorta. The biochemical changes were interpreted as a repair response of the arterial connective tissue to injury. Gross arteriosclerosis of the aorta developed simultaneously with the biochemical alterations.

KEY WORDS
connective tissue aortic wall wound healing protocollagen proline hydroxylase LDH isoenzymes hypoxia

The importance of nonspecific injury to the arterial wall in the development of arteriosclerosis has been stressed by various authors (1, 2). In animals, experimental arteriosclerosis can be induced by injuring the arteries by various techniques (3), the reaction of the arterial wall being highly independent of the nature of the damaging factors.

In previous studies, Helin et al. (4, 5) demonstrated the development of arteriosclerotic changes in rabbit aorta following a single mechanical dilatation of the descending thoracic aorta with a balloon catheter. The alterations included extensive gross and microscopic changes as well as alterations in the aortic content of glycosaminoglycans and hydroxyproline. The alterations, which were highly dependent upon the time elapsed following the injury, were interpreted as nonspecific processes of repair of the vascular connective tissue.

The purpose of the present experiment was a further investigation of the biochemical alterations in the thoracic aorta of rabbits following a single mechanical injury. DNA, RNA, total protein, activity of procollagen proline hydroxylase (PPH), and activity of lactate dehydrogenase (LDH) and its isoenzyme pattern, which reflect adaptation of the tissue to anaerobic metabolism were analyzed.

Material and Methods

The study involved 64 male albino rabbits of the Danish Country strain, 5 months old and weighing about 3 kg. The animals were fed a uniform laboratory stock diet containing 16%...
protein, 54% carbohydrates, 3.6% vegetable fat, and only traces of cholesterol.

In 34 rabbits, the descending thoracic aorta was dilated once by pulling an inflated 5F Fogarty arterial embolectomy catheter from the arch of the aorta to the diaphragm, as described previously by Helin et al. (4, 5). In 30 control animals, the same procedure was followed with the exception that the balloon of the catheter was uninflated. The animals were killed by intravenous injection of 300 mg sodium pentobarbital 3, 6, 13, and 30 days after the dilatation. The aortas were removed, cut longitudinally, and inspected for gross alterations. The intima-media layer of the aorta from the first intercostal arteries to the celiac artery was peeled off and used for the histochemical analysis of the tissue. The weighed, frozen with liquid nitrogen, and stored at −20°C. The analyses were carried out within a few days.

Specimens were first minced with scissors for 10 minutes and then with an Ultra-Turrax homogenizer three times for 5 seconds while suspended in cold 0.9% NaCl (1 part in 10 volumes). A sample of the tissue homogenate was taken for determination of hydroxyproline. Another sample of the homogenate was centrifuged at 15,000 g for 30 minutes. The supernatant fluid was used for determination of PPH and LDH. The remaining part of the homogenate was used for analyses of RNA, DNA, and total protein.

Assay of DNA and RNA.—DNA and RNA were assayed by a modification of the Schmidt-Thannhauser procedure (6). The homogenate was precipitated with equal volume of cold (0°C) 10% (w/v) trichloroacetic acid (TCA). The precipitate was washed twice with cold 5% TCA and once with 2% sodium acetate in 90% (v/v) ethanol. RNA was freed by incubation in 1M NaOH for 18 hours at 37°C. A specimen of the alkali digest was taken for determination of α-amino nitrogen. After acidification of the digest with HCl and TCA, the final concentration of which were 1M and 5%, respectively, the sample was centrifuged and the supernatant fluid used to determine RNA by an orcinol method (7) using d-ribose (Fluka AG) as standard. The precipitate was washed twice with cold 5% TCA, and DNA was extracted by heating the sample in 5% TCA at 90°C for 30 minutes. DNA was determined by a modification of the Schmidt-Thannhauser method described by Prockop and Ebert (14). Radioactivity was measured with a three-channel liquid scintillating (Packard Tricarb, Packard Instruments Co.) and the radioactivities were expressed as disintegrations per minute.

Presentation of the Results.—All values are expressed either per unit wet weight of tissue (concentration) or as the total amount of the substance in the aortic sample (content).

