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Vasodilating Effect and Tissue Accumulation of Prostaglandin E_1 Incorporated in Lipid Microspheres on the Rat Ductus Arteriosus

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ABSTRACT—Prostaglandin E_1 incorporated in lipid microspheres (lipo PGE_1) was administered to the umbilical vein of neonatal rats. Morphological measurement and quantitative autoradioluminography assessed the relationship between the vasodilating effect and tissue accumulation of lipo PGE_1 in the ductus arteriosus. In the morphological measurement under microscopy, the inner diameter ratio of the ductus arteriosus to the main pulmonary artery after infusion of ^{3}H-labeled lipo PGE_1 (^{3}H-lipo PGE_1) continued to remain significantly higher than that of free ^{3}H-PGE_1. Autoradioluminography of the frozen frontal section of neonates after intravenous infusion of ^{3}H-lipo PGE_1 for 2 h revealed that the ductus levels of radioactivity were higher than those of free ^{3}H-PGE_1 in saline solution, although the blood levels were almost equal. Localization of lipo PGE_1 labeled with a lipophilic fluorescent probe, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (diI), in the endothelial cells of the ductus arteriosus was confirmed by confocal laser scanning microscopy. These findings suggest that the incorporation of lipid microspheres by the endothelial cells is one of the mechanisms that enables lipo PGE_1 to accumulate to higher levels in the ductus tissue and to act more efficiently than free PGE_1 in neonatal rats.

Keywords: Prostaglandin E_1, Lipid microsphere, Ductus arteriosus, Drug delivery, Autoradioluminography

Infants with severe congenital heart disease depend on the postnatal patency of the ductus arteriosus for maintenance of adequate pulmonary blood flow and systemic oxygenation (1, 2). Since prostaglandin E_1 (PGE_1) dilates the ductus arteriosus in infants and in many neonatal animals, PGE_1 has been used for emergency treatment in preparation for surgery (2–6). However, PGE_1, which is rapidly inactivated in the lung, must be administered at a high dose (7–9). Lipo PGE_1 is a lipid emulsion produced by dissolving PGE_1 in lipid microspheres (10, 11). By means of incorporation in lipid microspheres, PGE_1 is able to increase its effectiveness and to reduce toxicity (10–12). The ductus-dilating effect of lipo PGE_1 is more potent and continues longer than that of free PGE_1 (inclusion complex with cyclodextrin) on the ductus arteriosus of the infant (12–14).

Lipid microspheres are stable drug carriers. The possibility that they increase the effectiveness of incorporated drugs has been suggested. Although the assumed benefits of lipid microspheres are avoidance of inactivation in the lung and preferential incorporation by endothelial cells (15, 16), there is no clear evidence for the relationship between the effect and tissue accumulation of lipo PGE_1.

Rat fetuses were used to determine the ductus-constricting effect by maternal ingestion of nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin (17). Because development of the fetal rat is very rapid, and ductal constriction is induced in only one hour, this animal model is useful for evaluating the ductus-dilating effect (18).

In this study, we have investigated the relationship between the ductus-dilating effect determined by morphological measurement and tissue accumulation of lipo PGE_1 determined by quantitative autoradioluminography using ^{3}H-labeled lipo PGE_1 in neonatal rats. Additionally, fluorescence microscopy was used to determine the distribution of fluorescence-labeled lipo PGE_1 into the tissue of the ductus arteriosus.

MATERIALS AND METHODS

Chemicals

[5,6-^{3}H(*N*)]-Prostaglandin E_1 (^{3}H-PGE_1, 2.22 TBq /mmol) was obtained commercially from Du Pont Co., Ltd. (Wilmington, DE, USA). Unlabeled PGE_1 was synthesized at Taisho Pharmaceutical Co., Ltd. (Tokyo). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (diI; Sigma, St. Louis, MO, USA) was obtained commercially. Soybean oil (Ajinomoto Co., Inc., Tokyo), egg yolk lecithin (Asahi Chemical Industry Co., Tokyo), and glycerin (Kozakai Pharmaceutical Co., Tokyo) were of reagent grade and were obtained commercially. All other chemicals were of reagent grade.

Preparation of lipo PGE_1

A mixture of 500 mg of soybean oil containing 25 μg or 187.5 μg of PGE_1, 90 mg of egg yolk lecithin and 12 mg of oleic acid were prepared. The radioassay was conducted with ^{3}H-PGE_1. To the mixture were added 110.5 mg of glycerol and a sufficient volume of purified water to make a final volume of 5 ml. The obtained mixture was then treated with a high pressure homogenizer (French Miniature Pressure Cell; American Instrument Company, Urbana, IL, USA). The diI-labeled lipo PGE_1 (diI-lipo PGE_1) was prepared in the same manner as described above, with the soybean oil containing 1 mg of diI per gram (15). The mean particle sizes of ^{3}H- and diI-lipo PGE_1 were confirmed as 0.2 μm by a light scattering spectrometer (model 370; Nicomp Instruments Division Pacific Scientific, Silver Spring, MD, USA) (11).

