Functional and Structural Changes in the Rabbit Ear Artery after Sympathetic Denervation

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SUMMARY We studied the tissue weight, dimensions, contractility, elasticity, and sensitivity to exogenous norepinephrine (NE) of denervated and innervated segments of the central ear arteries of white New Zealand rabbits. Three different age groups received unilateral superior cervical ganglionectomy, "growing" at 3-4 weeks, "young adult" at 9-11 weeks, and "mature" at 16-20 weeks. In the growing group, 8 weeks after ganglionectomy, the denervated arteries showed mean decreases in tissue weight (11%), total wall thickness (12%), cross-sectional area of media (17%), contractility (16%), and increases in the tangential modulus of elasticity and sensitivity to NE (2.3-fold) compared to the contralateral control vessels. The change in medial cross-sectional area was significant in the growing and young adult but not the mature animals. The other changes, however, although consistently seen, differed quantitatively among the groups. These results indicate that an intact innervation is necessary for normal development and maintenance of the artery wall. However, the precise consequences of this influence vary at different ages. Whether this influence involves a special trophic factor is not known.


DENERVATION produces remarkable morphological and functional changes in skeletal muscle (Guth, 1968; Drachman, 1976). In several mammalian species studied, diversification of muscle fibers occurs during postnatal development and is partially dependent on the innervation (Gutmann, 1976). It also has been reported that the sympathetic nervous system exerts a trophic influence on skeletal and smooth muscle (Mendez et al., 1970; Luco and Luco, 1971). However, it is generally believed that denervation of smooth muscle produces little morphological change (Thoenen and Tranzer, 1963; Levi-Montalcini, 1972). More recently, several apparently conflicting observations have been made regarding morphological changes associated with denervation of smooth muscle, using different methods or species and tissues. Following sympathetic denervation of the growing rabbit ear artery, there was less 3H-thymidine uptake (DNA precursor) and there were fewer labeled smooth muscle cell nuclei in the tunica media than in the control artery, suggesting that sympathetic innervation influences proliferation of vascular smooth muscle (Bevan, 1975). In contrast, Chamley and Campbell (1976) observed that the presence of sympathetic nerve fibers or homogenates of sympathetic chain delayed by several days the normal dedifferentiation and proliferation of isolated medial smooth muscle cells from the aorta and ear artery of "young" rabbits in tissue cultures. In addition, Campbell et al. (1977) have shown that sympathetic denervation of chicken extensor secundarium longus muscle by a total brachial plexus lesion in the 2-week and adult chicken produced a significant increase in dry weight, due to hyperplasia of the smooth muscle cells.

In the present paper, effects of sympathetic denervation on contractility, dimensions, elasticity, weight, and sensitivity to exogenous norepinephrine were examined in the course of a study of sympathetic nervous influence on the blood vessel wall. A preliminary report has appeared elsewhere (Bevan and Tsuru, 1978).

Methods

Three groups of New Zealand white male rabbits were used: (1) growing rabbits of 3-4 weeks of age, (2) young adult rabbits of 9-11 weeks, and (3)
mature rabbits of 16–20 weeks. They were fed an unlimited standard pellet diet.

Denervation of the Central Artery of the Ear

After sedation with chlorpromazine (10 mg/kg, im), the rabbits were anesthetized by intraperitoneal injection of an anesthetic mixture containing per 100 ml: chloral hydrate, 4.25 g; pentobarbital sodium, 0.972 g; magnesium sulfate, 2.126 g; propylene glycol, 42.5% and alcohol, 11.5%. The dose used was 2 ml/kg. The central artery of the ear was denervated by removing the ipsilateral superior cervical ganglion under sterile conditions (de la Lande and Rand, 1965).

Tissue Preparation

Eight weeks after denervation, animals were stunned by a blow on the head and rapidly exsanguinated. The mature rabbits were killed 10 weeks following surgery. Both the denervated artery and contralateral control ear arteries were cut in situ by use of three parallel blades held at a fixed standard distance to obtain two segments of equivalent lengths (3.0 mm) from each vessel. In addition, segments were removed from both the proximal and distal ends of artery adjacent to the measured segments for the study of catecholamine histofluorescence and wall dimensions.

