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Characterization and ontogenesis of nitric oxide synthase activity in pig enterocytes

Hamida M'Rabet-Touil, François Blachier*, Marie-Thérèse Morel, Béatrice Darcy-Vrillon, Pierre-Henri Duée

Unité d'Ecologie et de Physiologie du Système Digestif, Institut National de la Recherche Agronomique, F-78352 Jouy-en-Josas Cedex, France

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Nitric oxide has been implicated as a local modulator of several gastrointestinal functions. In this study, we have measured nitric oxide synthase activity in homogenates of enterocytes isolated from post-weaned pigs. The enzyme required the presence of NADPH and 6-(R,S)-5,6,7,8-tetrahydro-L-biopterin. Conversely exogenous FAD and FMN did not appear to be necessary for enzyme activity. The enzyme activity was not affected by added Ca^{2+} or EGTA and was inhibited by the arginine analogs N^G-monomethyl-L-arginine and N^{ω}-nitro-L-arginine. NO synthase activity was not detectable in enterocytes isolated at birth and increased slightly in suckling animals. NO synthase activity was found to be present mostly in the cytosolic fraction isolated from post-weaned pigs enterocytes.

Enterocyte; Nitric oxide; Development

1. INTRODUCTION

Many mammalian cell-types including macrophages produce nitric oxide (NO) and L-citrulline through a common biochemical pathway: the oxidation of L-arginine [1,2]. The enzyme which catalyses this pathway (i.e. NO synthase) appears to participate in a variety of physiological processes among which the protection of the gastrointestinal mucosa [3–6], the regulation of intestinal motility [7,8], and the modulation of intestinal epithelial permeability [9].

We have previously demonstrated the presence of a D-glucose modulated NO synthase in enterocytes isolated from post-weaned pigs [10]. The aim of the present work was to further investigate NO synthase activity in enterocytes from biochemical and ontogenic viewpoints.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Hyaluronidase, N^{ω}-nitro-L-arginine, nitrate reductase were obtained from Sigma Chemicals (St. Louis, USA). FAD, FMN and NADPH were from Boehringer Mannheim (Germany).

L-[guanido-^{14}C]Arginine was purchased from New England Nuclear (Boston, USA), 6-(R,S)-5,6,7,8-tetrahydro-L-biopterin · 2HCl (6-(R,S)-BH_4) from Serva (Heidelberg, Germany) and N^G-monomethyl-L-arginine monoacetate from Calbiochem (La Jolla, USA).

2.2. *Animals and intestinal absorptive cells isolation*

Pigs from the Large white strain were used in this study. Animals were weaned 4 weeks after birth. In one group, 4-month-old animals (mean age 135 ± 4 days, $n = 14$) were anaesthetized with halothane after an overnight fasting. Enterocytes were isolated from a portion of mid-jejunum and colonocytes were isolated from a portion of proximal colon. In another group, 1-month-old animals (mean age 40 ± 4 days, $n = 6$) were anaesthetized with sodium thiopental (Nesdonal; Rhone Merieux, France; 25 mg/kg in saline) and enterocytes were isolated from the jejunoileum.

The newborn animals used immediately after birth (0-day-old animals) or after 2–9 days suckling were anaesthetized with sodium thiopental (25 mg/kg in saline) and the enterocytes were isolated from the jejunoileum. The methods used to prepare enterocytes and colonocytes have been previously described [10–12].

The viability of the isolated enterocytes or colonocytes when tested by Trypan blue exclusion was always higher than 85%, and more than 90% of lactate dehydrogenase activity was recovered in the cell pellet at all stages of development studied.

The protein content of the isolated cells or subcellular fractions were determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

2.3. *Assay of enzyme activities*

Isolated enterocytes were pelleted (250 g, 3 min), frozen in liquid nitrogen and stored at −80°C before analysis. The cell pellets were resuspended in Tris-HCl buffer (100 mM, pH 7.2) and homogenized at 4°C using an ultrasonic cell disruptor (Sonifier B15, Branson, Soest, The Netherlands) for NO synthase and arginase assays.

The activity of NO synthase was determined in 100 μl by incubating the cell homogenate for 60 min at 37°C in the presence of 50 μM L-[guanido-^{14}C]arginine, 1 mM DTT and various cofactors.

