Guinea pig ductus arteriosus. II. Irreversible closure after birth

FREDRIC S. FAY AND P. H. COOKE

Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115, and The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02139

The ductus arteriosus from postnatal guinea pigs was perfused in vitro through the thoracic aorta and changes in flow conductance were measured as a function of oxygen pressure. Within 24–72 hr after birth, ducts no longer dilate in response to hypoxia and remain “irreversibly” closed thereafter. Parallel experiments were carried out in which changes in circumferential muscle tension induced by oxygen, acetylcholine, and K+ were measured. Irreversibility of ductal closure does not reflect loss of oxygen sensitivity of ductal smooth muscle since it persists for up to 4 days postpartum. Microscopy revealed major changes in the inner part of the vascular wall unique to irreversibly closed ducts, involving necrosis and loss of an intact endothelium. It is proposed that irreversibility reflects a mechanical restraint imposed on the ductus by these cellular changes in the central region of the vessel. Furthermore, localization of necrosis to the inner wall suggested that cell death was triggered by interruption of luminal blood flow. Support for both hypotheses was obtained from observations on ducts from neonates raised under hypoxic conditions. In these ducts, which remained patent for 3–4 days after birth, necrosis and cellular rearrangement was not observed during the first few days after birth, and reversible responses to changes in oxygen pressure were obtained at 80 hr postpartum.
FIG. 1. A: perfusion apparatus and organ bath. Fluid reservoirs and organ bath are constructed of Lucite. Stopcocks allow for rapid rinse of organ bath with solution from either reservoir equilibrated with 95% O2-5% CO2 or 95% N2-5% CO2. Perfusion apparatus consists of 10 ft of silicone rubber tubing (.020 inch x .037 inch) inside a polyethylene tube (.085 inch x .128 inch); perfusate is brought to temperature in a 6-inch length of 18-gauge stainless steel tubing connecting silicone tubing to preparation. B: tension-measuring apparatus. Ring of ductus arteriosus is slipped over 2 stainless steel pins (27 gauge stainless steel tubing) and placed into organ bath shown in A. Resting tension under anaerobic conditions is adjusted to about 0.5 g with micrometer; tension is measured isometrically with FT 03 force transducer (Grass Instruments Co; nominal displacement 0.02 mm/g).

and outer tubes (Fig. 1A). Complete equilibration between liquid and gas phases across the silicone rubber membrane was verified with an oxygen electrode (Instrumentation Laboratory, Inc.). Perfusion fluid was driven through the central tube by a fixed pressure head between 100 and 140 mm Hg. Thermal equilibration of the perfusate was accomplished in the stainless steel tube leading to the ductus preparation (Fig. 1A). As seen in Fig. 1A, an oxygen electrode in the organ bath monitored Po2. The perfusion fluid was Krebs-Ringer-bicarbonate saline containing 1 μg/ml streptomycin sulfate (Squibb) and 250 U/ml potassium penicillin G (Pfizer). The outputs from the pressure transducer, Po2 electrode, and force transducer (FT 03, Grass Instrument Company) (Fig. 1, A and B) were recorded on a polygraph (Grass).

The perfusion pressure at the aorta was used as an indicator of ductal flow conductance. A high perfusion pressure at the aorta was indicative of low flow conductance within the ductus. Ductal flow conductance was calculated explicitly using the expression \( P_1 \times R_1 / P_2 \) where \( P_1 \) = the measured pressure difference between the fluid reservoir and aorta, \( P_2 \) = the measured pressure difference between the aorta and organ bath, \( R_1 \) = the flow resistance of the perfusion apparatus as measured separately (7.4 \( \times 10^5 \) mm Hg·min·ml\(^{-1}\)). This expression is based on Ohm's law for two resistors in series. In this manner, 37 ducts obtained from animals ranging in age from 0 to 102 hr were studied.

