FULL PAPER *Pharmacology*

Effects of TAK-044, a Nonselective Endothelin Receptor Antagonist, on the Spontaneous and Indomethacin- or Methylene Blue-Induced Constriction of the Ductus Arteriosus in Rats

Tatsuya TAKIZAWA, Emiko HORIKOSHI, Ming-Hao SHEN[1)], Toshio MASAOKA[1)], Hirotaka TAKAGI[2)], Masako YAMAMOTO[2)], Keiichiro KASAI[3)] and Kazuyoshi ARISHIMA[2)]

Departments of Developmental Biotechnology, [1)]Comparative Toxicology, [2)]Anatomy II, Azabu University School of Veterinary Medicine, Sagamihara, Kanagawa 229–8501, and [3)]Department of Neurosurgery, Toho University School of Medicine, Meguro, Tokyo 153–8515, Japan

(Received 19 October 1999/Accepted 20 January 2000)

ABSTRACT. We studied the effects of TAK-044, a nonselective endothelin (ET) receptor antagonist, on the indomethacin- or methylene blue-induced constriction of the ductus arteriosus (DA) in rats and compared them with the effects on spontaneous DA constriction. Injection of TAK-044 into 21-day-old fetuses *in utero* was performed through the uterine wall of laparotomized mother rats under light ether anesthesia. The fetuses were autopsied 3 hr after treatment with TAK-044 (10 mg/kg) *in utero* and simultaneous administration to the laparotomized mother rats of indomethacin (3 mg/kg, po) or methylene blue (100 mg/kg, ip). In the second experiment, pregnant rats were decapitated on day 21 of gestation to obtain newborn rats by cesarean delivery. Newborn rats which were given TAK-044 (2, 10 mg/kg) immediately after or 1 hr before cesarean delivery were autopsied at various times after birth. In both experiments, pups were rapidly frozen in an acetone-dry ice mixture at autopsy to evaluate the DA constriction by the whole-body freezing and shaving method. TAK-044 injection into the fetus 3 hr before autopsy completely inhibited the DA constriction induced by maternal treatment with indomethacin or methylene blue. TAK-044 caused dose-dependent inhibition of the spontaneous closure of the DA after birth. The inhibitory effect was more pronounced in pups which were given TAK-044 *in utero* 1 hr before birth; however, the inhibitory effect was incomplete in newborn pups. These results, together with the previous finding that BQ-123, an ET_A-specific receptor antagonist, inhibits the ductal constriction induced by oxygen *in vitro* [Coceani *et al.*, 1992], indicate that the ET_A receptor plays a significant role in the indomethacin- or methylene blue-induced DA constriction as well as in the spontaneous DA constriction after birth, and also indicate that the inhibition of ET_A receptor by TAK-044 was more easily achieved in fetuses than in neonates.—KEY WORDS: ductus arteriosus, endothelin receptor, indomethacin, methylene blue, TAK-044.

J. Vet. Med. Sci. 62(5): 505–509, 2000

The ductus arteriosus (DA) connects the main pulmonary artery and the descending aorta during the fetal period, allowing blood to flow from the right ventricle to bypass the lungs. Ductal patency *in utero* is an active state principally maintained by the potent dilator effect of prostaglandins, particularly PGE_2 [9], and the nitric oxide-cGMP system [15, 30]. Closure at birth occurs because of contraction of the smooth muscle of the DA. The major factor actively stimulating contraction is probably the effect of increasing oxygen tension. After birth, the ductus is exposed to arterial blood because of the reversal of the direction of flow [14], and arterial oxygen tension rises rapidly after delivery [17].

There is a large body of evidence obtained from studies on the lamb ductus arteriosus which supports the cytochrome P_{450}/endothelin (ET)-1 hypothesis [8]. In this hypothesis, cytochrome P_{450} is the oxygen sensor, and its activation promotes contraction. The effect of oxygen on the enzyme is proposed to lead to the release from endothelial and smooth muscle cells of ET-1, which causes contraction of the ductus arteriosus via the action of the ET_A receptor in the lamb's ductal strips [11]. Indomethacin and methylene blue are known to cause DA constriction [1, 24, 30] through the inhibition of the dilator effect of PGE_2 [18, 21, 23] or cGMP [12, 16, 20]. However, the role of the endothelin receptor in the DA constriction induced by these agents is still unknown.

