

**Fig. 1.** Frontal cuts of the fetal and neonatal DA in the near-term rat on D21. **a** Control fetus without medication. The ductus was open. **b** Control neonate 60 min after birth. The ductus was closing. **c** The fetus exposed to indomethacin on D19 and D20. The ductus was dilated. **d** The neonate exposed to indomethacin on D19 and D20, 60 min after birth. The ductus was constricted mildly. AoA = Aortic arch; DA = ductus arteriosus; LPA = left pulmonary artery.

varying between 70 and 85% [4, 5]. In obstetrics, indomethacin is used for tocolysis [6]. Early in utero exposure to indomethacin is known clinically to increase the incidence of PDA and reduce the ductal sensitivity to indomethacin in premature babies [7–9]. Experimentally, the same effects of indomethacin and other inhibitors of COX have been previously identified [10–12]. Many clinical questions concerning this problem remain unsolved. For example, what dose of prenatal indomethacin is necessary to induce appreciable delay of neonatal ductus closure? There are many COX inhibitors [13–15] other than indomethacin, and these are used as analgesics and antipyretics. Do these COX inhibitors also delay neonatal closure of the ductus arteriosus (DA)? Is the delay induced by decreased prostaglandins and a decreased stimulus of the EP<sub>4</sub> receptor [16] in DA? Is the delay related to fetal DA constriction and ischemia of the fetal DA tissue [12]? To answer these questions, animal disease models may be helpful, and as a consequence, we decided to perform the following study in the rat.

## Methods

### Animals

Virgin Wistar rats were obtained and maintained in an environmentally controlled room, acclimatized to a 12/12-hour dark/light cycle, and sustained on commercial solid food and tap water ad libitum. Treatment conformed to the guiding principles of the American Physiological Society. The experiments were approved by the Ethical Committee of Animal Experiments of our institute. The rats (pregnancy period 21.5 days) were mated overnight from 17.00 to 09.00 h; the presence of sperm in vaginal smears was confirmed as day 0 of pregnancy. We studied near-term fetuses and neonates on the 21st gestation day (D21). The fetuses were fixed by whole-body freezing, following atlas dislocation of the mother rat and immediate cesarean section, with an intact umbilical cord. In studies of the neonatal rat, following atlas dislocation and immediate cesarean section of the mother, newborn pups were incubated in room air at 33°C for 15–180 min until freezing.

### Measurements

To study the in situ morphology and inner diameter of the fetal and neonatal DA, a rapid whole-body freezing method was used as described in earlier studies [17, 18]. This method was established to study in situ morphology of the DA in experimental animals [17]. Briefly, fetuses and neonates were frozen in acetone cooled to –80°C by dry ice. The body weight of the frozen fetus and neonate was measured. The frozen thorax was cut on a freezing microtome (Komatsu Solidate Co. Ltd., Tokyo, Japan) in the frontal plane, and the inner diameters of the ascending aorta, main pulmonary artery and DA were measured every 100 μm with a microscope (Nikon Binocular Stereoscopic Microscope; Nihon Kogaku Co., Tokyo, Japan) and a micrometer (Nikon Ocular Micrometer; Nihon Kogaku Co.; fig. 1). The narrowest DA diameter was used as the indicator of constriction.

The plasma indomethacin concentration (PIC) of the mother and neonates in studies E, F, G, H, M, P in table 1 was measured as follows. Immediately after cesarean section, maternal blood was drawn by cardiac puncture with a heparinized syringe. Following cesarean section, the neonates were incubated at 33°C, and heparin (400 U/kg, in 0.02 ml) was injected intraperitoneally. One sample of neonatal blood was collected from 10 to 14 littermates by making a deep cut in the neck 30 min after birth. Plasma was separated by centrifugation and frozen until the measurement of indomethacin. The PIC was determined by high-performance liquid chromatography with ultraviolet detection [19].

### Drugs

Indomethacin (Sigma Chemical, St. Louis, Mo., USA) in addition to other drugs was diluted with lactose, suspended in 1 ml of water, and administered through an orogastric tube to the mother at 09.00 h. L-NAME (Sigma Chemical), a nitric oxide synthase (NOS) inhibitor was dissolved with 1 ml of physiological saline and injected subcutaneously.

### Protocol

In the rat, the embryonic stage ends at D14 and the fetal stage ends from D15 to D21.5 [20]. In the fetal stage of the rat, the sensitivity of the DA to constrict in response to indomethacin administered to the mother changes from mildly sensitive on D19 and D20 to very sensitive on D21 [21]. As a consequence, near-

term fetal and neonatal studies were performed following cesarean section at 13.00 h on D21. DA constriction and the inner diameters were studied using 8–12 fetuses and neonates from 4 to 5 litters at each dose and time on D21. We measured the neonatal ductal diameter at 15, 30, 60, 120 and 180 min after birth.

#### Control

Two control groups were used to study ductal diameter changes. In control group 1, lactose (0.2 gm in 1 ml water) was administered through an orogastric tube to the pregnant rat on D19 and D20, and fetuses and neonates were studied on D21. The results in control group 1 were compared with the results in experimental groups with indomethacin and other drugs, except those with indomethacin administered on D21. In control group 2, lactose was administered through an orogastric tube to the pregnant rat on D21, and fetuses and neonates were studied 4 h later. The results in control group 2 were compared with the results in experimental groups with indomethacin administered on D21 (studies G, M, P).

#### Timing Study with a Large, Single Dose of Indomethacin

The effects of indomethacin (10 mg/kg) with variable timing to fetal and postnatal DA were studied by orogastric administration of indomethacin 10 mg/kg (single dose) on D16–D21 (5 to 0 days before birth). Indomethacin was administered at 09.00 h each day, and the ductus was studied following cesarean section at 13.00 h on D21 (table 1, studies B–G).

#### Dose Study on D19 and D21

In addition to a large dose (10 mg/kg), the fetal and postnatal DA effects of moderate and small doses of indomethacin (1 and 0.1 mg/kg) were studied, following administration of 1 of these doses of indomethacin on D19 or D21 (–4 h; table 1, studies K, M, N, P).

#### Multiple Doses of Indomethacin

To see the effects of multiple doses of 10 mg/kg of indomethacin, the rats who had received 2–3 doses on D18–D21 were studied (table 1, studies H–J), and the results were compared with the control (no early treatment) and rats given a single dose of indomethacin 10 mg/kg on D19. The effects of moderate and small doses (1 and 0.1 mg/kg) of indomethacin on D19 and D20 were studied (table 1, studies L, O).

#### Nonselective COX Inhibitors

In addition to indomethacin, the fetal and postnatal DA effects of ibuprofen (100 mg/kg; Kaken Pharma, Tokyo, Japan) and aspirin (100 mg/kg; Bayer, New York, N.Y., USA) were studied following orogastric administration of 2 doses, on D19 and D20, to the mother by studying the ductus on D21 (table 1, studies Q, R).