After establishing if a ductus preparation exhibited Po2-dependent conductance changes, the ductus was cut from the aorta and incubated for 10 min in Krebs-Ringer solution equilibrated with 95% N2-5% CO2 to allow it to dilate. Two stainless steel pins were then inserted through its lumen. Since ducts from animals older than 3 days did not dilate at all in response to low Po2, the stainless steel pins had to be forced through the luminal “area” of these vessels. The ductus was then mounted as shown in Fig. 1B and changes in circumferential muscle tension were measured isometrically in response to changes in Po2, acetylcholine (Sigma), and substitution for normal Krebs-Ringer-bicarbonate saline of an identical solution save for the molar replacement of all Na+ by K+. Maximal responses to oxygen and acetylcholine were obtained by using supramaximal levels of each agent (20 μg/ml acetylcholine, 680 mm Hg Po2) after determination of its dose-effect curve on a separate series of preparations.

Specimens for light and electron microscopes. Ducts were fixed: 1) in situ, by topical application for 10–15 min of 3% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.3); 2) in vitro, by injection slowly of 10 ml of 25% glutaraldehyde into the vigorously aerated organ bath to yield a final glutaraldehyde concentration of 3% in the Krebs-Ringer-bicarbonate saline (pH 7.4). The ductus specimens were then removed and further fixed for 2 hr in 3% glutaraldehyde in the same buffer used in the initial fixation. They were then postfixed in 1% osmic acid in phosphate buffer (pH 7.3), dehydrated in ethanol, and embedded in Epon. No differences in the appearance of cells fixed by the two methods were noted in the light or electron microscope. Cross sections, 1-μ thick, of the ductus were cut along the entire length of 16 ductus preparations whose
response to oxygen and nitrogen had been monitored during perfusion in the organ bath. Sections were cut from the aortic to the pulmonary end of the ductus, i.e., in the same direction as perfusate flow. Several consecutive sections at 0.1-mm intervals along the duct were stained according to the method of Richardson et al. (24) and examined under the light microscope. Electron-microscope observations were also made on 33 preparations. Thin sections were stained with 2% uranyl acetate and lead citrate (23).

**IRREVERSIBLE CLOSURE OF DUCTUS ARTERIOSUS**

**RESULTS**

Reversibility of ductal closure as a function of age. Table 1 lists the average flow conductance of 37 perfused ducts obtained from animals from 0 to 96 hr postpartum. In the presence of oxygen all preparations exhibited conductances close to zero, indicating complete obstruction of the lumen. Under anaerobic conditions, however, ducts from newborn animals (0-6 hr) developed high flow conductances, indicating that they were widely dilated. The ability to increase flow conductance in response to anaerobic conditions declined progressively with age and by 36 hr after birth flow conductance under anaerobic and aerobic conditions were statistically indistinguishable.

The decline in mean conductance in response to anaerobic conditions in the first few days after birth was due to: 1) increased frequency of preparations that failed to dilate at all (no conductance change), and 2) lesser magnitude of conductance change in those preparations that did respond. The frequency with which irreversibly closed ducts (no conductance change in response to anaerobiosis) were encountered at different ages is shown in Fig. 2. All preparations obtained from animals 72 hr or older were irreversibly closed.

**Effect of age on oxygen sensitivity of smooth muscle of ductus arteriosus**. The maximum increment in tension of the muscle in the wall of the ductus in response to increasing PO2 from 0 to 680 mm Hg decreased from 2.96 ± 0.44 g (mean ± se) in ducts from newborn animals to 0.98 ± 0.26 g (mean ± se) in ducts from animals 3- to 4-days postpartum. To determine if the decrease in response to oxygen with age represents a failure of the oxygen-sensing mechanism or merely a loss of the contractile capacity of the ductal smooth muscle, the response to oxygen was compared to the response to acetylcholine over the same time period. The maximum contractile response of the ductus to acetylcholine also decreased, from 1.32 ± 0.34 g in ducts from newborns to 0.36 ± 0.10 g in ducts from animals 3-4 days of age. As can be seen in Fig. 3, the ratio of the maximum contractile response to oxygen and acetylcholine remained constant during the first 4 days after birth. The ratio averaged 2.56 ± 0.29 (SEM) in ducts from newborns and 2.47 ± 0.28 in ducts from animals 0-6 hr.

