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Methods paper

Comparative analysis of caveolins in mouse and tammar wallaby: Role in regulating mammary gland function

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ABSTRACT

Recent studies using the mouse showed an inverse correlation between the Caveolin 1 gene expression and lactation, and this was regulated by prolactin. However, current study using mammary explants from pregnant mice showed that while insulin (I), cortisol (F) and prolactin (P) resulted in maximum induction of the β-casein gene, FP and IFP resulted in the downregulation of Caveolin 1. Additionally, IF, FP and IFP resulted in the downregulation of Caveolin 2. Immunohistochemistry confirmed localisation of Caveolin 1 specific to myoepithelial cells and adipocytes. Comparative studies with the tammar wallaby showed Caveolin 1 and 2 had 70–80% homology with the mouse proteins. However, in contrast to the mouse, Caveolin 1 and 2 genes showed a significantly increased level of expression in the mammary gland during lactation. The regulation of tammar Caveolin 1 and 2 gene expression was examined in mammary explants from pregnant tammars, and no significant difference was observed either in the absence or in the presence of IFP.

1. Introduction

Caveolae are small flask-shaped invaginations found on the plasma membrane (Palade and Bruns, 2004) and other organelles like the Golgi apparatus (Dupree et al., 1993; Kurzchalia et al., 1992) and caveosomes (Pelkmans et al., 2001). They are mainly involved in regulating different cellular functions like endocytosis (Pelkmans and Helenius, 2002), cholesterol homeostasis (Ikonena and Parton, 2000; Maxfield and Wüstner, 2002), calcium homeostasis (Saliez et al., 2008), vesicular transport and signal transduction (Razani et al., 2002; Smart et al., 1999). These proteins are also reported to be involved in regulating vascular reactivity and blood pressure (Chidlow and Sessa, 2010; Yu et al., 2006). The main structural components of these caveolae are caveolins which are a family of three proteins; Caveolins 1, 2 and 3. Caveolae are distributed differentially across cell types with Caveolin 1 (*CAV1*) and Caveolin 2 (*CAV2*) genes having a higher level of expression in endothelial cells and adipocytes (Kandror et al., 1995; Stan, 2005; Thorn et al., 2003) whereas Caveolin 3 (*CAV3*) gene expression is muscle specific (Tang et al., 1996; Way and Parton, 1995).

Reports during the past decade have shown a correlation between caveolin expression and lactation (Park et al., 2001; Sotgia et al., 2009). In mice, the expression of the Caveolin 1 and 2 genes was elevated in the mammary gland before and during early pregnancy, downregulated during lactation and subsequently upregulated with the onset of involution to levels similar to tissue from non-pregnant animals (Park et al., 2001). Subsequent studies reported that the genetic ablation of Caveolin 1 in mice resulted in enhanced mammary gland development and premature lactation through hyper-activation of *STAT5* (Park et al., 2002; Sotgia et al., 2006). Recently, Sotgia et al. (2009) showed that the loss of Caveolin 3 expression, a muscle specific protein of the same family, induced a lactogenic phenotype in virgin mammary gland. Moreover, the implantation of mammary tumour cells in the primary duct of Caveolin 3 null mammary gland resulted in tumour mass reduction which was assumed to be due to the local paracrine effects of lactogenic luminal mammary epithelial cells. Related studies have demonstrated that the caveolin family of proteins plays a role in regulating lactation in mice by prolactin via a Ras–p42/44 MAPK-dependent pathway (Park et al., 2001, 2002). However, it's not clear whether this regulation of mammary gland function by caveolins is mediated by a complex interaction of lactogenic hormones that includes insulin and cortisol together with prolactin. In addition, there are reports showing the localisation of Caveolin 1 in endothelial and myoepithelial cells in