At the end of incubation, the samples were centrifuged (2 min, 16,000 × *g*), then 60 μl of supernatant were incubated for 5 min at 37°C in the presence of 4.25 units of arginase and 2 mM $MnCl_2$ in a final volume of 80 μl. In such conditions, 97 ± 1% ($n = 5$) of labelled L-arginine was converted to labelled urea. This treatment minimizes the background due to labelled L-arginine in L-citrulline HPLC fraction. The reaction was then stopped in liquid nitrogen and the generation of radioactive citrulline was determined after *o*-phthaldialdehyde derivatization [14] and separation by reverse-phase liquid chrom-

*Corresponding author.

atography on a partisphere C18 column (Whatman, Clifton, NJ) using the gradient program described [10].

In certain experiments, arginase activity was measured in 100 μl by incubating the cell homogenate for 60 min at 37°C in the presence of 50 μM or 1 mM L-[guanido-^{14}C]arginine. The reaction was halted with 20 μl perchloric acid (PCA 12%) and labelled urea was separated from labelled L-arginine by HPLC [15].

2.4. *Determination of nitrite and nitrate*

Because NO ultimately decomposes to nitrite and nitrate, these anions were assayed in the incubation medium. 600 μl of homogenates obtained from 10–30 × 10^6 enterocytes were incubated for 60 min at 37°C in a Tris-HCl buffer (100 mM, pH 7.2) containing 1 mM DTT, 1 mM NADPH, 10 μM FAD, 10 μM FMN and 10 μM 6-(R,S)-BH_4 in the absence or the presence of 50 μM L-arginine. The homogenates were centrifuged (16,000 × *g*, 5 min), and the amount of nitrite and nitrate was determined in homogenate supernatant: 500 μl of supernatant was either incubated 10 min at room temperature with Griess reagent [16] and nitrite was then measured at 546 nm, or either incubated 60 min at 25°C in the presence of 6.7 mU nitrate reductase and 0.18 mM NADPH in order to reduce nitrate to nitrite and then treated with Griess reagent. Nitrate was then estimated by difference.

Aqueous samples of $NaNO_2$ and $NaNO_3$ ranging from 0.2 to 20 μM were treated exactly as cell extracts and used as standards.

A relatively high endogenous amount of nitrite and nitrate were found in post-weaned pig enterocytes (0.28 ± 0.08 nmol · 10^6 $cells^{-1}$, n = 5). Therefore no increase of nitrite and nitrate could be recorded in the presence of 50 μM L-arginine and all cofactors when compared to the level of nitrite and nitrate measured in the absence of the amino acid. The basal content of nitrite in enterocytes represented 90 ± 8% (n = 5) of the sum of nitrite and nitrate.

2.5. *Subcellular fractions from enterocytes*

Isolated enterocytes were frozen in liquid nitrogen and stored at −80°C. Cells were homogenized as described above and then centrifuged at 4°C (100,000 × *g*, 90 min). The supernatant used as the cytosolic fraction was collected and pellet fraction was washed threefold with Tris-HCl buffer (100 mM, pH 7.2) and then resuspended by sonication.

The cytosolic marker enzyme lactase dehydrogenase was measured in parallel with NOS.

2.6. *Statistical calculations*

Results are expressed per mg of protein except when NO synthase activity was determined during development. In that case, the results have been expressed per 10^6 cells. Results are given as the mean (± S.E.M.), together with the number of animals used (n). For statistical analysis, a two-tailed Student's t-test for paired or unpaired observations was used. P-values of less than 0.05 were considered to be significant.

3. RESULTS

The NO synthase activity as judged by the production of radioactive L-citrulline from 50 μM L-[guanido-^{14}C]arginine was close to the limit of detection (4.7 ± 2.6 pmol · mg $protein^{-1}$ · 60 min^{-1}, n = 9) in homogenates of enterocytes isolated from 1-month- or 4-month-old post-weaned pigs and incubated in the basal conditions (i.e. in Tris-HCl buffer containing 1 mM dithiothreitol (DTT)). When 1 mM NADPH was added to the incubation medium, a measurable amount of radioactive L-citrulline was detected (26.7 ± 9.7 pmol · mg $protein^{-1}$ · 60 min^{-1}, n = 8).

