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Determination of asymmetric dimethylarginine, an endogenous nitric oxide synthase inhibitor, in umbilical blood

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Abstract

Endothelial cells produce nitric oxide (NO), a potent vasodilator. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO synthase. Little is known about the potential physiological roles of ADMA in a perinatal setting. This study measures concentrations of ADMA in umbilical blood using enzyme-linked immunosorbent assay and those of NO as nitrite/nitrate (NOx^-) using the Griess assay. Their relationship to the degree of prematurity and maternal clinical condition is examined. Results show that ADMA concentrations in umbilical blood from control newborns were about twice as high as those of lactating women, healthy children, and healthy adults. Umbilical blood NOx^- concentrations from control newborns were about half of those of lactating women, healthy children, and healthy adults. Consequently, the levels of ADMA relative to NOx^- were about 4-fold higher in umbilical blood from control newborns than in blood from lactating women, healthy children, and healthy adults. Furthermore, the umbilical blood ADMA concentrations and the ratios of ADMA to NOx^- in newborns were higher according to their birth prematurity and lower birth weight. The umbilical ADMA concentrations were independent of the delivery mode and maternal preeclampsia. We infer that the high ADMA levels play physiological roles in maintaining vascular tone and blood redistribution to vital organs during birth, thereby favoring the circulatory transition from fetal to neonatal life.

1. Introduction

The endothelium plays a crucial role in the maintenance of vascular tone and structure. Nitric oxide (NO) is a major endothelium-derived vasoactive mediator [1]. In fact, NO is an active but short-lived molecule that is released from endothelial cells into circulating blood. It is a potent vasodilator that regulates vascular tone and tissue blood flow and inhibits platelet aggregation and leukocyte adhesion on the endothelial surface. In endothelial cells, NO is synthesized by endothelial nitric oxide synthase (NOS), which converts the amino acid L-arginine to L-citrulline and NO. Accumulated evidence strongly suggests that many of the features of endothelial dysfunction are intimately linked to the altered expression and function of the L-arginine/NO pathway [2].

Nitric oxide synthesis is inhibited competitively by guanido-substituted arginine analogues, including $N^G N^G$-dimethyl-L-arginine (asymmetric dimethylarginine [ADMA]) and N^G-monomethyl-L-arginine. Both ADMA and N^G-monomethyl-L-arginine are found in human blood and urine; the former is present at about 10-fold higher concentrations [2-4]. For that reason, it is of greater potential clinical interest. Like NO, ADMA is synthesized and released by endothelial cells. The released amounts are sufficient to inhibit endogenous NO production. Recent clinical and experimental investigations have indicated that ADMA is both a strong marker and a mediator of many aspects of endothelial dysfunction syndrome, including not only cardiovascular and renal diseases but also preeclampsia [3-6].

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At present, little information is available about the potential physiological roles of ADMA in a perinatal setting. Determination of ADMA concentrations in umbilical blood might be important to elucidate the regulatory mechanisms of the circulatory system in this particular period. Only 3 studies have measured umbilical blood concentrations of ADMA [7-9]. Asymmetric dimethylarginine in umbilical blood appears significantly higher than the maternal levels when delivery occurs at term or preterm. However, these studies did not examine the balance between ADMA and NO formation. Therefore, the present study was intended to examine ADMA and NO formation during the birth process and its relationship to the degree of prematurity and the maternal clinical condition. Enzyme-linked immunosorbent assay (ELISA) was selected for determination of ADMA in umbilical blood because it is well validated, is ubiquitously available, and can be performed simply and rapidly [10,11].

