Nitric oxide and nitric oxide synthases in the fetal cerebral cortex of rats following transient uteroplacental ischemia

Juan Antonio Gonzalez-Barrios, Bruno Escalante, Jesus Valdés, Bertha A. León-Chávez, Daniel Martinez-Fong

Departamento de Fisiología, Biofísica y Neurociencias, Cinvestav-I.P.N., Apartado postal 14-740, 07000, México D.F., Mexico
Departamento de Biomedicina Molecular, Apartado postal 14-740, 07000, México D.F., Mexico
Departamento de Bioquímica, Cinvestav-I.P.N., Apartado postal 14-740, 07000, México D.F., Mexico
Urgencias Adultos del Hospital Regional 1° de Octubre, ISSSTE, Avenida Instituto Politécnico Nacional no. 1669, 07760, México D.F., Mexico

Abstract

The effect of transient uteroplacental ischemia on nitric oxide (NO) levels, enzymatic activity, and expression of NO synthase (NOS) isoforms was studied in fetal rat brains. Fetuses were subjected to ischemia by clamping the uterine arteries for 5 min on gestational day 17 (GD17). At different times after ischemia, fetuses were delivered by Cesarean section under anesthesia to obtain the brains. Transient uteroplacental ischemia produced a time dependent increase in nitrite levels in the brain, reaching a maximum value (300 ± 25% of baseline) 24 h after uterine artery occlusion and remaining elevated as long as 48 h. Significantly increased nitrite levels were found in the cerebral cortex but not in the mesencephalon and cerebellum. The ischemia-induced increment in nitrite levels was totally blocked by either L-NAME (10 mg/kg) or AMT (0.65 mg/kg) administered i.p. 1 h before uterine artery occlusion. Both Ca²⁺-dependent and Ca²⁺-independent NOS activities in the cerebral cortex remained significantly increased with respect to controls after 24 h following the ischemia. Reverse transcriptase-polymerase chain reaction showed augmented levels of mRNAs for both nNOS and iNOS when compared with controls at 8 h after ischemia. At 36 h, nNOS mRNA returned to basal levels whereas eNOS mRNA levels increased and iNOS mRNA remained elevated. Our results show that the three NOS isoforms participate in increasing NO levels after transient ischemia and suggest a biphasic and differential regulation of the expression of constitutive NOS isoforms in the rat cerebral cortex.

Theme: Development and regeneration

Topic: Nutritional and prenatal factors

Keywords: Nitric oxide; Nitric oxide synthase; Hypoxia; Asphyxia; Perinatal period; Brain injury

1. Introduction

Nitric oxide (NO) is synthesized from the terminal guanidine group of L-arginine in two steps catalyzed by the nitric oxide synthase (NOS) enzymes under both physiological and pathological conditions [38]. In the brain, there are two major isoforms of NOS, the neuronal isoform (nNOS) present in neurons and the endothelial isoform (eNOS) present primarily within the vascular endothelium [35,40]. Expression of the inducible isoform (iNOS) has been well characterized in astrocytes, microglia cells, neurons and at a lesser extent in endothelial cells in diverse pathological conditions [8,12,33]. The existence of a variety of NOSs in the brain, even in similar cell type, addresses the importance of the physiological role of NO and suggests the presence of a regulatory control determining the participation of NOS isoforms in providing increased NO levels in pathological conditions [38].

The brain is particularly vulnerable to the failure of its blood supply; a transient ischemia can cause serious damage to selective neurons in adult and fetal brains. It has been reported that in the absence of cerebral blood flow, and therefore of the oxygen, the energy reserves of the adult brain are capable of sustaining ATP levels for about
1 min [34]. The compensating period seems to be longer, less than 5 min, in fetal brains due to placental reserves [42]. A large body of evidence coming from adult animal models sustains increment of NO levels in the brain during ischemic–hypoxic states, but the information of NO in perinatal asphyxia is still limited [4,10]. In a noninvasive animal model where asphyxia was induced by submerging Cesarean-delivered pups still in patent membranes in a waterbath at 37°C, NO production, nNOS mRNA levels and NOS activity were found unaffected for up to a 20-min period [30]. On the contrary, increments in NO production and NOS at the transcriptional and activity levels were found in fetal and neonatal brains in response to perinatal asphyxia [1,6,20]. Expression of iNOS in brains of adult and fetal rats has been consistently found in several models of transient ischemic insults. Such expression of iNOS is associated with production of iNOS protein and enzymatic activity [12]. It has been suggested that NO produced by iNOS contributes to ischemic brain damage since the infarct and the motor deficit produced by middle cerebral artery occlusion is smaller in iNOS-knockout than in wild type mice [23].