#### Selective COX inhibitors

The fetal and postnatal DA effects of SC 560 (10 mg/kg, a selective COX1 inhibitor; Taisho Pharma, Tokyo, Japan) and rofecoxib (10 mg/kg, a selective COX2 inhibitor; Merck, Readington Township, N.J., USA) were studied following orogastric administration of 2 doses on D19 and D20 to the mother by studying the ductus on D21 (table 1, studies S, T).

#### Prostanoid EP<sub>4</sub> Receptor Antagonist

The fetal and postnatal DA effects of ONO-AE3–208 (10 mg/kg, a prostanoid EP<sub>4</sub> receptor antagonist; Ono Pharma, Osaka,

**Table 1.** Protocols of administration of drug to the pregnant rat as well as study of neonatal ductal closure and blood indomethacin concentration

Study: drug	Date and dose, mg/kg						Follow-up time, min	Indomethacin concentration
	D16	D17	D18	D19 –52 h	D20 –28 h	D21 –4 h		
A: control							180	
B: indomethacin	10						120	
C: indomethacin		10					120	
D: indomethacin			10				120	
E: indomethacin				10			180	+
F: indomethacin					10		180	+
G: indomethacin						10	30	+
H: indomethacin				10	10		180	+
I: indomethacin			10	10	10		180	+
J: indomethacin				10	10	10	180	+
K: indomethacin				1			120	
L: indomethacin				1	1		120	
M: indomethacin						1	120	+
N: indomethacin				0.1			120	
O: indomethacin				0.1	0.1		120	
P: indomethacin						0.1	120	+
Q: aspirin				100	100		120	
R: ibuprofen				100	100		120	
S: SC560				10	10		120	
T: rofecoxib				10	10		120	
U: ONO-AE3-208				10	10		120	
V: L-NAME				30	30		120	

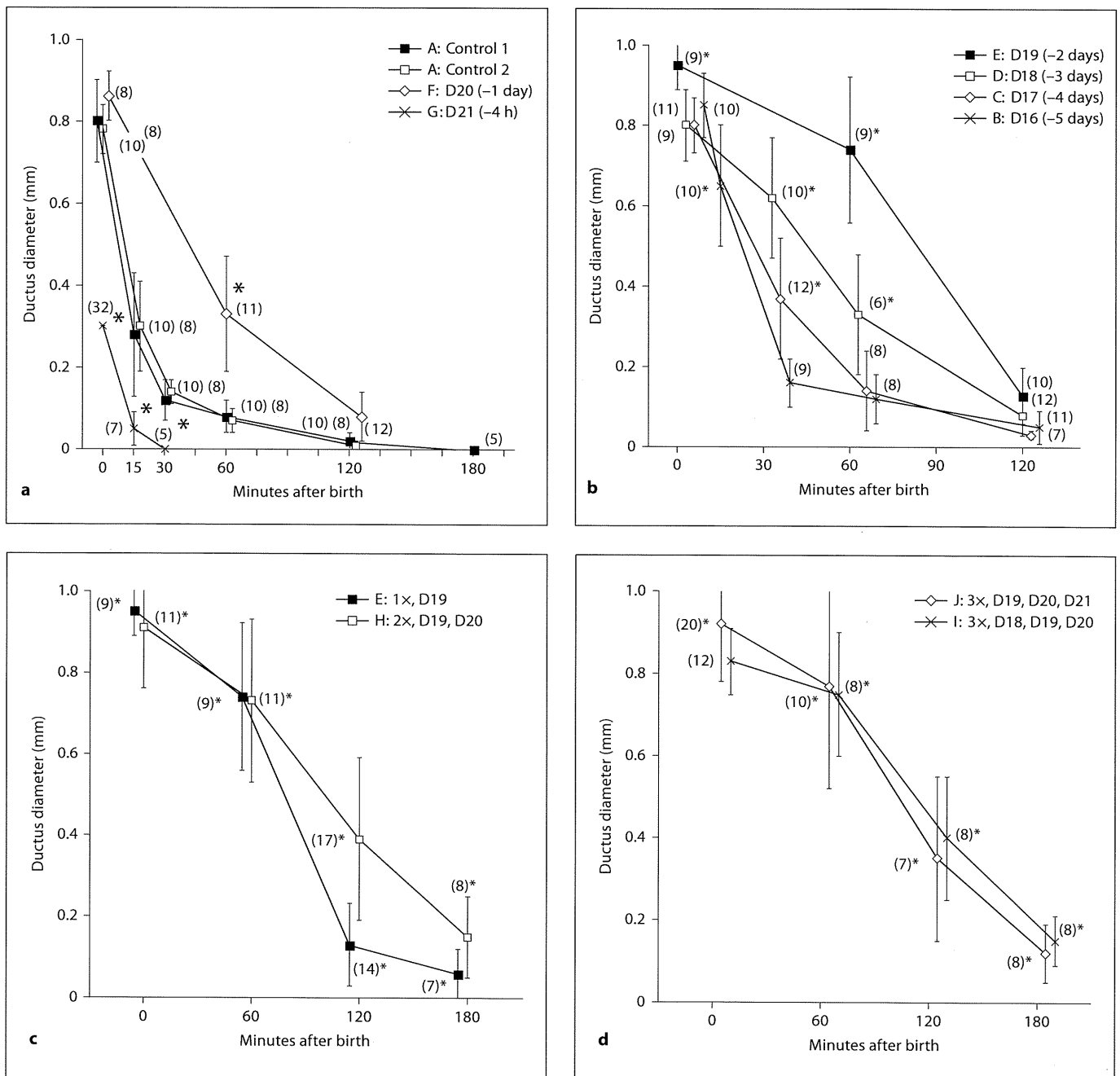
Japan) was studied following orogastric administration of 2 doses on D19 and D20 to the mother and studying the ductus on D21 (table 1, study U).

#### Fetal Ductal Constriction with L-NAME

L-NAME inhibits NOS, and induces severe fetal ductal constriction in preterm rat transplacentally on D19 and D20 [21]. To see the effects of severe fetal ductal constriction without inhibition of COX, L-NAME (30 mg/kg) was injected subcutaneously into the mother on D19 and D20, and fetal and neonatal ductus were studied on D21 (table 1V).

