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ScienceDirect

Journal of Nutritional Biochemistry 26 (2015) 1589–1598

Journal of Nutritional Biochemistry

Amino acid starvation induced by protease inhibition produces differential alterations in redox status and the thiol proteome in organogenesis-stage rat embryos and visceral yolk sacs

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Received 7 March 2015; received in revised form 23 July 2015; accepted 29 July 2015

Abstract

The process of embryonic nutrition in rodent conceptuses during organogenesis has been shown to involve a dominant histiotrophic mechanism where essential developmental substrates and micronutrients are supplied as whole maternal proteins or cargoes associated with proteins. The histiotrophic nutrition pathways (HNP) responsible for uptake and initial processing of proteins across maternal-conceptal interfaces involve uptake via receptor mediated endocytosis and protein degradation via lysosomal proteolysis. Chemical inhibition of either process can lead to growth deficits and malformation in the embryo (EMB), but selective inhibition of either HNP component will elicit a different subset of developmental perturbations. In vitro, whole embryo culture exposure of GD10 or GD11 rat conceptuses to the natural protease inhibitor, leupeptin, leads to significant reductions in all measured embryonic growth parameters as well as a myriad of other effects. Leupeptin doses of 10 μM or 20 μM over a 26-h period (GD10-GD11) and 50 μM over a 3 h pulse period produced significant decreases in the clearance of FITC-albumin from culture media. The near complete loss of acid soluble fluorescence and increased total visceral yolk sac (VYS) protein content confirmed the selective inhibition of proteolysis. Inhibition of lysosomal proteolysis thus deprives the developing EMB of essential nutrient amino acids producing conditions akin to amino acid starvation, but may also cause direct effects on pathways critical for normal growth and differentiation. Following leupeptin exposure for 26 or 6 h, total glutathione (GSH) concentrations dropped significantly in the VYS, but only slightly in yolk sac (YSF) and amniotic (AF) fluids. Cys concentrations increased in VYS and EMB, but dropped in YSF and AF fluids. Redox potentials (E_h) for the glutathione disulfide (GSSG)/glutathione (GSH) redox couple trended significantly toward the positive, confirming the net oxidation of conceptual tissues following leupeptin treatment. Analysis of the thiol proteome showed few alterations to specific pathways mapped to the Kyoto Encyclopedia of Genes and Genomes Pathway database, but did reveal significant increases in concentrations of proteins associated with glycolysis/gluconeogenesis in the VYS and decreased concentrations proteins associated with ribosome biogenesis and function in the EMB. A subset of proteins elevated by >2–23-fold in the VYS were identified as serum (blood) proteins and represent the maternal-side proteins captured by the VYS and which are not degraded in the lysosomes as a result of leupeptin's inhibitory action. The observed constellation of proteins decreased in the EMB by leupeptin represent proteins from several adaptive pathways that are commonly altered in responses to amino acid starvation. These studies show clear differential responses to protease inhibition in VYS and EMB during organogenesis and suggest the possibility for additional roles of redox regulation, cellular adaptations and metabolic insufficiency caused by protease inhibition.

Keywords: Embryo; Visceral yolk sac; Protease inhibition; Leupeptin; Redox potential; Thiol proteome; Amino acid starvation

1. Introduction

Embryonic nutrition during the growth and differentiation-sensitive phase of rodent organogenesis has been shown to be exclusively dependent on the capture and uptake of nutrients through histiotrophic nutrition pathways (HNP) by the visceral yolk sac (VYS) endoderm [1–6]. All mammalian conceptuses possess a functional VYS during this critical phase of development where programmed rapid growth and differentiation guide the structural and functional biogenesis of all systems required for a normal embryo and fetus. Studies in rodents, which have a non-vestigial inverted VYS that completely encompasses the developing embryo throughout gestation, have shown that the HNP functions of receptor-mediated endocytosis (RME), primary vesicular formation, and lysosomal proteolysis are necessary to capture bulk protein from the immediate surrounding maternal fluids for degradation in the lysosomes in order to supply the growing embryo with required amino acids, as well as to supply other lipids, cofactors, and growth factors, bound to their appropriate proteins carriers as cargoes [7–11]. Exclusive reliance on

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http://dx.doi.org/10.1016/j.jnutbio.2015.07.026
0955-2863/

the HNP axis for nutrient supply and processing raises the possibility that the transporters, structural proteins, regulators and enzymes of this pathway could be potential targets for mediating embryotoxicity and the elicitation of structural and functional birth defects.

