Behaviour of nitric oxide synthase in rat cerebellar granule cells differentiating in culture

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Abstract The possible relation between nitric oxide synthase (NOS) activity and neural differentiation was investigated using primary cultures of rat cerebellar granule cells differentiating in culture. NOS activity was measured in the cytosolic and particulate fractions obtained from cell homogenate. In the experimental conditions used the optimal pH for NOS activity was about 6.4, the activity being about 3-fold higher than at pH 7.4. Cerebellar granule cell differentiation was associated with marked increases in NOS activity. In undifferentiated cells the enzyme was almost evenly distributed between the cytosolic and particulate fractions, during differentiation there was a 12-fold increase in activity in the cytosolic enzyme and a 3-fold increase in the particulate one. This indicates a marked preferential enrichment of the cytosolic enzyme during differentiation. Cerebellar granule cells produced and released NO in the culture medium; NO formation being markedly higher in differentiated cells (7–12 DIC) than in undifferentiated (2–3 DIC) ones. These data demonstrate a relationship between NOS expression and NO production and the differentiation of cerebellar granule cells, supporting the notion that NO may play a role in this process.

Key words: Nitric oxide; Nitric oxide synthase; Cerebellar granule cell; Neuronal differentiation

1. Introduction

Nitric oxide (NO), a highly diffusible and short-lived free radical, represents the first of a new class of mammalian inter- and intracellular messenger molecules acting in the vascular, immune and nervous systems (for review see [1–4]). In the nervous system NO appears to be involved in neurotransmitter release [5,6], long-term potentiation and depression (for a review see [3]), neuronal differentiation [7,8], synaptogenesis [9,10], cell death and neurodegeneration [4]. NO biosynthesis is expected to be tightly controlled, primarily through a NO synthesizing enzyme (NOS). The neuronal isoform of this enzyme, first purified from the cytosolic fraction [11] and more recently from the particulate fraction of cerebellum [12], is a constitutive and Ca^{2+}-regulated protein [3], whose expression may be regulated in both the peripheral and central nervous system [13–16]. In adult rat cerebellum NOS expression appears to be characteristic of a well-defined cell type; it is abundant in granule and basket cells but not in Purkinje cells. In mature cerebellum granule cells NOS expression seems to be influenced also by exogenous factors [17].

In order to have a greater understanding of the possible role of NO in neuronal differentiation we measured NO production and NOS subcellular distribution in rat cerebellar granule cells, differentiating in culture. These cells are considered a good model of homogeneous neurons that replicate in vitro many of the morphological and physiological characteristics of native cerebellar development. In fact, during the first week in culture, the cerebellar granule cells develop a rich network of fasciculated fibers [18] that interconnects the cells, express voltage dependent Na^+ channels [19], glutamate receptors [20], and surface sialoglycoproteins [21], and accumulate gangliosides [22] that are characteristic components of neuronal plasma membrane.

2. Materials and methods

2.1. Materials

Basal modified Eagle's medium (BME), fetal calf serum (FCS), NADPH tetrasodium salt, (6R)-5,6,7,8 tetrahydrobiopterin dihydrochloride (BH_4), fetal calf thymus DNA (type I), arginine, HEPES, EGTA were from Sigma (St. Louis, MO); HPTLC silica gel plates from Merck (Darmstadt, Germany); L-[2,3,4,5-^{3}H]arginine monohydrochloride (64 Ci/mmol) from Amersham (UK); N^G-monomethyl L-arginine monoacetate (L-NMMA) and Cayman's Nitrite/Nitrate assay kit from Alexis Co. (San Diego, CA); Commassie Protein assay reagent and bovine serum albumin (BSA) from Pierce Chemical Co (Rockford, IL).