The preparations were submerged in Krebs bicarbonate solution [composition in mM: NaCl, 117; KCl, 4.9; CaCl₂, 1.6; MgSO₄, 1.2; NaHCO₃, 25; glucose, 11; calcium disodium ethylenediaminetetraacetic acid (EDTA), 0.025; and acetic acid, 0.11] equilibrated with 95% O₂ and 5% CO₂, and dissection was continued under a microscope to obtain clean segments.

Catecholamine Histofluorescence

Whole mounts and transverse sections of the adjacent proximal and distal segments from both denervated and control ear arteries were used to study catecholamine histofluorescence. Either the fluorescence histochemical formaldehyde method of Falck et al. (1962) or the glyoxylic acid method of Lindvall and Bjorklund (1974) was used.

Tissue Weight

Artery segments were blotted on filter paper and weighed on a Mettler balance (H54) as quickly as possible prior to the in vitro studies. We compared the tissue weights of 3-mm lengths, of the control artery segments. These were derived from initial pilot active tension length studies. After equilibration for 1 hour, the effectiveness of denervation was demonstrated by the absence of tetrodotoxin (TXX)-sensitive contractile response to transmural nerve stimulation (TNS) using biphasic pulses 0.2 msec in duration and supramaximal voltage at 8 Hz from Grass stimulator model S4.

After pretreatment with desmethylimipramine (DMI, 3 X 10⁻⁷ M), propranolol (3 X 10⁻⁷ M), and hydrocortisone (8.7 X 10⁻⁶ M) for 30 minutes to eliminate factors influencing β-adrenoceptor sensitivity, neuronal and extraneuronal uptake, and β-adrenoceptor response (Furchgott, 1972), norepinephrine was added cumulatively to the bath until the maximum response was obtained.

At the end of each experiment, the maximum contraction was obtained using L-norepinephrine (10⁻⁴ M), followed by histamine (10⁻³ M). Alternatively, Krebs solution was replaced with a depolar-
The elasticity of the vessel was determined from the length-stress relationship. Length-stress curves were obtained for 3.0-mm ring segments of ear artery, set up as described above. Tissues were equilibrated for 1 hour without load. The segments were stretched in steps of 50 μm every 5-10 minutes by turning a fine screw adjustment attached to the force displacement transducer. The distance between the outer edges of the wires suspending the segment was determined at each equilibrium level, through the tissue bath wall, using a microscope with 10× micrometer eyepiece. This was regarded as half internal circumference. Wall stress was expressed as passive stress (dynes/cross-sectional area in cm²), for each experimental length.

Measurement of Elasticity

The following drugs were used: L-norepinephrine bitartrate (l-Arterenol Bitartrate, Sigma Chemical Co.), desmethylimipramine HCl (DMI: USV Pharmaceutical Co.), propranolol HCl (Ayerst Laboratories), hydrocortisone sodium succinate (Solu-Cortef, Upjohn), lidocaine HCl (Xylocaine, Astra Pharmaceutical Products, Inc.), histamine dihydrochloride (Calbiochem), tetrodotoxin (TTX; Sankyo-Calbiochem), and NaN0₂ (Mallinckrodt).

Statistical Analysis

Paired observations on the control and denervated ear arteries in each age group were compared using the paired t-test. Differences between the different age groups were analyzed by means of the unpaired t-test (Dixon and Massey, 1957). An inference of statistical significance was made when \( P < 0.05 \).

Results

The mean body weights of growing, young adult, and mature rabbits at the time of operation were: (1) 410 ± 45 g (8) [mean ± se, \( n = 8 \)], (2) 2.30 ± 0.19 kg (8), and (3) 3.62 ± 0.40 kg (5), respectively. At the time of final study mean body weights of each group were: (1) 2.53 ± 0.14 kg (8), (2) 3.24 ± 0.33 kg (8), and (3) 3.81 ± 0.42 kg (5), respectively.

