LIS1 and platelet-activating factor acetylhydrolase (Ib) catalytic subunits, expression in the mouse oocyte and zygote

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Abstract Platelet-activating factor is a phospholipid with several documented roles in the pre-implantation embryo. Enzymes that belong to the platelet-activating factor acetylhydrolases family inactivate platelet-activating factor. Cytosolic platelet-activating factor acetylhydrolase (Ib) is a heterotetramer composed of two catalytic subunits (α1/α2) and two regulatory LIS1 subunits. The expression of these components was monitored in the mouse oocytes and zygotes using reverse-transcribed PCR and Western blot analysis. Interestingly, these proteins are expressed in the oocyte and zygote and their expression increases after fertilization, probably due to stabilization of maternal RNA. *Lis1* mRNA transcription also increases after fertilization. However, assaying for expression of a specific paternal LIS1 isoform detected no zygotic translation in the one cell stage. These findings suggest a potential role for platelet-activating factor acetylhydrolase (Ib) components in the early mouse embryo.

Key words: LIS1; Lissencephaly; Platelet-activating factor; Platelet-activating factor acetylhydrolase; Fertilization

1. Introduction

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) is a potent phospholipid that is important in many biological processes including pre- and post-fertilization (reviewed in [1,2]). PAF increases the sperm motility and improves the acrosome reaction [3–6]. This phospholipid is produced by zygotes but not by unfertilized ova [7]. Additionally, production of PAF is believed to be important for the development of the embryos. PAF elicits activation of signal transduction pathways through PAF receptors that are coupled to guanine nucleotide-binding proteins [8–12]. The presence of the PAF receptor has been documented already at the two cell stage embryo [13]. Under in vitro culture conditions, addition of exogenous PAF enhanced the human and mouse embryo viability [14,15]. PAF was shown to act as a survival factor during the two cell stage of development, protecting embryos from subsequent cell death [16]. Most of the information available regards extracellular secreted PAF. Within cells, PAF is synthesized by at least two pathways: the remodeling route and de novo synthesis (reviewed by [17,18]). It is widely believed that PAF has important intracellular functions as well [19] and the existence of intracellular PAF-binding sites shown in rat cerebral cortex cells [20] supports this notion. However, to date, the pathways in which intracellular PAF functions are not clear.

Deacetylation of PAF by PAF-acetylhydrolase (AH) results in a biologically inactive molecule, lyso-PAF. This enzymatic activity is considered to be important during the early development of the embryo. A decreased activity of PAF-AH in follicular fluids was correlated with more successful pregnancies [21]. Relatively low levels of PAF-AH activity were measured in the mouse uterus during the pre-implantation phase [22]. A reduced activity is found irrespective of the presence of the embryo, as similar values were measured from pseudo-pregnant mice [22].

PAF-AH composes a group of enzymes that are products of different genes (reviewed by [23]). The main PAF-AH in the serum is a 45 kDa monomer. In mammalian brain as well as in other tissues, three cytoplasmic isoforms were identified, Ia, Ib and II, from which only Ib and II were cloned [24–26]. The Ib and II isoforms differ in several aspects, while the Ib isoform is a heterotetramer composed of 30 (α2) and 29 (α1) kDa subunits and two 45 kDa subunits (β, LIS1), the II isoform is a 40 kDa monomer [27]. The Ib isoform is expressed at the highest levels in bovine brain, while isoform II is expressed most abundantly in bovine liver and kidney. The deduced amino acid sequence of isoform II has no homology with any subunit of isoform Ib, but showed 41% identity with that of plasma PAF-AH [27]. The focus of our study is isoform Ib, which is composed of two highly homologous α subunits forming a catalytic dimer and two regulatory β subunits. The β subunit is the product of the *LIS1* gene [28], which, when mutated in humans, results in abnormal brain patterning known as lissencephaly. We have demonstrated an additional role for LIS1 in its interaction with tubulin, a component of the cytoskeleton [29]. Addition of LIS1 modulates microtubule dynamics in vitro [29]. Recent gene targeting of *Lis1* demonstrated that the homozygotes are early lethal ([30] and A.C. unpublished results). In view of the importance of PAF and of LIS1 during early developmental processes, we set out to investigate the activity of PAF-AH and the expression of PAF-AH(Ib) subunits in mouse oocytes and zygotes.