2. Subjects and methods

2.1. Subjects and blood collection

Umbilical venous blood samples were obtained from 33 Japanese singleton newborns (male/female, 14/19) with gestational ages of 24.4 to 41.4 weeks (mean ± SD, 35.3 ± 5.2 weeks) and birth weights of 614 to 3350 g (2316 ± 810 g) (Table 1). Of them, 11 newborns were born preterm and 22 newborns were born at term; 8 newborns were born by vaginal delivery and 25 by cesarean delivery. Their mothers had not presented with preeclampsia, hypertension, diabetes mellitus, renal diseases, infectious diseases, or other critical underlying diseases. These newborns were taken as *control newborns*. Umbilical blood samples were also obtained from another group of 11 Japanese singleton newborns (male/female, 5/6) with gestational ages of 31.0 to 40.7 weeks (35.2 ± 3.1 weeks) and birth weights of 1444 to 4972 g (2374 ± 1057 g). Three newborns were born by vaginal delivery, and 8 were born by cesarean delivery. The mothers of the newborns had presented with preeclampsia (n = 7), chorioamnionitis (n = 2), or abruptio placentae (n = 2) (Table 1). *Preeclampsia* was defined as both blood pressure greater than 140/90 mm Hg and urinary protein excretion greater than 30 mg/dL, according to the criteria of the Japan Society of Obstetrics and Gynecology. The mothers with preeclampsia in the present study had no laboratory features of kidney or liver dysfunction. None of the studied newborns had congenital infections or major anomalies (such as brain, cardiac, pulmonary, renal, or gastrointestinal anomalies). Immediately after delivery, an umbilical cord segment was double clamped; and blood was drawn gently from the umbilical vein with an 18-gauge needle and syringe.

For comparison, venous blood samples were collected from 7 unrelated healthy lactating Japanese women during postpartum days 4 to 7 (6 ± 1) who had delivered healthy term newborns (gestational age at birth, 38.0-41.7 weeks [39.5 ± 1.3 weeks]; birth weight, 2596-3474 g [3117 ± 333 g]; male/female, 3/4), 19 healthy Japanese children (male/female, 10/9) aged 4.6 to 16.0 years (11.1 ± 3.8 years), and 10 healthy Japanese adults (male/female, 6/4) aged 24.0 to 46.0 years (34.3 ± 7.5 years) (Table 1).

Table 1
Asymmetric dimethylarginine and NOx^- concentrations in umbilical blood

Control newborns (n = 33, M/F = 14/19)	
Gestational age	35.3 ± 5.2 (24.4-41.4) wk
Birth weight	2316 ± 810 (614-3350) g
ADMA	1.71 ± 0.47 (1.01-3.04) μmol/L
NOx^-	23.3 ± 8.3 (10.0-40.9) μmol/L
ADMA/NOx^-	0.085 ± 0.045 (0.034-0.216)
Newborns of mothers with complicated pregnancies (n = 11, M/F = 5/6)	
Gestational age	35.2 ± 3.1 (31.0-40.7) wk
Birth weight	2374 ± 1057 (1444-4972) g
ADMA	1.66 ± 0.33 (1.24-2.26) μmol/L (preeclampsia, n = 7)
	1.65, 1.94 μmol/L (chorioamnionitis, n = 2) [a]
	1.83, 2.08 μmol/L (abruptio placentae, n = 2) [b]
NOx^-	28.5 ± 12.4 (14.3-52.4) μmol/L (preeclampsia, n = 7)
	107.0, 13.6 μmol/L (chorioamnionitis, n = 2) [a]
	29.8, 26.4 μmol/L (abruptio placentae, n = 2) [b]
ADMA/NOx^-	0.071 ± 0.042 (0.024-0.158) (preeclampsia, n = 7)
	0.015, 0.143 (chorioamnionitis, n = 2) [a]
	0.061, 0.079 (abruptio placentae, n = 2) [b]
Lactating women (n = 7)	
ADMA	0.71 ± 0.06 (0.63-0.82) μmol/L
NOx^-	42.3 ± 19.2 (23.1-77.7) μmol/L
ADMA/NOx^-	0.020 ± 0.007 (0.010-0.029)
Healthy children (n = 19, M/F = 10/9)	
ADMA	0.71 ± 0.11 (0.54-0.90) μmol/L
NOx^-	43.4 ± 22.9 (21.9-100.0) μmol/L
ADMA/NOx^-	0.020 ± 0.009 (0.006-0.039)
Healthy adults (n = 10, M/F = 6/4)	
ADMA	0.52 ± 0.12 (0.40-0.75) μmol/L
NOx^-	40.8 ± 27.8 (18.1-107.0) μmol/L
ADMA/NOx^-	0.019 ± 0.012 (0.004-0.041)

Data are presented as mean ± SD and range. The ADMA concentrations and the ADMA to NOx^- ratios are significantly higher ($P < .001$ in all) and the NOx^- concentrations are significantly lower ($P < .005$ in all) in umbilical blood from control newborns than in blood from lactating women, healthy children, and healthy adults. M/F indicates male/female.