**TABLE 1. Effect of age on flow conductance of perfused ductus arteriosus under anaerobic and aerobic conditions**

<table>
<thead>
<tr>
<th>Age, hr</th>
<th>No. of Exps</th>
<th>Flow Conductance × 10⁻³ ml min⁻¹ mm Hg⁻¹</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N₂</td>
<td>O₂</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>16.47 ± 4.65</td>
<td>.14 ± .11</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>5.80 ± 1.84</td>
<td>.05 ± .01</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>3.10 ± 1.07</td>
<td>.03 ± .04</td>
</tr>
<tr>
<td>36</td>
<td>5</td>
<td>.72 ± .41</td>
<td>.21 ± .11</td>
</tr>
<tr>
<td>48</td>
<td>6</td>
<td>.30 ± .16</td>
<td>.20 ± .20</td>
</tr>
<tr>
<td>72</td>
<td>4</td>
<td>.04 ± .02</td>
<td>.05 ± .02</td>
</tr>
<tr>
<td>96</td>
<td>5</td>
<td>.04 ± .02</td>
<td>.04 ± .02</td>
</tr>
</tbody>
</table>

Flow conductance values are means ± se.

**FIG. 2. Effect of postnatal age on percentage of perfused ductus arteriosus preparations which were irreversibly closed.** Ducts were considered irreversibly closed if under aerobic and anaerobic conditions flow conductance remained unchanged and close to zero (<2 × 10⁻⁴ ml min⁻¹ mm Hg⁻¹). Flow conductance was calculated using pressures at 2 points in perfusion system as described in Methods. Number of animals shown in parentheses. Animals were gathered into 12-hr age groups (±6 hr) for graphic representation.

**FIG. 3. Effect of postnatal age on ratio of contractile responses of ductus arteriosus to O₂ and acetylcholine.** Ratio was calculated using maximum tension increment of ductus rings, measured as shown in Fig. 1B, in response to increasing PO2 from 0 to 680 mm Hg and in response to 20 μg/ml acetylcholine under anaerobic conditions. This ratio averaged 2.56 ± 0.29 (SEM) and 2.47 ± 0.28 (SEM) in ducts from newborn and 3- to 4-day-old animals, respectively.
Effects of age on morphological characteristics of ductus arteriosus. A perfusion-pressure record for a ductus obtained from a newborn guinea pig, shown in Fig. 4A, demonstrates the ability of the vessel to dilate in response to anoxia and to constrict so that flow is blocked in the presence of oxygen. Figure 5 shows a cross section of the same ductus near the middle of its length after fixation under anaerobic conditions. The lumen of the vessel is patent. The vascular wall is lined with a typical endothelium. The major part of the wall is a muscular coat which merges gradually at the periphery into the loose connective tissue of the adventitia.

The cross-sectional profile of the vessel shown in Fig. 5 is representative of all sections cut along the length of ducts from all newborn guinea pigs studied.

Physiological and morphological features of the ductus of an animal 19 hr of age are shown in Figs. 4B and 6. Physiologically, this ductus is little changed from that of a newborn guinea pig; the ductus still dilated in response to anoxia, although the increase in flow conductance was not as great as seen in the newborn. Morphologically, the vessel shows no significant changes (Fig. 6) except that the lumen is somewhat narrower and the subendothelial region and muscularis are thicker compared to ducts from newborn animals (Fig. 5).

