DISSERTATION

LONG CHAIN POLYUNSATURATED FATTY ACIDS AND OXYTOCIN SIGNALING IN PREGNANT HUMAN MYOMETRIAL SMOOTH MUSCLE CELLS

Submitted by

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In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PAUL Y KIM ENTITLED LONG CHAIN POLYUNSATURATED FATTY ACIDS AND OXYTOCIN SIGNALING IN PREGNANT HUMAN MYOMETRIAL SMOOTH MUSCLE CELLS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF THE DISSERTATION

LONG CHAIN POLYUNSATURATED FATTY ACIDS AND OXYTOCIN SIGNALING IN PREGNANT HUMAN MYOMETRIAL SMOOTH MUSCLE CELLS

Since the suggestion by Olsen and colleagues that the pattern of exceptionally high birth weight in the Faroe Islands is related to a high intake of seafood, a number of epidemiological studies and interventional clinical trials have explored the possibility that consumption of long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) abundant in fish and fish oil, such as 20:5 eicosapentaenoic acid (EPA) and 22:6 docosahexaenoic acid (DHA), may lengthen gestational duration. A dose response relation between dietary LC n-3 PUFA and gestational duration has emerged wherein expectant women who consume small amounts of fish stand to benefit the most from LC n-3 PUFA supplementation. The mechanisms are not well understood. Although prostaglandins (PG) of the 2-series are known to play a role in the initiation and progress of labor, an interpretation based on modulation of PG biosynthesis appears unlikely since idiopathic preterm labor is accompanied by low PG concentrations. Consequently, we investigated one potential PG-independent mechanism of LC n-3 PUFA action using a pregnant human myometrial smooth muscle cell line, PHM1-41, as a model.

Our primary goal was to characterize the effect of DHA treatment on the signaling pathway of oxytocin, a potent uterotonic hormone involved in labor. The addition of 10 μM to 100 μM DHA to the culture media for 48 hrs resulted in

incorporation and dose dependent enrichment of DHA in membrane lipid. DHA significantly inhibited IP_3 elaboration (at 30 μM and 100 μM) and $[Ca^{2+}]_i$ mobilization (at 100 μM) in response to oxytocin stimulation compared with bovine serum albumin (BSA) control and equimolar 18:1 oleic acid (OA). DHA at 30 μM and 100 μM significantly reduced receptor density in the membrane (B_{max}) without altering the binding affinity (K_d) or rate of receptor internalization. These findings may be relevant to the reports of dietary fish and fish oil consumption prolonging gestation.

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Literature Review

1.1 Preterm Birth

Because preterm birth (defined as less than 37 completed weeks of gestation) is the leading cause of neonatal morbidity and mortality, its treatment and prevention pose a significant public health concern (1-3). Common health complications of immaturity at birth include respiratory distress, necrotizing enterocolitis, and sepsis. Intraventricular hemorrhage occurs in up to 40% of infants born before 35 weeks of gestation (4). Long-term morbidity includes cerebral palsy, impaired vision and hearing, neurocognitive deficits, and possible cardiovascular disease in adulthood (2, 3, 5-8). A recent Swedish study has even shown a progressive increase in psychiatric hospital admissions with an increasing degree of preterm birth for adolescents and young adults (9). Besides these grave health consequences, babies born prematurely can take an emotional and financial toll on families and also represent a significant economic burden to society. Preterm birth-related costs in the U.S. for 2005 have been estimated at a minimum of $26.2 billion dollars (10).

The National Center for Health Statistics report for 2006 indicates that 12.8% of all U.S. births were premature (10). Social and economic factors contribute to a racial disparity in the rate of preterm birth, as it is highest for non-Hispanic African American women (18.5 % in 2006), compared to the rate among white women of 11.7% in 2006. The overall rate of preterm birth in the U.S. continues to rise unabated and has climbed

more than 36% since 1981. A remarkable surge in multiple births (71% increase since 1981), attributable perhaps to increased use of assisted reproductive technologies, may account for part of that increase but Davidoff et al (1) have recently reported changes in gestational duration among singleton births. They have found that the duration of gestation has shifted over the 1992 to 2002 time frame, with birth at 39 weeks becoming the most common length of gestation in 2002 compared to 40 weeks in 1992. Late preterm births, that is, delivery occurring between 34 full weeks gestation and 36 weeks and 6 days gestation (11), have risen sharply over the 1992 to 2002 period (1) and comprised 75% of all singleton preterm births in 2006 (10). Although these late preterm infants tend to be healthier than very preterm infants, their health problems are not limited to transient issues such as hypoglycemia or hypothermia. In 2002, late preterm infants were 3 times more likely than term infants to die before their first birthday (7.9 versus 2.4 deaths per 1000 live births) and 6 times more likely to die within the first week of life (2.8 versus 0.5 deaths per 1000 live births) (12). These fold differences in mortality have remained more or less constant since 1995 and are well known. However, there is growing evidence indicating that late preterm infants are also at greater risk of long-term morbidity (13) such as cerebral palsy (14), speech impairment (15), and educational and behavioral problems (16, 17) as a result of incomplete neurodevelopment.

1.2 Fatty Acids and Gestation

Evidence from observational and clinical studies indicate that essential fatty acids of the omega 3 (n-3) and n-6 series may potentially modify gestational duration (18). An

early epidemiological study in Denmark and the Faroe Islands by Olsen et al linked high levels of long-chain n-3 polyunsaturated fatty acid (LC n-3 PUFA) intakes in Faroese mothers, owing to a diet rich in marine fat, with high average birthweights (19). The higher birthweight (194 g) observed in Faroese mothers compared to their Danish peers was partly attributable to a 4-day increase in gestational duration. In a similar study, erythrocyte fatty acid profiles were obtained from randomly selected groups of Faroese and Danish mothers within 2 days of delivery (20). The longer gestational duration (2.0 days) and higher birthweight (140 g) observed in the Faroese group were not significantly different from the Danish group, but a 20% increase in the ratio of maternal erythrocyte LC n-3 PUFAs to 20:4 n-6 arachidonic acid (AA) was associated with a significant increase in gestational duration of 5.7 days in the Danish women. The suggestion is that once LC n-3 PUFA levels exceed a certain threshold, further increases may not have an effect on pregnancy outcomes (discussed below and 25). A randomized, controlled fish-oil supplementation trial from the 30th week of pregnancy until delivery, showed that gestation was significantly prolonged by approximately 4 days when pregnant women consumed a fish oil supplement (Pikasol) providing a total of 920 mg 22:6 n-3 docosahexaenoic acid (DHA) and 1.3 g 20:5 n-3 eicosapentaenoic acid (EPA) per day (21).

A cross-sectional case-control study in 22 women with pre-eclampsia (pregnancy-induced hypertension with proteinuria, which carries risk of premature delivery) found that women with the lowest levels of erythrocyte n-3 fatty acids were 7.6 times more likely to have pre-eclampsia than women with the highest levels of n-3 fatty acids (22). A 15% increase in the n-3:n-6 fatty acid ratio was associated with a 46% reduction in risk

for pre-eclampsia. On the other hand, in a randomized, prospective, double-blind trial, providing 3 g per day supplementary EPA commencing at 12 to 14 weeks of gestation to pregnant women with a history of intrauterine growth retardation did not prevent the recurrence of pregnancy-induced hypertension or intrauterine growth retardation (23).

A randomized clinical trial focusing on fish oil administration in high-risk pregnancies and comprising both prophylactic and therapeutic trials in pregnant women has been reported by Olsen's group in Denmark (24). The four prophylactic trials enrolled women with uncomplicated pregnancies who were deemed to have higher than average risk because they had experienced preterm delivery (< 37 weeks of gestation), intrauterine growth retardation (< 5^{th} percentile), pregnancy-induced hypertension (diastolic blood pressure > 100 mm Hg) in an earlier pregnancy, or were currently carrying twins. The two therapeutic trials enrolled women with an existing pregnancy complication, either signs or symptoms of preeclampsia (with or without intrauterine growth retardation) or suspected intrauterine growth retardation (< 10^{th} percentile by ultrasonography). These trials were mutually exclusive: the previous preterm delivery subset did not contain any women who had either previous intrauterine growth retardation or previous pregnancy-induced hypertension and the previous intrauterine growth retardation subset did not contain any women who had previous pregnancy-induced hypertension. The remaining three subsets were defined independently of any previous pregnancy history.

The women were randomly assigned to receive either a fish oil supplement (Pikasol) or olive oil commencing at 20 weeks of gestation for the prophylactic trials and 33 weeks of gestation for the therapeutic trials. Fish oil, at the prophylactic dose (total of 920 mg DHA and 1.3 g EPA per day), significantly reduced the recurrence risk of preterm delivery from

33% to 21%, a reduction of approximately 36%. This was accompanied by significant increases in gestational duration of 8.5 days and birthweight of 209 g. The recurrence risks of intrauterine growth retardation or pregnancy-induced hypertension were similar between the fish oil and olive oil groups however. In the twin pregnancy group, the prophylactic fish oil dose had no effect on the occurrence of preterm delivery, intrauterine growth retardation, or pregnancy-induced hypertension. The therapeutic fish oil dose (total of 2.1 g DHA and 2.9 g EPA per day) had no effect on the mean duration of pregnancy in the pre-eclampsia group, or on the mean birthweight adjusted for gestational age in the suspected intrauterine growth retardation group.

This study (24) has since been reinterpreted in relation to habitual fish intake (25). This reinterpretation determined that prophylactic fish oil supplementation significantly reduced the hazard rate of spontaneous preterm delivery by 44% and 39%, respectively, in low and middle fish consumers but there was no effect in high fish consumers. Thus, the earlier observations by Olsen's group of an effect of fish oil supplementation on singleton pregnancies (21) and in high-risk pregnancies (24) depend on the baseline fish intake of the pregnant women. A prospective cohort study in Denmark has shown that low consumption of fish is a strong risk factor for preterm delivery and low birthweight (26). Occurrence of preterm delivery and low birthweight were found to decrease significantly and progressively with increasing exposure to dietary LC n-3 PUFA, with adjusted odds ratios of 3.60 for preterm delivery and 3.57 for low birthweight in the lowest exposure group relative to the highest exposure group. The essential fatty acid linolenic acid (LnA) can be converted to DHA through microsomal desaturation and elongation. This conversion, however, is very limited in humans, unaffected by the dietary linoleic acid (LA) to LnA ratio, and

increasing dietary LnA intake mostly increases phospholipid EPA content (27, 28). Therefore, the most effective way to increase DHA in plasma membrane phospholipids is to consume preformed DHA from sources such as fish and fish oils (29).

Most of the studies on fish oil supplementation and pregnancy duration have employed large doses of fish oil – gram quantities on a daily basis. However, a randomized, double-blind, placebo-controlled clinical trial using DHA enriched eggs, providing an additional 100 mg DHA per day in the last trimester of pregnancy, reported a significant 6 day increase in gestational duration (30). Infant birthweight and length, and head circumference were not significantly different. Participants in this study were 73% African Americans, a group known to be at heightened risk for shortened gestation (10) and in this group, modest amounts of supplementary DHA (approximately 100 mg per day) were effective in prolonging gestation.