Results obtained from adult animal models of brain ischemia and reperfusion have suggested the existence of a regulatory control that determines the participation of the NOS isoform in NO production. Brain NO levels increase rapidly in less than 5 min after the onset of focal ischemia, return to baseline within 1 h, and rise again between 12 and 24 h during reperfusion [33,39]. The rapid and immediate increase in NO levels after the onset of ischemia has been attributed to constitutive NOS isoforms, whereas the delayed increase may be due to the sustained expression of iNOS [14,21]. A differential control of NOS isoforms seems to be also present at least at the end of the perinatal period, since it has been reported an increase in the immunoreactive eNOS 135-kDa protein and a decrease in the immunoreactive nNOS 150-kDa protein parallelling the length of asphyxia [30]. We therefore decided to explore the nitrite levels, NOS enzymatic activity, and expression of the three NOS isoforms (nNOS, eNOS and iNOS) in rat fetal brains after transient uteroplacental ischemia in an attempt to provide further understanding to the role of NO in the brain.

2. Materials and methods

2.1. Animals

Experiments were conducted on pregnant Wistar rats on gestational day 17 (GD17) bred in our facilities. Animals were maintained under constant room temperature (23°C) and light–dark cycle (12:12 h); with food and water ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care as approved by the Cinvestav Animal Care Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

2.2. Surgical procedures

Pregnant rats on GD17 were anesthetized with chloral hydrate (350 mg/kg, i.p.) and subjected to medial laparotomy to expose the uterine horns. Clamping the uterine arteries induced uteroplacental ischemia for different times (0, 5, 10, 15 and 20 min); during the ischemia, the uterine horns were kept wet with phosphate buffered solution, pH 7.4 (PBS). Upon ischemia completion, the uterus was replaced in the abdominal cavity, which was sutured with silk 3(0) and the skin with nylon 3(0). At different times (8, 24 and 36 h) after occlusion of the uterine arteries, fetuses were delivered by Cesarean section under chloral hydrate anesthesia (350 mg/kg, i.p.) to obtain the brains. Each brain was placed upside down on an ice-cold stage of a stereoscopic microscope with a 6× objective to localize the cerebral cortex limits, which were separated by using a half curved Dumont tweezers no. 5 (Roboz, Rockville, MD, USA) to the degree that the cortex was completely unfolded. At this point, the remainder of the cerebrum was removed to dissect out the mesencephalon and the cerebellum. The dissected tissue was immediately frozen in liquid nitrogen and kept at −70°C until use.

2.3. Nitric oxide determination

NO production was assessed by the accumulation of nitrites (NO$_2$) in homogenates from different brain regions. Each brain region was mechanically homogenized in PBS (1:3; w:v) and the homogenates were centrifuged at 12 000 rpm in a Sorval MRC-14 microcentrifuge (Dupont, Newtown, CT, USA) at 4°C for 15 min. Samples of supernatants with similar protein concentration (200 μg) were completed to 900 μl with Milli-Q water and used to determine NO levels. The nitrite levels was measured as described previously [36]; briefly, nitrite concentration in 100 μl of supernatant was measured by induction of a colorimetric reaction by the addition of 100 μl of Griess reagent, which was composed of equal volumes of 0.1% N-1(naphthy)ethylenediamine dihydrochloride and 1.32% sulfanilamide in 60% acetic acid. The absorbance of the samples was determined at 540 nm in a DU-650 spectrophotometer (Beckman Instruments, Palo Alto, CA, USA) and interpolated to a standard curve of NaNO$_2$ (1–20 mM) to calculate the nitrite content [36].
2.4. Enzymatic activity of NO synthase