[a] Patients are placed in the same order.

[b] Patients are placed in the same order.

Blood samples were centrifuged, and the supernatants were stored at −30°C until analysis. The methods and purpose of the study were explained to the parents. Their informed consent was obtained before enrollment. Approval of the project was obtained from the institutional medical ethics committee.

2.2. Determination of ADMA and NO

Serum concentrations of ADMA were measured using a recently developed ELISA method (DLD Diagnostics, Hamburg, Germany). Competitive ADMA ELISA uses the microtiter plate format. The wells of a microtiter plate are

coated with ADMA, which in the samples is acylated and competes with solid-phase bound ADMA for a fixed number of rabbit anti-ADMA antiserum binding sites. After equilibrium is reached, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid-phase ADMA is detected by antirabbit/peroxidase. The substrate tetramethylbenzidine/peroxidase reaction is monitored at 450 nm. The amount of antibody bound to the solid-phase ADMA is inversely proportional to the ADMA concentration of the sample. This methodology was validated by Schulze and coworkers [10,11], and the validation data have been published elsewhere. Serum concentrations of NO were also measured as its stable metabolites, nitrite/nitrate (NOx^-), using the Griess method (Nitrate/Nitrite Colorimetric Assay; Cayman Chemical, Ann Arbor, MI) [12].

All analyses were performed in duplicate. The examiner was blinded to clinical and laboratory results. Intraassay and interassay coefficients of variation were less than 10% for both measurements.

2.3. Statistical analyses

Data were presented as mean ± SD and range. Differences between groups were examined for statistical significance using an unpaired *t* test. Correlations between variables were assessed using linear regression. A *P* value of less than .05 was inferred as statistically significant.

3. Results

Table 1 shows the concentrations of ADMA and NOx^- in umbilical blood at the time of birth. The ADMA concentrations were significantly higher (about 2-fold) in umbilical blood from control newborns than in blood from lactating women, healthy children, and healthy adults ($P < .001$ in all). In contrast, the NOx^- concentrations were significantly lower (about half) in umbilical blood from control newborns than in blood from lactating women, healthy children, and healthy adults ($P < .005$ in all). Consequently, the ratios of ADMA to NOx^- were much higher (about 4-fold) in umbilical blood from control newborns than in blood from lactating women, healthy children, and healthy adults ($P < .001$ in all).

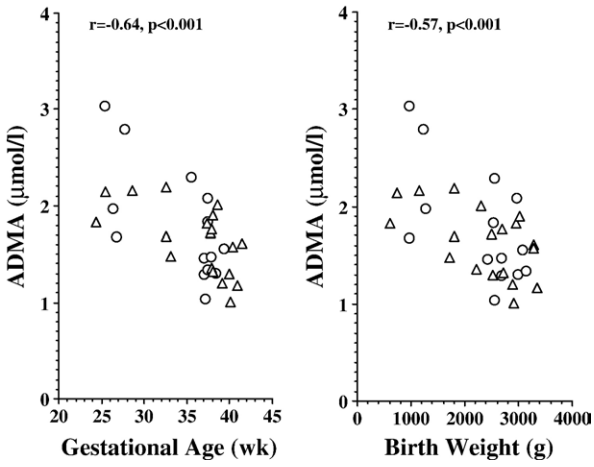

Fig. 1. Relationships between umbilical blood concentrations of ADMA (in micromoles per liter) and gestational age or birth weight in control newborns (n = 33, male/female = 14/19). Circles and triangles, respectively, denote boys and girls. The ADMA concentrations have significant inverse correlations with gestational age ($r = -0.64$, $P < .001$) and birth weight ($r = -0.57$, $P < .001$) when all data are combined.

