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Nitric oxide synthase in chick embryo retina during development

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Abstract High levels of nitric oxide synthase were found in the early stages of developing chick embryo retina. The enzyme activity sharply decreased up to 13-day-old chick embryo retina, when the level of the last embryonic day was reached. The results show that nitric oxide is synthesized in chick embryo retina prior to synaptogenesis. The incubation of chick embryo retinas in presence of NMDA increased the synthesis of nitric oxide, thus, the appearance of nitric oxide production before the synaptogenesis in the retina as well as in the brain may be considered as signal for the development and shaping of neuronal and non-neuronal cells.

Key words: Nitric oxide synthase; Chick embryo; Retina; Development; NMDA

1. Introduction

In the mammalian brain receptor agonists, e.g. glutamate and acetylcholine, which enhance intracellular Ca^{2+}, activate nitric oxide (NO) synthase (NOS) which in turn converts L-arginine into citrulline with stoichiometric production of NO. NOS inhibitors, such as N^{ω}-nitro-L-arginine (L-NA) and its methyl ester (L-NAME), have been shown to prevent the formation of long-term potentiation [1], the proposed electrophysiological correlate of memory formation [2]. Such inhibitors have also been reported to prevent sound evoked brain cortical responses [3,4] and to possess antinociceptive effects [5]. Interestingly, NOS-positive cells have been identified in the retina where the enzyme seems to be differentially modulated by light and dark [6–8]. Altogether, these data strongly suggest a role for NO in synaptic plasticity and in the processing of sensory stimuli. In addition, these roles played by NO may be important for the processes underlying neuronal differentiation during development. Indeed, in the brain of rat and guinea pig NOS displays a precise pattern of activity during the perinatal development of the nervous tissue [9]. Here, we report the original observation that in chick retina basal and *N*-methyl-D-aspartate (NMDA) receptor-stimulated NOS activity changes according to the stage of the embryo development.

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Abbreviations:: NO, nitric oxide; NOS, nitric oxide synthase; NMDA, *N*-methyl-D-aspartate; L-NA, N^{ω}-nitro-L-arginine; D-NA, N^{ω}-nitro-D-arginine; EGTA, ethylene glycolbis(β-aminoethyl ether) *N,N,N',N'*-tetracetic acid.

2. Materials and methods

L-NA was purchased from Research Biochemicals International (Natick, MA), tetrahydrobiopterin was from ICN Biomedicals (Aurora, OH). L-[^{14}C]arginine was obtained from Amersham; leupeptin, aprotinin and other chemicals were from Sigma (St Louis, MO).

Fertilized eggs of White Leghorn chickens were incubated at 38 ± 2°C at a relative humidity of 70–80%. At the indicated days, chick embryos were decapitated and their retinas rapidly removed. During dissection, the eyes were kept at 0–4°C but, once removed, the retinas were maintained at 25°C.

NOS activity was measured by monitoring the conversion of L-[U-^{14}C]arginine into L-[U-^{14}C]citrulline, according to the method described by Salter et al. [10]. After removal, the retinas were cut into thirds and suspended (2 retinas/ml) in cold (12–14°C) modified Krebs--Henseleit solution containing 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4 and 11 mM glucose. Each pool of retinal segments was preincubated in 5 ml of Krebs solution equilibrated with 95% O_2/5% CO_2, in a shaking water bath at 37°C for 60 min. After the preincubation period, the retinas were placed in 1 ml of Krebs solution enriched with arginine (20 μM L-arginine and L-[U-^{14}C]arginine 100,000 dpm), L-citrulline (1 mM, to inhibit reuse of [U-^{14}C]citrulline formed) and 10 μM tetrahydro-biopterin. After 30-min incubation with L-[U-^{14}C]arginine, the reaction was terminated by removal of the retinal segments, dilution and removal of substrate by addition of 2 ml of 1:1 (v/v) H_2O/Dowex resin (Na^+ form). The resin was left to settle for 10 min and 2.5 ml of supernatant was then removed and examined for the presence of L-[U-^{14}C]citrulline by liquid scintillation counting [10].

NOS activity in chick embryo retina was also determined after homogenization at 0°C in 5 vols. of buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor and 2 μg/ml aprotinin, brought to pH 7.0 at 20°C with HCl. The crude homogenate was centrifuged at 0°C at 20,000 × *g* for 20 min. Enzyme activity was then determined in the postmitochondrial supernatant and pellet.

Protein content was determined by the method of Bradford [11], using bovine serum albumin as the standard. The data are expressed as mean ± S.E.M. and evaluated for differences using Student's *t* test.

