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Patent Ductus Arteriosus and Neonatal Death in Prostaglandin Receptor EP4-Deficient Mice

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The physiological role of the prostaglandin E_2 receptor EP4 subtype was investigated by generation of EP4-deficient mice by gene targeting. Loss of the EP4 receptor was not lethal *in utero,* but most EP4 (−/−) neonates became pale and lethargic approximately 24 h after birth and died within 72 h. Less than 5% of the EP4 (−/−) mice survived and grew normally more than a year. Histological examination revealed that the ductus arteriosus in dead neonates remained open, while it was partially closed in the survivors. *In situ* hybridization study showed that EP4 mRNA was strongly expressed in the ductus. These results suggest that neonatal death is at least partly due to patent ductus arteriosus and that the EP4 receptor plays a role in regulation of the patency of this vessel. They also indicate that normal function of the EP4 receptor is essential in neonatal adaptation of the circulatory system.

Prostanoids comprising prostaglandins (PGs) and thromboxanes are the cyclooxygenase metabolites of arachidonate, and act as local mediators in various physiological and pathophysiological processes, such as the generation of fever and pain, and the stimulation of inflammatory responses (1, 2). Involvement of a prostanoid in a physiological process can be inferred by *in vivo* and *in vitro* actions of prostanoids added exogenously, and by actions of aspirin-like drugs which inhibit PG biosynthesis(3). However, their roles in many of these processes have not yet been fully defined.

Among the prostanoids, PGE_2 in particular exhibits a broad range of actions in diverse tissues; PGE_2 causes contraction or relaxation of vascular and nonvascular smooth muscles, and stimulates or inhibits the release of neurotransmitters and hormones. The actions of PGE_2 are mediated by specific rhodopsin-type receptors on target cells (4). There are four subtypes of PGE receptors, EP1, EP2, EP3 and EP4, which are encoded by different genes and differ in tissue expression. They also differ in signal transduction mechanism; they are coupled to Ca^{2+} mobilization (EP1), and the stimulation (EP2, EP4), inhibition (EP3) of adenylate cyclase (4, 5). In spite of this information, functions of each receptor subtype are not clear.

To elucidate the physiological importance of PGE_2 actions in the body and to identify the receptor subtypes responsible, we disrupted the genes encoding the EP receptor subtypes by homologous recombination. In this report, we present our initial findings of EP4-deficient mice.

MATERIALS AND METHODS

Construction of the targeting vector and electroporation of ES cells. A 9.6 kb SpeI-(SpeI)-BamHI genomic fragment spanning the area starting 6.2 kb 5′-upstream of the EP4 transcription initiation site to 1.7 kb 3′-downstream of the end of exon 2 was subcloned into pBluescript (Stratagene, La Jolla). A 1.6 kb fragment containing the coding region from Asn-32 in the N-terminal region to Val-317 in the sixth transmembrane domain (6) was replaced by the neomycin-resistant (*Neo*) gene. The MC1-herpes simplex virus thymidine kinase (HSV-TK) gene (7) was inserted into the Sal I site at the 3′ end of the homologous region. The targeting vector was electroporated into E14-1 ES cells as described (8). Homologous recombination was screened by the polymerase chain reaction (PCR) and subsequently by genomic Southern blot hybridization.

Generation of EP4-deficient mice. The generation of chimera mice was essentially as described (9). Chimeric males were mated to C57BL/6 females. ES clones were found to transmit the mutant gene through the germline. The resulting agouti offspring were analyzed for the mutated EP4 allele by PCR and Southern blotting. Homozy-