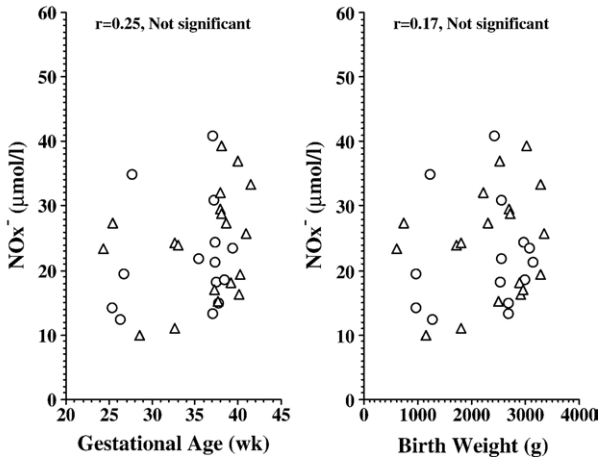

Fig. 2. Relationships between umbilical blood concentrations of NOx^- (in micromoles per liter) and gestational age or birth weight in control newborns (n = 33, male/female = 14/19). Circles and triangles, respectively, denote boys and girls. The NOx^- concentrations have no significant correlations with gestational age ($r = 0.25$) or birth weight ($r = 0.17$) when all data are combined.

In the control group, the umbilical ADMA concentrations and the ratios of ADMA to NOx^- in preterm newborns (n = 11) were significantly higher than those of term newborns (n = 22) (2.12 ± 0.47 μmol/L vs 1.51 ± 0.31 μmol/L and 0.120 ± 0.055 vs 0.067 ± 0.025, respectively; $P < .001$ in both). However, the umbilical NOx^- concentrations in preterm newborns were not significantly different from those of term newborns (20.3 ± 7.7 μmol/L vs 24.8 ± 8.3 μmol/L). The ADMA concentrations demonstrated significant inverse correlations with gestational age ($r = -0.64$, $P < .001$) and birth weight ($r = -0.57$, $P < .001$) when all data of control newborns were included in the analyses (Fig. 1). The ratios of ADMA to NOx^- also showed significant inverse correlations with gestational age ($r = -0.57$, $P < .001$) and birth weight ($r = -0.50$, $P < .005$), although the NOx^- concentrations showed no significant correlations with gestational age ($r = 0.25$) or birth weight ($r = 0.17$) (Fig. 2). The umbilical concentrations of ADMA and NOx^- did not correlate significantly with each other ($r = -0.07$).

In the control group, the gestational age and birth weight of newborns born vaginally (n = 8) were not significantly different from those of newborns born by cesarean delivery (n = 25) (36.4 ± 6.6 weeks vs 34.9 ± 4.7 weeks, 2540 ±

915 g vs 2244 ± 780 g, respectively). No significant differences were identifiable between the 2 groups either in the concentrations of ADMA (1.75 ± 0.61 μmol/L vs 1.70 ± 0.43 μmol/L) or NOx^- (24.5 ± 10.6 μmol/L vs 22.9 ± 7.6 μmol/L) or in the ratios of ADMA to NOx^- (0.089 ± 0.062 vs 0.083 ± 0.039). The gestational age and birth weight of male newborns (n = 14) were not significantly different from those of female newborns (n = 19) (34.3 ± 5.2 weeks vs 36.0 ± 5.1 weeks, 2279 ± 806 g vs 2343 ± 834 g, respectively). No significant differences were observed between male and female subjects in the concentrations of ADMA (1.80 ± 0.59 μmol/L vs 1.64 ± 0.35 μmol/L) or NOx^- (22.1 ± 8.4 μmol/L vs 24.2 ± 8.3 μmol/L) or in the ratios of ADMA to NOx^- (0.092 ± 0.046 vs 0.079 ± 0.044) in umbilical blood (Figs. 1 and 2).

We also examined the possible influences of maternal clinical conditions (such as preeclampsia, chorioamnionitis, or abruptio placentae) on the umbilical ADMA and NOx^- concentrations (Table 1). Apparently, the ADMA concentrations of these newborns were not different from those of the control newborns (Table 1). The gestational ages of the newborns whose mothers had had preeclampsia were 31.0 to 38.7 weeks (35.1 ± 3.1 weeks). The umbilical concentrations of ADMA and NOx^- and the ratios of ADMA to NOx^- in the preeclampsia group were not significantly different from those of the control group when the gestational age was matched (ie, control newborns who were born between 31 and 39 weeks [n = 19] were selected from the latter group) (data not shown).