Animals

Pregnant Wistar rats 12- to 14-week-old, were obtained from Shizuoka Laboratory Animal Center (Shizuoka) on days 14−17 of pregnancy. Mating was confirmed by vaginal smear tests, and the day of mating was defined as day 0 of pregnancy. On day 21 of pregnancy, animals were sacrificed by cervical dislocation and fetuses were delivered quickly by cesarean section. Live fetuses together with the placentas were maintained on an incubator at 38℃ for 1 h.

Administration of drug to rat neonates

For the experiments of bolus administration, ^{3}H-lipo PGE_1 diluted with 4 vol of isotonic saline and free ^{3}H-PGE_1 dissolved in isotonic saline were injected rapidly with a microlitersyringe (Hamilton Company, Reno, NV, USA) into the umbilical vein of neonatal rats 1 h after cesarean section at a dose of 10 μg as PGE_1/kg. For the experiments of constant rate infusion, ^{3}H-lipo PGE_1 and free ^{3}H-PGE_1 were prepared in the same manner as that of bolus administration and infused at 1 h after cesarean section for 2 h to the neonatal rats with a microliter-syringe and infusion pump (model 22; Harvard Apparatus, South Natick, MA, USA) at a dose of 2.5 μg as PGE_1/kg per minute.

Measurement of ductus arteriosus inner diameter

The neonates administered ^{3}H-labeled drugs were rapidly frozen and fixed by placing them in isopentane with dry ice (about −80℃) (4). The frozen chest was trimmed and mounted on a tissue holder to obtain a sectioning surface perpendicular to the ductus arteriosus. A frozen section (6-μm-thick) was cut in a frozen microtome (HM-500; Microm Co., Heidelberg, Germany) and thaw-mounted on a glass slide (Fig. 1) (19). After exposure to the imaging plate (described in the following section, measurement of tissue radioactivity), the section was stained with hematoxylin and eosin (H.E.). The morphology of the section was photographed with a microscope (Leitz DM RBE; Leica Mikroskopie und Systeme GmbH, Watzlar, Germany) and 35 mm color film (Super G 400; Fuji Photo Film, Co., Ltd., Tokyo). The inner diameters (I.D.) of the main pulmonary artery and the ductus arteriosus were measured in the photograph of the section. The I.D. of the ductus arteriosus was recorded from between the branch of the left and right pulmonary arteries and the origin of the descending aorta. Dilation of the ductus arteriosus was expressed as the ratio of the I.D. of the ductus arteriosus to that of the main pulmonary artery (I.D. ratio), which was used to determine the effect of the drugs (17).

Measurement of tissue radioactivity by bio-imaging analyzer

Radioactivity in the ductus arteriosus and whole blood were determined by a bio-imaging analyzer (BAS-3000, Fuji Photo Film) (20). The chest section mounted on a glass slide was dried in the microtome at −15℃ overnight, and was exposed to an imaging plate (IP; BAS-TR, Fuji Photo Film). After exposure for 72 h, IP was scanned by a bio-imaging analyzer (gradation: 4096, resolution: 50 μm, sensitivity: 10,000, latitude: 4), and the scanned image data was measured by image processing with an analytical program BAStation (Fuji Photo Film) to photostimulated luminescence value (PSL/mm^2). The histological area of individual sections for quantification was determined by referring to the tissue photograph of the same exposed section stained with H.E. Radioactivity in the ductus arteriosus and whole blood was determined in the area at the arterial wall and at the right atrium or right ventricle, respectively. The individual concentration value of radioactivity was expressed as the mean value of the three sequential sections.

Calibration of tissue radioactivity

The calibration coefficient of the PSL value to concentration of radioactivity was calculated based on the densities of ^3H-standard tissues. The neonatal hearts collected at 30 min after dosing with ^3H-lipo PGE_1 (1, 5 and 20 μg/kg) and the densities of ^3H-PGE_1 added to the mature male rat blood were used as the ^3H-standards for ductus arteriosus and blood, respectively. The heart samples or the parts of the blood were weighed and dissolved with 0.5 ml of Soluen-350 (Packard Instruments Co., Meriden, CT, USA) in glass vials. After decolorization and neutralization of the samples by addition of 30% (w/v) H_2O_2 and 1 N HCl, a scintillator (Instagel, Packard) was added to each vial, and the radioactivity was determined in a liquid scintillation counter (LS6000TA; Beckman Instruments, Inc., Fullerton, CA, USA). The radioactivity of each sample was converted to the nM-equivalent of PGE_1/g of the tissue (wet weight) or /ml of blood, based on the specific radioactivity of the dose administered. Other parts of the heart samples or the blood were frozen and sectioned. After drying the sections in the microtome at -15℃ overnight, their radioactivity was also determined by a bio-imaging analyzer, as described above.