3. Results

The pattern of NOS activity is shown in Fig. 1 as a function of chick embryo retina developmental age. The enzyme-specific activity was relatively high in the cytosolic fraction from retinas at the 8th and 9th day of incubation; thereafter, NOS activity sharply decreased up to 13th day, when the level of the last embryonic day was reached. To better characterize whether NOS was associated to membrane preparations of visual cells, we assayed the enzyme activity in the later stage of development. Indeed, NOS activity was also measurable in the particulate fraction and, in the presence of 1.0 mM L-NA, there was an almost total inhibition (94 ± 2.4%, $n = 3$) of the basal values. Furthermore, as reported in Fig. 2, in the particulate fraction the enzyme activity resulted Ca^{2+}-calmodulin-dependent, because EGTA (1.0 mM) was able to inhibit it and calmodulin

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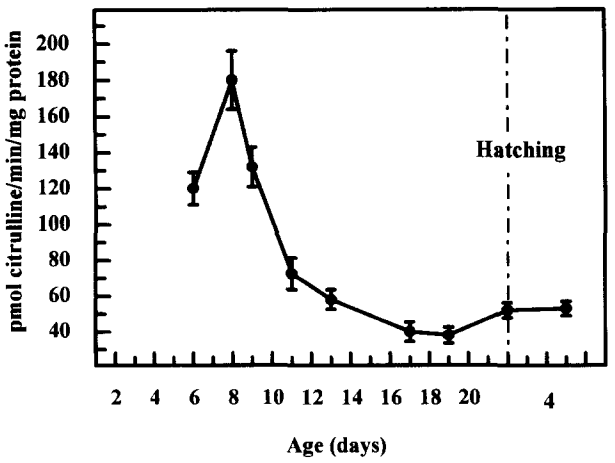

Fig. 1. NO synthase activity of developing chick retina. The data represent mean values ± S.E.M. from 4 experiments. Enzyme activity was measured by the conversion of L-arginine to L-citrulline.

was required to demonstrate the enzyme activity. In the crude retina supernatant preparation, calmodulin also increased the enzyme activity in a concentration-dependent manner and trifluoperazine, a calmodulin antagonist, inhibited the enzyme activity of both supernatant and particulate preparation (see Fig. 2). In another series of experiments, NOS activity was evaluated in retinal segments from chick embryos at various stages of development. Similarly to the results reported for the cytosolic fraction, as shown in Fig. 3, high levels of L-[U-^{14}C]citrulline production were observed in intact segments of embryonic retinas of 8–9th day of incubation and these dramatically decreased up to 17–18th day of incubation. Under these experimental conditions, the activation of NMDA subtype of glutamate receptors by exposure to 1 mM NMDA, yielded a strong Ca^{2+}-dependent increase in L-[U-^{14}C]citrulline production at 8–9th and at 17–18th day of incubation; whereas less significant changes were seen at day 13–14th. Indeed, NMDA-induced increase in citrulline production is due to enhanced NOS activity because 1 mM L-NA; but not 1 mM D-NA, was able to prevent it.

4. Discussion

The present experiments have shown that in chick embryo retina NOS activity undergoes changes during development. In fact, in intact retina segments as well as in the cytosolic preparations NOS activity was high at 8–9th day and at 15–18th day of incubation it reached the level of activity typical of the chick retina after hatching. In all instance, the enzyme activity was reduced by L-NA, a competitive inhibitor of NOS [12], thus, confirming the specificity of the enzyme activity measured. In addition, several evidences exist demonstrating that the conversion of L-[U-^{14}C]arginine in L-[U-^{14}C]citrulline in intact tissue preparations, such as which embryo retina segments (present data), correlates well with the rate of NO synthesis [12]. Quite importantly, similar developmental changes have been recently reported in brain neuronal NOS in rat and guinea pigs [9]. The present observation of Ca^{2+}-dependent NOS activity in both cytosolic and particulate fraction in chick embryo retina suggests that multiple forms of NOS are present during development of the retina and possibly of other tissues of neuroectodermal origin. Interestingly, Ca^{2+}-dependent NOS activity has been measured in the cytosolic and particulate fraction of adult bovine retina [7].

