Placenta 30 (2009) 241–249

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Transcriptomic Analysis of Polyamine-Related Genes and Polyamine Levels in Placenta, Yolk Sac and Fetus During the Second Half of Mouse Pregnancy

C. Lopez-Garcia [a], A.J. Lopez-Contreras [a], A. Cremades [c], M.T. Castells [b], R. Peñafiel [a,*]

[a] Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain
[b] Department of Cell Biology, Faculty of Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain
[c] Department of Pharmacology, Faculty of Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain

ARTICLE INFO

Article history:
Accepted 5 December 2008

Keywords:
Polyamines
Ornithine decarboxylase
Diamine oxidase
Antizymes
Fetus
Placenta
Yolk sac
Pregnancy

ABSTRACT

In mammals, polyamines are essential for the maintenance of cell growth. Although early studies reported the highest values of mammalian ornithine decarboxylase (ODC) activity, a key enzyme in polyamine biosynthesis, in rodent placenta, the role of this enzyme in the second half of rodent pregnancy is still controversial. In order to get new insights on polyamine metabolism during this period of pregnancy, we studied polyamine levels, ODC expression and activity and transcript profile of different polyamine-related genes in mouse placenta, fetus and yolk sac. Results indicated that ODC activity and protein levels were higher in placenta than in fetus and yolk sac, especially in the labyrinth, although no correlation between ODC activity and polyamine levels were observed. The half-life of placental ODC (~190 min) was also higher than the fetal one (~24 min). Messenger RNAs of all biosynthetic and retroconversion enzymes of polyamine metabolism were present in the three gestational compartments analyzed, as well as those of antizymes 1 and 2 and antizyme inhibitor 1. However, no expression of antizyme 3 and antizyme inhibitor 2 was detected. The catabolic enzyme diamine oxidase was expressed only in the maternal part of placenta but not in the fetal part or in the fetus. The expansion of polyamine pools in the fetus was markedly higher than in placenta, in spite of its lower biosynthetic activity. Our results suggest that the elevated polyamine biosynthetic activity of mouse placenta is required to satisfy the high demand of polyamines required by the growing fetus, during the later period of pregnancy.

1. Introduction

Polyamines (putrescine, spermidine and spermine) are ubiquitous organic cations synthesized and stored by mammalian cells, which play a relevant role in the regulation of cell growth and differentiation [1–3]. These cations also appear to play a dual role in cell survival and death, depending on their concentration and on the presence of different extra-cellular signals [4,5]. The molecular mechanisms by which polyamines affect cellular functions are mostly unknown. However, since they are positively charged under physiological conditions, electrostatic interactions with negative charged molecules are most likely to occur. In fact, numerous results indicate that polyamines interact with nucleic acids and proteins, affecting DNA conformation and chromatin condensation [1,2], gene transcription [6], and protein synthesis [7], as well as the function of different ion channels and receptors [8,9]. Spermidine also participates in the formation of hypusine, a specific post-translational modification of one specific lysine residue, in the eukaryotic translation initiation factor 5A [10].

The intracellular levels of polyamines in mammalian cells are tightly controlled by biosynthesis, degradation, uptake and excretion [4]. These processes in each tissue are finely regulated by many different factors, including hormones and growth factors, as well as by the polyamines themselves [2,4]. The first enzyme in the biosynthetic pathway is ornithine decarboxylase (ODC), which catalyzes the transformation of L-ornithine into putrescine. The addition of an aminopropyl group from decarboxylated S-adenosylmethionine (dcSAM) to putrescine to give spermidine requires of spermidine synthase (SpdST), whereas the formation of spermine from spermidine is catalyzed by another aminopropyltransferase named spermine synthase (SpnST). dcSAM is formed by S-adenosylmethionine decarboxylase (SAMDC). ODC and SAMDC are key regulatory enzymes in the biosynthetic process [11,12], whereas SSAT (spermidine/spermine N1-acetyltransferase) is the rate-controlling enzyme in the back-conversion pathway that

* Correspondence to: Dr. Rafael Peñafiel, Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain. Tel.: +34 968367174; fax: +34 968398601.
E-mail address: rapegar@um.es (R. Peñafiel).

0143-4004/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.placenta.2008.12.004

transforms spermine into spermidine, and spermidine into putrescine [13]. This pathway also requires of polyamine oxidase (PAO) that participates in the oxidation of acetylated polyamines formed by SSAT [14]. Another enzyme, spermine oxidase (SMO) can directly convert spermine into spermidine, but is unable to oxidate spermidine [15,16]. ODC is highly inducible by many trophic stimuli that increase ODC gene transcription or the translation efficiency of its mRNA [12]. The enzyme has a short half-life (about 15 min) and is negatively regulated by polyamines through the induction of a regulatory protein termed antizyme, which inhibits ODC activity and stimulates its degradation by the 26S proteasome [17,18]. There are at least three antizyme isoforms (AZ1, AZ2 and AZ3) coded by three different genes [19] and two different proteins highly homologous to ODC, but devoid of ODC activity, that act as antizyme inhibitory proteins, named AZIN1 and AZIN2 [20,21]. It must be noted that L-arginine used for the synthesis of polyamines is also the substrate of nitric oxide synthase (NOS), the enzyme producing the important bioregulatory molecule nitric oxide, and that different NOS isoforms are expressed in placenta and fetus [22,23].

