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Das Dilemma der Frühgeburt-
Neue molekulare Präventionsansätze

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1. Thema

Das Dilemma der Frühgeburt - Neue molekulare Präventionsansätze

2. Einführung in die Thematik

Die Frühgeburt ist definiert als die Entbindung vor der vollendeten 37. Schwangerschaftswoche (SSW). Das Dilemma der Frühgeburt entsteht durch die Tatsache, dass die weltweite Rate an (zu) früh geborenen Kindern einerseits nur etwa 5-10% beträgt, andererseits für etwa 70-80% der perinatalen Mortalität und Morbidität verantwortlich ist. In der Bundesrepublik Deutschland sind nach wie vor zwei Drittel aller Kinder, die perinatal versterben, Frühgeborene mit einem Geburtsgewicht unter 1500g und 20-30% der überlebenden Kinder weisen Spätmorbiditäten auf, welche unter anderen durch bronchopulmonale Dysplasien, intra- und periventrikuläre Hämorrhagien und Leukomalazien sowie weitere Folgen der Unreife bedingt sind (Munz et al. 2005).

Grundsätzlich muss man zwischen indizierter und spontaner Frühgeburt unterscheiden. Bei der indizierten Frühgeburt wird die Schwangerschaft aufgrund von fetalen oder maternalen Pathologien, beispielsweise einer manifesten Plazentainsuffizienz oder exazerbierender mütterlicher Grunderkrankung, vorzeitig beendet. Spontane Frühgeburtsbestrebungen können u. a. durch eine Zervixinsuffizienz, einen vorzeitigen Blasensprung und/oder eine vorzeitige Wehentätigkeit bedingt sein, deren Ursachen häufig in verschiedenen Formen von Plazentapathologien oder aszendierenden Infektionen zu finden sind. Etwa ein Drittel aller Frühgeburten ist Folge vorzeitiger Wehentätigkeit ohne erkennbare mütterliche oder fetale Pathologie (Keirse und Kanhai 1981, Schneider et al. 1994), welche es bis zum Erreichen der Lebensreife des Ungeborenen zu verhindern gilt. Eine vorzeitige Wehentätigkeit muss gegenüber einer physiologischen Zunahme der Kontraktilität des Uterus im Laufe der Schwangerschaft abgegrenzt werden. Dies erfolgt durch Palpation und vaginalsonographische Vermessung des Muttermundes. Sind die gemessenen Kontraktionen Zervix-wirksam so kommen wehenhemmende Medikamente zum Einsatz. Die Therapie der vorzeitigen Wehentätigkeit durch Einsatz so genannter Tokolytika (gr. τοκος λυτικος ; Geburt, fähig zu lösen) stellt jedoch lediglich eine symptomatische Therapie dar und keinen kausalen Ansatz im Sinne der Beseitigung der Wehen induzierenden Ursachen. Dies zeigt sich auch in

der bislang nur begrenzten Wirksamkeit dieser Medikamente. Der größte Benefit der Tokolytika besteht in der kurzfristigen Schwangerschaftsverlängerung um 2-7 Tage (Gyetvai K. et al. 1999), die zur Induktion der Lungenreife mittels Steroiden und der intrauterinen Verlegung des Feten in ein Perinatalzentrum genutzt werden kann. Eine langfristige Hemmung Zervix wirksamer Wehen bei anderweitig unauffälliger Schwangerschaft - gerade am Rande der Lebensfähigkeit - wäre jedoch wünschenswert, vor allem um einen Schwangerschaftszeitraum zu erreichen, der ein Überleben der früh geborenen Kinder wahrscheinlicher werden lässt (Abbildung 1).

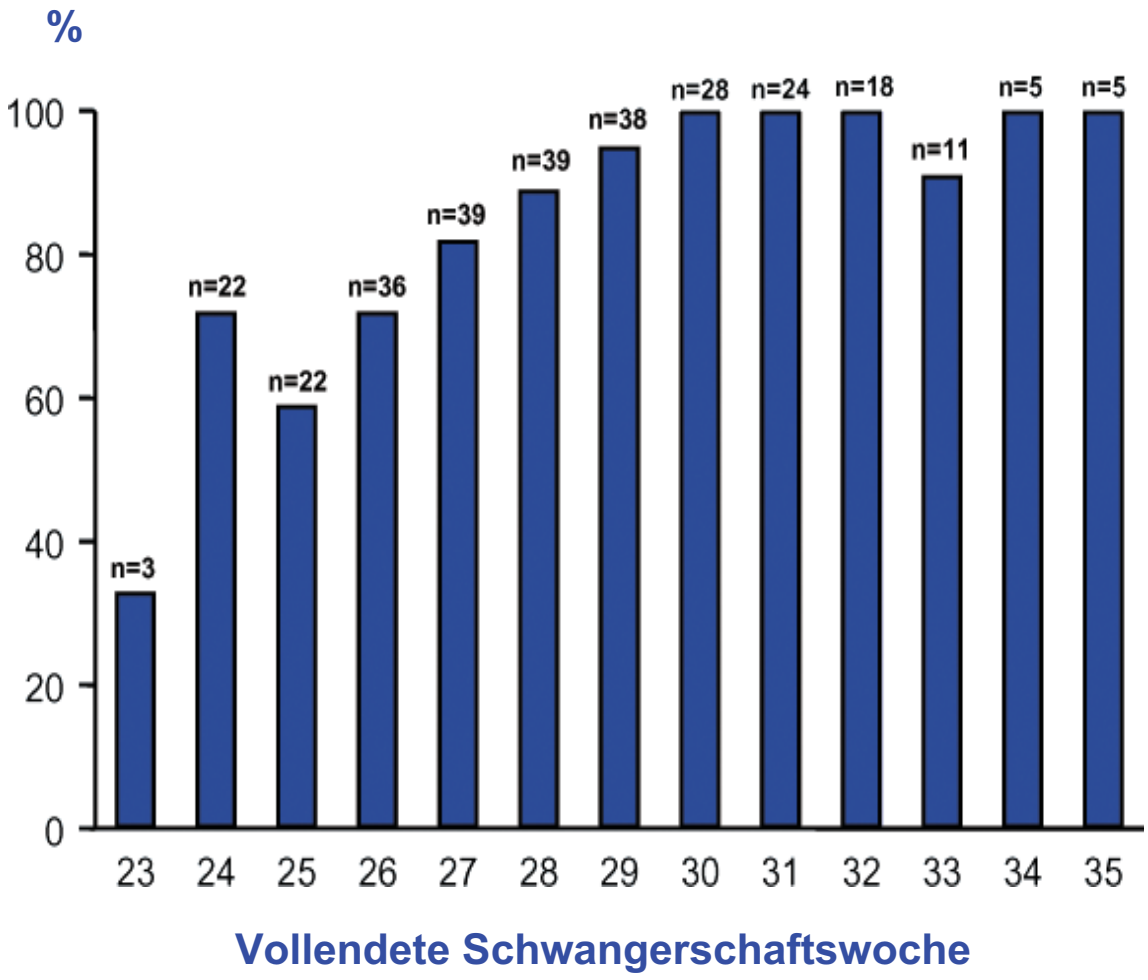


Abbildung 1: Überlebensrate der „very low birth weight“ – Neugeborenen (< 1500g) am Universitätsklinikum Düsseldorf (UKD) in den Jahren 2002-2006: Angabe in %. Mit freundlicher Genehmigung von Dr. H. Stannigel, Oberarzt der Neonatologie, UKD.

Zur Behandlung vorzeitiger Wehen (Tokolyse) stehen verschiedene Pharmazeutika zur Verfügung - neben den weit verbreiteten β-Mimetika auch Kalziumantagonisten,

Prostaglandinsynthesehemmer, NO-Donatoren und Oxytozinrezeptorantagonisten. Die genannten Substanzgruppen unterscheiden sich nicht nur in ihrem Wirkmechanismus, sondern auch im Nebenwirkungsprofil und den Kosten [s. Leitlinie der DGGG: Medikamentöse Wehenhemmung bei drohender Frühgeburt AWMF 015/025 (2006), sowie Cochrane database systemic reviews: CD 001992, 002255, 002860, 004071, 004352, 004452 (2002-2005)]. Allen Tokolytika gemein ist jedoch die mangelnde Effektivität, eine Schwangerschaft über die Zeit von 2-7 Tagen hinaus zu verlängern. Die mit der Einführung tokolytischer Medikamente verbundenen Erwartungen in Bezug auf eine effektive Senkung der Frühgeburtenrate haben sich damit bislang nicht erfüllt und der in den letzten Jahren zu verzeichnende Rückgang der Perinatalsterblichkeit beruht im Wesentlichen auf einer Abnahme der Neonatalsterblichkeit.

Um neue Therapieansätze zu Vermeidung unerwünschter vorzeitiger Wehentätigkeit entwickeln zu können, benötigen wir ein fundiertes Verständnis über die der uterinen Kontraktion zugrunde liegenden Mechanismen. Die in der vorliegenden kumulativen Habilitationsschrift zusammengefassten Originalarbeiten geben neue Einblicke in das komplexe Zusammenspiel systemischer sowie lokaler, sich im graviden Uterus befindlicher Signalkaskaden, die einer uterinen Kontraktion zugrunde liegen, und eröffnen damit möglicherweise neue molekulare Präventionsansätze zur Verhinderung vorzeitiger Wehentätigkeit.

Nachfolgend werden grundlegende Mechanismen vorgestellt, die für den Vorgang physiologischer, zeitgerechter und unphysiologischer, vorzeitiger Wehentätigkeit verantwortlich sind.

Physiologie der Kontraktion

Die Grundlage uteriner Kontraktionen besteht in der Interaktion zwischen den Muskelproteinen Myosin und Aktin. Kommt es zu einer Steigerung der intrazellulären Kalzium-(Ca^{2+}) Konzentration auf ca. 10^{-6}-10^{-7} M, bindet dieses an Kalmodulin (CaM) und führt als Komplex zur Aktivierung der Myosin-Leichtketten-Kinase (MLCK). Durch enzymatische Phosphorylierung der Myosin-Leichtkette ist Myosin in der Lage an Aktin zu binden und unter nachfolgender Hydrolyse von Adenosintriphosphat (ATP) zu Adenosindiphosphat (ADP) zur Kontraktion der Muskelzelle zu führen.

Ca^{2+} wird weitgehend durch spannungsvermittelnde Ca^{2+}-Kanäle von extra- nach

intrazellulär transportiert, wobei drei verschiedene Ca^{2+}-Kanäle unterschieden werden: ein so genannter langer, ein transienter und ein neuronaler Typ. Ersterer ist unter anderem Angriffspunkt kontraktionsinhibierender Ca^{2+}-Kanalblocker. Diese Kanäle werden durch Depolarisierung der Zellmembran sowie durch hormonelle und neuronale Stimulation aktiviert.

Das Absinken des intrazellulären Ca^{2+}-Spiegels und die Dephosphorylierung des Myosins durch die Myosin-Leichtketten-Phosphatase (MLCP) beenden die Kontraktion und die Muskelzelle erschlafft.

Die Erregungsbildung im Myometrium entsteht multifokal. Um die entstandenen Erregungen in synchrone Kontraktionen weiterzuleiten, ist während der Schwangerschaft der Zuwachs an uterinen Zell-Zell Verbindungen, den so genannten Gap Junctions (GJ), von maßgeblicher Bedeutung (Miller et al 1989). GJ bestehen aus verschiedenen Connexin Proteinen, die sich zwischen den einzelnen Muskelzellen zu kleinen Kanälen aufbauen, um den Austausch von Ionen und Molekülen des Second-Messenger-Systems zu ermöglichen. Im Uterus werden verschiedene Connexine exprimiert, wobei Connexin 43 am häufigsten vorzukommen scheint (Ciray et al. 2000). Ihre Anzahl sowie der Expressionsgrad ihrer Ausbreitung an der Zelloberfläche nehmen unmittelbar vor und während der Geburtwehen zu, zum Ende der Geburt hingegen wieder ab. Die Anzahl sowie die Funktion der GJ kann durch cAMP, Ca^{2+} und Steroidhormone modifiziert werden.

Heptahelikale Rezeptoren

Für die Stimulation der glatten Uterusmuskulatur ist das Zusammenspiel zwischen membranständigen Hormonrezeptoren und G-Protein-vermittelten Einflüssen auf sekundäre Botenstoffe wichtig. Heptahelikale Guanylnukleotid-Protein (G-Protein) aktivierende Rezeptoren gehören zu einer Familie von Rezeptorproteinen, welche u.a. die myometriale Zellmembran in transmembranöser Konfiguration überspannen. Sie stehen mit einem heterodimeren G-Protein in Verbindung, welches Guanosintriphosphat (GTP) mit hoher Affinität bindet. Durch Bindung eines Liganden an seinen Rezeptor wird dieser aktiviert und bewirkt durch Bindung von GTP an die sogenannte $G\alpha$ Untereinheit eine Abspaltung des $G\alpha$-GTP-Komplexes; ein $\beta\gamma$-Komplex verbleibt. Der $G\alpha$-GTP-Komplexe reguliert Effektorproteine wie zellmembrangebundene Enzyme, Adenylzyklase (AC), Phospholipase C (PLC) und Ionenkanäle. Die externe Hydrolyse des GTP zu GDP durch hormonaktivierte

GTPase bewirkt die erneute Bindung des Komplexes an den verbliebenen βγ–Komplex und damit sein Inaktivierung (Abbildung 2).

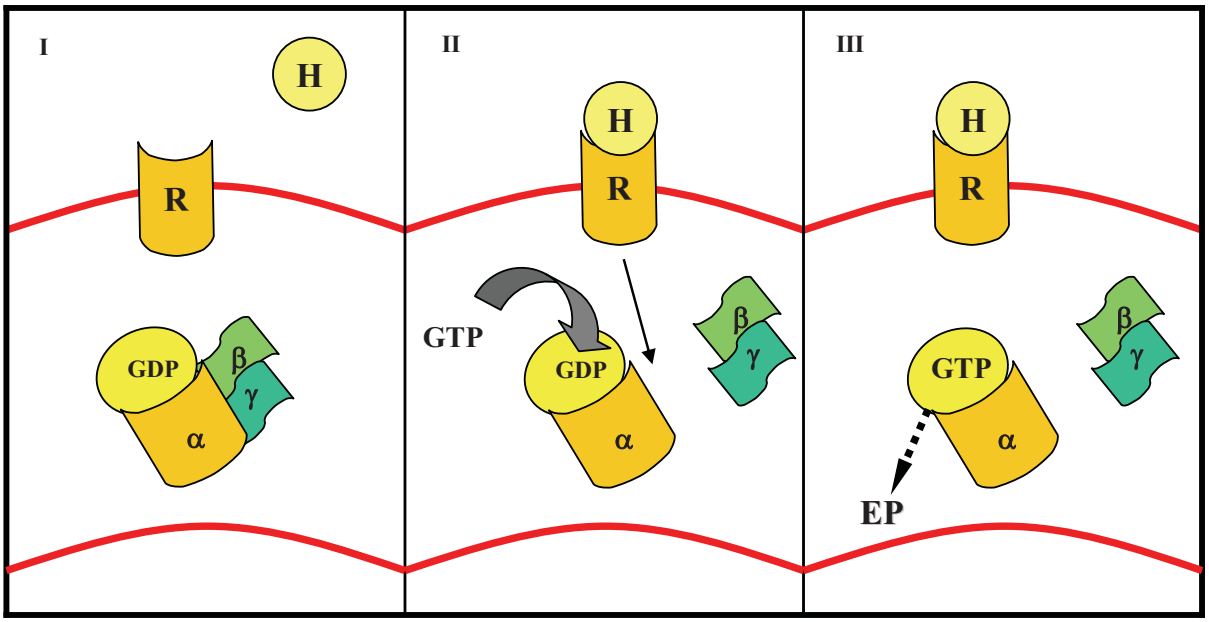


Abbildung 2: Guanylnukleotid-regulatorische Proteine (G-Proteine) bestehen aus drei Untereinheiten: α, β und γ. **I.** In Ruhe ist Guanosindiphosphat (GDP) an die α Einheit gebunden. **II.** Bindet das Hormon (H) an den Rezeptor (R), so führen diese als Komplex zur Abspaltung des βγ-Komplexes und Guanosindiphosphat wird durch Guanosintriphosphat (GTP) ersetzt. **III.** Gα-GTP stimuliert verschiedene Effektorproteine (EP) wie zellmembrangebundene Enzyme, Adenylzyklase, Phospholipase C oder Ionenkanäle
(Abbildung aus Friebe-Hoffmann und Klockenbusch: Molekulare Mechanismen der Geburt. In: M. W. Beckmann, P. Dall, J. S. Krüssel, D. Niederacher, B. Tutschek (Hrsg.). Molekulare Medizin in der Frauenheilkunde, Steinkopffverlag, Darmstadt, 2001).

Es werden verschiedene Untergruppen des Gα-Proteins unter Berücksichtigung ihrer Wirkung auf die glatte Muskulatur des Uterus unterschieden. Beispielsweise wirken $G\alpha_{i/q}$ Untereinheiten durch eine Inhibition der AC, eine Aktivierung von PLC und eine durch sekundäre Botenstoffe vermittelte Steigerung der intrazellulären Ca^{2+}-Konzentration kontraktionsfördernd, während die $G\alpha_s$-Untereinheit über eine

Aktivierung der Adenylzyklase mit nachfolgender Konzentrationserhöhung von cAMP eine relaxierende Wirkung auf die glatte Muskulatur hat. Es wird angenommen, dass es am Ende der Schwangerschaft zu einem Wechsel der Dominanz der G-Protein-assoziierten Systeme kommt: vom $G\alpha_s$-verbundenen System (Uterus relaxierend) zum $G\alpha_{i/q}$-assoziierten System (kontraktionsfördernd) (Europe-Finner et al. 1996, Lajat et al. 1996). Auch der am Ende der Schwangerschaft auf die Expression des G-Proteins ansteigende Einfluss von Östrogen wird zur Erklärung für den Wechsel von der Ruhephase des Myometriums zur anstehenden Kontraktionsphase angeführt.
Als humorale Botenstoffe erreichen Hormone ihre jeweiligen Zielzellen auf extrazellulärem Wege, um an der Außenseite der Zielzellen an spezifische, hoch affine Hormonrezeptoren zu binden. Lipophile Hormone (z. B. Steroidhormone) sind hiervon ausgenommen. Als Liganden der heptahelikalen Rezeptoren, die für die Funktion des Myometriums bedeutsam sind, fungieren Hormone und Neuropeptide wie Östrogen (E), Progesteron (P), Oxytozin (OT), Vasopressin (VP), Relaxin (RLX) sowie verschiedene Prostaglandine (PG). Durch die Hormon-Rezeptor-Bindung kommt es intrazellulär zur enzymatischen Bildung von sekundären Botenstoffen wie zyklischem Adenosin- und Guanosinmonophosphat (cAMP, cGMP), Inositol-triphosphat (IP_3) oder 1,2-Diacylglycerol (DAG). Da die Spezifität der Hormonwirkung durch die Rezeptorausstattung der einzelnen Zelle gewahrt bleibt, können viele Hormone die gleichen Rezeptorklassen benutzen als auch dieselben sekundären Botenstoffe verwenden.

Oxytozin

Oxytozin (OT), ein aus 9 Aminosäuren zusammengesetztes Hormon von 1007 Dalton Größe, wird im Hypothalamus gebildet und über den Hypophysenhinterlappen ins Blut sezerniert. Veränderungen des zirkulierenden OT im mütterlichen Blut unterliegen pulsatilen sowie zirkadianen Schwankungen.
Während der Schwangerschaft wird OT jedoch nicht nur im Hypothalamus, sondern auch im Uterus und in den Eihäuten gebildet. Östrogen sowie verschiedene inflammatorische Zytokine steigern die Expression von OT mRNA in humaner Choriondezidua sowie im Myometrium. Lokal vermittelt OT seine biologische Aktivität autokrin bzw. parakrin über spezifische lokale Rezeptoren (OTR). Der Oxytozinrezeptor (OTR) setzt sich aus 388 Aminosäuren zusammen, welche ein Molekulargewicht von 43kDa haben. OTR werden auf Zellen des Amnions, des

Chorions, der Dezidua und des Myometriums gefunden (Fuchs et al. 1982, Benedetto et al. 1990, Soloff 1993, Kimura 1993). Während der Schwangerschaft findet eine vermehrte Expression uteriner OTR statt, die unmittelbar vor Wehenbeginn nochmals um das 2-3fache ansteigt und mit einer Sensibilisierung des Myometriums gegenüber OT einhergeht. Zu diesem Zeitpunkt genügen schon geringe OT Mengen, um uterine Kontraktionen auszulösen. Das OTR Verteilungsmuster im Uterus zeigt eine entsprechend den physiologischen Anforderungen hohe Dichte an OTR im Fundus- und Korpusbereich und eine niedrigere Expression an Rezeptoren im unteren Uterinsegment sowie in der Zervix.
Während die Bindung von OT an den OTR in der Dezidua überwiegend über die Produktion von Diacylgycerol (DAG) und Arachidonsäure (AA) zur Produktion von uterotonen Prostaglandinen führt, kommt es im Myometrium im wesentlichen durch Bildung von IP_3 zur Freisetzung von intrazellulärem Kalzium und damit zur direkten Induktion von Kontraktionen (Abbildung 3).
Prostaglandine erhöhen die myometriale Sensitivität gegenüber OT, induzieren uterine Kontraktionen und sind darüber hinaus an der Zervixreifung beteiligt (Fuchs et al. 1981, Wilson et al. 1988, Soloff et al. 1993).
Leake et al. (1981) sowie Thornton et al. (1992) konnten nachweisen, dass die Serumkonzentration von OT im Menschen bereits vor Beginn der Wehentätigkeit deutlich ansteigt. Zum Zeitpunkt der Geburt wird OT sowohl zentral, als auch lokal im Amnion, Chorion und in der Dezidua in erhöhtem Maße produziert (Ikeda et al. 1987, Fuchs et al. 1991). Die myometriale Sensibilität gegenüber OT ist abhängig von der Rezeptordichte und der Affinität der OTR (Alexandrova und Soloff 1980, Fuchs et al. 1984), welche sowohl zum Zeitpunkt des berechneten Entbindungstermins, als auch bei Patientinnen mit vorzeitiger Wehentätigkeit deutlich erhöht sind (Bossmar et al. 1994, Takemura et al. 1994).

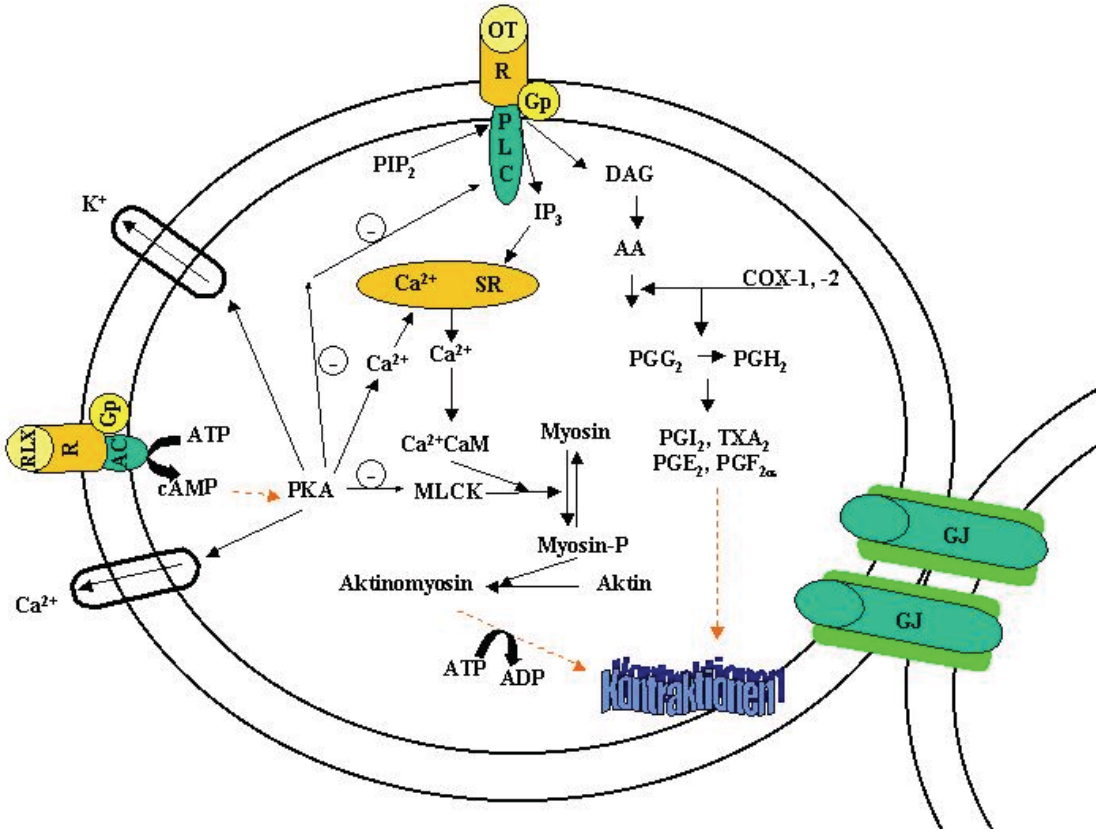


Abbildung 3: Signalkaskaden zur Relaxation und Kontraktion der glatten uterinen Muskelzelle. (AA) Arachidonsäure, (AC) Adenylzyklase, (ATP, ADP, cAMP) Adenosintriphosphat, -diphosphat, zyklisches -monophosphat, (Ca^{2+}) Calcium, (CaCaM) Kalzium-Kalmodulin-Komplex, (COX) Cyclooxygenase, (DAG) Diazylglycerol, (GJ) Gap Junctions, (Gp) G-Protein, (H) Hormon, (IP_3) Inositoltriphosphat, (K^+) Kalium, (MLCK) Myosin-Leichtketten-Kinase, (PKA) Phosphokinase A, (PLC) Phospholipase C, (PG) Prostaglandin, (R) Rezeptor, (SR) Sarkoplasmatisches Retikulum, (TXA_2) Thromboxan.
(Abbildung aus Friebe-Hoffmann et al. 2007b).

Cyclooxygenase und Prostaglandine

Die Cyclooxygenase (COX) spielt als Schlüsselenzym der Prostaglandinsynthese eine wichtige Rolle bei der Stimulierung der Wehentätigkeit. COX metabolisiert durch Phospholipase A_2 freigesetzte Arachidonsäure, die in der Phospholipidschicht der Zellmembran als Ester vorliegt, zu Prostaglandin G_2 (PGG_2) und Prostaglandin H_2 (PGH_2). Diese werden durch verschiedene Synthetasen zu aktiven Metaboliten wie PGD_2, PGE_2 und $PGF_{2\alpha}$, Thromboxan und Prostazyklin umgewandelt. Es existieren 2 Formen des Enzyms: eine konstitutiv exprimierte Form (COX-1) und eine

induzierbare Form (COX-2). Beide Formen werden sowohl im Myometrium als auch in materno-fetalen Membranen exprimiert. Während die Expression von COX-2 bei fortlaufender Wehentätigkeit signifikant ansteigt, kommt es lediglich zu einer moderaten Steigerung von COX-1 (Slater et al. 1999). Diese und andere Beobachtungen weisen darauf hin, dass COX-2 hauptverantwortlich für die im Rahmen der Wehentätigkeit auftretende gesteigerte Produktion von Prostaglandinen zu sein scheint. Die COX-2 Expression wird durch Serum, Phorbolester oder verschiedene Zytokine induziert und durch Glukokortikoide inhibiert.