2. Materials and methods

2.1. Animals

MF1 mice were housed under a 12 h light/12 h dark cycle and had access to food and water ad libitum. Superovulation and synchronization of 4 week old females was achieved by intraperitoneal injection of 5 IU of pregnant mare serum followed by injection of 5 IU of human chorionic gonadotropin (hCG) after 46 h. To achieve timed pregnancies, females were mated overnight and the presence of vaginal plugs was examined (0.5 days post-coitum). Generation of Lis1 −/+ (heterozygote knockout) mice will be described elsewhere (Cahana et al., manuscript in preparation). Briefly, the first encoding exon of *Lis1* was flanked by two loxP sites. Heterozygote mice were crossed

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with PGK-Cre mice on an MF1 background [31], resulting in the removal of the first encoding exon. The mutated allele gave rise to a shorter protein due to internal initiation.

2.2. Oocytes and zygotes collection

In the morning following hCG injection, females (mated or not) were killed and oviducts were dissected and placed in M2 medium (as described in [32]). The oocytes or zygotes were then released from the swollen ampulla. The oocytes or zygotes were separated from the associated cumulus cells by incubation for a few minutes in 300 mg/ml hyaluronidase (Sigma H 3884). Immediately after the dissociation of cumulus cells, the oocytes or zygotes were washed twice in M2 medium and collected.

2.3. Isolation of total RNA and reverse-transcribed (RT)-PCR

Total RNA from 400 oocytes or zygotes per batch was extracted with Tri-reagent (TR 118, Molecular Research Center, OH, USA), after addition of 10 μl glycogen as a carrier. Oligonucleotide primers were designed for *Lis1* RT-PCR that will yield a 240 bp product: 5′-GATGACAAGACCCTCCGTGT-3′; 5′-GAGCTCAAATGGGGTAACCA-3′. For PAF-AH α1, primers for RT-PCR that will yield a 293 bp product: 5′-ACACAGCATGTACTCTGGCG-3′ 5′-GCATCTAAGAAGTGGGCTCG-3′ and for PAF-AH α2, primers for RT-PCR that will yield a 127 bp product: 5′-AGAATGCCAAGGTGAACCAG-3′; 5′-AAATCAAACATGTCGTGGCA-3′. Primers were designed to be from separate exons yielding a specific RT product.

Reverse transcription and amplification were performed using the access RT-PCR system (Promega, Madison, WI, USA), according to the manufacturer's recommendations. PCR amplification was carried out during 40 cycles of denaturing, annealing and extension steps for 30 s at 94°C, 1 min at 55°C and 1 min at 68°C, with a final extension step for 10 min at 68°C. A negative control was made without reverse transcriptase in the reaction mixture. RT-PCR fragments were analyzed by electrophoresis on TAE agarose gels.

Quantitation of RT-PCR was done by using IMAGE MASTER VDS-CL (Dinco and Rhenium Marketing, Jerusalem, Israel) or by MacBASV2.5, the results using either quantitation methods were similar.

Statistical analysis of RT-PCR products was done using one factor ANOVA-repeated measures.

2.4. Protein extraction

Proteins were extracted in a minimal volume of PBS supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and phosphatase inhibitors (10 mM tetra-sodium diphosphate decahydrate and 50 mM sodium fluoride). The samples were freeze-thawed and vortexed three times, followed by 15 min centrifugation in an Eppendorf centrifuge at 14 000 rpm. The supernatant was frozen in aliquots.

2.5. Western blots

Proteins were separated using 12.5% SDS-PAGE gels run at 100 mV for 90 min. Proteins were transferred onto nitrocellulose (BioTrace NT, GelmanSciences) for 45 min at 90 mV. The efficiency of transfer was determined by Ponceau red stain (Ponceau S concentrate, Sigma). The filters were then blocked with 5% lyophilized milk in PBS for 60 min. First antibodies were added (polyclonal anti-α1/α2 [33], 1:300 dilution or monoclonal anti-LIS1 clone 210 [29], 1:1000 dilution) for 60 min at room temperature. The filters were washed three times with PBS and 0.05% Tween 20 and then incubated with a second antibody (goat anti-mouse HRP 1:10 000) for 1 h. The filters were then washed with PBS and 0.05% Tween 20. The chemiluminescence reaction was developed using SuperSignal substrate (Pierce, USA) according to the manufacturer's instructions. The autoradiograms were analyzed by a densitometer.

