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ORIGINAL ARTICLE

Acute effect of a dual ETA–ETB receptor antagonist on pulmonary arterial vasculature in preterm lamb fetuses with surgically induced diaphragmatic hernia

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Abstract
Purpose To study the effects of tezosentan, a dual ETA and ETB receptor antagonist on the cardiopulmonary profile in a fetal lamb model of CDH in utero.
Methods A diaphragmatic hernia was surgically created at day 75 of gestation. During a 45 min of tezosentan perfusion (1 mg/kg), hemodynamic parameters (pulmonary and aortic pressures, left pulmonary and aortic flows, left auricle pressure, heart rate) were measured at day 135 of gestation. Age-matched fetal lambs served as control animals. Secondarily, parietal tension of vessels rings of pulmonary arteries was assessed in organ baths under increasing concentration of tezosentan.
Results In CDH group, under perfusion of tezosentan, pulmonary artery pressure decreased from 45.8 ± 4.1 to 37.6 ± 5.9 mmHg ($P < 0.05$). Pulmonary artery flow and pulmonary vascular resistance remained constant. In control group, pulmonary artery flow increased from 153.9 ± 15.8 to 233.4 ± 26 ml/min ($P < 0.05$). Pulmonary artery pressure did not vary. Subsequently calculated pulmonary vascular resistance decreased. In organ bath, no significant relaxation was observed.
Conclusion In this fetal lamb model of CDH, tezosentan decreased pulmonary artery pressure but did not modify pulmonary blood flow. Endothelin may play a role in the regulation of pulmonary vascular tone in utero.

Keywords Pulmonary hypertension · Congenital diaphragmatic hernia · Fetal · Endothelin

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Introduction

Congenital diaphragmatic hernia (CDH) syndrome is a birth defect combining pulmonary hypertension with bilateral pulmonary hypoplasia [1] and abnormal pulmonary vascular development [2, 3]. In normal newborns pulmonary vascular resistance decreases dramatically with the onset of respiration during the transition from fetal to neonatal circulation. In fetuses with CDH, pulmonary vascular resistance remains elevated in the postnatal period [4] even after surgical correction [5]. In severe case a return to fetal circulation may occur with right-to-left shunting, hypoxemia and acute right heart failure [6].

Several studies have presented evidence implicating the endothelin-1 (ET-1) system in pulmonary hypertension in human neonates with CDH [7, 8], in lamb model of surgically CDH [9, 10] and in nitrofen-induced diaphragmatic hernia in newborn rats [11, 12]. Endothelin-1 is a 21-amino-acid isopeptide with strong vasoconstriction properties [13] and promitogenic activity [14]. Produced by endothelial cells, ET-1 is released into the basolateral compartment thus suggesting that it acts in a paracrine fashion [15]. The biological effects of ET-1 are mediated

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by two distinct receptor subtypes, i.e., ETA and ETB. The ETA subtype located mainly on vascular smooth muscle cells in systemic and pulmonary vessels [16] mediates vasoconstriction. The ETB subtype located on the vascular endothelium can mediate both vasodilation through release of nitric oxide and/or prostacyclin [17] and vasoconstriction [18]. Subtype ETB receptors are involved in the clearance of ET-1 [19].

In normal ovine fetal lungs, ET-1 may probably favor high pulmonary vascular resistance [20]. However, his role in adaptation to extra-uterine life remains controversial [21].

ET-1 and its receptors have been shown to contribute significantly to pulmonary arterial hypertension in human patients. The introduction of endothelin receptor antagonists to clinical medicine has substantially improved the approach toward severe pulmonary arterial hypertension. Tezosentan is a unique short half-life combined ETA and ETB receptor antagonist designed for the parenteral use [22]. It has been shown to induce pulmonary vasodilatation in a lamb model of drug-induced pulmonary hypertension [23] and to decrease pulmonary artery pressure in a pig model of acute lung injury [24].

We speculated that acute blockade of endothelin receptors in utero could prevent further acute deterioration of cardiopulmonary hemodynamics by decreasing pulmonary vascular resistances. To test this hypothesis, we investigated the effects of tezosentan delivery in the pulmonary artery on the cardiopulmonary profile in a preterm lamb model of CDH in feto-maternal circulation.