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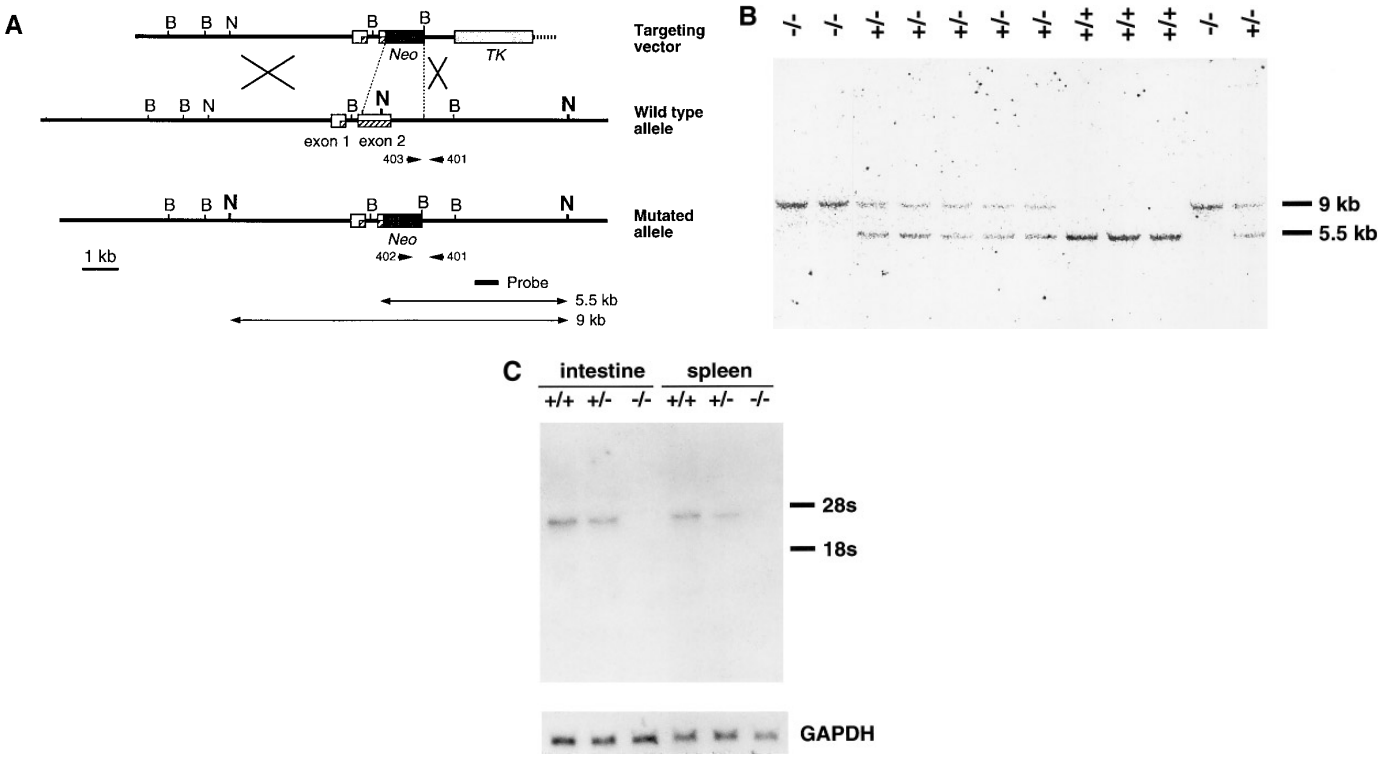

FIG. 1. Disruption of the gene encoding the EP4 receptor. (A) Strategy used for EP4 gene targeting. Construct of the targeting vector, organization of the EP4 gene, and the structure of the targeted genome are shown. Restriction sites are indicated: B, Bam HI, and N, Nsi I. Arrowheads indicate PCR primers. The mutated allele is identified by using the primers 401 and 402. The wild type allele is identified by using the primers 401 and 403. The thick line indicates the DNA probe used in Southern hybridization. TK, thymidine kinase gene; Neo, neomycin-resistant gene. (B) Southern blot analysis of Nsi I-digested genomic DNA from a representative litter of 12 pups. +/+, wild type; +/−, heterozygote; −/−, homozygote. (C) Northern blot analysis. Total RNA was isolated from the intestine and spleen of newborn mice and was used for hybridization with a specific probe. Hybridization signals for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in the lower panel.

gous mutant mice were obtained by interbreeding of heterozygous mice. In the following experiments, mice homozygous for the mutated allele or the wild-type allele in the background of (129/Ola × C57BL/6) F2 were examined. Two independent mutant lines displayed indistinguishable phenotypes.

PCR. PCR analysis was performed on DNA from the tails of embryos or neonates. Following an initial denaturing step (85°C for 10 min), amplification was carried out through 35 cycles of incubation at 94°C for 30 s, at 65°C for 30 s, and at 75°C for 60 s. The following oligonucleotide primers were used to identify the rearranged EP4 locus. Primer 401 with the sequence 5′-dTCTACTTGCTCCCAGTGGAC-ATAGATGG-3′ was complementary to the genomic DNA upstream of the short arm of homology. Primer 402 with the sequence 5′-dTGCCG-AATATCATGGTGGAAAATGGCCG-3′ was complementary to sequences at the 3′ terminus of the *Neo* cassette. A third primer, primer 403 with the sequence 5′-dGAACAGACTCCTGAACTGG-GTATGG-TTC-3′, complementary to the EP4 sequences 3′ of the site of disruption was used in conjugation with primer 401 and primer 402 to identify the wild-type and homologous mutant allele.