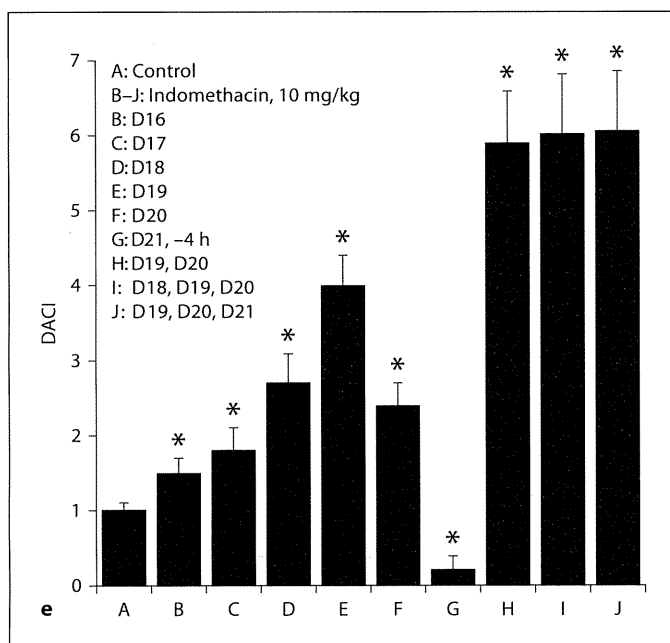
#### DA Closure Index

To show the closure speed of the neonatal DA, the DA closure index (DACI) was calculated as follows: the closure speed of the neonatal ductus was inversely related to the area under the ductus diameter curve (fig. 2a). The DACI was defined as the area under the ductus diameter curve, and is expressed as the ratio to the area of the control. For example, in figure 2a, the neonatal ductal closure was much delayed following a large dose of indomethacin (10 mg/kg) on D19 (study E), the area under the ductus diameter curve was 4 times larger than the area under the control ductus diameter curve (study A) and the DACI was 4.0, indicating a 4-fold delay in closure.



**Fig. 2.** In utero exposure to indomethacin (10 mg/kg, per os) and postnatal ductal closure of near-term rats. Data are means  $\pm$  SD. Figures in parentheses are numbers of animals. \*  $p < 0.05$  vs. control. **a** Ductal diameters in those neonatal rats without drugs (study A: control 1 and control 2) and in those with maternal administration of indomethacin (10 mg/kg) on 1 day before or 4 h before birth (studies F, G). **b** Ductal diameters in those neonatal rats with maternal administration of the same medication on

D19–D16 (2–5 days before birth, studies B–E). **c** Ductal diameters in those neonatal rats with repeated administration of the same medication on D19 and D20 (study H), compared to one maternal administration of the same medication on D19 (studies E, H). **d** Neonatal ductal closure of rats with 3 doses of indomethacin (10 mg/kg) on D18, D19, D20 or D21 (studies I, J). **e** DACI in the control neonatal rat (A), and in the neonatal rat with in utero exposure to 10 mg/kg indomethacin (B–J).



## Results

### Indomethacin Concentration

PIC in studies E, F, G, H, M and P in table 1 are shown in table 2. Following orogastric administration of a large dose (10 mg/kg) of indomethacin, the maternal PIC increased 4 h after administration, decreased by half, albeit remaining high 28 h later, and subsequently decreased 52 h later. Neonatal PIC increased up to 8 µg/ml, 4 h after administration, and remained at a high level (7 µg/ml) by 28 h, decreasing to 1 µg/ml at 52 h, consistent with a more rapid decrease in the second 24-hour period. Smaller doses of indomethacin induced proportionately smaller plasma concentrations of indomethacin (table 2).

### Timing of Indomethacin Administration and Neonatal Ductal Closure

Maternal administration of indomethacin 10 mg/kg on D21 induced constriction of the fetal ductus 4 h later, and the neonatal ductus closed more rapidly than the control ductus (fig. 2a, study G). In contrast, the neonatal ductus showed delayed closure following administration of indomethacin on D16–D20 (5–1 day before). The delay of the postnatal DA closure induced by administration of indomethacin on D18 or earlier or on D20 was significantly smaller than the delay with administration on D19 (2 days before; fig. 2a, b, e). Even the administration of indomethacin on D16, 5 days before term, showed a small but significant delay in ductal closure (fig. 2b).

### Multiple Doses of Indomethacin and Neonatal Ductal Closure

Effects of multiple doses of indomethacin on neonatal ductal closure are shown in figures 2c and d. Administration of indomethacin 10 mg/kg, given on both D19 and D20, induced a greater delay in neonatal ductal closure than 1 dose on D19 (fig. 2c, study H), and the DACI increased to 6 (fig. 2e). An additional dose on D18 did not cause a further increase in the delay in ductal closure (fig. 2d, study I).

### Additional Dose of Indomethacin Given 4 h before Birth on D21 following 2 Doses on D19 and D20

One dose of indomethacin on D21 in addition to the doses on D19 and D20 (table 1, study J) did not constrict the ductus or change the delayed closure induced by 2 doses of indomethacin (fig. 2d). This was in sharp contrast with the constricting effect of indomethacin on the fetal and neonatal ductus on D21 without early treatment with indomethacin (fig. 2a).

