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Modulation of total ceramide and constituent ceramide species in the acutely and chronically hypoxic mouse heart at different ages

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ABSTRACT

Ceramide has been implicated in regulatory processes vital for cell survival under different stressors, most notably hypoxia. Little has been done to investigate the contributions of the different ceramide species to the regulation of cell survival. This study aims to highlight the patterns of variation in total ceramide and its species in the growing and hypoxic mouse heart. *Mus musculus* mice were placed in a hypoxic environment at birth. Control animals remained in room air. The hearts were extracted at different time points: 1 day, 1 week, 4 weeks, and 8 weeks. The total ceramide content and the amounts of component species were assayed by a modified diacylglycerol kinase assay and high-performance liquid chromatography–tandem mass spectroscopy, respectively. Data was collected from both ventricles in hypoxic and control conditions. There was significant polycythemia in the hypoxic versus control animals with a nearly twofold increase in hematocrit levels. Hypoxic right ventricle (RV) mass significantly increased over that of controls at different age groups. When ceramide content was compared in the hypoxic versus control animals, there was a significant increase at day 1 and a significant decrease at week 4 in the left ventricle, whereas a significant decrease was found in the RV at 1 week, 4 weeks, and 8 weeks. There was also a differential involvement of the RV with regard to levels of *N*-palmitoyl-D-erythro-sphingosine (C16-Cer) and its synthetic precursor dihydro-*N*-palmitoyl-D-erythro-sphinganine (DHC-16-Cer). The decrease in C16-Cer observed in both hypoxic and control RV's over time was paralleled by a significant increase in DHC-16-Cer in hypoxic (142.1 ± 15.0 pmol; $p < 0.05$) but not control (52.8 ± 4.0 pmol) RV's suggesting a role for DHC-16-Cer in the RV adaptive response to hypoxia. Another species, *N*-arachidoyl-D-erythro-sphingosine (C20-Cer), was specifically and significantly decreased in the hypoxic RV. These studies support the presence of distinct roles for different ceramide species and their precursors. A better assessment of cyanotic congenital heart disease in light of the mechanism and timing of cardiomyocyte death, will lead to punctual interventions and even novel cardioprotective strategies.

1. Introduction

Cardiac myocytes rapidly proliferate during fetal life but exit the cell cycle soon after birth in mammals. Although the extent to which adult cardiac myocytes are capable of cell cycle reentry is controversial and species-specific differences may exist, it appears that for the vast majority of adult cardiac myocytes the predominant form of growth postnatally is an increase in cell size (hypertrophy) not number [1].

Increased myocardial workloads due to systemic hypertension, chronic hypoxia, or carbon monoxide exposure in fetal or early neonatal life lead to cardiac enlargement by causing an increased rate of hyperplasia of myocardial cells or continuation of hyperplasia beyond the normal period of hyperplastic growth [2].

Programmed cell death is an important physiological response to many forms of cellular stress. The signaling cascades that result in programmed cell death are as elaborate as those that promote cell survival, and it is clear that coordination of both protein- and lipid-mediated signals is crucial for proper cell execution. Sphingolipids are a large class of lipids whose diverse members share the common feature of a long-chain sphingoid base, e.g., sphingosine. Many sphingolipids were shown to play essential roles in both death signaling and survival. Ceramide, an *N*-acylsphingosine, has

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been implicated in cell death following a myriad of cellular stresses. Sphingosine itself can induce cell death but via pathways both similar and dissimilar to those of ceramide. Sphingosine-1-phosphate, on the other hand, is an anti-apoptotic molecule that mediates a host of cellular effects antagonistic to those of its pro-apoptotic sphingolipid siblings [4].

Changes in ceramide content and in activities of enzymes regulating its synthesis and breakdown were documented in models of ischemic-reperfused myocardium [5–7]. However, sphingosine, spingosine-1-phosphate, and ceramide are only a few of the myriad sphingolipid species that exist and potentially carry a regulatory role in the cell.

In this study, we aim to examine the effects of chronic hypoxia on selected sphingolipids in cardiac tissue in a neonatal mouse – *Mus musculus* – model. We previously showed that myocardial mass is increased in a rat model of chronic hypoxia mimicking cyanotic heart disease [8]. This increase was mainly due to cardiac proliferation in the right ventricle (RV) during the first week of life and was associated with the lack of apoptosis and a relative decrease in ceramide content in the hypoxic RV [3]. We chose to extend those findings to the mouse model in order to replicate the findings in a different animal model, examine in more detail the effects of chronic hypoxia on different ceramide species and some ceramide precursors and metabolites as well as the normal variation of these molecules with age using high-performance liquid chromatography–tandem mass spectrometry (HPLC–tMS), and to lay the cornerstone for future studies on enzyme expression and function using knock-out mouse models that will lead to a better understanding of sphingolipid regulation under stresses like hypoxia.