The present study addressed the averaged mean hemodynamic values which are commonly used in the clinical settings, while the pulsatile modelized fundamental hemodynamics in this fetal lamb model of CDH have already been reported [25].

Materials and methods

Surgical techniques

The Animal Use and Care Committee at the Marseille School of Medicine reviewed and approved all procedures used in this study. Thirty-one pregnant ewes (Merinos, NIAR 868, Montpellier, France) with fetuses between 81 and 82 days of gestation were deprived of food for 24 h before surgery. At the time of surgery, prophylactic antibiotic treatment with penicillin G (500 mg) and streptomycin (1 g) was administered. Anesthesia was performed using intravenous pentobarbital sodium (250 mg) and intrathecal bupivacaine [1%, 1 ml (10 mg)]. The dose of pentobarbital sodium was adjusted to maintain anesthesia under mechanical ventilation throughout the procedure.

Surgical procedures were carried out under sterile conditions. During the initial procedure, the fetal lamb's left forelimb was exposed through a uterine incision, the fetal diaphragm was exposed via thoracotomy in the left ninth intercostal space, and the stomach was pulled into the thorax through a short incision in the left hemi-diaphragm. After closure of the thorax, the fetal lamb was placed back into the uterus. Penicillin G (250 mg) and streptomycin (500 mg) were added to the amniotic liquid before hysterectomy closure. The abdominal incision was closed in separate layers.

A second procedure was carried out between 135 and 136 days of gestation. A skin incision was made under the left fetal forelimb after local infiltration with xylocaine 1% (2 ml). Polyvinyl catheters were inserted into the axillary artery and vein and advanced into the ascending aorta and superior vena cava, respectively. The heart and great vessels were exposed via left thoracotomy in the fifth intercostal space. Using a 16-gauge intravenous placement unit (Angiocath, Travenol Laboratories, Deerfield, IL), catheters were inserted into the left pulmonary artery (LPA), main pulmonary artery and left atrium by direct puncture through purse-string sutures. A 4-mm and 6-mm ultrasonics flow transducer (Transonics, Ithaca, NY) was secured around the LPA and ascending aorta, respectively, to measure LPA and aortic blood flows, respectively. Insofar as possible the fetal lamb was maintained inside the uterine cavity and exposed surfaces were kept warm with wet towels. Catheters were flushed with 2 ml of heparinized saline (2.5 UI/ml) to avoid obstruction.

Age-matched control animals underwent only the second surgical procedure. Just before in vitro study at the end of the protocol, fetal lambs were euthanized with potassium chloride (15%, 10 ml) and midazolam (20 mg) iv bolus infusions.

Drug preparation

Acetylcholine (Ach) at a dose of 15 μg/ml and tezosentan at a dose of 120 μg/ml were dissolved in normal saline and directly infused into the main pulmonary artery. Infusion of Ach was carried out over a 2-min period at a rate of 1 ml/min and tezosentan over a 45-min period at a rate of 67 μg/min for a total dose of 1 mg/kg. Indomethacin 10^{-5} M, phenylephrine 10^{-5} M, and tezosentan dilution 10^{-9} to 10^{-5} M were prepared extemporaneously.

Physiologic measurements

Measurements included the following hemodynamic parameters: pulmonary arterial pressure (PAP), aortic blood pressure (AoP), left atrial pressure (LAP), LPA flow, aortic (Ao) flow and arterial blood gas tension. The

pulmonary artery, left pulmonary artery, and left atrial catheters were connected to a computer-driven system to record mean pressures. Flow transducers were connected to an internally calibrated flowmeter (Transonic) for continuous measurement of aortic and LPA flow. Probe calibrations, data recordings, and treatment were performed as previous described [10]. Left lung vascular resistance (PVR, mmHg/ml/min) was calculated by dividing mean PAP minus mean LA pressure by mean LPA flow. Mean pressure or flow values expressed per minute. Blood samples for determination of pH, arterial pCO_2, and arterial pO_2 were drawn from the Ao and measured at 37°C.