A prospective study (31) evaluated the fatty acid status of women who delivered prematurely. In this study, 37 preterm (gestational age < 37 weeks) and 34 control mother-baby dyads (gestational age ≥ 37 weeks) were examined. Gestational age at delivery and birthweight were significantly lower by 16% and 38%, respectively, in the preterm group compared to the control term deliveries. Fatty acid analyses of samples from women who delivered at ≥ 37 weeks were conducted both at 34 weeks of gestation and at delivery in order to control for gestational duration effects on maternal pools. Percent of total AA in the maternal erythrocyte and plasma were significantly higher in preterm than in controls sampled at comparable gestational age (34 weeks) and at delivery. Erythrocyte AA in preterm samples were increased a remarkable 278% compared to controls at delivery and 149% compared to controls at 34 weeks. Plasma

total lipids AA were increased 42% and 18% respectively, in preterm births compared to controls at delivery and at 34 weeks. The AA enrichment in both maternal erythrocytes and plasma total lipids is quite striking, and suggests that the diet consumed by these women is high in n-6 fatty acids and low in n-3 fatty acids. The higher LA in preterm erythrocytes, 190% and 47% increase, respectively, compared to controls at delivery and at 34 weeks, supports this interpretation. Differences in maternal pools of LC n-3 PUFA were also observed in preterm versus controls. Percent of total DHA in plasma phospholipids (32%) and plasma total lipids (29%) as well as EPA in erythrocytes (129%) were significantly higher in the term deliveries. Al et al have suggested that pregnancy imposes increased demands on maternal DHA supply because of increased provision to the fetus (32). Thus, low maternal LC n-3 PUFA status at the onset of pregnancy is probably magnified by the demands of the fetus.

Not all n-3 intake studies in human pregnancy have reported an effect on gestational duration. A case-control nested cohort study in Danish women of dietary intake of marine n-3 fatty acids, estimated by food frequency questionnaire 6 months to 3 1/2 years after delivery, showed no relationship with pregnancy duration (33). A combined self-administered questionnaire and interview in the 30th week of gestation to assess n-3 intake in Danish women also found no association with gestational length (34). However, both of these studies estimated n-3 intake by retrospective questionnaires and did not employ controlled experimental designs. Since the questionnaire and interview in the 30th week of gestation focused on marine foods and fish oil, some of the women may have increased their consumption of marine foods in the third trimester under the impression that it would be beneficial.

Some n-3 or fish oil supplementation trials have also failed to demonstrate an effect on gestational duration (35-38). A double-blinded, randomized study in pregnant women providing supplements of either 10 mL per day cod liver oil (providing 803 mg EPA and 1183 mg DHA) or 10 mL per day corn oil (providing 4747 mg LA) from weeks 17 to 19 of gestation to delivery showed no differences in gestational duration or birthweight (35). The women in this Norwegian study habitually consumed 550 mg per day of EPA and DHA in contrast to an average consumption of just 121 mg per day for women in the United States. This high baseline intake of n-3 PUFAs may have reached saturation level in the dose response relation between dietary fish oil and gestational duration reported by Olsen et al (21). A randomized, placebo-controlled, double-blinded study in Australia provided 4 g fish oil (56% DHA and 28% EPA) daily from 20 weeks gestation until delivery (36). No differences were detected in pregnancy outcomes such as gestational duration, infant birthweight, length, head circumference, and 5 minute Apgar score. A large Danish study examined a range of supplementary EPA plus DHA doses (0.1, 0.3, 0.7, 1.4, and 2.8 g per day) and 2.2 g per day LnA, provided to pregnant women from weeks 17 to 22 of gestation until delivery (37). Again, there were no differences in gestational duration. However, this study measured maternal erythrocyte DHA, EPA, and AA in only a small subset (< 5% in each group) and non-compliance (failure to take the supplementary capsules until delivery) was 40%. A recent European randomized multicenter trial examined fish oil and folate supplements, providing 500 mg DHA and 150 mg EPA, 400 μg methylenetetrahydrofolic acid, or both, supplements per day to pregnant women from 22 weeks of gestation until delivery (38). While the fish oil supplements improved fetal LC n-3 PUFA status and attenuated depletion of maternal

stores, there were no effects on gestational duration, pregnancy outcomes, or fetal development.

Four recent reviews and meta-analyses of marine oil (n-3) effects on pregnancy outcomes have been published (39-42). Three of these reviews were limited to randomized, controlled trials (39, 40, 42) and the other included observational studies (41). Two of these reviews concluded that LC n-3 PUFA supplementation lengthened gestational duration, but that the mean effect size was small (40, 41). It is important to note that these meta-analyses may have decreased sensitivity for detecting an effect because they do not take into account the variable baseline LC n-3 PUFA status among the study populations. The meta-analysis by Horvath et al determined that supplementation with LC n-3 PUFAs reduced the relative risk of early preterm delivery (< 34 weeks of gestation) in high-risk pregnancies to 0.39 but did not detect other changes in pregnancy outcomes (42). The Cochrane Database Review (39) concluded that there was insufficient evidence to support the routine use of marine oil and individual n-3 fatty acids such as DHA and EPA during pregnancy to reduce the risk of preterm birth, pre-eclampsia, or low birthweight. However, the 6 trials reviewed showed that mean gestational duration was increased significantly by 2.6 days with LC n-3 PUFA supplementation. As the authors of one meta-analysis note, an effect of this magnitude may not be clinically important to most infants but such a shift in the distribution of a population may decrease the proportion of babies born prematurely (39).

Inflammation appears to be a principal characteristic of human labor. Investigators have demonstrated leukocyte infiltration into the myometrium with onset of labor (43-45) and data from suppressive subtraction hybridization (46) and gene arrays

(47-51) indicate that labor induces the expression of genes primarily involved in the acute inflammatory response. Cytokine concentrations are elevated in both term and preterm labor with approximately 3-6 fold increases in IL-1β, IL-6, and IL-8 in amniotic and chorionic-decidual tissues (52). As potent lipid mediators of the inflammatory pathway derived from essential fatty acids, prostaglandins represent an attractive and often invoked mechanism (21, 25, 30) for the effect of LC n-3 PUFA on gestational duration.

1.3 Prostaglandins and Parturition

Prostaglandins (PG) belong to a family of 20-carbon signaling molecules (eicosanoids) derived primarily from AA. Nearly all nucleated cells generate PGs and the biosynthetic pathway is well characterized. In the first step of PG production, AA is liberated from cellular membrane phospholipids by the action of phospholipase (PL) A_2, PLC, and PLD. The second, rate-limiting step consists of the oxygenation and reduction of AA by prostaglandin H synthase (PGHS) also known as cyclooxygenase (COX) to form an intermediate endoperoxide, PGH_2. The final step of PG synthesis is the conversion of PGH_2 to one of the biologically active prostaglandins by terminal PG synthases.

Although membrane phospholipid AA serves as the principal substrate for PGHS, giving rise to the 2-series PGs, PGHS are also capable of processing EPA, leading to formation of the anti-inflammatory 3-series PGs and reducing production of the 2-series. Suppression of 2-series PG synthesis by LC n-3 PUFAs may involve downregulation of PGHS expression (53, 54), displacement of AA from membrane phospholipids (55, 56), or in the case of EPA, competition with AA for PGHS activity. DHA is not a substrate for PGHS but it has been shown to competitively inhibit PGHS in vitro (57, 58) and data from

in vivo studies indicate that DHA alone can inhibit 2-series PG production. In neonatal pigs, a diet containing 0.7 g DHA per 100 g of total fatty acids significantly increased lung phospholipid DHA and tended to suppress the production of lung 6-keto-$PGF_{1\alpha}$ (a stable metabolite of prostacyclin) and TXB_2 (a stable metabolite of thromboxane A2) (59). A similar study in mice has demonstrated that supplementing with 3 g DHA per 100 g of diet more than doubles phospholipid DHA content and significantly decreases basal small intestine PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations by nearly 50% (60). In pregnant rats, DHA in the diet at 0.7 energy % from conception to day 20 of gestation significantly increased uterus and placenta DHA content at the expense of AA and significantly reduced uterus and placenta PGE_2 and $PGF_{2\alpha}$ production rates (61). These effects may be based in part on retroconversion of DHA to EPA, estimated to occur at a rate of 9.4% in one human study (62). In the neonatal pig (59) and pregnant rat studies (61), DHA supplementation was accompanied by a negligible increase in EPA however. Furthermore, Ikeda et al have reported that dietary DHA, but not EPA decreases aortic prostacyclin production, therefore DHA alone may be sufficient to modulate PG synthesis (63).

Numerous studies suggest that PGs play a key role in each of the five major physiological events of labor: membrane rupture, cervical ripening, myometrial contractility, placental separation, and uterine involution (64). PGs may contribute to the rupture of fetal membranes by inducing matrix metalloproteinase (MMP) expression. Studies in various systems such as rat mesangial cells (65), cultured human pancreatic cancer cells (66), fetal rat hepatocytes (67), and murine macrophages (68) have established that PGE_2 promotes MMP expression and the same has been shown for $PGF_{2\alpha}$ in cultured human ciliary smooth muscle cells (69, 70). Fetal bovine serum-stimulated PGE_2 production has

been shown to increase latent MMP-9 expression in fetal membrane tissue culture (71). Similarly, exogenous $PGF_{2\alpha}$ stimulation causes a significant increase in MMP-2 and MMP-9 production in tissue cultures of decidua, but not amnion or chorion, and this is accompanied by a large decrease in tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) (72). Both of these in vitro studies determined that inhibition of COX using indomethacin suppresses the PG-mediated increase in MMP expression by 35-80% depending on the PG, MMP, and fetal tissue type.

Zinc-dependent endopeptidases of the MMP family degrade extracellular matrix components and thereby remodel the reproductive tissues as parturition approaches. MMP-1, MMP-2, and MMP-9 break down collagen types I-V (73), which predominate in the extracellular matrix of fetal membranes (74). MMP-9 in particular has been implicated in the premature rupture of membranes (75), defined as membrane rupture before the onset of labor. An increase in the ratio of MMP-9 to TIMP-1 correlates with decreasing tensile strength of fetal membranes (76). Amniotic fluid obtained from women at term with normal labor exhibit greater MMP-9 activity (~2.3-fold higher) than nonlaboring women, but samples from women with premature rupture of membranes show a combination of conspicuously increased MMP-9 activity (~5.3-fold higher) and significantly decreased concentrations of TIMP-1 (77). The women with premature rupture of membranes in this study (77) experienced membrane rupture at gestational ages between 28 and 36 weeks. Given the swift progression from membrane rupture to delivery (78), it is likely that most, if not all, of these women delivered prematurely. These observations are consistent with reports by Athayde et al that the amniotic fluid MMP-9 concentration is elevated 2-fold in women with *preterm* premature rupture of membranes (rupture prior to 37 weeks of

gestation) compared to women with premature rupture of membranes at term (79). These data suggest that a shift in the equilibrium of matrix metalloproteinases and their inhibitors may facilitate premature rupture of the membranes leading to preterm delivery.