The present study was designed to obtain information about the effect of TAK-044 [3, 19], a nonselective endothelin receptor antagonist, on the indomethacin- or methylene blue- induced constriction of the fetal DA in order to determine the physiological role of the ET receptor in the DA constriction by these agents. We also examined the effects of TAK-044 on the spontaneous closure of the DA in newborn pups as compared with those in fetuses.

MATERIALS AND METHODS

Animals: Female Wistar rats (Charles River Japan Inc., Tokyo), 10–12 weeks old at the time of mating, were used. They were maintained on a commercial diet (CE-2, Clea Japan, Tokyo) and tap water *ad libitum*, and kept in a room at 22 ± 3°C, with relative humidity of 55 ± 10%. Three females were placed with a male overnight and examined the next morning for the presence of sperm in a vaginal smear. The day on which sperm were found was designated as day 0 of gestation, and the females were caged individually thereafter.

Effects of TAK-044 on the indomethacin- or methylene blue-induced constriction of the DA in utero: The effects of TAK-044 on the ductal constriction induced by maternal treatment with indomethacin or methylene blue were examined in 21-day-old fetuses. The pregnant rats were subjected to mid-ventral laparotomy under light ether anesthesia on day 21 of gestation. Three fetuses in a uterine horn in each litter were given a subcutaneous injection of 10 mg/kg of TAK-044 (Takeda Chemical Ind., Ltd., Osaka, Japan) dissolved in 50 μl of saline through the uterine wall, while uninjected and saline-injected littermate fetuses in the contralateral uterine horn in each litter served as controls. Indomethacin (3 mg/kg, po) or methylene blue (100 mg/kg, ip) was administered to the laparotomized pregnant rats just after the operation. Autopsy was performed 3 hr later.

Effects of TAK-044 on the spontaneous closure of the DA: The effects of TAK-044 on the spontaneous closure of the DA were examined in newborn rats. Pregnant rats from day 21 of gestation were decapitated to obtain newborn rats by cesarean section. Then 2 or 3 newborn rats in each litter were given a subcutaneous injection of 2 or 10 mg/kg of TAK-044. Pups injected with the same volume of saline served as controls.

In addition, because of the possible latent period of the drug, the effects of TAK-044 on the spontaneous closure of the DA were also examined in newborn rats which were injected with 10 mg/kg of TAK-044 subcutaneously *in utero* 1 hr before cesarean section. In these experiments, 2 or 3 fetuses in a uterine horn in each litter were given a subcutaneous injection of 10 mg/kg of TAK-044 as described above, while uninjected and saline-injected littermate fetuses in the contralateral uterine horn in each litter served as controls. Newborn pups were obtained by cesarean section just after maternal decapitation. The pups were maintained in a humidified chamber at 37°C until sampling. Autopsy was performed at 0.5, 1, 1.5 and 3 hr after cesarean delivery.

Caliber determination: In both the first and second series of experiments, each pup was rapidly frozen in an acetone-dry ice mixture at sampling. The frozen pups were weighed and stored for a few days at –20°C until caliber determination. The DAs and pulmonary arteries (PAs) of the pups were calibrated by the whole-body freezing and shaving method described elsewhere [1, 2], and the DA/PA ratio was obtained by a previously described method [29].

Data analysis: Results are expressed as the mean ± S.E.M. The differences among groups of pups were assessed using analysis of variance (ANOVA). If a significant difference among the groups was demonstrated, Scheffe's test was applied to assess the difference between groups. A p value of less than 0.05 was considered statistically significant.

RESULTS

Effects of TAK-044 on the constriction of the DA induced by indomethacin or methylene blue in utero: Indomethacin or methylene blue caused significant constriction of the fetal DA 3 hr after treatment when it was administered to pregnant rats on day 21 of gestation. Subcutaneous injection of TAK-044 to fetuses at a dose of 10 mg/kg 3 hr before autopsy completely inhibited the DA constriction induced by maternal treatment with indomethacin (3 mg/kg) or methylene blue (100 mg/kg) (Fig. 1A, B).