2.2. Cell cultures

Primary cultures of granule cells were prepared from cerebella of 8-day-old rats (Sprague-Dawley) and cultured in BME medium containing 10% FCS, as previously described [18]. In these experimental conditions cultures consist of ≥95% granule neurons and ≤5% glial cells. Cell morphology was assessed by phase-contrast microscopy. At different days in culture (DIC) the cells were rinsed twice with 50 mM HEPES, 2.7 mM KCl, 100 mM NaCl (pH 7.4), scraped off the plates and homogenized by gentle pipetting up and down for 50 times. Homogenates were then centrifuged at 100 000 × g (30 min at 4°C) and the cytosolic and total particulate fractions collected and used.

2.3. Determination of NOS activity

NOS activity was determined on the fresh homogenate, cytosolic and particulate fractions by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline. The assay was performed according to Bredt and Snyder [23], with some modifications. The assay mixtures, containing 10 μM [^{3}H]arginine (5 Ci/mmol), 0.45 mM $CaCl_2$, 2 mM NADPH, 10 μM BH_4, 50 mM HEPES buffer at the optimal pH (see Section 3), 25–50 μg protein in a final volume of 50 μl, were incubated at 37°C for 60 min. The reaction was terminated by heating samples at 90°C for 10 min [24]. Blank samples were mixtures heated at 90°C for 10 min before incubation at 37°C for 60 min. After cooling, TCA was added to a final concentration of 6% (w/v) and precipitates removed by centrifugation (17 000 × g, 30 min). Two microliters (carrying 14 000 dpm) of the supernatant were spotted on HPTLC silica gel plates. The HPTLC plates were developed in the solvent system chloroform/methanol/conc. ammonia/water 1:9:4:2 (v/v/v/v). Spots for [^{3}H]arginine and [^{3}H]citrulline were quantitated by radiochromatoscanning using a Digital Autoradiograph (Berthold, Germany). The quantity of [^{3}H]arginine converted to [^{3}H]citrulline was calculated from the specific activity of [^{3}H]arginine added to the assay mixture after subtraction of the blank values. The enzyme activity was ex-

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pressed as nmol [^{3}H]citrulline mg^{-1} protein h^{-1}. Under the described conditions the coefficient of variation of the method was lower than ±6%. The optimal pH for NOS activity in cultured granule cells was determined using HEPES-based buffers in the pH range 5.0–7.9. The dependence of NOS on Ca^{2+} ions was determined using the reaction mixture described above in which 0.45 mM $CaCl_2$ was replaced by EGTA (1–4 mM). The specificity of NOS activity was determined by adding 0.4 mM L-NMMA, a specific NOS inhibitor [23].

2.4. Measurement of NO in the culture media

NO produced by cerebellar granule cells at different DIC was evaluated from the amount of NO_3^- and NO_2^- (the stable oxidation products of NO) present in the culture medium [7]. In these experiments granule cells were cultured in supplemented BME containing 10% FCS without Phenol Red; NO_3^- and NO_2^- were determined using Cayman's Nitrate/Nitrite assay kit and 160 µl of culture medium (previously centrifuged, 1000×*g* for 15 min); standard curves, using $NaNO_2$ and $NaNO_3$, were included in each assay.

2.5. Other methods

Proteins were determined with the Commassie Protein assay reagent using BSA as the standard; DNA according to Burton [25] using calf thymus DNA as the standard.

3. Results

The optimal pH for NOS assay in cerebellar granule cells was first established in preliminary experiments. As shown in Fig. 1 the pH optimum of NOS was about 6.4, the NOS activity being about 3-fold higher than at the more commonly used pH 7.4. This increased activity was found for homogenates, cytosolic and particulate fractions and at different DIC. Thus, all NOS activity measurements were made at pH 6.4.

