Hydrolase Activities in the Rat Aorta

II. Effects of Hypertension Alone and in Combination with Diabetes Mellitus

HARVEY WOLINSKY, LOIC CAPRON, SIDNEY GOLDFISCHER, FREDERIQUE CAPRON, BERNICE COLTOFF-SCHILLER, AND LISA E. KASAK

SUMMARY Hypertension is an important risk factor for atherosclerosis and often occurs in association with diabetes mellitus. Specific activities of hydrolases in homogenates of aortas from rats with renal-clip hypertension, normotension following a period of hypertension, and hypertension combined with streptozotocin-induced diabetes mellitus were measured. Enzymes included: neutral α-glucosidase, and lysosomal N-acetyl-β-glucosaminidase, β-galactosidase, cathepsin C, acid α-glucosidase, and acid cholesteryl esterase. After 6 or 12 weeks of hypertension, specific activities of all enzymes measured were significantly increased, levels ranging from 24% above normal for cathepsin C to 351% above normal for N-acetyl-β-glucosaminidase. Six weeks of normotension following 6 weeks of hypertension resulted in restoration to normal of four of the six enzyme activities; the remaining two enzymes were significantly below normal levels. Combined hypertension and diabetes mellitus showed smooth muscle cell levels of four of the five hydrolases measured to be significantly lower than those present with hypertension alone. In every instance, histochemical studies of aortas showed acid phosphatase and N-acetyl-β-glucosaminidase activities which corresponded to the biochemical findings. These findings indicate profound and discrete effects of two clinical risk factors on vascular smooth muscle cell lysosomes.

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acid cholesteryl esterase, which is involved in lipoprotein metabolism. We also determined whether removal of hypertension lowered lysosomal levels. Second, it was of interest to study the effects, at the cellular level, of combining experimental hypertension and diabetes mellitus in the same animal model to determine whether the resulting hydrolyase activities reflected the contribution of each.

Methods

Male Sprague-Dawley rats (Marland Farms), age 6 weeks and weighing 175–200 g at the outset of the experiment, were made hypertensive by placing a silver clip, internal diameter 0.25 mm, on the left renal artery. In the Sprague-Dawley rat, hypertension, defined as a systolic blood pressure greater than 150 mm Hg, generally ensued 2 weeks after surgery. Blood pressures were taken every week for the first 4 weeks and every 2 weeks thereafter. The method of taking blood pressures by tail cuff is described elsewhere. To restore elevated blood pressure to normal, the previously placed renal artery clip was opened fully with clamps, and restoration of bounding pulsations in the renal artery could be palpated immediately. In most rats, a sharp fall in systolic blood pressure to near normal occurred within 1 week; in some, a moderate drop in the first week was followed by a further slight decline to near normal in the second. No further change was seen after this. These rats will be called the hypertensive-normotensive group. All surgical procedures involving the clips were matched in time by sham operations in all other rats.

Rats were made diabetic by injection with a single dose of 10 mg of streptozotocin (Upjohn) (approximately 55 mg/kg body weight) dissolved in 0.5 ml of 0.9% saline with 0.02 m sodium citrate, pH 4.5. Injections were made into the jugular veins of nonfasted animals. Details of criteria used for diabetes mellitus, bleeding schedules, and methods of blood glucose measurement are given in the preceding paper. All rats were killed by exsanguination via the abdominal aorta while under ether anesthesia.

Experimental Designs

Hypertension and Hypertension Followed by Normotension

The experimental design consisted of two 6-week periods. After documentation of hypertension for 6 weeks in the clipped group, two subgroups, controls (C) and hypertensives (H), were killed. The remaining hypertensive rats were further divided into sustained hypertensive (HH) and hypertensive-normotensive (HC) groups. After a documented 6-week period of stable reduced blood pressure in the latter group, three groups, HH, HC, and an age-matched control group (CC), were killed. The H group sustained the same duration of hypertension as the HC group (6 weeks).