NOS activity was assessed by quantifying \([^{3}H]L\)-citrulline resulting with NO from the enzymatic process of \([^{3}H]L\)-arginine [36]. Each brain region was homogenized at 1:1 ratio \((w/v)\) in Tris–HCl buffer \((50 \text{ mM}, \text{pH} \ 7.4)\) containing the detergent Tergitol NP-40 \((0.1\%, \text{v/v})\) and protease inhibitors \((0.1 \text{ mM EDTA, } 0.1 \text{ mM EGTA, } 0.1\% \beta\text{-mercaptoethanol, } 1 \mu\text{M leupeptin, } 100 \mu\text{M phosphate-methylphenyl-sulfoxide, } 1 \text{ mg/ml trypsin inhibitor from soybean, and } 2 \text{ mg/ml aprotinin}.\) The homogenates were centrifuged at 14,000 rpm in a Sorval MRC-14 microcentrifuge \((\text{Dupont})\) at 4°C for 5 min. Supernatants with similar protein concentration \((500 \mu\text{g})\) were incubated with the reaction mixture \((1 \mu\text{M} \ [^{3}H]L\text{-arginine, } 1 \text{ mM NADPH, } 30 \mu\text{M tetrahydrobiopterin, } 2.5 \text{ mM CaCl}_{2}, \text{ and } 10 \mu\text{M calmodulin})\) yielding 1 ml of final volume, for 30 min at 37°C. The enzymatic reaction was stopped by adding 0.1 ml of the stop buffer \((20 \text{ mM HEPES, } 2 \text{ mM EDTA, and } 2 \text{ mM EGTA, } \text{pH} \ 5.5.\) The whole volume of 1.1 ml was added on cation-exchange resin Dowex AG 50WX-8 equilibrated with the stop buffer to purify \([^{3}H]\)-citrulline whose radioactivity was determined by liquid scintillation counting. NOS activity was expressed as production of nmol \([^{3}H]L\text{-citrulline per mg protein per min. Total protein was measured with the BCA microassay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard.}\)

2.5. Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction \((\text{RT-PCR})\) was performed essentially as described elsewhere [9]. Total RNA was isolated from 100 mg of liquid nitrogen-frozen tissue in 1 ml of Trizol reagent \((\text{Gibco-Brl, Grand Island, NY, USA})\), quantified by spectrophotometry at 260 nm, and analyzed by 2%-agarose gel electrophoresis. The RNA preparations were treated with RNase-free DNase before their use in the reverse transcription to amplify\(\text{eNOS, nNOS and iNOS using specific primers modified from Keilhoff et al. [27] and glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) as housekeeping gene. To amplify a 742-bp fragment of eNOS, the sense primer was 5'-ACG ACT TGG 3', and the antisense primer was 5'-TCA TGC TTG CGG 3'; these primers flank the 3191–3214 sense and 3584–3561 antisense bases of the mRNA sequence for iNOS [15]. To amplify a 420-bp fragment of GA3PDH, the sense primer was 5'-ACC ACA GTG CAT GCC ATC AC-3' and the antisense primer was 5'-TCC ACC CTG TTG CGG 3' \((\text{Gene bank access: AF-10686}).\) A 1-\(\mu\text{g}\) amount of total RNA was transcribed with SuperScript II reverse transcriptase \((200 \text{ units})\) using 0.1 \(\mu\text{g}\) of random hexamer \((\text{Gibco-Brl}).\) A 2-\(\mu\text{g}\) volume of the reverse transcribed product were amplified in a temperature gene cycler \((\text{Bio-Rad, Richmond, CA, USA})\) using 0.4 \(\mu\text{g}\) of each sense and antisense primers and 2.5 units of Taq DNA polymerase, in a final volume of 100 \(\mu\text{l.}\) After an initial denaturation at 94°C for 2 min, amplification was carried out for 35 cycles as follows: 94°C for 1 min \((\text{denaturation}), 60°C \text{ for 1 min (annealing), and 72°C for 1 min (extension). PCR products were analyzed by restriction assays and fractionated on 2% agarose gel. Upon completion of electrophoresis, ethidium bromide-stained-PCR products were photographed with a Polaroid camera. The densitometric analysis was accomplished with the software LABWORKS 4.0 DNS 51N40176-21104 from Ultra Violet Products \((\text{Upland, CA, USA})\) and the densitometric values were expressed as arbitrary units.}\)