TABLE 1

Genotypes of Progenies of Heterozygote Intercrosses

	Genotype		
	+/+	+/−	−/−
E14.5 fetuses	16	41	20
E18.5 fetuses	40	67	37
3-day-old neonates	181	309	5

Note. The genotypes and numbers of living mice and fetuses are shown.

Southern blots. Genomic DNA (10 μg) was digested with Nsi I and subjected to agarose gel electrophoresis. DNA was transferred onto nylon membranes (Pall, East Hills), according to the protocol of the manufacturer. Filters were hybridized with radiolabeled probes overnight, and then washed in 2× SSC containing 1% SDS at 65°C several times before autoradiography.

Northern blots. For Northern blot analysis, intestine and spleen were isolated from neonates and subjected to RNA preparation. A Hinc II/Sac II fragment (680 bp) of the EP4 receptor cDNA (6) was used as an exon-2 specific probe.

Histological examination. Whole embryos and neonates were fixed in Bouin's solution for 3 days and transferred to 70% ethanol for examination of the lung. The lungs were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Embryos and neonates were also frozen in isopentane cooled to −80°C (10). The thorax was horizontally sectioned in sequence by a cryostat and stained with hematoxylin and eosin for examination of the ductus arteriosus.

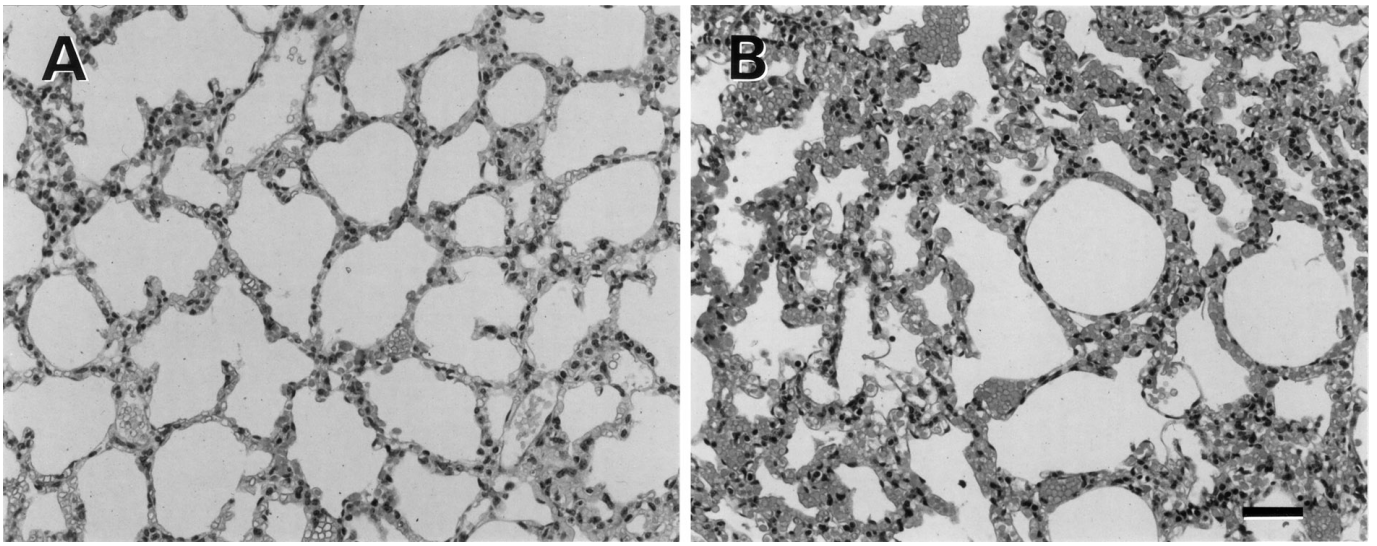

FIG. 2. Histological analysis of lungs from a wild type (A) and a homozygous (B) mouse sacrificed at 30 h after birth. Marked congestion of the pulmonary capillaries and disorganized alveolar structures are seen in the homozygous lung. Bar, 50 μm.