The results were evaluated using Student's t-test. The differences were regarded as significant at the level of P ≤ 0.05.
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TABLE 1

<table>
<thead>
<tr>
<th>Day after injury</th>
<th>Group</th>
<th>Wet wt of aortic sample (mg)</th>
<th>DNA concentration (mg/g wet wt of tissue)</th>
<th>DNA content (µg/aortic sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>I</td>
<td>180.3 ± 43.0 (10)</td>
<td>0.747 ± 0.057 (10)</td>
<td>136.3 ± 41.4 (10)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>157.1 ± 37.1 (9)</td>
<td>0.955 ± 0.112 (9)</td>
<td>143.8 ± 31.3 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>I</td>
<td>218.4 ± 35.3 (10)</td>
<td>0.927 ± 0.071 (10)</td>
<td>201.4 ± 31.7 (10)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>159.5 ± 15.9 (9)</td>
<td>0.953 ± 0.046 (9)</td>
<td>152.2 ± 17.5 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>I</td>
<td>237.6 ± 92.5 (8)</td>
<td>1.051 ± 0.203 (8)</td>
<td>245.7 ± 87.3 (8)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>158.5 ± 29.3 (6)</td>
<td>1.018 ± 0.118 (6)</td>
<td>159.7 ± 15.9 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>I</td>
<td>297.6 ± 64.6 (6)</td>
<td>0.971 ± 0.077 (5)</td>
<td>292.1 ± 67.5 (5)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>127.5 ± 9.6 (6)</td>
<td>0.973 ± 0.064 (6)</td>
<td>124.8 ± 15.6 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± sd. I = injured aortas; C = control aortas. Number of samples is given in parentheses.

Results

The wet weight of the injured aortas had increased to 137% of that of the control aortas by 6 days and increased further to 233% by 30 days (Table 1).

At 3 days the DNA concentration in the injured aortas was significantly decreased and was 78% of the control value. The DNA concentration returned to the control level at 6 days and remained at this level for the rest of the experiment (Table 1).

In spite of the decrease in DNA concentration, the total DNA content of aorta on the third day was at the same level in the experimental and control aortas. After 6 days, the total DNA content of the injured aortas had increased significantly and was 132% of that of the controls. A further increase to 234% of the control values was observed at 30 days (Table 1).

The RNA concentration was already significantly increased in injured aortas at 3 days and increased to a maximum of 120% of the control value at 13 days (Table 2).

The total RNA content in the injured aortas was 152% of the control values at 6 days, and increased to 176% and 256% at 13 and 30 days, respectively (Table 2).

The RNA-DNA ratio of the injured aortas was elevated after 3 and 13 days, being highest at 3 days. At 30 days the ratio did not differ from that of the controls (Table 2).

Hydroxyproline was determined at 3, 6, and 13 days. The total content of hydroxyproline in the gelatinized fraction of the injured aortas was 143% of the control value at 13 days, but there was no difference in the total hydroxyproline content of the nongelatinized fraction between these two groups (Table 3).

The total protein content in the injured aortas did not differ from that of the controls after 3, 6, and 13 days, but after 30 days it was 174% of the control values (Table 3).

At 6 days, the total LDH activity in the injured aortas was increased to 140% of the control values and remained at this level until 30 days (Fig. 1).

A marked change in LDH isoenzyme pattern was already seen at 3 days, the cathodically migrating isoenzymes which are composed of M subunits being increased in the electropherograms (Fig. 2). At 3 days the subunit M percent, calculated from the isoenzyme pattern, was 52.7% in the injured aortas, whereas in the control aortas it was 27.0% (Table 4). At 30 days the subunit M percent in the injured aortas was still higher than in the control aortas. In the injured aortas the ratio of LDH activity in high versus low pyruvate was elevated at 3 days and at 6...
TABLE 2
Concentration and Total Content of RNA and RNA-DNA Ratio in Injured and Control Aortas