Effects of TAK-044 on the spontaneous closure of the DA: TAK-044 injection of newborn pups just after cesarean delivery at a dose of 2 mg/kg caused a slight but significant inhibition of spontaneous closure of the DA at 3 hr after birth ($p<0.05$, Fig. 2A). The inhibitory effect of TAK-044 on the spontaneous closure of the DA was more marked when the dose of TAK-044 was increased to 10 mg/kg. Injection of TAK-044 into fetuses *in utero* at a dose of 10 mg/kg 1 hr before birth did not cause any change of the DA caliber *in utero*; however, it caused significant inhibition of spontaneous closure of the DA at 1.0 and 1.5 hr after birth ($p<0.05$, Fig. 2B). The littermate controls were not affected in any case.

DISCUSSION

The present findings revealed that TAK-044 completely inhibited the fetal DA constriction induced by maternal treatment with indomethacin or methylene blue. In newborn pups, TAK-044 inhibited the spontaneous closure of the DA in a dose-dependent manner. The inhibitory effect of TAK-044 on the DA was more pronounced in newborn rats which were injected with TAK-044 1 hr before birth in order to allow for a possible latent period of the drug action. However, the inhibitory effect was incomplete. Considering the present findings together with the report of Coceani *et al.* [11] showing that BQ-123, an ET_A-specific antagonist, inhibits the DA constriction induced by increasing oxygen tension *in vitro*, we conclude that the DA constriction induced by indomethacin or methylene blue and increasing oxygen tension after birth results from the actions of the ET_A receptor, and that the contractive effect results from the actions of the ET_A receptor exerted in the rat fetuses and neonates.

It is known that the oxygen tension is low in fetuses but rises rapidly after birth [17], whereas dilator prostaglandin concentrations are high in fetuses but decline in neonates [4, 6, 7, 22, 26]. Comparison of the present results in fetuses and neonates clearly indicated that TAK-044 caused complete inhibition of the DA constriction induced by indomethacin or methylene blue in fetuses (low oxygen tension) and incomplete inhibition of the DA constriction in neonates (high oxygen tension) *in vivo*. These findings are consistent with the report of Coceani *et al.* [11] in which it was shown that BQ-123 caused an inhibition of constriction of the vessel that was inversely proportional to the neonatal oxygen tension *in vitro*.

Ductal caliber is maintained by a balance of opposing actions by oxygen and dilating effectors, especially circulating PGE_2 [5] and nitric oxide [15]. Indomethacin

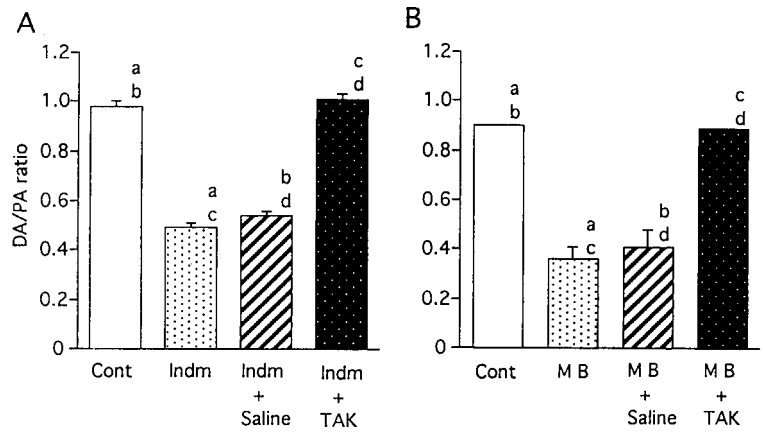

Fig. 1. Changes in the DA/PA ratio of fetuses in which 10 mg/kg of TAK-044, a nonselective endothelin receptor antagonist, was subcutaneously injected through the uterine wall of laparotomized pregnant rats which were then immediately administered (A) 3 mg/kg of indomethacin or (B) 100 mg/kg of methylene blue. Open columns represent controls from intact pregnant mothers, dotted and hatched columns represent the untreated and saline-injected littermate controls, respectively, and shaded columns represent the TAK-044-injected fetuses. The columns with vertical bars represent the mean ± S.E.M (n=12 fetuses from 4 litters). Groups marked with the same letters had significantly different DA/PA ratios (ex., a: $p<0.001$).