In contrast, exogenous FAD (10 μM) used alone or in combination with FMN (10 μM) did not affect the enzyme activity measured in the presence of NADPH representing 90 ± 6% (n = 5) of the value recorded when NADPH was the sole cofactor.

When 10 μM of 6-(R,S)-BH_4 was added to the incubation medium containing already NADPH, FAD and FMN, the activity of NO synthase was increased significantly (P < 0.0025 vs. no 6-(R,S)-BH_4) representing 50.3 ± 10.5 pmol · mg $protein^{-1}$ · 60 min^{-1} (n = 4) in 1-month-old animals; and 44.3 ± 13.4 pmol · mg $protein^{-1}$ · 60 min^{-1} (n = 6) in 4-month-old animals.

Arginase activity, the other L-arginine catabolic enzyme, was not affected by NADPH or 6-(R,S)-BH_4 when estimated by the production of urea from 50 μM L-[guanido-^{14}C]arginine. Indeed, the production of radioactive urea represented respectively in the presence of 1 mM NADPH, or in the presence of 1 mM NADPH and 10 μM 6-(R,S)-BH_4, 106 ± 6% (n = 5) or 114 ± 11% (n = 5) of the basal arginase activity (i.e. in Tris/DTT buffer containing no $MnCl_2$; 552.8 ± 33.3 pmol · mg $protein^{-1}$ · 60 min^{-1}, n = 5).

The L-arginine analogs N^{ω}-nitro-L-arginine (L-NNA) and N^{G}-monomethyl-L-arginine (L-NMMA) inhibited markedly NO synthase activity (Fig. 1). The enzyme activity was inhibited by 59 ± 12% (n = 5) with L-NNA and by 73 ± 11% (n = 4) by L-NMMA. The addition of those NOS inhibitors had no effect on arginase activity which represented respectively 114 ± 14% (n = 4) and 117 ± 13% (n = 4) of basal values.

The NO synthase activity measured in enterocyte ho-

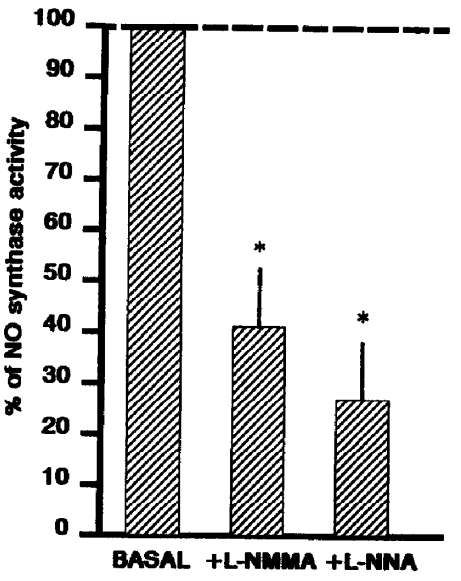

Fig. 1. Effect of N^{G}-monomethyl-L-arginine (L-NMMA) and N^{ω}-nitro-L-arginine (L-NNA) on the NO synthase activity in homogenate of enterocytes isolated from post-weaned pigs. NO synthase activity was determined by measuring the production of radioactive citrulline from 50 μM L-[guanido-^{14}C]arginine in the presence of 1 mM NADPH, 10 μM FAD, 10 μM FMN and 10 μM 6-(R,S)-BH_4 and in the absence or presence of 100 μM L-NMMA or L-NNA. Results are expressed as the mean value (± S.E.M.). The number of animals used is n = 4–5. *P < 0.01 vs. no inhibitor.