<table>
<thead>
<tr>
<th>Day after injury</th>
<th>Group</th>
<th>RNA concentration (mg/g wet wt of tissue)</th>
<th>RNA content (µg/aortic sample)</th>
<th>RNA-DNA (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>I</td>
<td>3.267 ± 0.199 (10)</td>
<td>607.5 ± 163.2 (10)</td>
<td>4.36 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.075 ± 0.141 (9)</td>
<td>481.4 ± 105.5 (9)</td>
<td>3.25 ± 0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>P &lt; 0.05</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>I</td>
<td>3.485 ± 0.200 (10)</td>
<td>760.2 ± 133.0 (10)</td>
<td>3.76 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.130 ± 0.173 (9)</td>
<td>498.9 ± 48.4 (9)</td>
<td>3.29 ± 0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>P &lt; 0.001</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>I</td>
<td>3.801 ± 0.312 (8)</td>
<td>881.8 ± 266.0 (8)</td>
<td>3.85 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.166 ± 0.116 (6)</td>
<td>499.5 ± 78.5 (6)</td>
<td>3.13 ± 0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>P &lt; 0.001</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>I</td>
<td>3.465 ± 0.124 (5)</td>
<td>1032.7 ± 233.5 (5)</td>
<td>3.55 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.197 ± 0.202 (6)</td>
<td>408.2 ± 43.9 (6)</td>
<td>3.28 ± 0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>P &lt; 0.025</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± sd. I = injured aortas; C = control aortas. Number of samples is given in parentheses.

days. At 30 days, the ratio was 117% of that of the controls (Table 4).

PPH activity per unit wet weight of tissue was significantly elevated at 6 days, being 157% of the control values. The activity increased further, and was 185% and 286% of the control values at 13 and 30 days, respectively (Fig. 3).

Discussion

Two weeks after the single dilatation injury to the aorta, gross alterations developed, consisting of plaques surrounding the intercostal arteries and bean-shaped bulgings of the aortic wall. These alterations were identical to those reported by Helin et al. (4, 5). The typical microscopic changes in the aortic wall,
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FIGURE 1

LDH activity, expressed as IU/g wet weight of tissue in injured (○) and control (●) aortas. Mean ± so of 6 to 10 samples. P ≤ 0.025 (*); P ≤ 0.001 (**).

described in the previous publications (4, 5), are subendothelial thickening, fragmentation or straightening of the elastic membranes or both, necrotic foci in the media, and accumulation of amorphous intercellular substance surrounding the foci, and cellular proliferation.

The earliest biochemical analyses were made 3 days after the injury to the aorta. By this time, the DNA concentration was decreased, possibly being a dilution phenomenon due to edema and deposition of intercellular substance. This interpretation is supported by the fact that the total aortic DNA content in the dilated aortas remained unchanged. After 6 days the total DNA content increased in the aortic wall and paralleled the weight gain, suggesting that the increase in aortic weight was partly due to increase in cell mass. Injury, which stimulates mitotic activity in tissues (16), seems to have caused cellular proliferation. This is in accord with the intimal hyperplasia seen histologically in the mechanically dilated aortas (4, 5).

Increase in RNA is a common reaction during the repair process after injury (17, 18).

FIGURE 2

LDH isoenzyme pattern in aortic tissue 3 days after dilatation injury. The electropherogram shows a change in isoenzyme pattern in injured aortas (a) to contain more cathodic isoenzymes than the controls (b).

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We found that RNA concentration and the RNA-DNA ratio were already increased three days after the dilatation of the aorta. This might be a sign of increased protein synthesis related to the repair process, reflected later in elevated protein content, elevated enzyme activities, and increased connective tissue components.

At 3 days after the injury, aortic LDH isoenzymes changed to a pattern composed more of cathodic isoenzymes and M subunits. LDH isoenzymes have been shown to indicate changes in the oxygen tension in the ambient air. Both in vivo (19, 20) and in vitro (20) hypoxia causes an increase in isoenzymes containing subunit M. Consequently, the change in LDH M percent can be interpreted as a sign of hypoxic condition in the arterial wall. The possibility (21) that the change in isoenzyme pattern is connected to mitosis regardless of oxygen tension cannot be excluded. However, this hypothesis has been challenged (22) and it further seems unlikely because in the present study changes in the LDH pattern could be observed before any increase in DNA. The metabolism of the aorta is dependent on glycolysis even in normal conditions (23, 24). The energy demands of the increased synthetic activities elicited by the primary injury may be met by enhanced anaerobic glycolysis. The change in LDH isoenzymes may be favorable in this respect, because isoenzymes composed of M subunits are better suited to the formation of lactate from pyruvate (25).

