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How do eggs get fat? Insights into ovarian fatty acid accumulation in the shortfinned eel, *Anguilla australis*

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ABSTRACT

Previous research using eels has shown that 11-ketotestosterone can induce ovarian triacylglyceride accumulation both *in vivo* and *in vitro*. Further, accumulation is dramatically enhanced in the presence of very-low density lipoprotein. This study examined the involvement of the low density lipoprotein receptor and vitellogenin receptor in oocyte lipid accumulation. Specific antisera were used in an attempt to block the vitellogenin receptor and/or the low density lipoprotein receptor. Accordingly, incubation with the low density lipoprotein receptor antiserum clearly reduced the oocyte diameter and the amount of oil present within the oocyte. In contrast, blocking the vitellogenin receptor had little effect on either oocyte surface area or the abundance of oil droplets in the cytosol. In keeping with birds, we conclude that the low density lipoprotein receptor is a major player involved in mediating ovarian fatty acid accumulation in the eel. However, lipoprotein lipase-mediated fatty acid accumulation also remains conceivable, for example through interactions between this enzyme and the low density lipoprotein receptor.

1. Introduction

Teleost eggs contain very high levels of fatty acids stored in the ooplasm in esterified form as neutral or polar lipids. These fatty acids are thought to be sourced from very-low density lipoprotein (VLDL) and low density lipoprotein (LDL) (Patino and Sullivan, 2002; Wiegand, 1996), which are both lipid-rich particles. In fish, the mechanisms of lipid accumulation are not well understood, a conundrum recognized nearly 20 years ago by Wiegand (1996) that has seen limited progress since then – hence mammalian paradigms are often employed. The most-supported lipid accumulation pathways in mammals centre around activation of receptors from the low density lipoprotein receptor (LDLr) family or around the enzymatic action of lipases, especially lipoprotein lipase (LPL). In mammals, lipids can be taken up via two main receptors; thus, VLDLr (a classical LR-8 receptor containing eight LDLr class-A ligand-binding repeats) and LDLr (a classical LR-7 receptor containing seven LDLr class-A ligand-binding repeats) bind and endocytose VLDL and LDL (Mulder et al., 1993; Truong et al., 1999) and acquired lipids are then broken down for use within the target tissue (Brown and Goldstein, 1976, 1979; Goldstein et al., 1975), i.e., for storage or for β-oxidation. When applying mammalian paradigms to fish oocytes, the lipoprotein particles would be stripped of fatty acids during post-endocytotic processing and free fatty acids would subsequently be sequestered into lipid stores (Wiegand, 1996). Pathways involving LPL are more complicated; LPL that is bound to either the endothelial cells of the capillaries or to the ovarian wall would hydrolyze triacylglycerides from VLDL and LDL into free fatty acids (Braun and Severson, 1992; Cryer, 1981; Wang et al., 1992), which would then be taken up by the oocyte. Alternatively, LPL would form a complex with the lipoprotein and its receptor (Saxena et al., 1989) and facilitate accumulation via a non-catalytic bridging function (Argraves et al., 1995; Takahashi et al., 1995). Lastly, LPL would mediate structural changes to the apolipoproteins on the surface of the lipoprotein particles leading to increased uptake via endocytosis by the lipoprotein receptors (Takahashi et al., 1995).

In oviparous vertebrates, the presence of an additional very-high density lipoprotein, vitellogenin (vtg), adds further complications. Two receptors with high homology to mammalian LR-8 and LR-7 have been isolated in the chicken (George et al., 1987); in this species, both receptors bind more than one ligand, the former binding VLDL and vtg (Barber et al., 1991; Bujo et al., 1994; George et al., 1987; Stifani et al., 1990) and the latter binding VLDL and LDL (Hummel et al., 2003). Classical LR-7 and LR-8 receptors

Abbreviations: 11-KT, 11-ketotestosterone; apoB, apolipoprotein B; BSA, bovine serum albumin; LPL, lipoprotein lipase; LDL, low density lipoprotein; LDLr, low density lipoprotein receptor; PBS, phosphate-buffered saline; PFA, paraformaldehyde; VLDL, very-low density lipoprotein; VLDLr, very-low density lipoprotein receptor; vtg, vitellogenin; vtgr, vitellogenin receptor.

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with high homology to their mammalian counterparts have also been isolated in fish. A membrane protein (putative LR-7) has the ability to bind both VLDL and LDL and has been designated as the teleostean LDLr (Tyler and Lubberink, 1996). However, the LR-8 has strong vtg binding properties and has been designated as the vtgr in teleosts (Davail et al., 1998; Hiramatsu et al., 2003; Prat et al., 1998).

In eel (*Anguilla* spp.), ovarian culture has been a tremendously helpful tool to study the regulation of lipid accumulation in developing oocytes *in vitro*; indeed, this approach has revealed that VLDL is the major lipoprotein class that enables lipids to accumulate as oil droplets in previtellogenic eel oocytes (Lokman et al., 2007; Endo et al., 2011). Moreover, we have identified a key role for the androgen 11-ketotestosterone (11-KT) in stimulating VLDL-supported lipid accumulation, at least in part by increasing LPL gene expression in the eel ovary (Divers et al., 2010). Using this proven *in vitro* culture system, we therefore set out to investigate the involvement of LR-7 and LR-8 in ovarian esterified/free fatty acid accumulation and downstream oil droplet formation; accordingly, this study aimed to firstly evaluate the involvement of the teleost LR-7 (LDLr) and LR-8 (vtgr) in the accumulation of lipids by the oocytes of the eel, *Anguilla australis*, and to then provide insights into the predominant pathway that may be employed.