4. Discussion

In the body, ADMA is generated by the degradation of methylated proteins because of protein arginine methyltransferase 1 activity and subsequent protein turnover. Although excreted in the urine, ADMA is mainly metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [3,4]. That enzyme hydrolyzes ADMA to L-citrulline and dimethylamine. Two isoforms of DDAH exist, DDAH-1 and DDAH-2, which show different tissue expression patterns. The former, DDAH-1, is present in many tissues that express neural NOS, whereas DDAH-2 is mainly present in vascular tissues that express endothelial NOS. In fact, ADMA inhibits endothelial NOS by competitive displacement of the physiological substrate, L-arginine, from the enzyme. That inhibition decreases NO production in the endothelium of vessel walls [2]. Therefore, when ADMA levels are elevated, endothelial dysfunction might result. To date, elevated ADMA levels have been detected in numerous diseases associated with an impaired L-arginine/NO pathway, including hypertension, atherosclerosis, diabetes mellitus, renal insufficiency, hypercholesterolemia, and hyperhomocysteinemia [3-6]. Furthermore, ADMA has recently been shown to be an independent risk factor for cardiovascular diseases. Moreover, by experimental administration of ADMA to healthy human subjects, symptoms of endothelial dysfunction, including elevated blood pressure, reduced cardiac output, and impaired renal perfusion, can be evoked [13].

Fetal and neonatal vascular tones are modulated through a balance between vasoconstrictor and vasodilator stimuli, including endogenous mediators such as arginine vasopressin, endothelin-1, adrenomedullin, and NO [12]. Taking into account the emerging role of ADMA as a potent regulator for NOS, increasing interest exists in measuring ADMA concentrations in umbilical blood to elucidate the molecular mechanisms for circulatory adaptation to extrauterine life. So far, quantification of ADMA using high-performance liquid chromatography (HPLC) analysis has been the most widely applied method [14]. Very recently, a more rapid and feasible ELISA method has been developed and validated by Schulze and coworkers [10,11]. We selected that ELISA method for determination of ADMA in umbilical venous blood serum from newborns with various gestational ages and birth weights in the present study. We simultaneously measured the NOx^- concentrations in umbilical blood using the Griess method.

The results of our study demonstrated that ADMA concentrations in umbilical blood from control newborns were about 2-fold higher than those in blood from lactating women, healthy children, and healthy adults. In contrast, the NOx^- concentrations in umbilical blood from control newborns were about half of those in blood from lactating women, healthy children, and healthy adults. Consequently, the ratios of ADMA to NOx^- were about 4-fold higher in umbilical blood from control newborns than in blood from lactating women, healthy children, and healthy adults. Furthermore, the results of our study showed that the umbilical ADMA concentrations and the ADMA to NOx^- ratios were increased in newborns concomitant with their prematurity of birth and the lower birth weight. The results also showed that the umbilical concentrations of ADMA and NOx^- and the ADMA to NOx^- ratios were independent of the mode of delivery. The origins of the increased ADMA in umbilical blood have not yet been clarified. Asymmetric dimethylarginine is a naturally occurring amino acid that is widely distributed in different tissues and organs [4]. It is likely that the high levels of ADMA are derived from several tissue sources such as the vessels, kidney, and liver in the fetus. Some factor of increased levels of ADMA in umbilical blood may also originate from the maternal side, possibly from enhanced accumulation of ADMA in the placenta.

Only 3 studies in the literature have measured umbilical blood concentrations of ADMA. Maeda et al [7] showed that ADMA concentrations in umbilical venous blood were significantly higher than the maternal values, but that L-arginine concentrations were not significantly different from the maternal values. Mittermayer et al [8] reported that male preterm newborns had significantly higher umbilical venous blood concentrations of ADMA than did female preterm newborns and term newborns of both sexes. Vida

et al [9] reported that ADMA concentrations in umbilical blood from term newborns were elevated with a consistent venoarterial difference, suggesting that the high levels of ADMA in umbilical blood are mainly generated by the placenta. These 3 groups of investigators used HPLC for analyses. They did not simultaneously measure NOx^- concentrations in umbilical blood. In addition, they did not compare the ADMA concentrations in umbilical blood with those of children or adults. Our results did not show consistency with the findings by Mittermayer et al [8]: we found no sex-specific differences in the umbilical ADMA concentrations. The subjects were few and rather inhomogeneous in both studies. More data might be necessary to clarify this matter.