Confocal microscopy

Fluorescence derived from diI-lipo PGE_1 was visualized using a confocal laser scanning microscope (CLSM Fluovert Argon Laser; Leica Lasertechnik GmbH, Heidelberg, Germany) on an inverted microscope (Leitz FLUOVERT FU; Leica Mikroskopie und Systeme GmbH, Ernst-Leitz-Straße, Germany) equipped with a 40× objective (NPL Flouter 1.30 Oil Fluoreszenz; Leitz Wetzlar GmbH, Wetzlar, Germany), a 514-nm excitation filter, a 580-nm dichroic mirror and a 515-nm emission filter. The illumination sources were 488- and 514-nm lines from a 25-mW argon laser. Photographs were printed with a full color digital printer (Pictrography 3000, Fuji Photo Film).

Statistics

Values were expressed as the mean±S.E.M. Statistical significance was accepted at the level of $P<0.05$, calculated by Student's *t*-test or the Aspin-Welch's *t*-test after the equality of variance was examined by the *F*-test with the SAS system (version 6.12).

RESULTS

Effect of lipo PGE_1 and PGE_1 on ductal dilatation

The photographs of the ductus arteriosus and the I.D. ratio of the ductus arteriosus to the main pulmonary artery (I.D. ratio) following rapid injection and infusion of ^3H-lipo PGE_1 or ^3H-PGE_1 to neonatal rats are shown in Figs. 1 and 2, respectively. The ductus arteriosus of the untreated control animal was already constricted at 1 h after cesarean section, and its I.D. ratio was 0.19. Five minutes after rapid injection of ^3H-lipo PGE_1, the I.D. ratio was immediately increased to 0.78 and decreased to 0.31 at 1 h. When ^3H-PGE_1 was injected rapidly, the I.D. ratio, which was almost similar to that of ^3H-lipo PGE_1, was 0.77 at 5 min and decreased to 0.23 at 1 h (Fig. 2A).

In the cases of constant rate infusion of ^3H-lipo PGE_1 for 2 h, the I.D. ratio was 0.99 at the termination of infusion, which indicated that the ductus artery was fully dilated, and it remained higher than 0.5 over 30 min. On the other hand, 30 min after termination of infusion of free ^3H-PGE_1, the I.D. ratio decreased rapidly to 0.3 (Fig. 2B).

