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Nitric oxide mediates brain mitochondrial maturation immediately after birth

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Abstract The possible role of nitric oxide (•NO) in brain mitochondrial maturation was studied. Within the first 5 min after birth, a sharp increase in ATP concentrations was observed, coinciding with an increase in mitochondrial complex II-III (succinate-cytochrome *c* reductase) activity, while complex I (NADH-CoQ_1 reductase) and complex IV (cytochrome *c* oxidase) activities remained unchanged. Under the same circumstances, cGMP concentrations were increased by 5 min after birth, correlating significantly with ATP concentrations. Since ATP concentrations also correlated significantly with mitochondrial complex II-III activity, these three parameters may be associated. Inhibition of •NO synthase activity brought about by the administration of N^{ω}-nitro-L-arginine monomethyl ester to mothers prevented the postnatal increase in cGMP and ATP levels and complex II-III activity. These results suggest that early postnatal mitochondrial maturation in the brain is a •NO-mediated process.

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Key words: Nitric oxide; Brain; Mitochondrion; Perinatal

1. Introduction

During the postnatal period the rat brain undergoes a period of rapid growth and maturation which requires a good supply of energy as the cells divide, enlarge in size and develop their full adult metabolic activity (for a review, see [1]). Under these circumstances, the provision of energy in the form of ATP is a prime requirement for the developing brain to carry out its normal functions. Consequently, the acquisition of fully developed mitochondria after birth is essential to enable newborn mammals to successfully adapt to extrauterine life. Brain mitochondrial function has been studied during the first month of extrauterine life [2–5], but no evidence concerning the mechanisms and factors involved in mitochondrial maturation in the brain is available.

Within the CNS, •NO appears to be an important signalling molecule in the transduction pathway leading to increased intracellular cGMP concentrations. This ability to increase brain cGMP levels suggests that •NO is involved in the regulation of important metabolic pathways in the brain (for a review, see [6–8]). In addition, a role for •NO in the development of the nervous system has also been suggested [9–11]. In this context, nitric oxide synthase (NOS) is present in brain tissues during the embryonic and early postnatal stages [10–13]. Thus, Western blot analysis of NOS isoform proteins has revealed that the amount of brain NOS protein increases around birth in the rat [10], together with brain NOS activity [10,11,13] and •NO biosynthesis [14].

The mitochondrion is known to be a target organelle for the action of •NO (reviewed in [15,16]). Under certain pathological circumstances •NO synthesis may be excessive and then •NO becomes neurotoxic, causing mitochondrial dysfunction and hence a state of energy deficiency [15].

The aim of this work was to investigate the possible relationship between •NO synthesis around birth and the abrupt onset of mitochondrial function in the brain immediately after delivery.

2. Materials and methods

2.1. Materials

N^{ω}-Nitro-L-arginine monomethyl ester (L-NAME) and ubiquinine-5 (coenzyme Q_1) were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Cytochrome *c* (Boehringer Mannheim, Germany) was reduced with sodium ascorbate immediately before use and passed through Sephadex G-25M (PD-10 columns, Pharmacia LKB, Uppsala, Sweden) to remove ascorbate. Other substrates, enzymes, coenzymes and standard analytical laboratory reagents were purchased from Sigma, Boehringer or Merck (Darmstadt, Germany).

2.2. Animals

Albino Wistar rats fed on a stock laboratory diet (w/v, carbohydrate 58.7%, protein 17.0%, fat 3.0%, plus added salts and vitamins) and of known gestational age were used for the experiments. Virgin females weighing 225–250 g were caged overnight with males. Conception was considered to occur at 01.00 h and was confirmed the next morning by the presence of spermatozoa in vaginal smears. To inhibit NOS, L-NAME was given to pregnant rats in their drinking water at a concentration of 0.1, 0.3 or 1 mg/ml throughout the last week of gestation, as previously described [17]. Fetuses were delivered on day 21.5 of gestation (21.7 day for full gestation) by rapid hysterectomy after cervical dislocation of the mother.

Immediately after delivery, newborns were carefully wiped, their umbilical cords were tied and cut and they were kept in an incubator at 37°C under a continuous stream of water-saturated air without feeding. All newborns were killed by decapitation at the times indicated in the figures (5 min, 1 h or 2 h) and their whole brains were rapidly (<2 s) freeze-clamped in liquid nitrogen and kept frozen (−20°C) until analysis.