Prostaglandine (PG) haben im Uterus vielfältige Wirkungen: sie lösen Kontraktionen im Myometrium aus, induzieren GJ und sind an der Zervixreifung beteiligt (O'Brien 1995). Die im Chorion exprimierte PG-metabolisierende Dehydrogenase verhindert während des überwiegenden Teils der Schwangerschaft den Übertritt der in den Membranen gebildeten PG zum Myometrium. Im Rahmen aszendierender Infektionen, bei denen es zu einer vermehrten Synthese von proinflammatorischen Zytokinen in den Eihäuten kommt, ist die Synthese dieses Enzyms vermindert, was zu einem vermehrten Durchtritt der PG zum Myometrium führt und Wehen auslösend wirkt. Dieser Mechanismus könnte auch bei der nicht infektinduzierten physiologischen Wehentätigkeit eine Rolle spielen.
Vom zweiten Trimenon an bis zur Geburt steigt die Konzentration von PG im Fruchtwasser kontinuierlich an. Ihre Produktion im Uterus wird durch Steroidhormone, eine Erhöhung der Estradiol/Progesteron Ratio und einer vermehrten Dehnung uteriner Gewebe im Rahmen des fetalen Wachstums, aber auch durch die myometriale Kontraktion selbst stimuliert.
Die selektive wie auch die unselektive Hemmung des Cyclooxygenase-Komplexes findet seine klinische Anwendung in der Tokolyse mit z.B. Indomethacin oder Nimensulid; eine effektive Therapie zur Hemmung der Wehentätigkeit, deren langfristiger Einsatz jedoch von erheblichen Nebenwirkungen für Mutter und Fetus begleitet wird (Monga et al. 1995).

Zytokine

Das reproduktive Gewebe des Menschen ist eine Quelle für inflammatorische Zytokine.
Bakterielle Lipopolysaccharide, aber auch andere Faktoren, wie die Dehnung des

Amnions, stimulieren die Produktion Zytokine wie Interleukin-1 beta (IL-1β), Tumor-Nekrose-Faktor alpha (TNFα) und Interleukin-6 (IL-6) in Dezidua, Chorion und Amnion. Zytokine sind intrazellulär produzierte Proteine mit Mediatorfunktion, die unter anderem von entzündungsaktiven Leukozyten freigesetzt werden. Im Rahmen der Schwangerschaft werden sie auch von Plazenta und den intrauterinen Membranen freigesetzt (Dudley et al. 1996). Neuere Studien konnten zeigen, dass Zytokine auch im Myometrium produziert werden (Friebe-Hoffmann et al. 2001 u. a.). Sie vermitteln ihre Wirkung in autokriner, parakriner oder juxtakriner Weise. Während IL-10, IL-1ra (IL-1 Rezeptorantagonist) und TGFβ Wehen hemmend wirken, haben IL-1β, -6, -8, -10, TNFα und GM-CSF eine kontraktionsfördernde Wirkung.

Bei vorzeitiger Wehentätigkeit werden inflammatorische Zytokine häufig in hohen Konzentrationen im Fruchtwasser gefunden (Romero et al. 1989, 1990, 1998) und führen dann zu einer Frühgeburt innerhalb von 7 Tagen. Wird jedoch kein IL-1β nachgewiesen, erfolgt die Entbindung erst zu einem späteren Zeitpunkt (Hillier et al. 1993). In Mäusen konnte eine Frühgeburt durch die alleinige systemische Gabe von IL-1 hervorgerufen werden (Romero et al. 1991). *In vitro* Studien von Romero et al. 1989 konnten zeigen, dass Deziduagewebe durch die Zugabe von Bakterien zur Produktion von IL-β angeregt werden kann.

Neben IL-1β scheint auch IL-6 eine wichtige Rolle bei der Regulation von Kontraktionen im graviden Uterus einzunehmen. Hohe Konzentrationen des Zytokins in Amnionflüssigkeit sowie mütterlichem Serum haben einen positiven Voraussagewert für das Vorliegen eines Amninoninfektionssyndroms, dem Versagen tokolytischer Maßnahmen und einer daraus resultierenden nachfolgenden Entbindung (Greig et al. 1993).

Neuere Studien zeigen, dass Zytokine auch bei der Induktion der regelrechten Geburt am Termin eine Rolle spielen (Steinborn et al. 1996). So konnte zum Zeitpunkt der Entbindung eine erhöhte Genexpression von IL-6 in den Eihäuten und im Myometrium gefunden werden, auch ohne dass Infektionszeichen vorlagen (Arntzen et al. 1997). Man muss sich somit die Frage stellen, welche Rolle Zytokinen bei der Regulation von Kontraktionen zukommt: sind sie Ursache oder Folge der Wehentätigkeit?

Als hochpotente Stimulatoren des Arachidonsäure-Stoffwechsels wirken Zytokine im Uterus durch Stimulation des Cyclooxygenase-Komplexes mit nachfolgender Produktion von uterotonen PG kontraktionsfördernd (Mitchell 1991). Verschiedene

Zytokine wie IL-1β sind in der Lage die Produktion von Kollagenasen, Metalloproteinasen und Hyaluronidasen in Chorion und der Zervix zu induzieren. Diese sind sowohl an der Induktion eines Blasensprungs als auch an der Zervixreifung beteiligt, welche beide gehäuft mit Wehentätigkeit assoziiert sind.

Relaxin

Relaxin (RLX), ein Polypeptid von 6kDa Größe gehört zur *Insulin-like-growth-factor-family*. Wie Insulin besteht es aus zwei Disulfid-Ketten A und B, die miteinander verbunden sind und von denen die B-Kette die für die Rezeptorbindung relevante Interaktionssequenz enthält. Die Synthese des Polypeptidhormons erfolgt bei der Frau hauptsächlich im Corpus luteum des Ovars. Während der Schwangerschaft wird RLX zusätzlich in der Plazenta und der Dezidua produziert. Erst kürzlich wurde die Identifikation der RLX spezifischer Rezeptoren veröffentlicht (Hsu et al. 2002), worüber RLX ein breites Spektrum an biologischen Aktivitäten vermittelt (Bani 1997). Es handelt sich hierbei um eine Gruppe von Leucin-reichen G-Protein assoziierten Rezeptoren, die seit 2006 die Namen RXFP-Rezeptoren (Relaxin-Peptid-Familie) tragen (Bathgate et al. 2006) und durch Bindung ihres Liganden zu einer Aktivierung der Adenylatzyklase führen mit nachfolgender Produktion von zyklischem Adenosin-Monophosphat (cAMP) (Sanborn et al. 1995) (Abbildung 3).

Während der Schwangerschaft spielt RLX eine wichtige Rolle in multiplen Adaptationsprozessen des mütterlichen Organismus. Im Verlaufe einer Gravidität stimuliert dieses Peptid das Wachstum und die Differenzierung der mütterlichen Brustdrüsen. Neben einer Lockerung der Bänderstrukturen im Bereich des Beckens bzw. Geburtskanals scheint es auch eine unterstützende Aufgabe bei der Zervixreifung zu haben (Goldsmith 1995). In tierexperimentellen Untersuchungen zeigte sich eine fundamentale Rolle des RLX bei der Vaskularisierung und Differenzierung des Endometriums zur Vorbereitung der Embryoimplantation (Vasilenko et al. 1986, Bani et al. 1995), als auch eine ausgeprägte Uterus relaxierende Wirkung (Sherwood et al. 1994). Für den Menschen ist ein Einfluss von RLX auf die PKA beschrieben, unter dem es zu einer Hemmung der OT induzierten PLC und damit einer Inhibition Uterus kontrahierender Signaltransduktionswege kommt (Zhong et al. 2005) (s. Abbildung 3).

In einer kürzlich erschienenen Studie zeigt sich eine Hemmung der durch proinflammatorische Zytokine stimulierten Aktivierung von humanen Neutrophilen

durch RLX (Masini et al. 2004). Somit scheint RLX einen hemmenden Einfluss auf die Wirkung von Entzündungsmediatoren zu haben, was die Frage aufkommen lässt, inwieweit RLX die durch inflammatorische Mediatoren verursachte vorzeitige Wehentätigkeit beeinflussen kann.

3. Zielsetzung der Untersuchungen

Geburtsinduktion und –progression haben ihren Ursprung im komplexen Zusammenspiel systemischer sowie lokaler, sich im graviden Uterus befindlicher Mediatoren. Sowohl die Oxytozinrezeptor (OTR)-Signalkaskade als auch das Cyclooxygenase (COX)-System mit der nachfolgenden Produktion von kontraktionsfördernden Prostaglandinen nehmen hierbei eine zentrale Rolle ein.
In einer Serie von *in vitro* Experimenten sollte der Einfluß von inflammatorischen Zytokinen (IL-1β, IL-6) auf den OTR und seine nachfolgende Signalkaskade in humanen Myometriumzellen untersucht werden (Teil 1).
Da IL-1β während der Schwangerschaft hauptsächlich von den in die Dezidua einwandernden Makrophagen sowie dezidualen Stromazellen produziert wird, wurden Teile der im ersten Abschnitt durchgeführten Untersuchungen auch auf kultivierte Deziduazellen übertragen (Teil 2).
Schließlich sollte im dritten Teil der Experimentalserie mit Hilfe des in der Schwangerschaft lokal produzierten Polypeptidhormons Relaxin überprüft werden, ob ähnliche Regulationsmechanismen für die OTR Signalkaskade bzw. das COX-Systems auch außerhalb inflammatorischer Prozesse gelten (Teil 3).

Die Ergebnisse der hier vorgestellten Originalarbeiten stellen Bausteine innerhalb der im graviden Uterus kontraktionsregulierenden Signalkaskaden dar. Ihre Kenntnis dient als Grundlage der Entwicklung geeigneter Interventionstherapien mit dem Ziel der Hemmung kontraktionsfördernder Prozesse und damit der Regulation unerwünschter vorzeitiger Wehentätigkeit und der damit verbundenen perinatalen Mortalität und Morbidität.

4. Material und Methoden

4.1. *Untersuchungen zum Einfluss von IL-1β und IL-6 auf die OT Sekretion und die Expression des OTR in humanen Myometriumzellen.*

4.1.1 *Primärkultur und Zytokine*

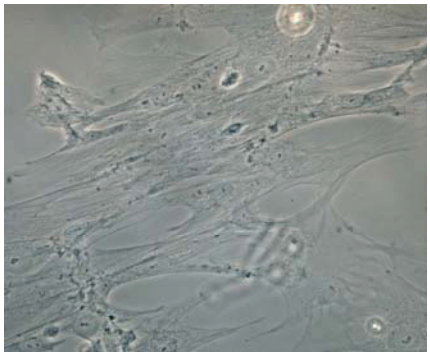
Nach zuvor eingeholter schriftlicher Einverständniserklärung wurde Myometriumgewebe gemäß den Bedingungen des Institutional Review Board (IRB) des Magee-Womens Hospital, Pittsburgh, PA, USA sowie der Ethikkommission des Universitätsklinikums Düsseldorf im Rahmen elektiver Kaiserschnitte am Termin (37.-42. SSW) gewonnen.

Nach Zerkleinerung und Verdauung des Gewebes mittels Kollagenase P und DNAse wurden die Zellen in die Kultur gebracht und mit Nährmedium MEM versetzt. Etablierte Primärkulturen wurden dann über verschiedene Zeiträume (0 -24h) je nach Versuchsanordnung mit rhIL-1β (5ng/ml), rhIL-6 (1ng/ml) versetzt. Als Kontrollen dienten Zellen, die über verschiedene Zeiträume allein mit Nährmedium kultiviert wurden.

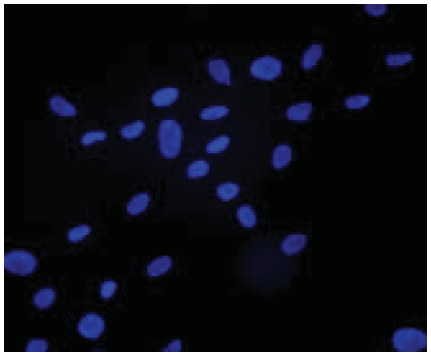
Die eingesetzten Zytokinkonzentrationen orientieren sich an denen, die im Fruchtwasser Schwangerer mit vorzeitiger Wehentätigkeit und intrauterinen Infektionen gefunden wurden und nehmen Bezug auf zuvor veröffentlichte Stimulationsversuche (Hertelendy et al. 1993, Todd et al 1996).

4.1.2 *Myometriumkultur*

Die Reinheit der Zellkulturen für Myometrium wurde mit Hilfe eines monoklonalen Antikörpers für Muskel α-Aktin überprüft (Santa Cruz Biotechnology, Santa Cruz,CA) (Abbildung 4).



A



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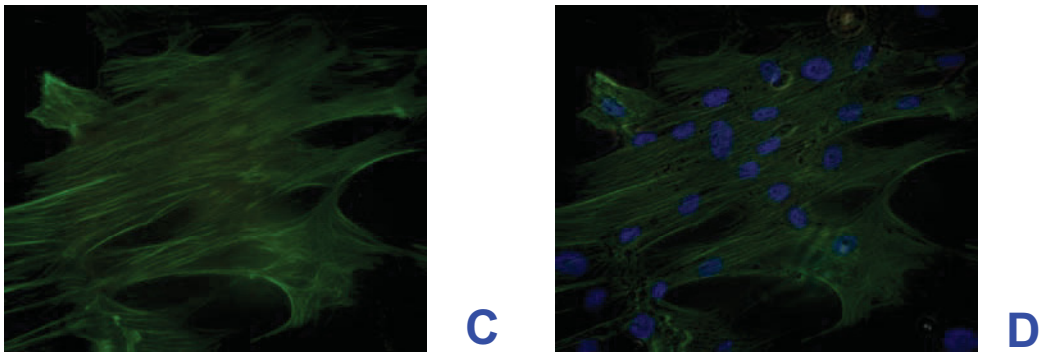


Abbildung 4: Immunhistochemie von Primärkulturen humanen Myometriums: A) MC im Phasenkontrastmikroskop 40x. B) DAPI Färbung. C) α-Actin Färbung. D) „Doppelfärbung".

4.1.3 *OT im Überstand*

Die OT-Peptidsekretion wurde im Überstand der Myometriumzellen unter dem Einfluss von IL-1β und IL-6 mittels Radioimmunoassay (Peninsula Laboratories Inc., Belmont, CA, USA) bestimmt. Das Prinzip des Radioimmunoassays beruht auf der kompetitiven Bindung von ^{125}I-markiertem OT sowie zellulär sezernierten Peptids an einen spezifischen OT-Antikörper (Friebe-Hoffmann et al. 2001a).

4.1.4 *IL-6 mRNA*

Modifiziert nach Chirgwin et al. (1979) erfolgte die Bestimmung der myometrialen IL-6 mRNA nach Stimulation der Zellen mit IL-1β mittels Guanidin-Isothiozyanat-Extraktion sowie anschließender RT-PCR und Southern Blotting (Friebe-Hoffmann et al. 2001a).

4.1.5 *IL-6 Protein Sekretion*

Zur Messung der IL-1β induzierten IL-6 Sekretion wurden Myometriumzellen über verschiedene Zeiträume mit IL-1β versetzt. Die Sekretion von IL-6 Protein in den Überstand wurde mittels IL-6 ELISA-Assay (Quantikine, R&D, Minneapolis, MN, USA) nach Herstellerangaben durchgeführt (Friebe-Hoffmann et al. 2001a).

4.1.6 *OTR Immunzytochemie*

Der qualitative Nachweis der OTR Expression an der Zelloberfläche nach Inkubation

mit IL-1β oder Kontrollmedium erfolgte mit Hilfe eines primären monoklonalen Maus-OTR-Antikörpers (O-2F8, Rohto Laboratories, Japan) (Rauk und Friebe-Hoffmann 2000).

4.1.7 *OTR Anzahl und Bindungskapazität*

Der Einfluss von IL-1β und IL-6 auf die Regulation der OTR Anzahl pro Zelle sowie die OTR Bindungskapazität wurde mittels Radioliganden-Bindungs-Assay bestimmt. Hierbei konkurriert ein radioaktiv markierter, spezifischer und kompetitiver OTR Antagonist (^{125}I-Ornithin-Vasotocin, New England Nuclear, USA) mit endogenem OT um die auf der Zelloberfläche lokalisierten OTR Rezeptoren (Rauk und Friebe-Hoffmann 2000; Rauk, Friebe-Hoffmann et al. 2001).

4.1.8 *OTR mRNA*

Die kultivierten Myometriumzellen wurden zunächst mit den beiden o. g. inflammatorischen Zytokinen inkubiert. Für die Bestimmung eines möglichen Einflusses von Phophorylierungswegen wurden zur Untersuchung der OTR mRNA in einigen Versuchen den Zellen neben IL-6 der Tyrosinkinaseinhibitor Genistein (100µl/ml) und der Phosphokinase C Inhibitor H7 (100µM) H7 zugesetzt.

Modifiziert nach Chirgwin et al. (1979) erfolgte die Bestimmung der myometrialen OTR mRNA nach Stimulation der Zellen mittels Guanidin-Isothiozyanat-Extraktion sowie anschließender RT-PCR und Southern Blotting sowie radioaktiver ^{32}P-*in vitro* Hybridisierung (Rauk und Friebe-Hoffmann 2000, Rauk, Friebe-Hoffmann et al. 2001).

4.2. *Untersuchungen zum Einfluß von IL-1β auf die OT/OTR Signalkaskade in humanen Deziduazellen.*

4.2.1 *Primärkultur*

In einer der Myometriumgewinnung (s. Kapitel 4.1.1) entsprechender Weise wurde Dezidua im Rahmen elektiver Kaiserschnitte am Termin (37.-42. SSW) gewonnen.

Nach Zerkleinerung und Verdauung des Gewebes mittels Kollagenase III und DNAse wurden die Zellen in die Kultur gebracht und mit Nährmedium DMEM versetzt. Etablierte Primärkulturen wurden dann über verschiedene Zeiträume (0-24h) mit rhIL-1β (5ng/ml) versetzt. Als Kontrollen dienten Zellen, die über verschiedene

Zeiträume allein mit Nährmedium kultiviert wurden.

4.2.2 *Deziduakultur*

Die Reinheit der Zellkulturen für Dezidua wurden mit Hilfe monoklonaler Antikörper gegen Vimentin überprüft (Dako Cytomation, Hamburg) (Abbildung 5).

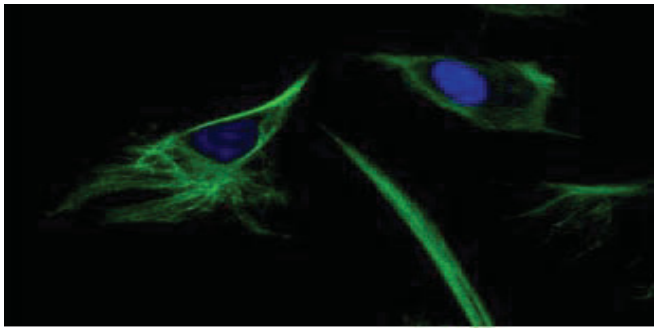


Abbildung 5: Immunhistochemie von Primärkulturen humaner Dezidua mit Vimentinfärbung.

4.2.3 *OT Peptid*

Sowohl intrazelluläres als auch sezerniertes OT Peptid im Überstand wurden unter dem Einfluss von IL-1β mittels Radioimmunoassay (Peninsula Laboratories Inc., Belmont, CA, USA) bestimmt.

4.2.4 *OTR mRNA*

Zunächst erfolgte eine Inkubation kultivierter Deziduazellen mit IL-1β über verschiedene Zeiträume (0, 2, 4, 6, 8, 16, 24h). Die Bestimmung der dezidualen OTR mRNA wurde modifiziert nach Chirgwin et al. (1979) mittels Guanidin-Isothiozyanat-Extraktion, RT-PCR, Southern Blotting sowie radioaktiver ^{32}P-*in vitro* Hybridisierung durchgeführt.

4.2.5 *OTR Anzahl und Bindungskapazität*

Der Einfluss von IL-1β auf die Regulation der OTR Anzahl pro Zelle sowie der OTR Bindungskapazität wurde wie unter **4.1.7** beschrieben mittels Radioliganden-Bindungs-Assay bestimmt.

4.2.6 *Inositoltriphosphat*

Zur Bestimmung der OT vermittelten Produktion des „second messengers“ IP_3 unter dem Einfluss von IL-1β wurden etablierte deziduale Primärkulturen für 24h mit IL-1β sowie radioaktivem ^{3}H-Myoinositol (Substrat für die intrazelluläre Produktion von IP_3) inkubiert. Im Anschluss erfolgte eine Inkubation der Zellen mit verschiedenen OT Konzentrationen (1-5000nM). Nicht eingebaute Radioaktivität wurde mit Hilfe einer Säulenextraktion ausgewaschen und die eingebaute Radioaktivität, die der Menge an neu produziertem IP_3 entsprach, mittels Szintillationszähler ermittelt.

4.2.7 *Arachidonsäure*

Für die Messung der OT vermittelten Arachidonsäure (AA) Freisetzung wurden die Primärkulturen für 24h mit IL-1β sowie radioaktiv markierter ^{3}H-AA versetzt. Es folgte die Auswaschung nicht inkorporierter AA. Nach Inkubation der Zellen mit verschiedenen Konzentrationen an OT (1-500nM) erfolgte die Ermittlung der freigesetzten AA nach mehreren Waschschritten mit Hilfe eines Szintillationszählers.

4.2.8 *Prostaglandin E_2*

Nach 24stündiger IL-1β Inkubation wurden die Deziduazellen über 3h mit verschiedenen OT Konzentrationen (1-500nM) versetzt. PGE_2 wurde mit einem ELISA-Assay detektiert (Amersham Pharmacia Biotech, Airlington Heights,IL, USA). Es ist bekannt, dass IL-1β im Myometrium die Produktion von Prostaglandinen über die induzierbare Form der Cyclooxygenase (COX-2) beeinflusst. Zur Überprüfung dieser Hypothese, wurden die hier beschriebenen Versuche zusätzlich unter Inkubation der Zellen mit dem selektiven COX-2 Enzyminhibitor NS-398 durchgeführt.

Alle hier genannten Methoden sind ausführlich in der Arbeit von Friebe-Hoffmann et al. (2007a) beschrieben.

4.3 *Untersuchungen zur Regulation des OTR in humanen Myometriumzellen durch das schwangerschaftsassoziierte Polypeptidhormon RLX.*

4.3.1 *Primärkultur und RLX*

Etablierte myometriale Primärkulturen wurden über verschiedene Zeiträume (0, 2, 4, 6, 8, 16, 24, 48, 72, 96h) mit rhRLX H2 (5µg/ml) versetzt. Als Kontrollen dienten

Zellen, die über verschiedene Zeiträume allein mit Nährmedium kultiviert wurden.
Über die lokalen Konzentrationen von RLX im uterinen Gewebe in der Schwangerschaft gibt es bislang in der Literatur keine Angaben. Die bei den beschriebenen Stimulationsversuchen gewählten Konzentrationen orientieren sich an vorausgegangenen Publikationen (z.B. Lopez-Bernal et al. 1987).

4.3.2 *OTR mRNA*

Kultivierte Myometriumzellen wurden zunächst über verschiedene Zeiträume (0-96h) mit RLX inkubiert. Die Bestimmung der myometrialen OTR mRNA erfolgte wie unter **4.1.8** beschrieben mittels Guanidin-Isothiozyanat-Extraktion, RT-PCR, Southern Blotting sowie radioaktiver ^{32}P-*in vitro* Hybridisierung.

4.3.3 *OTR Anzahl und Bindungskapazität*

Der Einfluss von RLX auf die Regulation der OTR Anzahl pro Zelle sowie der OTR Bindungskapazität wurde wie unter **4.1.7** beschrieben mittels Radioliganden-Bindungs-Assay bestimmt.

4.3.4 *OTR Protein*

Die Detektion der OTR Proteinexpression an der Zelloberfläche der Myometriumzellen nach Inkubation mit RLX bzw. Kontrollmedium erfolgte durchflußzytometrisch unter Verwendung von Cy3-Fluoreszenzfarbstoff konjugierten Antikörpern (Jackson Immuno Research Inc. West Grove, PA, USA) wie in Friebe-Hoffmann et al. (2007b) beschrieben.

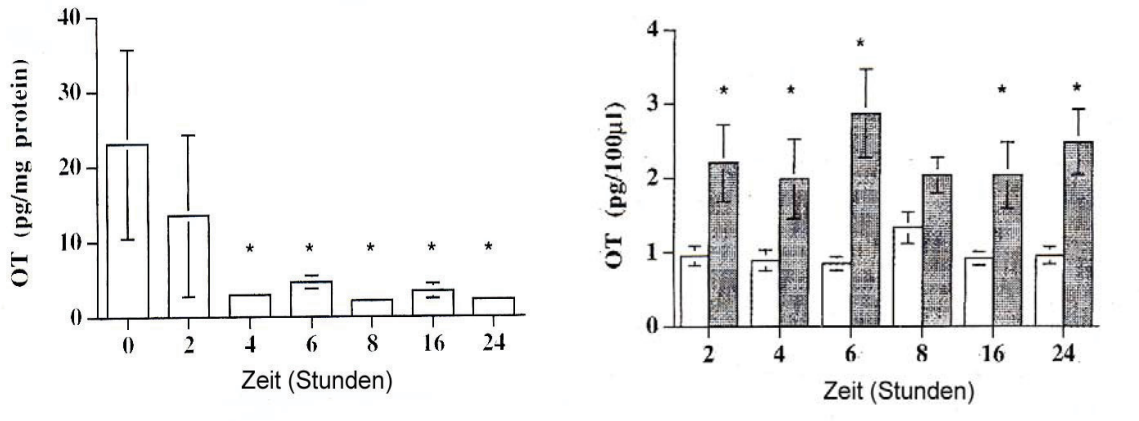
Statistik

Die statistische Auswertung erfolgte in allen Arbeiten für die wiederholten Messungen der einzelnen Inkubationen mittels ANOVA (ANOVA Microsystems Inc. San Jose, CA, USA), zur Evaluation der Differenzen der unterschiedlichen Inkubationsbedingungen mittels Student`s *t*-test. Ein signifikanter Unterschied bestand ab einem $P < 0.05$.

5. Ergebnisse und Diskussion

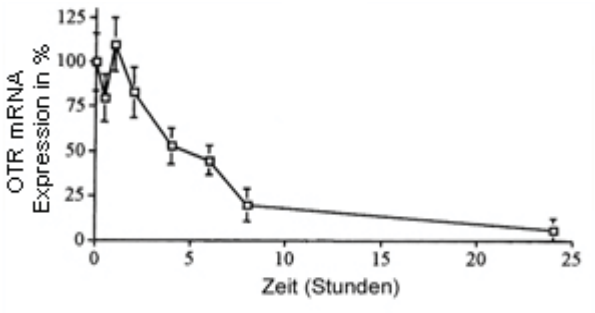
5.1 *Untersuchungen zum Einfluss von IL-1β und IL-6 auf die OT Sekretion und die Expression des OTR in humanen Myometriumzellen.*

Im Nachfolgenden werden die ersten drei Arbeiten der Habilitationsschrift zusammenfassend beschrieben und diskutiert. Die Stimulation der humanen Myometriumzellen mit IL-1β führte zunächst zu einer Steigerung der OT-Peptid Sekretion in den Überstand bei gleichzeitiger Reduktion der intrazellulären OT Konzentration (Abbildung 6). Dieser folgte eine zeitabhängige Minderung der OTR mRNA Expression, welche in einer Verminderung der OTR-Bindungskapazität durch Minimierung der OTR der Zahl pro Zelle resultierte (Abbildung 7). Die OTR Bindungsaffinität für den einzelnen Rezeptor gegenüber seinem Liganden OT änderte sich hingegen nicht.