PPH is an enzyme that participates in the biosynthesis of collagen by catalyzing the hydroxylation of proline in peptide form, a

<table>
<thead>
<tr>
<th>Day after injury</th>
<th>Group</th>
<th>Subunit M percent of LDH</th>
<th>Substrate inhibition of LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>I</td>
<td>52.7 ± 7.9 (10)</td>
<td>0.807 ± 0.045 (10)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>27.0 ± 3.7 (9)</td>
<td>0.683 ± 0.046 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>6</td>
<td>I</td>
<td>53.7 ± 5.5 (10)</td>
<td>0.784 ± 0.036 (10)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>26.0 ± 3.6 (9)</td>
<td>0.710 ± 0.070 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.002</td>
</tr>
<tr>
<td>13</td>
<td>I</td>
<td>47.1 ± 9.6 (9)</td>
<td>0.832 ± 0.086 (8)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>22.9 ± 4.4 (6)</td>
<td>0.733 ± 0.119 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*P &lt; 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>30</td>
<td>I</td>
<td>35.4 ± 3.1 (6)</td>
<td>0.830 ± 0.086 (6)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>22.2 ± 2.0 (6)</td>
<td>0.711 ± 0.072 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SD. I = injured aortas; C = control aortas. Number of samples is given in parentheses.

**TABLE 4**

_Subunit M Percent and Substrate Inhibition of LDH in Injured and Control Aortas_

![Figure 3](http://circres.ahajournals.org/static/circresimages/20.jpg)
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precursor of collagen called procollagen (26). The PPH activity has been found to increase parallel with collagen formation in experimental granuloma (27). In experimental pulmonary fibrosis an increase in PPH activity preceded collagen accumulation (28). In the present work the elevation in PPH activity preceded the increase in collagen content, the elevation being significant at 6 days after the dilatation procedure. Fuller and Langner, who induced experimental arteriosclerosis with epinephrine-thyroxine injections, found a sixfold increase in PPH activity in the presence of gross plaquing (29). This agrees with our results and emphasizes the similarity between the two types of experimental arteriosclerosis (4, 5). The similarity is in agreement with the conception of a uniform reaction pattern of the arterial wall following injury (1).

Comstock and Udenfriend have suggested that lactate may be an activator of a preexisting inactive PPH in fibroblast culture (30). In our recent experiment dealing with experimentally induced fibrosis of the lung, we found an increase in LDH activity preceding the increase in PPH activity, and we suggested that the early events, preceding collagen accumulation in the lung tissue, consist of metabolic adaptation to anaerobic conditions (31). In the present study, the increase in LDH activity and change in isoenzyme composition, suggesting anaerobic conditions and lactate accumulation, preceded the increase in PPH activity. Our results are in agreement with the hypothesis that increase in PPH activity may be partly due to lactate accumulation in vivo.

The total hydroxyproline content in the gelatinized fraction of the aorta was significantly elevated 13 days after the dilatation of the aorta. This is in accord with reports by Helin et al. (4, 5) in which increased total hydroxyproline content in the aortic wall after injury was found. No differences in the contents of hydroxyproline in nongelatinized fractions were found in the aortas of experimental and sham-operated animals. The fractionation method used gave higher values of hydroxyproline than reported for the aortic elastin hydroxyproline (32), which indicates that the nongelatinized fraction contained some nongelatinized collagen in addition to elastin.

The mechanical injury to the aortic wall seems to induce a repair reaction, which consists of a metabolic adaptation to hypoxic conditions, increased cellular proliferation and protein synthesis and deposition of connective tissue components. Simultaneously with these processes the aortic wall developed extensive gross changes representing a type of experimental arteriosclerosis.

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