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Systemic and placental α-klotho: Effects of preeclampsia in the last trimester of gestation

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ABSTRACT

Introduction: α-klotho is an anti-aging protein, potentially important in preeclampsia (PE). Produced by kidney, brain and placenta, and by mRNA splicing is both a full-length membrane-bound and a truncated soluble protein in the circulation. The membrane-bound protein is an obligate co-receptor for fibroblast growth factor 23 (FGF23) and its action on receptor (FGFR), but ADAM proteinases also cause its shedding. The aims of this study were to investigate levels of maternal plasma, placental, and fetal membrane α-Klotho and their association with placental accelerated villous maturation (AVM) in PE. In addition, placental and membrane levels of ADAM17 and FGFR were measured in the same patients.
Methods: Maternal blood, placenta and fetal membranes from 61 women (31 with PE and 30 controls) between 32 and 40 weeks gestation were collected. Plasma α-klotho was measured by ELISA, and quantitative immunohistochemistry used for α-klotho, ADAM17 and FGFR1 in tissues. Placental AVM was histologically assessed.
Results: Maternal plasma levels of α-Klotho were higher in PE compared to controls ($p = 0.01$) and patients with the highest levels had significantly less AVM ($p = 0.03$). α-Klotho, ADAM17, and FGFR were all present in syncytiotrophoblast and cytotrophoblast of membranes. Between 32 and 40 weeks gestation, all placental levels decreased in controls respectively ($p = 0.04$, $p = 0.004$, $p = 0.05$), but not in PE. Fetal membrane levels were unchanged.
Discussion: Maternal plasma α-Klotho was increased in PE and its levels associated with reduced placental AVM. Changes in placental α-Klotho, ADAM17, and FGFR suggest their involvement in the pathophysiology of PE.

1. Introduction

Hypertensive disorders of pregnancy are still a significant cause of maternal and perinatal morbidity and mortality. Preeclampsia (PE) complicates 10% of all pregnancies and is responsible for 20% of maternal deaths in the United States [1]. Several mechanisms, such as abnormal placentation [2,3], oxidative stress [4,5], placental ischemia [6] and endothelial dysfunction [7,8] are all pathophysiologic aspects of PE. The placenta has a central role, producing bioactive factors, which act both locally and in the maternal compartment, with the potential for involvement in the genesis of hypertension [9]. An inadequacy in the placenta is the most likely reason for the increased placental oxidative stress, which in turn generates greater cellular stress, causing inflammatory effects within and beyond the placenta [10]. Prolonged oxidative damage in the placenta gives rise to characteristic placental histology [11,12], defined as accelerated villous maturation (AVM) correlated with PE [11,12] and preterm birth [13]. However, there are few

Abbreviations: PE, preeclampsia; HELLP, hemolysis, elevated liver enzymes, and low platelets; sFlt-1, soluble vascular endothelial growth factor receptor-1; SGA, small for gestational age; ELISA, Enzyme-linked immunosorbent assay; FGFR, fibroblast growth factor receptor; FGF23, bone derived fibroblast growth factor 23; AVM, accelerated villous maturation; ADAM, a disintigrin and metalloproteinase.

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studies to date which attempt to link placental histological profiles with upstream molecular events.

α-Klotho is an anti-aging protein, which when disrupted in mouse models gives rise to a syndrome closely resembling human aging. Transgenic knockout mice show skin atrophy, atherosclerosis, osteopenia, cognitive impairment and motor neuron degeneration at 3–4 weeks and die prematurely at two months [14]. At the cellular level, α-klotho regulates senescence by repressing the p53/p21 pathway [15]. In humans, a functional variant of the *Klotho* gene has been shown to be associated with human survival [16]. In cross-sectional and prospective studies in human populations, *Klotho* has been implicated in the etiology of cardiovascular disease and mortality [17]. α-klotho is produced in the kidney and brain and to a lesser degree the placental syncytiotrophoblast [18,19]. The klotho gene generates both a full-length membrane-bound protein and a truncated soluble secreted (systemic) form by alternative mRNA splicing [20]; the major human gene product is the secreted protein [21]. However, the trans-membrane protein is also shed into blood, urine and cerebrospinal fluid [21]. This is accomplished by cleavage and release by the disintegrin and metalloproteinases; ADAM10 and 17 [22]. ADAM17 is a major sheddase for placental TNFα, and increases in preeclampsia [23]. The receptor for soluble α-klotho is unknown, whereas the trans-membrane form interacts with three fibroblast growth factor receptor (FGFR) isoforms (FGFR 1c, 3c and 4). It serves as an obligate co-receptor for bone derived fibroblast growth factor 23 (FGF23), which is secreted from bone into the circulation [24,25], but is not produced by the placenta [26]. Thus, α-klotho significantly increases the affinity of specific FGFRs for FGF23 [27].