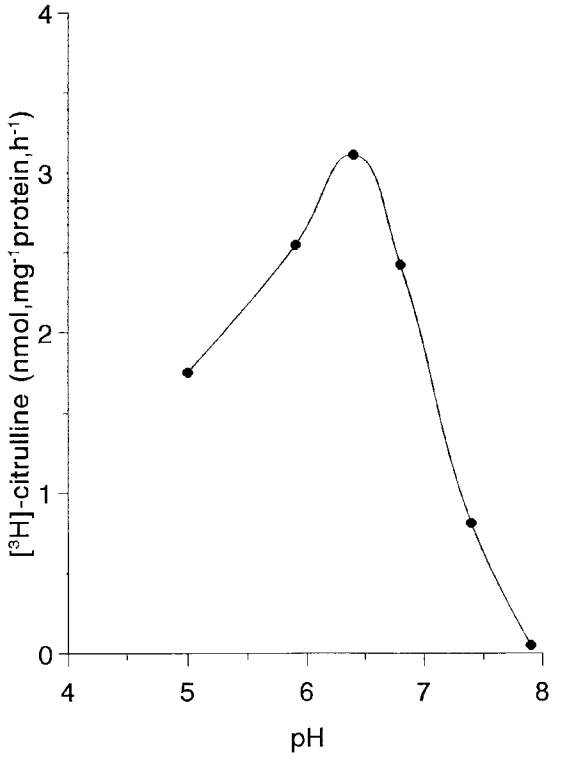

Fig. 1. Influence of pH on NOS activity in differentiated cerebellar granule cells. The enzyme was determined as reported in Section 2, in the presence of 10 µM arginine, 0.45 mM Ca^{2+}, 2 mM NADPH 10 µM BH_4, using 50 mM HEPES based buffers in the pH range 5.0–7.9, and expressed as nmol [^{3}H]citrulline h^{-1} mg^{-1} protein. Data are the mean of four experiments: SD values never exceeded 12% of the mean.

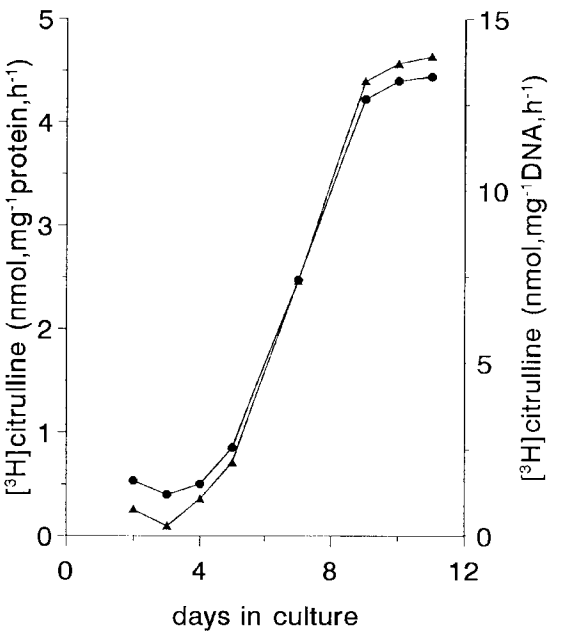

Fig. 2. Changes of NOS activity in cerebellar granule cells during differentiation in culture. NOS activity was determined as indicated in the legend to Fig. 1 at pH 6.4 and expressed as nmol [^{3}H]citrulline h^{-1} mg^{-1} of protein (●) or DNA (▲). In blank samples [^{3}H]citrulline accounted for 1.1 ± 0.2% (mean ± SD) of total radioactivity and was independent of the protein concentration and the incubation mixture used. Data are the mean values of at least three experiments; SD values never exceeded 15% of the mean.

NOS was determined in homogenates of cerebellar granule cells during differentiation in culture (Fig. 2). Under the experimental conditions used granule cells undergo differentiation during the first week in culture, in agreement with previous studies [18]. The completeness of differentiation was reached at 7–9 DIC when most of the cells are grouped in large clumps connected by a rich network of fasciculated fibers. In the first DIC the granule cells showed low but measurable NOS activity referred to both mg DNA and mg protein; at day 2 in culture the enzyme activity was 0.76 ± 0.08 and 0.53 ± 0.06 (mean ± SD) nmol mg^{-1} DNA and protein, respectively. The activity then rose sharply, reaching a maximum value of 13.19 ± 1.80 and 4.22 ± 0.60 nmol mg^{-1} DNA and protein, respectively, at day 9, remaining unchanged thereafter.