Combined Diabetes Mellitus and Hypertension

Four groups were studied: controls (C), diabetic (D), hypertensive-diabetic (HD), and hypertensive (H). Rats in groups HD and H had a renal artery clip placed at age 6 weeks. One week later (before an increase in systolic blood pressure could be detected in those clipped), rats in groups HD and D were injected with streptozotocin; overt diabetes ensued in 3 days. The experimental period was 8 weeks of documented hypertension and/or diabetes mellitus, after which all rats were killed. Studies on vessels of all groups in this experiment were done simultaneously.

Morphological and Cytochemical Studies

Light and electron microscopic studies were done exactly as described elsewhere.

Biochemical Studies

These were carried out on intima-media fragments of thoracic aortic segments as described elsewhere with the exception that, in the hypertension and hypertension-normotension experiments only, three aortas, pooled and homogenized in 4.5 ml of sucrose-EDTA solution, represent each experimental sample.

Enzyme Assays

Glycosidases and cathepsin C were assayed fluorometrically in the same manner as previously discussed. Optimal incubation conditions for neutral α-glucosidase, β-galactosidase, cathepsin C, N-acetyl-β-glucosaminidase, and acid α-glucosidase in vessels from H and HD groups were identical to those given elsewhere for C and D groups. Incubation periods ranged from 15 to 150 minutes at 37°C depending on the enzyme studied. The activity of α-mannosidase, measured previously in C and D aortas at pH 5.9 and thought to be a Golgi enzyme, was not assayed here because pH optimum curves obtained on H and HD aortas showed an additional prominent peak of activity at pH 4.5, probably due to the lysosomal α-mannosidase. The presence of two enzymes with overlapping pH optima prevented unambiguous determinations of the activity of each. Acid cholesteryl esterase in H aortas showed linear activity and the same optimum conditions as were shown to be necessary for this enzyme in aortas from C and D groups. The incubation period used was 2 hours. In HD vessels, however, kinetics for this enzyme were not linear under these conditions. Therefore, this enzyme was not measured in the hypertensive-diabetic experiment.

Under the conditions described, all enzyme activities were linear with time and protein concentration. (See accompanying paper for details of criteria, units of activity, DNA, and protein determinations.)

Results

Characteristics of Animal Groups

For rats in the hypertension and hypertension-normotension experiments, final body weights, blood pressures, and DNA contents per pooled sample of three aortas are shown in Table 1. After both the 6- and 12-week experimental periods, body weights of hypertensive rats were
less than those of controls \( (P < 0.001) \), blood pressures and heart-body weight ratios were greater \( (P < 0.001) \), and aortic DNA contents were larger \( (P < 0.01) \). Hypertensive-normotensive rats achieved final body weights greater than sustained hypertensive rats \( (P < 0.01) \) but still less than controls \( (P < 0.02) \). Mean systolic blood pressure of hypertensive-normotensive rats did not differ from controls during the normotensive period \( (0.1 > P > 0.05) \). Heart weight-to-body weight ratios reflected these pressure changes (Table 1). Interestingly, despite the normotensive period in reversed rats, aortic DNA contents remained high and did not return toward normal.

For the hypertension-diabetes experiment, all the above measurements as well as blood glucose values of the four groups are shown in Table 2. The mean body weight differed significantly in each group \( (P < 0.001) \). Blood glucose tended to be higher in hypertensive groups than in corresponding normotensive groups, but not significantly so \( (P > 0.05) \).

Blood pressures of hypertensive and hypertensive-diabetic groups were similar \( (P > 0.2) \), as were those of control and diabetic groups \( (P > 0.05) \). Heart weight-body weight ratios reflected these pressures. That this ratio was greater in diabetic groups than in nondiabetic groups \( (P < 0.001 \text{ for C vs. D and P < 0.05 for H vs. HD}) \) may reflect loss of body fat in diabetics. DNA contents of aortic pools from hypertensive rats, but not from hypertensive-diabetic rats, were greater than normal \( (P < 0.02) \).

### Biochemical Findings

The kinetic behavior of all aortic enzymes studied from both hypertensive and hypertensive-diabetic groups is summarized in Figure 1 (refer to Figure 1 of the accompanying paper\(^\text{a}\) for similar data on control and diabetic animals). A linear relationship was found between substrate hydrolyzed and protein-time product\(^{16}\) for each enzyme studied. Differences between activities of aortas from H and HD groups for most enzymes were noted during kinetic studies.