None of the ducts taken from animals during the 1st day of life were irreversibly closed (see Fig. 2). Several ducts studied on the 2nd postnatal day, however, were no longer able to dilate in response to anoxia. Figure 4C shows a perfusion record obtained for an irreversibly closed ductus from a guinea pig 45 hr postpartum. When cross sections were cut along this vessel proceeding in the direction of perfusion, i.e., from the aortic end of the ductus, the lumen was found to be patent for approximately 1 mm, and then became occluded. A section through the vessel where the lumen is occluded is shown in Fig. 7. A distinct endothelial layer is no longer present and what was once the area of the lumen is now filled with a mass of pleomorphic cells with large nuclei (Fig. 8). Some cells have vacuolated cytoplasm and pycnotic nuclei. Myelin figures are abundant in the cytoplasm of the cells and in the extracellular spaces. By contrast to this irreversibly closed duct, vessels that were not irreversibly closed but fixed under aerobic conditions so that the lumina were occluded, contain a typical endothelium (Figs. 9 A and B). Four irreversibly closed ducts from animals in the 2nd postnatal day were sectioned serially from aortic to pulmonary end. Each one contained an area where the lumen was occluded and replaced by a loosely organized mass of cells similar to that described above.

By the 4th day after birth, ducts from all animals studied were irreversibly closed. Figure 4D shows a typical perfusion record for a duct obtained from an animal 93 hr old indicating constant and low flow conductance under aerobic and anaerobic conditions. The lumen of this vessel was occluded over most of its length. Cross sections (Fig. 10) taken near the aortic and pulmonary ends of the occluded portion of the ductus were similar to those shown in Figs. 7 and 8 for the irreversibly closed duct of a 2-day-old animal. There is
no endothelium and numerous cells containing pycnotic nuclei are present in the central area of the vessel. Necrosis appears to be limited to the central region and innermost layers of the muscularis. Cross sections taken midway along the occluded length of the ductus from this 4-day-old animal (Fig. 11) also revealed the absence of an endothelium and the presence of numerous necrotic cells in the central area. By contrast to sections (Fig. 10) obtained near the ends of the occluded region, however, the cells in the central area of Fig. 11 are more loosely arranged, cell fragments are more numerous, and a number of cells involved in phagocytosis are found (Fig. 12).

Loss of a distinct endothelium and the presence of a central mass of cells showing varying degrees of necrosis were characteristic features of all 12 irreversibly closed ducts from neonates between 24 and 96 hr postpartum which were systematically studied. The results suggested that irreversible closure might be causally related to the presence of this central mass of cells and cellular debris. In order to test this possibility, an attempt was made to prevent or delay these transitions within the inner wall of the ductus. If irreversibility was directly related to the presence of this central plug, then delay in its formation would also be expected to delay the onset of irreversibility of closure.

Postnatal guinea pigs at low Po2. When newborn guinea pigs were reared under hypoxic conditions the ductus remained patent for 3-4 days after birth as judged by histological observations on ducts fixed in situ. Furthermore, the endothelium remained intact over the first 3-4 days after birth and cellular necrosis did not occur (Fig. 13). A typical record of flow conductance for one of these ducts is shown in Fig. 4E. Even though obtained from an animal 79 hr of age, this ductus was fully capable of reversible closure and dilation in response to oxygen and anoxia, respectively. Similar changes in flow conductance were also observed in a littermate after 80 hr under hypoxic conditions. These
reversible changes in flow conductance are to be compared with the behavior observed in normal animals where all ducts obtained after 72 hr were irreversibly closed (see Fig. 3).

DISCUSSION

The time course for irreversibility of ductal closure found in the present study agrees well with that observed by Gilman and Burton (7) who also reported the loss of a dilatory effect of hypoxia 72 hr postpartum in the guinea pig. Angiographic studies on newborn children (21) and swine (25, 26) also indicate a tendency for the ductus to become irreversibly closed within the first few days after birth. Further comparison of the present in vitro results with angiographic studies in vivo is not warranted, however, since ductal flow measured in vivo is a complex function of aortic and pulmonary pressures and flows as well as tension within the ductal wall (20).