2.3. Enzyme activity determinations

For the determination of mitochondrial respiratory chain complex activities, brains were homogenized in 0.1 M potassium phosphate buffer, pH 7.4, to a final protein concentration of ∼8 mg/ml. Enzyme activities were determined in the homogenates using a Hitachi U2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Complex I (NADH-CoQ_1 reductase; EX 1.6.99.3) activity was measured as described in Ragan et al. [18]. The activity of complex II-III (succinate-cytochrome *c* reductase; EC 1.8.3.1) was determined following the method of King [19]. Complex IV (cytochrome *c* oxidase; EC 1.9.3.1) activity was determined as described by Wharton and Tzagoloff [20]. All enzyme activities were expressed as nmol per min per mg of protein,

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Abbreviations: L-NAME, N^{ω}-nitro-L-arginine monomethyl ester; NOS, nitric oxide synthase

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except for cytochrome *c* oxidase, which was expressed as the first-order rate constant (*k*, per min per mg of protein).

2.4. ATP and cGMP determinations

For nucleotide determinations, frozen brains were weighed and immediately homogenized in 30 volumes of 0.3 M $HClO_4$. Extracts were neutralized with 0.5 M $KHCO_3$ and centrifuged, the supernatants being used for analysis. cGMP was measured using a commercially available radioimmunoassay kit (Amersham International, UK) and following the manufacturer's instructions. ATP was determined by the luciferin/luciferase chemiluminescence method using a commercially available kit (Sigma) following the manufacturer's instructions. Nucleotide concentrations were expressed as pmol of cGMP or μmol of ATP per g of brain wet weight.

2.5. Protein determinations

Protein concentrations were determined in brain homogenates by the method of Lowry et al. [21], using bovine serum albumin as standard.

2.6. Statistical analysis

Results are expressed as means ± S.E.M. for the number of newborns, indicated in the legends, coming from at least three different pregnant rats. Statistical analysis of the results was determined by Student's *t* test.

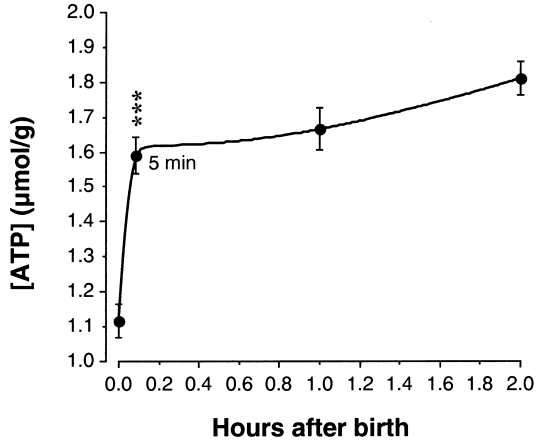

Fig. 1. ATP concentrations in rat brain during the early postnatal period. Term fetuses (0 h; without breathing) or 5 min, 1 h or 2 hold newborns were killed and their brains were immediately removed and frozen for ATP analysis as described in Section 2. Results are mean ± S.E.M. values from 14–26 newborns from 4–10 different pregnant rats. ***$P < 0.001$ compared to previous value.

3. Results

Immediately after birth (5 min), we observed a significant increase (31%, $P < 0.001$) in ATP concentrations in the rat brain (Fig. 1), reaching 75% of the ATP concentration found at 2 h of extrauterine life (Fig. 1). This prompted us to investigate the activities of the mitochondrial respiratory chain complexes under the same circumstances. Fig. 2 shows that complex II-III (succinate-cytochrome *c* reductase) activity increased by about 29% at 5 min after birth, following a developmental pattern similar to that found for ATP concentrations (Fig. 1). In fact, a linear correlation ($r = 0.982$) between