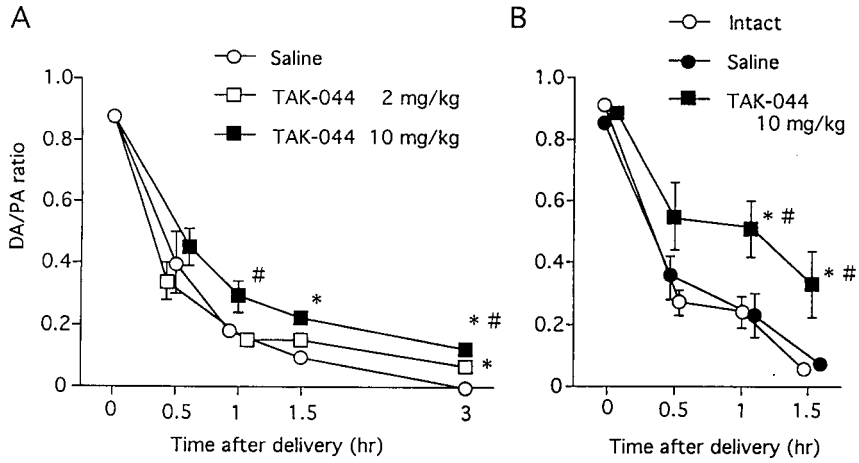

Fig. 2. Time-course of changes in the DA/PA ratio of the neonatal ductus arteriosus after subcutaneous injection of TAK-044 (2, 10 mg/kg), a nonselective endothelin receptor antagonist, to (A) newborn rats immediately after cesarean delivery or to (B) fetuses *in utero* through the uterine wall of laparotomized pregnant rats 1 hr before cesarean delivery. Open circles represent saline-injected pups and open and solid squares represent pups injected with 2 and 10 mg/kg of TAK-044, respectively (Fig. 2A). Open and solid circles represent uninjected and saline-injected littermates, respectively, and solid squares represent pups injected with 10 mg/kg of TAK-044 (Fig. 2B). Each symbol with vertical bar represents the mean ± S.E.M. (n=6–19 pups from 4–8 litters for Fig. 2A and n=7–10 pups from 7–10 litters for Fig. 2B). *, #: Significantly different from the saline control or intact control (*: $p<0.05$) and the low-dose or saline control group (#: $p<0.05$).

and methylene blue are known to cause DA constriction [1, 24, 30] via the inhibition of PGE_2 [18, 21, 23] or cGMP [12, 16, 20] synthesis. PGE_2 elevates intracellular cAMP in the lamb DA [32]. Increased levels of cAMP and cGMP dilate the lamb DA [13]. Therefore, the present findings indicate that the ET_A receptor plays a significant role in the DA constriction induced by decreased dilating actions of PGE_2 or cGMP and increased contractile action of oxygen after

birth.

Recently, the EP_4 receptor was proposed to mediate the PGE_2-induced dilation of the ductus arteriosus in rabbits on the basis of the selectivity of several PGE analogues [28]. Nyugun *et al*. [25] reported that PGE_2 receptor (EP_4)-lacking mice (EP_4-/-) failed to constrict the DA after birth, and they suggested that the EP_4 receptor triggers remodeling of the cardiovascular system at birth. Similar results were also reported in another type of EP_4-lacking mice [27]. Based on these reports, the present findings are consistent with the hypothesis that the decline in the plasma PGE_2 concentration is a key factor in the spontaneous constriction of the DA. Other mechanisms may also be involved in the spontaneous closure of the DA, such as oxygen-sensitive delayed rectifier potassium channels [31].

In conclusion, the ET_A receptor plays a significant role in the indomethacin- or methylene blue-induced and spontaneous DA constriction after birth, and the inhibition of the ET_A receptor by TAK-044 was more easily achieved in fetuses than in neonates.