Denervation of one ear artery was confirmed in all unilaterally sympathectomized rabbits by the absence of catecholamine fluorescence at the adventitial-medial junction (Bevan, 1975). The innervation pattern in the control ear artery was similar in brightness and distribution in all three age groups.

Tissue Weight and Dimensions

When the data from individual rabbits were paired for comparison in each age group, tissue weight and total wall thickness were significantly reduced in the denervated as compared with the contralateral control vessel. The results are summarized in Table 1.

The only significant difference between the weight and wall thickness of innervated vessel segments among age groups was in the wall thickness of the young adult vessel, which was significantly greater than that of the mature group (\( P < 0.05 \)). Weights of the denervated vessel segments expressed as a percent of control were in the growing rabbit 89.05 ± 2.54% (8) [mean ± se, \( n = 8 \)] in the young adult rabbit, 87.47 ± 0.03% (8), and in the mature rabbit 84.08 ± 2.58% (5) (see Figs. 1 and 2). The total wall thickness of the denervated vessels expressed in the same way was in the growing rabbit 88.23 ± 2.27% (8), in the young-adult rabbit, 91.49 ± 2.70% (8), and in the mature rabbit 93.37 ± 2.09% (5). There was no significant difference in the percentage of decrease of tissue weight and total wall thickness of the denervated compared with the innervated vessels among the three groups.

Denervation was associated with changes in cross-sectional area of the media. Expressed as a percentage of control ear arteries, cross-sectional area in the growing rabbits was 72.8 ± 5.48 (8), in the young adult group 86.63 ± 4.14 (14), and in the mature group 95.1 ± 5.38 (8), respectively (see Figs. 1 and 2). Thus, effects of denervation on the amount of vascular smooth muscle in the vessel wall diminished with age. In the mature animal, there was no significant decrease in cross-sectional area of the media with denervation.

Cross-Sectional Area of Media

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Responses to Transmural Electrical Stimulation

The effectiveness of denervation, demonstrated by the absence of catecholamine histofluorescence, was confirmed by eliciting contractile responses to electrical field stimulation. Stimulation frequency-response curves of vessels from the young adult group are shown in Figure 3. Only at frequencies greater than 2 Hz were consistent, but small, responses observed in the denervated vessel. The contractile response of the denervated vessel at a stimulation frequency of 16 Hz was 10.9 ± 3.5% (8).
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TABLE 1 Effets of Denervation on the Ear Artery Weight, Wall Thickness, Maximum Force Development, Maximum Stress Development, Cross-sectional Area of Media, Norepinephrine ED₅₀, and Tangential Modulus of Elasticity in the Growing, Young Adult, and Mature Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Growing rabbits (n = 8)</th>
<th>Young adult rabbits (n = 8)</th>
<th>Mature rabbits (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Denervated</td>
<td>Control</td>
</tr>
<tr>
<td>Tissue weight (mg)</td>
<td>1.76±0.06</td>
<td>1.57±0.06†</td>
<td>2.13±0.22</td>
</tr>
<tr>
<td>Total wall thickness (µm)</td>
<td>212±8.8</td>
<td>188±11.2</td>
<td>225±10.2</td>
</tr>
<tr>
<td>Cross-sectional area of media (mm²)</td>
<td>0.1965±0.0150</td>
<td>0.1725±0.0075§</td>
<td>0.1940±0.0077</td>
</tr>
<tr>
<td>Maximum force development (g)</td>
<td>2.25±0.32</td>
<td>1.90±0.34*</td>
<td>2.56±0.16</td>
</tr>
<tr>
<td>Maximum stress development × 10⁵ (dynes/cm²)</td>
<td>1.74±0.08</td>
<td>1.68±0.16</td>
<td>1.96±0.22</td>
</tr>
<tr>
<td>Norepinephrine ED₅₀ × 10⁻⁸ m</td>
<td>2.69±0.41</td>
<td>1.15±0.309§</td>
<td>6.57±1.74</td>
</tr>
<tr>
<td>Tangential modulus of elasticity × 10⁹ (dynes/cm²)</td>
<td>5.28±0.28</td>
<td>10.96±0.95§</td>
<td>5.35±0.14</td>
</tr>
</tbody>
</table>