**Table 2.** PIC (µg/ml) of neonatal and maternal rats on D21

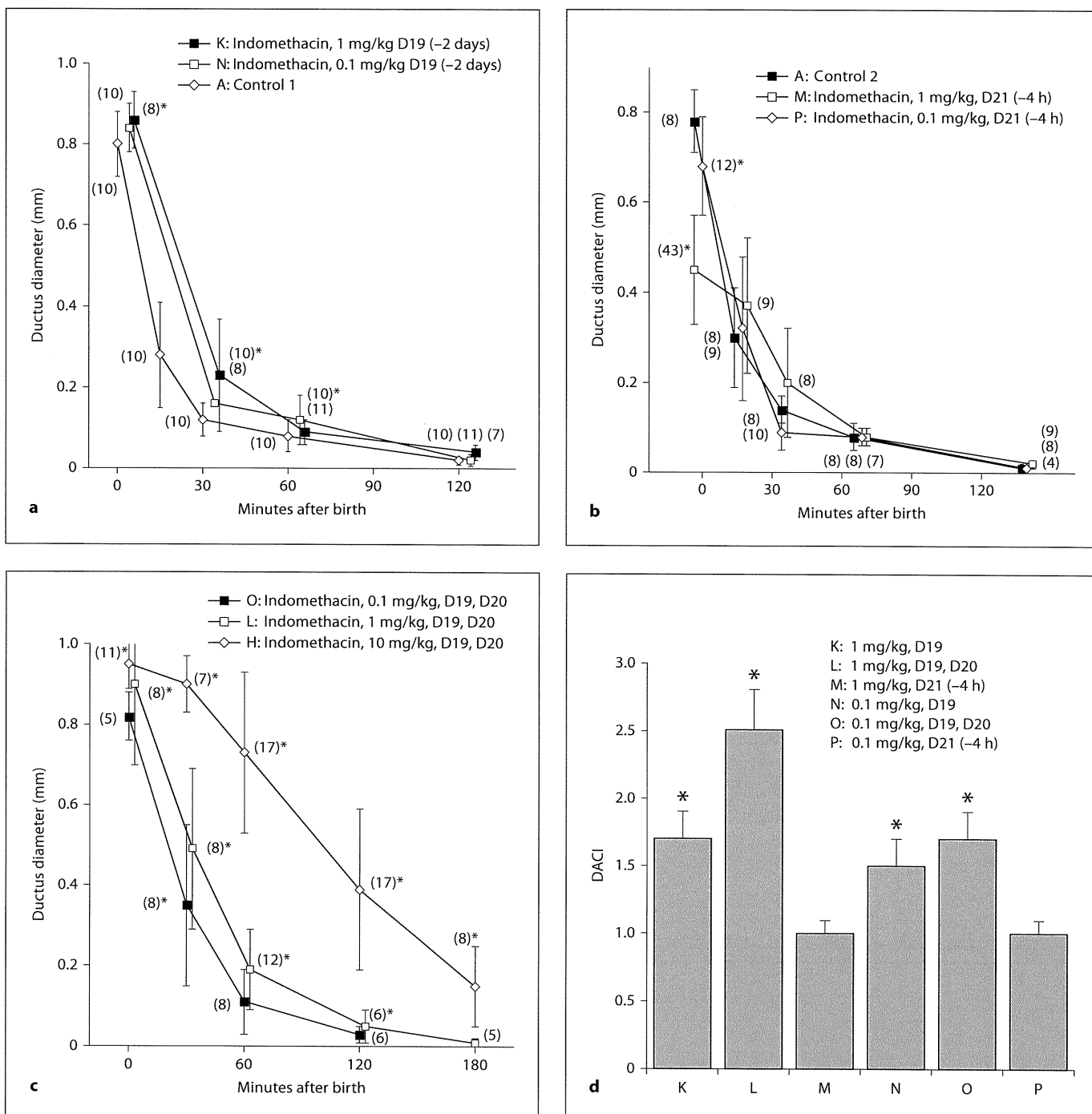
Dose and date	n	Neonate	Mother	Neonate/mother
P: 0.1 mg/kg, D21, -4 h	5	0.08 ± 0.06	0.30 ± 0.14	0.26 ± 0.10
M: 1 mg/kg, D21, -4 h	5	0.76 ± 0.16	2.70 ± 0.54	0.27 ± 0.04
G: 10 mg/kg, D21, -4 h	4	8.19 ± 2.35	33.53 ± 7.58	0.22 ± 0.04
F: 10 mg/kg, D20, -28 h	3	6.94 ± 2.52	16.03 ± 4.63	0.43 ± 0.05
E: 10 mg/kg, D19, -52 h	5	0.90 ± 0.55	1.81 ± 1.23	0.51 ± 0.08
H: 10 mg/kg, D19 and D20	4	8.85 ± 2.32	17.01 ± 2.96	0.51 ± 0.22

### Photographs

The frontal section of the DA was photographed to demonstrate ductal constriction with a binocular stereoscopic microscope (Wild M400 Photomicroscope; Wild Heerbrugg Ltd., Heerbrugg, Switzerland) using color film (Reale; Fuji Film Co., Tokyo, Japan; fig. 1).

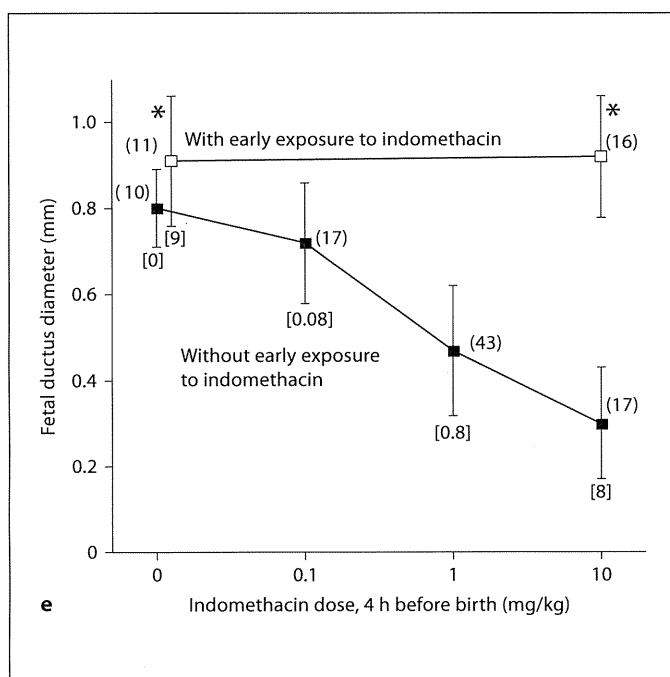
### Statistics

The results are expressed as means ± standard deviation. The statistical significance of differences between group means was determined by ANOVA and the Bonferroni methods [22]. The difference was considered to be significant if the p value was less than 0.05.



**Fig. 3.** Data are means  $\pm$  SD. Figures in parentheses are numbers of animals. \*  $p < 0.05$  vs. control. **a** Neonatal ductal closure following moderate (1 mg/kg) and small (0.1 mg/kg) doses of indomethacin on D19 (studies A, K, N). **b** Neonatal ductal closure in control group 2 and in respective study groups following administration of moderate and small doses of indomethacin on D21 (-4 h, studies A, M, P). **c** Postnatal ductal closure following 2 administrations (D19, D20) of various doses (10, 1, 0.1 mg/kg) of

indomethacin (studies H, L, O). **d** DACI in the neonatal rat with in utero exposure to 1 mg/kg indomethacin (studies K, L, M) and to 0.1 mg/kg indomethacin (studies N, O, P). **e** Fetal ductal diameters and PIC 4 h after orogastric administration of indomethacin to D21 mother rats without early exposure (studies A, G, M, P), and in those rats with early exposure to 10 mg/kg indomethacin on D19 and D20 (studies H, J).



(10 mg/kg) of indomethacin induced a prolonged delay in DA closure (fig. 2b) and smaller dose induced more minor, albeit significant delays in DA closure (fig. 3a). The 3 doses of indomethacin were administered repeatedly on D19 and D20 (studies H, L, O) and the delay of the neonatal DA closure was studied on D21. These studies also revealed dose-dependent delay (fig. 3c, d).

#### Fetal Ductal Constriction with Indomethacin Administered 4 h before Birth on D21

In those fetuses without early exposure to indomethacin, this drug induced fetal ductal constriction dose dependently (fig. 3d, studies G, M, P). In those fetuses with early exposure to indomethacin (10 mg/kg, D19 and D20), the fetal ductus was dilated with high PIC (8 µg/ml), and additional administration of indomethacin (10 mg/kg, -4 h) did not constrict the fetal ductus whatsoever (fig. 3e, studies H, J). This study was associated with a high fetal and neonatal mortality (more than 80%), and fetal PIC could not be measured, but was assumed to be very high.