As shown in Fig. 3 particulate NOS activity gradually increased, from 0.55 ± 0.08 to 1.6 ± 0.2 nmol mg^{-1} protein between days 2 and 9; cytosolic NOS activity was 0.6 ± 0.1 nmol mg^{-1} protein at days 2–4 and had increased to 7.3 ± 0.8 nmol mg^{-1} protein by day 9. Between days 9 and 11, neither particulate nor cytosolic NOS displayed any significant change. The ratio between the cytosolic and particulate enzyme activity changed from 1:1 at days 2–4 to 4:1 at day 9.

At all DIC, the cytosolic NOS activity was almost exclusively Ca^{2+} dependent being markedly inhibited by 1 mM EGTA (Fig. 4). On the contrary, most of the particulate enzyme appeared to be Ca^{2+} independent, being measurable in the presence of 1 mM EGTA (Figs. 3 and 4). Increasing concentrations of EGTA, up to 4 mM, did not modify NOS activity determined in the particulate fraction (data not shown). The calcium-independent NOS activity associated to the particulate fraction progressively increased with time in

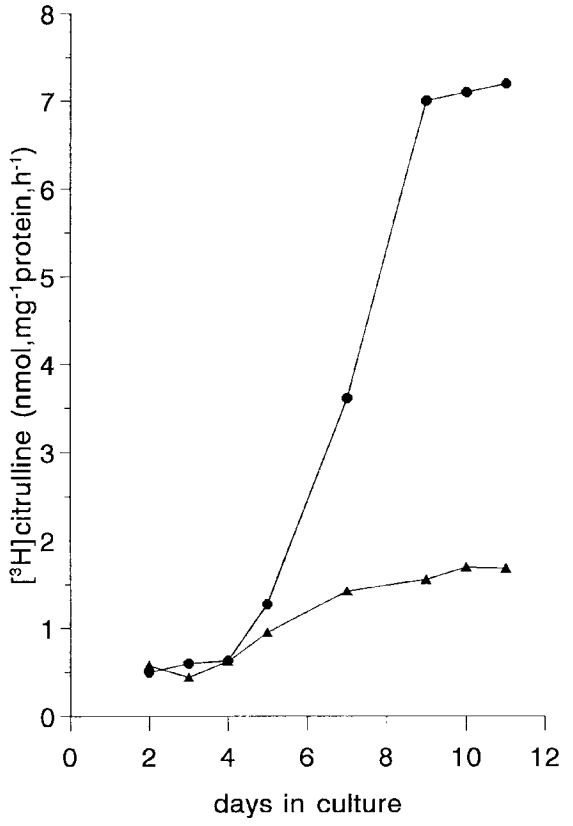

Fig. 3. Changes of NOS activity in the cytosolic (●) and particulate (▲) fractions of cerebellar granule cells during differentiation in culture. NOS activity was determined and expressed as indicated in the legend to Fig. 1, at pH 6.4. Blank values were as reported in legend of Fig. 2. Data are the mean values of at least three experiments; SD values never exceeded 15% of the mean.

culture: a 5-fold enhancement of this activity was observed from day 2 to day 9. K_m values of 2.75 ± 0.25 and 2.5 ± 0.2 μM and V_{max} values of 7.7 ± 0.7 and 1.46 ± 0.13 nmol, mg^{-1} h^{-1} were obtained at 8 DIC for NOS cytosolic Ca^{2+}-dependent and particulate Ca^{2+}-independent activity, respectively. Both cytosolic and particulate NOS activities were completely inhibited by 0.4 mM L-NMMA.