### Hypertension and Hypertension-Normotension Experiments

Figure 2A shows the biochemical findings in pooled aortas of control and hypertensive groups at the 6-week point. Specific activity (calculated on DNA basis) of each hydrolytic enzyme studied was greater in hypertensive vessels than in controls, with the percent increase above controls ranging from 24\% for cathepsin C to 351\% for N-acetyl-β-glucosaminidase.

Figure 2B summarizes the second 6-week period of the experiment. Control and hypertensive aortas again showed differences for each enzyme studied with the percent increase above control ranging from 41\% for cathepsin C to 231\% for N-acetyl-β-glucosaminidase.

Following return of blood pressure to normal levels, the specific activity of each enzyme fell significantly below hypertensive levels measured after 6 or 12 weeks. Al-

### Table 1 Characteristics of Animal Groups

<table>
<thead>
<tr>
<th></th>
<th>6-Week experimental period</th>
<th>12-Week experimental period</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (18)</td>
<td>Hypertensive (18)</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>435 ± 35(^t)</td>
<td>360 ± 59</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>119 ± 9</td>
<td>197 ± 32</td>
</tr>
<tr>
<td>Heart weight-body weight (%)</td>
<td>0.31 ± 0.02</td>
<td>0.49 ± 0.10</td>
</tr>
<tr>
<td>Total DNA/3 aortas (μg)</td>
<td>138 ± 13</td>
<td>168 ± 16</td>
</tr>
</tbody>
</table>

\(^t\) Men ± SD.

* Numbers in parentheses = number of rats in each group. DNA measurements were made on pooled samples of three aortas each.

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### Table 2 Characteristics of Animal Groups

<table>
<thead>
<tr>
<th></th>
<th>Control (24)(^*)</th>
<th>Diabetic (24)</th>
<th>Hypertensive diabetic (24)</th>
<th>Hypertensive (24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>470 ± 48(^t)</td>
<td>331 ± 44</td>
<td>282 ± 59</td>
<td>407 ± 63</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>153 ± 14</td>
<td>543 ± 86</td>
<td>590 ± 102</td>
<td>162 ± 21</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>123 ± 9</td>
<td>128 ± 9</td>
<td>202 ± 31</td>
<td>191 ± 28</td>
</tr>
<tr>
<td>Heart weight-body weight (%)</td>
<td>0.32 ± 0.03</td>
<td>0.36 ± 0.03</td>
<td>0.49 ± 0.08</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td>DNA/4 aortas (μg)</td>
<td>216 ± 20</td>
<td>199 ± 23</td>
<td>197 ± 13</td>
<td>252 ± 21</td>
</tr>
</tbody>
</table>

\(^*\) Numbers in parentheses = number of rats in each group. DNA measurements were made on pooled samples of four aortas each.

\(^t\) Mean ± sd.
though four or six enzymes measured returned to levels no different from controls, interestingly, specific activities of cathepsin C and N-acetyl-β-glucosaminidase fell to levels significantly below controls (79% of controls for both). The reasons for this remain unknown.

Mixing experiments were carried out to test the possibility that the increased enzyme activities in homogenates of hypertensive aortas were due to an activator. Table 3 shows specific activities of N-acetyl-β-glucosaminidase after mixing different proportions of homogenates from control and hypertensive rats. Activities observed fell close to those calculated from the original homogenates.

Hypertension-Diabetes Experiment

After 8 weeks of hypertension alone or diabetes alone (Fig. 3) increases or decreases, respectively, in all enzymes were seen, which corresponded to our observations in the previous experiment (Fig. 2, A and B) and in the accompanying paper11 (P < 0.001 for every comparison of control vs. hypertensive or control vs. diabetic, except cathepsin C in the latter, where P < 0.05). In diabetics, levels of aortic enzymes compared to normals ranged from 55% for N-acetyl-β-glucosaminidase to 80% for acid α-glucosidase. In hypertensives, increases in all aortic enzymes above control values ranged from 70% for cathepsin C to 293% for N-acetyl-β-glucosaminidase.