In situ hybridization. Using the linearized EP4 receptor cDNA as a template, an antisense riboprobe was transcribed with T3 RNA polymerase (Stratagene) in the presence of [^{35}S]CTPαS. The resultant riboprobe was degraded to 150 bases by alkaline hydrolysis. The 10-μm thick thoracic sections containing the ductus arteriosus were fixed with 4% formaldehyde, treated with 0.25% acetic anhydride and dehydrated. Hybridization was carried out essentially as described previously (11). The specificity of the signal with an EP4 antisense probe was verified by its disappearance with the addition of an excess of unlabeled probe. The slides were air-dried and dipped in NTB3 emulsion (Kodak). After exposure for 5 weeks at 4°C, the dipped slides were developed in a Kodak D-19 developer, fixed, and counterstained with hematoxylin and eosin.

RESULTS AND DISCUSSION

A targeting vector was constructed so that the coding region of the 2nd exon could be replaced with the *Neo* gene (Fig. 1A). Of 400 resistant clones, 5 were true recombinants, two of which were used to generate mutant mice. Heterozygous mutant mice grew normally and had no obvious defects. Homozygous mutant mice were generated by the interbreeding of heterozygous mice, and offspring genotypes were determined by Southern blotting of genomic DNA from fetuses and neonates (Fig. 1B). Disruption of the EP4 receptor was verified by Northern blot analysis in intestine and spleen from neonates (Fig. 1C).

Homozygous mutant embryos were present at the expected ratio *in utero*, and appeared normal by inspection (Table 1). The EP4 (−/−) neonates began to breath after birth and were indistinguishable with the wild type neonates in their suckling behavior and general appearance. The EP4 (−/−) neonates, however, became pale and their movement was significantly weakened approximately 24 h after birth and almost all of them died within 72 h (Table 1). When the dying EP4 (−/−) neonates were sacrificed for pathological examination, marked congestion in the pulmonary capillaries and disorganized and shrunken alveolar structures were observed (Fig. 2). Dilation of the left ventricle and pulmonary arteries was also found (data not shown). These observations suggested that the homozygous neonates had left-sided heart failure before death. To find out the cause of this failure, we examined the expression of EP4 receptor mRNA by *in situ* hybridization, and found that it was highly expressed in the ductus arteriosus (Fig. 3); the expression was already detected at E15.5 and continued up to at least 6 h after birth. The ductus arteriosus is a blood vessel connecting the main pulmonary artery and the descending aorta, and serves to shunt the pulmonary blood flow to the aorta in the fetal circulation (12). It closes just after birth to adapt to the onset of respiration. In the fetal period, the ductus arteriosus of both the wild type and the EP4 (−/−) mice were widely open (data not shown). After birth, the ductus in wild type mice thickened within 30 min and usually closed in about 3 h, at about the same time as previously reported in the rat (13). On the other hand, the ductus arteriosus remained fully open in 6 h-old EP4 (−/−) animals that still appeared healthy (Fig. 4), and it did not close at all in the EP4 (−/−) neonates until their death. Less than 5% of the EP4 (−/−) mice that were born survived and grew normally for more than a year (Table 1). The ductus arteriosus of these mice were either closed or severely narrowed (Fig. 5). The observation by a stereomicroscope revealed that, whereas the closure was observed from end to end of the ductus in the wild type animals, only a part of the vessel was closed in the homozygous animals (data not shown). Al-