2. Methods

2.1. Experiment I

In a pilot trial, two previtellogenic eels (body weights of 800 g and 671 g) were captured in fyke nets in Lake Ellesmere (South Island, New Zealand) and transported to our facilities at the University of Otago (Dunedin) where they were held in a recirculating tank (200 L) at ambient autumn photoperiod and temperature. Eels were euthanised (0.03% benzocaine) and drained of blood by tail transection before the body was submerged in 70% ethanol for sterilisation. Ovarian fragments were used for Western blotting (Section 2.3), organ culture (Section 2.4) and histological examination (Section 2.5).

2.2. Experiment II

Experiment I was repeated with an additional four previtellogenic eels (body weight = 755 ± 18 g); all parameters were kept identical to those in Experiment I.

2.3. Antisera validation

As previously reported, Mizuta et al. (2011, 2013) generated antisera against recombinant proteins that encompassed the ligand binding repeats of the vtgr or LDLr of cutthroat trout (*Oncorhynchus clarkii*). The specificity of the resulting antisera and their suitability for use on eel was examined by Western blotting. To this end, ovarian tissue from a female shortfinned eel in the oil droplet stage was homogenised in 1× proteinase inhibitor and phosphate buffered saline (PBS) as described by Damsteegt et al. (2014). Sodium-dodecyl sulphate polyacrylamide gel electrophoresis using 50 μg of ovarian homogenate was followed by Western blots using the vtgr and LDLr antisera (1:1000 dilutions) exactly as described in Damsteegt et al. (2014).

2.4. Organ culture

Ovarian culture was conducted based on the method developed for eel testicular organ culture (Miura et al., 1991). In brief, the basal culture medium consisted of Leibovitz L-15 medium (L15) (Sigma) supplemented with 0.5% BSA (MP Biomedicals, Auckland, New Zealand), 10 mM HEPES, 0.1 mM L-glutamic acid sodium salt, 0.1 mM L-aspartic acid, 1.7 mM L-proline and 1 μg/ml bovine insulin (Sigma) adjusted to pH 7.4. Agarose supports were made by suspending agarose in MQW (1% w/v) before autoclaving. After cooling to ~60 °C, 300 μl of agarose was pipetted into the wells of a 96-well plate (Laboindustria SPA, Arzergrande, Italy). Once set, agarose gels were removed and placed into 24-well tissue culture plates (Corning Incorporated, New York, USA) and a small piece of nitrocellulose membrane was placed on top (Miura et al., 2011; Ozaki et al., 2006). Fifteen hundred microliters of un-supplemented Leibovitz L-15 medium was added to each well and incubated at 4 °C overnight to allow the agarose gels and nitrocellulose membranes to absorb the medium.

Freshly removed ovaries were washed in eel ringer containing antibiotics (streptomycin: 100 mg/L; penicillin: 100,000 U/L) then cut into fragments approximately 2–3 mm and submerged in un-supplemented Leibovitz L-15 medium. One fragment was transferred into 4% paraformaldehyde (PFA) for later histological analysis (Section 2.5). The remaining ovarian fragments were placed onto individual squares of nitrocellulose membrane. The un-supplemented L15 was replaced with 1500 μl of incubation medium containing one of twelve treatments as described in Table 1. VLDL was isolated from serum as described by Damsteegt et al. (2014) and 1 mg/ml was added to the appropriate wells. 11-KT was obtained from Steraloids (Newport, USA) and used at a concentration of 30 ng/ml (100 nM). Wells without VLDL or antiserum (added at 1:1,000 dilution) were supplemented with an equal volume (*ca.* 5% of final incubation volume) of PBS (20 mM $NaH_2PO_4{\cdot}2H_2O$, 80 mM $Na_2HPO_4{\cdot}12H_2O$, 1.5 M NaCl, pH 7.6) and wells without 11-KT with an equal volume of ethanol (0.03% of final incubation volume). Tissues were cultured for 21 days at 18 °C and in 100% humidified air. Culture medium and treatments were renewed every seven days during the incubation period. At the end of the incubation period all fragments were fixed overnight in 4% PFA for histological analysis (Section 2.5).

2.5. Histology

Fixed tissues were embedded in Technovit 7100 resin (Heraeus Kulzer GmbH and Co., Hanau, Germany) following the manufacturers' instructions. Using glass knives, sections were cut on a Reichert Jung microtome at 2 μm and stained with Polychrome I (1.3% methylene blue and 0.2% azure II in distilled water), then counterstained with Polychrome II (2% basic fuchsin in distilled water). Microscope images were captured using an Olympus BX51 microscope coupled to an Olympus SC100 camera and analysed using

Table 1
Treatments applied *in vitro* to ovarian tissue from eel contained different combinations of very-low density lipoprotein (VLDL), 11-ketotestosterone (11-KT), vitellogenin receptor antiserum (α-vtgr) or low density lipoprotein receptor antiserum (α-LDLr).

Treatment	VLDL (1 μg/ml)	11-KT (30 ng/ml)	α-vtgr (1:1000)	α-LDLr (1:1000)
1				
2	+			
3	+		+	
4	+	+	+	
5	+			+
6	+	+		+
7	+	+	+	+
8	+		+	+
9	+	+		
10		+		
11			+	
12				+