Values represent mean ± SE. Growing, young adult, and mature rabbits were denervated at age 4 weeks, 9–11 weeks and 16–20 weeks respectively, and studied 8 weeks later. Statistical analyses were made by using the paired t-test between control and denervated ear artery in each group. A statistical inference of significance was made when P < 0.05; §P < 0.01. Values of tissue weights are of 1 segment of 30 mm in situ length.

that of the control (Fig. 3). This was not affected by tetrodotoxin (TTX) 10⁻⁷ g/ml, a treatment that reduced the control response by a mean of 95.0 ± 3.1% (4) at 16 Hz. The patterns of the tetrodotoxin-resistant responses of the denervated vessels in all three age groups were similar.

Sensitivity to Exogenous Norepinephrine

Norepinephrine (NE) concentration-response curves of innervated and denervated vessel rings from the young adult group in the presence of propranolol (3 × 10⁻⁷ M), desmethylimipramine (3 × 10⁻⁷ M), and hydrocortisone (8.7 × 10⁻⁷ M) are shown in Figure 4. The denervated vessel was 2.4-fold more sensitive to NE than the control. The denervated vessels of the growing and mature groups also showed an increased sensitivity to NE of 2.3 and 1.6-fold, respectively (Table 1 and Fig. 1).

Maximum Contractile Response

The contractile response of the ring segments induced by NE (10⁻⁴ M), followed by histamine (10⁻⁵ M) or depolarizing K⁺ solution was considered maximum. In a few instances, the contractile force initiated by NE (10⁻⁴ M) was increased by 2 to 3% by the addition of histamine (10⁻⁵ M). The response to the depolarizing K⁺ solution was never more than that to NE (10⁻⁴ M).

Although there was no significant difference in the maximum force developed among the innervated vessels of the three groups, denervation resulted in decreased maximum force development at all ages (Table 1). The maximum force development of the denervated vessels expressed as a percentage of control was: in the growing rabbit, 83.9 ± 5.34% (8); in the young adult rabbit, 70.3 ± 7.3% (8); and in the mature rabbit, 85.7 ± 1.84% (5).

Differences between the control and the denervated vessels became less marked (Table 1) when corrected for cross-sectional area so that the maximum stress rather than the maximum force developed was compared. Although this parameter was reduced significantly in the mature young adult rabbits, this was not true of the growing group.

Length-Stress Relationship

As illustrated in Figure 5 for the young adult group the length-stress curves of the denervated vessels of all three groups were shifted to the left of control. From each length-stress curve, tangential moduli of elasticity were calculated at the circumference corresponding to a blood pressure of 80 mm Hg in vivo calculated from the Laplace relationship. Values from innervated vessels had a narrow range. However, this parameter in the denervated vessel was significantly increased in comparison to control, indicating that the vessel wall was stiffer, i.e., less distensible.

Discussion

These results indicate that long-term denervation produces structural and functional changes in the central ear artery of growing, young adult and mature rabbits. Some significant differences were apparent when a comparison of the observed changes in the various parameters among three different age groups was made. Some of the changes are found only after denervation in the growing animal, whereas others are seen consistently at all ages, although differing quantitatively among the age groups.