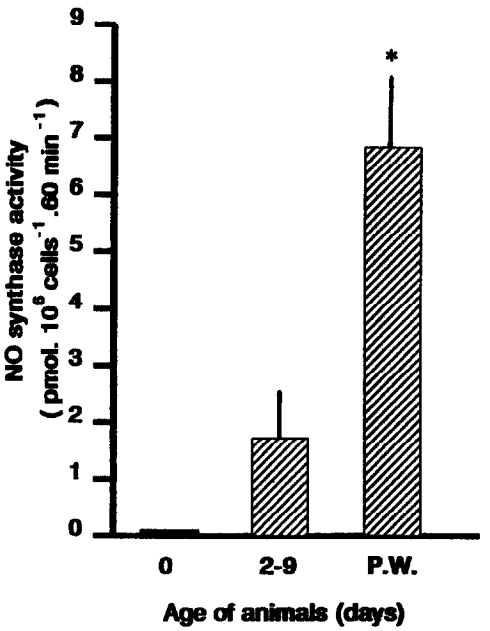

Fig. 2. NO synthase activity at different stages of development. NO synthase activity was determined as described in Fig. 1 by measuring the production of radioactive citrulline from 50 μM L-[guanido-^{14}C]arginine in the presence of all cofactors. Results are expressed as the mean value (± S.E.M.). The number of animals used is $n = 4$ at 0 days of age, $n = 7$ at 2–9 days of age and $n = 10$ for P.W. animals. P.W. = post-weaned. *$P < 0.01$ vs. 2–8 days value.

mogenates did not appear to be dependent on the presence of Ca^{2+} since the addition of either 1 mM Ca^{2+} or 5 mM EGTA did not significantly modify the generation of L-citrulline. Indeed, the NOS activity recorded in the presence of all cofactors (i.e. NADPH, FAD, FMN and 6-(R,S)-BH_4) was not affected by the presence of exogenous Ca^{2+} or EGTA representing respectively 92 ± 18% ($n = 4$) and 98 ± 9% ($n = 4$) of the enzyme activity measured in the sole presence of cofactors.

When homogenates of enterocytes isolated immediately after birth were incubated in the presence of 50 μM L-[guanido-^{14}C]arginine, NADPH, FAD, FMN and 6-(R,S)-BH_4, no production of L-citrulline was detected (Fig. 2). This activity was modest but measurable when the enterocytes were obtained from 2- or 8-day-old suckling animals. The activity in post-weaned animals represented 4-fold the value recorded at 2–9 days of age.

When the enterocytes isolated from post-weaned pigs were homogenized and then 100,000 × g supernatant and pellet were separated, approximately half of the protein cell content was recovered in the cytosolic fraction (Table I). Almost all LDH activity (used as a cytosolic marker) was recovered in the 100,000 × g supernatant fraction. After incubation of subcellular fractions in the presence of 50 μM L-[guanido-^{14}C]arginine and all cofactors, most of the NO synthase and arginase activities were recovered in the 100,000 × g supernatant fraction.

The presence of NO synthase activity was checked in another intestinal cell type, i.e. colonocytes from post-weaned pigs by measuring the production of radioactive L-citrulline from 50 μM L-[guanido-^{14}C]arginine in the presence of 1 mM NADPH, 10 μM FAD, 10 μM FMN and 10 μM 6-(R,S)-BH_4. In such conditions, this activity represented 132.1 ± 32.2 pmol · mg protein^{-1} · 60 min^{-1}, $n = 4$, a value significantly higher ($P < 0.005$) than the value recorded in enterocytes (i.e. 46.7 ± 8.7 pmol · 10^6 cells^{-1} · 60 min^{-1}). In contrast, arginase activity in colonocytes when measured in the presence of 2 mM $MnCl_2$ by the conversion of 1 mM L-[guanido-^{14}C]arginine to urea was not significantly different from that measured in enterocytes (30.9 ± 3.4 nmol · mg protein^{-1} · 60 min^{-1}, $n = 4$ in colonocytes and 19.0 ± 6.0 nmol · mg protein^{-1} · 60 min^{-1}, $n = 6$ in enterocytes).

4. DISCUSSION

We have previously demonstrated that villus entero-

Table I

NO synthase and arginase activities measured in post-weaned pig enterocyte crude homogenate and fractions obtained after centrifugation of homogenate at 100,000 × g for 90 min

	Homogenate	Cytosol	Pellet
		% of total	
Protein (mg · 10^6 cells^{-1})	0.160 ± 0.012(14)	48 ± 4 (5)[a]	52 ± 4 (5)
LDH (μmol · mg protein^{-1} · min^{-1})	1.09 ± 0.19(4)	90 ± 2 (4)[b]	10 ± 2 (4)
NO synthase[c] (pmol · mg protein^{-1} · 60 min^{-1})	46.7 ± 8.7(10)	94 ± 6 (4)[b]	6 ± 6 (4)
Arginase[d] (pmol · mg protein^{-1} · 60 min^{-1})	1792.0 ± 263.8(13)	88 ± 4 (4)[b]	12 ± 4 (4)