Previous studies have characterized the effects of known dysmorphogens and chemical inhibitors of RME and lysosomal proteolysis in the characterization of HNP in rodents [12–15]. Although embryotoxic agents such as ethanol have been shown to have a selective inhibitory effect on RME, the majority of studies have focused on the inhibition of lysosomal proteolysis by several different types of chemicals [16]. Lysosomal inhibitors such as chloroquine [14], selective cathepsin inhibitors, such as Z-Phe-Ala-CHN_2 [12], and broad-spectrum cysteine, serine and threonine inhibitors, such as leupeptin [10,17,18], have all been shown to disrupt HNP activity and alter normal embryonic growth and development. Gross developmental consequences of protease inhibition have been well documented at the biochemical level, but very little is known about the specific mechanisms and molecular pathways that are impacted by the induced amino acid starvation.

Leupeptin (*N*-acetyl-L-leucyl-L-leucyl-L-arginal) is a naturally occurring microbial protease produced by the Gram-positive bacteria (Streptomyces; actinomycetes), which are naturally found in the soil [19,20]. The endopeptidase, leupeptin, differs from most other natural protease inhibitors because it contains an acetyl group and possesses an arginal aldehyde in place of the more common terminal carbonyl. Unlike most of its synthetic protease counterparts, leupeptin is believed to be much less toxic to humans and has generated considerable interest as a possible therapeutic intervention for purposes as diverse as the treatment of hearing loss (10–100 μM) [21,22], treatment of malaria [23], HIV [24,25], or anywhere protease inhibition may be otherwise indicated [26]. Direct exposure of organogenesis-stage rat and mouse conceptuses grown in whole embryo culture (WEC) to leupeptin (10 μM -100 μM) resulted in a significant accumulation of undigested protein in the lysosomes (~80% increase) and the significant reduction of growth and widespread dysmorphogenesis [17]. The initial characterization of leupeptin action in the developing rodent conceptus focused on histiotrophic nutrition pathways (HNP) as the principal means through which bulk proteins, specialized carrier proteins, and their essential cargoes (vitamins, minerals, cofactors, growth factors) are captured and processed by the conceptus. With the primary focus on supplying amino acids for protein and nucleic acid biosynthesis, previous studies overlooked the extended developmental consequences of reduced proteolytic activity. It is expected that targeted compartmental disruption of the HNP-dependent nutrient supply pathways could have many other significant effects on the developing embryo. Sant et al. reported that exposure of rat conceptuses in WEC to leupeptin on GD10 and GD11 resulted in significant changes in the concentrations of substrates and cofactors required by the one carbon metabolism pathway (C_1) [10]. S-adenosylmethionine (SAM) concentrations were significantly reduced, as was global DNA methylation in EMB and VYS. These results suggest that disruption of HNP activities along with its subsequent nutritional consequences may be producing acute functional alterations as well as changes in epigenetic programming.

Multiple adverse developmental consequences of altered maternal nutrition during fetal growth have been well documented, with particular emphasis on the roles and functional activities of the placenta [27]. The biochemical and molecular understanding of nutritional fluctuations during early embryogenesis and organogenesis have not been well characterized. Most studies have focused heavily on maternal measurements of nutrient levels in serum. Much less is known about the actual mechanisms of nutrient uptake and utilization in the early embryo during a period that precedes establishment of full functional placental activity. Recent studies suggest that the observed mechanisms of rodent nutrient uptake and processing may also be important in humans during the first trimester [28,29].