Direct measurements of changes in tension exerted by the muscular wall of the ductus indicate that the cells of the muscularis retain their sensitivity to oxygen relative to that of other stimuli during the first 4 days after birth. A general loss of the contractile response to all stimuli was noted between 0 and 4 days postpartum, but this may result in part from the trauma involved in forcing stainless steel pins through irreversibly closed ducts of the 3- to 4-day old animals, and in part from necrosis of smooth muscle of the inner media in ducts from the older animals ((13), also Fig. 10 of the present study). Despite a significant loss of the contractile capacity of the muscle in the wall of the ductus, it is clear that, at a time when the perfused ductus has lost its ability to dilate in response to anoxia, the muscle cells of the vessel are still capable, albeit at a diminished level, of contracting and relaxing in response to high and low PO₂, respectively. Furthermore, papaverine, which causes an even greater reduction in tension than N₂, had no dilatory effect whatsoever on perfused ducts which were irreversibly closed (unpublished observations). All of these findings indicate that irreversibility of closure is not the result of sustained active force within the muscularis of the vessel wall.

The histological observations on ducts from animals of various ages provide some insight as to the mechanism that underlies irreversibility of closure. In all 12 irreversibly closed ducts systematically studied, the lumen was occluded at some point along the vessel. Cross sections through the occluded part revealed rearrangement of cells formerly in the area occupied by the endothelium and subendothelium and the apparent intermingling of cells from apposing walls. This appearance is not merely the result of apposition of the walls of the vessel, for no loss of an intact endothelium is apparent when ducts not yet irreversibly closed are fixed under aerobic conditions so that their walls are apposed. Before closure has become irreversible, the only difference between open and closed ducts is in the relative thickness of the endothelium and subendothelium which results from changes in the geometry of the vessel. A number of authors have recently reported similar changes as a result of muscular closure (10, 18).

The apparent death of many of the cells in the central region of the vessel was also a prominent and consistent feature of all cross sections through the occluded region of irreversibly closed ducts. In contrast, in ducts not yet irreversibly closed, necrosis was generally absent and when observed was confined to a few cells. When the onset of necrosis and cellular rearrangement was prevented or at least delayed by raising neonates under hypoxic conditions, irreversible closure was also prevented up to 80 hr postpartum.
The correlation between irreversible closure and cellular necrosis and rearrangement in ducts from normal and hypoxic neonates suggests an explanation for the mechanism underlying irreversible closure in the first few days postpartum. Ducts presumably lose their ability to reopen as a result of the presence of this central plug of cells and cell debris. In order for this central plug to effectively prevent flow through the ductus under anaerobic as well as aerobic conditions, it must somehow restrain movement of the vessel walls. Otherwise relaxation of the ductal smooth muscle in response to anoxia would result in the separation of the vessel wall from the plug and an increase in flow conductance. Alternatively, the plug itself may be elastic and expand during anaerobic conditions as the muscle in the vessel wall relaxes. In either case, the central mass of cells and cell debris must itself be cohesive and in addition must adhere to the inner walls of the ductus. These studies do not indicate what the basis for any "adhesiveness" might be. It is likely, however, that adhesiveness resides less in cell-cell interactions than in the properties of some extracellular material, since the ductus remains closed despite phagocytosis of

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**Fig. 9.** Cross section of a reversibly closed ductus obtained from an animal 26 hr postpartum. 

9A: note absence of a lumen. Dense line at center (arrow) represents apposed endothelium that is shown in greater detail in 9B. Ductus was fixed under aerobic conditions. X132. 

9B: two layers of endothelium are shown to be apposed (E). Subendothelial region (SE) and muscular coat (M) surround apposed endothelium. Pycnotic nuclei are largely absent in endothelial and subendothelial regions. X318.

**Fig. 10.** Cross section of irreversibly closed ductus from Fig. 4D obtained from an animal 96 hr postpartum. This section was obtained near aortic end of occluded portion of vessel. Note absence of an endothelium or lumen. Cells containing pycnotic nuclei (arrows) are evident in central area of vessel and in innermost parts of muscularis (M). Ductus was fixed under anaerobic conditions. X366.
FIG. 11. Cross section taken midway along occluded length of same ductus shown in Fig. 10. Note loosely arranged cells in central area. There are numerous pycnotic nuclei (arrows) and there is no endo-
thelium or lumen. ×382.