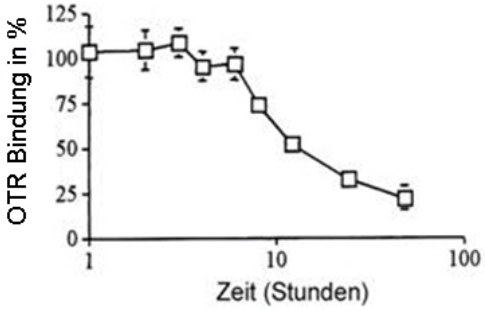


A. **B.**

Abbildung 6: **A**. Intrazelluläre OT Peptid Konzentration **B.** Extrazelluläre OT Peptid Konzentration (*helle Balken: Kontrollen, dunkle Balken: IL-1β Stimulation)* in humanen Myometriumzellen nach Inkubation mit IL-β (5ng/ml) über verschiedene Zeiträume (0-24h). Die Ergebnisse sind dargestellt als Mittelwert ± Standardabweichung (n=6). * $p < 0.05$.



A.



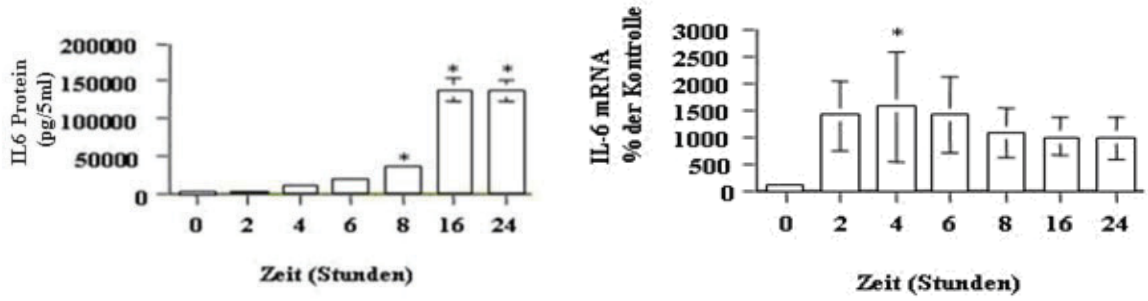
B.

Abbildung 7: **A.** OTR mRNA Expression **B.** OTR Bindung in humanen Myometriumzellen nach Inkubation mit IL-β (5ng/ml) über verschiedene Zeiträume (0-24h bzw. 0-48h). Die Ergebnisse sind dargestellt als Mittelwert ± Standardabweichung (n=5).

Die oben beschriebenen Ergebnisse könnten Ausdruck eines negativen „feedback"-Mechanismus zwischen dem OTR und seinem Liganden sein, bei dem die vermehrte OT Sekretion in den Extrazellularraum zu einer sekundären Herunterregulierung des OTR Rezeptors führt. In der Vergangenheit konnte gezeigt werde, dass IL-1β in humanen Myometriumzellen *in vitro* die OTR Signalkaskade durch Minderung der IP_3-Produktion und eine Hemmung der AA Freisetzung inhibiert (Rauk und Chiao 2000a). Andererseits ist IL-1β aber in der Lage über die Induktion der COX-2 Expression unabhängig von seinem Einfluss auf den OTR die PG Produktion in humanem Myometrium zu stimulieren (Rauk und Chiao 2000b).
Klinische Beobachtungen zeigen, dass Frauen mit einem Amnioninfektionssyndrom häufig auf die intravenöse Gabe von OT zur Geburtseinleitung nicht ansprechen. Bei den Betroffenen findet sich auch eine erhöhte Rate an postpartalen Atonien und Hämorrhagien, die durch exogenes OT kaum beeinflussbar ist. Die in den vorliegenden *in vitro* Experimenten beobachtete Herunterregulierung der OTR im Myometrium durch IL-1β ist mit dieser klinischen Beobachtung in Einklang zu bringen.

Parakrine und autokrine Zytokininteraktionen sind Vorgänge die auch im reproduzierenden Gewebe eine große Rolle spielen. Es konnte gezeigt werden, dass IL-1β die Produktion des IL-6 reguliert (Dinarello et al. 1991), was in der vorliegenden Arbeit auch für das myometriale Zellkulturmodell nachgewiesen werden konnte. IL-1β

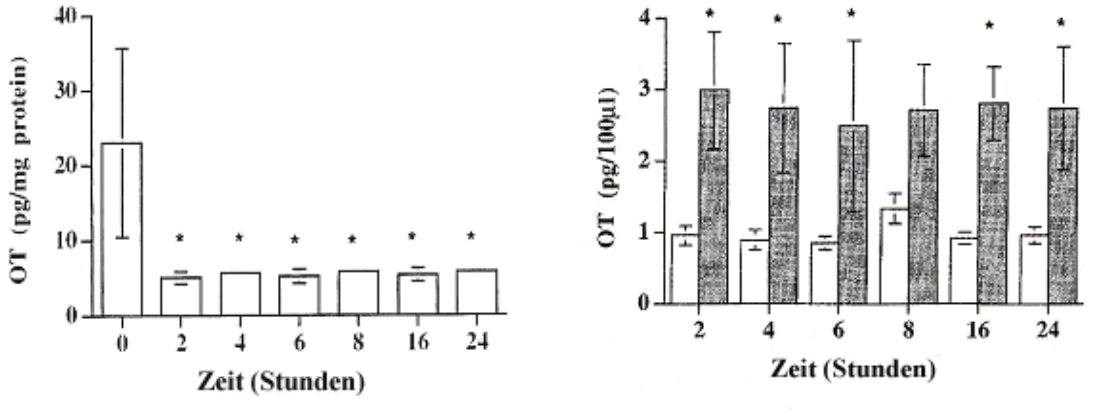
stimulierte die Sekretion von IL-6 in den Überstand der glatten Muskelzellen bei gleichzeitiger Stimulation der IL-6 mRNA Expression (Abbildung 8).



A. **B.**

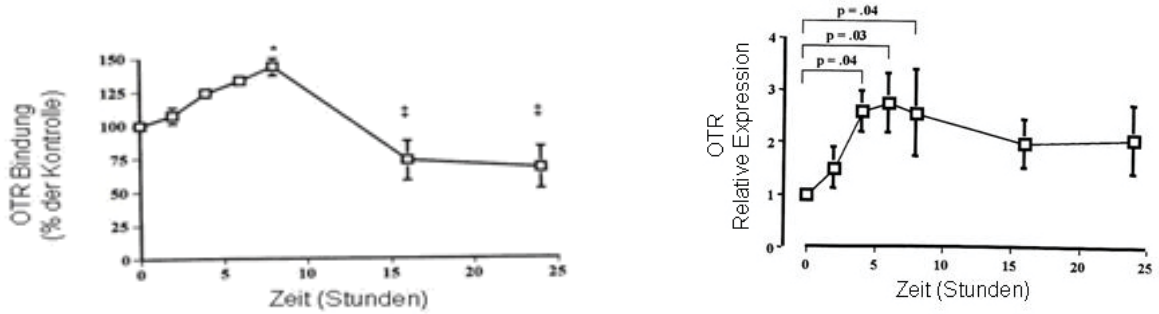
Abbildung 8: **A**. IL-6 Proteinsekretion in den Zellüberstand und **B**. IL-6 mRNA Expression humaner Myometriumzellen nach Inkubation mit IL-1β (5ng/ml) über verschiedene Zeiträume (0-24h). Die Ergebnisse sind dargestellt als Mittelwert ± Standardabweichung (n=6). * $p < 0.05$.

Unter dem Einfluss von IL-6 steigerte sich die OT Sekretion in den Überstand bei gleichzeitiger Verminderung der intrazellulären OT Konzentration (Abbildung 9). Dies steht in Einklang mit den Ergebnissen zum Einfluss von IL-1β auf OT. Entgegen den Erwartungen und im Gegensatz zu der Wirkung von IL-1β auf den OTR erhöhte sich jedoch die OTR mRNA Expression im zeitlichen Verlauf nach Stimulation mit IL-6 signifikant. Auch die OTR-Bindungskapazität nahm zu (Abbildung 10), wobei sich auch hier die Affinität des einzelnen Rezeptors im Scatchard Plot nicht veränderte.



A. **B.**

Abbildung 9: **A**. Intrazelluläre OT Konzentration **B**. Extrazelluläre OT Konzentration (*helle Balken: Kontrollen, dunkle Balken: IL-1β Stimulation)* nach Inkubation mit IL-6 (1ng/ml) in humanen Myometriumzellen über verschiedene Zeiträume (0-24h). Die Ergebnisse sind dargestellt als Mittelwert ± Standardabweichung (n=6). * $p < 0.05$.



A. **B.**

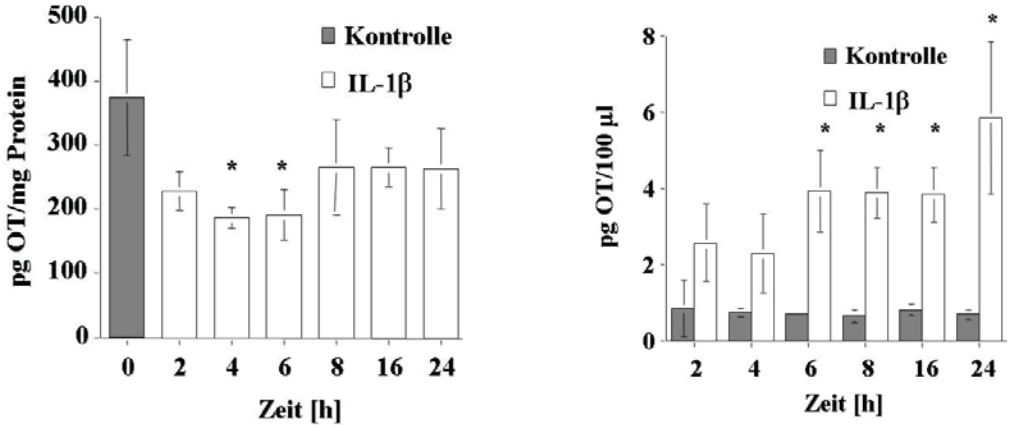
Abbildung 10: **A**. OTR Bindung *(* $p < 0.02$ verglichen mit 0h; ‡ $p < 0.02$ verglichen mit 8h)* **B**. OTR mRNA Expression *(Signifikanz der Ergebnisse wie mittels ANOVA evaluiert)* nach Inkubation mit IL-6 (1ng/ml) in humanen Myometriumzellen über verschiedene Zeiträume (0-24h). Die Ergebnisse sind dargestellt als Mittelwert ± Standardabweichung (n=6).

Um festzustellen, ob Phosphorylierungswege einen Einfluss bei der Steigerung der OTR Expression haben, wurden die Versuche unter der Zugabe des Thyrosinkinaseinhibitors Genistein (100µl/ml) und dem Proteinkinase C Inhibitor H7

(100µM) wiederholt. Beide Faktoren führten zu einer Verminderung der IL-6 vermittelten OTR Expressionssteigerung, was darauf hindeutet, dass IL-6 die uterine OTR mRNA Expression durch Tyrosin- als auch Serinphosphorylierungswege stimuliert, die den nukleären Faktor Stat-3 mit einschließen könnten.
Die ansteigende Sensitivität des Uterus gegenüber OT am Entbindungstermin hängt vor allem von der vermehrten OTR Expression unmittelbar vor Beginn der Wehentätigkeit ab (Fuchs et al. 1984). Diese findet sich nicht nur bei Kontraktionen am Termin, sondern auch bei Wehen weit vor dem errechneten Entbindungsdatum, wie eine retrospektive Analyse zeigen konnte (Takahashi et al. 1980). Anders als IL-1β stimuliert IL-6 sowohl die Verfügbarkeit von OT im Gewebsverband als auch die Expression des OTR. Dies könnte darauf hinweisen, dass IL-6 möglicherweise noch eine bedeutendere Rolle bei der Induktion von Wehen im Rahmen intrauteriner Infektionen zukommt als IL-1β. Die *in vivo* Stimulation der myometrialen OTR Expression durch IL-6 mag ein Grund dafür sein, dass vermehrte IL-6 Konzentrationen im Fruchtwasser einen hohen prädiktiven Wert für das Fehlschlagen tokolytischer Maßnahmen sowie für die daraus resultierende Frühgeburt haben (Romero et al 1993).
Alle hier vorgestellten Ergebnisse sind ausführlich in den Originalarbeiten von Rauk und Friebe-Hoffmann 2000; Friebe-Hoffmann et al. 2001 und Rauk, Friebe-Hoffmann et al. 2001 beschrieben.

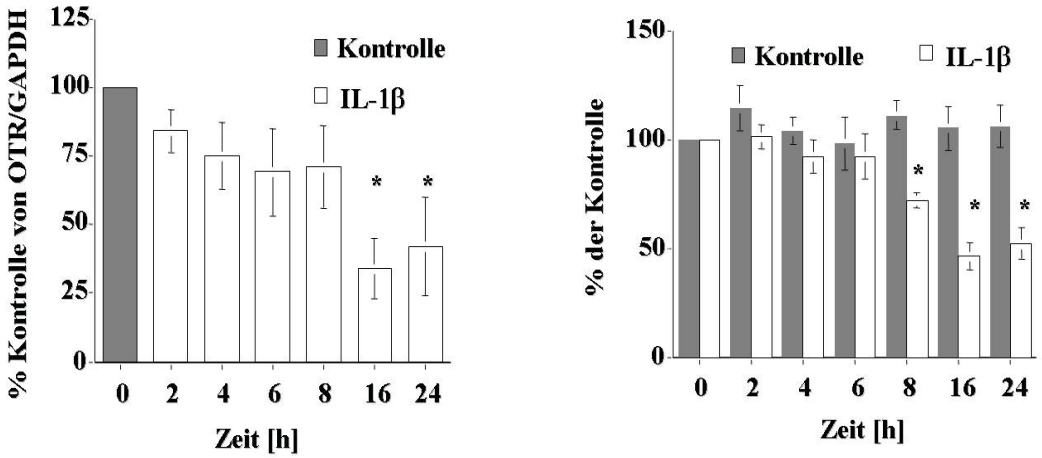
5.2 *Untersuchungen zum Einfluß von IL-1β auf die OT/OTR Signalkaskade in humanen Deziduazellen.*

In diesem Teil der Arbeit wurden die Untersuchungen zur Wirkung von IL-1β auf die OT/OTR Signalkaskade in Zelllinien humaner Dezidua durchgeführt. Die in die Dezidua einwandernden Makrophagen sind die Hauptproduktionsstätte von IL-1β im schwangeren Uterus. Analog zu den Ergebnissen in Myometrium zeigte sich in den Versuchen eine inkubationsabhängige Stimulation der OT Sekretion bei gleichzeitiger Minderung des intrazellulären OT Peptid Anteils (Abbildung 11). Nachfolgend kam es zu einer Hemmung der OTR mRNA über die Zeit, welches sich in einer Einschränkung der OTR Bindungskapazität ohne Verlust der Bindungsaffinität des einzelnen Rezeptors wieder spiegelte (Abbildung 12).



A. **B.**

Abbildung 11: **A**. Intrazelluläre und **B**. extrazelluläre Konzentration von OT Peptid nach Inkubation mit IL-1β in humanen Deziduazellen.



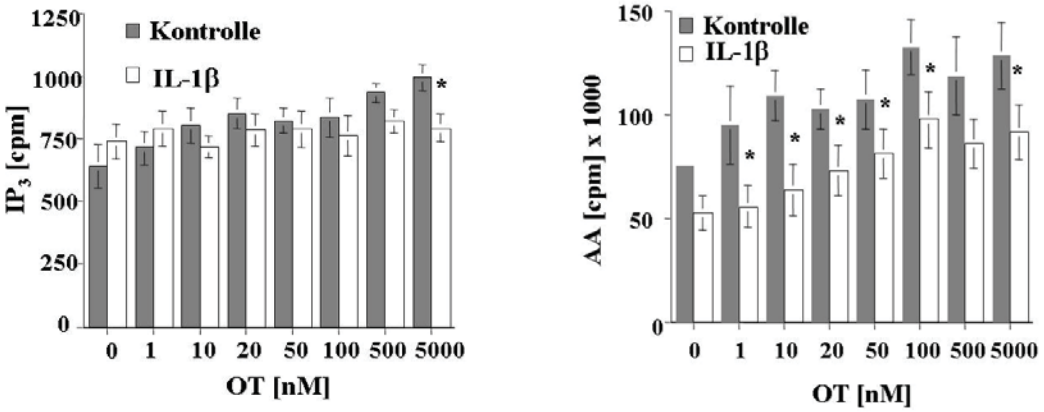
A. **B.**

Abbildung 12: **A**. OTR mRNA Expression und **B**. OTR Bindung nach Inkubation mit IL-1β in humanen Deziduazellen.

Der Minderung der OTR Expression durch IL-1β folgte mit zunehmender OT Konzentration eine Inhibition der OTR Signalkaskade. Während ansteigende OT Konzentrationen die Produktion von IP_3 marginal steigerten, veränderte sich die IP_3-Produktion in Deziduazellen unter dem Einfluss von IL-1β nicht. Die Freisetzung von AA hingegen sank in den Deziduazellen unter dem Einfluss von IL-1β mit zunehmender OT Konzentration signifikant (Abbildung 13). Dies unterstreicht, das

der IP_3 Signalweg mit nachfolgender Freisetzung von intrazellulärem Ca^{2+} eher von Relevanz für die kontraktionsfähigen Muskelzellen ist, während die AA Freisetzung mit nachfolgender PG Produktion von größerer Wichtigkeit für die mütterliche Eihaut zu sein scheint.

Entgegen den eigenen Erwartungen nahm die PGE_2 Produktion in den Deziduazellen mit zunehmender OT Konzentration unter IL-1β zu (Abbildung 14). Demgegenüber führte die alleinige Inkubation mit OT zu keiner Steigerung der PG Synthese. Dies steht in Einklang mit einer Studie von Fuchs et al. (1984), die nach OT Inkubation keinen relevanten Anstieg der $PGF_{2\alpha}$ sowie PGE_2 Produktion in humanen Deziduazellen beschrieb. Die Produktion von PG konnte im vorliegenden Modell durch den spezifischen COX-2 Inhibitor NS-398 vollständig geblockt werden, woraus sich schließen lässt, dass COX-2 für die dezidуale PG Produktion essentiell zu sein scheint.



A. **B.**

Abbildung 13: **A**. IP_3 Produktion und **B**. AA Freisetzung unter verschiedenen OT Konzentrationen nach 24h Inkubation mit IL-1β.

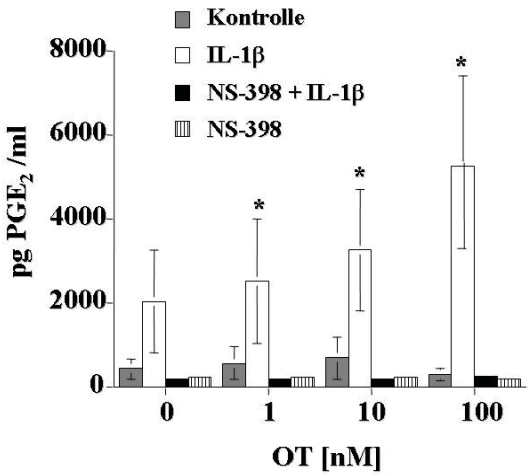


Abbildung 14: PGE2 Produktion in humanen Deziduazellen nach Inkubation mit IL-1β über 24h und verschiedenen OT Konzentrationen.

Diese Ergebnisse sind kongruent zu den Resultaten, die unter **5.1** für humane Myometriumzellen beschrieben wurden. Sie unterstützen die dort formulierten Thesen zur Unterhaltung vorzeitiger Wehentätigkeit über die OTR Signalkaskade. Somit gelten in Bezug auf einen der zentralen Signalwege der uterinen Muskelkontraktion vergleichbare Regulationsmechanismen in der dem Myometrium anliegenden mütterlichen Eihaut und man muss davon ausgehen, dass die in der Dezidua produzierten PG über parakrine Mechanismen am Myometrium wirksam werden. Wann jedoch der eine oder andere Signalweg an- bzw. abgeschaltet wird bleibt weiterhin ungeklärt.

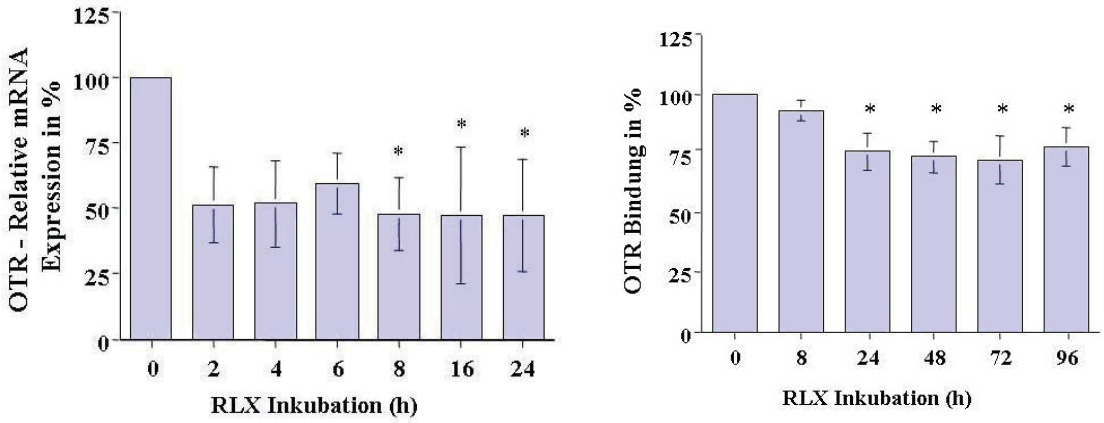
Alle hier beschriebenen Ergebnisse sind ausführlich in der Arbeit von Friebe-Hoffmann et al. (2007a) beschrieben.

5.3 *Untersuchungen zur Regulation des OTR in humanen Myometriumzellen durch das schwangerschaftsassoziierte Polypeptidhormon RLX.*

In humanen *in vitro* Versuchen sowie im Tiermodell konnte gezeigt werden, dass das Polypeptidhormon Relaxin (RLX) über das cAMP-Proteinkinase-A-System eine relaxierende Wirkung auf Myometriumzellen vermittelt (Dodge und Sanborn 1998). Angenommen wurde eine direkte Inhibition der PLC mit nachfolgender

Spaltungshemmung von PIP_2 in IP_3 und DAG (siehe Abbildung 3). In den nachfolgend beschriebenen Versuchen wurde nun die mögliche Regulation des OTR durch das schwangerschaftsassoziierte Polypeptidhormon RLX in humanen Myometriumzellen untersucht. Bindungsstudien zur Evaluation der OTR Bindungskapazität unter dem Einfluss von RLX wurden wie oben beschrieben durchgeführt und die OTR mRNA Expression mittels RT-PCR sowie Southern Blotting evaluiert. Erste durchflusszytometrische Untersuchungen gaben Aufschluss über die Proteinexpression des OTR in den untersuchten Zelllinien.

Ähnlich den inflammatorischen Zytokinen IL-1β und IL-6 führte die Inkubation mit RLX über die Zeit zu einer Verminderung der OTR mRNA Expression (Abbildung 15), welche zu einer verminderten OTR Proteinexpression in der Durchflußzytometrie führte (Abbildung 16). Dies spiegelte sich auch in einer verminderten Bindungskapazität, nicht aber der Bindungsaffinität des Rezeptors wieder (Abbildung 15).



A. **B.**

Abbildung 15: **A.** OTR mRNA Expression und **B.** OTR Bindung nach Inkubation mit RLX in humanen Myometriumzellen.

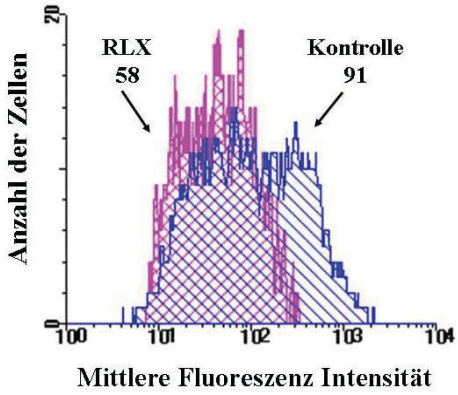
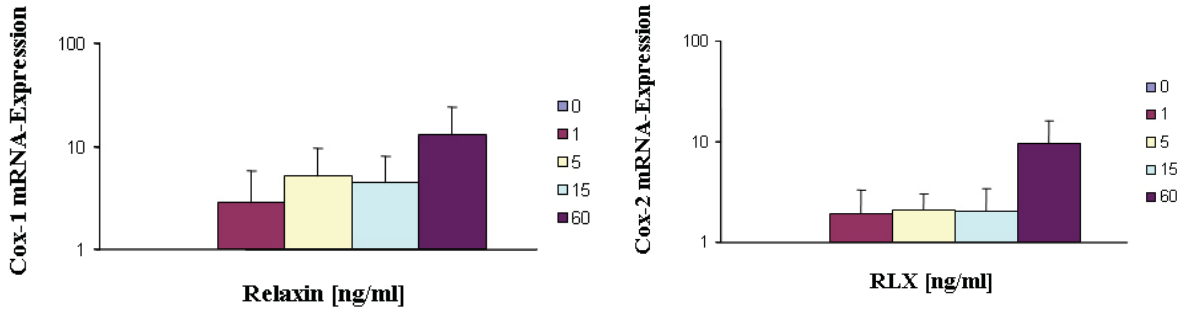


Abbildung 16: Repräsentatives Histogramm einer Myometriumzelllinie nach durchflusszytometrischer Messung der OTR Proteinexpression unter Inkubation mit RLX.

Alle hier beschriebenen Ergebnisse sind ausführlich in der Arbeit von Friebe-Hoffmann et al. (2007b) beschrieben.

In einem derzeit laufenden DFG Projekt (Fr 104/3-1) mit dem Titel „Untersuchungen zum Einfluss von Relaxin auf das humane Cyclooxygenasesystem im Rahmen vorzeitiger Wehentätigkeit“ konnte in ersten Versuchen gezeigt werden, dass es unter *in vitro* Bedingungen in humanen Myometriumzellen unter RLX Stimulation zu einer Erhöhung der COX-1 sowie COX-2 mRNA und Protein Expression kommt (Abbildungen 18, 19).



A. **B.**

Abbildung 18: **A.** COX-1 und **B.** COX-2 mRNA Expression in humanen Myometriumzellen nach Stimulation mit RLX Konzentrationen von 0-60 ng/ml.