Cervical ripening is a phenomenon akin to membrane rupture in that it is also an MMP-dependent process. MMPs carry out extensive remodeling of the cervical extracellular matrix to turn a firm and rigid organ soft and compliant in preparation for delivery. In addition to promoting collagen degradation via MMPs, PGE_2 and its n-6 fatty acid precursors LA and AA, have been found to impair collagen synthesis (80, 81). Application of exogenous PGE_2 into the cervical canal has been used clinically to induce ripening for decades (82, 83). Since Liggins first likened cervical ripening to an inflammatory process in 1981 (84), considerable literature has accumulated in support of this concept. The influx of inflammatory cells such as neutrophils and monocytes into the cervical stroma has been well documented (43-45). Cervical ripening appears to be associated with the up-regulation of inducible nitric oxide synthase, a common feature of inflammation (85). IL-8 levels in the cervix have been shown to increase 6-fold at term and 11-fold just before the onset of labor (86). These proinflammatory factors function in concert with each other. IL-8 recruits and activates neutrophils (87), which themselves are a rich source of proinflammatory cytokines and eicosanoids. PGE_2 is able to induce IL-8 expression through the cAMP signaling pathway and can act synergistically with IL-8 to enhance IL-8-mediated chemotaxis of neutrophils (88, 89). PGE_2 may also play an indirect role in cervical ripening through its vasoactive properties. Dilation and increased permeability of blood vessels would facilitate the infiltration of leukocytes into the cervix (90).

There is some evidence to suggest that PGs are involved in the postpartum period. $PGF_{2\alpha}$ levels in the maternal circulation increase during labor but do not peak until after delivery, reaching their maximum at (91, 92) or within minutes after (93) placental separation. Data from animal studies show a correlation between uterine involution and plasma concentrations of $PGF_{2\alpha}$ in cows (94) and ewes (95), but not in mares (96). Treatment with $PGF_{2\alpha}$ enhances uterine involution in cows (97-99) and buffalos (100), but again, not in mares (101).

A large body of literature exists on the role of PGs in myometrial contractility. Bygdeman and Eliasson were the first to study the effect of PGs on the human uterus using isolated myometrial strips (102, 103). Bygdeman and colleagues were able to demonstrate in subsequent in vivo work that intravenous infusion of PGE_1 stimulates uterine contractions (104). Further in vivo research by Karim et al found that intravenous, subcutaneous, intramuscular, and oral administration of PGE_2 or $PGF_{2\alpha}$ excites pregnant human myometrium (105, 106). Conversely, inhibition of COX with indomethacin has been shown to diminish uterine contractions and delay labor (107, 108). Since these actions of PGs may be pharmacologic rather than physiologic, other studies have focused on the levels of PGs (and their precursor AA) in the amniotic fluid during pregnancy and found that these are elevated in labor (109-115). There is evidence for a causal relationship between PGs and labor i.e. that increased bioavailability of PGE_2 and $PGF_{2\alpha}$ precedes labor rather than being a consequence of labor (116). Together these data support a central role of PGs in initiating and maintaining normal labor.

The role of PGs in preterm labor is less well defined. The relationship between intrauterine infection, accompanied by large increases in PGs, and preterm labor has long

been recognized. Knox and Hoerner were among the first to investigate the etiologic role of infection in the premature rupture of membranes (117). Since that classic paper in 1950, a consistent picture of how infections cause preterm labor has developed (118). Endotoxins and exotoxins secreted by the infective organisms stimulate the maternal decidua and fetal membrane tissues to produce a number of proinflammatory cytokines (119-122). Other processes at work may include an increase in corticotropin-releasing hormone secretion by the fetus and a decrease in prostaglandin dehydrogenase activity in the chorion (123). These pathways all contribute to increased PG production and decreased PG metabolism as a result of intrauterine infection, culminating in PG-mediated preterm labor (124-126).

In the absence of infections however, reproductive tissue PG production is lower in women delivering preterm compared with women delivering at term (127-129). For instance, Reece et al reported significantly lower PGE_2 and $PGF_{2\alpha}$ concentrations in amnion and placenta samples from women with preterm compared to term births (128). Sadovsky et al found that the levels of PGE_2 in the amnion, chorion, decidua, and myometrium from women in labor were two-fold to five-fold higher than from nonlaboring women (129). However, both amniotic COX-2 expression and PGE_2 levels in these tissues were much lower in preterm than in term births, consistent with earlier observations (127, 128). Differences in the mobilization of reproductive tissue AA stores between preterm and term labor could account for differences in PG concentrations but fetal membrane phospholipids contain high concentrations of esterified AA (130) making an interpretation based on depleted AA pools unlikely (31). Studies indicate that AA bioavailability is not rate limiting for PG synthesis in term placenta (131, 132) but this has not been thoroughly examined in preterm reproductive tissues.

In view of the low PG concentration and synthetic capacity in idiopathic preterm labor, it is difficult to reconcile the well known effect of LC n-3 PUFAs on reducing PG production (56-62, 133-135) as the sole mechanism of action for prolonging gestation. Olson et al have demonstrated in the mouse that preterm labor induced by various means is associated with an increase in uterine $PGF_{2\alpha}$ receptor mRNA (136-138). Interestingly, uterine $PGF_{2\alpha}$ concentrations did not increase in preterm mice relative to gestational age-matched controls, similar to the situation of preterm birth absent infection in humans. Based on these data, Olson has suggested that enhanced sensitivity to PGs, rather than a rise in PG concentrations, may be an important mechanism in mediating idiopathic preterm labor (64). Increased responsiveness to low PG concentrations in idiopathic preterm labor may reflect differential expression and function of PG receptors as proposed by Sadovsky et al (129).

As fatty acid derivatives, PGs were initially thought to exert their effects through direct interaction with the plasma membrane. However, observations that the actions of PGs were associated with the generation of second messengers such as cAMP or Ca^{2+} (139) along with studies comparing the potency of the various prostanoids and their antagonists in a range of tissues suggested that each prostanoid has its own distinct receptor (140-142). Eight G protein-coupled receptors (GPCR) have been cloned and characterized to date - one each for PGD_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, and four for PGE_2 - designated as DP, FP, IP, TP, and $EP_{1\text{-}4}$, respectively. These receptors can be classified as relaxatory (DP, IP, EP_2, EP_4) or contractile (FP, TP, EP_1, EP_3) on the basis of their action in smooth muscle.

Only one study has compared the expression of PG receptors in preterm birth with term birth in pregnant human myometrium (143). Subjects in this study comprised preterm or term pregnancies that were delivered by cesarean section in the absence or presence of labor. The expression of EP_2 in the lower myometrium declined with advancing gestational age, consistent with a role of EP_2 in maintaining uterine quiescence through coupling to G_s, resulting in cAMP-dependent relaxation. The expression of FP mRNA in the lower myometrium was higher at preterm with and without labor compared to term without labor. In the absence of labor, the high expression of contractile FP receptors at preterm may have been offset by high expression of relaxatory EP_2 receptors. In the presence of labor, the relative expression of FP to EP_2 was lower at preterm than at term, consequently providing no evidence that differential expression of PG receptors mediates enhanced PG responsiveness in preterm labor. The expression of EP_2 and FP receptors in the lower myometrium is unlikely to represent total uterine function however. The topographical pattern of receptor expression may be important, as studies in baboons have demonstrated higher expression of contractile receptors (EP_1, EP_3) and lower expression of relaxatory receptors (EP_2, EP_4) in fundal tissue compared with myometrium from the lower uterine segment, where relaxation of the uterus may facilitate delivery (144). The balance of PG receptors beyond the lower myometrium may shift inappropriately in preterm labor such that low levels of PGs are sufficient for initiating and maintaining labor. Other events influencing PG responsiveness in preterm labor may include differential coupling of PG receptors to effector proteins, which may also involve alternative splicing of the receptors.

1.4 Membrane effects of LC n-3 PUFAs

The mechanism of LC n-3 PUFA action is not limited to replacing AA as an eicosanoid substrate and inhibiting AA metabolism; LC n-3 PUFAs can act at the plasma membrane level as well. The original demonstration of an effect of LC n-3 PUFAs on a membrane-associated process, and one that has progressed from initial observation through mechanistic understanding to clinical application is the antiarrhythmic effect of fish and fish oil consumption (145-147). McLennan et al were the first to show, in rats (148, 149) and marmoset monkeys (150), that feeding of n-3 PUFA enriched diets significantly reduced coronary artery ligation-induced ventricular fibrillation. Leaf et al confirmed these findings in a dog model of sudden cardiac death using intravenous infusion of free n-3 PUFAs rather than feeding experiments (151-152). Studies in isolated neonatal rat cardiomyocytes have shown that n-3 PUFA administration to the culture medium could both prevent and terminate arrhythmias induced by Ca^{2+} (153), ouabain (154), isoproterenol (155), lysophosphatidylcholine, acylcarnitine, the Ca^{2+} ionophore A23187 (156), and thromboxane (157). The addition of delipidated bovine serum albumin caused the extraction of free fatty acids from the myocytes and the return of induced arrhythmia, indicating that the antiarrhythmic action of n-3 PUFAs does not involve incorporation into the plasma membrane phospholipids, but rather simple partition into the lipid bilayer. In animal feeding studies as well as clinical trials, the n-3 PUFAs are incorporated into membranes at the sn-2 position of phospholipids but the free n-3 fatty acids can be readily released during myocardial infarction by phospholipase A2. LC n-3 PUFAs stabilize the electrical activity of cardiomyocytes primarily by inhibiting the fast, voltage-dependent sodium current and the L-type calcium current

(158). The precise site of action in cardiomyocytes still needs to be fully elucidated, but data from Andersen and colleagues (159-162) have led to the current thinking that LC n-3 PUFAs modulate ion channels through changes in plasma membrane fluidity and elasticity in the microdomain bordering the transmembrane protein.

A recent examination of the *Shaker* K channel has considered another potential mechanism based on electrostatic interaction (163). This lipoelectric hypothesis proposes that upon insertion of the lipophilic PUFA into the lipid bilayer (or hydrophobic pocket of the channel protein), the negatively charged carboxyl group acts electrostatically on the ion channel voltage sensor. Uncharged PUFA methyl esters do not affect the voltage dependence of *Shaker* K channels suggesting that PUFAs may not act purely through membrane distortion. LC n-3 PUFAs have also been shown to exert their anti-inflammatory properties through eicosanoid-independent membrane effects. The successful application of LC n-3 PUFAs in the treatment of immune-inflammatory diseases is based largely on their ability to suppress T lymphocyte activation (164-167). This suppression in turn results from the displacement of key signaling proteins from T cell membrane lipid rafts. Lipid rafts are sphingolipid and cholesterol-rich subdomains within the plane of the plasma membrane. The term raft illustrates the concept of these tightly packed, liquid-ordered domains floating in a less densely packed, liquid-disordered sea where they are thought to function as signaling platforms (168). As such, a number of proteins involved in signal transduction are known to associate with lipid rafts, targeted there by acylation with saturated fatty acids (169). LC n-3 PUFAs have been proposed to disrupt T cell activation by displacing raft-resident signaling proteins such as Src family kinases and transmembrane linker for activation of T cells from lipid rafts

(170-172). LC n-3 PUFAs are thought to act by altering raft composition and structure, although changes in protein fatty acylation may also contribute to the displacement of proteins from lipid rafts (173). Recent data from MDA-MB-231 human breast cancer cells indicate that n-3 PUFAs inhibit tumor growth by modifying raft lipid composition and selectively decreasing epidermal growth factor receptor localization to lipid rafts (174). To confirm these in vitro findings, Chapkin et al have demonstrated in vivo that dietary LC n-3 PUFAs remodel mouse T cell lipid rafts and suppress T cell proliferation (175, 176).