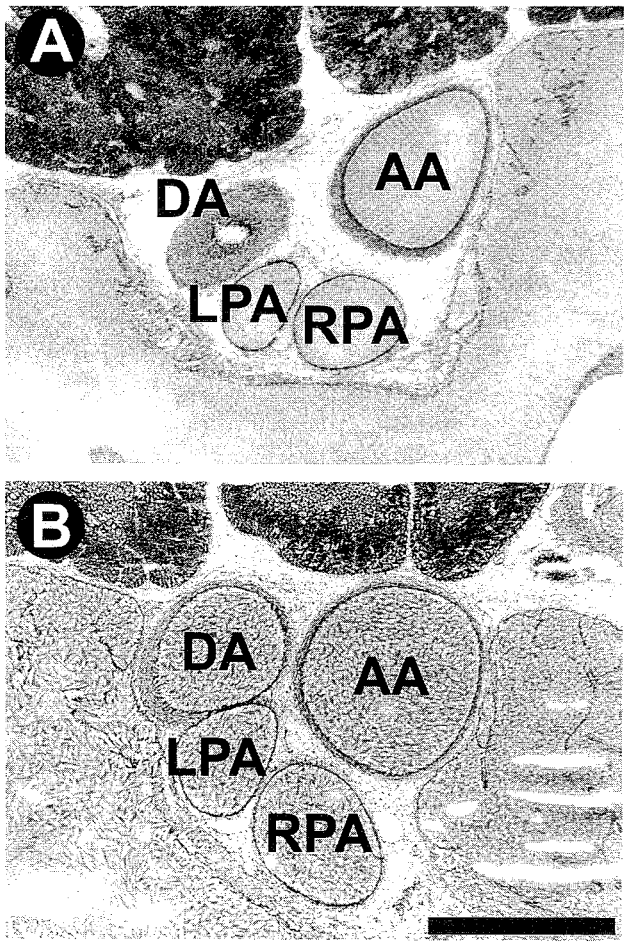

Fig. 1. Frontal sections of a typical ductus arteriosus stained with hematoxylin and eosin from non-treated (A) and ^3H-lipo PGE_1-treated (B) neonatal rats. Photo A shows the ductus arteriosus from a non-treated rat 1 h after cesarean section. Photo B shows the ductus arteriosus at 5 min after rapid injection of ^3H-lipo PGE_1 at 10 μg/kg. AA, aortic arch; DA, ductus arteriosus; LPA and RPA, left and right pulmonary artery, respectively. Scale bar indicates 500 μm.

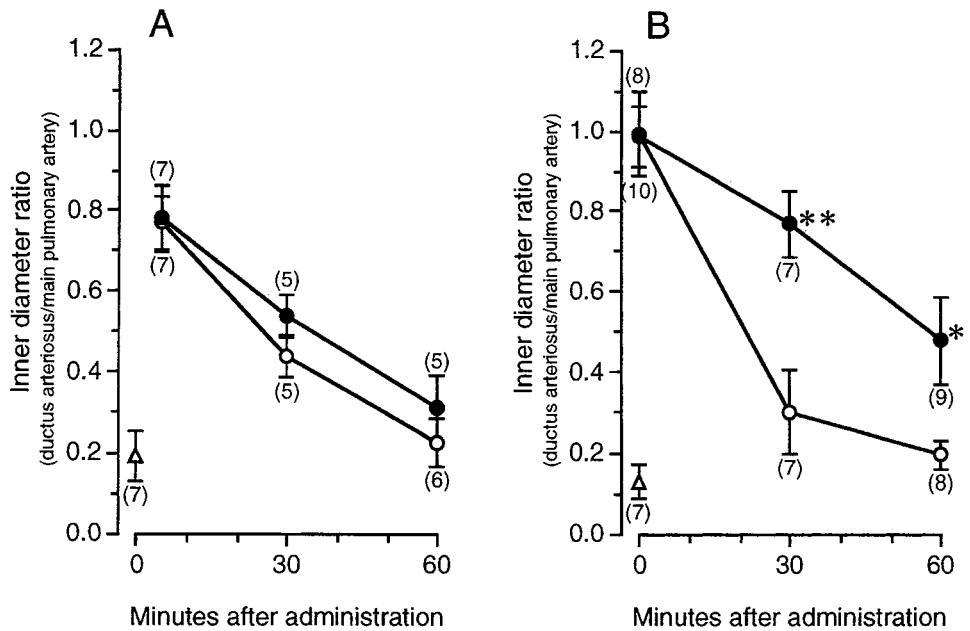

Fig. 2. Inner diameter ratios of ductus arteriosus to main pulmonary artery after rapid injection (10 μg/kg, A) and constant rate infusion (2.5 μg/kg per minute for 2 h, B) of ^{3}H-lipo PGE$_1$ (●) or ^{3}H-PGE$_1$ (○) to neonatal rats. △ indicates the values of untreated animals. Values are expressed as the mean±S.E.M. Values in parentheses are the number of animals. Significant difference from the value of ^{3}H-PGE$_1$: *P<0.05, **P<0.01 (Student's *t*-test).

Tissue concentration of ^{3}H-lipo PGE$_1$ and ^{3}H-PGE$_1$

Taking advantage of the quantification properties of the bio-imaging analyzer, we could quantify the ductus arteriosus and blood concentrations of radioactivity in rat neonates. The calibration coefficient of PSL values (Y) to radioactivity (X) were calculated based on the densities of ^{3}H-standard tissues. The calibration curves, Y=10.290X (r=0.999, n=6) and Y=10.729X (r=0.999, n=9) were obtained for the ductus arteriosus and blood, respectively (Fig. 3).

Ductus arteriosus and blood concentrations of radioactivity following rapid injection of ^{3}H-lipo PGE$_1$ or ^{3}H-PGE$_1$ at 10 μg/kg to neonatal rats are shown in Fig. 4. Ductus levels of ^{3}H-lipo PGE$_1$ and ^{3}H-PGE$_1$ at 5 min were 10.05 and 14.25 ng, equivalent to those of PGE$_1$/g, followed by a decrease to 4.16 and 4.18 ng eq./g within