Nonspecific postjunctional denervation supersensitivity of smooth muscle to agonists has been recognized for many years (Trendelenburg, 1963).
In our studies, the denervated ear artery showed an increased sensitivity to exogenous NE of 1.6- to 2.4-fold, a value which is in agreement with previous observations (Fleming et al., 1973) and of similar order of magnitude to that found in the rat portal vein following 6-hydroxy dopamine administration (Aprigliano and Hermans, 1977). The NE ED₅₀ of both innervated and denervated vessels increased with age to maturity. In the present experiments, drug sensitivity was determined in the presence of propranolol, desmethylimipramine, and hydrocortisone to eliminate consequences of β-adrenoceptor activation, and neuronal and extraneuronal uptake, which have been shown to influence the concentration of NE at α-adrenoceptors (Furchgott, 1972). Other evidence of the increased sensitivity of the denervated vessel to agonists is the increased magnitude of its contractile response to transmural nerve stimulation in comparison to an innervated vessel in the presence of tetrodotoxin (10⁻⁷ g/ml). It has been well documented that TTX blocks exclusively the nerve-induced contractile response (Bevan and Su, 1975). Thus, the direct muscle response was increased after denervation. In addition, the denervated ear artery was more sensitive to K⁺-induced depolarization than the control (unpublished observation). This phenomenon is consistent with the conclusions of Fleming et al. (1973) for the guinea pig vas deferens, and Aprigliano and Hermans, (1977) for the chemically denervated
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Figure 4 Cumulative norepinephrine concentration-response curves of the control and denervated ear artery segments from young adult rabbits. Experiments were done in the presence of propranolol (3 × 10⁻⁷ M), desmethylunipramine (3 × 10⁻⁷ M), and hydrocortisone (8.7 × 10⁻⁶ M). Vertical bars refer to standard error of mean: (n = 8). ** P > 0.005. * P > 0.01.

Figure 5 Passive length-stress curves of paired innervated and denervated ear artery segments from young adult rabbits. Vertical bars refer to standard error of mean: (n = 8). * P > 0.01. ** P > 0.05.

rat portal vein, that the denervation-induced partial depolarization of vascular smooth muscle cells contributes to their hypersensitivity (Fleming, 1976).

Among the three different age groups, with one exception, there was no significant difference in tissue weight and total wall thickness of the control vessels—although there was a tendency for these measurements to be greatest in the young adult group. Denervated vessel segments in comparison to their innervated controls showed mean decreases in weight of 11-16%, and in wall thickness of 7-12% (Table 1). As would be expected, in general, the changes in the weight and total wall thickness were comparable.

After chronic sympathectomy, the reduction in cross-sectional area of the media is age related, being greatest in the youngest group but not significantly changed in the mature animal. Replication of vascular smooth muscle is marked in blood vessels during the early postnatal period, decreases with increasing age, and in the normal mature animal is infrequently seen (R.D. Bevan, unpublished observations). Consequently, the greatest effect of denervation on wall thickness would be expected in the youngest group of animals provided the vascular smooth muscle cell, once formed, is either retained in the wall or else has a very slow turnover rate. Our results are also compatible with previous observations that vascular smooth muscle proliferation was less in the 2-week denervated rabbit ear artery at 6 weeks of age (Bevan, 1975). It is possible, however, that the apparent differences in cross-sectional area of the media, determined in vitro on a histological section, reflect a change in wall structural components which results in an alteration in the ability of the segment to retract after removal from the body. However, the data do indicate that, in all age groups, a structural change has occurred as identical in situ lengths of vessels weighed less after denervation and the change in total wall thickness was, in general, proportional to the change in tissue weight. Although there was no significant difference between innervated and denervated arteries in the internal diameter of the vessel in the young adult group, this parameter was not measured in the growing group where the greatest change in structure occurred.

Alterations in activity of the sympathetic nervous system have been shown to influence cell proliferation in other tissues, the rat parotid gland (Schneyer, 1974), and the epithelium of the small intestine (Tutton, 1975). Electrical nerve stimulation increased cell size and number in the adult parotid gland (Muir et al. 1975), and the mitotic rate of the jejunal epithelial cells (Tutton, 1975). In contrast, chemical and surgical sympathectomy resulted in prolongation of the jejunal cell cycle time (Tutton, 1975) and a decrease in the number of mitotic cells (Klein and Torres, 1978).