These observations raise the possibility that dietary LC n-3 PUFAs prolong gestational duration through changes in the lipid bilayer properties. The neurohypophysial hormone oxytocin is one example of an important membrane-mediated signaling pathway in parturition. Oxytocin is best known for its potent uterotonic activity (177) but data from some animal studies (178-181) suggest that oxytocin may be involved in the initiation of labor. In humans, myometrial sensitivity to oxytocin is significantly increased in women delivering preterm and decreased in women delivering post-term (182). The oxytocin receptor belongs to the class A G protein-coupled receptor (GPCR) family characterized by seven transmembrane domains; hence, it may potentially be influenced by the lipid composition and architecture of the plasma membrane in which it embeds. Fahrenholz et al have demonstrated that membrane cholesterol acts as an allosteric modulator of the oxytocin receptor, stabilizing it in a high-affinity state and protecting it from thermal denaturation (183-186). Gimpl and Fahrenholz have measured 10-15% of the total oxytocin receptor with a two-fold enrichment of the high-affinity

binding sites in the cholesterol-rich low-density membrane fractions of HEK293 fibroblasts stably expressing the oxytocin receptor (187).

The classical function of oxytocin is linked to reproduction but it can also stimulate or inhibit cell growth depending on the cell line (188). The oxytocin receptor appears to mediate these opposite effects through different signaling pathways depending on its localization in different membrane domains. In stably transfected MDCK and HEK293 cells, the oxytocin receptor is largely excluded from caveolae (a specialized subset of lipid rafts) and oxytocin inhibits cell proliferation (189, 190). When the oxytocin receptor is targeted to caveolae by fusion with caveolin-2, oxytocin becomes proliferative. Extraction of cholesterol with methyl-β-cyclodextrin (MβCD) results in redistribution of oxytocin-caveolin-2 chimeric receptors outside caveolae and reverses the mitogenic effect of oxytocin. In prostatic stromal cells, Whittington et al have reported that oxytocin-induced inhibition of cell growth is eradicated by MβCD treatment suggesting that this effect is mediated by oxytocin receptors localized within caveolae (191). The discrepancy between these studies probably reflects differences in the experimental systems. Taken together these data suggest that while the oxytocin receptor is not strictly a lipid raft resident, perturbation of the cholesterol-rich low-density membrane subdomains (lipid raft or caveolae) may have consequences for oxytocin-mediated signaling.

Besides receptors, other signal transducing proteins are known to segregate to lipid rafts, notably heterotrimeric G proteins (192-198). Fatty acylation appears to play a role in targeting Gα subunits to lipid rafts (199-201). S-acylation predominantly involves palmitoylation but PUFAs can also act as substrates for S-acyltransferases (202-204).

Moffet et al have shown that acylation by unsaturated rather than saturated fatty acids reduces the localization of G_i in lipid rafts (193). Not only proteins but also signaling lipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2) partition to lipid rafts. Pike et al have demonstrated that at least 50% of the PIP_2 in A431 cells compartmentalizes to caveolae and that this pool is the primary site of hormone-stimulated PIP_2 turnover (205). Furthermore, cholesterol depletion by MβCD specifically delocalizes PIP_2 from caveolae and results in the inhibition of PIP_2 turnover in response to agonists (206). In cells that lack caveolae such as Neuro 2a, FRT, and KB, a large proportion of PIP_2 still localizes to cholesterol-rich low-density membrane fractions suggesting that PIP_2 compartmentalization is a general feature of many cells (207). In any event, myometrial smooth muscle cells are very rich in caveolae, which may represent another possible site of LC n-3 PUFA action. Manipulation of plasma membrane cholesterol using MβCD remains controversial (208) but dietary LC n-3 PUFAs have been shown to reduce cholesterol levels in caveolae/lipid rafts in vivo (209, 210). These membrane-associated LC n-3 PUFA actions intimate plausible mechanisms whereby dietary LC n-3 PUFAs may influence parturition independent of PGs, particularly in idiopathic preterm labor where the PG status unexpectedly favors the maintenance of pregnancy.

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Chapter 2

Introduction

The Centers for Disease Control reports that the percentage of infants delivered preterm (< 37 weeks of completed gestation) increased from 10.6% in 1990 to 12.8% in 2006. The percentage of infants born low birth weight (< 2500 g) comprised 8.3% of births in 2006 (1). Shortened gestation and low birth weight are major risk factors for death and morbidity in infants (1-3). Evidence from observational and clinical studies indicate that essential fatty acids of the n-3 and n-6 series may potentially modify gestational duration (4). Early epidemiological studies in Denmark by Olsen and colleagues found a significant increase in gestational duration with increasing maternal erythrocyte long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) (5, 6).
In subsequent randomized control trials, LC n-3 PUFA supplementation during pregnancy has been shown to increase gestational duration by 4 to 6 days (7-11). The mechanism remains largely unknown.

Evidence indicates that prostaglandins (PGs) play a key role in initiating and maintaining normal labor (review 12). PGE_2 and $PGF_{2\alpha}$ increase at term in laboring women compared with nonlaboring women (13, 14), can activate tissue remodeling matrix metalloproteinases (15-23), mediate cervical ripening (24-26), and induce uterine contractions (27-33). These PG_2-mediated actions coupled with markedly elevated arachidonic acid (AA) in maternal erythrocytes of women delivering prematurely (34-40), apparent LC n-3 PUFA deficiency in these women (41), and the opposing effects of

LC n-3 PUFAs and AA on PG_2 biosynthesis (42-52) have pointed investigators toward a mechanism in which LC n-3 PUFAs prolong pregnancy by suppressing the AA cascade (7, 8, 11). Elevated reproductive tissue cytokine and PG production have been implicated in preterm birth involving intrauterine infection (review 53), but the role of PGs in idiopathic preterm labor is less well defined. In the absence of infection, PG production is lower in women delivering prematurely compared with women delivering at term (54-56). This seems at odds with a mechanism that invokes PG_2 suppression for increased gestational duration with DHA supplementation in expectant women.

Independent of PG biosynthesis, LC n-3 PUFAs have been shown to exert their cardioprotective (57-59) and immunosuppressive (60-64) effects at the level of the plasma membrane. DHA-induced changes in the membrane structure and architecture leading to modulation of ion channels and the displacement of key proteins from lipid raft signaling platforms appear to be the mechanisms of action. Manipulation of the lipid environment has also been shown to modulate the action of oxytocin (65-72), a potent uterotonic hormone (73) involved in the initiation of labor. To complement these observations and demonstrate one potential PG-independent mechanism for lengthened gestation with DHA supplementation, we examined the effect of DHA on oxytocin signaling in pregnant human myometrial smooth muscle cells. The goal of our first specific aim was to define the cell membrane lipid composition and characterize changes in response to the addition of micromolar concentrations of fatty acids (OA, AA, DHA) into the culture media. Our second specific aim was to determine whether incorporation of exogenous fatty acids into the membrane affects oxytocin-mediated signaling events involved in smooth muscle contraction. This aim tested the hypothesis that treatment with

DHA inhibits elaboration of IP3 and mobilization of Ca^{+2} in response to oxytocin stimulation. Our third specific aim was to examine the effect of DHA treatment on the properties of the oxytocin receptor. This aim tested the hypothesis that DHA does not affect the membrane density or binding affinity of the oxytocin receptor.

Chapter 3

Materials and Methods

3.1 Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin, and other cell culture reagents were acquired from HyClone (Logan, UT). Fatty acid-free, cell culture-tested bovine serum albumin (BSA) (A8806) was obtained from Sigma (St. Louis, MO). Fatty acids and fatty acid methyl ester standards were from Nu-Chek Prep (Elysian, MN). Oxytocin and other compounds were from Sigma. [2-^{3}H(N)]-myoinositol and [tyrosyl-2,6-^{3}H]-oxytocin were from NEN (Boston, MA).

3.2 PHM1-41 cell culture

Immortalized pregnant human myometrial cells (PHM1-41) were kindly provided by Dr. BM Sanborn. PHM1-41 cells maintain the morphological characteristics of proliferating smooth muscle cells in culture, express smooth muscle-specific α-actin, and retain estrogen and oxytocin receptors (74). Cells were grown in DMEM supplemented with 10% heat-inactivated FBS in a humidified atmosphere of 5% CO_2 at 37°C. The cells were subcultured at 4-day intervals with 0.05% trypsin, 53 mM EDTA in Hank's Balanced Salt Solution (HBSS). Cells were between passages 20-25 in our experiments.

3.3 Fatty acid supplementation

Stock solutions of the fatty acids 18:1 oleic acid (OA), 20:4 arachidonic acid (AA), and 22:6 docosahexanoic acid (DHA) were prepared in absolute ethanol at a concentration of 0.5 M, purged in argon gas, and stored at -20°C. 100 μM fatty acid media was prepared by adding fatty acid free BSA to DMEM to a final BSA concentration of 50 μM then incubating overnight with 2 μl of 0.5 M fatty acid stock solution per 10 ml media. 30 μM and 10 μM fatty acid media were prepared by dilution of 100 μM fatty acid media. 0 μM fatty acid media (BSA-only) served as our control. The final ethanol concentration was adjusted to 0.02% as necessary. For all experiments, the cells were grown in supplemented media 48 hrs after subculture and the treatment continued for 2 more days until the cells were nearly confluent.

3.4 Membrane fatty acid extraction and analysis

PHM1-41 cells were seeded in 100 mm plastic tissue culture dishes at ~5.5 x 10^5 cells/dish. The cells were harvested after 4 days in culture by rinsing twice with chilled phosphate buffered saline (PBS) and scraping into ice-cold hypotonic buffer (20 mM HEPES, 10 mM EDTA, pH 7.4). Cells were sheared by dounce homogenization and centrifuged at 1000 x g for 20 min at 4°C. The supernatant was centrifuged at 27,000 x g for 20 min at 4°C. Lipids were extracted from the membrane pellet by chloroform:methanol (2:1 v/v) according to the method of Folch et al (75). Methyl esters were prepared by 14% boron trifluoride methanolysis according to the method of Morrison and Smith (76).

Fatty acid composition was determined by gas chromatography on an Agilent 6890 Series GC (Santa Clara, CA) equipped with an autosampler, a J&W Scientific DB-225 (30 m x 0.25 mm I.D., 0.15μm) capillary column (Folsom, CA), and flame ionization detector. The oven temperature was ramped from an initial temperature of 100°C to 167°C at a rate of 10.65°C/min, then raised from 167°C to 206°C at a rate of 2.58°C/min and held for 8 min. Fatty acids were identified by comparing their retention times with those of fatty acid methyl ester standards.