α-Klotho functions in several biological processes involved in the pathophysiology of PE including endothelial nitric oxide production, angiogenesis, antioxidant enzyme production and protection against endothelial dysfunction [28–30]. Maternal soluble α-klotho is higher in pregnant compared to non-pregnant women. However, PE together with a small-for-gestational age infant was associated with lower than normal levels [31]. There have been no studies to date, which concomitantly measured maternal α-klotho and placental α-klotho, ADAM17 and FGFR1, in placenta and fetal membranes of a single set of patients. In this study, we therefore aimed to gain new insights into the α-Klotho system in the maternal circulation and in the placenta in PE.

2. Materials and methods

2.1. Study participants and sample collection

PE was defined according to current guidelines from the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy [32]. All patients were preterm (≤36 weeks 6 days) or term (≥37 weeks 0 days). Controls had no medical comorbidities, no illicit drug or tobacco use. Preterm controls had spontaneous preterm labor or preterm premature rupture of membranes (PPROM) and were delivered during the same admission. These cases accounted for the majority of preterm controls (12/15 = 80%). In cases of preterm PPROM ≥34 weeks, labor was augmented with Pitocin in accordance with ACOG recommendations. The other three preterm controls were admitted for scheduled cesarean delivery due to placenta previa (3/15 = 20%). Preterm neonates were classified as small-for-gestational-age (SGA) using gender specific Fenton growth curves [33]. There are currently no validated ethnic-based fetal/neonatal growth curves and ten percent of our controls were SGA, likely due to our high prevalence of Asian patients, specifically Japanese and Filipino [34].

After approval from Hawaii Pacific Health and Western Institutional Review Board, pregnant women admitted to Kapi'olani Medical Center for Women and Children (Honolulu, HI) meeting study criteria were recruited with informed consent in 2013–2014. Exclusion criteria included maternal connective tissue/autoimmune disease, renal disease, pre-gestational or gestational diabetes mellitus, active infection, chronic corticosteroid use, illicit drug or tobacco use, anemia (hemoglobin < 10 mg/dl), obstructive sleep apnea, multiple gestation, uterine malformations, fetal chromosomal/congenital anomalies, or histologic evidence of placental infection. Prior to delivery, maternal blood samples were collected and plasma stored at −80 °C. Immediately after delivery, one full-thickness sample of placenta, excluding basal plate, was collected near the cord insertion site, fixed in neutral-buffered formaldehyde for 72 h and embedded in paraffin.

2.2. Maternal plasma analyses

Maternal plasma sFlt-1 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Inter-assay and intra-assay coefficients of variation were 7.4% and 3.2% respectively with sensitivity of 3.5 pg/mL. Significantly higher levels in PE compared to controls were found (15,599.0 ± 11,949.7 pg/mL vs. 3423.2 ± 2417.3 pg/mL; $p < 0.0001$) consistent with other studies. α-Klotho was measured by ELISA (Immuno-Biological Laboratories Inc., Minneapolis, MN) with inter-assay and intra-assay coefficients of variation of 4.7% and 2.9% and sensitivity of 6.16 pg/mL. All samples were assayed in duplicate, with personnel blinded to clinical information and outcomes.

2.3. Placental histopathology, immunohistochemistry and quantitation

Histologic evaluation was performed by a placental pathologist (KT) blinded to gestational age and clinical outcomes. H&E stained placental sections were evaluated for AVM (narrow terminal villi and/or syncytial knotting out of proportion for gestational age) and scored positive if the finding was present in multiple 10× objective fields [11,12,35]. Immunohistochemistry was performed for quantitative assessment [36], using serial sections (5 μm) heated in sodium citrate buffer (10 mM, pH 6.0) for 20 min for antigen retrieval. The Vectastain Elite kit (Vector Labs, Burlingame, CA) was used according to the manufacturer's protocol. Non-specific binding was blocked with 2.5% normal serum for 20 min before incubation for 60 min with antibodies: polyclonal goat IgG to α-klotho (Santa Cruz Biotechnology, Santa Cruz, CA, sc-22218, 0.75 μg/mL), polyclonal rabbit IgG to FGFR1 (Santa Cruz Biotechnology, sc-121, 0.25 μg/mL), or monoclonal mouse IgG2b to ADAM17 (ABCAM, Burlingame, CA, ab 57484, 1.25 μg/mL). Negative controls were species-specific non-immune IgG at equivalent concentrations with 3,3-diaminobenzidine (DAB) substrate solution used for visualization. Slides were rinsed, counterstained with Gill's hematoxylin, cleared and mounted.

A multispectral imaging system with an Olympus BX51 microscope (Olympus America Inc, Mellville, NY) and a CRI Nuance spectral analyzer (Caliper Life Sciences, Hopkinton, MA) was used to obtain brightfield image cubes between 420 and 700 nm wavelength at 20 nm intervals. The Inform Tissue Finder software (version 2.0.2; PerkinElmer, Waltham, MA) segmented tissues into cell types, unmixed the spectral components, and quantified staining levels [37]. Average signal intensity per pixel, was obtained from five different fields from each patient, as mean optical density units (ODU). Signal intensities for α-klotho, FGFR1, and ADAM17 were quantified at 400× magnification. Laboratory personnel were blinded to clinical information.