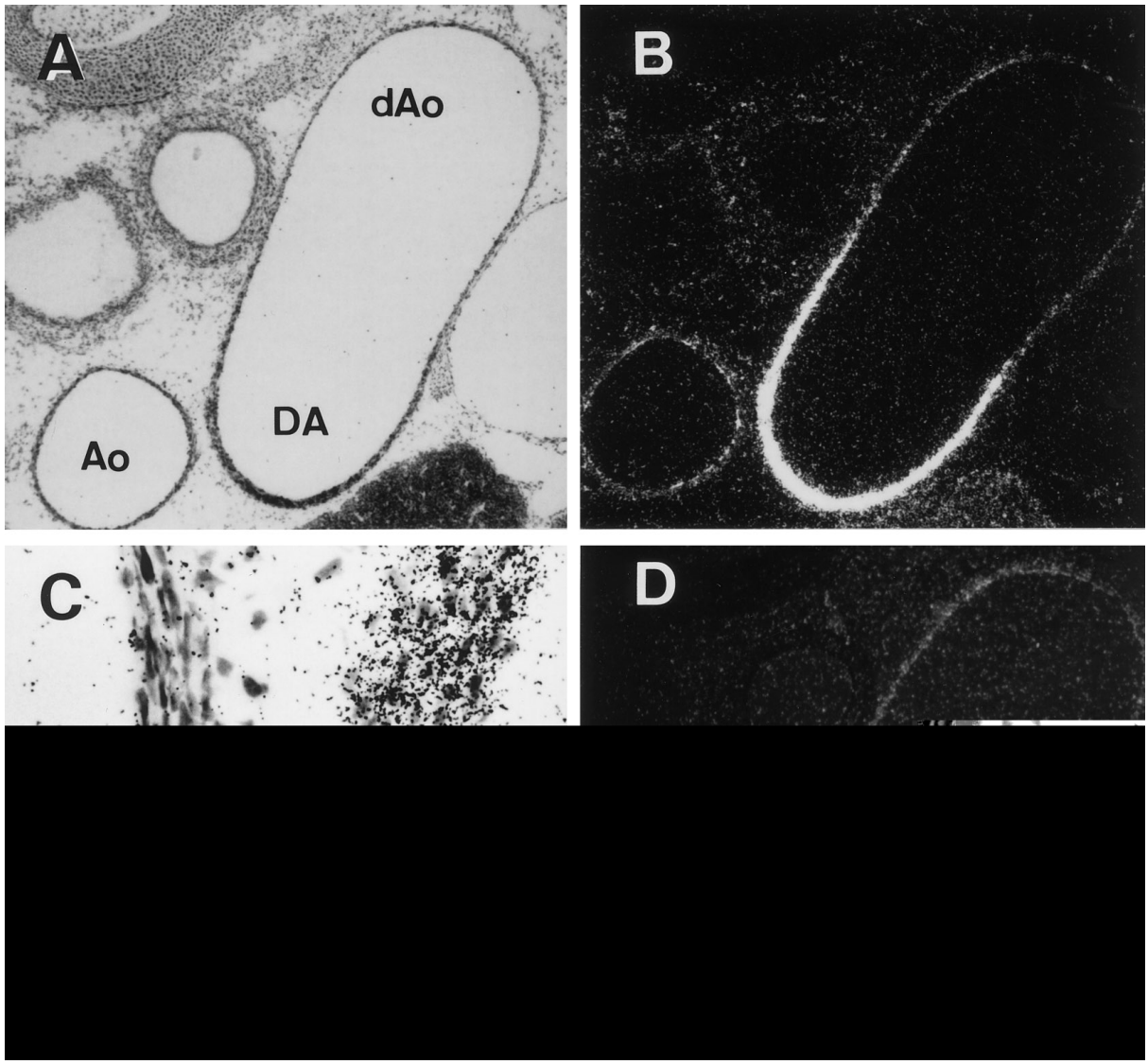

FIG. 3. *In situ* hybridization of the EP4 transcripts. Photomicrographs are thoracic sections of a wild type E18.5 fetus. These sections were hybridized with a ^{35}S-labeled antisense RNA probe for the EP4 receptor in the absence (A, B and C) or presence (D) of an excess amount of unlabeled probe (A and C, bright-field; B and D, dark-field). Strong signals are observed in the smooth muscle layer of the ductus arteriosus. Ao, aorta; dAo, descending aorta; DA, ductus arteriosus. Bars, 200 μm (D) and 25 μm (C). The magnifications of micrographs A, B and D are the same.

though the survived EP4 (−/−) mice were fertile, interbreeding of these mice did not increase the survival rate of the homozygous animals. We have thus found neonatal death and patent ductus arteriosus in EP4 (−/−) mice. The above pathological findings suggest that the left-sided heart failure caused by the ductus remaining open after birth may be a major, if not only, cause of neonatal death in these animals.