3.5 Intracellular Ca^{2+}

PHM1-41 cells were plated into 35 mm uncoated glass-bottom dishes (MatTek, Ashland MA) at ~8 x 10^4 cells/dish. After 4 days in culture, the following procedures were carried out in the dark at room temperature. The cells were washed three times with fluorescence buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4) and placed in 1 ml of fluorescence buffer. Cells were loaded with 5 μl of DMSO containing 5 μg of Fura-2-acetoxymethylester (Fura-2 AM, Invitrogen, Carlsbad, CA) for 30 min. Cells were then washed three times with fluorescence buffer and allowed to hydrolyze Fura-2 AM for 30 min. Cells were washed three more times with fluorescence buffer and finally placed in 1 ml of fluorescence buffer. An area of the glass-bottom well was selected for imaging and 20-25 individual cells were analyzed for changes in intracellular free calcium $[Ca^{2+}]_i$ in response to 25 nM oxytocin. $[Ca^{2+}]_i$ was measured using an InCyt2 Im2 imaging system with excitation at 340 nm and 380 nm and emission at 510 nm (Intracellular Imaging,

Cincinnati OH). Data are expressed as the average peak increase in $[Ca^{2+}]_i$ per dish and unresponsive cells were excluded from the analysis where indicated.

3.6 Phosphatidylinositol turnover

PHM1-41 cells were seeded into six-well tissue culture plates (Corning, Acton, MA) at ~1.2 x 10^5 cells/well. After 3 days in culture, cells were labeled with 0.4 μM [2-^{3}H(N)]-myoinositol in serum-free DMEM for 18 hours at 37°C. The cells were washed twice with PBS then incubated in 1 ml of HBSS containing 10 mM LiCl and 0.1% BSA for 30 min at 37°C. Cells were removed from the incubator and given either 100 nM oxytocin in HBSS or HBSS vehicle. Following 30 min incubation at 37°C, the media was aspirated and 1 ml of 20 mM cold formic acid was added. The cells were incubated for 30 min at 4°C then the acid was neutralized with 370 μl of 150 mM NH_4OH. Samples were loaded onto AG1-X8 resin (100-200 mesh formate form, Bio-Rad, Hercules, CA) columns and glycosylphosphatidyl inositol was eluted with 4 ml of 5 mM sodium borate/60 mM sodium formate. Inositol 1-phosphate (IP) and inositol 1,4-bisphosphate (IP_2) were eluted together with 4 ml of 0.4 M ammonium formate in 0.1 N formic acid. Inositol 1,4,5-trisphosphate (IP_3) was eluted with 4 ml of 1 M ammonium formate in 0.1 N formic acid. 0.5 ml of each fraction was counted in 5 ml of Scintisafe Econo 1 scintillation cocktail (Fisher, Fairlawn, NJ). Data are expressed as percent increase in dpm of total IPs over basal.

3.7 Oxytocin receptor ligand binding assay

PHM1-41 cells were seeded in 100 mm plastic tissue culture dishes at ~5.5 x 10^5 cells/dish. After 4 days in culture, the cells were washed twice with chilled PBS and scraped into ice-cold hypotonic buffer (20 mM HEPES, 10 mM EDTA, pH 7.4). Cells were mechanically disrupted by 4 x 15 sec 25,000-rpm pulses with an Ultra Turrax T8 homogenizer at 4°C. Cell homogenates were centrifuged at 500 x g for 10 min at 4°C and the post-nuclear supernatants were spun at 27,000 x g for 30 min at 4°C. The resulting crude membrane pellets were suspended in 20 mM HEPES buffer (pH 7.4) and stored at -80°C until use.

On the day of the assay, crude membrane pellets were quickly thawed at 37°C and centrifuged at 20,000 x g for 30 min at 4°C. Membrane pellets were resuspended into membrane buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4) by homogenizing on ice. [tyrosyl-2,6-^{3}H]-oxytocin was diluted in ligand buffer (50 mM Tris, 0.1% BSA, 10 mM $MgCl_2$, pH 7.4). 96-well Multiscreen FB filter plates (Millipore, Bedford, MA) were pre-wet with 100 µl of membrane buffer for 30 min. The wetting buffer was removed by vacuum filtration and 100 µl of membrane sample was added to each well along with 100 µl of appropriate concentrations of [tyrosyl-2,6-^{3}H]-oxytocin. Plates were incubated on a slow speed reciprocal shaker for 2 hr at room temperature. The binding reaction was stopped by rapid filtration under vacuum to separate bound ligand from free ligand. The plates were washed twice with 200 µl of cold wash buffer (50 mM Tris, 0.1% BSA, pH 7.4). The filters containing the bound ligand were punched out and total binding was determined by liquid scintillation counting (Scintisafe Econo 1 in Packard Tri-carb

2900TR). Nonspecific binding was determined by carrying out the binding with 10 μM of unlabeled oxytocin in the reaction mixture. The level of specific binding was calculated as the difference between total and nonspecific levels of binding.

3.8 Oxytocin receptor internalization assay

PHM1-41 cells were seeded into six-well tissue culture plates (Corning) at ~1.2 x 10^5 cells/well. After 4 days in culture, cells were washed twice with chilled PBS and incubated for 3 hr at 4°C in 1 mL of Ca^{2+} free HBSS containing 5 mM $MgCl_2$, 0.1% BSA, and 10 nM [tyrosyl-2,6-^{3}H]-oxytocin. Following the 3 hr equilibration at 4°C, the cells were incubated at 37°C for specific time points, the HBSS was removed, the cells were washed twice with chilled HBSS (Ca^{2+} free, Mg^{2+} free, 0.1% BSA), then washed with 400 μL of membrane wash solution (150 mM NaCl, 50 mM acetic acid, pH 2.8) to collect membrane bound receptors. To collect internalized receptors, cells were next lysed with 400 uL of 1 M HCl and then the HCl was neutralized with 400 μL of 1 M NaOH. Total binding was determined by liquid scintillation counting (Scintisafe Econo 1 in Packard Tri-carb 2900TR). Nonspecific binding wells contained an additional 1 μM of unlabeled oxytocin. The level of specific binding was calculated as the difference between total and nonspecific levels of binding. For each time point, the internalized receptor values are expressed as a percent of total cell-associated (internalized and membrane bound) receptor.

3.9 Data and Statistical Analysis

The maximal binding capacity (B_{max}) and affinity (K_d) were estimated using a GraphPad Prism (GraphPad Software, San Diego CA) one-site binding model. Data were analyzed using a SAS/STAT 9.1 (SAS Institute, Cary NC) linear mixed model procedure with day of experiment as a random effect. Numbers of experiment replications are noted in the figure legends. Significance was defined as $p \leq 0.05$. Data are expressed as mean ± SEM.

Chapter 4

Results

4.1 FA incorporation into membrane

To our knowledge, the present study is the first to apply LC n-3 PUFAs to the culture medium of human myometrial myocytes. Various investigators have incubated isolated cardiomyocytes (77-83), vascular myocytes (84-86), and colonocytes (87, 87) using LC n-3 PUFAs bound to albumin at concentrations ranging from 5 μM to 214 μM and exposure durations ranging from 24 h to 96 h. Our treatment regimen of 10 μM to 100 μM fatty acid incubation for 48 h falls within the range of these previous reports and we detected minimal impact on cell viability or morphology at these low micromolar concentrations (data not shown).

The addition of exogenous fatty acids to the growth media resulted in incorporation and enrichment of that particular fatty acid in the membrane lipid of PHM1-41 cells (Figure 1). Supplementing cells with 10 μM AA resulted in a small, yet significant increase in membrane AA as a proportion of total fatty acids as compared to equimolar BSA supplementation. With 10 μM DHA supplementation there was a marked increase in membrane DHA because endogenous DHA is nearly absent in these cells under normal culture conditions. Increasing the concentration of exogenous AA or DHA to 30 μM and 100 μM further enriched the respective fatty acid in the membrane as compared to equimolar BSA supplementation. OA is among the most common membrane fatty acid species in PHM1-41 cells (and the fetal bovine serum in the growth

media), therefore significant enrichment with OA supplementation was not observed at concentrations below 100 μM. The incorporation of exogenous fatty acids appeared to be dose dependent as membrane lipids were more enriched at 100 μM supplementation than at 10 μM supplementation (Figure 2).

Obviously, an increase in the relative proportion of one fatty acid is accompanied by a decrease in the relative proportion of one or more other fatty acids. The relative increases in membrane AA or DHA in polyunsaturated fatty acid (PUFA) supplemented cells came at the expense of endogenous membrane OA. Increases in membrane 16:0 palmitic acid (PA) and 18:0 stearic acid (SA) also contributed to the decrease in membrane OA in PUFA supplemented cells (data not shown). OA accounted for 30 ± 2.7% of total membrane fatty acids in cells grown in DMEM + 10% FBS. In comparison, OA accounted for 18.2 ± 1.6% and 16.7 ± 1.6% of total membrane fatty acids in 30 μM AA and DHA treated cells and 15.9 ± 0.2% and 16.4 ± 0.3% in 100 μM AA and DHA treated cells, respectively. There is no substantial difference in membrane OA content between 30 μM and 100 μM PUFA treated cells, suggesting that the differences in oxytocin signaling observed between these two concentrations are not due to depletion of OA, but rather due to enrichment of AA or DHA in the membrane.

We are not aware of any fish oil supplementation trials in pregnant women that have characterized myometrial membrane fatty acid composition. Data for maternal erythrocyte phospholipids does exist for a rough comparison. Dunstan et al have reported increases in maternal erythrocyte DHA as a percentage of total fatty acids at 37 weeks gestation, from 6.6 ± 0.2% in the control group to 11.0 ± 0.2% with fish oil consumption equivalent to one fatty fish meal per day (total of 2.2 g DHA and 1.1 g EPA per day) (89).

In the present study, PHM1-41 membrane DHA composed 6.7 ± 0.5% of total fatty acids with 30 μM DHA treatment and 10.2 ± 0.5% with 100 μM DHA treatment. Relative proportions of other fatty acids were similar between erythrocytes from women fed fish oil and DHA treated PHM1-41 cells. SA and OA each composed 14-16% of total fatty acids in both erythrocytes and in myocytes treated with 30 μM and 100 μM DHA.

4.2 Intracellular Ca^{2+}

We assessed the effect of the changes in membrane composition of PHM1-41 cells by measuring $[Ca^{2+}]_i$ mobilization in response to oxytocin stimulation (Figure 3). The increase in $[Ca^{2+}]_i$ evoked by 25 nM oxytocin was 39% and 57% lower with 100 μM AA and 100 μM DHA treatment, respectively, as compared to equimolar BSA treatment. Treatment with 10 μM and 30 μM fatty acids had no significant effect on oxytocin-stimulated $[Ca^{2+}]_i$.

Not all cells selected for imaging responded to oxytocin with an increase in $[Ca^{2+}]_i$ (Figure 4). We observed considerable heterogeneity in the response of PUFA treated cells in particular, i.e., some cells in the dish responded robustly, some weakly, and some not at all. When individual cells that exhibited no response to oxytocin were excluded from the analysis, the effect of 100 μM DHA treatment was less pronounced, but still represented a significant decrease of 25% compared to equimolar BSA treatment, whereas 100 μM AA treatment was not significantly different (Figure 5). Thus, the decrease in oxytocin-induced elevation of $[Ca^{2+}]_i$ observed with 100 μM PUFA could be partially attributed to a decrease in the proportion of oxytocin-sensitive cells as well as attenuated oxytocin-mediated Ca^{2+} signaling in responsive cells.