2.6. Statistical analysis

All values are means±standard error of the mean \((\text{S.E.M.})\) obtained from at least six independent experiments. After testing for normality of the data by the Snedecor’s \(F\) test, ANOVA and the difference between each group with Student’s \(t\) test were used for analysis with significance assumed at the \(P<0.05\) level.

2.7. Chemicals

TRIzol, primers, 100-bp DNA ladder, Taq DNA polymerase, SuperScript II RNase H\(^{+}\) reverse transcriptase, DNase I were purchased from Gibco-Brl. Bovine serum albumin, sodium nitrite, Tergitol NP-40 \((\text{nonylphenoxoy polyethoxy ethanol}), N-1(\text{naphthyl})\text{ethylenediamine dihydrochloride, sulfanilamide, ethidium bromide, tri- s(hydroxyethyl)aminomethane hydrochloride (Tris–HCl), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethyl)-N,N',N'-tetraacetic acid (EGTA), \(\beta\text{-mercaptoethanol, calcium chloride anhydrous (CaCl}_{2}), N^{\text{6}}\text{-nitro-L-arginine methyl ester (L-NAME) hydrochloride, leupeptin hemisulfate, phosphate-methyl phenyl-sulfoxide, trypsin inhibitor from soybean (STB), aprotinin, dihydronicotinamidealanine dineudioleotide (NADPH), tetrahydrobiopterin, calmodulin and Dowex AG 50WX-8 were from Sigma (St. Louis, MO, USA). \([^{3}H]L\)-arginine \((66 \text{ Ci/mmol})\) were purchased from Amersham (Buckinghamshire, UK), BCA microassay from
Pierce and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) from Tocris Cookson (Ballwin, MO, USA). All other chemicals were of analytical reagent grade quality and obtained from usual commercial sources.

3. Results

3.1. Duration of uterine artery occlusion affected fetal viability

To determine the effect of ischemia duration on the survival of the fetuses, the uterine arteries of 17-day-pregnant rats were clamped for 0, 5, 10, 15 and 20 min (six different rats for each time point); 24 h after the clamping the number of fetuses that survived or died were counted. Fetal viability was calculated by dividing the number of fetuses alive by the number of total (alive+death) fetuses present in the pregnant rat (8–12 fetuses per pregnancy). As Fig. 1 shows, fetal viability decreased depending on the duration of uteroplacental ischemia. The lethal half-time was 7.6±0.2 min, n=6. Therefore, the occlusion span of uterine arteries selected to use in this work was 5 min since the fetal viability was less affected (95±5%) during this ischemic period (Fig. 1) and due to the finding that fetal–placental reserves in rats are 2±0.5 min [42].

3.2. Transient uteroplacental ischemia increased NO production in the cerebral cortex

Uteroplacental ischemia produced a time-dependent increase in nitrite levels in the brain, reaching a maximum value (300±25% of baseline) 24 h after uterine artery occlusion (Fig. 2A). Furthermore, nitrite levels remained elevated as compared with control and sham-operated animals as long as 48 h after ischemia (Fig. 2A).
significant increase in nitrite levels (80±8%) was observed 24 h after surgery in sham-operated animals in comparison with control animals. However, nitrite levels returned to basal line after 24 h postsurgery and nitrite values were not different from those of control rats (Fig. 2A).