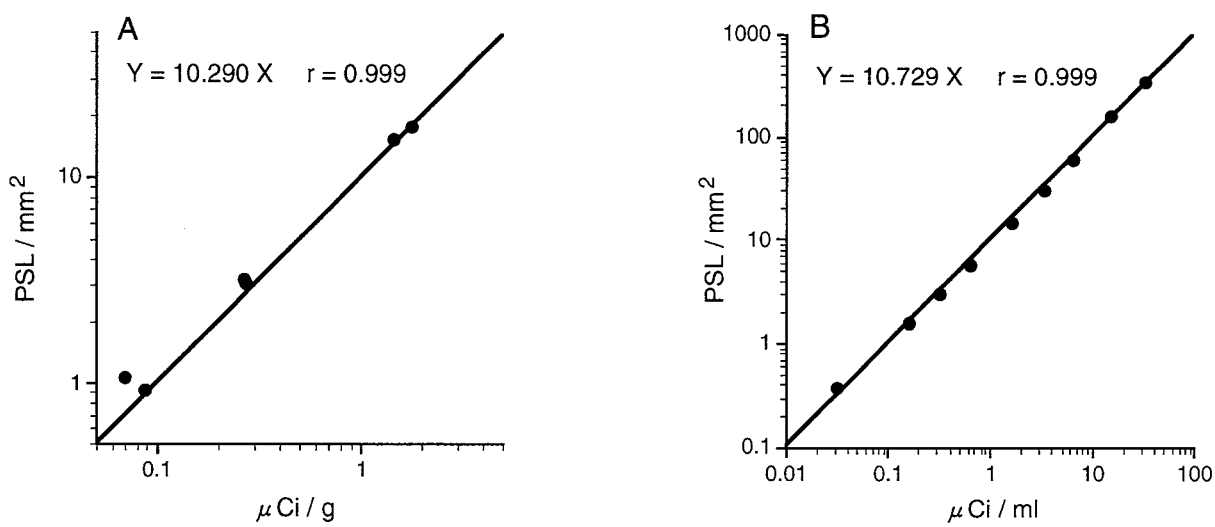

Fig. 3. Relationships between photostimulated luminescence value after 72 h exposure and concentration of radioactivity given by ^{3}H standard tissues. A, heart; B, blood.

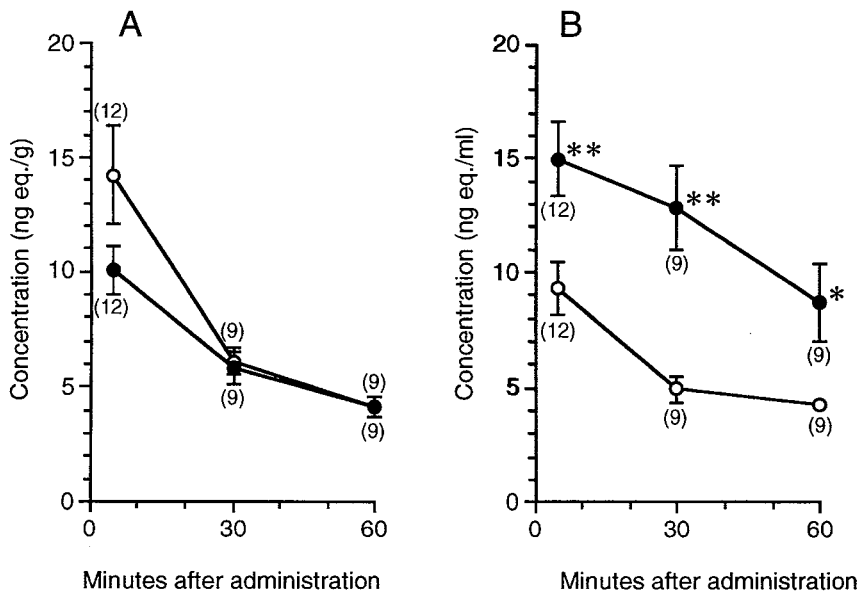

Fig. 4. Tissue concentrations of radioactivity after rapid injection of ^{3}H-lipo PGE_1 (●) or ^{3}H-PGE_1 (○) at the dose of 10 μg/kg to neonatal rats. A, ductus arteriosus; B, blood. Values are expressed as the mean±S.E.M. Values in parentheses are the number of animals. Significant difference from value of ^{3}H-PGE_1: *P<0.05, **P<0.01 (Student's *t*-test).

1 h, respectively. No significant difference of ductus levels was observed between the ^{3}H-lipo PGE_1 and ^{3}H-PGE_1 groups. Blood levels of ^{3}H-lipo PGE_1 and ^{3}H-PGE_1 were 14.95 and 9.29 ng eq./g at 5 min, 8.66 and 4.30 ng eq./g at 1 h, respectively. A significant difference was observed between the ^{3}H-lipo PGE_1 and ^{3}H-PGE_1 groups at all points.