3. Results and discussion

The presence of PAF-AH(Ib) subunits in oocytes and zygotes was investigated using RT-PCR. We were able to detect RNA expression of all three PAF-AH(Ib) subunits (Fig. 1). While it is known that the oocyte genome is transcriptionally very active, the majority of the maternal mRNA synthesized is translationally silent or masked [34]. Therefore, the existence of mRNA does not necessarily correspond to protein expression. Western blot analysis (Fig. 2A,C) revealed that LIS1 and the catalytic subunits are expressed both in the oocyte and in the zygote. As our antibodies react with both the αl and α2 subunits and the RT-PCR revealed expression of the transcripts of both subunits, we suggest that both subunits are expressed also at the protein level. Interestingly, the expression of LIS1 and of the catalytic subunits increase after fertilization 40% and 50%, respectively (Fig. 2B,D). The increase is not contributed by the sperm, as Western blot analysis of 10 μg of sperm protein revealed a very weak signal (data not shown). Our study demonstrates for the first time the presence of PAF-AH(Ib) subunits in mammalian eggs. Oocytes are known to contain a large store of dormant mRNAs that may serve as a source of genetic information during early development. These maternal transcripts are translated selectively during early development. One of the most common mechanisms of selective maternal mRNA translation is cytoplasmic polyadenylation. A class of mRNAs that have relatively short poly(A) tails undergo poly(A) extension in a message-specific manner that results in translational activation (reviewed by [35]). The *cis* elements necessary for cytoplasmic poly(A) elongation, at least in vertebrates, are located in the 3′-untranslated regions of mRNAs. These elements include the hexanucleotide AAUAAA (reviewed in [36–38]) and usually a cytoplasmic polyadenylation element [39–43]. A preliminary specific RT-PCR poly(A) reaction (described by [43]) suggests that the *Lis1* transcript is indeed polyadenylated in the zygote (data not shown). We then examined whether there is any zygotic transcription. We performed a semi-quantitative RT-PCR from 10 oocytes or zygotes and quantified products from cycles that were within the linear range of the reaction (cycles 19–24) (Fig. 3). Quantitation of the *Lis1* mRNA product demonstrates that it is double (95% more, $P = 0.02$) in the zygotes versus the oocytes. Therefore, we concluded that *Lis1* mRNA is transcribed in the fertilized embryo. It has been demonstrated that the fertilized mouse eggs can delay expression of zygotic genes by uncoupling the translation from transcription (reviewed in [44]). In order to investigate the possibility of translation of the zygotic transcript, we utilized Lis1 −/+ mice (gene targeting in the Lis1 locus, A.C. manuscript in preparation). The mutated allele of these mice translates a shorter LIS1 protein (Fig. 4, right panel, two LIS1 proteins that differ in size are visible in a brain extract). This allows us to differentiate between the products of two alleles. The exist-

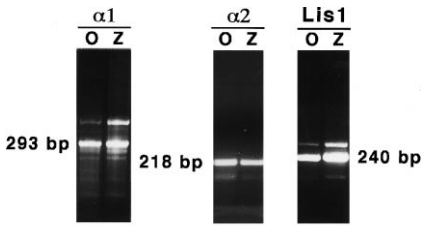

Fig. 1. PAF-AH(Ib) subunits RNA expression. RT-PCR demonstrates the presence of transcripts of Lis1, α1 and α2 in both oocytes (O) and zygotes (Z). The size of cDNA products from the three pairs of primers corresponded to the expected sizes and are indicated Lis1: 240 bp, α1: 293 bp, α2: 127 bp. Total RNAs from 100 oocytes or zygotes were used per reaction. The primers designed from different exons allow to differentiate between amplification from cDNA and DNA.