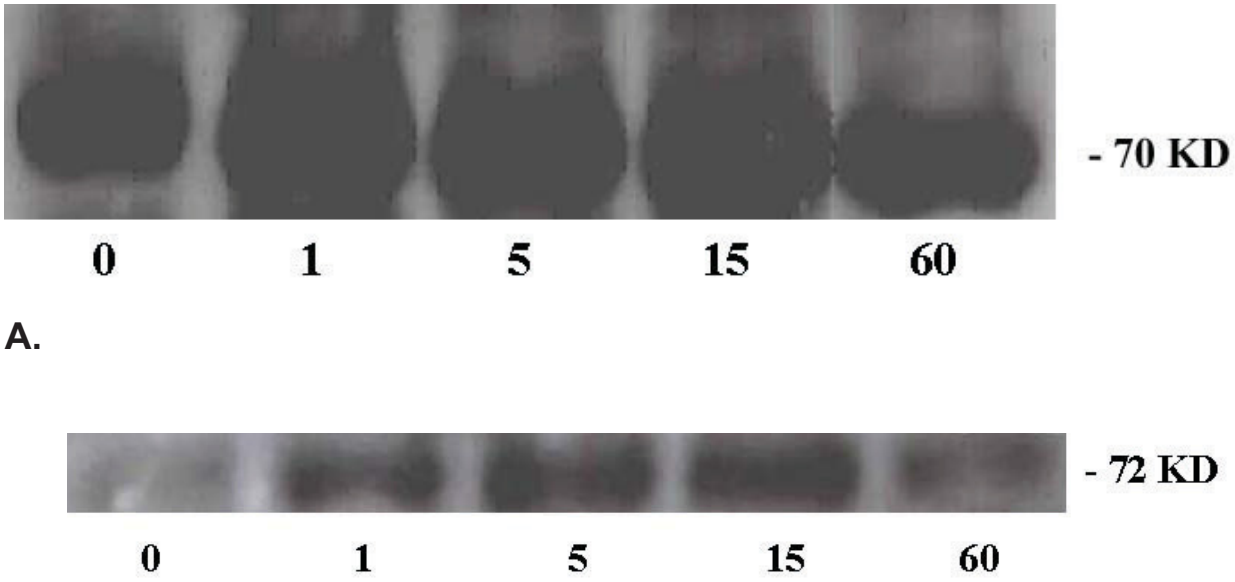


Abb. 19: Repräsentativer Western Blot für **A**. COX-1 Protein und **B**. COX-2 Protein in humanen Myometriumzellen nach Inkubation mit verschiedenen RLX (ng/ml) Konzentrationen.

Auch in Deziduazellen kam es unter RLX Stimulation zu einer ähnlichen, wenn auch nicht ganz so ausgeprägten Steigerung der COX-1 sowie COX-2 Expression auf mRNA bzw. Proteinebene. Dies lässt vermuten, dass RLX im Myometrium eine größere Rolle zu spielen scheint als in der mütterlichen Eihaut und die dort produzierten Mediatoren auf parakrinem Wege wirksam werden.

Die Zusammenschau dieser Ergebnisse zeigt Parallelen zu den oben beschriebenen Ergebnissen der IL-1β Stimulation. Auch hier kommt es zu einer Reduktion der OTR Zahl und nachfolgender Beeinträchtigung der OTR Signalkaskade, während durch eine Art „Bypass“ gleichzeitig die Stimulation des COX-Systems angeregt wird. Ob dies auch zu einer vermehrten Produktion kontraktiler PG führt, bleibt Gegenstand andauernder Forschungen.

6. Zusammenfassung und Ausblick

Die weltweite Rate an (zu) früh geborenen Kindern liegt bei etwa 5-10% und ist für den Großteil der perinatalen Mortalität und Morbidität verantwortlich. Etwa ein Drittel aller Frühgeburten ist Folge vorzeitiger Wehentätigkeit ohne erkennbare mütterliche oder fetale Pathologie. Im Rahmen infektgetriggerter Kontraktionen, aber auch bei Geburtswehen am Termin konnten proinflammatorische Zytokine vermehrt im Fruchtwasser gefunden werden und scheinen bei der Initiierung der Wehentätigkeit eine Rolle zu spielen.

Das Polypeptidhormon Relaxin ist an multiplen Adaptationsvorgängen des mütterlichen Organismus im Rahmen der Schwangerschaft beteiligt. In tierexperimentellen Untersuchungen konnte eine ausgeprägte Uterus relaxierende Wirkung von Relaxin nachgewiesen werden. Für den Menschen ist sein Einfluss auf die Proteinkinase A beschrieben, unter der es zu einer Inhibition Uterus kontrahierender Signaltransduktionswege kommt.

Oxytozin (OT) sowie sein spezifischer Rezeptor OTR spielen eine Schlüsselrolle im Prozess der Wehentätigkeit. Zum einen wird über den Rezeptor-Liganden-Komplex die Freisetzung von intrazellulärem Kalzium für die Muskelkontraktion bereitgestellt, zum anderen vermittelt er die Produktion kontraktiler Prostaglandine.

Vor diesem Hintergrund wurde in den vorliegenden Arbeiten der Einfluss der proinflammatorischen Zytokine IL-1β und IL-6 sowie dem Polypeptidhormon Relaxin (RLX) auf die OTR-Signalkaskade in humanen uterinen Muskelzellen (Effektororgan) sowie Deziduazellen (Hauptproduktionsstätte von Zytokinen im graviden Uterus) untersucht.

IL-1β führte in humanen Myometrium- sowie Deziduazellen zu einem Anstieg der OT Sekretion mit zeitversetzter Inhibition der OTR Expression und nachfolgender Signalkaskade. IL-6 stimulierte neben der OT Sekretion in den Überstand humaner Myometriumzellen jedoch auch die Expression des OTR.

Die Inkubation der Muskelzellen mit Relaxin führte wie unter IL-1β Stimulation zu einer Sekretionssteigerung von OT bei nachfolgender Inhibition der OTR Expression. Studien zu IL-1β und erste Ergebnisse eigener laufender Versuche mit Relaxin im humanen Myometrium zeigen, dass beide Substanzen über eine Stimulation des Cyclooxygenase-Komplexes zu einer vermehrten Produktion von kontraktilen Prostaglandinen führen. So werden auf der einen Seite Wehen hemmende Signale

vermittelt (OTR Inhibition), auf der anderen Seite die myometriale Kontraktion unterstützt (Stimulation von Prostaglandinen). Dies könnte erklären, warum im klinischen Alltag der Einsatz des OTR-Antagonisten Atosiban als Tokolytikum bei Infekt getriggerter vorzeitiger Wehentätigkeit versagt. Die Tatsache, dass diese Signalwege durch weitere Faktoren wie proinflammatorische Zytokine beeinflusst werden, verdeutlicht auch die Komplexität der molekularen Wehenregulation und muss bei der Planung zukünftiger Interventionstherapien zur Hemmung unerwünschter Wehentätigkeit berücksichtigt werden.

Die Zukunft der Wehenhemmung liegt mit großer Wahrscheinlichkeit in einer Kombination verschiedener Therapieansätze. Diese parallel oder in zeitlicher Abfolge eingesetzt, könnten simultan mehrere Wege der Wehenstimulation unterbinden. Denkbar wäre eine Kombination aus dem neuen hochspezifischen OTR Antagonisten Barusiban®, der derzeit in Phase III Studien getestet wird, und antiinflammatorischen Zytokinen wie dem IL-1Rezeptorantagonist zur Blockade der durch IL-1β getriggerte Produktion von Prostaglandinen.

Die weitere Identifikation von molekularen Wirkprinzipien der Wehentätigkeit, wie sie in der vorliegenden Habilitationsschrift für die Zytokine IL-1β, IL-6 sowie das Polypeptidhormon Relaxin und ihre Auswirkungen auf die OTR Signalkaskade durchgeführt wurde, wird hierfür Grundlage sein.

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Studium

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Zusatzqualifikationen

- Fachkunde *Sonographie der Brustdrüse*
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- *A-Diplom* für Akupunktur

Preise

- **3. Vortragspreis** für den Beitrag „In-vitro Untersuchungen zum Einfluss von IL-1β auf die Oxytozin-Oxytozinrezeptor Regulation und nachfolgende Signalkaskade in humanen Deziduazellen“: U. Friebe-Hoffmann, J. P. Chiao, P. N. Rauk anlässlich des 53. Kongresses der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe, München 14.-17. Juni, 2000
- **SGI Young Investigators Award** für den Beitrag “Secretory leukocyte protease inhibitor (SLPI) blocks interleukin-1 induced cyclooxygenase-2 expression and prostaglandin production in amnion cells” : D. C. Streitman, J.-P. Chiao, U. Friebe-Hoffmann, P. N. Rauk anlässlich des 48. Annual Meeting der Society for Gynecologic Investigation, Toronto, Canada 14.-17. März, 2001
- **AACR-AFLAC Scholar In Training Award** für den Beitrag "T cells specific for the wild-type sequence $p53_{264-272}$ epitope in the peripheral circulation of cancer patients-implications for immunoselection of epitope-loss tumor variants”: T. K. Hoffmann, A. D. Donnenberg, S. Finkelstein, H. Bier, K. Chikamatsu, U. Friebe-Hoffmann, V. Donnenberg, A. B. DeLeo, T. L. Whiteside anlässlich des 92. Annual Meeting der American Association of Cancer Research, New Orleans, USA, 24.-26. März, 2001
- **Milupa-Forschungsstipendium** für den Antrag „Untersuchungen zur Interaktion verschiedener Zytokine bei der Induktion der Oxytozinrezeptor-Signalkaskade“: U. Friebe-Hoffmann anlässlich des 54. Kongresses der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe, Düsseldorf 10.-14- September 2002

Mitgliedschaften

- Deutsche Gesellschaft für Gynäkologie und Geburtshilfe (DGGG)
- Arbeitsgemeinschaft Materno-fetale Medizin (AGMFM) der DGGG
- Deutsche Gesellschaft für Perinatale Medizin (DGPM)
- Niederrheinisch Westfälischen Gesellschaft für Gynäkologie und Geburtshilfe (NRWGGG)
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11. Eine Auswahl der Originalarbeiten

Im Anhang sind fünf Originalarbeiten angefügt, die im engen inhaltlichen Zusammenhang miteinander stehen und in der vorliegenden kumulativen Habilitationsschrift zusammengefasst wurden.

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Effect of IL-1β and IL-6 on Oxytocin Secretion in Human Uterine Smooth Muscle Cells

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PROBLEM: Infection-mediated preterm labor results in the production of inflammatory cytokines, including interleukin-1β (IL-1β) and interleukin-6 (IL-6). Oxytocin (OT) plays a key role in the process of labor. This study investigates the effect of IL-1β and IL-6 on intra-and extracellular OT in human smooth muscle cells and evaluates IL-1β induced changes in IL-6 production.

METHOD OF STUDY: Primary cultures of human myometrium, obtained from term pregnant women ($n = 7$) were incubated with either IL-1β or IL-6 for 0–24 hr. Intra- and extracellular OT peptide concentrations were measured by radioimmunoassay and IL-6 mRNA and protein were evaluated by reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay.

RESULTS: Both, IL-1β and IL-6 led to an increase in OT secretion, which was accompanied by a reduction of the intracellular OT peptide pool. IL-1β significantly induced IL-6 mRNA expression and protein secretion, which did not further enhance IL-1β induced OT secretion.

CONCLUSIONS: The induction of OT secretion by proinflammatory cytokines in human myometrium *in vitro*, supports the concept of a thus regulated infection-triggered preterm labor process *in vivo*.

Key words:
Human, interleukin-1β, interleukin-6, myometrium, oxytocin

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INTRODUCTION

Delivery before 37 weeks of gestation occurs in about 5–10% of pregnancies[1] and accounts for as much as 90% of all neonatal morbidity and mortality. Preterm delivery can result from premature activation of cells in the cervix, decidua, and fetal membranes in response to local mediators of maternal stress, fetal stress, or ascending genital tract infection.[2] Those local mediators include inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-6 (IL-6), which promote the production of factors capable of eliciting uterine contractions, e.g., prostaglandins.[3,4]

IL-1β has a wide range of biological activities, but it is mainly involved in the initiation and maintenance of inflammatory response. The major physiological source of IL-1β are activated macrophages, which invade the decidua during pregnancy in high numbers.[5] But IL-1β mRNA expression is also found in fetal membranes and in the myometrium.[6,7] Several studies have linked increased levels of amniotic fluid IL-1β with human parturition which predicts preterm birth within 7 days.[3] A connection between infection, cytokine production, and preterm labor has also been demonstrated in animal models[8,9] and premature delivery was induced by systemic application of IL-1β in mice.[10]

IL-6 was originally identified as a T-cell derived lymphokine that induces the final maturation step of B cells. Subsequent studies revealed that IL-6 possesses pleiotropic activities that play a central role in host defense and acts on a wide variety of tissues.[11] In the pregnant uterus, IL-6 is produced by decidua, chorion, and amnion.[6] Increased IL-6 concentrations in amniotic fluid and maternal serum are highly predictive of intra-amniotic infection, failed tocolysis, histological chorioamnionitis, and preterm birth.[12] Paracrine and autocrine interactions between cytokines are important in gestational tissues and IL-1β has been shown to be an important regulator of IL-6 production.[13]

Oxytocin (OT), a nine amino acid peptide, is synthesized within myometrium and maternal–fetal membranes during pregnancy. It plays a key role in the process of term and preterm labor as part of an autocrine/paracrine system that regulates myometrial contractility.[14] OT directly stimulates uterine contractions but also releases arachidonic acid from gestational tissue with subsequent production of decidual prostaglandins (PGE_2, $PGF_{2\alpha}$).[15] Prostaglandins induce uterine contractions and increase myometrial sensitivity to OT. OT-stimulated prostaglandin production by decidua as well and endocervix also contribute to labor by inducing ripening of the cervix. OT and OT receptor (OTR) expression in the pregnant uterus strikingly increases around the time of parturition.[16,17]

Cytokines are thought to play a role in the regulation of the OT system. While IL-1β is known to induce OT release from various tissues, the influence of IL-6 on OT is controversially described in animal experiments.[18,19] However, the OT system and proinflammatory cytokines seem to play an important role in the chain of events that result in the initiation of infection-triggered preterm labor. To obtain a better understanding of the role of IL-1β and IL-6 in OT regulation in human myometrium, we treated primary cultures of uterine smooth muscle cells with rhIL-1β and rhIL-6 in order to determine their effect on OT secretion. We also examined the time course of IL-1β induced increase in IL-6 mRNA and protein to determine whether the effect of IL-1β on OT may be partially mediated through IL-1β induced increases in IL-6.

MATERIAL AND METHODS

Study Population

Segments of the upper margin of the lower uterine segment were obtained from term (37–42 weeks) pregnant women undergoing elective cesarean section. Subjects were excluded for maternal metabolic diseases (e.g., diabetes), labor, rupture of membranes, or multifetal gestation. Informed consent was obtained from all patients in accordance with the Magee-Womens Hospital Institutional Review Board.

Cell Culture Conditions

Cell cultures of human myometrium were established as previously described by Rauk and Friebe-Hoffman.[20] Culture medium contained minimal essential medium supplemented with 580 mg/L glutamine, 4.5 g/L glucose, 1% non-essential amino acids, 1 mM pyruvate, 26 mM $NaHCO_3$, 10% charcoal-stripped fetal bovine serum, 100 U/mL penicillin/streptomycin, 2.5 μg/mL amphotericin B and 40 μg/mL gentamycin. Cells were maintained at 37°C in humidified 5% CO_2, and medium was changed every 2 days. Sub-culturing was performed in 100-mm^2 culture plates after short trypsination (0.25% trypsin/0.1% EDTA). All cell culture agents were purchased through Mediatech Inc., Cellgro (Herndon, VA, USA).

Proinflammatory Cytokines

At confluence, the myometrium cells were incubated for 2, 4, 6, 8, 16, or 24 hr with 5 ng/mL rhIL-1β (R&D, Minneapolis, MN) or 1 ng/mL rhIL-6 (R&D, Minneapolis, MN, USA) as previously described.[20,21] Cells incubated with medium alone served as control.

OT-Radioimmunoassay

Uterine smooth muscle cells were incubated with IL-1β, IL-6 or medium alone for different periods of time (0–24 hr) and intracellular ($n = 6$) as well as extracellular ($n = 7$) OT peptide were determined by radioimmunoassay according to the manufacturer's instruction (Peninsula Laboratories Inc., Belmont, CA, USA). The sensitivity of the assay ranged from 1 to 128 pg per 100 μL media and the inter- and intra-assay coefficients of variation were < 15 and $< 5\%$, respectively.

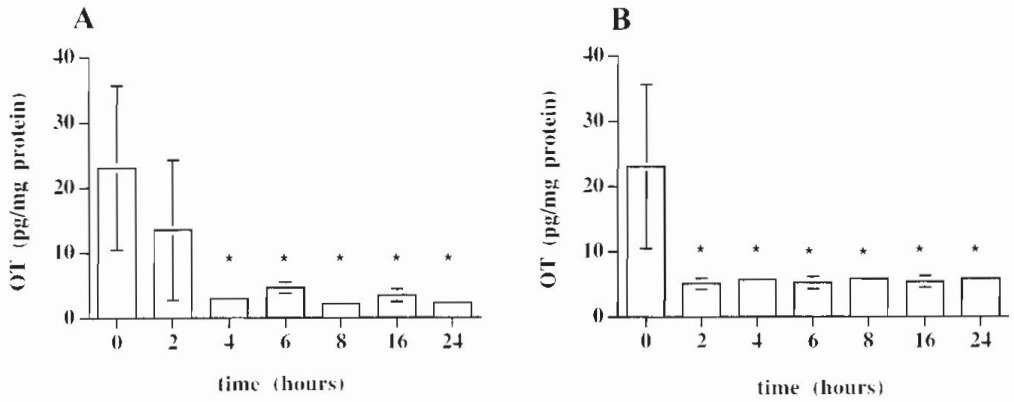

Fig. 1. Intracellular OT peptide expression after IL-1β (5 ng/mL) (**A**) and IL-6 (1 ng/mL) (**B**) exposure for different time periods (0–24 hr). Results are represented as mean pg OT per mg protein ± SEM ($n = 6$). $^*P < 0.05$.

After cytokine incubation, the cell media was collected and stored at −20°C until assayed for OT peptide secretion. For the determination of intracytoplasmatic OT, the cells were harvested with 1% phenylmethanesulfonyl fluoride (Boehringer Mannheim, Indianapolis, IN, USA) and 1% leupeptin (Sigma, St. Louis, MO, USA) in phosphate-buffered saline and stored overnight at −80°C to lyse the cells while preserving cytoplasmatic OT peptide. The lysate was centrifuged, free cytoplasmatic OT assayed and compared to total cell protein, as determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). Since intracellular proteins (e.g., proteolytic enzymes) could potentially reduce antibody-hapten binding and consequently would give false results for the OT competition assay, we compared standard curves of the provided OT standard dissolved in assay buffer with the OT standard dissolved in unstimulated cell extracts. As both standard curves were identical, we concluded that cellular proteins did not interfere with the radioimmunoasssay. All experiments were performed in duplicate.

IL-6 mRNA Detection

Total RNA from six different patients was isolated according to a method described by Chirgwin et al.[22] Spectrophotometric absorbance at 260 and 280 nm was used to estimate total RNA and purity, which was within the acceptable range of 1.6–1.8 (260/280 nm). The integrity of RNA was assessed by visualization of a 1% ethidium bromide-stained agarose gel. Equal amounts of RNA (2 μg) were used to perform semi-quantitative reverse transcription polymerase chain reaction (rtPCR) of IL-6 and GAPDH as control.

For IL-6, rtPCR oligonucleotide primers were used to amplify a 628-bp (36–663) fragment of the IL-6 cDNA. Oligonucleotide primers for GAPDH that amplify a 500-bp fragment of the human GAPDH cDNA were used as control. Briefly, cDNA was synthesized using total RNA, 20 IU avian myeloblastoma virus reverse transcriptase (Promega, Madison, WI, USA) and 30 ng of oligo-dT. One-fifth of the rtPCR reaction was further amplified for 30 cycles with 1 IU taq polymerase (Promega, Madison, WI, USA) and 100 pmol of IL-6 and GAPDH primers. The linearity of our IL-6 and GAPDH rtPCR products was established prior to the experiments using 0.5–6 μg total RNA and cycle lengths of 20–40 cycles.

The amplified products of IL-6 and GAPDH were separated in a 2% agarose gel and transferred to a nylon membrane (GeneScreen, New England Nuclear, Boston, MA, USA) by overnight capillary transfer. The membranes were hybridized with randomly nick-labeled IL-6 and GAPDH cDNA probes (Clontech, Palo Alto, CA, USA) labeled with ^{32}P-CTP (Amersham Pharmacia, Arlington Heights, IL, USA). Filters were developed by autoradiography and the band density of IL-6 was normalized to the corresponding band for GAPDH by scanning densitometry with the

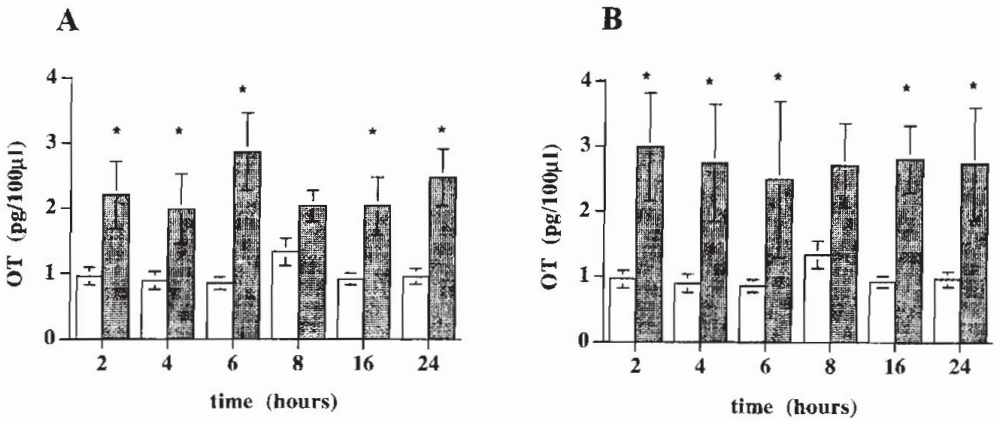

Fig. 2. Extracellular OT peptide concentrations after IL-1β (5 ng/mL) (**A**) and IL-6 (1 ng/mL) (**B**) stimulation for different time periods (0–24 hr) (*light bars*: no treatment, *dark bars*: cytokine treatment). Results are shown as mean pg OT per 100 μL supernatant ± SEM ($n = 7$). $^*P < 0.05$.

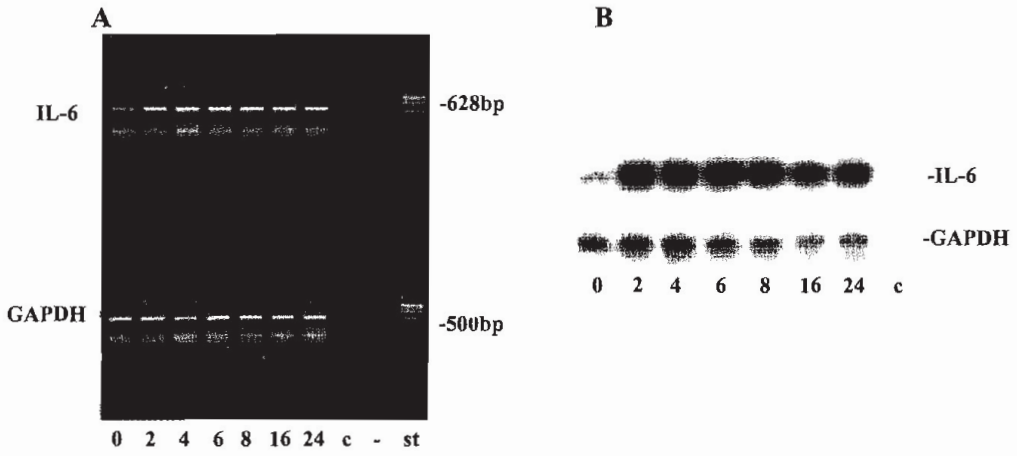

Fig. 3. A representative agarose gel (*left*) and Southern Blot (*right*) showing the PCR products for IL-6 and GAPDH after stimulation with IL-1β for different time periods (0–24 hr).

Videk Harmony Program v. 4.03 (Videk Corporation, Rochester, NY, USA).

IL-6 Protein Secretion

Uterine smooth muscle cells were grown to confluence and treated with IL-1β (5 ng/mL) for different time periods. IL-6 was measured in the supernatant by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (Quantikine, R&D, Minneapolis, MN, USA). The sensitivity of the assay for IL-6 was 0.7 pg/mL and the inter- and intra-assay coefficients of variation were < 3.7 and $< 4.4\%$, respectively. The experiments were performed in duplicate with cells isolated from six different patients.

Statistical Analysis

Repeated measures of time course experiments were analyzed using ANOVA (ANOVA Microsystems Inc., San Jose, CA, USA) and differences among treatment groups were evaluated by Student's *t*-test. Significance was concluded for $P < 0.05$.

RESULTS

Intracellular OT Expression

Fig. 1A shows the mean concentrations of intracellular OT peptide concentrations of 6 different human uterine smooth muscle cell cultures after stimulation with IL-1β. Compared with untreated controls, there was a significant decrease of up to 80% after 4 hr of IL-1β treatment and this decrease was maintained up to 24 hr of cytokine incubation. Similar results were observed for the treatment with IL-6. As shown in Fig. 1B, incubation with IL-6 resulted in a 75% reduction of cytoplasmatic OT peptide concentrations in human myometrial cells after 2 hr and continued out to 24 hr of stimulation.

Extracellular OT Expression

While IL-1β decreased intracellular OT peptide concentrations, a parallel increase in OT secretion of uterine smooth muscle cells was observed (Fig. 2A). With the exception of the 8-hr treatment period this increase was found to be significant with a maximum increase of three-fold maximum compared to untreated control groups. Similarly, IL-6 incubation led to a two- to three-fold increase of OT secretion compared to control for all times tested (Fig. 2B).

mRNA Analysis of IL-6

IL-6 mRNA expression was detected in all cell cultures tested. Fig. 3 shows a representative agarose gel (A) and Southern blot (B) of PCR products of IL-6 and the corresponding GAPDH after treatment with

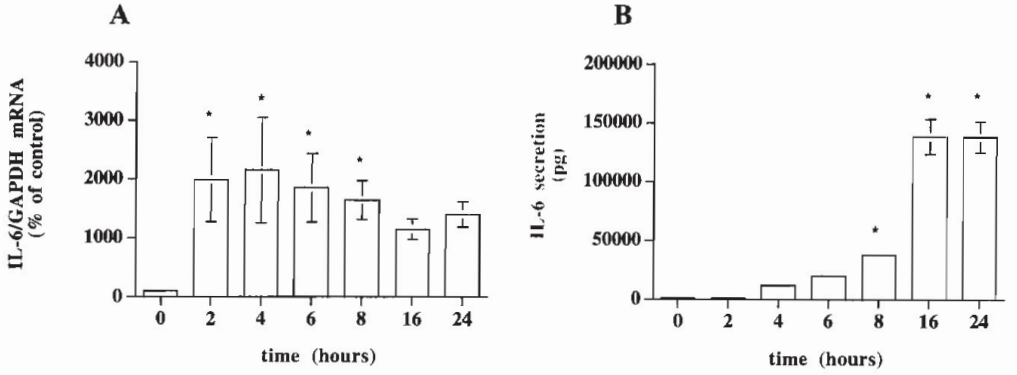

Fig. 4. IL-6 mRNA expression (**A**) and IL-6 peptide secretion (**B**) into the supernatant of uterine smooth muscle cells after the incubation with IL-1β (5 ng/mL) for different times (0–24 hr). Data are represented as mean ± SEM ($n = 6$) and shown as percent of control for the mRNA and as pg per 5 mL supernatant for extracellular IL-6 peptide secretion. $^*P < 0.05$.