Chamley and Campbell (1976), observed that a diffusible substance from sympathetic nerves delayed the onset of modification accompanied by proliferation of cultures of smooth muscle cells isolated from “young” rabbit aorta and ear artery. However, the processing of the tissues with trypsin and collagenase to obtain isolated cells might well have changed the membrane characteristics so that, at this stage, they are not comparable to cells in situ. These substances have been shown to stimulate cell division in fibroblast cultures (Blumberg and Robbs, 1975). The sympathetic ganglia homogenate does appear to contain a factor enabling a partial “repair” process to occur in the smooth muscle cells, thus delaying the onset of dedifferentiation. The basis of differences in response to denervation of vascular smooth muscle in situ and in culture, and also smooth muscle in other tissues, remains to be elucidated.
Maximum force development was decreased in the denervated ear artery in comparison to its control (Table 1 and Fig. 1). When the force development had been converted to stress development by correction for differences in cross-sectional area, the differences between the control and the denervated vessels became less and, in the growing rabbit, were not significant. In the growing rabbit, it seems likely that the dimensional and contractility changes result predominantly from the loss of vascular smooth muscle mass. However, the diminution in contractility cannot be accounted for by loss of tissue mass in the young adult and in the mature groups (Table 1 and Fig. 1). Thus, a qualitative change in the contractile machinery may be involved in the loss of ability to develop tension after denervation of these groups.

The functional and structural changes in the artery wall after denervation could be attributed to loss of a "trophic" factor or to the absence of sympathetic drive. If the age-related effect of denervation on medial thickness could be extrapolated to other blood vessels, the level of sympathetic drive during growth might be a significant factor in determination of blood pressure. This could have both a genetic and environmental component.

In a genetic model of hypertension, the spontaneously hypertensive rat, blood pressure elevation occurs mainly between 4 and 12 weeks of age, a period of growth. There is evidence that transmission in a sympathetic ganglion from normal rodents is not functionally mature before 3-4 weeks of age (Black et al., 1971), although this may have regional variations. In this animal model there is evidence of increased peripheral sympathetic nerve activity during growth (Judy et al., 1976; Nagatsu et al., 1976) and, in the 6-week and adult animal, an increased thickness of the medial smooth muscle layer has been found in resistance vessels (Mulvany and Halpern, 1977; Warshaw et al., 1979). Although many factors are known to induce proliferation in vascular smooth muscle, it is interesting to speculate that it may be influenced by increased nerve activity. Because vascular resistance is proportional to the 4th power of the internal radius, even a small change in medial thickness would have a pronounced effect on blood pressure.

The length-stress curves of the denervated vessels were shifted to the left in comparison to their controls in all three groups. As seen in Table 1, it is of interest that the values of the tangential modulus of elasticity of the denervated vessels are inversely proportional to age, although the control values are similar. The shape of the length-stress curve of an artery is complex, reflecting the relative importance of the different structural components of the wall to stretch at different tissue lengths. The observed increase in elastic modulus probably is due to a relative increase in extracellular material. The differences in the tangential moduli of elasticity of the denervated vessels among the three different ages may reflect the relatively greater loss of vascular muscle at younger compared to older age groups combined with different changes in extracellular components.

In conclusion, our data suggest that loss of sympathetic innervation may result in structural as well as functional alterations in an artery. It is known that the presynaptic innervation to the superior cervical ganglion is not mature until 3-4 weeks of age in the mouse (Black et al., 1971), which has a temporal pattern of postnatal growth similar to that of the rabbit. The ability of the sympathetic innervation to transmit impulses of high frequency is not mature until 4-6 postnatal weeks in the rabbit (Schweiler et al., 1970). It is likely that neuronal activity would be more effective in influencing the vascular wall only after the neuronal influence has functionally matured. The sympathetic innervation may either specifically influence cell replication or may modulate metabolic processes in the vascular smooth muscle cell. Age-related differences in the various parameters investigated following denervation may reflect a change in the synthetic capacity and role of the vascular smooth muscle cell during maturation of the arterial wall.

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