Abbreviations: %, Percentage; °C, Degree Celsius; BLG, Beta lactoglobulin; BSA, Bovine serum albumin; CAV1, Caveolin 1; CAV2, Caveolin 2; CAV3, Caveolin 3; cDNA, Complementary deoxyribonucleic acid; CO_2, Carbon dioxide; F, Cortisol; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; h, Hour; I, Insulin; μg, Micro gram; μl, Microlitre; μm, Micrometre; ml, Millilitre; ng, Nano gram; P, Prolactin; PBS, Phosphate buffered saline; qPCR, Quantitative polymerase chain reaction; RNA, Ribonucleic acid; RPS15, Ribosomal protein S15; RPY, Removal of pouch young; SEM, Standard error of the mean; STAT5, Signal transducer activator of transcription 5; WAP, Whey acidic protein.

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mouse mammary glands (Hue-Beauvais et al., 2007) and not in epithelial cells which may indicate a paracrine regulation of the lactation process.

This recent study correlating the caveolin protein family with the process of lactation was focused on the mouse, a eutherian model wherein milk composition and milk production do not change significantly across lactation apart from the initial colostrum produced immediately after postpartum (Kruse, 1983). To further evaluate the role of caveolin in milk synthesis and production, the current study utilised the unique reproductive strategy of tammar wallaby (*Macropus eugenii*) by examining the expression level of caveolin genes during different phases of lactation. Compared to eutherian mammals (e.g. human, mouse), marsupials such as the tammar wallaby have a different reproductive strategy comprised of a short gestation of 26.5 days followed by a long lactation cycle of about 300–330 days. Lactation is divided into three different phases during which the milk composition and rate of milk production change progressively (Nicholas et al., 1997; Tyndale-Biscoe and Janssens, 1988). During the early and mid-phases of lactation, the mammary gland produces small amounts of milk rich in carbohydrates but low in protein and lipids. Later during the third phase of lactation, large volumes of milk are secreted which are rich in protein and lipids but low in carbohydrates (Nicholas et al., 1997). There is limited understanding of the regulation of milk composition and production in tammar wallaby, and the question of whether these changes are correlated with the expression profile of caveolin genes would provide new information on the potential role of the caveolins in regulating a complex lactation cycle. Therefore, the current study compared the gene organisation and expression pattern of caveolins in two different species, mouse and tammar wallaby, and further employed the mammary explant model to gain a better understanding of the role of insulin, cortisol and prolactin for the regulation of this group of genes.

2. Materials and methods

2.1. Animals

BALB/c mice were obtained from Monash Animal Services, Melbourne, Australia. The inguinal and abdominal mammary glands were dissected under sterile conditions from mice at day 12 pregnancy.

Tammar wallabies were maintained in the Deakin University Marsupial Facility, Victoria, Australia. Mammary glands were dissected from pregnant — phase 1 (9, 21, 23 and 25 days), lactating — phase 2A (parturition, 1, 2, 3, 5, 10, 22, 37, 40, 62, 80 and 100 days), lactating — phase 2B (110, 114, 133, 135, 150, 151, 171 and 193 days), lactating — phase 3 (216, 240, 260, 266 and 293 days) and involuting (1, 3, 5 and 10 days) tammar wallabies under sterile conditions after the animals were euthanized. The tissues were stored at −80 °C and later used for examining the expression profile of different genes in the present study. All experiments were approved by the Deakin University Animal Ethics Committee.

The reactivation of pregnancy in the tammar wallaby was performed by removal of pouch young (RPY) and injecting bromocriptine (5 mg/kg body weight) to reinitiate the development of the dormant blastocyst (Gordon et al., 1988; Tyndale, 1978). The day of RPY was considered as day 0 pregnancy and the animals were sacrificed at day 24 of pregnancy to collect mammary tissue for explant culture experiments.