Hemodynamic effects of tezosentan delivery

Tezosentan was infused directly into the main pulmonary artery over a 45-min period at a rate of 67 μg/min (total dose of 1 mg/kg). Pulmonary vascular reactivity was tested before and after tezosentan delivery by a 2-min perfusion of Ach. Pulmonary reactivity was considered to be intact if Ach infusion vasodilatation occurred after tezosentan delivery. A 60-min recovery period was allowed for return to steady state. In case of no Ach perfusion response, animal was excluded. Hemodynamic parameters were recorded under baseline conditions and throughout Ach and tezosentan delivery. During tezosentan delivery measures were taken for 30 s every 2 min.

Organ bath experiments

Pulmonary arteries harvested from the lungs of lambs with and without CDH were dissected up to the third-generation bifurcation. These vessels were then cut into 2–3 mm rings and suspended between two 25 μm tungsten wires. Each organ chamber was filled with 20 ml of Krebs solution. The organ chambers were maintained at 37°C and continuously oxygenated with 95% O_2 and 5% CO_2 mixture. One of the tungsten wires used to suspend the rings was connected to a force transducer to allow for isometric tension recording (EMKA Technologies, France and *Maclab Computer 6300*, Apple, Madford, MA). Before experiments vascular rings were stretched to optimize the length–tension relationship (predetermined optimal passive load of 2 g) and organ chambers were rinsed out with Krebs solution. A 60-min equilibration period was allowed for recovery after stretching.

Effect of tezosentan on contracted vessels rings

At the beginning of each protocol vessels rings were precontracted with phenylephrine 10^{-5} M and indomethacin 10^{-5} M was added. The endothelium was considered as intact if tension increased more than 50% in comparison with the basal value. The concentration of tezosentan was increased from 10^{-9} to 10^{-5} M. The pharmacological effect of tezosentan was assessed by measuring the decrease in tension from the baseline and expressed as reversal (in %) of the tension generated by contracting agents.

Statistical analysis

Data were presented as mean ± SD. Statistical analysis was performed with Systat 12 Software (SPSS Inc, Chicago, IL). Analysis of variance was made with the Kruskal–Wallis nonparametric test and Fisher's test for post hoc comparisons. A P value of <0.05 was considered as significant.

Results

Technical outcome

A total of 32 fetuses were investigated. Seventeen fetuses (10 with CDH and 7 controls) adequately fulfilled the entire protocol, and 15 were excluded due to either ischemic status (7), weak vascular reactivity to Ach (2), surgically induced bleeding (4), or abortion (2).

Baseline

LPA flow was lower and pulmonary vascular resistance significantly higher in CDH lambs than in control lambs (Table 1). All fetuses presented significant vasodilatation (>20%) under Ach perfusion (unpublished data). Aortic pressure and flow were not significantly different between the two groups.

Acute hemodynamic effects of tezosentan perfusion (Fig. 1)

Heart rate remained constant during tezosentan infusion in both groups and no significant difference was observed in arterial blood gas tension throughout the study. In control lambs both LPA and aortic flow increased during tezosentan delivery but pharmacological response varied according to the vascular bed. Aortic flow peaked after 15 min while LPA flow remained constant for the first 10 min, and then increased progressively to a maximal value by the end of tezosentan delivery. On balance tezosentan delivery led to a significant drop in pulmonary vascular and aortic resistances in control lambs. Drug delivery did not significantly alter mean LPA flow and pulmonary vascular resistance in CDH lambs. In CDH lambs mean PAP decreased during tezosentan delivery and a decrease in aortic pressure was observed.