4.3 Phosphatidylinositol turnover

Although oxytocin can mobilize $[Ca^{2+}]_i$ through the production of the second messenger IP_3, we evaluated *total* inositol phosphates (IP_3, IP_2, IP) in our analysis (Figure 6). This was due to the difficulty in recovering the transient IP_3 signal before it was dephosphorylated. Treatment with 30 μM PUFA significantly decreased the total inositol phosphates generated in response to 25 nM oxytocin as compared to equimolar OA, but not BSA (AA, $p = 0.12$; DHA, $p = 0.11$) treatment. Treatment with 100 μM AA and 100 μM DHA significantly decreased phosphatidylinositol (PI) turnover by 53% and 50% respectively as compared to equimolar BSA treatment. Treatment with 10 μM fatty acids had no effect on PI turnover and there were no significant differences between BSA and OA treatment at any concentration.

4.4 Oxytocin receptor ligand binding assay

Representative binding curves are shown in Figure 7. Treatment with 30 μM and 100 μM PUFA significantly decreased the maximum number of oxytocin receptor binding sites (B_{max}) in a dose dependent manner without affecting affinity (K_d) as compared to equimolar BSA treatment (Figure 8). Treatment with 10 μM PUFA had no effect on B_{max} and there were no significant differences between BSA and OA treatment at any concentration. The B_{max} (3-4 pmol/mg protein) and K_d ($\approx$ 1 nM) of controls were within the range of previously published values for the oxytocin receptor in human term myometrium (74, 91).

4.5 Oxytocin receptor internalization assay

Treatment with PUFAs did not significantly alter the internalization of the oxytocin receptor (Figure 9). There was less ligand bound overall in 100 μM AA and 100 μM DHA treated cells (data not shown), reflecting the reduced number of oxytocin receptor binding sites, but the rate of internalization between 0 min and 30 min time points did not change as compared to equimolar BSA treated cells. Maximum internalization was observed between 30 min and 60 min time points.

Chapter 5

Discussion

Considering the low PG concentration and synthetic capacity in idiopathic premature delivery, it is difficult to reconcile the inhibitory effect of LC n-3 PUFAs on PG biosynthesis (48-51, 91-93) as the sole mechanism of action for DHA prolonging gestation. Besides their effects on production of PGs, LC n-3 PUFAs can act directly at the plasma membrane level. This was first demonstrated by Leaf et al regarding the antiarrhythmic effect of fish and fish oil consumption (57-59). The precise site of action in cardiomyocytes remains to be fully elucidated, but data from Anderson and colleagues (94-97) have led to the current thinking that LC n-3 PUFAs electrically stabilize cardiomyocytes by modulating ion channels through changes in membrane fluidity and elasticity in the microdomain bordering the transmembrane protein.

LC n-3 PUFAs have also been shown to exert their anti-inflammatory properties through eicosanoid-independent membrane effects. The successful application of LC n-3 PUFAs in the treatment of immune-inflammatory diseases is based largely on their ability to suppress T lymphocyte activation (98-101). A number of key proteins involved in signal transduction are known to associate with lipid rafts, targeted there by acylation with saturated fatty acids (102). LC n-3 PUFAs have been proposed to disrupt T cell activation by displacing raft-resident signaling proteins such as Src family kinases and transmembrane linker for activation of T cells from lipid rafts (103-105). LC n-3 PUFAs are thought to act primarily by altering raft composition and structure, although changes

in protein fatty acylation may contribute to the displacement of proteins from lipid rafts (106).

These observations raise the possibility that dietary LC n-3 PUFAs prolong gestational duration through changes in the lipid bilayer properties. The neurohypophysial hormone oxytocin activates an important membrane-mediated signaling pathway in parturition. Oxytocin is best known for its uterotonic activity (73) and data from some animal studies (107-110) suggest that it may be involved in the initiation of labor. In humans, myometrial sensitivity to oxytocin is significantly increased in women delivering preterm and decreased in women delivering post-term (111). The oxytocin receptor belongs to the class A G protein-coupled receptor (GPCR) family characterized by seven transmembrane domains; hence, it may potentially be influenced by the lipid composition and architecture of the plasma membrane in which it embeds.

In the present study we demonstrated that both AA and DHA significantly alter the membrane lipid composition of PHM1-41 pregnant human myometrial smooth muscle cells (Figures 1 and 2) and suppress oxytocin-stimulated $[Ca^{2+}]_i$ mobilization (Figures 3 and 4). The cells were washed several times during the Ca^{2+} dye loading procedure, thus an interpretation based on a free fatty acid effect as proposed in cardiomyocytes (112-116) and rat aortic smooth muscle cells appears unlikely. Inhibition of the oxytocin-stimulated rise in $[Ca^{2+}]_i$ was associated with near abolition of PI turnover (only 7.9 ± 9.7% and 14.2 ± 8.8% over basal for 100 μM AA and 100 μM DHA treated cells respectively) (Figure 6). Upstream of PI turnover, we found that 100 μM PUFA drastically reduced the number of oxytocin receptor binding sites in the membrane

without affecting binding affinity (Figure 7). Fahrenholz et al have demonstrated that membrane cholesterol acts as an allosteric modulator of the oxytocin receptor (117-120). High-affinity state receptors are stabilized three-fold ($t_{1/2}$) and enriched two-fold in cholesterol-rich, caveolin-containing membrane domains (121). The range of oxytocin concentrations used in this saturation binding study (10^{-10} to 10^{-7} M) would not detect the presence of a second, low affinity binding site in the 10^{-7} to 10^{-6} M range as reported by Zhong et al (122). Since Chen et al have shown that DHA incorporation into membrane phospholipids is accompanied by depletion of cholesterol from caveolae (123), the possibility that DHA in the present study shifts a population of receptors into the low affinity binding state may warrant further investigation. PI turnover and receptor binding sites decreased with 30 μM PUFA treatment, but apparently remained at levels sufficient for oxytocin-stimulated $[Ca^{2+}]_i$ mobilization.

The decreased oxytocin response observed in the present study appears predominantly attributable to decreased number of oxytocin binding sites. Fatty acids are known to modulate the expression of a number of genes, mostly encoding proteins involved in fatty acid transport or metabolism (review 124). Fatty acids can influence gene expression through direct binding to GPCRs (125) and transcription factors (126-128), regulation of the nuclear abundance of transcription factors (129-131), and regulation of protein kinases (131, 132). Still, it is unclear how, or indeed whether, PUFAs regulate expression of the oxytocin receptor in myometrial cells. DHA can activate PKC, at least in hepatocytes (review 124), and melatonin-dependent PKC activation has been shown to inhibit oxytocin receptor transcription (133) in human myometrial cells. Prolonged exposure to agonists desensitizes many GPCRs, protecting

the cell from overstimulation. Following receptor activation, GPCRs are commonly subject to a cycle of internalization and sequestration, resensitization, and recycling back to the plasma membrane. We did not detect any differences in the rate of oxytocin receptor internalization upon ligand binding however (Figure 9). Phaneuf et al have reported that the number of oxytocin receptor binding sites decreases by nearly 90% in human myometrial cells exposed to oxytocin for 20 h, however, they detected no change in the level of oxytocin receptor protein measured by Western blotting (134). This data suggests the possibility that PUFAs might regulate oxytocin receptor function post-translationally through phosphorylation or uncoupling from G proteins.

In view of the frequently antagonistic actions of dietary AA and DHA, our observation that both n-6 and n-3 LC PUFAs inhibit oxytocin signaling was unexpected. AA in the body originates from either synthesis through desaturation and elongation of 18:2 linoleic acid (LA), an essential fatty acid, or from the diet. The intake of LA contributes 5-7% of dietary energy in western nations. A recent human study has demonstrated that increasing the contribution of LA to as much as 20% of dietary energy does not increase plasma phospholipid AA (135). Meat, fish, and eggs are the major food sources of AA. Studies comparing the fatty acid profile of omnivores and vegetarians have reported only slight differences in the level of AA (136-138). These data suggest that a moderate variation in intake of AA has no major effect on AA accumulation, thus an effect of AA supplementation observed in cell culture may not be relevant to a physiological diet. In conclusion, we have shown a generalized effect of LC PUFAs on inhibiting oxytocin-stimulated $[Ca^{2+}]_i$ mobilization. These findings may be relevant to the reports of dietary fish and fish oil consumption prolonging gestation.

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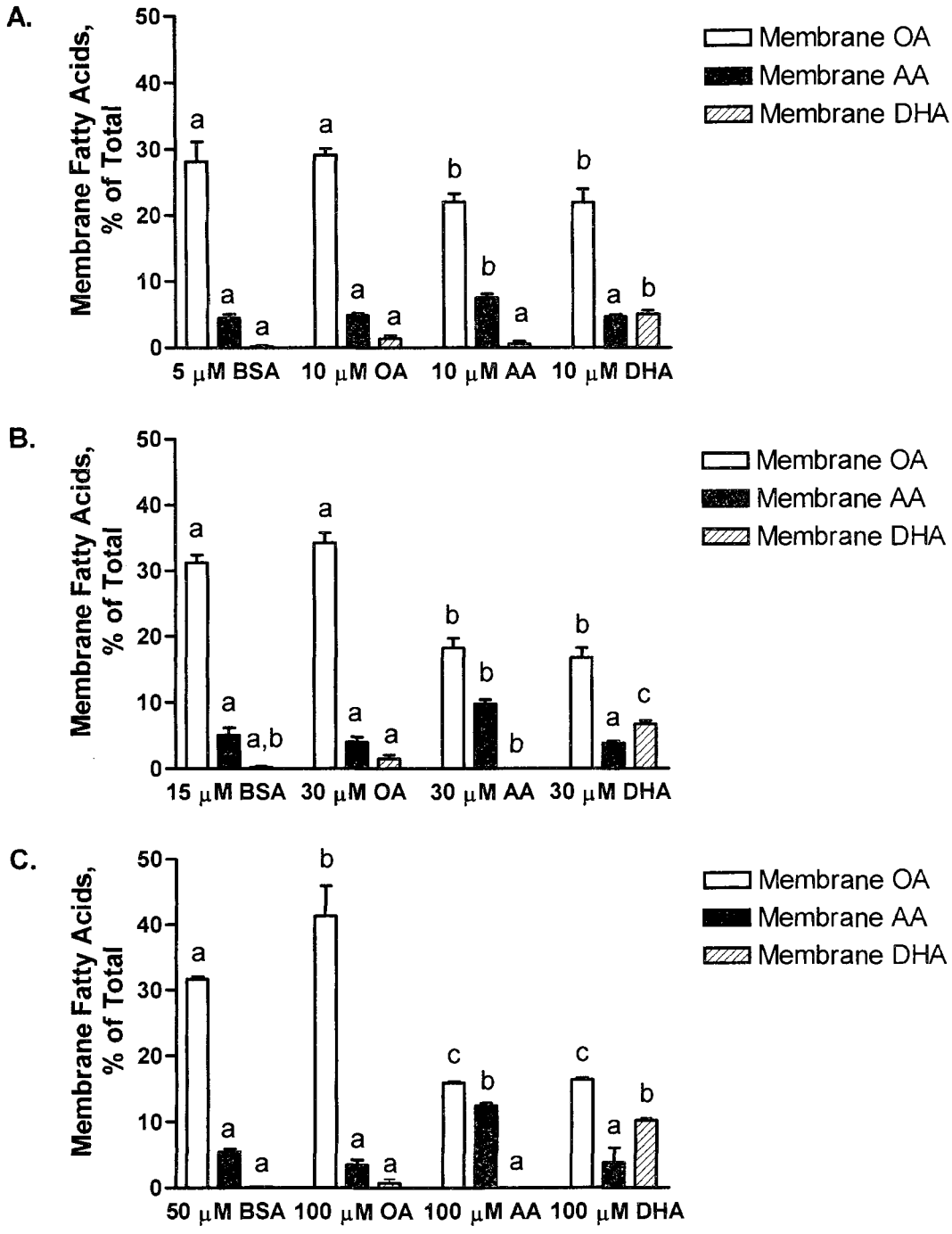


Figure 1. Membrane fatty acid composition (18:1 OA, 20:4 AA, 22:6 DHA) of PHM 1-41 cells treated with (A) 10 μM, (B) 30 μM, (C) 100 μM OA, AA, DHA. Data are expressed as a percent of total membrane fatty acids. Values are mean percent ± SEM (n = 3 experiments). Bars within a particular membrane fatty acid series that do not share common letters are significantly different among treatment groups, $p < 0.05$.