ACKNOWLEDGMENT. We are grateful to Takeda Chemical Ind., Ltd., Osaka, Japan, for the kind donation of TAK-044.

REFERENCES

1. Arishima, K., Yamamoto, M., Takizawa, T., Ueda, Y., Kusanagi, M. and Eguchi, Y. 1991. Onset of the constrictive effect of indomethacin on the ductus arteriosus in fetal rats. *Acta Anat.* 142: 231–235.
2. Arishima, K., Yamamoto, M., Takizawa, T., Somiya, H., Eguchi, Y. and Shiota, K. 1993. Effect of acute maternal alcohol consumption on the fetal ductus arteriosus. *Biol. Neonate* 63: 40–43.
3. Awane, Y., Kusumoto, K., Kubo, K., Kawata, A., Kikuchi, T., Wakimasu, M., Watanabe, T. and Fujino, M. 1994. Pharmacological profile *in vivo* of a new endothelin receptor antagonist, TAK-044. *Jpn. J. Pharmacol.*: 64: suppl. I, 167.
4. Challis, J. R. G., Dilley, S. R., Robinson, J. S. and Thorburn, G. D. 1976. Prostaglandins in the circulation of the fetal lamb. *Prostaglandins* 11: 1041–1046.
5. Clyman, R. I. 1987. Ductus arteriosus: current theories of prenatal and postnatal regulation. *Semin. Perinatol.* 11: 64–71.
6. Clyman, R. I., Mauray, F., Roman, C., Rudolph, A. M. and Heymann, M. A. 1980. Circulating prostaglandin E_2 concentrations and patent ductus arteriosus in fetal and neonatal lamb. *J. Pediatr.* 97: 455–461.
7. Clyman, R. I., Wong, L., Heymann, M. A. and Rudolph, A. M. 1980. Responsiveness of the lamb ductus arteriosus to prostaglandins and their metabolites. *Prostaglandins* 15: 325–331.
8. Coceani, F. 1994. Control of the ductus arteriosus –A new function for cytochrome P_{450}, endothelin and nitric oxide. *Biochem. Pharmacol.* 48: 1315–1318.
9. Coceani, F. and Olley, P. M. 1988. The control of cardiovascular shunts in the fetal and perinatal period. *Can. J. Physiol. Pharmacol.* 66: 1129–1134.
10. Coceani, F., Armstrong, C. and Kelsey, L. 1989. Endothelin is a potent constrictor of the lamb ductus arteriosus. *Can. J. Physiol. Pharmacol.* 67: 902–904.
11. Coceani, F., Kelsey, L. and Seidlitz, E. 1992. Evidence for an effector role of endothelin in closure of ductus arteriosus at birth. *Can. J. Physiol. Pharmacol.* 70: 1061–1064.
12. Coceani, F., Kelsey, L. and Seidlitz, E. 1994. Occurrence of endothelium-derived relaxing factor-nitric oxide in the lamb ductus arteriosus. *Can. J. Physiol. Pharmacol.* 72: 82–88.
13. Coceani, F., Kelsey, L. and Seidlitz, E. 1996. Carbon monoxide-induced relaxation of the ductus arteriosus in the lamb: evidence against the prime role of guanylyl cyclase. *Br. J. Pharmacol.* 118: 1689–1696.
14. Dawes, G. S., Mott, J. C. and Widdicombe, J. G. 1955. The patency of the ductus arteriosus in newborn lambs and its physiological consequences. *J. Physiol.* 128: 361–383.
15. Fox, J. J., Ziegler, J. W., Ivy, D. D., Halbower, A. C., Kinsella, J. P. and Abman, S. H. 1996. Role of nitric oxide and cGMP system in regulation of ductus arteriosus tone in ovine fetus. *Am. J. Physiol.* 271: H2638–H2645.
16. Gruetter, C. A., Gruetter, D. Y., Lyon, J. E., Kadowitz, P. J. and Ignarro, L. J. 1981. Relationship between cyclic 3', 5'-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. *J. Pharmacol. Exp. Ther.* 219: 181–186.
17. Heymann, M. A. and Rudolph, A. M. 1975. Control of the ductus arteriosus. *Physiol. Rev.* 55: 62–78.
18. Heymann, M. A., Rudolph, A. M. and Silverman, N. H. 1976. Closure of the ductus arteriosus in premature infants by inhibition of prostaglandin synthesis. *New Engl. J. Med.* 295: 530–544.
19. Ikeda, S., Awane, Y., Kusumoto, K., Wakimasu, M., Watanabe, T. and Fujino, M. 1994. A new endothelin receptor antagonist, TAK-044, shows long-lasting inhibition of both ET_A- and ET_B-mediated blood pressure responses in rats. *J. Pharmacol. Exp. Ther.* 270: 728–733.
20. Kontos, H. A. and Wei, E. P. 1993. Hydroxyl radical dependent inactivation of guanylate cyclase in cerebral arterioles by methylene blue and by LY83538. *Stroke* 24: 427–434.
21. Levin, D. L. 1980. Effects of inhibition of prostaglandin synthesis on fetal development, oxygenation, and the fetal circulation. pp 35–44. *In*: Prostaglandins in the Perinatal Period (Heymann, M. A. ed.), Grune & Stratton, New York.
22. Mitchell, M. D., Brunt, J., Clover, L. and Walker, D. W. 1980. Prostaglandins in the umbilical and uterine circulations during late pregnancy in the ewe. *J. Reprod. Fertil.* 58: 283–287.
23. Moise, K. J. Jr., Huhta, J. S., Sharif, D. S., Ching-Nan, O., Kirshon, B., Wasserstrum, N. and Cano, L. 1988. Indomethacin in the treatment of premature labor: effects on the ductus arteriosus. *New Engl. J. Med.* 319: 327–331.
24. Momma, K. and Takao, A. 1987. *In vivo* constriction of the ductus arteriosus by nonsteroidal anti-inflammatory drugs in near-term and preterm fetal rats. *Pediatr. Res.* 22: 567–572.
25. Nguyen, M. T., Camenisch, T., Snouwaert, J. N., Hicks, E., Coffman, T. M., Anderson, P. A. W., Malouf, N. N. and Koller, B. H. 1997. The prostaglandin receptor EP_4 triggers remodeling of the cardiovascular system at birth. *Nature (Lond.)* 390: 78–81.
26. Piper, P. J., Vane, J. R. and Wyllie, J. H. 1970. Inactivation of prostaglandins by the lungs. *Nature (Lond.)* 225: 600–604.
27. Segi. E., Sugimoto, Y., Yamasaki, A., Aze, Y., Oida, H., Nishimura, T., Murata, T., Matsuoka, T., Ushikubi F., Hirose, M., Tanaka, T., Yoshida, N., Narumiya, S. and Ichikawa, A.