[a] Calculated as the ratio between cytosolic protein and the sum of cytosolic and pellet protein.
[b] Calculated as the ratio between cytosolic activity and the sum of cytosolic and pellet activity, each of them being multiplied by the amount of protein recovered in each subcellular fraction.
[c] Determined as the production of radioactive L-citrulline from 50 μM L-[guanido-^{14}C]arginine in a Tris-HCl buffer (pH 7.2) containing 1 mM DTT, 1 mM NADPH, 10 μM FAD, 10 μM FMN and 10 μM 6-(R,S)-BH_4.
[d] Determined as the production of radioactive urea from 50 μM L-[guanido-^{14}C]arginine in a Tris-HCl buffer (pH 7.2) containing 2 mM $MnCl_2$.

cytes isolated from post-weaned pigs express an NO synthase activity generating citrulline from L-[guanido-^{14}C]arginine [10]. The absolute requirement observed for NADPH is in agreement with previous studies on several tissues including brain and macrophages [17,18]. We have recently proposed that the metabolization of D-glucose in the pentose phosphate pathway, by providing NADPH for NO synthase, could explain the increased flux of L-arginine through NO synthase pathway in intact enterocytes [10].

In contrast, in our experiments, the exogenous FAD and FMN does not seem to be necessary for enzymatic catalysis since they did not affect the activity measured in the presence of NADPH. Enzyme purification [19,20] and cDNA cloning [21–23] have demonstrated that brain, macrophage and hepatocyte NO synthase contain binding sites for FAD and FMN in addition to NADPH in C-terminal half of both proteins. Based on these reports, the absence of stimulation of enterocyte NO synthase by added flavins could be due to the presence of endogenous flavins bound to NO synthase already present in crude homogenate of enterocytes.

The addition of 6-(R,S)-BH_4 also stimulates enterocyte NO synthase activity. In macrophages, the amount of NO produced is limited by the amount of tetrahydrobiopterin present in the cells [24]. Furthermore, in cerebellum, it has been shown that tetrahydrobiopterin does not function as a reactant in NO synthase catalysis but in that case, the role of the cofactor may be allosteric [25].

Chelation of Ca^{2+} in the medium by addition of a high concentration of EGTA had no inhibitory effect on NO synthase activity. This result indicates that enterocyte NO synthase does not appear to be Ca^{2+}-dependent. Different NO synthase isoenzymes have been described. For example, macrophage NO synthase, unlike that in the brain, does not depend on Ca^{2+} [26].

When the enterocytic NO synthase activity was measured on cytosolic and membranes fractions, the enzyme appeared to be present mostly in the cytosol. Results obtained with brain, N1E-115 neuroblastoma cells and macrophages [27–29] suggest also a cytosolic localization of the enzyme.

The arginine analogs L-NMMA and L-NNA described as the inhibitors of NO synthase in macrophages [30] and a number of other cells and tissues including adrenal glands and human platelets [31,32] also inhibited enterocyte NO synthase. N^{ω}-nitro-L-arginine failed to inhibit the hepatic microsomal cytochrome P450 which is described as a citrulline and NO generating enzyme [33]. The present data showing a marked inhibition by L-arginine analogs together with a cytosolic localization of our enzyme activity rules out the possibility of citrulline generation by cytochrome P450 in our assay.

Cellular NO synthase ontogenesis has not been reported yet. In enterocytes, we have measured the NO synthase activities at different stages of development. The enzyme activity was not detectable at birth, increased slightly at 2–9 days and represented in post-weaned pigs 4-fold the value measured in 2–9-day-old pigs. This increase could be related to the rapid replacement after birth of foetal cells by adult type enterocytes [34]. The ontogenesis of the other catabolic enzyme activity of L-arginine, i.e. arginase, follows the same pattern of development [15].

NO synthase activity in colonocytes is nearly 3-fold higher when compared with the NO synthase activity measured in enterocytes. NO biosynthesis from large bowel mucosa has been suggested in a recent work [35].

The physiological role of NO in gastrointestinal tract is generally considered to be beneficial [3]. Since the endogenous source of NO is not clearly identified yet, we propose intestinal absorptive cells as candidate.

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