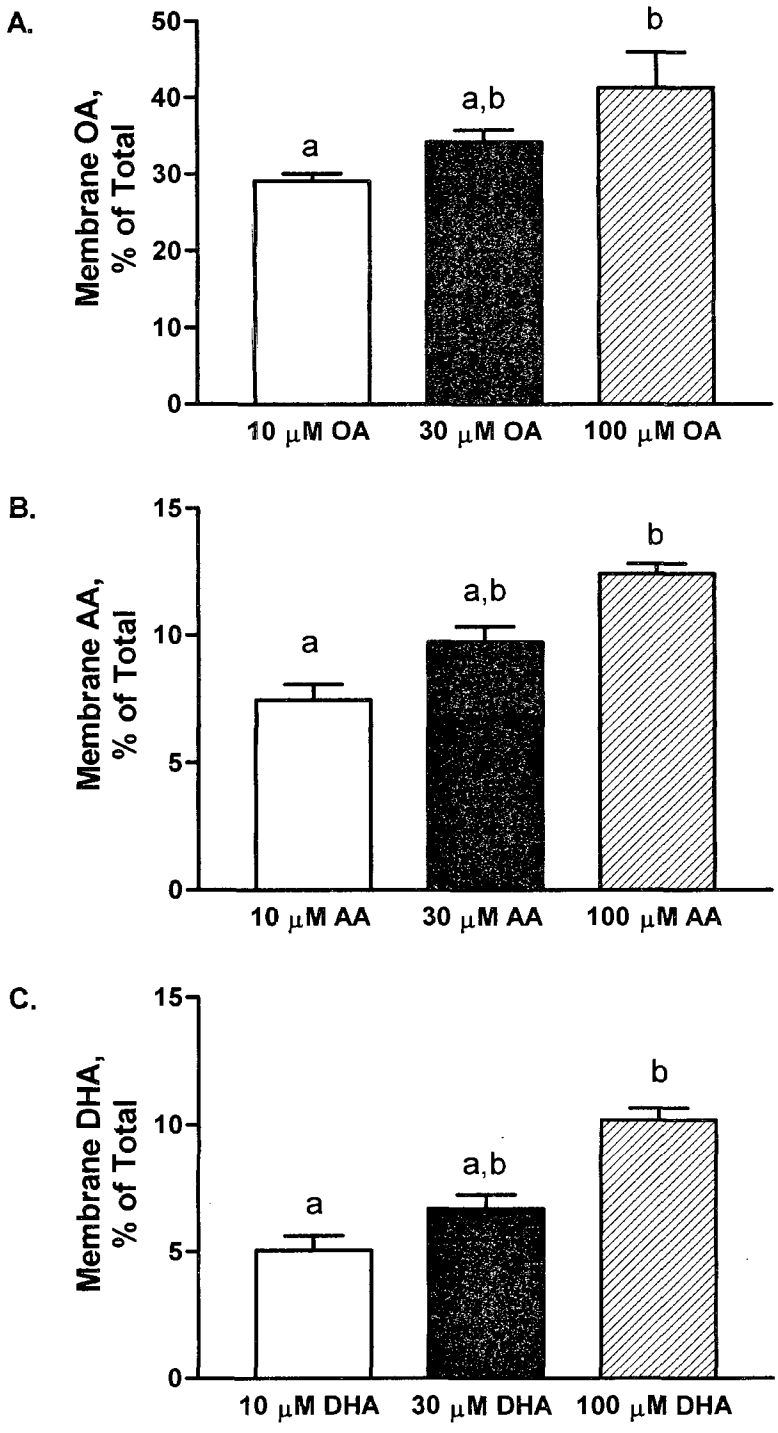


Figure 2. Membrane fatty acid composition of PHM 1-41 cells treated with 10 μM to 100 μM (A) 18:1 OA, (B) 20:4 AA, or (C) 22:6 DHA. Data are expressed as a percent of total membrane fatty acids. Values are mean percent ± SEM (n = 3 experiments). Bars that do not share common letters are significantly different among treatment levels, $p < 0.05$.

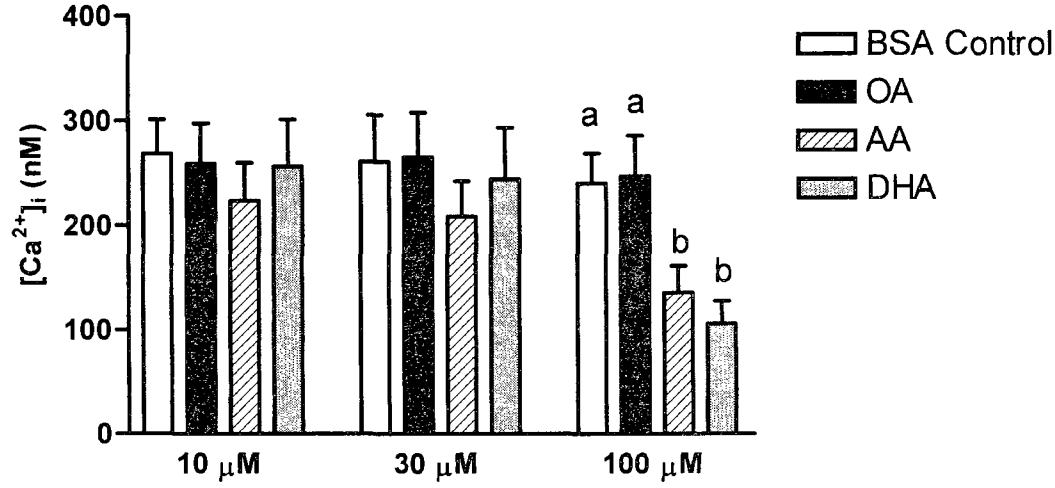


Figure 3. The change in $[Ca^{2+}]_i$ of PHM1-41 cells in response to 25 nM oxytocin. Data are expressed as the average peak increase in $[Ca^{2+}]_i$ per dish. Values are mean ± SEM (n = 7 experiments at 10 μM and 30 μM; n = 12 experiments at 100 μM). Bars not sharing common letters are significantly different among treatment groups, $p < 0.05$.

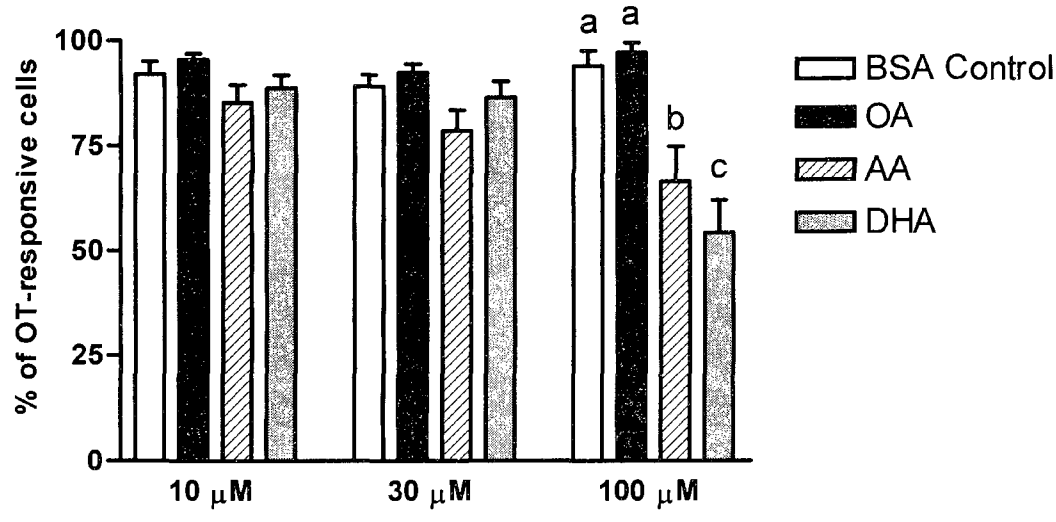


Figure 4. The percentage of PHM1-41 cells per dish that responded to 25 nM oxytocin with an increase in $[Ca^{2+}]_i$. Values are mean ± SEM (n = 7 experiments at 10 μM and 30 μM; n = 12 experiments at 100 μM). Bars not sharing common letters are significantly different among treatment groups, $p < 0.05$.

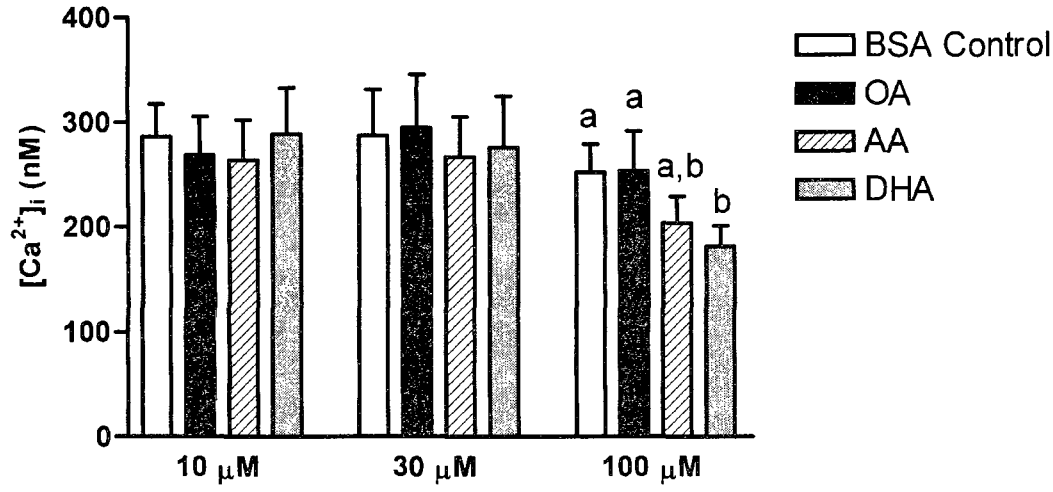


Figure 5. The change in $[Ca^{2+}]_i$ of PHM1-41 cells in response to 25 nM oxytocin. Completely unresponsive cells were excluded from the analysis. Data are expressed as the average peak increase in $[Ca^{2+}]_i$ per dish. Values are mean ± SEM (n = 7 experiments at 10 μM and 30 μM; n = 12 experiments at 100 μM). Bars not sharing common letters are significantly different among treatment groups, $p < 0.05$.