It is well known that aspirin-like drugs given to pregnant women induce closure of the ductus of the fetus (14). This action of the drugs is also utilized to stimulate the ductus closure after birth (15). PGE_1, on the contrary, is capable of opening this vessel and is used to keep the ductus open for neonates who depend on the ductus patency for survival from cardiac malformations (16). Aspirin-like drugs and PGE_2 can constrict and relaxes the ductus, respectively, both *in vivo* and *in vitro* in experimental systems (17, 18). Recently, the EP4 receptor was proposed to mediate the PGE_2-induced dilation of the ductus arteriosus in rabbits on the basis of selectivity of several PGE analogues (19). These findings implicate that the PGE_2/EP4 system works to keep the ductus open in fetuses. The present finding of patent ductus arteriosus in EP4 (−/−) mice

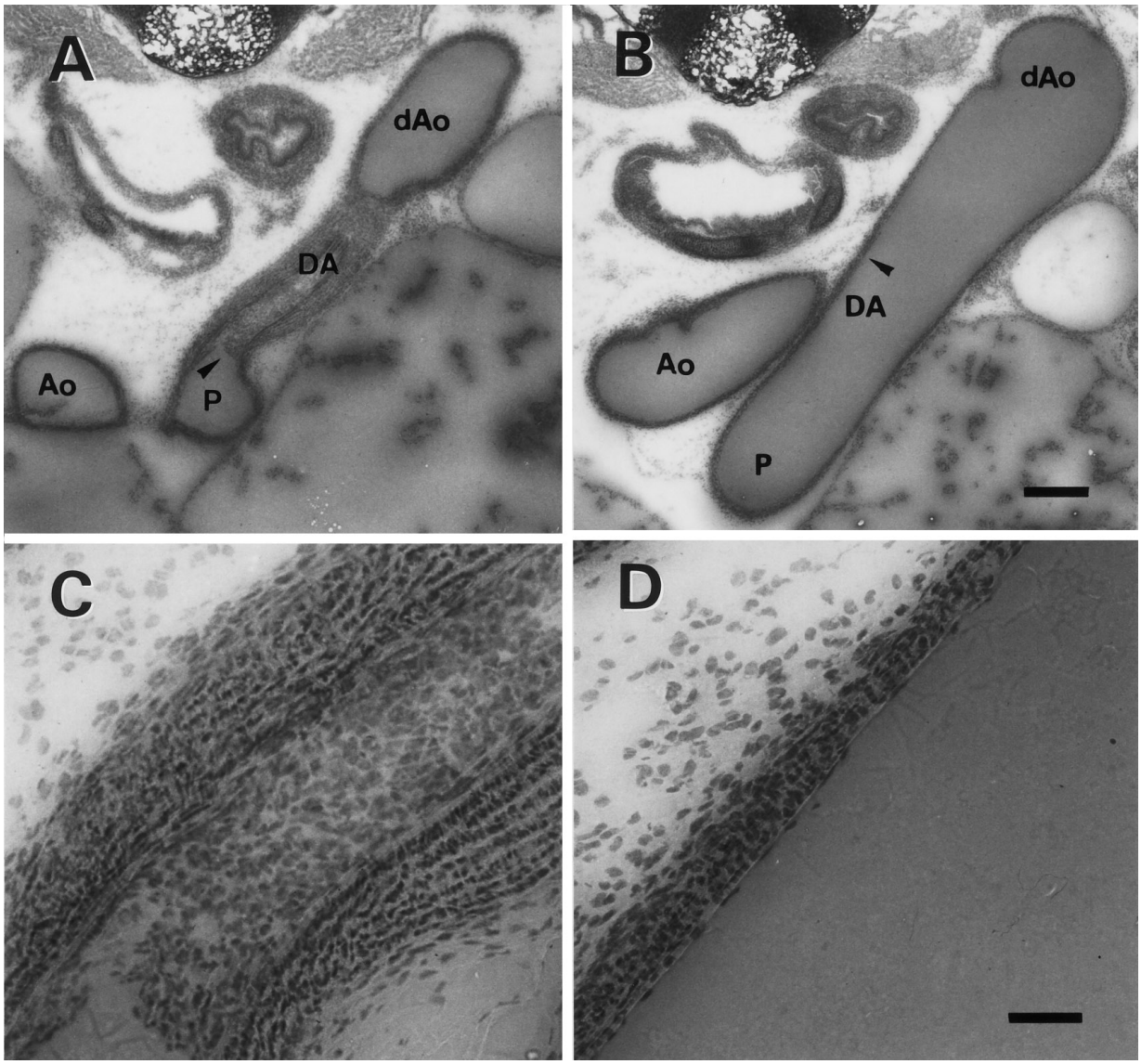

FIG. 4. Patency of the ductus arteriosus in EP4-deficient neonates. Thoracic sections of a wild type (A, C) and a homozygous (B, D) neonate sacrificed at 6 h after birth are shown. Bottom panels are magnifications of the arrowhead on each top panel. In the wild type, the ductus arteriosus is completely closed and its wall is thickened, but in the homozygous neonate, the ductus is open and its wall is thin. Ao, aorta; dAo, descending aorta; P, pulmonary artery; DA, ductus arteriosus. Bars, 200 μm (B) and 50 μm (D).