IL-1β for different time points (0–24 hr). Fig. 4A demonstrates the time course of IL-6 mRNA expression of six different primary uterine smooth muscle cell cultures after incubation with IL-1β. Even we observed considerable variation in mRNA expression in primary cell cultures obtained from different patients, a significant increase in IL-6 mRNA was noted after 2–8 hr of IL1-β treatment.

ELISA for IL-6

Fig. 4B shows IL-6 secretion into the supernatant of primary cultures of uterine smooth muscle cells treated with IL-1β for different time periods (0–24 hr). The increase in IL-6 mRNA expression after 2–8 hr of IL-1β treatment (Fig. 4A) was followed by a significant increase of IL-6 secretion at 8–24 hr of incubation.

DISCUSSION

As many as 70% of all women delivered at mid-gestation secondary to preterm labor have evidence of chorioamnionitis.[23] Intrauterine infections are known to be associated with increased inflammatory cytokines in the amniotic fluid including IL-1β and IL-6.[24] Human gestational tissues are potentially rich sources of proinflammatory cytokines and while maternal–fetal membranes are known to produce cytokines constitutively,[6] there is now growing evidence that myometrium itself may also be a production site for proinflammatory cytokines as well as for OT in response to cytokine exposure as shown in the present study.

While cytokine induced OT release has been described for different tissues, there are no studies about the effect of IL-1β and IL-6 on OT secretion in human uterine smooth muscle cells. In the current study, we administered rhIL-1β and rhIL-6 to primary cultures of human myometrial cells *in vitro* and determined their effect on OT over time. Our data show that human uterine smooth muscle cells respond to treatment with IL-1β and IL-6 with a significant increase in OT secretion at various time intervals. Human myometrium has not previously been considered a source of cytokine-stimulated OT production. We previously demonstrated that beginning after 8 hr of IL-1 treatment, myometrial OTR binding decreases to 10%.[20] The early and prolonged release of OT by myometrial cells may down-regulate the receptor by an autocrine mechanism. In addition, our laboratory has observed a defined increase in OT peptide secretion from human decidual cells after incubation with IL-1β (manuscript in preparation). Supportive data also comes from animal studies showing that IL-1β enhances OT peptide release in hypothalamic explants[18] and OT plasma levels increase after systemic infusion of IL-1β.[19] Although in the current study we have observed a substantial stimulatory effect of IL-6 on OT release from myometrial cells, previous animal experiments report conflicting data on the influence of IL-6 on OT secretion and production. IL-6 increases hypothalamic OT secretion at high concentrations *in vitro* but does not change OT plasma concentrations of freely moving rats after systemic infusion.[18,19]

In the present study, IL-1β and IL-6 induced increase of extracellular OT was accompanied by a reduction of intracellular OT peptide. Intracellular OT peptide concentration decreased after 2 hr of IL-1β and IL-6 stimulation, followed by a subsequent OT secretion into the media of the cells. We observed a consistent release of OT over a period of 24 hr but did not detect any further decrease of intracellular concentrations. Therefore, we concluded that an intracellular de novo synthesis of OT was taking place in order to maintain intracellular stores. We attempted to demonstrate this by northern blotting, however, OT mRNA levels were below the lower limit of detection. The evaluation of this and other mechanisms is currently ongoing in our laboratory.

Several studies have demonstrated that IL-1β is able to stimulate IL-6 mRNA expression and protein production in human chorion and decidual cells.[25,26] As both IL-1β and IL-6 induced secretion of OT from myometrial cells we sought to determine whether the time course of IL-1β induced changes in IL-6 mRNA expression and protein secretion may explain the prolonged effects of IL-1β on OT secretion. IL-1β stimulation increased IL-6 mRNA expression and protein secretion over time in uterine myocytes, but a significant increase in IL-6 secretion into the media occurred just after 8 hr of incubation. If IL-1β mediates its effect on OT through IL-6, we would have expected to see another peak of OT secretion after 8 hr of IL-1β incubation when extracellular levels of IL-1β induced IL-6 were high. However, the remaining OT pool in the cytoplasm of the cells as well as the amount of OT secreted into the media did not change over time. These results suggest that in the our model, IL-1β-induced increases in OT secretion were not mediated in part by a secondary induction of IL-6. An alternative explanation would be that under the influence of IL-1β maximal levels of OT secretion were already reached, which were not further inducible by another cytokine. However, it is noteworthy that human myometrium itself is a potential source of proinflammatory cytokines as shown here for IL-6.

In summary, our results demonstrate that human uterine smooth muscle cells are an important source of OT and IL-6 and that their production is regulated

by proinflammatory cytokines. Therefore, our study supports the concept that proinflammtory cytokines could have a significant impact on infection-triggered preterm labor processes *in vivo* through the induction of OT in myometrium.

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Interleukin-1β Down-Regulates the Oxytocin Receptor in Cultured Uterine Smooth Muscle Cells

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Rauk PN, Friebe-Hoffmann U. Interleukin-1β down-regulates the oxytocin receptor in cultured uterine smooth muscle cells. AJRI 2000; 43:85–91 © Munksgaard, Copenhagen
PROBLEM: Intrauterine infection accounts for 20% of preterm labor and results in the production of decidual inflammatory cytokines, including interleukin-1 (IL-1). The oxytocin receptor plays a key role in the onset of preterm labor. Cytokines likely regulate oxytocin receptor expression through several cytokine-induced DNA-binding proteins.
METHOD OF STUDY: The objective of this study was to evaluate the effect of the IL-1 alone on oxytocin receptor number as measured by radioligand binding and immunocytochemistry, and oxytocin receptor mRNA as measured by reverse transriptase-polymerase chain reaction (RT-PCR) in cultured uterine myocytes.
RESULTS: Unexpectedly, IL-1 treatment decreased oxytocin receptor number from 111,067 to 23,941 receptors/cell. Loss of oxytocin receptor binding began after 8 hr of IL-1 treatment and was reversible after IL-1 removal. Immunocytochemistry confirmed a loss of cellular oxytocin receptors. Oxytocin receptor mRNA decreased beginning after 2 hr of IL-1 treatment.
CONCLUSIONS: IL-1 down-regulates the uterine oxytocin receptor in a time- and dose-dependent fashion.

Key words:
Human uterus, interleukin-1β, oxytocin receptor

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INTRODUCTION

Delivery before 37 weeks of pregnancy occurs in 7% of all pregnancies and accounts for as much as 90% of all neonatal morbidity and mortality. Intraamniotic infection and chorioamnionitis account for as much as 20% of all cases of preterm labor.[1] The pathophysiology of infection-mediated preterm labor has been extensively studied. Intraamniotic infection is associated with the production of cytokines including interleukin-1 (IL-1) by decidua, chorion, and amnion.[2,3] Although it is not known which cytokines or bacterial products initiate labor, IL-1 alone stimulates preterm labor in many animal models of infection-mediated labor. IL-1 treatment results in the up-regulation of cyclooxygenase type 2 (COX-2) in decidua and my-

ometrium and increased COX-2 enzyme activity accounts for increases in decidual prostaglandin production and the onset of labor.[4,5]

In addition to the role of prostaglandins, oxytocin also contributes to the maintenance of both term and preterm labor.[6–8] Oxytocin receptors increase in the myometrium and decidua prior to the onset of both term and preterm labor in all animals studied including humans. The role of inflammatory cytokines on the regulation of myometrial and decidual oxytocin receptor has not been investigated. Sequencing of the 5′ upstream regulatory region of the human and rat oxytocin receptor genes has identified many DNA-binding regions likely to be regulated by cytokine-induced nuclear proteins.[9] Multiple DNA-binding sites are present for the proteins, CEBPβ (also referred to as NF-IL-6) and STAT-3 (also referred to as acute phase regulatory element). Both of these DNA-binding proteins are increased in the nuclear cytoplasm in cells in response to IL-1, IL-6, and lipopolysaccharide stimulation. To date, these sites have not been studied as either promoters or inhibitors of oxytocin receptor (OTR) gene transcription.

The aims, therefore, of this study were to investigate the effect of IL-1 treatment on oxytocin receptor binding and on oxytocin receptor mRNA in primary culture of human uterine myometrial cells. The findings of this study demonstrate a time- and dose-dependent down-regulation of oxytocin receptor binding and a decrease in oxytocin receptor mRNA. Down-regulation of cellular oxytocin receptor was also confirmed by immunocytochemistry. These data suggest that in the setting of preterm labor caused by intrauterine infection, decidual IL-1 production may actually limit the premature up-regulation of the oxytocin receptor. The down-regulation of prematurely-induced uterine oxytocin receptors by IL-1 may be another "protective" mechanism limiting the preterm labor process in early infection.

MATERIALS AND METHODS

Study Population

Segments of the upper margin of the lower uterine segment were used for primary uterine smooth muscle cell culture. Tissue was obtained from 14 term (37–42 weeks), uncomplicated, pregnant women undergoing elective cesarean section. Subjects were excluded for evidence of labor and/or rupture of membranes at the time of cesarean section. Consent was obtained from all subjects in accordance with the Magee-Womens Hospital Institutional Review Board.

Human Uterine Smooth Muscle Cell Culture

Segments ($0.5 \times 0.5 \times 3$ cm) of the upper margin of the lower uterine segment incision were obtained from 14 women at the time of cesarean section and placed in modified Hank's balanced salt solution (HBSS) (500 mL of calcium and magnesium-free HBSS with 1000 U/mL heparin) and kept at 4°C prior to processing. Using sterile technique, the tissue samples were minced, washed with Earle's balanced salt solution, and placed in a 50-mL conical tube with collagenase type P (Boehringer Mannheim, Indianapolis, IN; 1 mg/mL). The suspension was incubated at 37°C for 2–3 hr with occasional pipetting. The suspension was then passed through fine meshed gauze and individual cells collected by centrifugation at 500*g* for 5 min. The cells were washed twice with media (minimal essential media with nonessential amino acids, sodium bicarbonate [26 mM], pyruvate [1 mM], gentamicin [50 mg/mL], penicillin G [100 U/mL], streptomycin [100 mg/mL], L-glutamine [2 mM], and charcoal stripped fetal calf serum [10%, v/v]). The cells were then plated at 5×10^5 cells in 75-cm^2 flasks and maintained at 37°C in humidified 5% CO_2. The media was changed every 2–3 days and the cells subcultured at confluence after 5–7 days.

Oxytocin Receptor Binding Assay in Cultured Cells

Oxytocin receptor binding was measured in a competitive-binding assay using the oxytocin analogue, ornithine vasotocin (OVT). Cells plated in 24-well plates were studied at confluence. The plates were washed with HBSS. The cells were then incubated with 6 pM ^{125}I-labeled OVT (New England Nuclear, Boston, MA) and 0.05–50 nM-unlabelled OVT in HBSS with 0.1% bovine serum albumin and 5 mM $MgCl_2$ at room temperature. After 3 hr, the cells were washed with HBSS with 0.1% bovine serum albumin and the cells lysed with 1 N NaOH. The lysate was counted on a gamma counter. Data were analyzed using a scatchard plot with determinations of B_{max} (receptors/cell) and K_d (nM). For dose-response experiments to various agents, incubations were performed with 6 pM ^{125}I-OVT with and without 50 nM-unlabelled OVT and total, specific, and nonspecific binding compared. Differences between IL-1 and control-treated cells were compared using the Student's *t*-test. Time-course and dose-response experiments were compared using ANOVA.

Immunocytochemistry for Oxytocin Receptor

Myometrial cells were grown to confluence on chamber slides. Cells were treated with media alone or media with IL-1 (5 ng/mL) for 24 hr. Cells were then

fixed with 2% paraformaldehyde. Nonspecific binding was blocked with 10% normal rabbit serum in phosphate-buffered saline with 0.05% Tween 20 and 0.3% Triton X-100. The primary mouse monoclonal OTR antibody, O-2F8 (Rohto Laboratories, Japan), was added at 1:400 dilution for 2 hr at room temperature. The secondary fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin antibody (Jackson ImmunoResearch, Inc., West Grove, PA) was added for 1 hr at room temperature. After a final wash with the incubation buffer, the slides were mounted and examined using a fluorescence microscope (Zeiss, Germany).

RNA Isolation

Cells were homogenized in 4 M guanidine thiocyanate, 25 mM Na-acetate (pH 7.0), 0.5% sarcosyl, 50% water saturated-phenol, and 0.7 mM 2-mercaptoethanol. Chloroform (1/5 volume) was added and the solution incubated on ice for 15 min. The solution was then centrifuged at 2000*g* and the upper aqueous phase collected. RNA was precipitated with an equal volume of cold 2-propanol and stored at $-80°C$.

Reverse Transriptase-Polymerase Chain Reaction for Oxytocin Receptor

Oligonucleotide primers developed by Takemura[10] were used. These primers amplify a 391-bp (1215–1602) fragment of oxytocin receptor cDNA. The primed region spans both exons 3 and 4 of the oxytocin receptor gene. Oligonucleotide primers for GAPdH that amplify a 500-bp fragment of human GAPdH cDNA were used as control. Briefly, cDNA was synthesized using 2.5 mg total RNA, 20 U avian myeloblastoma virus reverse transcriptase (Promega, Madison, WI), and 30 ng of oligo-dT. One-sixth of the reverse transriptase-polymerase chain reaction (RT-PCR) reaction was further amplified for 30 cycles with 1 U taq polymerase (Promega, Madison, WI) and 100 pmol of the forward and reverse primers for oxytocin receptor. The GAPdH was amplified for 25 cycles. After amplification, the samples were separated in 1% agarose gels, transferred to nylon membrane, and hybridized to the oxytocin receptor and GAPdH cDNA probe randomly nick-labeled with ^{32}P. The linearity of oxytocin receptor PCR products were established as done by Zingg[9] using 0.5–10 μg total RNA and cycle lengths of 20–40 cycles.

RESULTS

Oxytocin Receptor Binding

Fig. 1 demonstrates data from experiments on six sets of cells. The binding of OVT decreases to 20% of control with addition of IL-1 for 24 hr ($P < 0.01$) (Fig. 1A). The K_d was unchanged between control and IL-1-treated cells and analysis of the data revealed only one binding site for OVT (Fig. 1B). Receptor concentration returned to 35 and 80% of pretreatment levels at 48 and 72 hr after removal of IL-1, respectively.

The dose response and time course of loss of oxytocin receptor binding was then determined. Radioligand-binding assays were modified and binding was

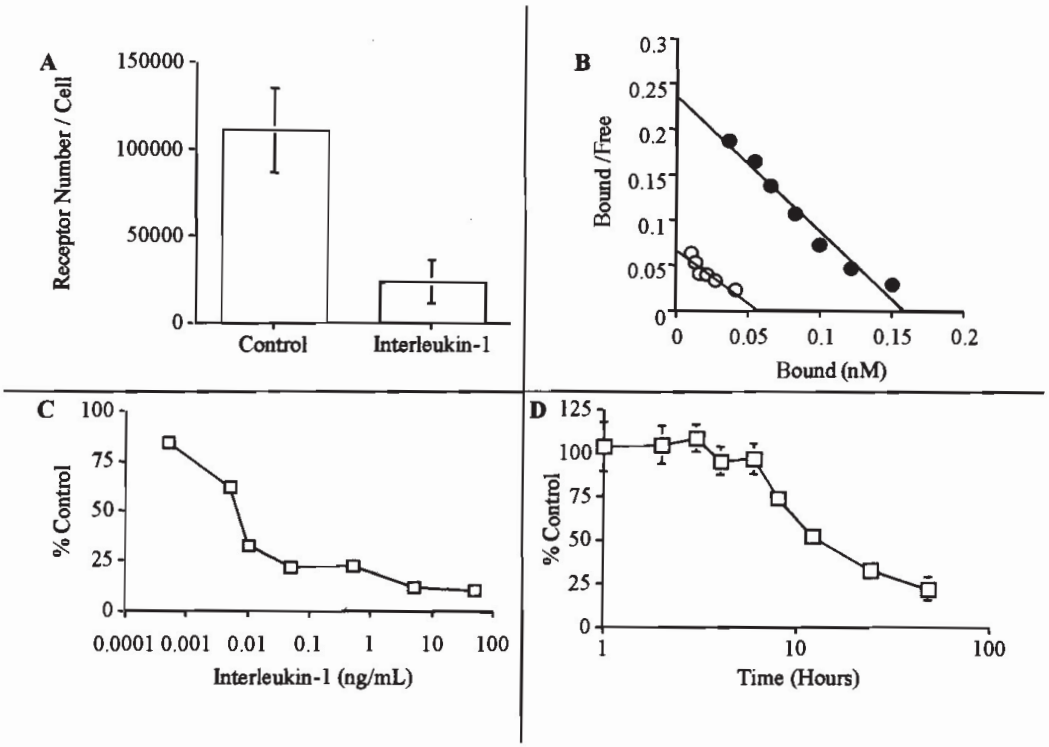

Fig. 1. (**A**) Oxytocin receptor binding with IL-1 at 48 hr. Data are expressed as mean ± SEM ($n = 6$). (**B**) Scatchard analysis of OVT binding. Results represent means for data obtained from binding experiments in control-treated (*filled circles*) and IL-1-treated (*open circles*) cells obtained from the six different uterine specimens represented in (**A**). (**C**) Dose response for oxytocin receptor binding with IL-1. Data are expressed as mean ± SEM ($n = 6$). (**D**) Time course of oxytocin receptor binding with IL-1 (5 ng/mL). Data are expressed as mean ± SEM ($n = 6$).

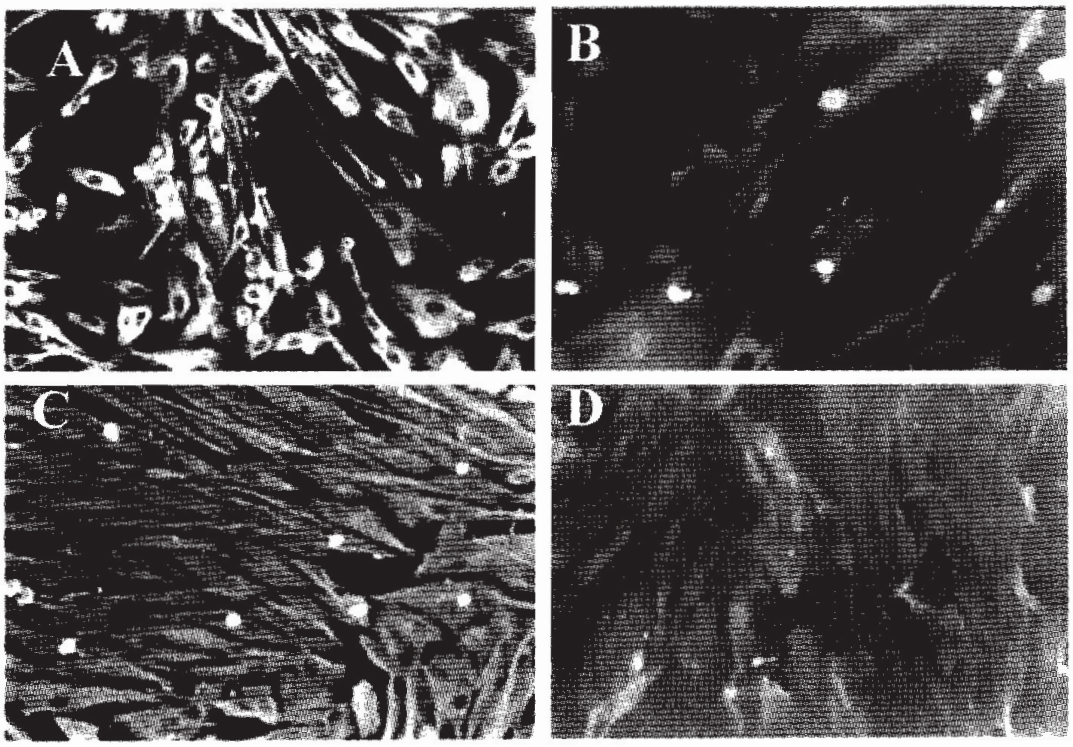

Fig. 2. Immunocytochemistry for oxytocin receptor in myometrial cells. (**A**) control-treated cells; (**B**) control-treated cells, secondary antibody only; (**C**) IL-1-treated cells; (**D**) IL-1-treated cells, secondary antibody only.

determined with a fixed concentration of ^{125}I-OVT and two concentrations of cold OVT, 0 and 50 nM ($0\times$ and $100\times$ K_d, respectively). The loss of specific OVT binding with IL-1 is dose dependent with a half-maximal dose of 5 pg/mL and maximal dose of 1 ng/mL (Fig. 1C). Time course data indicates that loss of receptor begins after 8 hr of IL-1 exposure and declines exponentially (Fig. 1D).

Immunocytochemistry for Oxytocin Receptor

IL-1 treatment of myometrial cells for 24 hr resulted in a marked loss of specific OTR cell surface staining using the mouse monoclonal OTR antibody (Fig. 2A, C). The staining for OTR was comparable to staining in cells treated with the secondary antibody alone (Fig. 2B, D).

Oxytocin Receptor RT-PCR

The effect of IL-1 on oxytocin receptor mRNA was then determined. As the concentration of oxytocin receptor mRNA is very low in human myometrial tissue, RT-PCR was used to measure changes in OTR mRNA. Cells were exposed to IL-1 for various times and at various concentrations for 24 hr. RT-PCR for GAPdH was also performed as an internal control and for semiquantitation. The oxytocin receptor PCR product was linear over a range of 0.1–10 μg of total RNA and for 25–45 cycles and the GAPdH PCR product was linear over a range of 20–35 cycles. Fig. 3B demonstrates the time course for decrease in OTR mRNA as measured by RT-PCR following exposure to IL-1 (5 ng/mL). A decline in mRNA begins after 2 hr and reaches a nadir of 5% of control mRNA by 24 hr. A representative photograph agarose gel showing PCR products as well as the corresponding Southern blot for a time-course experiment are shown in Fig. 4. Fig. 3A demonstrates the dose-response curve for OTR mRNA decrease. A significant decrease in OTR mRNA was observed at an IL-1 concentration of 0.5 ng/mL.

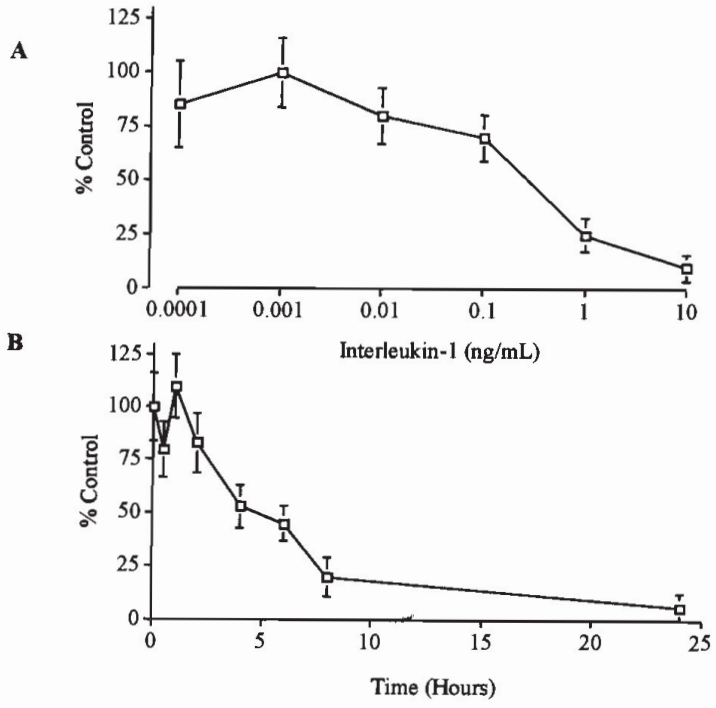

Fig. 3. (**A**) Oxytocin receptor RT-PCR product (normalized to GAPdH) measured by Southern blot after exposure to IL-1 (5 ng/mL) at various concentrations for 24 hr. The data are represented by mean ± SEM ($n = 5$). (**B**) Oxytocin receptor RT-PCR product (normalized to GAPdH) measured by Southern blot after exposure to IL-1 for various times. Data are represented by mean ± SEM ($n = 5$).

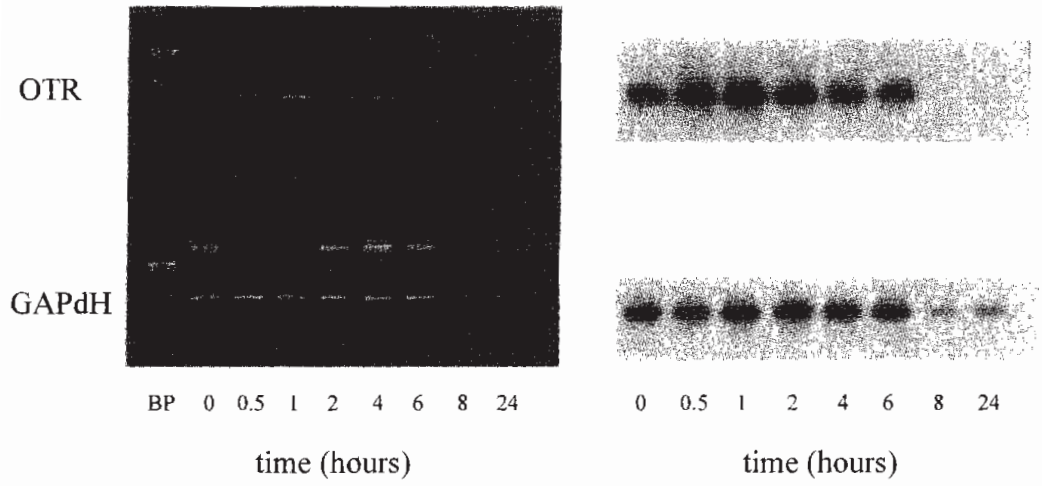

Fig. 4. Representative agarose gel (*left*) showing PCR products for the OTR and GAPdH for a time-course study after IL-1 treatment and the corresponding Southern blot (*right*).

DISCUSSION

Estrogen up-regulates and progesterone down-regulates the oxytocin receptor *in vivo* in animals and *in vitro* in myometrial explant culture. Other factors regulating myometrial and decidual oxytocin receptor expression are unknown. This study demonstrates that IL-1 down-regulates the oxytocin receptor in human myometrium in a dose- and time-dependent fashion. This study also demonstrates complete reversal of the loss in oxytocin receptor binding following removal of IL-1. Cytokine regulation of the oxytocin receptor has been studied in human placenta. Interferon (IFN)-γ treatment of human placental explants also results in down-regulation of the oxytocin receptor.[11] The mechanism of IFN-γ-mediated down-regulation has not been studied. Down-regulation by IFN-γ in early placentation is thought to inhibit oxytocin receptor up-regulation during implantation.