FIG. 13. Cross section showing central area of a ductus obtained
from a "hypoxic animal" 54 hr postpartum. Lumen (L) is patent, and
endothelium (E), subendothelium (arrow), and muscular coat (M) are
intact. Moreover there is no evidence of pycnosis. Ductus was fixed in
situ. ×350.

FIG. 12. Electron micrograph of a cell from central area of ductus
shown in Fig. 11. Cell completely encloses a dense mass of material
(arrow) that possibly corresponds to a cell fragment. Cell is surrounded
by large amounts of elastinlike intercellular substance (e). ×7,000.

many of the necrotic cells in the central area between the
middle and end of the 1st week postpartum (13), see also
Figs. 11 and 12).

Kennedy and Clark (13) first noted the necrotic changes
that take place in the guinea pig ductus during the first few
days postpartum. Mato and Aikawa (17) and Jones et al.
(12), using the electron microscope, have recently described
similar necrotic changes in the ductus arteriosus of the rat;
rearrangement of cells of the intima similar to that ob-
served in the present study has also been recently reported
(17, 19). Because necrosis and cell dislocation were limited
to the central regions of the vessel, and because they followed
muscular closure, several authors (9, 13, 18, 19) have sug-
gested that these processes result from interruption of
luminal blood flow. The present results with animals raised
under hypoxic conditions provide experimental evidence
supporting this suggestion. Ducts from hypoxic animals
remain patent for 3-4 days after birth and show neither
necrosis nor rearrangement of endothelial and subendo-
thelial cells characteristically seen in ducts from normal
animals of similar ages in which luminal flow had ceased.
It appears, therefore, that cellular rearrangement and
necrosis are triggered by metabolic disturbances associated
with the interruption of luminal blood flow which results
upon muscular closure of the ductus at birth. Ischemia
(8, 22, 29) and direct chemical trauma (11) have also been
shown to produce necrotic changes in the walls of other vessels. Furthermore, detachment and migration of endothelial cells following ischemia or direct mechanical trauma to other blood vessels have also been noted (16, 27). Thus the cellular transitions observed in the inner wall of the ductus appear to be typical for the response of a vascular wall to ischemic trauma. The confinement of necrosis to the outer media and adventitia, which do not undergo the inner wall of the ductus presumably reflects the metabolic dependence of this region on luminal flow. The cells of the outer media and adventitia, which do not undergo necrosis, presumably remain viable because blood flow through the vasa vasorum, located predominantly in the outer part of the vascular wall, is unaltered after birth (3).

Our inability to block closure of the ductus by hypoxia for more than 3 or 4 days prevented any longer tests of the relationship between cell death and rearrangement and the interruption of ductal flow. This inability to prevent closure suggests that, especially in cases of systemic hypoxia factors other than oxygen, perhaps circulating catecholamines as Dawes suggests (2, 4) initiate muscular closure of the ductus.

The authors are grateful to professors J. R. Pappenheimer, K. R. Porter, and H. M. Goodman for their advice and criticism in the preparation of this manuscript.

This investigation was supported by Public Health Service Grants 5-T07-GM 00707-07 and NGR 22-007-059 to K. R. Porter and continuing grants to J. R. Pappenheimer from the American Heart Association.

This work was carried out during the tenure by F. S. Fay of a research fellowship from the Massachusetts Heart Association.

Present address of F. S. Fay: Dept. of Physiology, University of Massachusetts Medical School, 419 Belmont Street, Worcester, Mass. 01604. Send reprint requests to this address.

Present address of F. H. Cooke: Dept. of Physiology and Cell Biology, University of Kansas, Lawrence, Kan. 66044.

Received for publication 1 October 1971.

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