The coexistence of hypertension and diabetes (HD, Fig. 3) resulted in significant depressions of four of the five enzymes measured, compared to levels with hypertension alone. In most cases, HD aortas had enzyme levels intermediate between controls and hypertensives and significantly different from both (all P < 0.001 except neutral α-glucosidase in HD vs. C, P < 0.05). However, in the case of cathepsin C, the combination of hypertension and diabetes resulted in levels no different from those of controls (0.4 > P > 0.3). Only acid α-glucosidase levels in HD vessels were not significantly different from those of H vessels (0.4 > P > 0.3), perhaps due to the large scatter of these values.

Morphological Findings

Grossly, the aortic wall from hypertensive rats was thicker than that of normotensive animals, but aortas from normotensive rats with preceding hypertension were more similar to controls. Cytomorphological results of incubation of aortic slices for acid phosphatase or N-acetyl-β-glucosaminidase showed the marked clustering of increased numbers of lysosomes previous described in the hypertrophic smooth muscle cells of hypertensive vessels (Figs. 4, A and B, compared to Fig. 1 in ref. 4). Lysosomes were clearly less numerous in aortas from hypertensive-normotensive rats (Fig. 4C) and were similar to control aortas.
FIGURE 2  A: Hydrolase activities in aortic homogenates of control (C) and hypertensive (H) vessels after the 6-week experimental period of the hypertension-normotension experiment are shown. Individual data points are presented, together with bars representing calculated means and standard deviations. Statistical significances of differences between groups are given at the base of each panel. B: Hydrolase activities in aortic homogenates from control (CC), hypertensive (HH), and reversed (HC) vessels after the 12-week experimental period of the hypertension-normotension experiment.
**Table 3** Enzyme Activity of Mixed Control and Hypertensive Homogenates

<table>
<thead>
<tr>
<th>Proportions*</th>
<th>Control Activity of</th>
<th>Predicted</th>
<th>Observed</th>
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<tbody>
<tr>
<td></td>
<td>N-acetyl-β-glucosaminidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mU/mg DNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.0</td>
<td>289.2</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.75</td>
<td>247.0</td>
<td>261.1</td>
</tr>
<tr>
<td>0.50</td>
<td>0.5</td>
<td>204.8</td>
<td>199.3</td>
</tr>
<tr>
<td>0.75</td>
<td>0.25</td>
<td>162.5</td>
<td>154.5</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>120.3</td>
<td></td>
</tr>
</tbody>
</table>

* Stated proportions of aortic homogenates from control and renal-clip hypertensive rats were mixed to a final volume of 0.1 ml and incubated with 0.1 ml of substrate. The activities of different mixtures were compared with those calculated from assays performed on original hypertensive and control homogenates. All observed activities were within the linear range of the assay.

Incubation of vessels from the four groups of rats of the hypertension-diabetes experiment showed a hierarchy of cytochemical activities which corresponded to the biochemical results (Fig. 5). Compared to controls (Fig. 5A), aortic lysosomal activities were depressed in the diabetic rats (Fig. 5B) and stimulated in hypertensive rats (Fig. 5D). Aortas from rats with the combination of hypertension and diabetes (Fig. 5C) had activities similar to those of the controls.

Electron microscopic examination of tissues incubated for acid phosphatase activity confirmed the localization of enzyme reaction product in lysosomes and supported light microscopic findings with respect to the numbers of reactive lysosomes. Other aspects of aortic morphology assessed ultrastructurally were consistent with previous observations. In diabetic aortas, no necrosis or other cellular abnormalities were seen. Muscle cells in hypertensive aortas were enriched in rough endoplasmic reticulum and Golgi structures, as well as lysosomes. Aortic cells of hypertensive-normotensive rats were smaller than cells of hypertensives and had many fewer of these organelles. Extracellular connective tissue matrix was very prominent in hypertensive, hypertensive-normotensive, and hypertensive-diabetic vessels. However, the latter two groups had fewer lysosomes than seen with hypertension alone. The most common lysosome was the dense body, often containing a clear droplet of lipid-like material.