2.2. Tissue culture

Mammary gland explants were prepared from day 12 pregnant mice and cultured in Medium 199 (11150-059, Invitrogen Gibco, Victoria, Australia) with bovine serum albumin (0.25 mg/ml) (A0281, Sigma Sydney, Australia) (Nicholas and Tyndale-Biscoe, 1985). Explants were incubated at 37 °C and 5% CO_2 in 5 ml of media per well in 6 well plates. Media was changed on alternate days during the four day experiment. Hormones were added at the following concentration: bovine insulin 1 μg/ml (I; I6634 Sigma Sydney, Australia), hydrocortisone 50 ng/ml (F; H4001 Sigma Sydney, Australia) and ovine prolactin 1 μg/ml (P; National Hormone and Pituitary Program, USA). Mammary glands from 4 groups of 3 mice were dissected and pooled and explants were maintained in the indicated hormone combinations for four days. The control explants were maintained in media without the addition of exogenous hormones (NH-No hormone). Explants at different time points were collected and stored at −80 °C for RNA extraction.

Tammar mammary glands from day 24 pregnant animals were cut into 1–2 mg sized explants and placed on siliconised lens paper floating on 5 ml of Medium 199 in 6 well plates. These explants were incubated at 37 °C and 5% CO_2 and media changed every second day. Hormones were added at the following concentrations: bovine insulin 1 μg/ml (I; I6634 Sigma Sydney, Australia), hydrocortisone 50 ng/ml (F; H4001 Sigma Sydney, Australia) and ovine prolactin 1 μg/ml (P; National Hormone and Pituitary Program, USA).

2.3. RNA extraction and reverse transcription PCR

Mammary tissue (~100 mg) was first homogenised in QIAzol lysis reagent using a polytron kinetamica PT 2100 homogeniser and total RNA was extracted using the RNeasy Lipid Tissue Kit (74804 Qiagen, Sydney, Australia) following manufacturer's instructions. The quantity and quality of RNA were assessed by spectrophotometric analysis at 230, 260 and 280 nm (Nanodrop ND-1000, Biolab, Victoria, Australia). The cDNA was synthesized from total RNA (1 μg) using Superscript III (18080-044 Invitrogen, Australia) and following manufacturer's protocol.

2.4. Quantitative polymerase chain reaction

Quantitative polymerase chain reactions (qPCR) were performed on a Bio-Rad CFX Manager using SsoFast Evagreen supermix (172-5200 Bio-Rad Laboratories). The PCR reactions (20 μl) contained 1x SsoFast Evagreen supermix, forward and reverse primers (Table 1) and 2 μl of 1 in 20 diluted cDNA. The qPCR thermal profile included an initial denaturation (95 °C for 3 min) and quantification for 40 cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s). All samples were assayed in duplicate. The ΔCt method was used to normalise transcripts to housekeeping genes GAPDH for mouse and RPS15 for tammar wallaby (Table 1). The housekeeping genes for both species were selected based on a low level of variation in the expression across samples (data not shown). Melting curve analysis was done to confirm the amplification comprised a single PCR product of correct size.

2.5. Sequence analysis

The tammar wallaby caveolin gene sequences were retrieved from tammar wallaby genome in NCBI and Ensembl using mouse (*Mus musculus*) and gray short-tailed opossum (*Monodelphis domestica*) caveolin sequence and the blast program (http://www.ncbi.nim.nih.gov/blast). Protein alignments were performed using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.6. Preparation of mouse mammary gland sections and immunohistochemistry

Mammary glands were collected from pregnant mice, washed in phosphate buffered saline (PBS) and were fixed in 4% paraformaldehyde solution overnight at 4 °C, processed and embedded in paraffin. Sections of 5 μm thickness were dewaxed, antigen retrieved by boiling in citric acid buffer (pH = 6) and endogenous peroxidase blocked by incubation in 0.5% H_2O_2 for 15 min. Subsequently, the sections were blocked in 5% skimmed milk for 1 h and incubated with primary antibody (1:500, diluted in PBS/1% BSA) (Polyclonal Rabbit Anti-Caveolin, 610059 BD Transduction Laboratories) overnight at 4 °C. The slides