Many pharmacological and genetic results have clearly shown that ODC and polyamines are essential for embryonic development in both invertebrate and vertebrate animals [24–31]. In rodents, α-difluoromethylornithine (DFMO), an ODC specific inhibitor, exerted marked contragestational effects when administered, during a critical period after implantation [26,27,32–34]. On the other hand, when this inhibitor was given during the second half of pregnancy no contragestational effect was produced, although small decreases in body and brain weight were observed [35–37]. Although early works extensively studied the changes in ODC activity and polyamine levels in reproductive tissues during rodent pregnancy [38–43], little is known about the expression pattern of other enzymes and proteins related with polyamine metabolism during different periods of gestation.

In the present study, we have carried out a systematic analysis of putrescine, spermidine and spermine levels in placenta, fetus, and yolk sac during the second half of mouse pregnancy. In parallel, we have also studied the expression pattern of different genes related with polyamine metabolism in all these gestational tissues. Our results suggest that polyamine biosynthesis in the mouse placenta is critical to support the high demand of polyamines required by the growing fetus.

2. Experimental procedures

2.1. Animals

Female Swiss CD1 mice bred in the Service of Laboratory Animals of University of Murcia were used. Animals were fed with standard chow (UAR A03; Panlab, Barcelona, Spain) and water *ad libitum*, and maintained at 22 °C and 55% relative humidity under a controlled 12:12 h light-dark cycle (lights on from 0700 h). Timed-pregnant mice were obtained by housing the females with males of proven fertility. The day when the vaginal plug was observed was designated as day 1. Pregnant animals at different days of pregnancy were sacrificed by cervical dislocation. Animal procedures were carried out according to the institutional guidelines of the University of Murcia that are in compliance with national (*RD 1201/2005*) and international laws and policies (European Union normative 86/009). To obtain the different tissues, the abdominal cavity was opened and the uteri were removed and opened. Fetuses, yolk sacs and placentas were dissected and processed for biochemical or histological analysis. At least 3–4 fetuses, placentas and yolk sacs from every pregnant mouse were analyzed by the different techniques. In all experimental groups, three to four pregnant animals were used at each day of pregnancy.

2.2. ODC enzyme activity measurements

To determine ODC activity, tissues were homogenized with the aid of a Polytron homogeniser in buffer containing 25 mM Tris (pH 7.2), 2 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 0.1 mM EDTA and 0.25 M sucrose. The extract was centrifuged at 20,000× *g* for 20 min, and ornithine decarboxylase (ODC) activity was determined in the supernatant. ODC activity was assayed basically as described elsewhere [44] by measuring $^{14}CO_2$ release from L-[1-^{14}C] ornithine. The incubation mixture contained 20 mM Tris pH 7.2, 0.1 mM pyridoxal phosphate, 0.1 mM EDTA, 2 mM dithiothreitol, 0.4 mM L-[1-^{14}C] ornithine (New England Nuclear, Boston, MA, specific activity 2.6 Ci/mol) in a total volume of 62.5 μl. The samples were incubated at 37 °C for 30 min and the reaction was stopped by adding 0.5 ml of 2 M citric acid. Activity was expressed as nmol CO_2 produced per hour and per g of wet weight.

2.3. Polyamine content analysis

Tissues were homogenized in 0.4 M perchloric acid and after centrifugation at 10,000× *g* for 5 min, the polyamines from the supernatant were dansylated according to standard method [45]. Dansylated polyamines were separated by HPLC, using a Lichrosorb 10-RP-18 column (4.6 × 250 mm) and acetonitrile:water mixtures (running from 70:30 to 96:4 ratio during 25 min of analysis) as mobile phase. 1,6-Hexanediamine was used as internal standard. Detection of the derivatives was achieved using a fluorescence detector, with a 340 nm excitation filter and a 435 nm emission filter. Polyamine content was expressed as nmol/g wet weight of tissue.

2.4. Western-blot analysis

Whole fetuses or placentas were homogenized in 50 mM Tris–HCl pH 8, 1% Igepal, 1 mM EDTA and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) containing additional mixture of protease inhibitors (13 μM bestatin, 1.4 μM E-64, 100 μM leupeptin and 30 nM aprotinin). The homogenates were centrifuged at 12,000× *g* for 20 min and the supernatants used to analyze ODC protein. Equal amounts of protein were mixed with Laemmli sample buffer, heated at 95 °C for 5 min and separated by electrophoresis in 10% polyacrylamide-SDS gels. The resolved proteins were electroblotted to PVDF membranes, and the resulting blots were incubated with 5% non-fat dry milk in PBS (0.01 M phosphate buffered saline pH 7.4) for 1 h. After washing in PBS + 0.1% Tween 20 (PBST), the blots were incubated for 1 h at room temperature with rabbit polyclonal antibody to ODC at a dilution 1:500 (Euro-Diagnostica, Malmö, Sweden). The blots were washed in PBST and incubated for 1 h at room temperature with a horseradish peroxidase-labeled goat secondary antibody (dilution 1:5000; Santa Cruz Biotechnology, CA). Immunoreactive bands were detected by using ECL + detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and commercial developing reagents and films (Amersham).

2.5. Semiquantitative RT-PCR analysis

Total RNA was extracted using Gen Elute® Total RNA Miniprep Kit (Sigma Chemical, St Louis, MO). Fresh whole tissues were homogenized by using a Polytron and the total RNA yield was determined by measuring the absorbance at 260 and 280 nm. Five μg of total template RNA were reverse-transcribed by adding 1 μl of 100 μM oligo dT, 1 μl of 10 mM dNTP mix and nuclease free water. The mixture was incubated for 10 min at 75 °C and chilled before addition of M-MLV reverse transcriptase (Sigma–Aldrich) and 2 μl of 10× buffer. The mixture was incubated at 37 °C for 1 h and the