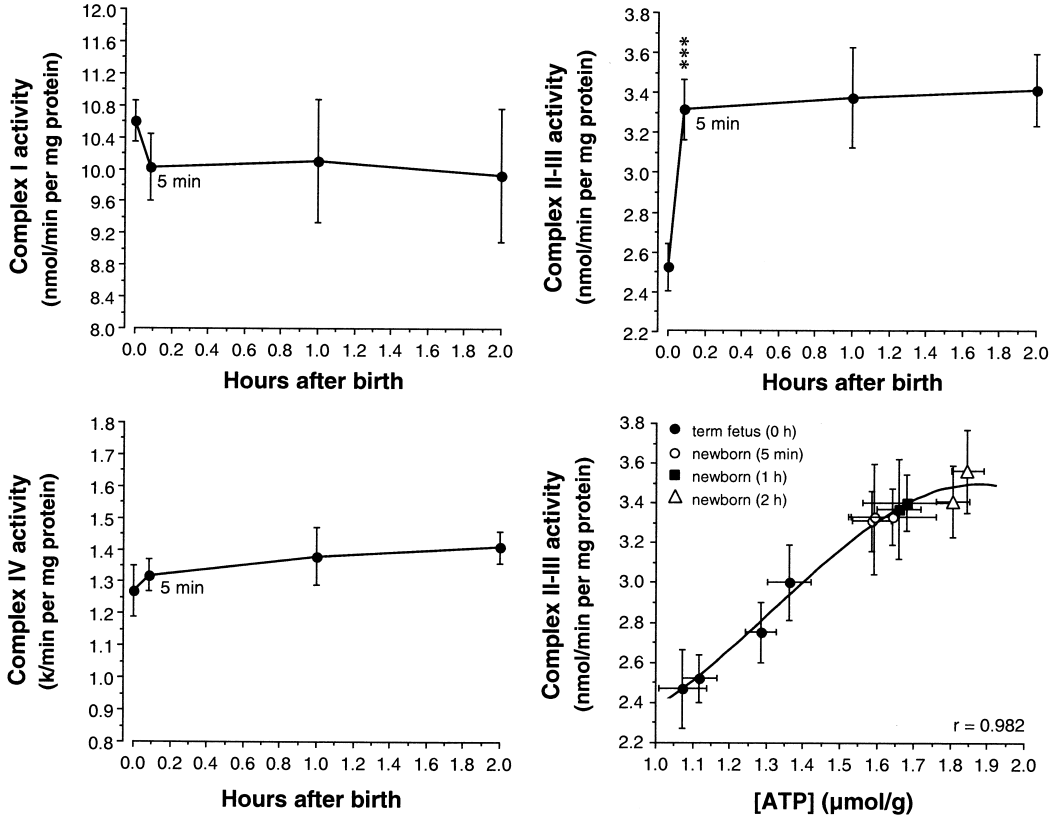

Fig. 2. Mitochondrial respiratory chain complex activities in rat brain during the early postnatal period. Term fetuses (0 h; without breathing) or 5 min, 1 h or 2 hold newborns were killed and their brains were immediately removed and frozen for enzymatic analysis as described in Section 2. The lower panel shows the correlation between complex II-III activities and ATP concentrations at different times around birth. Results are mean ± S.E.M. values from 10–17 newborns from 4–6 different pregnant rats. Each value in the correlation plot represents the mean ± S.E.M. from 4–6 newborns from each pregnant rat. ***$P < 0.001$ compared to previous value.

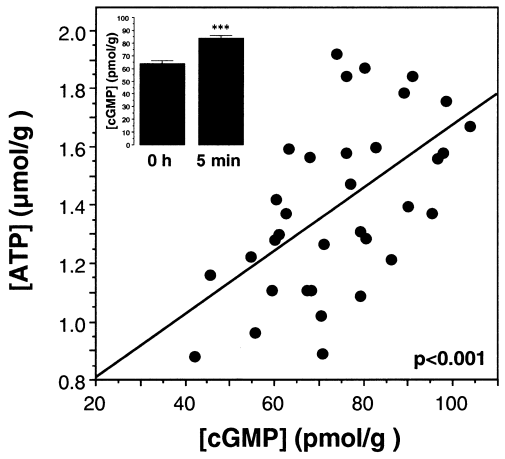

Fig. 3. cGMP concentrations in rat brain during the early postnatal period. Term fetuses (0 h; without breathing) or 5 min, 1 h of 2 hold newborns were killed and their brains were immediately removed and frozen for cGMP and ATP analysis as described in Section 2. In the inset, results on cGMP are mean ± S.E.M. values from 20–26 newborns from 7–10 different pregnant rats. ***$P < 0.001$ compared to 0 h value.

complex II-III activity and ATP concentration was found in the brain during the postnatal period, at least up to 1 h after birth (Fig. 2). In contrast, no changes in the activities of complex I (NADH-CoQ_1 reductase) and complex IV (cytochrome *c* oxidase) were found during the postnatal period (Fig. 2). Also, the activity of citrate synthase remained unchanged in these circumstances (170 ± 8.75 and 185 ± 5.35 nmol/min per mg protein at 0 h and 5 min, respectively), suggesting that the postnatal increase in complex II-III activity (Fig. 2) would not be due to an increase in the number of mitochondria.