The perinatal setting is characterized by the transition from intrauterine hypooxygenemia to extrauterine normooxygenemia under the stress of birth. The reason for high concentrations of ADMA in umbilical blood remains to be clarified, but might be a consequence of increased synthesis, decreased metabolism by DDAH, decreased clearance by fetal kidneys or the placenta, or some combination of those factors [3-6]. In any case, we consider that the high ADMA levels per se or relative to NOx^- are likely to represent one important mechanism that maintains blood pressure during the birth process. The array of potent vasoactive substances during critical environments is aimed primarily at preserving cardiocerebral perfusion; less priority is given to the splanchnic organs. It is therefore conceivable that the high ADMA levels contribute to redistribution of blood flow to the cardiocerebral axis and away from the splanchnic organs during birth. In our study, the high levels of ADMA and the ADMA to NOx^- ratios were more enhanced in preterm newborns than in term newborns. Interpretation of this phenomenon also remains elusive. We assume that the more increased ADMA levels might reflect a greater production as a consequence of accelerated protein turnover or an attenuated clearance of ADMA in premature subjects favoring prevention of circulatory shock immediately after birth. Arrigoni and coworkers [15] reported as "unpublished data" that ADMA was found in particularly high concentrations in the human fetus and in amniotic fluid. This observation might present one possibility: high levels of ADMA tonically inhibit the NOS activity in utero, thereby contributing to the maintenance of blood pressure in the fetus.

Our results on the umbilical NOx^- concentrations were consistent with previous findings by Endo et al [16,17] and Biban et al [18]. They used the Griess method for the analysis. The reported mean levels of NOx^- were 20 to 30 μmol/L. Our results showed no significant difference in umbilical NOx^- concentrations between preterm and term newborns. Biban et al [18] reported results that were similar to ours.

Preeclampsia remains a major cause of maternal morbidity and death. It is a leading indication for iatrogenic premature delivery [6]. Endothelial dysfunction is considered to be a crucial factor in the disease process. Ellis et al [19] measured ADMA in venous blood from pregnant women with nonpreeclamptic and preeclamptic pregnancies and found the ADMA concentrations to be elevated significantly in the preeclamptic group. Savvidou et al [20] found high blood ADMA concentrations in pregnant women with impaired placental perfusion, well before the clinical signs of preeclampsia developed. In contrast, Maas et al [21] found no significant difference in blood ADMA concentrations in women with preeclampsia compared with the nonpreeclamptic group. All these investigators adopted HPLC for determination of ADMA. In the present study, we measured umbilical blood concentrations of ADMA for the first time in newborns of mothers who had presented with preeclampsia. We identified no significant elevation in the ADMA concentrations or the ratios of ADMA to NOx^- in newborns of mothers with preeclampsia compared with the gestational-age–matched control newborns. These results were unexpected because, conceptually, we had assumed that maternal preeclampsia might impair placental circulation, thereby engendering endothelial dysfunction in the fetus [22,23]. In addition, we found no apparent alteration in umbilical ADMA in newborns of mothers who had had chorioamnionitis or abruptio placentae. We must admit that the study subjects were few. Further research is warranted for elucidation of the ADMA and NO pathways in complicated pregnancies and their adverse effects on the mother and fetus.

The enzyme DDAH hydrolyzes ADMA and plays a major role in its metabolism [4]. It is becoming increasingly apparent that DDAH activity is regulated by NOS through *S*-nitrosylation [24] and that the activity of NOS is controlled by DDAH through metabolization of ADMA and the consequent modulation of its levels [25,26]. Very recently, an attractive hypothesis has been presented: whole blood contains enzymatic systems that contribute to control of blood ADMA concentrations by acting as both a source (via degradation of ADMA-containing proteins) and a sink (via DDAH-mediated hydrolysis) for ADMA [27]. The DDAH/ADMA system and the NOS/NO system might be regulated mutually and strictly. We assume that the relative potency and interrelationship between ADMA and NO are important in the transition from fetal to neonatal life and that an imbalance of these mediators might be linked to transition failure states such as circulatory shock [28] and persistent pulmonary hypertension [15,29]. The present study examines only umbilical concentrations of ADMA and NOx^- and so has limited inference. Sequential analyses of ADMA and NOx^- might clarify the contribution of the DDAH/ADMA/NOS pathways to the dynamic postnatal changes in the circulatory system.

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