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Reproduction and maternal behavior in insulin-regulated aminopeptidase (IRAP) knockout mice

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ABSTRACT

During human pregnancy, a circulating form of insulin-regulated aminopeptidase (IRAP EC 3.4.11.3), often termed oxytocinase or placental leucine aminopeptidase (PLAP), is present in plasma. It is proposed that circulating IRAP plays an important role in regulating the circulating levels of oxytocin and/or vasopressin during pregnancy. We assessed the reproductive and maternal profile of global IRAP knock out mice. No differences in the reproductive profile were observed, with normal gestational period, litter size and parturition recorded. However, western blot analysis of pregnant mouse serum, failed to detect IRAP, a result which was confirmed by fluorimetric IRAP enzyme assay. A review of the literature revealed that the presence of IRAP in the maternal circulation during pregnancy has been only reported in humans. Moreover, the sequence, Phe^{154} Ala^{155}, identified as the cleavage site for the release of soluble IRAP, is restricted to members of the homindae family. Therefore the absence of IRAP from the circulation in mice, and other species during pregnancy, is due to the inability of a secretase to cleave placental IRAP to produce a soluble form of the enzyme. Given the expression of IRAP in areas of the brain associated with oxytocin modulated maternal behavior, we also investigated whether the IRAP global knockout mice had improved maternal responses. Using standard tests to assess maternal behavior, including pup retrieval, feeding and nurturing, no differences between knock out and wild type dams were observed. In conclusion, the physiological significance of circulating IRAP during human pregnancy cannot be addressed by investigations on mice.

1. Introduction

Insulin-regulated aminopeptidase (IRAP), also known as placental leucine aminopeptidase or oxytocinase (EC. 3.4.11.3), can degrade a range of structurally related (oxytocin and arginine vasopressin) and unrelated (lys-bradykinin, met-enkephalin, dynorphin A, neurokinin A and neuromedin B) bioactive peptides. The *in vivo* substrates have not been elucidated [11,16,19], but arginine vasopressin (AVP) has been put forward as the most promising candidate based on recent work utilizing IRAP knockout (KO) mice [31].

Historically, oxytocin (OT) was thought to be the favored substrate, with a critical role proposed for IRAP in the prevention of onset of preterm labor by the regulation of the peptide hormone levels during the later stages of pregnancy [25]. A soluble form of IRAP is present in the maternal serum, produced by cleavage of the enzyme from the apical membrane of placental syncytiotrophoblast cells, through the activity of an, as yet, unidentified secretase [24,26]. In humans, the serum level of the aminopeptidase increase during the third trimester and plateau just prior to parturition and is proposed by Mizutani and his co-workers to play an important role in the maintenance of OT homeostasis [22,32].

IRAP may also play a more localized role in the modulation of OT or AVP levels and activity in the primary sites of production, namely the neurosecretory magnocellular neurons of the hypothalamus which project to the posterior pituitary [8,23]. The neurohypophyseal release of OT into the blood stream is well known to be associated with uterine contraction and milk ejection [10]. In addition, OT is also synthesized in the parvocellular regions of the hypothalamus and other sites in the brain including the amygdala where it is released locally in response to social or stressful stimuli to influence social, sexual or maternal behaviors [10]. Other sites of OT production in humans include the uterus during the later stages of pregnancy, in amnion, chorion and decidua where it exerts a paracrine effect to regulate prostaglandin biosynthesis [6]. The studies outlined above suggest that there are

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multiple sites for IRAP to exert its action on the regulation of the peptide hormone levels. However, the functional significance of the catalytic domain of IRAP remains far from resolved, whether it is intraluminal in neurosecretory vesicles, on the extra-cellular surface of the cell membrane or in the circulation.

In this study we investigated the role of IRAP in (i) the maintenance of pregnancy and (ii) the establishment and maintenance of maternal behavior post partum using the IRAP KO mice and their wildtype (WT) littermates.

2. Materials and methods

2.1. Generation of IRAP knockout (Ozgene Pty Ltd. (Perth, Australia)

Genomic clones containing fragments of the IRAP gene were isolated from a mouse genomic DNA (C57Bl6) by PCR screening with two primers derived from the rat IRAP cDNA. The genomic clones were analyzed by restriction digestions, Southern blotting, and sequencing, and a detailed exon-intron map of the IRAP gene was obtained. Based on this information, a targeting vector was constructed containing Exon2 followed by the PGK-neo marker both flanked by *loxP* sites.