NOS is expressed transiently in the CNS during the perinatal period [13,22,23], coinciding with an increase in •NO biosynthesis [14]. We therefore investigated the role of •NO synthesis during the early postnatal period. In this sense, since the cGMP concentration is a well-known index of the rate of •NO synthesis [24], we measured cGMP concentrations during the first 5 min of extrauterine life. Fig. 3 shows that brain cGMP concentrations increased significantly from the fetal (0 h) to the neonatal (5 min) periods concurrently with those of ATP (Fig. 1). In order to quantitatively evaluate both phenomena, a linear regression analysis of the data was performed; this gave a highly significant ($P < 0.001$) correlation ($r = 0.694$) between ATP and cGMP concentrations in the brain under our experimental conditions (Fig. 3).

In order to further confirm the possible role of •NO in

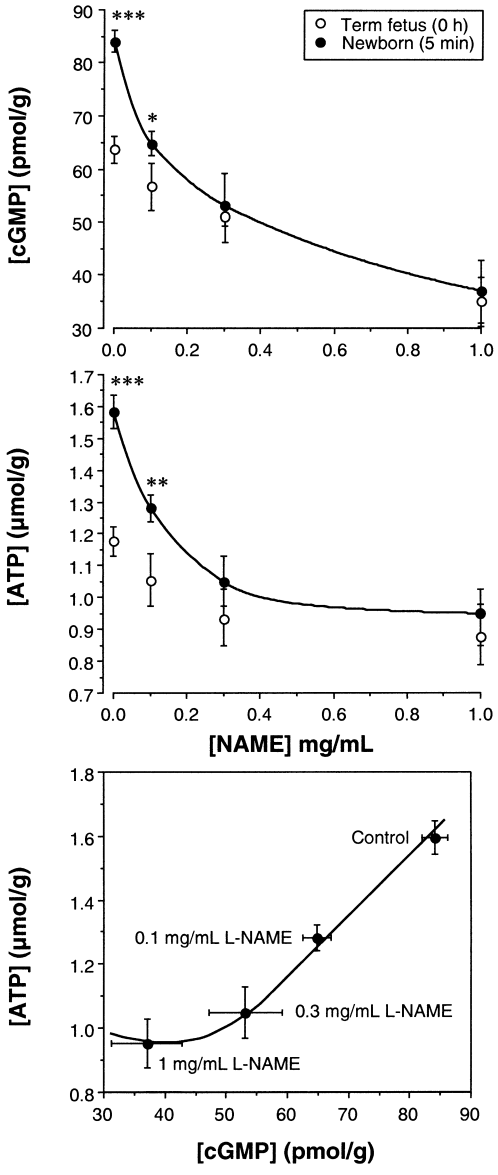

Fig. 4. Effect of L-NAME administration on ATP and cGMP concentrations in the newborn rat brain. Different concentrations (0.1, 0.3 or 1 mg/ml) of L-NAME were administered to pregnant rats in their drinking water during the last week of gestation. Term fetuses (0 h; without breathing) or 5 min old newborns were killed and their brains were immediately removed and frozen for cGMP and ATP analysis as described in Section 2. Results of cGMP and ATP are mean ± S.E.M. values from 12–26 newborns from 3–10 different pregnant rats. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared to term fetuses (0 h) from the same experimental group.

Table 1
Effect of L-NAME administration on mitochondrial respiratory chain complex activities in the brain of 5 min old newborn rats

Condition	Mitochondrial enzyme activities			
	Complex I	Complex II-III	Complex IV	CS
Control	10.02 ± 0.42	3.31 ± 0.15	1.32 ± 0.05	185 ± 5.39
L-NAME (0.1 mg/ml)	10.48 ± 0.62	2.75 ± 0.24*	1.38 ± 0.08	180 ± 3.88
L-NAME (1 mg/ml)	11.00 ± 0.97	2.46 ± 0.21**	1.40 ± 0.07	188 ± 7.27

Different concentrations (0.1 or 1 mg/ml) of L-NAME were administered to pregnant rats in their drinking water during the last week of gestation. Enzyme activities were measured in the brains of newborns as described in Section 2. Results are mean ± S.E.M. values from 7–17 newborns from 3–6 different pregnant rats and are expressed as nmol/min per mg of protein, except complex IV activity, which is expressed as *k*/min per mg of protein. CS, citrate synthase.
*$P < 0.05$, **$P < 0.01$ compared to control value.