To identify the brain region that receives the effect of transient uteroplacental ischemia, nitrite levels were evaluated in the cerebral cortex, mesencephalon and cerebellum of one fetus for each of six different pregnant rats. Nitrite levels increased in the cerebral cortex and no changes were seen in either the mesencephalon or cerebellum. Consequently, 24 h after occlusion of the uterine arteries nitrite levels in the mesencephalon were 0.08±0.01, 0.09±0.01 and 0.08±0.02 nmol/mg protein in tissue from control, sham-operated and ischemia rats, respectively. In the cerebellum, nitrite values were 0.040±0.002, 0.045±0.001 and 0.037±0.002 nmol/mg protein in tissue from control, sham-operated and ischemia rats, respectively. On the contrary, in the cerebral cortex from ischemia rats, nitrites increased 101±5 and 78±8% as compared with control or sham-operated rats, respectively, suggesting that this region is a target of transient uteroplacental ischemia (Fig. 2B). The ischemia-induced increase in nitrite levels in the cerebral cortex was completely prevented by either L-NAME (10 mg/kg of body weight, i.p.) or AMT (0.350 mg/kg kg of body weight, i.p.) injected to pregnant rats 1 h before clamping the uterine arteries (Fig. 2C).

3.3. Effect of transient uteroplacental ischemia on activity of NOS isoforms in the cerebral cortex

Sham operation produced an increase in Ca$^{2+}$-dependent and Ca$^{2+}$-independent NOS activity 24 or 36 h after surgery. However, ischemia by transient occlusion of the uterine arteries increased Ca$^{2+}$-dependent and Ca$^{2+}$-independent NOS activity higher than sham-operation at those time points. When compared with the control group, sham operation augmented Ca$^{2+}$-dependent and Ca$^{2+}$-independent NOS activity 30±4 and 12±2%, respectively, after 24 h, and 5±2 and 14±4%, respectively, after 36 h following the surgery. At 24 h, ischemia significantly (P<0.05) increased Ca$^{2+}$-dependent and Ca$^{2+}$-independent NOS activity 82±7 and 118±15%, respectively, from control, or 46±3 and 72±7%, respectively, from sham-operated rats. At 36 h, the ischemia effect on Ca$^{2+}$-dependent and Ca$^{2+}$-independent NOS activity was 40±9 and 32±8%, respectively, higher than control or 40±9 and 27±8%, respectively, higher than sham-operated rats (Fig. 3A and B).

3.4. Transient uteroplacental ischemia differentially affects expression of NOS isoforms in the cerebral cortex

To explore the NOS isoform involved in the increase of NO production following transient uteroplacental ischemia, the expression of eNOS, nNOS and iNOS was assessed by RT-PCR in the cerebral cortex of fetal rats at two time points after ischemia—8 and 36 h. At 8 h, the normalized values of densitometry for eNOS were 0.80±0.06, 0.80±0.04, 1.10±0.18 a.u., P<0.05, for control, sham-operated and ischemia fetuses, respectively; for nNOS were 0.52±0.03, 0.93±0.09 a.u., P<0.05, 1.27±0.12 a.u., P<0.05, for control, sham-operated and ischemia fetuses, respectively; and for iNOS were 0, 0, 0.37±0.09 a.u., P<0.05, for control, sham-operated and ischemia fetuses, respectively (Fig. 4A); at 36 h, the normalized values of densitometry for eNOS were 0.75±0.03, 0.64±0.06, 1.54±0.11 a.u., P<0.05, for control, sham-operated and ischemia fetuses, respectively; for nNOS were 0.67±0.05, 0.61±0.10, 0.61±0.08 a.u. for control, sham-operated and ischemia fetuses, respectively; and for iNOS were 0, 0, 0.55±0.08 a.u., P<0.05, for control, sham-operated and ischemia fetuses, respectively (Fig. 4B). The expression of
Fig. 4. Transient uteroplacental ischemia differentially affects the expression of NOS isoforms in the cerebral cortex of fetal rats. (A,B) Representative photographs of ethidium bromide-stained RT-PCR products fractionated on 2%-agarose gel and their respective densitometry analysis 8 and 36 h following 5-min occlusion of uterine arteries on gestational day 17 (GD17). Lanes: 1=100-bp molecular weight markers; 2, 5, 8 and 11=PCR products from control groups; 3, 6, 9 and 12=PCR products from sham-operated groups. 4, 7, 10 and 13=PCR products from ischemia groups. GA3DPH O.D. values 8 h after ischemia were: 138.2±13.2, 140.7±12.1, 127.7±9.3 for control, sham-operated and ischemia groups, respectively. GA3DPH O.D. values 36 h after ischemia were: 121.2±14.4, 120.1±17.5, 115.8±18.9 for control, sham-operated and ischemia groups, respectively. All values are the mean±S.E.M. of four living fetuses delivered from four independent mothers for each group. *, Significantly different from control group; †, significantly different from sham-operated group; P, 0.05. C, control; S, sham-operated; I, ischemia.