The targeting vector was linearized and introduced into mouse C57BL/6 embryonic stem cells by electroporation. Embryonic stem cell clones were selected for resistance to G418. Genomic DNA was isolated from resistant clones, digested with *Xba*I, and screened for homologous recombination by Southern blotting. The oligonucleotide probe used for screening corresponded to nucleotides (GenBank accession NT_039643). Four clones derived expected fragments of 12 and 8.5 kb characteristic for the endogenous intact and the disrupted IRAP allele, respectively. One of these clones was injected into Balb/C blastocysts and implanted into pseudopregnant recipients. Male chimeric offspring were bred with C57BL6 female mice and germ line transmission of the mutant IRAP allele was identified by Southern blotting of genomic DNA obtained from tail snips as described above.

IRAPlox (±) male mice were then mated with female Cre deletor mice [29]. Female offspring of this mating with the genotype IRAP(±)/Cre were then crossed with wild type C57BL6 male mice to remove the Cre from the line. IRAP(±) breeding pairs were then set up to produce IRAP(−/−), (±), (+/+) offspring. Offspring were genotyped by PCR using the oligonucleotides GATAAGATAGAAGTAGGGGAGA, CAATAGAGGTACAGTCACCA and GGAGAATAAGGGCTGTGAGAGA (GenBank accession NT_039643). Wildtype allele PCR product = 384 bp (A and B) knockout allele = 1041 bp. (B and C) [1].

All animals were housed under 12:12 h, light:dark, conditions with ad libiitum access to water and food (mouse breeder cubes, Barastoc, Australia).

2.2. Mating

Females aged 11–16 weeks old were paired with an age-matched male of the same genotype (−/− or +/+). $n = 13$ females (6−/− and 7+/+) and $n = 13$ males (6−/− and 7+/+) were used.

Females were checked each morning for the presence of a vaginal plug. When a plug was observed (pregnancy day 0), the pair was separated.

2.3. Gestation

Once mating had been established (by the presence of a vaginal plug) the female was housed individually and food and water intake and body weight were measured daily.

On the 8–10th day of gestation, the female was moved to a small fish tank cage to enable her to acclimate prior to parturition. The tank was used to enable clear observation of maternal behavior.

On the 15th day of gestation, the standard nesting material (tissue paper) was removed and replaced with a known weight of cotton pads [7]. Use and incorporation of this sheeting into a nest was scored 24 h later: 0 = no nest, 1 = flat nest with no walls,1.5 = nest with some walls or with walls but not all available nesting material incorporated, 2 = full nest with enclosed walls/roof.

2.4. Parturition

The presence of pups was recorded as post partum day 0. The condition of the nest was scored as follows: 0 = no nest, 1 = incomplete nest (no enclosing walls), 2 = complete nest (enclosing walls). The number of pups born was noted without disturbing the nest.

2.5. Post parturition/lactation

2.5.1. Pup development

From post partum day 2, pups were weighed daily until weaning. The following basic developmental milestones were recorded: pigmentation of skin, visible whiskers, appearance of lanugo, eruption of lower and upper incisors, palpebral opening.

2.5.2. Health of dam

From post partum day 2, the dam was weighed and food and water intake measured daily until weaning.

2.5.3. Care and suckling

On the afternoon (1600–1700 h) of post partum days 2 and 12, the dam and pups were moved (in the fish tank) to a quiet behavioral room where they were video taped under infra red light until 0900 h the following day. The taped behavior was reduced to three 30 min time periods beginning at: 2 h after dark, 6 h after dark, and 2 h prior to lights on, respectively. The 30 min of behavior in each of these three time frames was scored for the a range of parameters, including nursing bouts and duration, pup grooming and nest building, as described previously [21].

2.5.4. Pup retrieval

On post partum days 5 and 15, between 0900 and 1100 h, the dam and pups were moved (in the fish tank) to a quiet behavioral room and allowed to acclimate for 30–60 min.

The dam was then briefly removed from the tank and the pups randomly displaced (using gloves to handle them) throughout the two-thirds of the tank furthest from the nest. [3] The dam was then returned to the nest in the tank and the latencies to retrieve first, fourth and all pups to the nest was recorded and responses to each pup retrieved as previously described [3]

All animal experiments were approved by Howard Florey Institute animal ethics committee.

2.6. Enzymatic activity assay – [33]

IRAP enzymatic activity in plasma samples was determined by the hydrolysis of the synthetic substrate Leucine-7-amido-4-methylcoumarin (Leu-MCA) (Sigma–Aldrich, Missouri, USA) monitored by the release of a fluorogenic product, MCA, at excitation and emission wavelengths of 380 and 440 nm, respectively. Assays were performed in 96-well plates; each well containing 10 μL or 50 μL of plasma with 25 μM substrate in a final volume of 100 μL 50 mM Tris-HCl buffer (pH 7.4). Specific IRAP activity was determined by incubating plasma samples $\pm 10^{-5}$ HFI-419 [2].