**Discussion**

Hypertension results in marked stimulation of smooth muscle cell metabolism, including the lysosomal system. The increased vascular permeability which occurs in hypertension is thought to be a stimulus to the lysosomes, since it has been shown that intravenously injected tracers can be followed into the lysosomes of vascular smooth muscle cells. The present study shows that a broad array of hydrolases located in several differ-
FIGURE 4  Aortic lysosomes in the hypertension-normotension experiment after 12 weeks. Light microscopy of sections of rat thoracic aorta incubated 90 minutes for N-acetyl-β-glucosaminidase activity: (A) control, (B) hypertensive, and (C) reversed rats. Reactive lysosomes are markedly increased in the hypertensive aorta compared to the control. Large clusters of lysosomes are present in hypertensive smooth muscle cells. Reversed aortas (C) have fewer lysosomes and are comparable to control aortas (A). 650×.

FIGURE 5  Aortic lysosomes from rats in hypertension-diabetes experiment. Light microscopy of sections of thoracic aorta incubated for acid phosphatase activity for 30 minutes: (A) control, (B) diabetic, (C) hypertensive-diabetic, and (D) hypertensive. Reactive lysosomes (black dots) are fewer in the diabetic (B) and more numerous in the hypertensive (D) than in control (A) vessel. The hypertensive-diabetic vessel (C) has reaction product comparable to the control (A). 800×.
ent subcellular structures are markedly increased. Among
the several lysosomal enzymes studied here, the increase
in acid cholesteryl esterase is perhaps most interesting in
view of its important role in lipoprotein catabolism. 18, 20
Specific activity of this enzyme in hypertensive aortas was
increased approximately 65% above control values after
both 6 and 12 weeks of hypertension (Fig. 2). Increased
hydrolyase activities presumably reflect a cellular response
to increased influx of materials. In this heightened meta-
oblastic state, the hypertensive cell may be unusually vulner-
able to the introduction of other conditions which tend to
increase the net influx of a specific circulating material or
reduce the net hydrolytic capacity of the cell (see below).

The changes in hydrolyase levels seen here after blood
pressure was reduced to normal correspond well to pre-
vious physiological, 21 morphological, and biochemical 12
findings which showed marked regression of the smooth
muscle mass to near normal levels. The quick and dra-
matic responses of vascular cell metabolism to intravascula-
re pressure changes thus extends to subcellular levels of
hydrolytic enzymes. Indeed, it appears that several aspects
of metabolic activity or aortic cells, including their connect-
ive tissue synthesizing machinery, 17, 22 are quickly influ-
enced by changes in blood pressure acting on the vessel
wall. Despite the return of cellular hydrolyases to normal
levels per cell after a period of normotension, the number
of cells present remains at the increased level seen with
hypertension.

Most intriguing, perhaps, is our observation that the coex-
istence of hypertension and diabetes mellitus results in
significantly lower levels of most hydrolyases in the
vascular smooth muscle cell than are found with similar
degrees of hypertension alone. Thus, the depressing ef-
facts of diabetes mellitus on hydrolyase activities seen
under normotensive conditions 11 persist when another
stimulus, hypertension, simultaneously is influencing cel-
lar metabolism. These observations suggest that levels of
hydrolyase activities measured in this cell reflect a com-
plex interplay of hormonal, mechanical, and perhaps many
other influences present in the animal at that time.

Thus, the functional significance that can be attached to
the isolated finding of a "normal" level of a given hydro-
lytic enzyme remains unclear. This is evident in the
hypertensive-diabetic rats in which the level of cathepsin
C is "normal" (at the control level) despite the existence
of marked changes in two major determinants of that
level. Clearly, the situation here is hardly equivalent to
being nonhypertensive and nondiabetic. The implication
of this conclusion is that adequacy of a given hydrolytic
enzyme level for total clearance of incoming substrate
cannot be directly linked to the absolute cellular level of
that enzyme, be it "normal" or "abnormal." It is plausi-
ble, therefore, that accumulation of a substrate could
occur even if the measured level of the relevant hydrolytic
enzyme were increased above control levels.