Recent sequencing of both the rat and human oxytocin receptor gene 5′ up-stream regulatory region has, however, identified potential cytokine regulated pathways.[12–14] Six up-stream DNA-binding sites for cis-enhancer-binding protein-β (CEBP-β) are found. CEBP-β, previously named nuclear factor IL-6 (NF-IL-6), is the DNA-binding protein responsible for both IL-1 and IL-6-induced transcription of IL-6 mRNA. Although CEBP-β binding to DNA usually results in enhanced transcription of down-stream genes, CEBP-β is also a potent negative regulator of gene transcription.[15] Multiple sites for binding the acute phase reactant element (STAT-3) have also been identified in the 5′ up-stream sequence of the OTR gene.[14] Although tumor necrosis factor-α and lipopolysaccharide have been shown to increase nuclear STAT-3 protein in most cell types, IL-1 is known to signal through STAT-3 in some cell types as well. The results of this study do not identify through which mechanism oxytocin receptor mRNA levels fall after IL-1 treatment. If both CEBP-β and STAT-3-binding proteins are induced after IL-1 binding in myometrium, then the DNA-binding sites for these proteins in the oxytocin receptor gene are either inactive or inhibitory.

IL-1 may also reduce steady-state OTR mRNA levels by destabilizing mRNA and reducing mRNA half-life. Down-regulation of the OTR by oxytocin results in a decrease in OTR mRNA half-life.[16] The 3′ region of the OTR cDNA contains the destabilizing motif AUUUA as well as deadenylation sequence UUAUUUAUU. Both of the regions of the mRNA may be responsible for destabilization during desensitization processes in the regulation of the OTR mRNA.

The half-maximal dose for IL-1-induced down-regulation of oxytocin receptor binding is 100-fold lower than the half-maximal dose for IL-1-induced OTR mRNA loss. A decrease in OTR gene transcription or mRNA destabilization may not be the sole explanation for loss of OTR binding. The possibility exists that loss of binding may instead result from receptor protein modification that either inhibits oxytocin binding or results in oxytocin receptor protein degradation. Oxytocin down-regulates its own receptor in many cell types. Oxytocin receptor down-regulation also occurs in myometrium following prolonged exposure to oxytocin. Autologous down-regulation of the oxytocin receptor by oxytocin has been demonstrated in astroglial cells as well as myometrial explant culture.[17,18] Oxytocin down-regulates oxytocin receptor in astroglial cells by a mechanism involving protein kinase C (PKC).[18] The authors suggest that PKC regulates OTR binding through receptor phosphorylation, internalization, and degradation. PKC signaling also mediates IFN-γ-induced down-regulation of the oxytocin receptor in endometrium.[11] Although the second messenger pathways for IL-1 signaling in myometrial cells has been fully investigated, increase in PKC activity fol-

lowing IL-1 treatment have been demonstrated in other cell types.[19] The oxytocin receptor is a G-protein-binding receptor in the same family as the more extensively studied β-adrenergic receptor. Down-regulation of the β-adrenergic receptor by both β-adrenergic receptor kinase and PKC has been studied. Similarly, another G protein-coupled receptor, the type 1A angiotensin II receptor, has been shown to be down-regulated by phosphorylation by a specific G protein receptor kinase and PKC.[20] One could hypothesize that the oxytocin receptor, which belongs to this same family of receptors, may also have its own unique G protein-coupled receptor kinase and may also be phosphorylated by PKC. IL-1 is a potent stimulus for oxytocin release in hypothalamic explants from rats.[21] Similarly, IL-1 may increase the production of oxytocin by myometrium resulting in autologous down-regulation of the oxytocin receptor. Such a mechanism would explain the time course of oxytocin receptor binding loss observed in this study. The loss in oxytocin receptor binding and in mRNA is the same as that reported by Phaneuf[16] in his study of oxytocin-mediated down-regulation of the oxytocin receptor in human myometrium.

The results of this study are difficult to understand given that IL-1 induces labor in many animal models. Teleologically, there is likely to be an advantage to a species to not labor and deliver when infection is limited and easily controlled by the host defense mechanisms. IL-1, in high concentrations of 0.5–5 ng/mL, stimulates the production of uterotonic prostaglandins in myometrium. Production of these prostaglandins is secondary to cytokine induction of the enzyme COX-2. IL-1, at low concentration of 0.5–5 pg/mL, decreases oxytocin receptor binding and does not induce COX-2. Therefore, in the setting of low-grade infection neither oxytocin nor prostaglandin labor pathways are activated. With overt chorioamnionitis, however, IL-1 at high concentration increases prostaglandin production, thus stimulating labor. In the setting of early chorioamnionitis when infection is effectively limited by the host defense system, premature induction of oxytocin receptors may be blocked in a protective mechanism limiting the parturition process. Clinically, this theory is substantiated by the observation that the uteri of women with severe chorioamnionitis are less sensitive to infused oxytocin. Severe chrorioamnionitis results in a higher rate of dysfunctional labor and increased incidence of postpartum atony and hemorrhage unresponsive to oxytocin.[22]

The observation that IL-1 down-regulates the oxytocin receptor in human myometrium may provide information on other mechanisms of oxytocin receptor regulation in the human uterus. As the second messenger pathways for IL-1 signaling become clearer, possible mechanisms for down-regulation of the oxytocin receptor will be discovered. A more detailed understanding of the regulation of uterine oxytocin receptors is likely to increase our understanding of the mechanisms responsible for labor initiation. Novel approaches to preventing the premature induction of oxytocin receptors before term may also be investigated.

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Interleukin-6 Up-Regulates the Oxytocin Receptor in Cultured Uterine Smooth Muscle Cells

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Rauk PN, Friebe-Hoffmann U, Winebrenner LD, Chiao J-P. Interleukin-6 up-regulates the oxytocin receptor in cultured uterine smooth muscle cells. AJRI 2001; 45:148–153 © Munksgaard, Copenhagen

PROBLEM: Intra-uterine infection results in the production of inflammatory cytokines, including interleukin-6 (IL-6). Increased oxytocin-receptor (OTR) concentrations are associated with the onset of preterm labor. We hypothesize that infection up-regulates OTR expression through IL-6-induced transcription factors.

METHOD OF STUDY: Primary cultures of human myometrium were treated for various time periods or with different concentrations of IL-6 and OTR mRNA as well as OTR binding were measured by means of reverse transcription polymerase chain reaction and ^{125}I-ornithine-vasotocin-binding assay. To study underlying mechanisms of OTR changes with IL-6 treated, cells were also incubated with genistein or H7 (tyrosine and serine phosphorylation inhibitors), respectively.

RESULTS: OTR mRNA increased 2.5-fold after 4 hr of IL-6 treatment and OTR binding 1.4-fold after 8 hr of cytokine stimulation. The IL-6-induced increase in binding was blocked by genistein and H7.

CONCLUSIONS: IL-6 up-regulates uterine OTR mRNA expression and binding capacity in cultured human myocytes most likely through tyrosine and serine phosphorylation pathways involving the nuclear factor STAT-3.

Key words:
Human uterus, interleukin-6, oxytocin receptor

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INTRODUCTION

Delivery before 37 weeks of gestation occurs in 7% of all pregnancies and accounts for as much as 90% of all neonatal morbidity and mortality.

* Both first authors contributed equally to this work.

Intra-amniotic infection and chorioamnionitis are responsible for approximately 13% of all cases of preterm labor with intact membranes.[1] The pathophysiology of infection-mediated preterm labor has been extensively studied. Intra-amniotic infection is associated with the production of cytokines, including interleukin-6 (IL-6) by decidua, chorion, and amnion.[2–4] IL-6 also stimulates uterotonic prostaglandin production by decidua and amnion.[5] Increased IL-6 concentrations in amniotic fluid and maternal serum are highly predictive of intra-amniotic infection, failed tocolysis, histological chorioamnionitis, and preterm birth.[6,7]

In addition to the role of prostaglandins in labor initiation, the oxytocin–oxytocin receptor (OT–OTR) system significantly contributes to the maintenance of both term and preterm labor.[8] Myometrial and decidual OTR concentrations markedly increase prior to the onset of term and preterm labor[9]. Cytokines are thought to play a role in the regulation of OTR gene transcription. Sequencing of the 5′-upstream promoter region of the human OTR gene has identified many DNA-binding regions likely to be regulated by cytokine-induced nuclear proteins.[10] Among them are nuclear factor IL-6 (NF-IL-6) and STAT-3. Both NF-IL-6 and STAT-3 are cytoplasmic proteins that become phosphorylated by cytokine-activated protein kinases, mitogen-activated protein kinase (MAPK) and JAK-2, respectively.[11] Phosphorylation results in nuclear translocation of the protein and binding to specific promoter regions in various genes. Zingg et al.[10] have speculated that both NF-IL-6 and STAT-3 may be important regulators of the OTR gene given the predominance of binding sites of these proteins in the regulatory region of the OTR gene.[10]

Unexpectedly, however, we recently showed that the inflammatory cytokine IL-1 down-regulates the OTR mRNA expression as well as binding capacity in human myometrial cells.[12] Other cytokines, specifically IL-6, may alter the expression of myometrial OTR differently than IL-1 secondary to alternative signal transduction pathways. The difference between IL-1 and IL-6 nuclear signaling is that IL-1 induces the phophorylation of NF-IL-6, but not STAT-3, while IL-6 induces the phosphorylation of both NF-IL-6 and STAT-3. These transcription factors are known to act cooperatively in the promotion of gene expression. For example, IL-6-induced expression of the heat-shock protein 90 genes (hsp90) is known to occur through cooperative activity of NF-IL-6 and STAT-3, but does not occur through NF-IL-6 or STAT-3 alone.[11]

IL-6 has been shown to up-regulate OTR mRNA in pregnant rats,[13] but there have been no reports of the influence of IL-6 on OTR regulation in human myometrial cells. Therefore, the aim of this study was to investigate the effect of IL-6 treatment on OTR mRNA expression and on OTR binding in cultured human uterine myometrial cells.

MATERIALS AND METHODS

Study Population

Myometrial tissue from the upper margin of the lower uterine segment incision was obtained from term (37–42 weeks) pregnant women undergoing either repeat cesarean section or cesarean section for breech. Although the fundus of the uterus contains a greater concentration of OTR than the lower uterine segment, fundal tissue was not accessible during cesarean section. Subjects were excluded for evidence of labor and/or rupture of membranes, maternal metabolic disease, and multifetal gestation. Informed consent was obtained from all patients in accordance with the Magee-Womens Hospital Institutional Review Board.

Human Uterine Smooth Muscle Cell Culture

Segments ($0.5 \times 0.5 \times 3$ cm) of the upper margin of the lower uterine segment incision were obtained at the time of cesarean section and placed in modified Hank's balanced salt solution with 1,000 U/mL heparin and kept at 4°C until processing. Using sterile technique, the tissue samples were minced, washed with Earl's balanced salt solution, and placed in a 50-mL conical tube with 1 mg/mL collagenase type P (Boehringer Mannheim, Indianapolis, IN). The suspension was digested at 37°C for 2–3 hr with occasional pipetting. The suspension was then passed through fine meshed gauze and individual cells collected by centrifugation at $500 \times g$ for 10 min. The cells were washed twice with media (minimal essential media with non-essential amino acids, 26 mM sodium bicarbonate, 1 mM pyruvate, 50 mg/mL gentamicin, 100 U/mL penicillin G, 100 mg/mL streptomycin, 2 mM L-glutamine, and 10% (v/v) charcoal stripped fetal calf serum). The cells were then plated at 5×10^5 cells in 75-cm^2 flasks and maintained at 37°C in humidified 5% CO_2. Cultured cells were 95% smooth muscle cells by immunocytochemical staining for smooth muscle specific actin. The media was changed every 2–3 days and the cells sub-cultured at confluence after 5–7 days. For sub-culture, myometrial cells were harvested with 0.05% trypsin/0.53 mM EDTA and passaged to 60-mm^2 culture plates for RNA isolation or 24-well plates for the binding assay.

Experimental Conditions

For the evaluation of OTR mRNA expression, the myometrial cells were incubated with 1 ng/mL IL-6 for different time periods (0–24 hr). For the binding

assay, the cells were incubated with 1 ng/mL IL-6 for different time periods (0–24 hr) and treated with 0–1 ng/mL IL-6 for 8 hr in dose–response experiments. The cells were treated with 1 ng/mL IL-6 for 24 hr in combination with 100 μl/mL genistein, a tyrosine phosphorylation inhibitor, or 100 μM H7, a serine phosphorylation inhibitor, to measure the influence of phosphorylation pathways.

RNA Isolation

Cells were homogenized in 4 M guanidine thiocyanate, 25 mM Na-acetate (pH 7.0), 0.5% sarcosyl, 50% water-saturated phenol, and 0.7 mM 2-mercaptoethanol. After the addition of 1/5 volume chloroform, the solution was incubated on ice for 15 min, then centrifuged at 2000 × *g* for 10 min and the upper aqueous phase collected. RNA was precipitated with an equal volume of cold 2-propanol and stored at −80°C.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for OTR

Oligonucleotide primers developed by Takemura et al.[9] were used for this semi-quantitative RT-PCR. These primers amplify a 391-bp (1215–1602) fragment of OTR cDNA. The primed region spans both exons 3 and 4 of the OTR gene. Oligo-nucleotide primers for GAPdH that amplify a 500-bp fragment of human GAPdH cDNA were used as control.

Briefly, cDNA was synthesized using 2.5 μg total RNA, 20 U avian myeloblastoma virus reverse transcriptase (Promega, Madison, WI) and 30 ng of oligo-dT. One-sixth of the RT-PCR reaction was further amplified for 30 cycles with 1 U taq polymerase (Promega, Madison, WI) and 100 pmol of the forward and reverse primers for OTR. After amplification, the samples were separated in 1% agarose gels, transferred to nylon membrane, and hybridized to the OTR and GAPdH cDNA probe randomly nick-labeled with ^{32}P. The linearity of the dose–response curve of the OTR PCR was established using 0.5–10 μg total RNA and cycle lengths of 20–40 cycles. Filters were developed by autoradiography and band density for OTR was normalized to the corresponding band for GAPdH by scanning densitometry.

OTR-Binding Assay in Cultured Cells

OTR binding was measured in a competitive binding assay using the OT analogue, ornithine vasotocin (OVT). Cells plated in 24-well plates were studied at confluence. Cells were treated with IL-6 (1 ng/mL) for 8 hr. The plates were washed with Hank's balanced salt solution. The cells were then incubated with 6 pM ^{125}I-labeled OVT (New England Nuclear, Boston, MA) and 0.05–50 nM unlabeled OVT in HBSS with 0.1% bovine serum albumin and 5 mM $MgCl_2$ at room temperature. After 3 hr the cells were washed with HBSS with 0.1% bovine serum albumin and the cells lyzed with 1 N NaOH. The lysate was counted on a gamma counter. Data were analyzed using a Scatchard plot with determination of B_{max} (fmol/mg protein) and K_d (nM). For time course and dose–response experiments, incubations were performed with 6 pM ^{125}I-OVT with and without 50 nM unlabelled OVT and total, specific, and non-specific binding compared.

To evaluate the role of phosphorylation pathways in the regulation of OTR binding by IL-6, the cells were treated with IL-6 after a 2-hr preincubation with either genistein or H7.

Statistical Analysis

Differences between IL-6, control, and IL-6 plus inhibitor-treated cells were compared using the Student's *t*-test. Time course and dose–response experiments were compared using analysis of variance (ANOVA). Significance was concluded for $P < 0.05$. Results are expressed as mean $(n = 6) \pm$ SEM and graphically presented as fold increase for OTR/GAPdH and OTR binding, respectively.

RESULTS

OTR RT-PCR

As the concentration of OTR mRNA in our myometrial tissue was very low, RT-PCR was used to measure changes in OTR mRNA expression. A time-dependent increase in OTR mRNA expression was found after IL-6 treatment. Fig. 1 shows the time

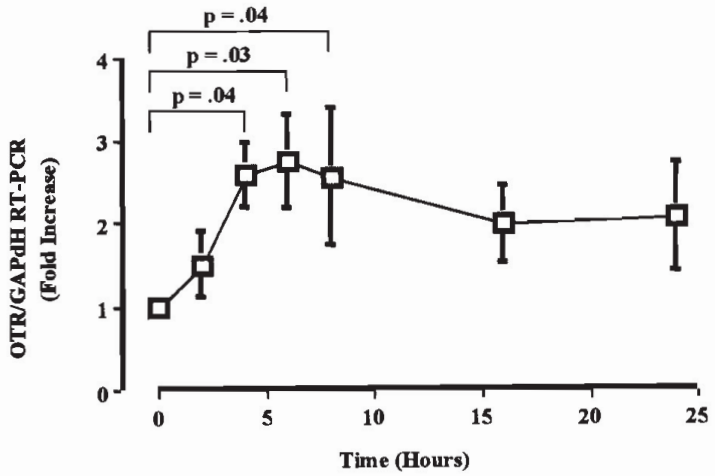

Fig. 1. Effect of duration of IL-6 treatment on OTR mRNA. OTR RT-PCR product (normalized to GAPdH) measured by Southern blot after exposure to 1 ng/mL IL-6 for various times. Data represent mean $(n = 6) \pm$ SEM. Significance is indicated as determined by ANOVA.

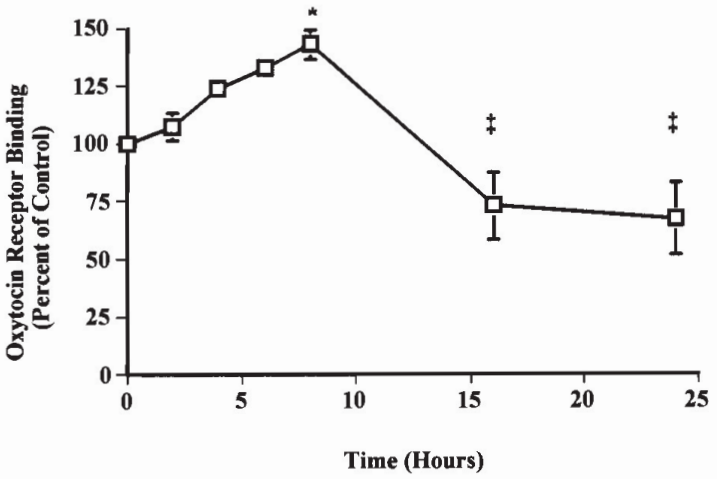

Fig. 2. Effect of duration of IL-6 treatment on OTR binding. Time course for OTR binding (expressed as percent of binding with control treatment) after exposure to 1 ng/mL IL-6 for various times. Data represent mean ($n = 6$) $\pm$ SEM. (*$P = 02$ compared with time 0, ‡$P = 0.02$ compared with time 8.)

course for OTR mRNA expression as measured by RT-PCR following exposure to IL-6 (1 ng/mL). A statistically significant increase in mRNA was detectable after 4, 6, and 8 hr of IL-6 treatment.

OTR Binding

Our results show a significant increase in OTR binding of 37% after stimulation with IL-6 (1 ng/mL) for 8 hr (Fig. 2). Maximal binding capacity was 775 ± 230 fmol/mg protein for control compared with 996 ± 293 fmol/mg protein for IL-6-treated cells ($P = 0.02$). The K_d was unchanged between control (1.83 ± 0.38 nM) and IL-6 (1.76 ± 0.35 nM)-treated cells. Scatchard analysis of the data revealed only one binding site for OVT. The OTR concentration returned to 75% of pretreatment levels after 24 hr of IL-6 exposure. IL-6 at concentrations as low as 10 pg/mL significantly increased OTR binding (Fig. 3).

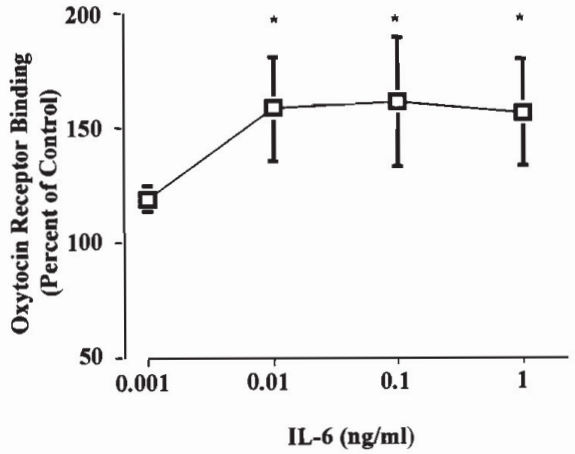

Fig. 3. Effect of concentration of IL-6 treatment on OTR binding. Dose–response for OTR binding (expressed as percent of binding with control treatment) after exposure to IL-6 for 8 hr at various concentrations. Data are expressed as mean ($n = 6$) $\pm$ SEM. (*$P =$ 0.02 compared with control treatment.)

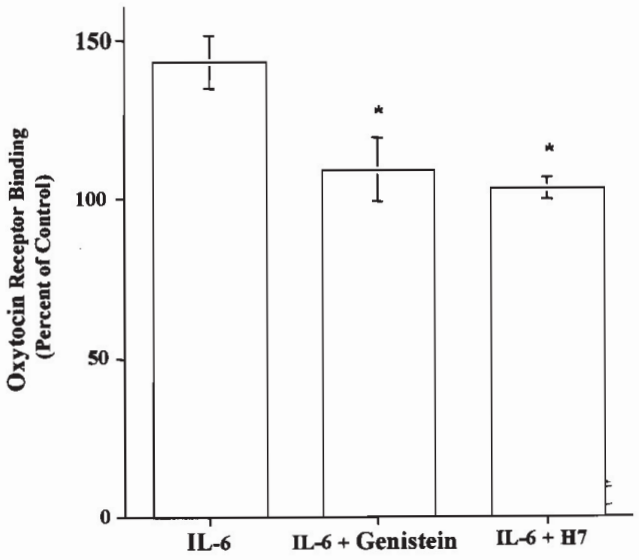

Fig. 4. Effect of kinase inhibitors, genistein and H7, on IL-6-induced changes in OTR binding. Data are expressed as mean ($n = 6$) $\pm$ SEM. (*$P = 0.01$ compared with IL-6 treatment alone.)

The role of phosphorylation pathways involved in the increase in OTR binding was then examined. Cells were treated with IL-6 in combination with genistein or H7, which both significantly blocked IL-6-induced increases in OTR binding (Fig. 4). Neither inhibitor nor vehicle (dimethylsulfoxide) had any effect on OVT binding in control cells.

DISCUSSION

Cytokine regulation of the OTR has been studied in both human myometrium and placenta, and we previously demonstrated that IL-1β dramatically down-regulates OTR mRNA and receptor binding in human uterine myocytes.[11] The present study demonstrates an IL-6-induced up-regulation of OTR gene expression and OTR binding in primary cultures of human myometrium cells. Estradiol is the only other substance known to up-regulate cultured myometrial cell OTR expression. Adachi and Masataka[14] demonstrated a 40 and 80% increase in OTR in human myometrial cells following incubation with 10^{-8} and 10^{-7} M estradiol, respectively. This increase is comparable with the 37% increase in OTR binding we observed in the present study.

Sequencing of the rat and human OTR gene 5′-upstream regulatory region has identified potential cytokine regulated pathways.[10,15] Six upstream DNA binding sites for NF-IL-6 were found in the OTR gene. NF-IL-6 represents the DNA-binding protein responsible for IL-1- and IL-6-induced transcription of IL-6 and cyclooxygenase-2.[16] Binding sites for STAT-3 have also been identified in the 5′-upstream sequence of the OTR gene.[15] IL-6 as well as tumor necrosis factor-α and lipopolysaccharides have been

shown to increase STAT-3 nuclear translocation and DNA binding. IL-1 is known to signal through NF-IL-6, but not STAT-3; therefore, it is likely that IL-6 stimulation of OTR transcription involves STAT-3 activation in possible synergism with NF-IL-6. This hypothesis is further supported by the observation that in our model IL-6-induced OTR up-regulation was completely blocked by the tyrosine kinase inhibitor, genistein, and the serine kinase inhibitor, H7. It is known that phosphorylation of STAT-3 by the tyrosine kinase JAK2 results in activation of the protein followed by its nuclear translocation and DNA binding.[17] Serine phosphorylation of STAT-3 has been shown to be necessary for STAT-3 homodimerization and activation.[18–20] Our study suggests that both a genistein-sensitive tyrosine kinase as well as H7-sensitive serine kinase is necessary for IL-6-induced OTR gene transcription. We have not determined whether a MAPK-mediated serine phosphorylation of the NF-IL-6 is cooperatively involved with STAT-3 in OTR transcription as suggested by others.[11,20] However, given that IL-1 alone does not increase OTR transcription, this suggests that NF-IL-6 binding alone is not sufficient to induce OTR gene transcription.

The IL-6 concentration required to up-regulate the OTR in myometrial cells is within the range of IL-6 concentrations found in serum, but significantly less than concentrations found in amniotic fluid from women in preterm labor with infection.[6,7] We observed an increase in the receptor with as little as 10 pg/mL IL-6. Greig et al.[7] measured IL-6 concentrations of 4.7 pg/mL in the serum of women with term labor. In this study, patients with preterm labor and failed tocolysis had a mean IL-6 concentration of 9.3 pg/mL, while those with chorioamnionitis had a mean concentration of 15.9 pg/mL. Even though fetal membrane tissue concentrations of IL-6 are not known, they are most likely higher than that found in serum, because decidua, chorion, and amnion are important local sources of IL-6.[2–4] IL-6 mRNA is detectable in the membranes from women in term and preterm labor and is markedly increased in the setting of chorioamnionitis.[21] The sources of IL-6 in membranes are macrophages and decidual stromal cells.[22] IL-6, therefore, may act in a paracrine fashion to up-regulate the OTR in adjacent myometrium *in vivo* in the setting of intra-amniotic infection.

Increasing sensitivity of the uterus to OT at term largely occurs as a result of enhanced expression of the OTR prior to the onset of labor.[8] Increased uterine sensitivity to OT is also observed in patients who deliver preterm. A retrospective analysis of OT challenge tests among complicated preterm pregnancies revealed that increased sensitivity to OT occurred immediately prior to preterm labor and delivery.[23] If IL-6 increases myometrial OTR expression *in vivo*, it may explain why elevated concentrations of IL-6 in the amniotic fluid are a strong predictor of failed tocolysis and the onset of preterm birth.[24] As IL-6 is significantly increased in plasma in patients with preterm labor with chorioamnionitis, it may play a key role in the regulation of the OTR expression in infection triggered labor. Furthermore, the observation that IL-6 up-regulates the OTR in human myometrium may provide important information necessary for the understanding of cellular signaling mechanisms responsible for OTR regulation in human uterus.