A myriad of molecular switches controlling signaling and regulatory pathways have been studied with respect to their possible roles in directing the complex and overlapping networks of development. None have proven adequate in isolation to describe developmental regulation and control. Widespread recognition of the importance of nutrient quality and quantity, as well as the roles other environmental regulatory factors, prompt the need to understand the mechanisms behind these signals. Incontrovertible evidence connects the transmission of environmental signals through redox signaling pathways, which are dependent on the regulated synthesis and turnover of soluble thiols such as glutathione (GSH) and cysteine (Cys) and the generation of reactive oxygen species (ROS) as second messengers [30–32]. This work explores the concept that inhibition of HNP activity and the resulting amino acid deficits elicited in the EMB has the potential to influence intracellular redox signaling and alter the thiol proteome as major consequences of HNP inhibition.

2. Materials and methods

2.1. Chemicals and reagents

Leupeptin hemisulfate salt, FITC-albumin (11.2 mol FITC/mol albumin), glutathione, glutathione disulfide, cysteine, cystine, γ-glutamyl-glutamate, iodoacetic acid, trichloroacetic acid, iodoacetamide, bicinchoninic acid and penicillin/streptomycin (10,000 U/ml penicillin, 10,000 μg/ml streptomycin sulfate) were purchased from Sigma/Aldrich (St. Louis, MO, USA). Hanks balanced salt solution (HBSS) was purchased from GIBCO/Life Technologies (Grand Island, NY, USA). Dansyl chloride was purchased from Fluka Chemie/Sigma-Aldrich (St. Louis, MO, USA). Cleavable Isotope Coded Affinity Tag (ICAT) reagent kits for protein labeling were purchased from AB Sciex/Applied Biosystems (Framingham, MA). MeOH (HPLC grade) was purchased from Honeywell Burdick & Jackson. All other chemicals were of reagent grade and were purchased from local sources.

2.2. Animals

All experiments in this study were conducted using viable, intact GD10–GD11 conceptuses grown in rat WEC. Primagravida specific pathogen-free Sprague–Dawley rats (Charles River, Portage, MI, USA) were time mated and shipped 4–5 days prior to use. A sperm-positive vaginal smear on the morning following mating was used to confirm pregnancy, this time was designated GD 0. Dams were maintained on a 12 h light–12 h dark cycle and allowed access to standard commercial rodent feed and water *ad libitum*.

Anesthesia, exsanguination and uteri removal were conducted as previously described and according to an approved institutional animal use and care protocols [33]. Conceptuses were explanted on GD10, prepared for culture and placed into 60-ml culture bottles containing 10 ml of control medium. Two different leupeptin exposure protocols were used, both starting with conceptuses explanted into culture on GD10. The conceptuses in the first protocol, designated as "26 h," were exposed to 10 μM, 20 μM or 50 μM leupeptin by direct addition to the culture medium on the morning of explant (GD10) and were assessed on GD11 after a total of 26 h in culture. In the second group, designated as "6 h," conceptuses were cultured for 22 h in control medium from GD10 and then treated with 50 μM leupeptin on GD11, immediately following the 95% O_2/5% CO_2 gas change, for a total treatment duration of 6 h.

2.3. Culture conditions, exposure, and sampling

At the time of explant, gravid uteri were placed in HBSS (pH 7.4 without indicator dyes) and each implantation site was removed using watchmaker's forceps and iridectomy scissors. Decidual masses were opened and intact conceptuses were removed. Reichert's membranes were torn away using fine forceps and the conceptus, consisting of an ectoplacental cone, intact inverted VYS, amnion, and EMB, were transferred into 10 ml of warmed (37°C) culture medium. A total of 6-10 intact viable conceptuses were placed in the bottles, not to exceed 1 conceptus per ml of medium. Culture medium was prepared from immediately centrifuged, heat inactivated rat serum (50%), HBSS (50%), and 43 μl penicillin/streptomycin (10,000 U/ml) [33]. Prior to the addition of conceptuses, culture medium and culture bottle headspace were saturated with 20% O_2, 5% CO_2, and 75% N_2, then the medium was warmed to 37°C. Gestational Day 10 embryos at the onset of culture had open anterior neuropores, complete dorsal flexion, and 8–12 somite pairs. Culture occurred in sealed sterile glass bottles on a roller apparatus in a constant 37°C incubator. After 22 h of culture, the culture medium and headspace were re-saturated with 95% O_2 and 5% CO_2. Leupeptin,