Table 1 Hemodynamic and metabolic data at baseline and after 45 min perfusion of tezosentan, in controls and in animals with congenital diaphragmatic hernia

	Control ($n = 7$)		CDH ($n = 10$)	
	Baseline	45 min	Baseline	45 min
LPA flow (ml/min)	153.9 ± 15.8	233.4 ± 26.0†	94.1 ± 21.9*	100.3 ± 32.3*
PA pressure (mmHg)	43.0 ± 6.3	42.1 ± 3.4	45.8 ± 4.1	37.6 ± 5.9†
Aortic flow (ml/min)	271.4 ± 35.1	370.9 ± 54.6†	266.2 ± 52.1	269.7 ± 73.5*
Aortic pressure (mmHg)	34.9 ± 5.6	35.4 ± 5.4	41.6 ± 5.6	36.9 ± 6.0†
LA pressure (mmHg)	23.1 ± 10.8	32.5 ± 11.7†	10.9 ± 1.7*	8.6 ± 2.7†,*
Aortic resistance (mmHg/ml/min)	0.13 ± 0.02	0.08 ± 0.02†	0.19 ± 0.1	0.17 ± 0.10*
Pulmonary vascular resistances (mmHg/ml/min)	0.15 ± 0.04	0.04 ± 0.06†	0.31 ± 0.07*	0.30 ± 0.13*
pH	7.27 ± 0.05	7.19 ± 0.13	7.23 ± 0.07	7.23 ± 0.10
pO_2 (mmHg)	17.3 ± 6.6	16.7 ± 3.4	22.5 ± 4.7	20.8 ± 4.5
pCO_2 (mmHg)	48.9 ± 5.0	51.8 ± 9.3	49.1 ± 10.7	51.9 ± 8.6

Values are mean ± SE

LPA left pulmonary artery, *PA* pulmonary artery, *LA* left atrial

* $P < 0.05$ versus control animals

† $P < 0.05$ baseline versus perfusion of tezosentan

Organ bath

An increasing dose of tezosentan did not induce significant relaxation of pulmonary vessel rings in either the CDH or control group (Fig. 2).

Discussion

In this study we showed that the dual ET-1 receptor blocker tezosentan decreased mean PAP and AoP in pre-term lamb fetuses with surgically created CDH. Despite lower pressure in the pulmonary and systemic vasculature, no change in flow was observed. In control animals, tezosentan induced an increase in systemic and pulmonary blood flows and in LAP with a subsequent decrease in calculated aortic and pulmonary vascular resistance.

Previous studies have shown that ET-1 is present in perinatal lungs [26] and contributes to regulation of vascular tone during late gestation [27]. However, many questions remain about its physiological role and regulation in normal fetal lamb pulmonary circulation. Some authors have suggested that the main physiological role of ET-1 is vasodilatation [28] while others have described vasoconstriction [27, 29]. Our findings in normal fetal lamb in utero support vasoconstriction.

Enhancement of ET-1 gene expression has been previously documented in an animal model of CDH [12]. Based on his data showing a correlation between plasmatic ET-1 levels, persistent pulmonary hypertension, and survival in newborns with CDH, Kobayashi [7] proposed that ET-1 as a pathophysiological mediator of pulmonary hypertension. This hypothesis is supported by our findings showing that tezosentan infusion decreased PAP in lamb fetuses with CDH.

ETA and ETB receptors have been shown to be present in pulmonary circulation of normal human [30, 31] and lamb fetuses [27]. Their protein expression did not differ between newborn lambs with or without CDH [32]. Tezosentan induces vasodilatation in an ovine model of hypertension [23] and decreases mean PAP and pulmonary vascular resistance after acute lung injury in a pig model [24]. An explanation is that this may be a fixed component of pulmonary vascular resistance, related to the small cross-sectional area of pulmonary vessels and structural vascular remodeling [33].

A fall in LAP associated with constant aortic and pulmonary flows and unchanged heart rate was observed in CDH lamb fetuses. This could suggest a decrease in fetal blood volume: one hypothesis is that the placental blood volume increases concomitantly. Previous authors have identified ETA and ETB receptors in the human placental vasculature [34] and suggested that ET may play a role in the control of fetoplacental circulation [35].

As intracardiac shunts and blood flow through aortic isthmus were not measured, in our experiment, interpretation of pulmonary blood flow has to be considered carefully. We measured pulmonary blood flow through the left pulmonary artery and therefore may have underestimated the total pulmonary blood flow.