Acknowledgments

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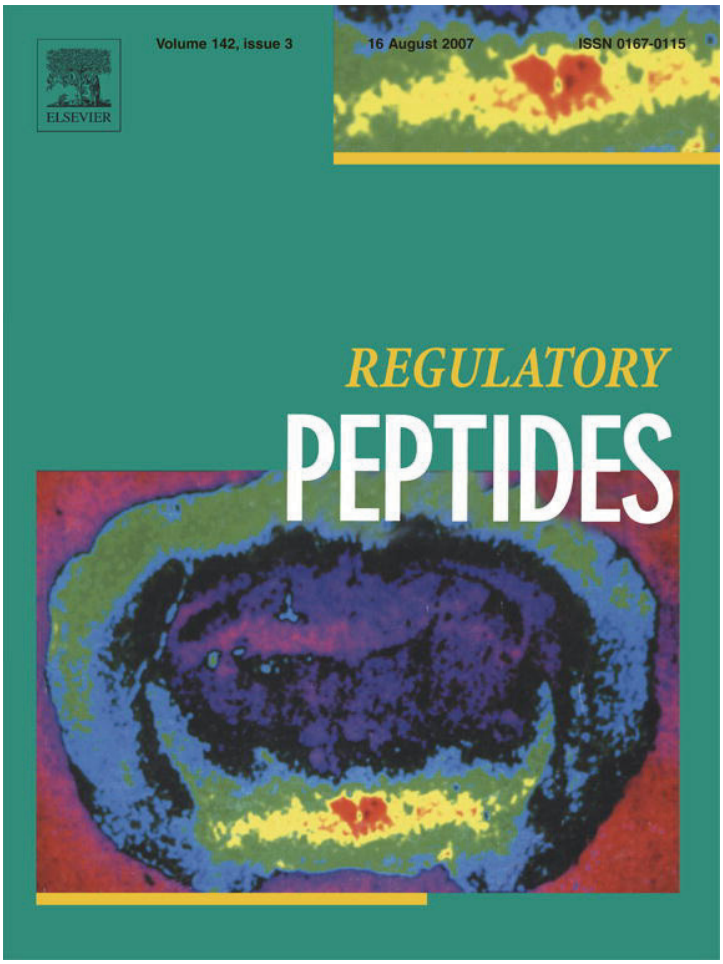

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The influence of interleukin-1β on oxytocin signalling in primary cells of human decidua

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Abstract

Objective: Oxytocin (OT) and its corresponding receptor (OTR), synthesized within the pregnant uterus, play a key role in the process of (preterm) labor as part of a paracrine system that regulates symmetrical contractility. In the setting of intrauterine infection, a major cause of preterm labour and birth, decidua serves as a major source of cytokine production. The present study evaluates the time-dependent effect [0–24 h] of the inflammatory cytokine Interleukin-1β (IL-1β) treatment on OT signalling and OT stimulated prostaglandin release in primary cultures of human decidua.
Study design: Primary cultures of human decidua ($n=6$) were treated with IL-1β [5 ng/ml] for 0–24h and or indomethacin [100 μM] – an inhibitor of the prostaglandin synthesis – for 0–24 h or for 24 h. OT peptide expression, OTR binding, Inositol triphosphate production (IP_3), and arachidonic acid (AA) as well as prostaglandin (PGE_2) release were measured.
Results: IL-1β transiently reduced cytoplasmic OT peptide at 4–6 h of IL-1β incubation, while its secretion into the media was increased after 6 h of stimulation. The later was completely blocked by indomethacin. A decrease in OTR mRNA expression and a loss of OTR binding were detected after 8 h and 16 h of IL-1β treatment, respectively. IL-1β also decreased IP_3 production and AA release, but significantly enhanced OT mediated PGE_2 production. This effect was completely suppressed by the cyclooxygenase-2 (COX-2) inhibitor NS-398.
Conclusion: Our data suggest, that IL-1β indirectly increases OT secretion in primary cultures of human decidua in a time dependent fashion through the production of prostaglandins through COX-2 and that this increase in OT peptide may secondarily down-regulate the OTR and its signalling cascade. These findings might explain the poor effectiveness of oxytocin receptor antagonists as tocolytic agents in the setting of intrauterine infection.

Keywords: Preterm labor; Cytokines; Prostaglandins; Maternal–fetal membranes; Contractions; Inflammatory processes; Cyclooxygenase

1. Introduction

Preterm Labor (PTL) with subsequent preterm birth (PTB) occurs in 5–10% of all pregnancies [1] and accounts for approximately 75% of all perinatal mortality and morbidity. Ten to twenty percent of PTL is caused by clinical or subclinical intrauterine infection [2]. The pathophysiology of infection-triggered PTL has been attributed to inflammatory cytokines as i.e. IL-1β, resulting in decidual prostaglandin release [3] and myometrial contractions. IL-1β has a wide range of biological activities but it is mainly involved in the genesis and maintenance of inflammatory responses. It binds to two different receptors (type I and II) and while both receptors are expressed in human decidua at the end of pregnancy [4], the type-I-receptor most likely transmits biological action via signal transduction [5]. The major physiological source of IL-1β are activated macrophages, which invade the decidua during pregnancy in high numbers [6]. Furthermore, this cytokine is also thought to be released from decidual stroma cells [7]. Several studies have linked IL-1β and human parturition [8,9]. Particularly, during PTL high amniotic fluid concentrations of IL-1β predict PTB within 7 days [10]; however, if IL-1β is absent, PTB occurs at a later time in pregnancy [11]. A connection between infection, cytokine production and PTL was also shown in various experimental animal models [12,13] and PTB was induced by systemic application of IL-1β in mice [14].

Binding of OT to OTR in human decidua, as in myometrium, activates phoshpolipase C, which converts phosphoinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 mediates mobilization of Ca^{2+} from intracellular stores, stimulating phospholipase A_2 activity and the

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DAG-mediated activation of proteinkinase C (PKC) leading to the release of AA from the cell membrane and activation of myosin light chain kinase (MLCK). Cyclooxygenase-1 (COX-1) and COX-2 catalyse the production of prostaglandins [15,16] from their precursor AA. Prostaglandins induce uterine contractions as well as they increase myometrial sensitivity to OT. OT expression in human decidua strikingly increases around the time of parturition [17] as does the expression of the OTR [18].

In former *in vitro* studies IL-1β induced down-regulation of the OTR and its signal transduction pathway in human myometrial primary cells could be seen [19].

The purpose of the present study was to evaluate the time-dependent influence of IL-1β on OT/OTR and their signalling pathway including IP_3 production, AA release and prostaglandin production in primary cells of human decidua.

2. Materials and methods

2.1. Patients and tissue preparation

Decidua was obtained within 20 min of delivery from term pregnant women (37–42 weeks of gestation) undergoing elective lower transverse Caesarean section. Subjects were excluded for maternal metabolic diseases, infection, evidence of labour and/or rupture of membranes, as well as multifetal gestation. Tissue collection was approved by the Labor and Delivery Research Specimen Collection Committee of Magee-Women's Hospital and the Magee-Women's Hospital Institutional Review Board.

Tissue culturing of decidual cells (DC) was modified after Delvin *et al*. 1985 [20]. Briefly, the decidua, separated from fetal membranes, was stored on ice for 20 min in Earl's salt solution supplemented with 250 mM $NaHCO_3$, 100 U/ml Penicillin/Streptomycin, 2.5 μg/ml Amphotericin B and 40 μg/ml Gentamycin. Digestion was performed in Earl's salt solution with 0.2% Collagenase Type III (Worthington Biochemical Corp., Lakewood, NJ) and 20 U DNAse grade II (Boehringer Mannheim, Indianapolis, IN) for 2 h at 37 °C. Digested cells were filtered serially through a sterile 100 μm nylon mesh (Sefar America Inc., Kansas City, MO) and centrifuged at 2200 rpm for 10 min at 4 °C. The cell pellet was resuspended in phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and layered over a discontinuous 50–10% Percoll gradient (Sigma, St. Louis, MO) in PBS. After centrifugation for 30 min at 3000 rpm and 4 °C, the cells in the 20–30% and 30–40% interface were collected, washed with PBS–BSA and the cell pellet then resuspended in DMEM medium supplemented with glutamine, 4.5 g/l glucose, 1× non-essential amino acids, 1 mM pyruvate, 26 mM $NaHCO_3$, 10% charcoal stripped fetal bovine serum (FBS), 100 U/ml Penicillin/Streptomycin, 2.5 μg/ml Amphotericin B and 40 μg/ml Gentamycin. Plated on 60 mm^2 culture dishes the cells were incubated at 37 °C in humidified 5% CO_2.

2.2. Experimental conditions

The culture medium was changed after 24 h and then every other day. For subculture, DC were harvested with 0.05% trypsin/0.53 mM EDTA and transferred to 60 mm^2 culture plates or 24-well-plates. All experiments were performed on passages 6–12.

The chosen concentration of 5 ng/ml IL-1β (R&D, Minneapolis, MN) used in our experiments is within the range of values [1–10 ng/ml] found in the amniotic fluid of women destined to deliver preterm due to infection [21].

For the choice of the OT concentration we refer to Schrey *et al.*, who used 1–1000 nM OT for their stimulation studies in DC [22].

2.3. Radioimmunoassay for OT peptide

Cultured DC were incubated with either DMEM or IL-1β [5 ng/ml] for different time periods [0–24 h]. To determine, if IL-1β-induced prostaglandin production has an impact on OT peptide secretion, we also stimulated the cells for 24 h with a combination of IL-1β and indomethacin [100 μM] – an inhibitor of the prostaglandin synthesis – (Merck, Rathway, NJ) or indomethacin alone.

OT peptide in the cells as well as OT peptide secretion into the supernatant of the cells were determined by radioimmunoassay (Peninsula Laboratories Inc., Belmont, CA) according to the manufacturer's instruction. The radioimmunoassay is based upon the competition of labelled ^{125}I-OT and unlabeled peptide, binding to a specific OT-antibody. The sensitivity of the kit ranged from 1–128 pg/100 μl supernatant. The inter-assay and intra-assay coefficients of variation were <15% and <5%, respectively.

Briefly, the cell supernatant was collected after IL-1β incubation and stored at −20 °C until assayed. For determination of OT peptide in DC, the cells were harvested with PBS supplemented with 1% phenylmethanesulfonyl fluoride (Boehringer Mannheim, Indianapolis, IN) and 1% leupeptin (Sigma, St. Louis, MO), stored over night at − 80 °C and centrifuged at 3000 rpm for 5 min. An aliquot of the supernatant was taken to determine OT peptide in the cells by radioimmunoassay. The amount of cytoplasmic OT was compared to the total amount of cell protein, determined by modified bradford assay (Bio-Rad, Hercules, CA). The experiments were performed in duplicate with cells isolated from 6 and 5 different patients, respectively.

2.4. OTR mRNA analysis

Total RNA was isolated after a method described by Chirgwin *et al*. [23]. Absorbance at 260 nm and 280 nm was used to estimate total RNA and the integrity of RNA was assessed by visualisation of a 1% ethidium bromide-stained agarose gel. Equal amounts of RNA [1–2.5 μg] were used to perform semi-quantitative RT–PCR of OTR and GAPDH.

For OTR RT–PCR oligonucleotide primers developed by Takemura were used [24]. The sequences of the primers were OTR-F 5′CCTTCATCGTGTGCTGGACG3′ and OTR-R 5′ CTAGGAGCAGAGCACTTATG3'. These primers amplify a 391 bp (1215–1602) fragment of OTR cDNA. The primed region spans parts of exons 3 and 4 of the OTR gene. Oligonucleotide primers for GAPDH amplifying a 500 bp fragment of GAPDH cDNA (kindly provided by Dr. A.

Westerhausen-Larson, Pittsburgh, PA) were used as control (GAPDH 1–5′GGCTGAGAACGGGAAGCTTG3′, GAPDH 2–5′TCTAGACGGCAGGTCAGGTC3′).

Briefly, cDNA was synthesized using total RNA, 20 U avian myeloblastoma virus reverse transcriptase (Promega, Madison, WI), 30 ng of oligo-dT, 5 mM $MgCl_2$, 1× RT-buffer and 200 μM dNTPs. One-fifth of the reverse transcription reaction was further amplified for 30 cycles with 1 U Taq polymerase (Promega), 100 pmol of the forward and reverse primers for OTR and GAPDH, 5 mM $MgCl_2$, 1×PCR-buffer and 200 μM dNTPs. The linearity of OTR RT–PCR products were established as described by Zingg *et al.* [25].

The amplified products of OTR and GAPDH were loaded on a 2% agarose gel (1:1 Standard/Nusieve GTG) and transferred to a nylon membrane (GeneScreen, New England Nuclear, Boston, MA) by overnight capillary transfer. The membranes were hybridized with randomly nick-linked OTR (kindly provided by Dr. Michael J. Brownstein, NIH, Bethesda, Maryland) and GAPDH (Clontech, Palo Alto, CA) cDNA probes labelled with ^{32}P-CTP (Amersham Pharmacia, Arlington Heights, IL) by random priming (Boehringer, Mannheim). Filters were developed by autoradiography and the band density of OTR was normalized to the corresponding band for GAPDH by scanning densitometry.

The experiments were performed with cells isolated from 6 different patients.

2.5. Radioligand-binding-assay

OTR binding was measured in a competitive binding assay using the OT analogue [^{125}I]-desGly(NH$_2$)^{9}d(CH$_2$)^5 [Tyr(Me)^{2}Thr^4]OVT (New England Nuclear). The specific radioactivity of the OVT was 2000 Ci/mmol or greater at the time of experiment. The cells were incubated with IL-1β [5 ng/ml] for 2–24 h for the time study and for 24 h for the determination of cell receptors per cell. Cells incubated with medium alone served as control.

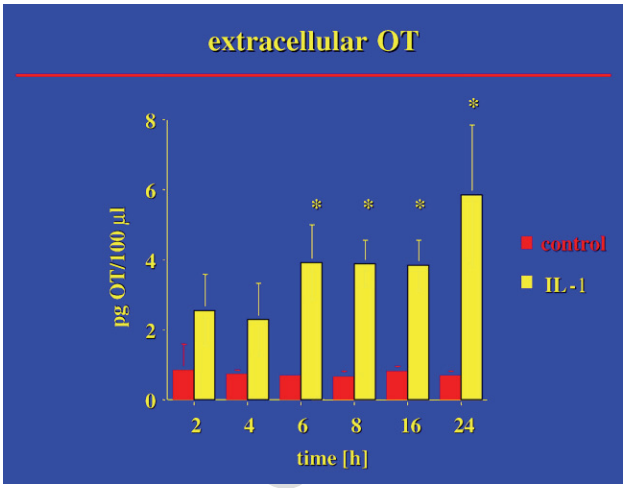

Fig. 2. *In vitro* effect of IL-1β exposure on OT peptide secretion of human DC. Data are expressed in pg OT per 100 μl supernatant, compared to control and represented as mean (n=6)±SEM (*dark bars*: control, *light bars*: IL-1β treatment). A significant increase of OT secretion was seen after 6, 8, 16 and 24 h (*, p<0.05).

After incubation the cells were washed with Hank's balanced salt solution (HBSS) and incubated with 6 pM ^{125}I-OVT with and without 50 nM unlabelled OVT in HBSS including 0.1% BSA and 5 mM $MgCl_2$ for 3 h at 25 °C. The cells were washed with HBSS with 0.1% BSA and lysed with 1 M NaOH. Standard curves were constructed according to the manufacturer's manual. The lysed cells were counted on a gamma counter (1277 Gammamaster, Wallac Inc., Gaithersburg, MD) and total, specific and non specific binding were compared. Incubation with 6 pM ^{125}I-OVT and 0.05 to 50 nM unlabelled OVT was performed for the determination of the cell receptors per cell. B_{max} (receptors/cell) and K_d [nM] were analyzed by scatchard plot using the computer program IGOR (WaveMetrics Inc., Lake Oswego, OR).

The experiments were performed in duplicate with DC isolated from 4 and 6 different patients, respectively.

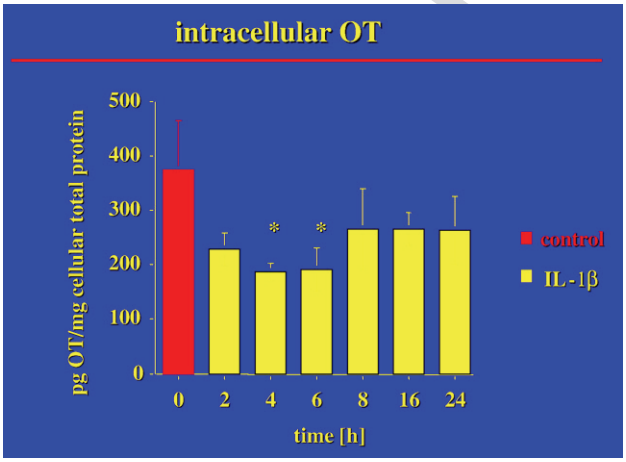

Fig. 1. Effect of IL-1β exposure on cytoplasmic OT peptide expression in human DC. Data are expressed in pg OT per mg total cell protein, and represented as mean (n=6)±SEM. A significant decrease in cytoplasmic OT peptide is seen after 4 and 6 h of IL-1β incubation (*, p<0.05).

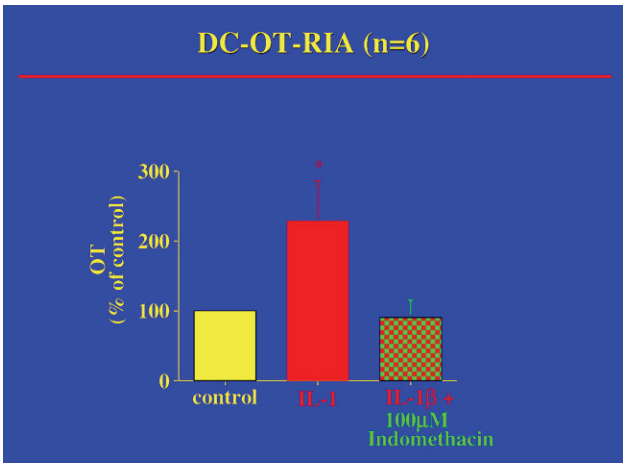

Fig. 3. Effect of 24 h exposure of vehicle (control), IL-1β, or IL-1β and indomethacin on OT peptide secretion in human DC. Data are expressed as percent of control and represented as mean (n=6)±SEM. A significant increase of OT secretion was seen after incubation with IL-1β alone (*, p<0.05).

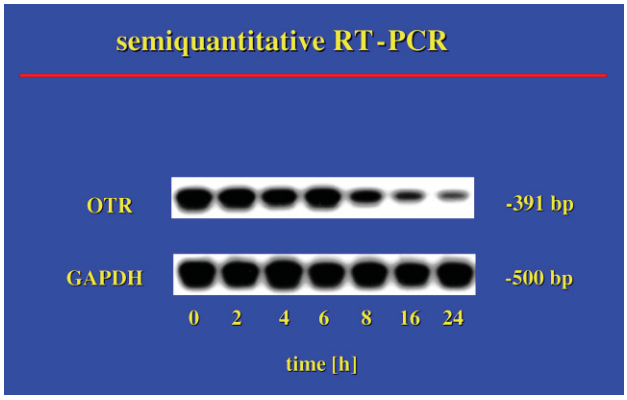

Fig. 4. Representative autoradiography of the radioactively probed OTR RT–PCR products of DC from one patient, corresponding to OTR/GAPDH mRNAs. Hours of IL-1β incubation are indicated [0–24 h].

2.6. IP_3 measurements

On the day of the experiment, at 95% cell confluence, the cells were washed with PBS and incubated with or without 5 ng/ml recombinant human IL-1β and [^{3}H]-myoinositol [3.5 μCi/ml] (New England Nuclear, Boston, MA) for 24 h in culture medium. The cells were harvested with trypsin/EDTA and washed with Hank's balanced salt solution and 5 mM cold unlabeled myoinositol and incubated in HEPES-Hank's buffer with 10 mM LiCl at 37 °C for 10 min. OT (Peninsula Laboratories, San Carlos, CA) was added at various concentrations [0–5000 nM] with 5 mM $MgCl_2$ at 37 °C for 10 min. The reaction was stopped by adding methanol/chloroform/hydrochloric acid (50:100:1 v/v) and after 10 min centrifugation at 2200 rpm aliquots of the upper phase were diluted with water and separated over Dowex resin columns (BioRad Laboratories, Hercules, CA). The final wash, representing total IP_3, was collected with 1 M ammonium formate/0.1 V formic acid and counted by liquid scintillation. All experiments were performed in triplicate with cells isolated from 6 different patients.

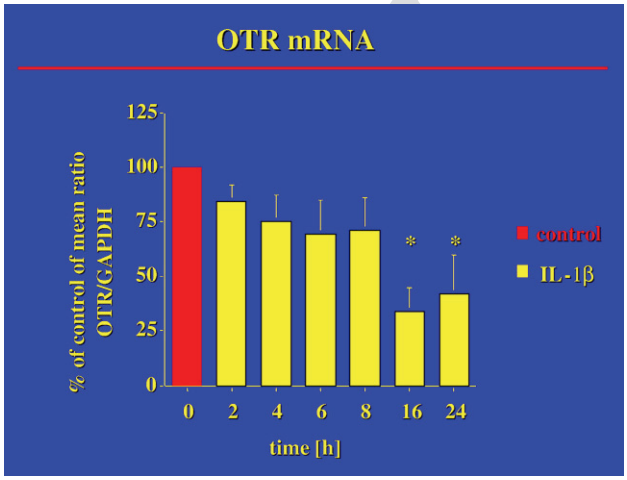

Fig. 5. Time dependent inhibitory effect of IL-1β on OTR mRNA expression in human DC ($n=6$). Data are expressed as percent of control of mean ratio (OTR/GAPDH)±SEM. OTR mRNA expression significantly decreases after 16 and 24 h of IL-1β incubation. (*, $p \leq 0.05$).

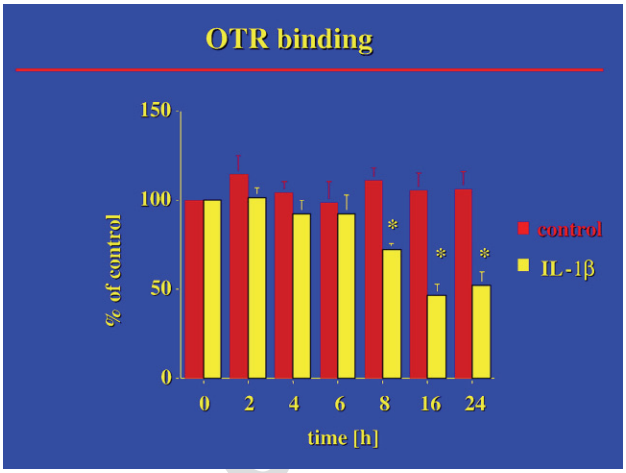

Fig. 6. Time dependent inhibitory effect of IL-1β on OTR binding in human DC. Data are expressed as percent of control ($n=6$)±SEM (*dark bars*: control, *light bars*: IL-1β treatment). A significant decrease of OTR binding is seen after 8, 16 and 24 h (*, $p \leq 0.05$).

2.7. AA release measurement

DC were grown to confluence in 24-well-plates in DMEM with 5% FBS. After incubation for 24 h with or without IL-1β [5 ng/ml] and [^{3}H]-AA [0.4 μCi/well] (New England Nuclear, Boston, MA) in 2% FBS-DMEM followed for 24 h, the cells were washed with HEPES/Hank's including 0.2% BSA. OT [0–5000 nM] with 5 mM $MgCl_2$ was added at 37 °C for 10 min. The reaction was terminated by the addition of 0.5 M NaOH and the release of radio labelled AA was measured by liquid scintillation as above. All experiments were performed in triplicate with cells isolated from 6 different patients.

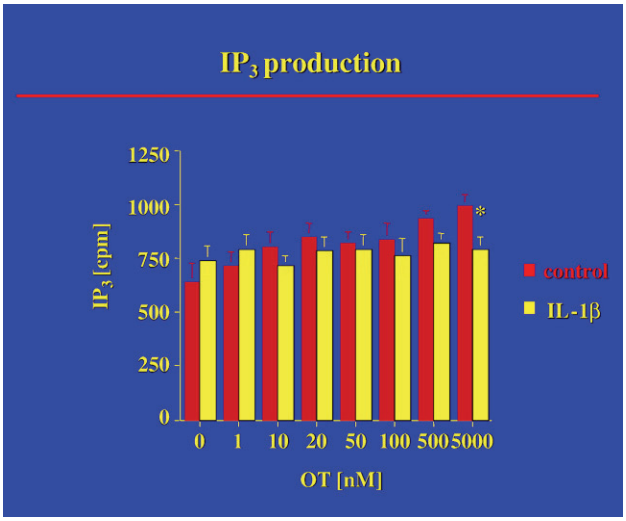

Fig. 7. Effect of 24 h IL-1β [5 ng/ml] exposure on OT [0–5000 nM] induced IP_3 production in human DC. Results as counts per minute (cpm) were obtained from 6 different patients and performed in triplicate. Data are represented as mean ($n=6$)±SEM (*dark bars*: control, *light bars*: IL-1β treatment). * indicate significant difference ($p<0.05$) between IL-1β induced responses compared to control group.

2.8. PGE_2 enzyme-linked immunosorbent assay

PGE_2 was measured in the supernatants of DC grown to confluence and treated with different concentrations of OT [0–100 nM] with or without pre-treatment with IL-1β [5 ng/ml], NS-398 [32 mM] (Biomo Research Laboratories, Inc., Plymouth Mission, PA), a selective COX-2 inhibitor or IL-1β and NS-398 in combination for 24 h. PGE_2 was measured in the media by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (Amersham Pharmacia Biotech, Arlington Heights, IL). The sensitivity of the assay for PGE_2 was 2.5–320 pg/well or 50–6400 pg/ml and the inter- and intra-assay coefficients of variation were <13% and <9%, respectively. The experiments were performed in duplicate with cells isolated from 3 different patients.

2.9. Statistical analysis

Repeated measures of time course experiments were analysed using ANOVA (ANOVA Microsystems Inc., San Jose, CA) and differences among treatment groups were evaluated by Student's *t*-test. Significance was concluded for $p<0.05$.

3. Results

3.1. Radioimmunoassay for OT peptide

A 50% decrease in cytoplasmic OT peptide expression was observed in DC after 4 and 6 h of IL-1β incubation compared to controls. However, this effect was reversed after 8 h of cytokine stimulation (Fig. 1). While IL-1β transiently decreased OT peptide in the cells, a significant increase in OT secretion into the supernatant of DC was seen after 6, 8, 16 and 24 h of IL-1β incubation (Fig. 2). This IL-1β triggered increase in OT peptide secretion was completely suppressed by co-treatment with indomethacin as shown in Fig. 3.

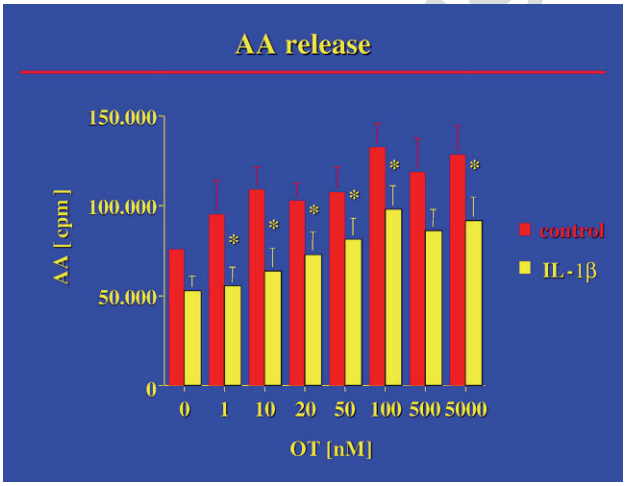

Fig. 8. Effect of 24 h IL-1β [5 ng/ml] exposure on OT [0–5000 nM] induced AA release in human DC. Results as counts per minute [cpm] were obtained from 6 different patients and performed in triplicate. Data are represented as mean ($n=6$)±SEM (*dark bars*: control, *light bars*: IL-1β treatment). * indicate significant difference ($p<0.05$) between IL-1β induced responses compared to control group.