Ductus arteriosus and blood concentrations of radioactivity following constant rate infusion of ^{3}H-lipo PGE_1 or ^{3}H-PGE_1 at 2.5 μg/kg per minute for 2 h to neonatal rats are shown in Fig. 5. Ductus levels of ^{3}H-lipo PGE_1 and ^{3}H-PGE_1 were 267 and 215 ng eq./g at 0 min,

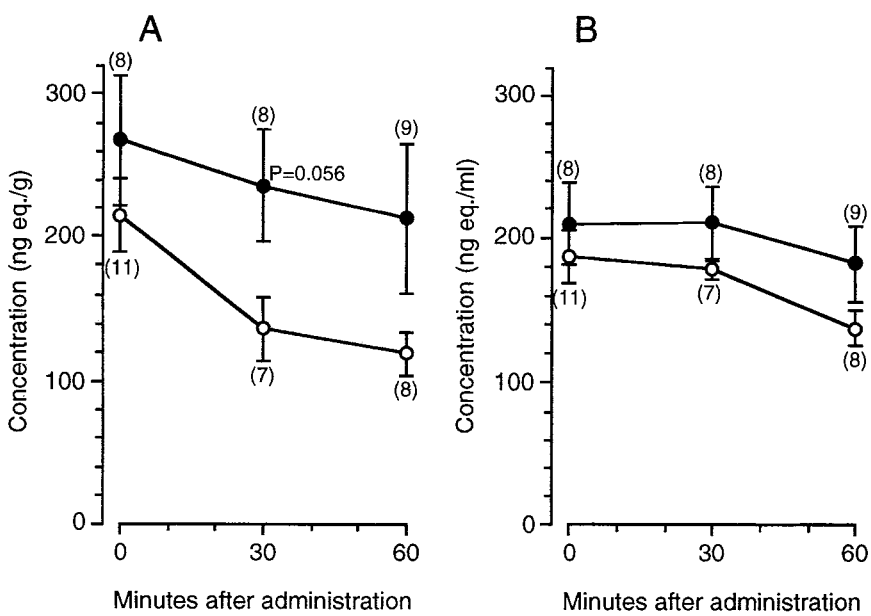

Fig. 5. Tissue concentrations of radioactivity after constant rate infusion of ^{3}H-lipo PGE_1 (●) or ^{3}H-PGE_1 (○) at the dose of 2.5 μg/kg per minute for 2 h to neonatal rats. A, ductus arteriosus; B, blood. Values are expressed as the mean±S.E.M. Values in parentheses are the number of animals. Significant difference was not detected (Student's *t*-test).