A limitation to our study is that it was only designed to measure the effect of tezosentan in utero and did not integrate the cardiovascular changes occurring at birth. In the normal newborn, birth leads to mechanical distension with increased O_2 tension and shear stress, thus causing a drop in pulmonary vascular resistance and a rise in pulmonary blood flow. In newborns with CDH these birth changes often fail to occur and pulmonary vascular resistance remains at suprasystemic levels, thus causing right-to-left extrapulmonary shunting and hypoxemia [36]. Our data cannot be extrapolated to postnatal conditions.

In organ bath experiments adjunction of tezosentan had no effect on the parietal tension of vascular rings in either the CDH or control group. Since tezosentan is a competitive

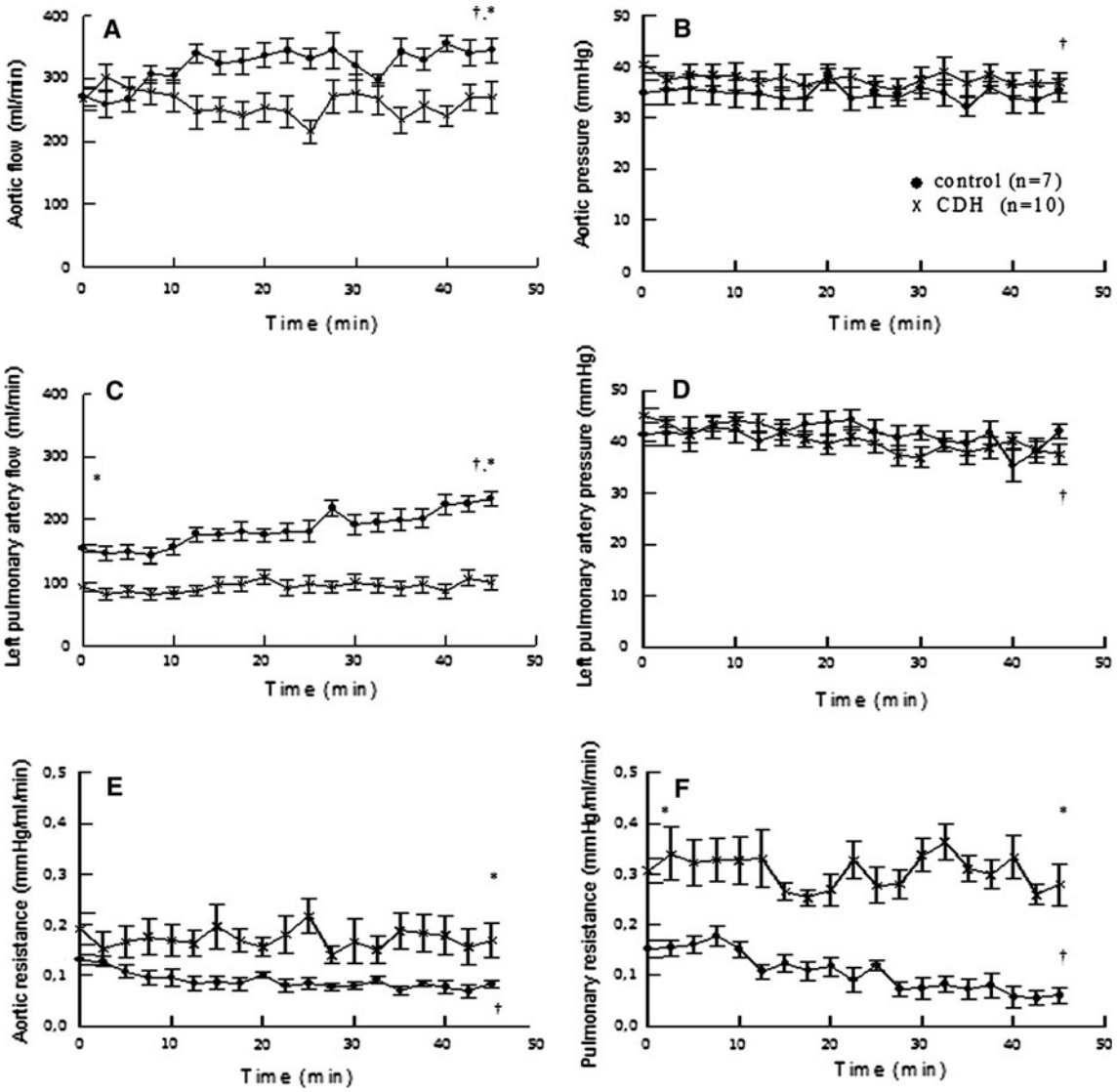

Fig. 1 Response to 45 min of tezosentan infusion on aortic flow (**a**), aortic pressure (**b**), left pulmonary artery flow (**c**), pulmonary artery pressure (**d**) and on calculated aortic resistance (**e**) and pulmonary vascular wedge resistances (**f**). *Filled circle* Control ($n = 7$), *times symbol* CDH ($n = 10$). *$P < 0.05$ versus control animals, $^{\dagger}P < 0.05$ baseline versus perfusion of tezosentan

antagonist [22], its inhibitory activity depends on the agonist concentration. We speculate that tezosentan concentration in organ bath is too high and induce receptor saturation despite bath rinsing.

Our study was restricted to third-generation pulmonary arteries. Differences in location and distribution of endothelin receptor subtypes in the pulmonary vasculature must be taken into account to understand the variable effects of endothelin and endothelin receptor antagonists between in vivo hemodynamic and in vitro studies. The location of ETA and ETB receptors in the normal rat pulmonary vasculature has been studied [37]. ETA receptors are located in the media of pulmonary arteries with a mean distribution in elastic and large muscular arteries and in veins. ETB receptors predominate in the media of distal segments of the pulmonary artery and in the intima of proximal segments.