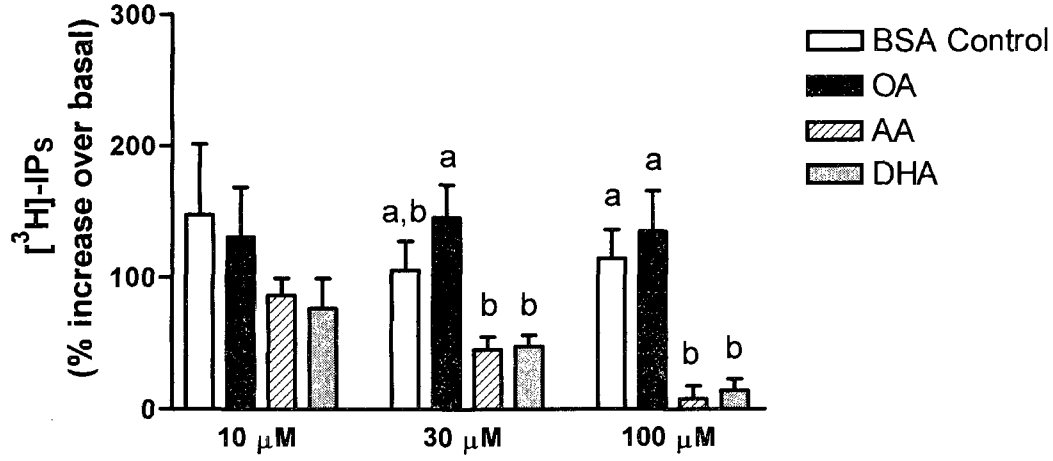


Figure 6. Total inositolphosphates (IPs) generated in response to 100 nM oxytocin in PHM1-41 cells. Data are expressed as a percent increase in dpm of total IPs over basal. Values are mean ± SEM (n = 4 experiments). Bars not sharing common letters are significantly different among treatment groups, $p < 0.05$.

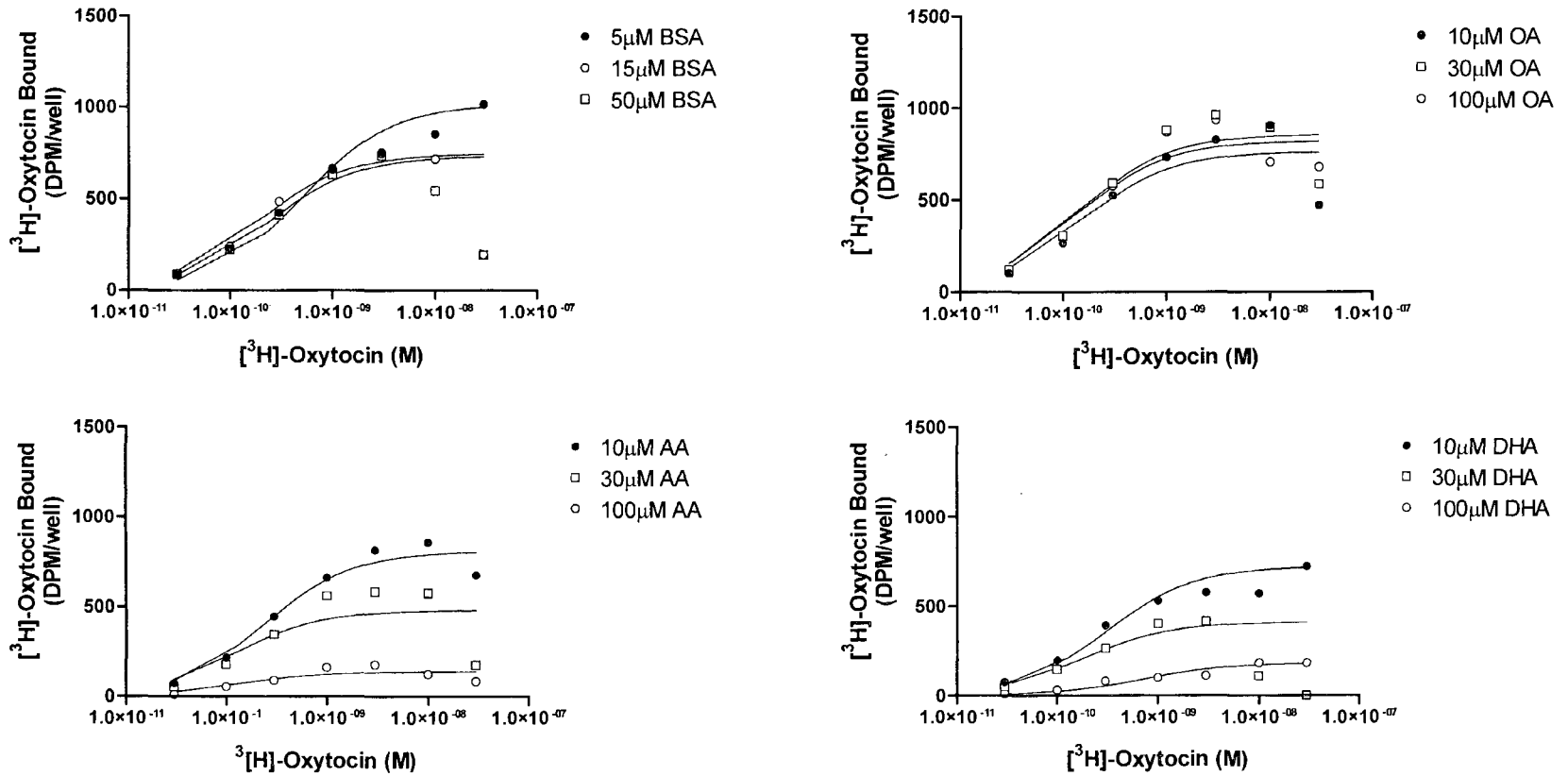
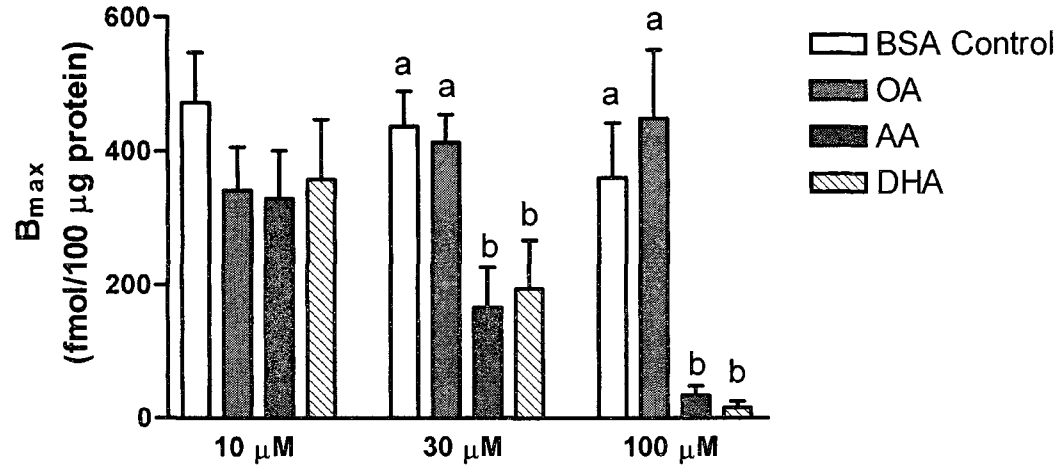


Figure 7. Representative saturation binding curves of oxytocin receptor in crude membrane preparations from PHM1-41 cells treated with (A) BSA, (B) OA, (C) AA, (D) DHA. Binding curves were fitted with nonlinear regression using a one-site binding model in GraphPad Prism.



	10 µM		30 µM		100 µM	
	Bmax	**K_d (nM)**	**Bmax**	**K_d (nM)**	**Bmax**	**K_d (nM)**
BSA	473 ± 75	0.53 ± 0.12	437 ± 53[a]	0.86 ± 0.43	360 ± 82[a]	0.43 ± 0.19
OA	342 ± 65	0.30 ± 0.13	413 ± 42[a]	0.48 ± 0.18	449 ± 103[a]	0.73 ± 0.36
AA	330 ± 71	0.38 ± 0.12	205 ± 66[b]	0.35 ± 0.26	22 ± 13[b]	0.25 ± 0.12
DHA	358 90	1.22 ± 0.53	166 ± 61[b]	1.02 ± 0.76	47 ± 3[b]	0.63 ± 0.05

Figure 8. Maximal oxytocin binding capacity (Bmax) and binding affinity (K_d) in crude PHM1-41 cell membrane preparations. Data are expressed as fmol/100 µg protein. Values are mean ± SEM (n = 3 experiments). Bars that do not share common letters and values in columns not sharing common letter superscripts are significantly different among treatment groups, $p < 0.05$.

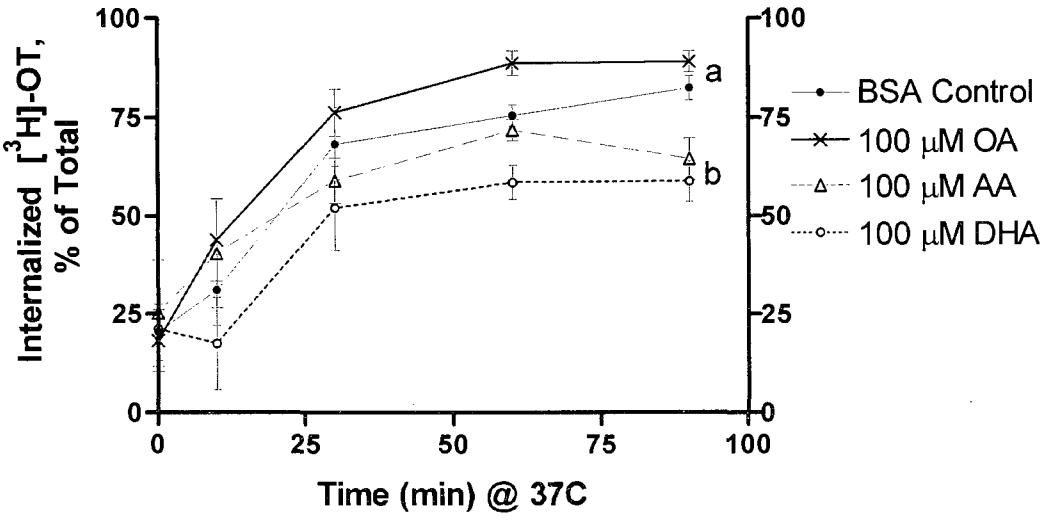


Figure 9. Internalized [^{3}H]-OT in PHM1-41 cells. Data are expressed as a percent of total cell-associated ligand (Membrane-bound + Internalized). Values are mean ± SEM (n = 3 experiments). Points not sharing common letters are significantly different among treatment groups, $p < 0.05$.