The housekeeping gene GA3PDH was very similar in the three experimental groups at 8 h, 138.2±13.1, 140.7±12.1, and 127.7±9.3 a.u. for control, sham-operated and ischemia fetuses, respectively (Fig. 4A, lanes 2, 3 and 4), or at 36 h, 121.2±17.4, 120.1±17.5 and 115.8±18.9 a.u. for control, sham-operated and ischemia fetuses, respectively (Fig. 4B, lanes 2, 3 and 4). In the cerebral cortex, eNOS expression was similar in the three experimental groups 8 h after surgery (Fig. 4A, lanes 5, 6 and 7), but it increased in ischemia animals 36 h after surgery (Fig. 4B, lane 7) as compared with control and sham-operated groups (Fig. 4B, lanes 5 and 6). nNOS expression increased in the cerebral cortex of sham-operated and ischemia animals (Fig. 4A, lanes 9 and 10) as compared with controls (Fig. 4A, lane 8) at 8 h after surgery, whereas its expression was very similar in the three experimental groups at 36 h (Fig. 4B, lanes 8 and 9). Finally, iNOS expression was not observed in the cerebral cortex of either control or sham-operated animals at 8 h (Fig. 4A, lanes 11 and 12) and 36 h after surgery (Fig. 4B, lanes 11 and 12), but there was a significant expression in ischemic cortex 8 h (Fig. 4A, lane 13) and 36 h (Fig. 4B, lane 13) after surgery.

4. Discussion

Our results showed that interruption of placental blood supply for 5 min on GD17 lead to increased NO production in the cerebral cortex associated with augmented NOS activity and NOS mRNA levels. Although our data showed that sham-operation also increased nitrite accumulation, NOS activity and nNOS mRNA levels, it was clear that ischemia by occlusion of the uterine arteries further potentiated the induction of the NO system, suggesting that ischemia does play a role in the regulation of cerebral NO synthesis. Our results agree and disagree with previous reports; for instance, no changes in NO production, NOS activity and expression in the immediate 20-min period following perinatal asphyxia in rats have been reported [30], whereas Bolanos et al. have shown that brain nitrates increase 1.4-fold after anoxia, an effect prevented by l-NAME administration to the mother [3]. Increased NOS activity as responsible for the elevated nitrite levels after ischemia is supported by the blockade of the nitrite increment with NOS inhibitors, suggesting that increased nitrite levels are indeed related to increased NO production in this animal model of transient ischemia. The total inhibitory effect of AMT, a selective iNOS inhibitor, could suggest the participation of this isofrom as the only responsible for the increased NO production. However, data showing increased Ca²⁺-dependent and Ca²⁺-independent NOS activity and augmented nNOS mRNA as well as eNOS mRNA levels suggest that NO production is depending on the activity of all the NOS isoforms and probably, under our experimental conditions, AMT is acting as a nonspecific NOS inhibitor.

The total overproduction of NO induced by transient
uteroplacental ischemia was not widespread throughout the brain, but it was localized only in the cerebral cortex. It is possible that the regional effect of transient uteroplacental ischemia could be due to the high density of NMDA receptors in the cerebral cortex [17]. An increasing amount of evidence shows that the activation of NMDA receptors during cerebral ischemia enhances NOS expression and activity [7,16,25]. The effect on Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent NOS activity and on constitutive and inducible isofrom expression found in the cerebral cortex supports the suggestion that this region is the main target of transient uteroplacental ischemia [2].