As reported in Fig. 5 cerebellar granule cells produced and released NO at all DIC. The amount of NO released in the culture medium in the first 2–3 DIC was about 0.14 nmol μg^{-1} DNA per day, this then increased markedly and rapidly, reaching, at the sixth DIC, 0.75 nmol μg^{-1} DNA per day, a value that was maintained thereafter.

4. Discussion

First it should be noted that the NOS activity in cerebellar granule cells differentiating in culture was determined at pH 6.4, unlike most literature reports where pH 7.4 is used. In fact, under our experimental conditions, the optimal pH for NOS was about 6.4, regardless of the cellular fraction used and the day in culture considered. This finding supports an earlier work that indicates a relatively acidic pH optimum as characteristic of neuronal NOS [26].

This study demonstrates that the differentiation of cerebellar granule cells in culture is characterized by a marked increase in NOS activity, that concerns not only cell number (DNA content) but also protein concentration. Since cultured granule cell differentiation is accompanied by an increase in protein content, the data indicate that NOS is one of the enzymes specifically expressed in granule cells during differentiation. Similar developmental changes in NOS expression were demonstrated in different rodent brain areas [13–16], in rat spinal cord [27] and in chick embryo retina [28].

In cerebellar granule cells the NOS activity was more or less evenly distributed between the cytosolic and particulate fractions up to day 5 in culture. Later on the cytosolic enzyme activity underwent a 12-fold increase, whereas the particulate enzyme showed only a 3-fold increase. Thus during differentiation there appears to be a marked and preferential enrichment of the cytosolic NOS activity. This is in agreement with evidence of a NOS cytosolic localization in adult rat cerebellum [11]. The increase in cytosolic NOS activity, which is essentially Ca^{2+}-dependent, is particularly evident after the first DIC when the cultured granule cells progressively acquire the biochemical and morphological properties of differentiated neuronal cells [18–22]. In addition, our data indicate that NOS activity is present also in the particulate fraction of cultured granule cells. In contrast to the cytosolic enzyme, particulate NOS is mainly Ca^{2+}-independent, being measurable in the presence of EGTA (up to 4 mM). Our results demonstrate that also the Ca^{2+}-independent activity in the partic-

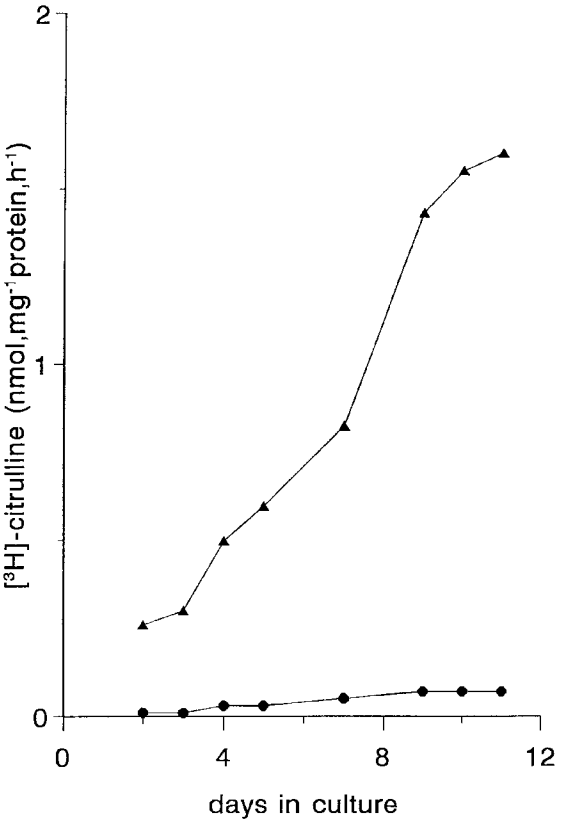

Fig. 4. Ca^{2+} dependence of NOS contained in the cytosolic (●) and particulate (▲) fractions of cerebellar granule cells during differentiation in culture. NOS activity was determined in the presence of 10 μM arginine, 1 mM EGTA, 2 mM NADPH 10 μM BH_4, 50 mM HEPES (pH 6.4) and expressed as nmol [^{3}H]citrulline h^{-1} mg^{-1} of protein. Blank values were as reported in legend of Fig. 2. Data are the mean values of at least three experiments; SD values never exceeded 15% of the mean.