therefore appears paradoxical. During preparation of this manuscript, generation of EP4 (−/−) mice with the same phenotype as reported here was reported (20). At present we do not know the reason behind this discrepancy. The ductus differs from the other embryonic great vessels; it is a muscular rather than an elastic artery and fully differentiated smooth muscle cells are already present at birth (21). The closure of the ductus is supposed to occur not only from the inhibition of vasodilation but also from strong contraction caused by elevation of oxygen tension. This contraction is followed by fibrous organization of the vessel. It is therefore likely that the presence of the PGE_2/EP4 system in the fetal period may affect the progress of these latter steps. The nature of a contractile substance involved and its mechanism remain unknown. However, it is often seen that the tonic inhibition such as chronic vasodilation induces the amplification of the contractile potential, and that a marked contraction is evoked by the abrupt cessation of this inhibition (22, 23). Whether the PGE_2/EP4 system is involved not only in dilation of the ductus arteriosus but also in amplification of its contractile machinery by tonic inhibition in the fetal period remains an interesting possibility to test.

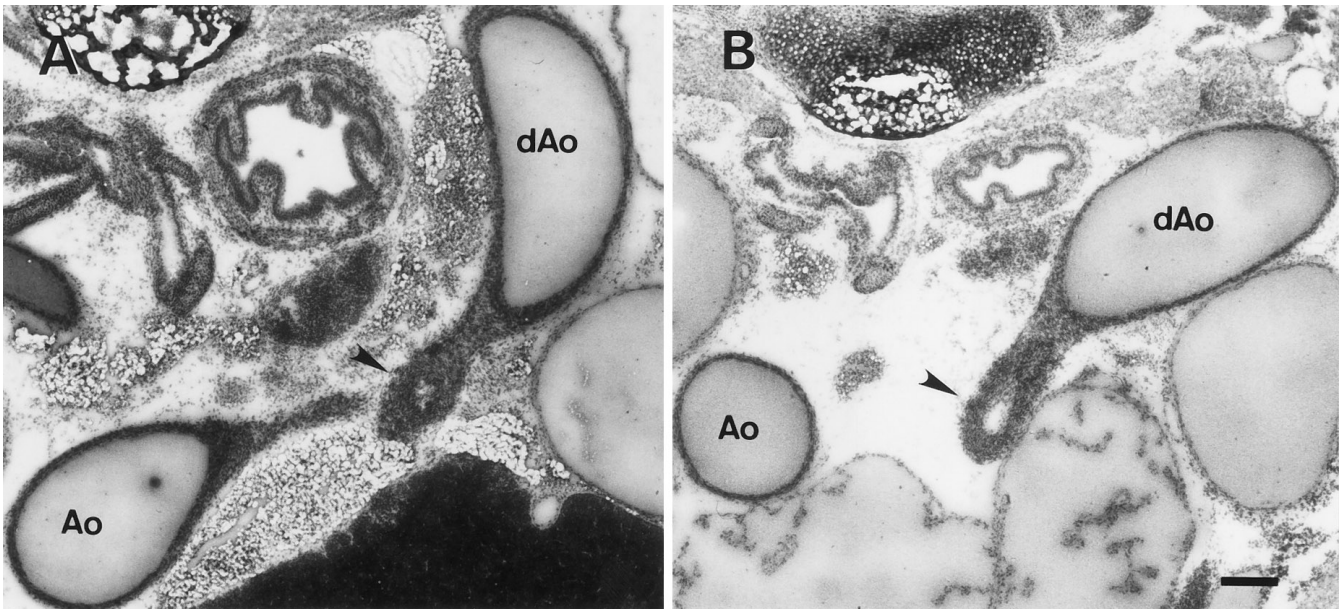

FIG. 5. The ductus arteriosus in the survived EP4-deficient neonate. Thoracic sections of a wild type (A) and a survived homozygous (B) neonate sacrificed at 3 days after birth are shown. Eight surviving homozygous mice at various ages were examined, and representatives are shown. The ductus arteriosus (arrowhead) in the survived homozygous animals are almost closed. Ao, aorta; dAo, descending aorta. Bar, 200 μm.

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