1998. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP_4-deficient mice. *Biochem. Biophys. Res. Commun.* 246: 7–12.
28. Smith, G. C. S., Coleman, R. A. and McGrath, J. C. 1994. Characterization of dilator prostaglandin receptors in the fetal rabbit ductus arteriosus. *J. Pharmacol. Exp. Ther.* 271: 390–396.
29. Takizawa, T., Arishima, K., Yamamoto, M., Kusanagi, M., Somiya, H. and Eguchi, Y. 1992. Studies on closure of the ductus arteriosus in perinatal rats. *J. Vet. Med. Sci.* 54: 1195–1198.
30. Takizawa, T., Horikoshi, E. and Kamata, A. 1999. Role of the nitric oxide-cGMP system in the regulation of ductus arteriosus patency in fetal rats. *J. Vet. Med. Sci.* 61: 1277–1280.
31. Tristani-Firouzi, M., Reeve, H. L., Tolarova, S., Weir, E. K. and Archer, S. L. 1996. Oxygen-induced constriction of rabbit ductus arteriosus occurs via inhibition of a 4-aminopyridine-voltage-sensitive potassium channel. *J. Clin. Invest.* 98: 1959–1965.
32. Walsh, R. S. and Mentzer, R. M. 1987. Role of cyclic nucleotides in relaxation of fetal lamb ductus arteriosus. *Surgery* 102: 313–318.