Investigation of the nitric oxide [NO]/cGMP signaling pathway in experimental CDH models has provided conflicting results. Findings in rats with nitrofen-induced CDH indicate that pulmonary endothelial NO synthase gene expression [38] and activity [39] decreased. In the ovine model of surgically created CDH, basal and stimulated NO release by fourth generation of pulmonary arteries was identical to that of control animals [40]. According to Thébaud et al. [10] who studied the same ovine model, the NO signaling pathway remained intact. While endothelin induces NO release by activation of ETB receptors, our

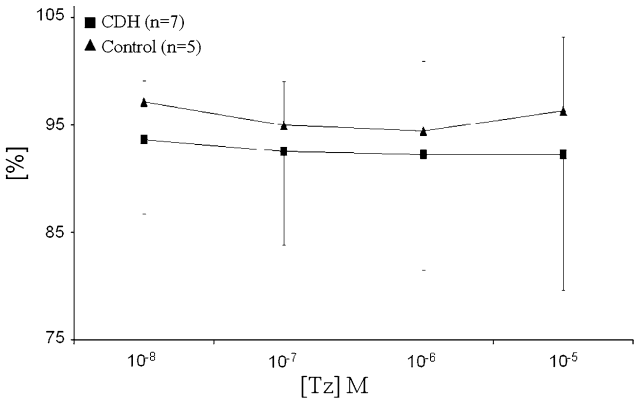

Fig. 2 Response to increasing concentration of tezosentan (Tz) on vessel rings of left pulmonary artery, expressed as reversal (in %) of the tension generated by phenylephrine. Values are mean ± SE. *Filled square* CDH ($n = 7$), *filled triangle* control animals ($n = 5$)

findings do not allow us to draw any conclusion since no changes in parietal tension were observed.

Conclusion

This study investigated the effect of the dual intravenous receptor blocker tezosentan in utero on the pulmonary vasculature of lamb fetuses with surgically created CDH. Findings showed that tezosentan decreased pulmonary artery pressure but did not modify pulmonary blood flow. Decreased aortic pressure can be considered as a possible deleterious effect. Endothelin may play a role in the regulation of pulmonary vascular tone in utero in normal preterm lamb fetuses and with surgically created CDH. More studies especially delivery study are foreseen to investigate the perinatal or postnatal effect of tezosentan on pulmonary circulation.

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