#### Ibuprofen (100 mg/kg) and Aspirin (100 mg/kg) Administered on D19 and D20

Neonatal ductal closure was significantly delayed on D21 following in utero exposure to ibuprofen and aspirin, and DACI was significantly prolonged to 3.1 and 2.2, respectively (fig. 4a, d).

#### Selective COX1 Inhibitor, SC 560 (10 mg/kg) and COX2 Inhibitor Rofecoxib (10 mg/kg) Administered on D19 and D20

Neonatal ductal closure was significantly delayed on D21 following in utero exposure to these COX1 and COX2 inhibitors (fig. 4b), and the closure index was 1.8 and 2.6, respectively (fig. 4d).

#### Prostaglandin EP<sub>4</sub> Receptor Blocker ONO-AE3-208 (10 mg/kg) Administered on D19 and D20

Neonatal ductal closure was significantly delayed on D21 following in utero exposure to the EP<sub>4</sub> blocker ONO-AE3-208 (fig. 4c), and the closure index was 2.3 (fig. 4d).

#### NOS Inhibitor L-NAME (30 mg/kg, Subcutaneously) on D19 and D20

Neonatal ductal closure was normal on D21 following in utero exposure to L-NAME, and the closure index was 1.0 (fig. 4c, d). However, all fetal vessels including the aorta, pulmonary artery and DA were small, and their diam-

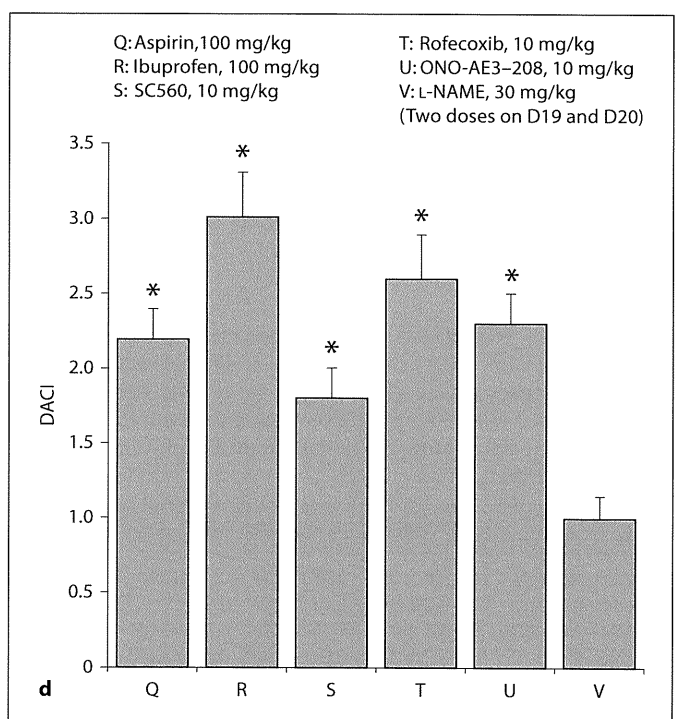
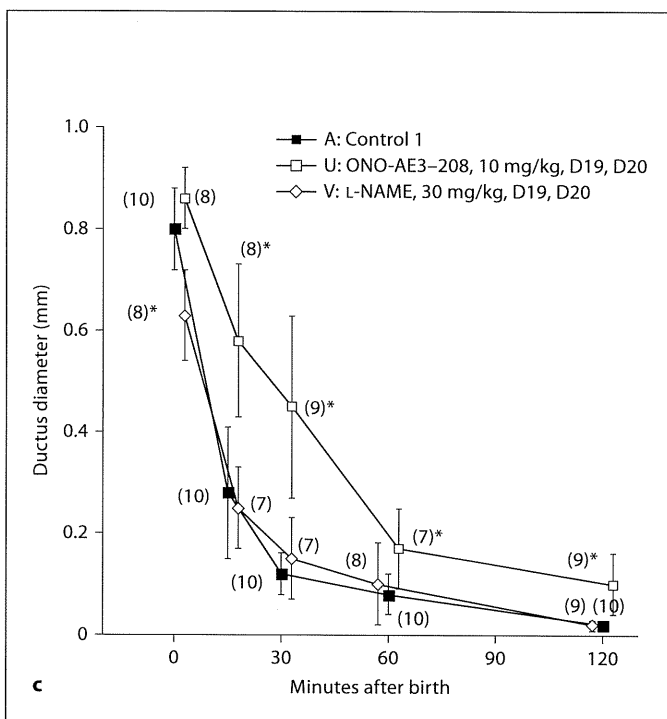
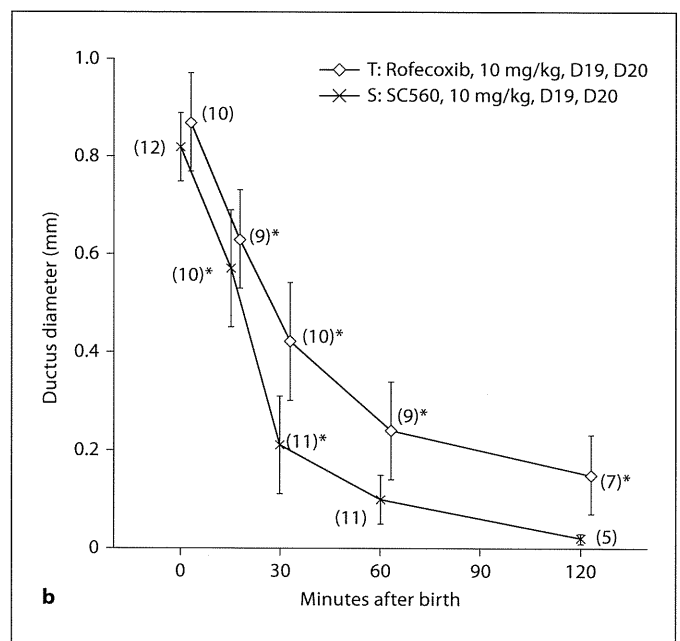
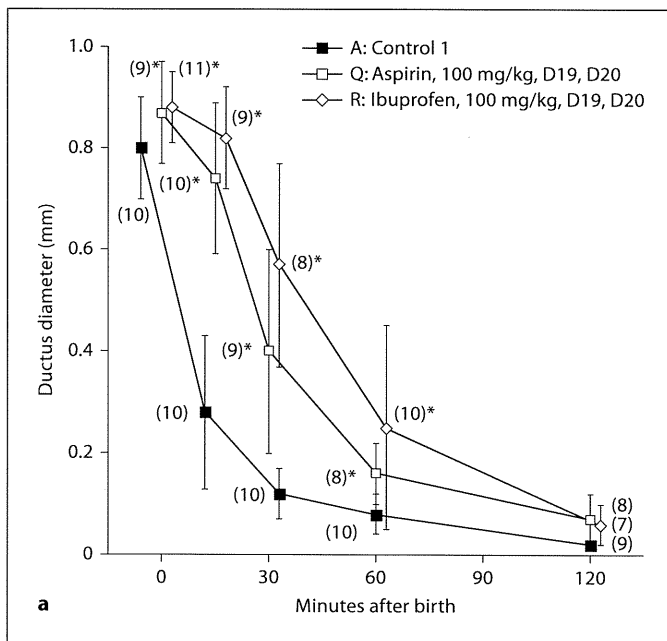
**Table 3.** Body weight and vascular diameters of the ascending aorta, main pulmonary artery and DA on D21 in control fetuses and those fetuses with early exposure to indomethacin (10 mg/kg), ONO-AE3-208 (10 mg/kg, a blocker of prostanoid EP<sub>4</sub> receptor) and L-NAME (30 mg/kg, subcutaneously, an NOS inhibitor) on D19 and D20