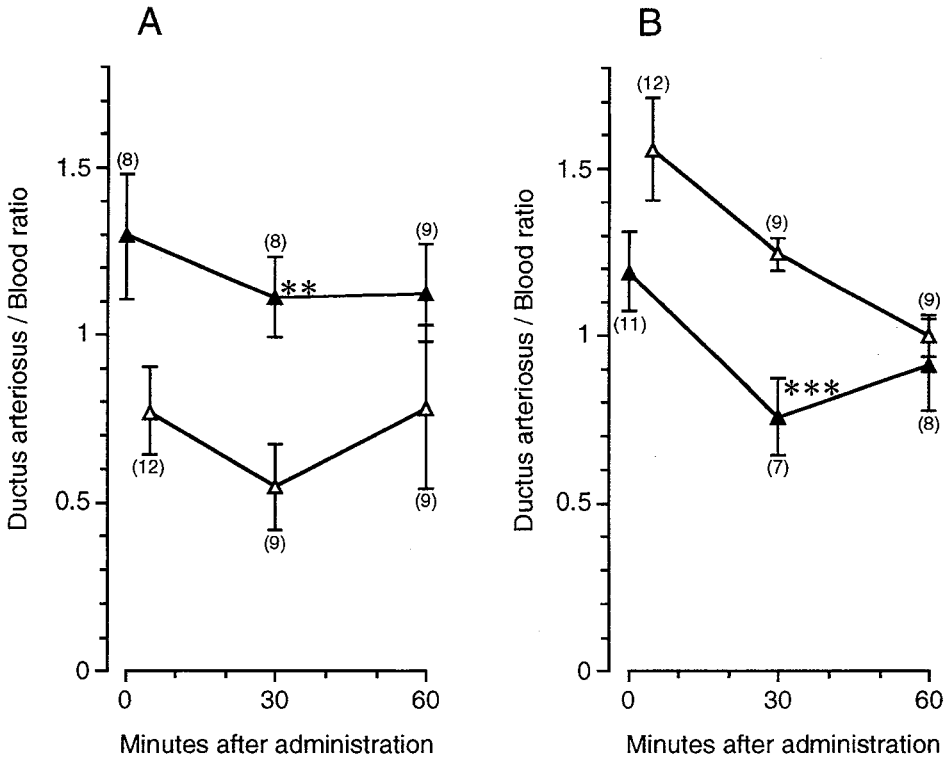

Fig. 6. Concentration ratios of ductus arteriosus to blood after rapid injection (10 μg/kg, △) and constant rate infusion (2.5 μg/kg per minute for 2 h, ▲) of ^{3}H-lipo PGE$_1$ (A) or ^{3}H-PGE$_1$ (B) to neonatal rats. Values are the mean±S.E.M. Values in parentheses are the number of animals. Significant difference from value of rapid injection: **P<0.01, ***P<0.001 (Student's *t*-test).

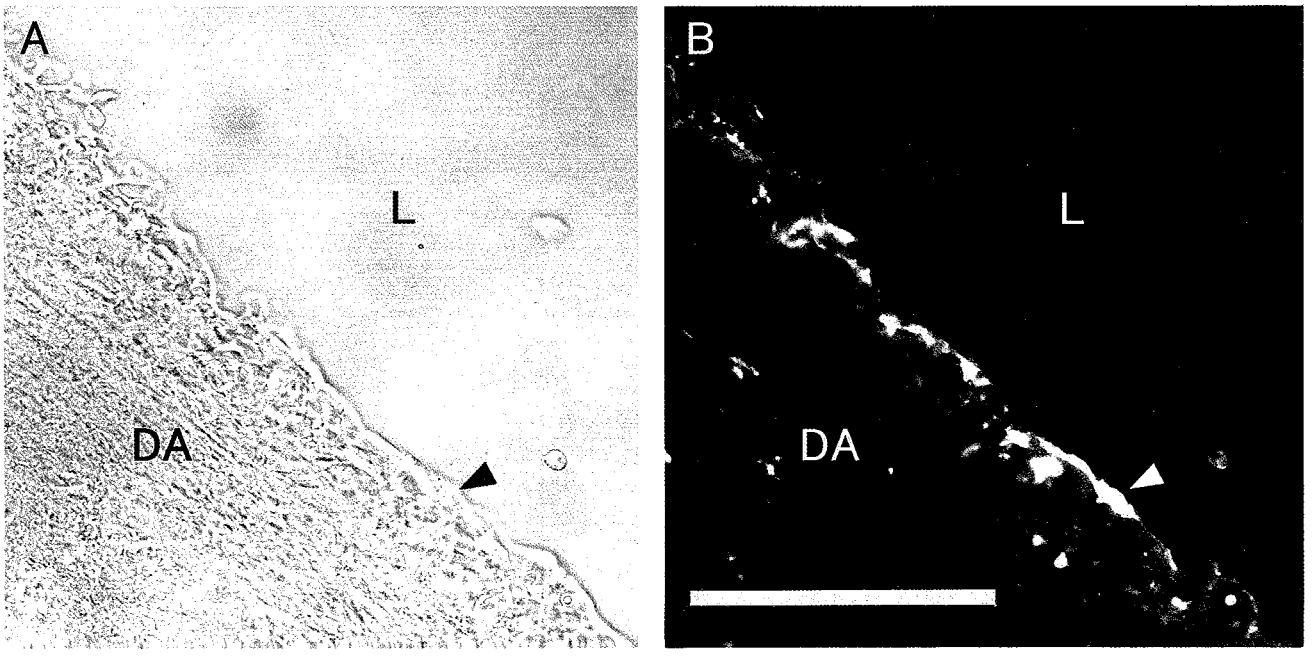

Fig. 7. Distribution of dil-labeled lipo PGE$_1$ in the tissue of ductus arteriosus after constant rate infusion (2.5 μg/kg per minute for 2 h). Both phase contrast (A) and fluorescence (B) microscopic views of the same fields are shown. DA, ductus arteriosus; L, lumen; arrowhead, endothelial cells. Scale bar indicates 50 μm.

236 and 137 ng eq./g at 30 min and 213 and 119 ng eq./g at 1 h, respectively. Although no significant difference was observed between ^{3}H-lipo PGE_1 and ^{3}H-PGE_1 at any point, the mean value of ^{3}H-lipo PGE_1 at 30 min had a tendency to be higher than that of ^{3}H-PGE_1, and the P value was 5.6%. Blood levels of ^{3}H-lipo PGE_1 and ^{3}H-PGE_1 were 210 and 187 ng eq./g at 0 min and 182 and 138 ng eq./g at 1 h, respectively. No significant difference was observed.