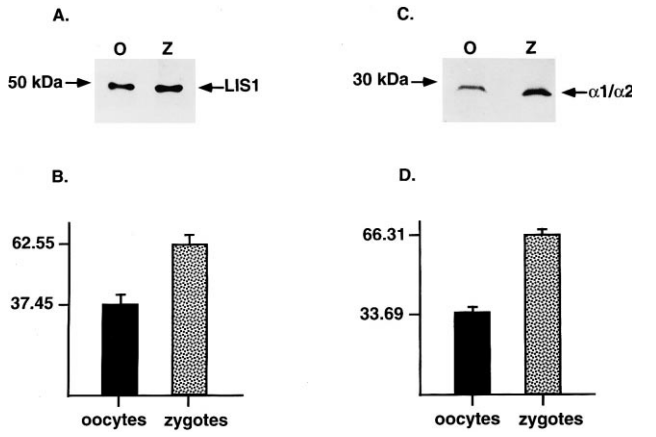

Fig. 2. PAF-AH(Ib) subunits protein expression. A Western blot analysis demonstrates an increased expression of LIS1 and α1/α2 subunits following fertilization. A: Representative Western blot of 100 oocytes (O) and zygotes (Z) using anti-LIS1 monoclonal antibody #210. Positions of a size marker of 50 kDa and of the LIS1 protein are indicated. B: The LIS1 Western blot experiment was repeated five times and quantified using a densitometer. The average ratio in Lis1 expression between oocytes and zygotes is 1:1.67 and is extremely significant ($P < 0.01$), analyzed by one factor ANOVA-repeated measures. C: Western blot of 520 oocytes or zygotes reacted with anti-α1/α2 polyclonal antibodies. The positions of a size marker (30 kDa) and of the α1/α2 proteins are indicated. D: The α1/α2 Western blot experiment was repeated three times and quantified using a densitometer. The average ratio between oocytes and zygotes expression is 1:2 and is statistical significant ($P < 0.01$).

ence of specific maternal or paternal isoforms has been used to study zygotic expression of glucose-phosphate isomerase [45,46]. We used normal female mice with their maternal LIS1 protein of the normal size and crossed them to Lis1 (−/+) heterozygote males that carry both a mutated and a normal allele. Half of the offsprings are expected to be heterozygote, when the mutant allele is totally of paternal origin. Therefore, we expected that if there is zygotic LIS1 translation in the one cell stage, it might be possible to detect a slower migrating band. As seen in Fig. 4 (left panel), no additional bands are visible. Therefore, zygotic translation of *Lis1* mRNA is most likely delayed until the two cell stage, as has been previously detailed in other experiments (reviewed in [44]). The sensitive luciferase transgene marker was found to be expressed in the one cell stage [47,48]. However, only a

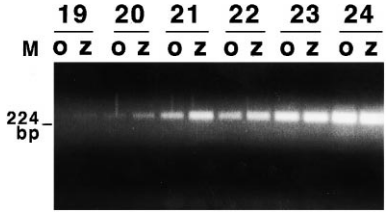

Fig. 3. Semi-quantitative Lis1 RT-PCR. RNAs from 10 oocytes or zygotes were used per RT-PCR reaction. RT-PCR products from oocytes (O) or zygotes (Z) generated in successive amplification cycles were separated on an agarose gel and products within the linear range of the reaction (cycles 19–24, indicated in the top row) are shown in this figure. The products were quantified and subjected to statistical analysis. Comparing the RT-PCR products from oocytes and zygotes suggests transcription of *Lis1* mRNA in the fertilized embryo. On the left side of the gel, the position of the size marker (M) is indicated.

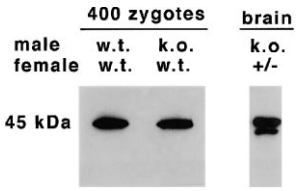

Fig. 4. Western analysis of the Lis1 isoform. Left panel, protein extracted from 400 zygotes using wild-type × wild-type or wild-type female × heterozygote (Lis1 −/+) male mating were separated on 10% PAGE and subjected to Western blot analysis using anti-LIS1 monoclonal antibody #210. Only one band is visible. Right panel, protein extracted from brain of heterozygotes (Lis1 −/+) was separated on 10% PAGE and subjected to Western blot analysis using anti-LIS1 monoclonal antibody #210. Two bands corresponding to the two LIS1 isoforms are visible. The position of a size marker of 45 kDa is indicated.

small portion of the resulting fertilized eggs produced luciferase activity and the level of activity was only 2% of that observed in two cell embryos [47,48]. A detailed quantitative analysis of protein synthesis in the early mouse embryo revealed a limited number of coordinately regulated protein sets that are responsible for most of the changes observed during the one and two cell stages [49]. Overall, our data demonstrate maternal expression followed by an increase in the protein components of PAF-AH(Ib) in the one cell stage, suggesting a potential function for these proteins in the early embryo. We can speculate about the putative roles of these proteins in the one cell stage. One possibility may be related to the enzyme. PAF is an autocrine factor in the one cell stage (reviewed in [2]) and perhaps intracellular enzymatic activity in the embryo is of importance. A second possibility may be related to LIS1-tubulin interaction. A homolog of LIS1 in *Aspergillus nidulans* NudF has a vital function in nuclear migration [50]. LIS1 is a highly conserved protein (reviewed in [51]), it may retain a conserved nuclear migration function and therefore may participate in pronuclear migration that is one of the essential processes following fertilization. Further analysis may allow us to dissect the relative importance of each of these roles.

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