Drug	n	Weight, g	Aorta, mm	MPA, mm	Ductus, mm
A: none (control)	24	5.8 ± 0.3	0.78 ± 0.04	0.79 ± 0.04	0.80 ± 0.05
H: indomethacin	11	4.2 ± 0.5*	0.73 ± 0.08*	0.84 ± 0.14*	0.91 ± 0.13*
U: ONO-AE3-208	8	5.4 ± 0.3*	0.75 ± 0.05	0.80 ± 0.05	0.86 ± 0.05*
V: L-NAME	8	4.7 ± 0.5*	0.64 ± 0.06*	0.67 ± 0.07*	0.63 ± 0.09*

MPA = Main pulmonary artery. \* p < 0.05 vs. control.

#### Doses of Indomethacin on D19 and Neonatal Ductal Closure

Three doses of indomethacin (10, 1 and 0.1 mg/kg) were administered on D19 (studies E, K, N), with observation for any dose-dependent effects. The largest dose



**Fig. 4.** Data are means  $\pm$  SD. Figures in parentheses are numbers of animals. \*  $p < 0.05$  vs. control. **a** Neonatal ductal closure of rats in control group 1 and of rats following administration of aspirin (100 mg/kg) and ibuprofen (100 mg/kg) to the mother on D19 and D20 (studies A, Q, R). **b** Neonatal ductal closure of rats following administration of rofecoxib (10 mg/kg, a selective COX2 inhibitor) and SC560 (10 mg/kg, a selective COX1 inhibitor). **c** Neonatal

ductal closure of rats in control group 1 and of rats following administration of ONO-AE3-208 (10 mg/kg, a blocker of prostanoid EP<sub>4</sub> receptor) and L-NAME (30 mg/kg, subcutaneously, an NOS inhibitor) on D19 and D20 (studies U, V). **d** DACI in the neonatal rat with in utero exposure to those drugs other than indomethacin (studies Q–V).

eters were 80% of the control (table 3). The fetal body weight was small and was  $4.7 \pm 0.5$  g (81% of the control; table 3).

#### *Fetal Vascular Diameter*

Delayed neonatal ductal closure was associated with significant fetal ductal dilatation on D21 in these studies. Indomethacin 10 mg/kg on D19 induced enlargement of the fetal ductus on D21 by 10–20% (studies E, H, I, J). A significant 8–10% ( $p < 0.05$ ) dilatation of the ductus was present following in utero exposure to aspirin, ibuprofen, rofecoxib and ONO-208.

The main pulmonary artery was also dilated mildly associated with fetal ductal dilatation, and this was significant and was 7% compared to the control following administration of 10 mg of indomethacin on D19 and D20 (table 3). The aorta was not dilated.

#### *Fetal Body Weight*

As is shown in table 3, early administration of indomethacin and ONO-AE1-208 were associated with reduced body weight of the near-term rat. Small body weight in near-term rats was observed in every study with inhibition of COX on D19 and D20.

#### *Maternal and Fetal Mortality*

Maternal and fetal mortality in the control was less than 1%. Indomethacin 10 mg/kg induced maternal mortality of 25 and 28% following administration on D16 and D17, respectively. Fetal fatality was increased to 10% following administration of indomethacin 10 mg/kg on D16, D19 and D20. No mortality was associated with indomethacin used at 1 or 0.1 mg/kg. Repeated administration of indomethacin 10 mg/kg increased maternal and fetal mortality. Following administration of indomethacin on D19 and D20, maternal mortality was 10% and fetal mortality was 30%. Following administration of indomethacin on D18, D19 and D20, maternal mortality was 80% and fetal mortality was 40%. No maternal or fetal mortality was induced by aspirin, ibuprofen, SC560, rofecoxib, ONO-AE3-208 or L-NAME.

## **Discussion**

#### *Fetal PIC and Ductal Constriction*

Orogastric administration of indomethacin to the near-term mother without pretreatment induced dose-dependent fetal ductal constriction in the rat in early studies [10, 14]. The present study reconfirmed this and

clarified dose-dependent fetal PIC (table 2), and showed rapid postnatal ductal closure following administration of indomethacin 10 mg/kg at 4 h prior to birth. In contrast to these responses, the near-term fetal and neonatal ductus showed dilatation and delayed closure following in utero exposure to large doses of indomethacin 1 or 2 days before birth. One day after the administration of 10 mg/kg indomethacin, the fetal ductus was dilated, neonatal ductal closure was delayed and PIC was very high (7  $\mu$ g/kg) at 28 h after the administration, indicating persistent and profound inhibition of COX. Two days after the administration of 10 mg/kg indomethacin, the fetal ductus was dilated, neonatal ductal closure was further delayed and PIC was about 1  $\mu$ g/ml, suggesting persistent inhibition of COX. Therefore, fetal ductal dilatation and delay of the neonatal ductal closure were due to some kind of inhibition to ductal constrictive mechanisms, and this inhibition was induced by the inhibition of COX for more than 1 day in the fetus.

Although the reason is not clear, the slow decline in the indomethacin plasma concentration of the mother rats during the initial 24 h in this study was different from the rapid decline in adult nonpregnant rats reported by Hucker et al. [23].

#### *Fetal Exposure to Indomethacin and Ductal Closure Delay: Dose and Timing*

Experimentally and clinically, it has now been established that in utero exposure to indomethacin induces late tolerance or insensitivity to indomethacin [7–11]. Clinically, very wide ranges of timing and doses of indomethacin inducing late neonatal patency of the DA in the premature human baby have been reported [7–9].