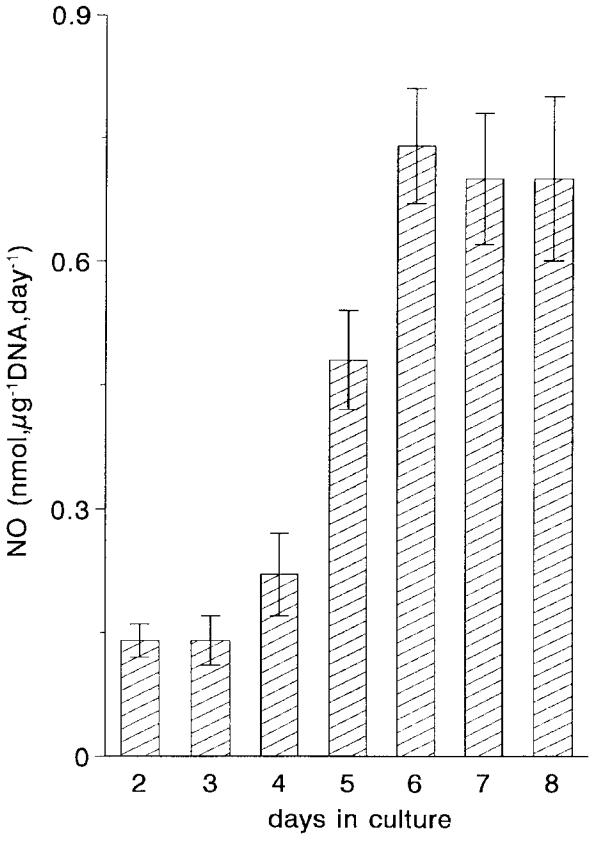

Fig. 5. NO released by granule cells during differentiation in culture. NO measured as the amount of NO_3^- and NO_2^-, accumulating daily in the culture medium, and expressed as nmol μg^{-1} DNA day^{-1}. The amount of $NO_3^-+NO_2^-$ in the culture medium alone was 0.43 ± 0.04 nmol/160 μl. Data are the mean ± SD values of at least three experiments.

ulate fraction increased along with granule cells differentiation. This NOS activity might be the expression of a calcium-independent isoform or the result of a modulatory influence; in fact Okada [29] recently demonstrated, in cerebellar slices from adult rats, that PKC modulates the calcium sensitivity of NOS. Instead, the possibility that particulate NOS represents the inducible Ca^{2+}-independent NOS isoform, characteristic of non neuronal (mainly glial) cells [30], can be excluded, since glial contamination in the used primary cultures of granule cells is extremely low and unstimulated rat cerebellar astrocytes do not express NOS activity ([31], and unpublished results from our laboratory). Furthermore, the time course profile of Ca^{2+}-independent NOS activity closely parallels the progress of granule cell differentiation, thus such activity appears to be a biochemical marker of this neuronal process. Also, the cerebellar granule cell production of NO increased during differentiation, supporting the concept that NO could play a role in neuronal differentiation [7,8]. It is worth noting that the maximal daily production of NO observed at 6 DIC, occurred just before the maximal expression of NOS activity.

In conclusion, the data presented suggest that NOS participates in granule cell differentiation and represents a specific expression of differentiated granule cells possibly participating to extra- and intracellular signal transduction mechanisms operating in such differentiated neuronal cells.

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