The second important finding of our research work is that NOS activity in chick embryo retina can be stimulated by NMDA and, under these circumstances, the level of enzyme activity varies according to developmental stage. In fact, a 6-fold increase in NOS activity was observed at 17–18th day of incubation whereas only modest effects were seen at day 13–14th. The effect of NMDA appears to be due to the stimulation of the NMDA-subtype of glutamate receptors because according to brain tissues MK-801, a selective NMDA receptor antagonist [13], prevented the increase in NOS activity (unpubl. data). In addition, in experiments in which chick embryo retina segments were incubated in Ca^{2+}-free medium, NMDA failed to stimulate NOS activity. Altogether, these data indicate that a functional NMDA receptor complex, including the associated cationic ionofore (reviewed in [14]), is expressed in developing chick retina, and the level of expression varies according to the developmental stage. It will be interesting to establish whether the NMDA receptor complex in chick embryo retina is sensitive to the positive modulation of polyamines as described in mammalian species [15]. This is of particular importance in view of the knowledge that arginine, the precursor of NO synthesis [16], also represents the precursor of ornithine and, hence, of polyamine synthesis [17]. Incidentally, the pattern of chick embryo retina NOS expression is superimposable to that of ornithine decarboxylase activity [18], the key enzyme of polyamine synthesis [17]. Thus, during development, in chick retina two arginine-requiring pathways appear to exist and these seem to be involved in differentiation processes and synaptogenesis. Interestingly, in chick embryo retina synapses have not been detected prior to the 13th embryonic day [19].

In conclusion, the present data demonstrate that developing

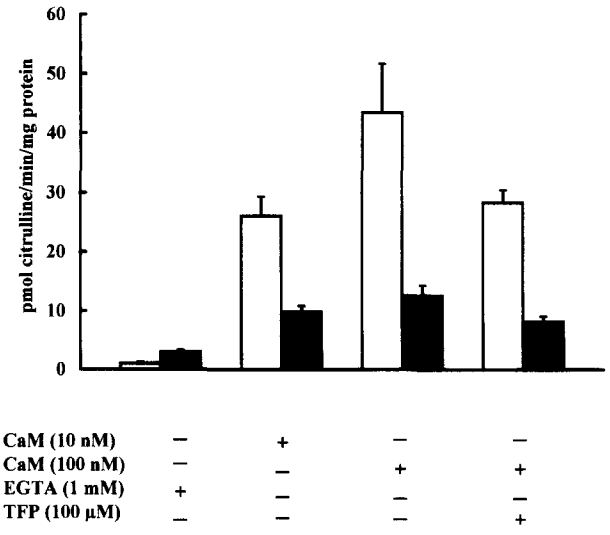

Fig. 2. Ca^{2+}-calmodulin dependent of NOS activity in the crude retina supernatant □ and particulate fraction ■ from homogenates of 15-day-old chick embryos. Basal enzyme activity was determined as reported in section 2, in the presence of 0.45 mM $CaCl_2$, 2 mM NADPH and 100 nM calmodulin (CaM). Ca^{2+}-calmodulin dependence was determined by adding EGTA, or trifluoperazine (TFP). Values are mean ± S.E.M. for 5 experiments, each done in triplicate.

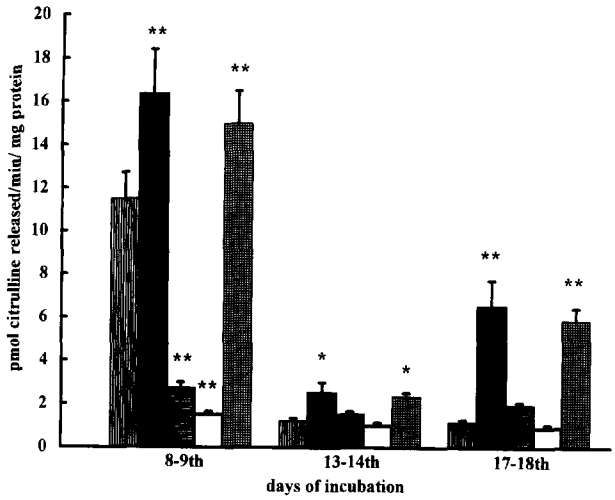

Fig. 3. Effect of 1 mM NMDA on the NO synthesis from chick embryo retina at various ages of development. The retinas were cut into thirds and incubated for 30 min in 1 ml of KREBS solution alone ▥. The incubation medium was enriched with 1 mM NMDA, in the presence ■ or absence ▤ of 2 mM $CaCl_2$, or in the presence of 1 mM N^{ω}-nitro-L-arginine (L-NA) □ or 1 mM N^{ω}-nitro-D-arginine (D-NA) ▦. Data are mean ± S.E.M. (bars) from 3–4 independent experiments performed in triplicate. The NOS activity was measured by monitoring the conversion of L-[U-^{14}C]arginine into [U-^{14}C]citrulline. $*P < 0.05$, $**P < 0.001$ in comparison to basal values.

retina preparations constitute excellent systems for studying functional changes in basal and NMDA-induced NO synthesis during maturation of neuroretina and visual cells.

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5. References

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