With regard to the dose-effect relationship, this study indicated clearly that the indomethacin-induced delay in neonatal ductal closure was dose dependent, and the larger the dose of indomethacin, the greater the delay in neonatal ductal closure.

With regard to the timing of fetal exposure to indomethacin, the most prominent delay in the neonatal ductal closure was induced with a large dose of indomethacin on D19 and D20 in this study. In the rat, the ductus is premature on D19 and D20, and the constrictive reaction to the large dose of indomethacin is small [10, 21]. This is in sharp contrast to the severe constriction of the fetal ductus induced by indomethacin on D21.

Another interesting aspect of the timing study was the smaller but significant effect of indomethacin administered on D16 and D17. A null effect of early fetal stage was reported in mice [11]. D16 and D17 are early fetal stages



in the rat, and may indicate that in early fetal stage, indomethacin induces only a weak delay in ductal closure. The ductal constriction in response to oxygen is sensed by the Kv channel which increases in late gestation [24]. Fetal prostaglandin E is essential in preparing for postnatal ductal remodeling associated with organic closure [25]. It is considered that prostaglandin E is essential in the development of the ductal Kv channel in late gestation, although this remains to be proved.

Another interpretation is that indomethacin decreases ductal contractility on D16, but contractility recovers on D21, 5 days later. If we accept the latter interpretation, this observation has clinical importance, as will be discussed below.

Clinically, some investigators have not noticed any increase in the incidence of PDA in premature infants who were exposed to in utero indomethacin [26–28]. There seem to be 2 reasons for this. The first is the relatively small dose of indomethacin used for tocolysis. Usually, only about 2 mg/kg of indomethacin is used as a daily dose, and if we extrapolate the results of the dosage study in this experiment, this dose has a rather small delaying effect on postnatal ductal closure. The second is the timing of indomethacin administration. We noticed recovery of the delayed closure when indomethacin was administered 3–5 days before birth, and the same recovery effects seem to work clinically when there is an interval of 3 days or more between the in utero exposure and postnatal administration of indomethacin.

#### *Mechanism of the Delay*

In this study, all tested COX inhibitors and a prostanoid EP<sub>4</sub> antagonist, ONO-AE3-208, induced delay in the postnatal ductal closure. This is concordant with the early reports that COX1 and COX2 knockout mice died postnatally with PDA [29], and that prostaglandin EP<sub>4</sub> receptor knockout mice died in the same way [25, 30]. Recently, Yokoyama et al. [31] showed that prenatal prostaglandins and stimulus of prostanoid EP<sub>4</sub> were essential for postnatal ductal remodeling and organic closure. These results indicate that prenatal prostaglandins play an essential role in postnatal ductal constriction, remodeling and organic closure.

#### *Fetal Ductal Constriction by L-NAME*

In this study, in utero exposure to L-NAME did not induce any delay in ductal closure. Administration of L-NAME on D19 and D20 to pregnant rats induced severe constriction of the fetal DA [21]. This indicates that NO is the main dilator of the fetal DA in the preterm rat [21].

Following in utero exposure to L-NAME on D19 and D20 in this study, the fetuses had low body weights and small aortas, main pulmonary arteries and DA on D21, suggesting generalized and vascular growth retardation. However, postnatal ductal closure was not delayed, indicating that the fetal ductal constriction was not essential to the cause of the delay in the postnatal ductal closure in the rat.

#### *Extrapolation of the Results to the Clinical Situation*

If we extrapolate the results of this timing study to the clinical situation, the premature neonate with fetal exposure to a large dose of indomethacin on days 1, 2 or 3 before birth may develop persistent PDA which does not respond to indomethacin. Prophylactic administration of indomethacin [32, 33] administered immediately after birth may inhibit ductal closure further, and is contraindicated, and conservative treatment [34] is indicated. If the pediatrician waits for several days before administering indomethacin, the ductus may recover from the inhibitory effect of in utero exposure to indomethacin and become responsive to the drug. A critical and unanswered question is what is the necessary interval between the fetal exposure to indomethacin and postnatal recovery of ductal response to indomethacin, and this remains to be studied. If therapeutic indomethacin is not effective in the premature infant with symptomatic PDA and early fetal exposure to indomethacin, surgical treatment rather than repeated medical therapy is indicated.

#### **Conclusions**

In utero exposure to indomethacin in the mid-to-late fetal stage induced delay of postnatal ductal closure in the near-term rat with an interval of 1 or more days. The delay with indomethacin was dose dependent. A single large dose (10 mg/kg) of indomethacin was most effective in inducing the delay on D19, 2 days before birth, and least effective on D16, 5 days before birth, possibly showing recovery. Two large doses of indomethacin on D19 and D20 induced the largest delay. Other COX inhibitors and an EP<sub>4</sub> antagonist also induced a delay, indicating that the delay was caused by withdrawal of the prostaglandin EP<sub>4</sub> stimulus. The delay was temporary and delayed neonatal ductal closure tended to return to baseline following an interval of 3 days or more after exposure. Fetal ductal constriction by inhibition of NOS 1 and 2 days before birth did not delay neonatal ductal closure.