Our finding that iNOS may be upregulated after perinatal ischemia is supported by the report showing upregulation of iNOS in cerebral ischemia [13]; the upregulation of iNOS may be critical to maintain elevated levels of NO after cerebral ischemia. In addition to iNOS upregulation, we found a time-dependent and differential upregulation of nNOS and eNOS, supporting the hypothesis that all the NOS isoforms participate in the regulation of NO production after transient uteroplacental ischemia. We observed an increase in eNOS expression in the cerebral cortex at 36 h following uteroplacental ischemia. This result suggests that eNOS-mediated NO production is triggered late in the cerebral cortex of fetuses subjected to transient uteroplacental ischemia. These data contrast with previous reports showing that eNOS-mediated NO production is triggered early in the global cerebral ischemia induced by cardiac arrest in adult animals [43]. Moreover, we found that nNOS was upregulated 8 h after ischemia and returned to basal levels after 36 h postischemia. Based on these data, we suggest that NO production depends on the activity of all the NOS isoforms and probably the differential expression of NOS isoforms maintains elevated levels of NO after uteroplacental ischemia by an initial and rapid increment of nNOS and iNOS expression. These isoforms might thus account for the increase in both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent NOS activity. On the other hand, later on after the ischemia the increased eNOS expression and the ongoing iNOS expression could explain the increase in both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent NOS activity and therefore the overproduction of NO. To the best of our knowledge, this is the first study that assesses the effect of transient uteroplacental ischemia on the three major isoforms of NOS present in the cortex of fetal brains.

Our RT-PCR results showed the existence of a regulation of the expression of constitutive isoforms (eNOS and nNOS), although we did not explore the mechanism for this regulation. It has been suggested that the NO itself can be the factor responsible to return the expression of nNOS to basal levels [11]. The upregulation of eNOS expression can be explained by the finding that this isoform has regulatory elements in its promoter region, which are characteristic of inducible genes [31]. It has been suggested that a redox-sensitive AP-1 mediated transcriptional control is one of the regulatory elements responsible for the hypoxia-induced upregulation of eNOS [18]. The expression of iNOS after transient cerebral ischemia appears to be a constant event in different animal models of ischemia [22]. Brain parenchyma damage present in transient ischemia produces several stimuli, most of them involving activation of cytokine-mediated gene expression, which induce iNOS expression resulting in a massive production of NO with cytoxic effects (for review see [29]).

The role of NO in brain ischemia during the perinatal period remains to be understood. It is thought that NO derived from nNOS in the ischemic brain is cytoxic since activation of NMDA receptors appears to be the mechanism leading to excessive nNOS activation [24,41]. This suggestion is supported by the finding that the size of infarct induced by local cerebral ischemia is smaller in nNOS knockout than in wild-type mice [37]. Amelioration of brain ischemic-induced brain damage by relatively selective iNOS inhibitors and reduced size of brain infarcts and motor deficits in iNOS knockout mice support the cytoxic effect of NO as iNOS source [32,44]. Conversely, exacerbation of brain infarct size induced by focal ischemia has been found in eNOS knockout mice suggesting the protective effect of NO synthesized by eNOS [19]. Based on this evidence, it can be suggested that NO generation from each isoform plays opposite roles in the cerebral cortex of fetuses deprived for 5 min of maternal blood flow in the perinatal period. Whereas NO synthesized by nNOS and iNOS might be cytoxic [4], eNOS might provide NO known to act as endothelium derived relaxing factor in an attempt to limit the extent of brain damage [28]. In conclusion, we suggest that this noninvasive animal model resembles clinical situations of perinatal asphyxia due to umbilical cord prolapse, premature placenta abruption and vasoconstriction of umbilical arteries in cocaine-addicted pregnant females, and therefore this model can be useful to understand the role of NO in the ischemic process.

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