postnatal mitochondrial maturation, L-NAME, a potent NOS inhibitor, was administered to pregnant rats at increasing doses during the last week of gestation [17] and brain ATP concentrations were measured in 5 min old newborns. Our results show that L-NAME administration prevented the postnatal increase in cGMP concentration in a dose-dependent fashion, the increase being abolished at 0.3 mg/ml L-NAME (Fig. 4). Likewise, the administration of 0.1 mg/ml L-NAME to the mothers prevented the postnatal increase in brain ATP concentrations as compared to the values found in newborns from untreated mothers (Fig. 4). The postnatal increase in brain ATP concentrations was completely prevented when L-NAME was administered at 0.3 mg/ml (Fig. 4). The correlation between ATP and cGMP concentrations in the brains of newborns from untreated and 0.1, 0.3 or 1 mg/ml L-NAME-treated mothers is shown in Fig. 4. A significant ($P < 0.001$) linear correlation ($r = 0.997$) was observed, although this disappeared when L-NAME was administered at the highest concentration of 1 mg/ml (Fig. 4).

Table 1 shows the activities of the mitochondrial respiratory chain complexes and citrate synthase in the brains of 5 min old newborns from untreated (control) and 0.1 or 1 mg/ml L-NAME-treated mothers. The administration of L-NAME to the mothers significantly decreased the activity of mitochondrial complex II-III as compared to the values found in newborns from untreated mothers. However, complex I, complex IV and citrate synthase activities remained unchanged by the treatment (Table 1).

4. Discussion

Although previous works have studied brain mitochondrial function during the first month of extrauterine life [2–5], no evidence concerning brain mitochondrial maturation during the first hours after birth, when neurons represent 95–98% of brain cells [25], is available. Our results show that complex II-III activity, but not complex I or complex IV activities, increased significantly at 5 min after birth and this activity persisted during the first 2 h of extrauterine life (Fig. 2). It is noteworthy that the activity found for complex II-III at 5 min after birth was similar to the observed in mature cultured neurons [26,27]. This suggests that the maturation of mitochondria occurs in the brain immediately after birth. The developmental pattern of complex II-III activity (Fig. 2) was similar to that found for ATP concentrations (Fig. 1). Thus, brain ATP concentrations increased sharply 5 min after birth and remained high throughout the early postnatal period (Fig. 1). In addition, a linear correlation between complex II-III activity and ATP concentrations (Fig. 2) was observed during the first hour after birth, suggesting that both phenomena might be related. If so, the increases in ATP concentrations and in complex II-III activity immediately after birth might be due to postnatal mitochondrial maturation. In fact, the activities of glycolytic enzymes are very low in the rat brain at birth [28–30], suggesting that under these circumstances ATP synthesis is mainly due to mitochondrial activity.

Since it is well accepted that cGMP concentrations can be regarded as an index of endogenous •NO formation [24], the increase in cGMP concentrations observed by us shortly after birth (Fig. 3, inset) suggests that •NO synthesis is enhanced under these circumstances. In agreement with this, it has been demonstrated that NOS is present in brain tissues during the embryonic and early postnatal stages [10–13]. In addition, brain NOS activity rises significantly at birth [10,11,13] together with •NO biosynthesis [14], suggesting that •NO may play a role in the transition from fetal to neonatal life.

The highly significant ($P < 0.001$) correlation observed between ATP and cGMP concentrations in the neonatal rat brain suggests that both parameters may be associated. In fact, the administration of L-NAME, a potent NOS inhibitor, decreased the postnatal increase in ATP concentration in a dose-dependent way, the increase being completely blocked when 0.3 mg/ml L-NAME was administered (Fig. 4). These results suggest that •NO synthesis is mandatory for the postnatal increase in ATP concentrations. Moreover, the administration of L-NAME also prevented the postnatal increase in complex II-III activity (Table 1; see Fig. 2 for 0 h value), suggesting that •NO mediates postnatal mitochondrial maturation in the brain.

Immediately after birth there is an important enhancement of oxygen availability that coincides with an increase in oxidative metabolism [31,32]. Since the synthesis of •NO from arginine in the reaction catalyzed by NOS requires oxygen as a compulsory co-substrate (reviewed in [33]), the early increase in oxygen availability [32] may trigger •NO synthesis. If so, oxygen may be the signal for mitochondrial maturation, •NO acting as a second messenger.

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