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## References

- 1 Rudolph AM: Congenital Diseases of the Heart, ed 2. Armonk, Futura, 2001, pp 155–196.
- 2 Van Overmeire B, Chemtob S: The pharmacologic closure of the patent ductus arteriosus. *Semin Fet Neonat Med* 2005;10:177–184.
- 3 Clyman RI: Developmental physiology of the ductus arteriosus; in Long WA (ed): *Fetal and Neonatal Cardiology*. Philadelphia, Saunders, 1990, pp 64–75.
- 4 Corbert AJ: Medical manipulation of the ductus arteriosus; in Garson A Jr, Bricker JT, Fisher DJ, Neish SR (eds): *The Science and Practice of Pediatric Cardiology*, ed 2. Baltimore, Williams & Wilkins, 1998, pp 2489–2513.
- 5 Brash AR, Hickey DE, Graham TP, Stahlman MT, Oates LA, Cotton RB: Pharmacokinetics of indomethacin in the neonate. *N Engl J Med* 1981;305:67–72.
- 6 Briggs GG, Freeman RK, Yaffe SJ: *Drugs in Pregnancy and Lactation*, ed 6. Philadelphia, Lippincott Williams & Wilkins, 2002, pp 705–715.
- 7 Norton ME, Merill J, Cooper BAB, Kuller JA, Clyman RI: Neonatal complications after the administration of indomethacin for preterm labor. *N Engl J Med* 1993;329:1602–1607.
- 8 Souter D, Harding J, McCowan L, O'Donnell C, McLeay E, Baxendale H: Antenatal indomethacin-adverse fetal effects confirmed. *Aust NZ J Obstet Gynaecol* 1998;38:11–16.
- 9 Suarez VR, Thompson LL, Jain V, Olson GL, Hankins GDV, Belfort ML, Saade GR: The effect of in utero exposure to indomethacin on the need for surgical closure of a patent ductus arteriosus in the neonate. *Am J Obstet Gynecol* 2002;187:886–888.
- 10 Momma K, Takao A: In vivo constriction of the ductus arteriosus by nonsteroidal anti-inflammatory drugs in near-term and preterm fetal rats. *Pediatr Res* 1987;22:567–572.
- 11 Reese J, Anderson JD, Brown N, Roman C, Clyman RI: Inhibition of cyclooxygenase isoforms in late- but not mid-gestation decreases contractility of the ductus arteriosus and prevents postnatal closure in mice. *Am J Physiol Regul Integr Comp Physiol* 2006;291:R1717–R1723.
- 12 Clyman RI, Chen YQ, Chemtob S, Mauray F, Kohl T, Varma DR, Roman C: In utero remodeling of the fetal lamb ductus arteriosus. Vasa vasorum proliferation, neointimal formation, and cell death. *Circulation* 2001;103:1806–1812.
- 13 Antman EM, Bennett JB, Daugherty A, Furberg C, Roberts H, Taubert KA: Use of non-steroidal anti-inflammatory drugs. An update for clinicians. A scientific statement from the American Heart Association. *Circulation* 2007;115:1634–1642.
- 14 Momma K, Takeuchi H: Constriction of fetal ductus arteriosus by non-steroidal anti-inflammatory drugs. *Prostaglandins* 1983;26:631–643.
- 15 Momma K, Hagiwara H, Konishi T: Constriction of fetal ductus arteriosus by non-steroidal anti-inflammatory drugs: study of additional 34 drugs. *Prostaglandins* 1984;28:527–536.
- 16 Momma K, Toyoshima K, Takeuchi D, Imamura S, Nakanishi T: In vivo constriction of the fetal and neonatal ductus arteriosus by a prostanoid EP<sub>4</sub>-receptor antagonist in rats. *Pediatr Res* 2005;58:971–975.
- 17 Hoernblad PY, Larson KS: Studies on closure of the ductus arteriosus I. Whole-body freezing as improvement of fixation procedures. *Cardiologia* 1967;51:231–236.
- 18 Momma K, Uemura S, Nishihara S, Ota Y: Dilatation of the ductus arteriosus by prostaglandins and prostaglandins' precursors. *Pediatr Res* 1980;14:1074–1077.
- 19 Sato J, Amizuka T, Niida Y, Umetsu M, Ito K: Simple, rapid and sensitive methods for determination of indomethacin in plasma by high-performance liquid chromatography with ultraviolet detection. *J Chromatogr* 1997;692:241–244.
- 20 Sissman NS: Developmental landmarks in cardiac morphogenesis: comparative chronology. *Am J Cardiol* 1970;25:141–148.
- 21 Momma K, Toyono M: The role of nitric oxide in dilating the fetal ductus arteriosus in rats. *Pediatr Res* 1999;46:311–315.
- 22 Wallenstein S, Zucker CL, Fleiss JL: Some statistical methods used in circulation research. *Circ Res* 1980;47:1–9.
- 23 Hucker HB, Zacchei AG, Cox SV, Brodie DA, Cantwell NHR: Studies on the absorption, distribution and excretion of indomethacin in various species. *J Pharmacol Exper Therap* 1966;153:237–249.
- 24 Thebaud B, Michelakis ED, Wu X-C, Moudgil R, Kuzyk M, Dyke JRB, Harry G, Hashimoto K, Haromy A, Rebecka I, Archer SL: Oxygen-sensitive Kv channel gene transfer confers oxygen responsiveness to preterm rabbit and remodeled human ductus arteriosus. Implications for infants with patent ductus arteriosus. *Circulation* 2004;110:1372–1379.
- 25 Nguyen MT, Camenisch T, Snouwaert JN, Hicks E, Coffman TM, Anderson PA, Malouf NN, Koller BH: The prostaglandin receptor EP<sub>4</sub> triggers remodelling of the cardiovascular system at birth. *Nature* 1997;390:78–81.
- 26 Abbasi S, Gerdes JS, Sehdev HM, Samimi SS, Ludmir J: Neonatal outcome after exposure to indomethacin in utero: a retrospective case cohort study. *Am J Obstet Gynecol* 2003;189:782–785.
- 27 Cordero L, Nankervis CA, Gardner D, Giannone PJ: The effects of indomethacin tocolysis on the postnatal response of the ductus arteriosus to indomethacin in extremely low birth weight infants. *J Perinatol* 2007;27:22–27.
- 28 Amin SB, Sinkin RA, Glantz JC: Meta-analysis of the effect of antenatal indomethacin on neonatal outcomes. *Am J Obstet Gynecol* 2007;197:486 e1–e10.
- 29 Loftin CD, Trivedi DB, Tiano H, Clark JA, Ebstein JA, Morham SG, Breyer M, Hawkins BM, Goulet JL, Smithies O, Koller BH, Langenbach R: Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2. *Proc Natl Acad Sci USA* 2001;98:1059–1064.
- 30 Segi E, Sugimoto Y, Yamazaki A, Aze Y, Oida H, Nishimura T, Murata T, Matsuoka T, Ushikubi F, Hirose M, Tanaka T, Yoshida M, Narumiya M, Ichikawa A: Patent ductus arteriosus and neonatal death in prostaglandin receptor EP<sub>4</sub>-deficient mice. *Biochem Biophys Res Comm* 1998;246:7–12.
- 31 Yokoyama U, Minamizawa S, Quan H, Ghatak S, Akaike T, Segi-Nishida E, Iwasaki S, Iwamoto M, Mizura S, Tamura K, Hori H, Yokota S, Toole BP, Sugimoto Y, Ishikawa Y: Chronic activation of the prostaglandin receptor EP<sub>4</sub> promotes hyaluronin-mediated neointimal formation in the ductus arteriosus. *J Clin Invest* 2006;116:3026–3034.
- 32 Fowlie PW, Davis PG: Prophylactic indomethacin for preterm infants: a systematic review and meta-analysis. *Arch Dis Child Fetal Neonatal Ed* 2003;88:F464–F466.
- 33 Cordero L, Nankervis CA, Delooye D, Giannone PJ: Indomethacin prophylaxis or expectant treatment of patent ductus arteriosus in extremely low birth weight infants? *J Perinatol* 2007;27:158–165.
- 34 Vanhaesebrouck S, Zonnenberg I, Vandervoort P, Bruneel E, Van hoestenbergh MR, Theyskens C: Conservative treatment for patent ductus arteriosus in the preterm. *Arch Dis Child Fetal Neonatal Ed* 2007;92:F244–F247.

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