2. Methods

2.1. Experimental design

We used an animal model to mimic conditions of some forms of cyanotic heart disease. *Mus musculus* mice raised in our established breeding colony were studied. They were placed in a hypoxic environment within the first few minutes of life. Control animals remained in room air. Nitrogen and oxygen tanks with gauged flow were attached into a specially built air-tight Plexiglas chamber at standard cage dimensions. Oxygen levels were monitored continuously by an oxymeter and maintained at 10% in the Plexiglas chamber. Air within the chamber was recycled after being passed through an anhydrous calcium sulfate and charcoal mixture to remove ammonia and moisture. Soda lime was placed inside the chamber to absorb CO_2 [9]. All animals received care in accordance with approved institutional animal care guidelines and according to the Guide for the Care and Use of Laboratory Animals of the National Academy of Science and the Principles of Laboratory Animal Care of the National Society of Medical Research.

Mice were randomly divided into eight groups. Group A included control mice raised in room air and studied at 1 day; group B included mice raised in hypoxic environment since birth and studied at 1 day of age. Groups A and B included 36 animals each due to the extremely small size of the heart at this age. Group C included control mice raised in room air and studied at 1 week, and group D included hypoxic mice raised in hypoxic environment since birth and studied at 1 week of age. Groups C and D included 24 animals each. The remaining groups E and F ($n = 5$), and G and H ($n = 3$) received similar treatments at 4 weeks and 8 weeks, respectively. The dam stayed with its mice pups in the Plexiglas chamber and tolerated the hypoxic environment.

The mice were sacrificed by spinal dislocation. The hearts were harvested after performing a midline sternotomy. The heart was excised, and the RV and LV were carefully identified, separated and individually weighed. Hematocrit levels were assessed in all studied groups.

2.2. Lipid extraction

Lipids were extracted by the method of Bligh and Dyer [10]. Briefly, cardiac tissue was homogenized after washing with PBS, then extracted with 3 mL of chloroform-methanol (1:2, v/v) in 13-mm × 100-mm screw-top glass tubes. The monophase was mixed, then 0.7 mL of water was added and the samples were rested for 10 min. The organic and aqueous phases were subsequently separated by addition of 1 mL of chloroform and 1 mL of water followed by vigorous shaking and centrifugation at 1000 rpm. The organic phase was carefully removed and transferred to a new tube, and the samples were dried under nitrogen. Lipids were then resuspended in 1 mL of chloroform.

2.3. Ceramide measurement

Ceramide levels were measured using a modified diacylglycerol kinase assay using external standards [11]. Briefly, 80% of the lipid sample was dried under nitrogen. The dried lipid was solubilized in 20 μL of an octyl-[beta]-D-glucoside-dioleoyl phosphatidylglycerol micellar solution (7.5% octyl-[beta]-D-glucoside, 25 mM dioleoyl phosphatidylglycerol) by two cycles of sonication in a bath sonicator for 60 s followed by resting at room temperature for 15–20 min. The reaction buffer contained 100 mM imidazole HCl, pH 6.6, 100 mM LiCl, 25 mM MgCl2, and 2 mM EGTA. To the lipid micelles, 50 μL of 2× reaction buffer was added, 0.2 μL of 1 M DTT, 5 μg of diglycerol kinase membranes, and dilution buffer (10 mM imidazole, pH 6.6, 1 mM diethylenetriaminepentaacetic acid, pH 7) to a final volume of 90 μL. The reaction was started by adding 10 μL of 2.5 mM [[gamma]-^{32}P] ATP solution (specific activity of 75,000–200,000 cpm/nmol). The reaction was allowed to proceed at 25 °C for 30 min. Lipids were extracted as described above, and a 1.5-mL aliquot of the organic phase was dried under nitrogen. Lipids were then resuspended in a volume of 100 μL of methanol–chloroform (1:20, v/v), and 20 μL was spotted on a 20-cm silica gel thin-layer chromatography plate. Plates were developed with chloroform–acetone–methanol–acetic acid–water (50:20:15:10:5), air-dried, and subjected to autoradiography. Radioactive spots corresponding to ceramide-phosphate, the phosphorylated product of ceramide, were scraped into a scintillation vial containing 4 mL of scintillation fluid and counted on a scintillation counter. Linear curves of phosphorylation were produced for a concentration range of 0–960 pM of external standards (dioleoyl glycerol and C-III ceramide; Sigma Chemical Co., St. Louis, MO, USA). Ceramide levels were always normalized to lipid phosphate.

2.4. Lipid phosphates

Lipid phosphates were measured according to the method of Rouser et al. [12]. Briefly, 20% of the lipid sample was dried under nitrogen and oxidized with 70% perchloric acid on a heating block at 160 °C for 45 min. The tubes were allowed to cool, then water was added, followed by 2.5% ammonium molybdate and 10% ascorbic acid with vortexing after each addition. The tubes were then incubated at 50 °C for 15 min and allowed to cool, and the absorbency was read at 820 nm and compared with standards.

2.5. High-performance liquid chromatography–tandem mass spectrometry (HPLC–tMS)

Protein level was determined using the Bio-Rad dye-binding assay as per company instructions with bovine serum albumin