The concentration ratios of ductus arteriosus to blood following rapid injection and constant rate infusion of ^{3}H-lipo PGE_1 or ^{3}H-PGE_1 are shown in Fig. 6. The ratio at 30 min after termination of infusion of ^{3}H-lipo PGE_1 was significantly higher than that after rapid injection. In the case of ^{3}H-PGE_1, the ratio after infusion was significantly lower than that after rapid injection.

Tissue accumulation of diI-lipo PGE_1 in rat ductus arteriosus

The frozen sections of the ductus arteriosus were preparated at the termination of constant rate infusion of diI-lipo PGE_1 for 2 h. The confocal laser scanning microscope visualized the tissue distribution of diI-lipo PGE_1. The bright fluorescence of diI-lipo PGE_1 was mainly detected in the vasolateral surface of the ductus arteriosus, whose region coincided with the endothelial cells (Fig. 7).

DISCUSSION

Lipo PGE_1 is widely used clinically for the treatment of several vascular diseases, because it has stronger effects and fewer side effects than free PGE_1 (inclusion complex with cyclodextrin) (12). Also, on the neonatal ductus arteriosus of cyanotic congenital heart disease, the ductus-dilating effect of lipo PGE_1 was stronger than that of free PGE_1, and the effect appeared to continue for longer than that of free PGE_1 after discontinuation of the drug infusion (12–14). Because its mechanism has been unclear, we have studied the relationship between the ductus-dilating effect and the tissue accumulation of lipo PGE_1, using the neonatal rat, a suitable animal model for the study.

The fetal ductus arteriosus is constricted mainly by an increase in oxygenation that occurs after birth (1). In rat neonates, the ductus arteriosus constricted almost completely at 1 h after delivery. Although there was no difference of the ductus-dilating effect between the drugs after intravenous rapid injection of lipo PGE_1 and free PGE_1, the effect of lipo PGE_1 infused for 2 h was significantly greater than that of free PGE_1. This result suggests that lipid microspheres enabled the PGE_1 to act more efficiently after infusion than after rapid injection.

Since the ductus arteriosus of the rat is too small for measuring drug concentration, we employed autoradioluminography using the imaging plate, BAS-TR that can detect weak β rays from ^{3}H due to the absence of a protection layer on its surface, and substituted the neonatal hearts for the ductus arteriosus as ^{3}H-standard tissue. After scanning the IP, which was exposed to the sections from neonates given ^{3}H-labeled PGE_1, by the bioimaging analyzer, the tissue radioactivity was calibrated using the calibration coefficient of the PSL value to the concentration of radioactivity obtained from ^{3}H-containing standard tissue. The blood level of ^{3}H-lipo PGE_1 was higher than that of ^{3}H-PGE_1 after bolus administration. This result seems to be related to the decrease of drug distribution volume and avoidance of inactivation in the lung or the liver, which might be due to the inclusion within lipid microspheres. As for ^{3}H-PGE_1, the blood level seems to be decreased, since PGE_1 is quickly metabolized in those organs. The ductus level of ^{3}H-lipo PGE_1 was higher than ^{3}H-PGE_1 after infusion, although the ductus level of ^{3}H-lipo PGE_1 was not different from that of ^{3}H-PGE_1 after rapid injection. These results indicate that the ductus-dilation effect was closely related to the ductus level of the drug, but not to the blood level.

The excellent lipophilic fluorescent probe diI has been used in the tracer study of LDL metabolism (21). Since diI incorporated into the cell remains in the lipid membrane even after the labeled objective has been metabolized, a low level of uptake can be detected from the fluorescence (21). As the advantages of lipid microspheres, the selective accumulation of lipid microspheres in endothelial cells has been reported (15, 16). Suzuki et al. reported that the cultured endothelial cells of human umbilical vein and the human endothelial cell line, ECV304 incorporated diI labeled lipid microspheres in 4 h (15). In our results, the cultured normal endothelial cells derived from the artery of a healthy donor also incorporated diI-lipo PGE_1, and detection of the fluorescence required about 30 min or more of incubation (data not shown). It was supposed that the accumulation of lipid microspheres in the arterial endothelial cells took place slowly but steadily. Coincidentally, in our in vivo studies, the concentration ratio of ductus arteriosus to blood after constant rate infusion of ^{3}H-lipo PGE_1 was higher than that after rapid injection, and the fluorescence of diI was detected in the endothelial cells of the ductus arteriosus after infusion of diI-lipo PGE_1. These results suggest that incorporation of lipid microspheres by the endothelial cells may be one of the mechanisms that enable the PGE_1 to accumulate in the arterial tissue and to act more efficiently than free PGE_1.

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