Appreciation of this point is critical to proper testing of
an hypothesis advanced by deDuve 23 and Peters et al. 24
They proposed that atherosclerosis might reflect a relative
deficiency of acid cholesteryl esterase in vascular tissue.
Cholesterol ester accumulations, first within the lysosome,
then spilling out into the extracellular matrix, would
reflect a subtle but persistent inability by the vascular cell
to hydrolyze completely all the poorly permeant esters.
This hypothesis does not require the demonstration of
absolute decreases in levels of this enzyme, but a defi-
ciency relative to amounts of incoming substrate. There-
fore, the report of an increased level of this enzyme in
experimental atherosclerosis 26 does not rule out the pos-
sibility that it still is inadequate. Accumulation of increased
lipid, particularly cholesteryl ester, within the lysosome
may be more revealing about adequacy of the hydrolytic
enzyme than is the measured level of the enzyme alone.

The most reasonable conclusion from the studies de-
scribed here is that two important clinical risk factors have
clear-cut effects on vascular smooth muscle cell metabo-
lism, specifically on cellular hydrolyases. Among the fea-
tures of these risk factors which seem to be important are:
the rates of influx of circulating materials into the wall,
the concentrations of circulating lipoproteins (or other
components) in the serum, and the levels of hydrolytic
enzymes within the cell itself. Interactions of these deter-
minants could result in impaired clearance of incoming or
locally synthesized materials by the cell and combinations
of deleterious effects could exacerbate the process. Care-
ful dissection of each of these events and the mechanisms
involved is needed to ascertain the validity of these
formulations in the pathogenesis of atherosclerosis and
other vascular diseases.

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phology of calf aortic smooth muscle cells. Studies on cells from whole
Postnatal Changes in the Circulation and Responses to Volume Loading in Sheep

H. Sidney Klopfenstein and Abraham M. Rudolph

SUMMARY We examined postnatal circulatory changes in three groups of lambs at 1, 4, and 6 weeks after birth. Five lambs in each group were instrumented chronically with electromagnetic flow transducers on the ascending aorta and catheters in the aorta, left ventricle, left atrium, and superior vena cava. After recovery for 2 days, measurements were made daily at rest and during intravenous infusion of 0.9% NaCl solution (25 ml/kg per min) for 2 minutes into the superior vena cava to increase mean left atrial pressure to about 25 mm Hg. Resting heart rate fell progressively, from 210 ± 27/min (mean ± SD) at 1 week to 147 ± 28 at 6 weeks. The influence of the autonomic nervous system on cardiovascular function in the resting state was assessed by administration of selective parasympathetic and /3-adrenergic blocking agents. Also, the response of the circulation to volume loading by rapid infusion of saline was studied in the lambs at various ages.

THE CARDIOVASCULAR changes that occur immediately after birth have been studied extensively. These changes are concerned with adaptation from fetal to extrauterine life and include elimination of the umbilical-placental circulation, establishment of an adequate pulmonary blood flow and closure of the ductus arteriosus and foramen ovale. The subsequent changes associated with postnatal development have not been examined in detail. It is well known that the pulmonary vascular resistance continues to decrease for several weeks after birth and that pulmonary arterial pressure progressively falls. Cross et al.1 reported that cardiac output falls after birth in the lamb, but their studies were performed on anesthetized animals and anesthesia may have affected the circulation profoundly. Woods et al.2 reported a progressive fall in heart rate in the first few weeks and a rapid increase in systemic arterial pressure to adult levels immediately after birth in lambs. However, sequential changes in heart rate, systemic arterial pressure, and cardiac output have not been studied carefully. We have measured several cardiovascular variables in three groups of unanesthetized newborn lambs with mean ages of 1, 4, and 6 weeks. The influence of the autonomic nervous system on cardiovascular function in the resting state was assessed by administration of selective parasympathetic and /3-adrenergic blocking agents. Also, the response of the circulation to volume loading by rapid infusion of saline was studied in the lambs at various ages.
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