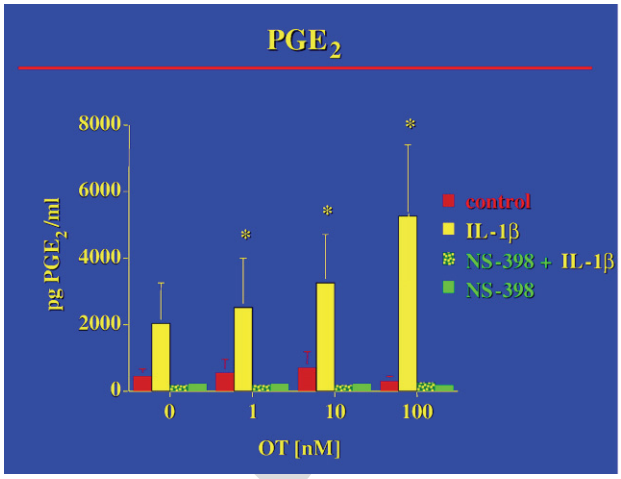

Fig. 9. Effect of 24 h IL-1β [5 ng/ml] exposure, combined with or without NS-398 on OT induced PGE_2 production in human DC. Results as pg/ml were obtained from 3 different patients and performed in duplicate. Data are represented as mean ($n=6$)±SEM (dark *bars*: control, *light bars*: IL-1β treatment, *dark grey bars*: IL-1β treatment and NS-398, *light grey bars*: NS-398). * indicate significant difference ($p<0.05$) between IL-1β induced responses compared to control groups.

3.2. OTR mRNA analysis

As OTR mRNA concentration in human decidua is low, we used RT–PCR to measure the effect of IL-1β on OTR steady state mRNA level. RT–PCR of GAPDH mRNA was performed as internal control and for semi-quantitation. OTR mRNA expression was detected in all samples tested. Fig. 4 shows a representative Southern blot of OTR vs. GAPDH RT–PCR bands in a time course experiment with IL-1β for DC from one patient.

Fig. 5 demonstrates a 50% decrease in OTR mRNA expression in primary cultures of DC cells ($n=6$) measured by RT–PCR following the exposure to IL-1β for 16 and 24 h.

3.3. Radioligand-binding-assay

No significant change of specific OVT binding over time was detected in the DC tested ($n=6$) under normal culture conditions. However, a significant loss of specific OVT binding was seen after 16 h and 24 h of IL-1β incubation (Fig. 6). Scatchard analysis of OVT binding at 24 h of IL-1β treatment showed remaining 40% of receptor binding sites compared to non-treated cells. The calculated K_d values were not significantly different from each other with 0.16 nM for control and 0.35 nM for IL-1β treated cells, respectively.

3.4. IP_3 production

OT-induced total IP_3 production was measured in DC treated with or without 5 ng/ml recombinant human IL-1β and [^{3}H]-myoinositol [3.5 μCi/ml] for 24 h (Fig. 7).

Under control conditions, IP_3 increases with increasing OT, however, under IL-1β treatment, there is no apparent increase in IP_3 with increasing OT ($p<0.001$). This leads to a significant difference in IP_3 production between IL-1β and control treated cells at 5000 nM OT.

3.5. AA release

OT-induced release of AA was measured in cells treated with or without IL-1β [5 ng/ml] and [^{3}H]-AA [0.4 μCi/well] in 2% FCS-DMEM for 24 h. We did not detect any difference in basal AA release between control and IL-1β treated cells but in both treatment groups OT increased AA release in a dose-dependent manner ($p<0.001$). Our regression analysis showed that the increase in AA release was the same in both groups ($p=0.94$). However, for any given concentration, the IL-1β group showed significantly less AA release than the control ($p>0.001$), which did not change with increasing OT concentrations (Fig. 8).

3.6. PGE_2 production

PGE_2 production was measured after 4 h of OT treatment [0–100 nM] in cells exposed to media alone or IL-1β for 24 h (Fig. 9). OT alone at any given concentration did not have any effect on PGE_2 production ($p=0.31$), but IL-1β markedly up-regulated prostaglandin expression. As IL-1β up-regulates the inducible COX-2, the contribution of COX-2 to prostaglandin production was measured by incubating the cells with the selective COX-2 inhibitor, NS-398 alone and in combination with IL-1β. NS-398 had little or no effect on PGE_2 production in our DC cultures compared to control groups, but NS-398 completely suppressed the OT-induced increase in prostaglandin production in IL-1β treated cells ($p=0.001$).

4. Discussion

The pathophysiology of infection-triggered (preterm) labour has been attributed to inflammatory cytokine release, e.g. IL-1β, by fetal–maternal tissues and high amniotic IL-1β levels found during preterm labour predicting preterm birth within 7 days [2].

OT–OTR interaction plays a key role in the process of (preterm) labour, which leads to myometrial [2] and decidual [26] prostaglandin production through the release of AA.

We previously demonstrated an IL-1β induced down-regulation of the OTR in human myometrial cells, followed by a marked impairment in OT–OTR signalling through IP_3 and AA [9,10]. In the present study, we were able to show, that the pro-inflammatory cytokine IL-1β effects OT and OTR signalling and regulation including IP_3 production, AA release and PGE_2 production in primary cultures of human decidua, too.

IL-1β incubation of DC evoked a significant, time-dependent increase in OT peptide secretion, while at the same time intracellular OT peptide concentration decreased.

Similar effects of IL-1β were seen in studies of our group with primary cultures of human myometrium [27]. Further support comes from animal studies showing IL-1β-induced increases of OT peptide release in hypothalamic explants as well as in electrically stimulated neurohypophysis of the rat [28,29]. Naito *et al*. demonstrated an increase in plasma OT levels after systemic infusion of IL-1β in rats [30]. These findings are standing in contrast to recent results by Terzidou *et al*. who found increases in OTR mRNA and ligand binding in human myometrial cells through IL-β activation of the transcription factors C/EBPbeta and NF-kappaB in the OTR promotor [31].

IL-1β is known to activate cyclooxygenase-2 which is followed by stimulation of the prostaglandin production in human decidua [32–34]. Prostaglandins have the capacity to release OT from various tissues [35] and to increase tissue sensitivity for OT. In the present study co-treatment of IL-1β with indomethacin – an inhibitor of the prostaglandin synthesis – resulted in a complete suppression of the IL-1β-induced increase in OT peptide secretion into the supernatant of human DC cultures after 24 h of incubation. Similarly, Bojanowska and Guzek demonstrated an indomethacin-induced inhibition of prostaglandin synthesis in rats resulting in a significant decrease of OT release from the neurohypophysis [36]. Summy-Long and co-workers could show that indomethacin prevented the enhancement of OT secretion induced by centrally administered IL-1β [37]. Plasma OT concentrations in pregnant women at term without and with labour are known to be approximately ≤20 nM and ≤45 nM OT, respectively [38,39], but literature assumes that local OT concentrations in the pregnant uterus are much higher. Mitchell and co-workers did not see any activation of the rat uterus during late gestation after intraperitoneal infusions of IL-β or TNF-α while maternal OT serum levels increased significantly [40]. This underlines that *local concentrations* of cytokines seem to be of relevance for the regulation of cellular peptides and their receptors.

Prolonged IL-1β stimulation not only resulted in up-regulation of OT secretion into the supernatant but also in a down-regulation of the corresponding receptor OTR. After 16 h of IL-1β incubation, 10 h after a significant increase in OT peptide secretion, we observed a pronounced down-regulation of the OTR ligand binding capacity. This down-regulation of OTR is due to a decrease in OTR binding sites as demonstrated by scatchard plot. Receptor binding affinities (K_d [nM]) for control and IL-1β treated cells were not significantly different from each other when measured at 24 h of incubation. Similar results were acquired by a group around Mitchell who found a significant decrease in OTR receptor mRNA measured by ribonuclease protection analysis after IL-1β treatment of human myometrial cells [41].

The present study further evaluated the effect of prolonged IL-1β incubation on the phosphoinositide pathway in human DC. 24 h treatment of IL-1β resulted in a slight impairment of OT-induced IP_3 production and in a marked decrease of cellular AA release in DC. In recent studies, we were able to show, that prolonged IL-1β exposure to human myometrial cells resulted in down-regulation of the OTR [42] with a subsequent decrease in IP_3 production and AA release, but an increase in $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production [19].

In the present study, we saw four different phenomenons by testing PGE_2 expression under the influence of IL-1β:

1. OT alone did not increase prostaglandin production in our DC cultures at any given concentration. Other studies (e.g. Fuchs *et al*.) have found a significant increase in $PGF_{2\alpha}$ and PGE_2 production in human decidual tissue after incubation with OT [18]. One theory might be that cofactors for this stimulation (e.g. endogenous IL-β or other cytokines) might be missing in isolated cultured cells but available in decidual tissue consisting of many different cell types.
2. IL-1β markedly increased PGE_2 production in comparison to control treated cells. Even though the differences are graphically apparent, however, they did not differ significantly from control treated cells at OT concentrations less than 100 nM. This might be due to the fact, that in primary cell cultures basal production rates of prostaglandin vary considerably between cells from different patients [43] and our n (=3) is small.
3. IL-1β-induced increase in prostaglandin production was completely blocked by NS-398, a specific COX-2 inhibitor. COX-2 most likely is the enzyme responsible for IL-1β-induced prostaglandin production in our DC cultures. This assumption is supported by previous studies that assessed IL-1β induces COX-2 expression in human decidua [44].
4. Increasing concentrations of OT enhanced IL-1β-induced prostaglandin production even though OT signalling through the phosphoinositide pathway was impaired. Mitchell *et al*. suggested an OT-induced activation of COX-2 through myosin activated protein kinase (MAPK), bypassing the OTR in human myometrial cells [45]. The mechanism by which OT enhances IL-1β-induced PGE_2 production in our model is not exactly known, but a bypass of the receptor would also explain the ineffectiveness of OTR antagonists in many patients with preterm labour.We conclude that despite a decrease in OT-mediated IP_3 production and AA release in decidua following IL-1β exposure, OT and IL-1β act cooperatively to increase prostaglandin production in human decidua in the setting of (preterm) labour associated with infection. Since prostaglandins induce as well as maintain uterine contractions during (preterm) labour, the use of selective COX-2 inhibitors – as clinically proven – might be a promising tool to prevent preterm labour in the future if side effects can be minimized.

Acknowledgements

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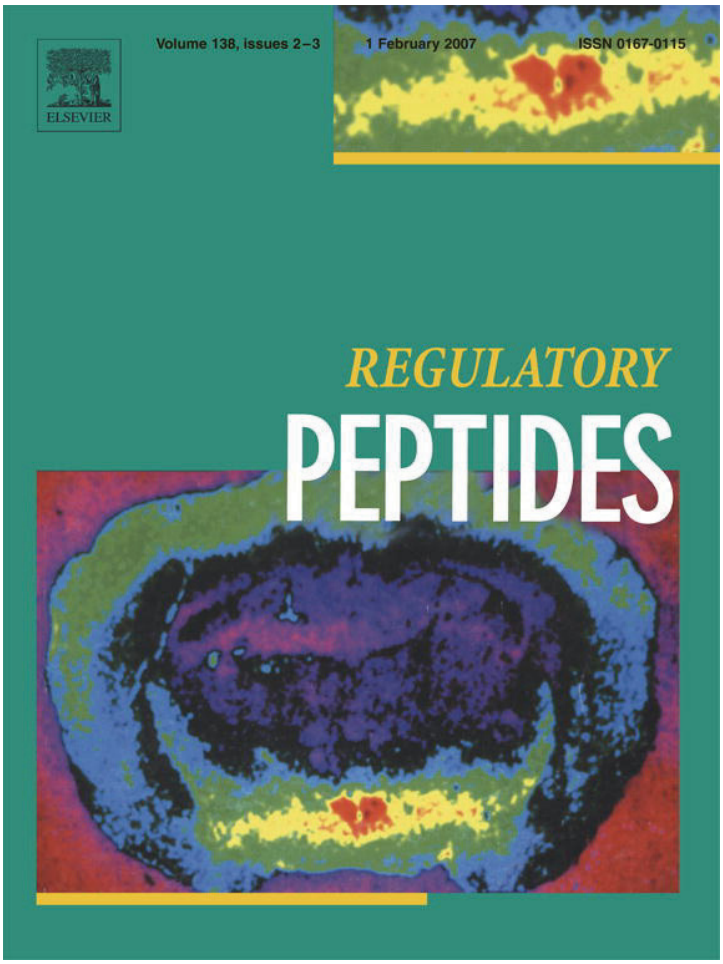

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The effect of relaxin on the oxytocin receptor in human uterine smooth muscle cells

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Abstract

Experimental objectives: Activation of the oxytocin receptor (OTR) induces phospholipase C induced PIP_2 turnover in the human uterus. Relaxin (RLX), a polypeptide hormone produced in the corpus luteum of pregnancy as well as in the placenta and decidua inhibits PIP_2 turnover and subsequent signaling in human myometrium. The purpose of this study was to evaluate a possible effect of RLX on OTR regulation in human uterine smooth muscle cells. Primary cultures of myometrium from term pregnant women undergoing elective caesarean section were incubated for different time periods (0–96 h) and with different concentrations of RLX [10 pg/ml–20 μg/ml]. The effects on OTR binding, mRNA and protein expression were evaluated by means of ^{125}I-OVT binding assay, RT-PCR and flow cytometry.
Results: Prolonged RLX incubation was able to inhibit 30–40% of OTR binding while binding affinity remained unchanged. Oxytocin receptor mRNA and protein expression were down regulated by RLX about 50% and 35% respectively.
Conclusion: We report for the first time an effect of RLX on OTR regulation in human uterine myometrial cells. The above results indicate that high local uterine RLX concentrations may be involved in uterine quiescence during human pregnancy by down regulating the OTR.

Keywords: Signaling; Peptide regulation; Preterm labor; Insulin-like growth factor family

1. Introduction

Relaxin (RLX), a polypeptide of 6 kDa size, belongs to the insulin-like growth factor family and was first described in 1926 by Frederick L. Hisaw [1]. This "pregnancy associated peptide" consists of 57 amino acids and two polypeptide chains. One intra- and two interchain disulfide bridges stabilize the structure of the protein in analogy to insulin [2]. The third chain C is removed during processing of the pre-hormone [3]. While the receptor interaction site is located in the B chain in a Arg–X–X–X–Arg–X–X–Ile motif, which is also necessary for biological activity, the A chain seems to be necessary to determine RLX's binding to its own receptors [2,3]. In 2002 Hsu et al. [4] detected the orphan receptors LGR7 and LGR8. Leucine-rich G-protein-coupled receptor 7 has been confirmed to bind relaxin 2 in an immortalized myometrial cell line prepared from pregnant females [5].

The human genome contains three distinct genes for RLX (H1, H2 and H3) located on chromosome 9 (H1, H2) [6] and chromosome 19 [7]. Relaxin 1 is expressed in human decidua, placenta and prostate [8], while relaxin 2 seems to be the circulating form [9].

Relaxin has numerous effects on the reproductive system including endometrial vascularization and remodeling of connective tissue leading to structural changes as related to the increase in breast and abdominal size, loosening of joints and tendons and softening of the cervix in preparation for birth. In

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pregnant rodents and pigs, RLX also performs uterine relaxation during pregnancy even after OT stimulated contractions [10–13] while its role in uterine quiescence of higher mammals including humans still remains unclear. Serum RLX is discussed inconsistently as a possible indicator for premature labor in women within 30 weeks of gestation [14]. Weiss et al. reported that elevated serum RLX concentration in pregnant women after ovarian stimulation in the first trimester predicts prematurity [15].

During human pregnancy RLX is mainly produced by the corpus luteum, placenta and decidua with highest serum concentrations [1 ng/ml] during the first trimester [16–18]. It is noteworthy that peripherally measured relaxin (H2 relaxin) is produced by the corpus luteum of pregnancy. On the contrary relaxin (H1 relaxin) produced by myometrium, decidua and placenta can only be detected locally. Both forms are encoded by different genes (*rln1* and *rln2*) and are thought to be the results of an evolutionary gene duplication [19]. In myometrium RLX induces cyclic AMP (cAMP) production by activating adenylate cyclase (AC). This results in the stimulation of Ca^{2+}-activated K^+-channels with K^+ transport to the extracellular space and activation of protein kinase A (PKA) as well as inhibition of phosphoinositolbiphosphate (PIP_2) turnover [20,21] by specific phospholipase C (PLC) (Fig. 1) [5,22–29]. Inhibition of PIP_2 turnover leads to a diminished liberalization of Ca^{2+} from the sarcoplasmic reticulum as well as Ca^{2+} entry from the extracellular space [30] stabilizing the membrane potential of the cells.

This way RLX seems to play a key role in controlling the ion content of muscle cells and therewith, uterine cell contractility. Sanborn et al. [31] showed in rats that RLX was able to stimulate the production of cAMP with subsequent inhibition of PIP_2 turnover followed by a reduced production of inositoltriphosphate (IP_3) and diacylglycerol (DAG) — second messengers of the OTR signaling cascade. Relaxin treatment also altered the kinetic properties of MLCK activity in cultured myometrial cells by cAMP-mediated phosphorylation which was associated with a decreasing affinity of calmodulin binding to MLCK promoting uterine relaxation [30].

The nonapeptide hormone OT has several physiological functions including milk ejection and induction of uterine contractions at birth. The physiologic response to OT is mediated by the OTR, a typical seven transmembrane G-protein-coupled receptor linked to the IP_3–PKC signal transduction pathway [32]. In myometrium OT binding to the receptor exerts the ligand's dual role in stimulating cell contractility by activating specific PLC to produce a.) IP_3, leading to the release of intracellular Ca^{2+} and b.) DAG, which stimulates the liberalization of arachidonic acid and subsequent production of contractile prostaglandins [33]. High Ca^{2+} concentrations contribute to the activation of calcium calmodulin and MLCK resulting in myometrial contractions.

While RLX is proposed to decrease PIP_2 turnover by inhibiting PLC, thus blocking OT action [31], the objective of the present study was to evaluate if high local RLX has any inhibitory effect on OTR regulation which might subsequently slow down PIP_2 turnover and further down stream signal cascades in human myometrium primary cells.

2. Material and methods

2.1. Study population

Segments of the upper margin of the lower uterine segment were obtained from term (37–42 weeks) pregnant women undergoing elective Caesarean section. Despite the fact, that the fundus of the uterus contains OTR in greater numbers than the lower uterine segment fundus tissue was not accessible during routine Caesarean section.

Subjects were excluded for maternal metabolic diseases (e.g. diabetes), labor, rupture of membranes or multifetal gestation. Informed consent was obtained from all patients in accordance with the Magee-Women's Hospital Institutional Review Board.

2.2. Cell culture

Cell cultures of human myometrium were established as previously described [34]. Culture medium contained minimal essential medium (MEM) supplemented with 580 mg/l glutamine, 4.5 g/l glucose, 1× non-essential amino acids, 1 mM pyruvate, 26 mM $NaHCO_3$, 10% charcoal-stripped fetal bovine serum, 100 U/ml Penicillin/Streptomycin, 2.5 μg/ml Amphotericin B and 40 μg/ml Gentamycin. Cells were maintained at 37 °C in humidified 5% CO_2, and medium was changed every 2 days. Subculturing was performed in 100 mm^2 culture plates after short trypsination (0.25% Trypsin/0.1% EDTA). All cell culture agents were purchased through Mediatech Inc., Cellgro (Herndon, VA).

2.3. Relaxin

At confluence (70–90%) (3rd to 6th passage), the myometrium cells were incubated with different concentrations [10 pg/ml–20 μg/ml] of recombinant human relaxin H2 (rhRLX H2) for 24 h or with 5 μg/ml RLX for different time points (0–96 h). Cells incubated with medium alone served as control.

2.4. Immunocytochemistry

The total number of cells were determined by staining with 4,6-diamidino-2-phenylindol (DAPI), the purity of isolated primary myometrial cells by staining with monoclonal mouse anti-smooth muscle α-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

At 90% confluence cells were centrifuged and 5×10^4 cells passed on Lab-Tek® Chamber Slides® (Nunc, Karlsruhe, Germany). After three days of cultivation the cells were washed twice with PBS and then fixed and permeabilized in − 20 °C methanol for 5 min. After incubation in 10% CS-FBS in PBS for 20 min to saturate non-specific binding, cells were incubated with monoclonal mouse anti-smooth muscle α-actin (1:5000) in PBS/0.5% BSA at 37 °C for 2 h. As negative control mouse monoclonal anti-von Willebrand factor (Dako, Hamburg, Germany) was used. After washing with PBS cells were incubated with the fluorescence coupled Alexa® Fluor 488 monoclonal rabbit anti-mouse IgG $F(ab')_2$ antibody (Molecular Probes, Karlsruhe, Germany) in a 1:400 dilution in PBS/0.5% BSA for 1 h at room temperature in the dark. Cells were then embedded

in VECTASHIELD® Mounting Medium with DAPI [1.5 µg/ml] (Vectorlabs, Burlingame, CA), stored at 4 °C to stabilize fluorescence. For evaluation Leica microscope (Leica, DC 300 F) and Leica IM500 Image Manager program were used.

2.5. Radioligand-binding assay

The availability of appropriate OTR ligands in particular the OT analogue [^{125}I]-9d(CH_2)$_5$-[Tyr(Me)^2,Thr^4,Tyr-NH_2^9]-ornithine–vasotocin (OVT) permits detailed studies of OTR pharmacology. OTR binding was measured in a competitive binding assay using OVT (New England Nuclear, Boston, MA). This assay is based upon the competition between labeled ^{125}I-OVT and unlabeled peptide binding to a limited quantity of specific antibody. As the concentration of unlabeled protein increases, the amount of labeled protein able to bind to the antibody decreases. A standard curve was constructed by measuring the amount of labeled protein bound as a function of the concentration of the unlabeled molecule in standard reaction mixtures. The specific radioactivity of the OVT was 2000 Ci/mmol or greater at the time of experiment. Cells from 5 different patients were grown to confluence and incubated in 24-well dishes in duplicate with increasing concentrations [10 pg/ml–20 µg/ml] of RLX for 24 h or with 5 µg/ml RLX for different time points (0–96 h) for the determination of cell receptors per cell. Cells incubated with medium alone served as control. After incubation the cells were washed with Hank's balanced salt solution (HBSS) and incubated with 6 pM ^{125}I-OVT with and without 50 nM unlabelled OVT in HBSS including 0.1% BSA and 5 mM $MgCl_2$ for 3 h at 25 °C. The cells were washed with HBSS with 0.1% BSA and lysed with 1 M NaOH. The lysate was counted on a gamma counter (1277 Gammamaster, Wallac Inc., Gaithersburg, MD) and total, specific and non-specific binding were compared. Incubation with 6 pM ^{125}I-OVT and 0.05 to 50 nM unlabelled OVT was performed for the determination of the cell receptors per cell. B_{max} (receptors/cell) and K_d (nM) were analyzed by scatchard plot using the computer program IGOR (WaveMetrics Inc., Lake Oswego, OR).

All experiments were performed in duplicate with cells of 5 different patients.

2.6. RNA isolation and RT-PCR for OTR

Since human myometrium cells express low but detectable mRNA levels of OTR we used RT-PCR to measure the effect of RLX on OTR transcription.

Total RNA was isolated from myometrial tissue after the single-step method described by Chirgwin et al. [35]. Tissues were homogenized in a 4 M solution of the protein denaturant guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol to break protein disulfide bonds. The RNA was isolated free of protein by ethanol precipitation. Absorbance at 260 nm and 280 nm was used to estimate total RNA and the integrity of RNA was accessed by visualisation of a 1% ethidium bromide-stained agarose gel. Equal amounts of an RNA [1–2.5 µg] were

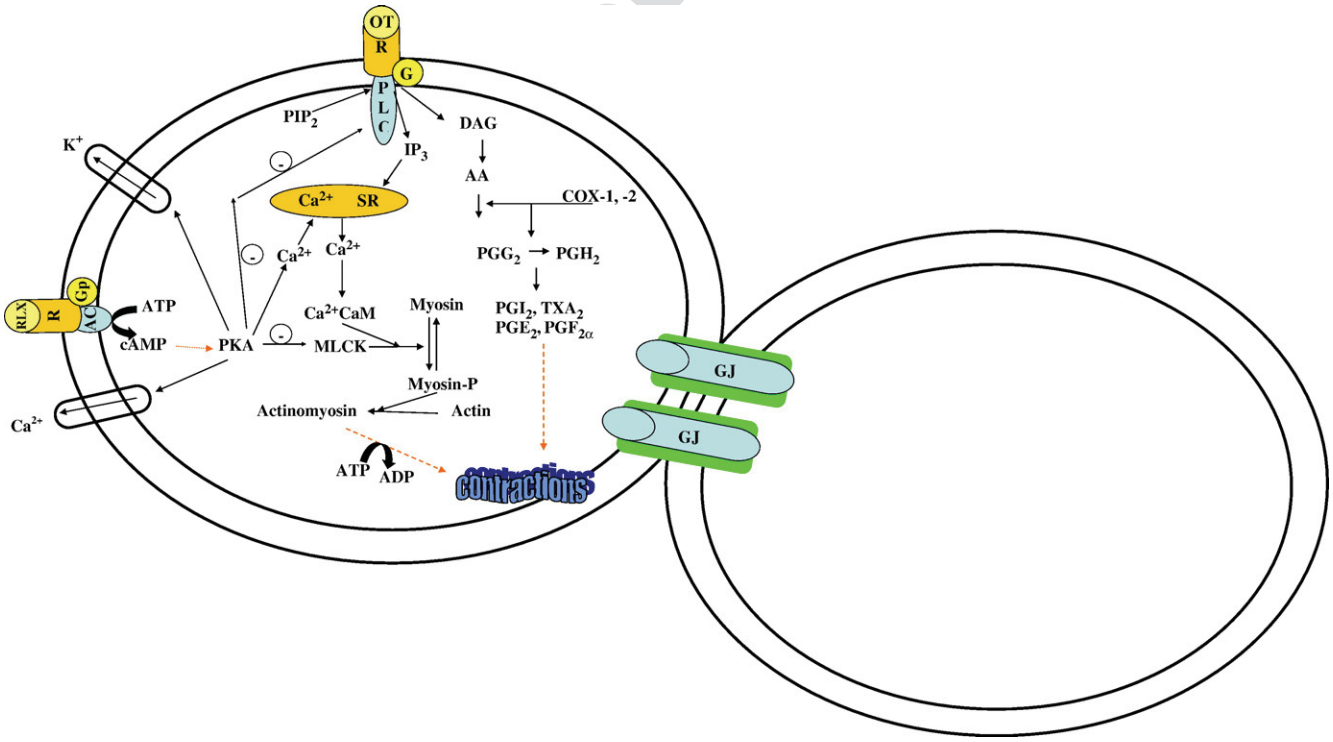

Fig. 1. Relaxation and contraction in uterine smooth muscle cells. (AA) arachidonic acid, (AC) adenylate cyclase, (ATP, ADP, cAMP) adenosin-triphosphate, -diphosphate, cyclic adenosin-monophosphate, (Ca^{2+}) calcium, (CaM) calcium–calmodulin-complex, (COX) cyclooxygenase, (DAG) diacylglycerol, (GJ) gap junctions, (Gp/G) G-protein-coupled receptor, (IP_3) inositol-triphosphate, (K^+) potassium, (MLCK) myosin–lightchain-kinase, (PIP_2) phosphatidylinositol-4,5-bisphosphate, (PKA) protein kinase A, (PLC) phospholipase C, (PG) prostaglandin, (R) receptor, (SR) sarcoplasmic reticulum, (TXA_2) thromboxane. — stimulation, - - - - inhibition.

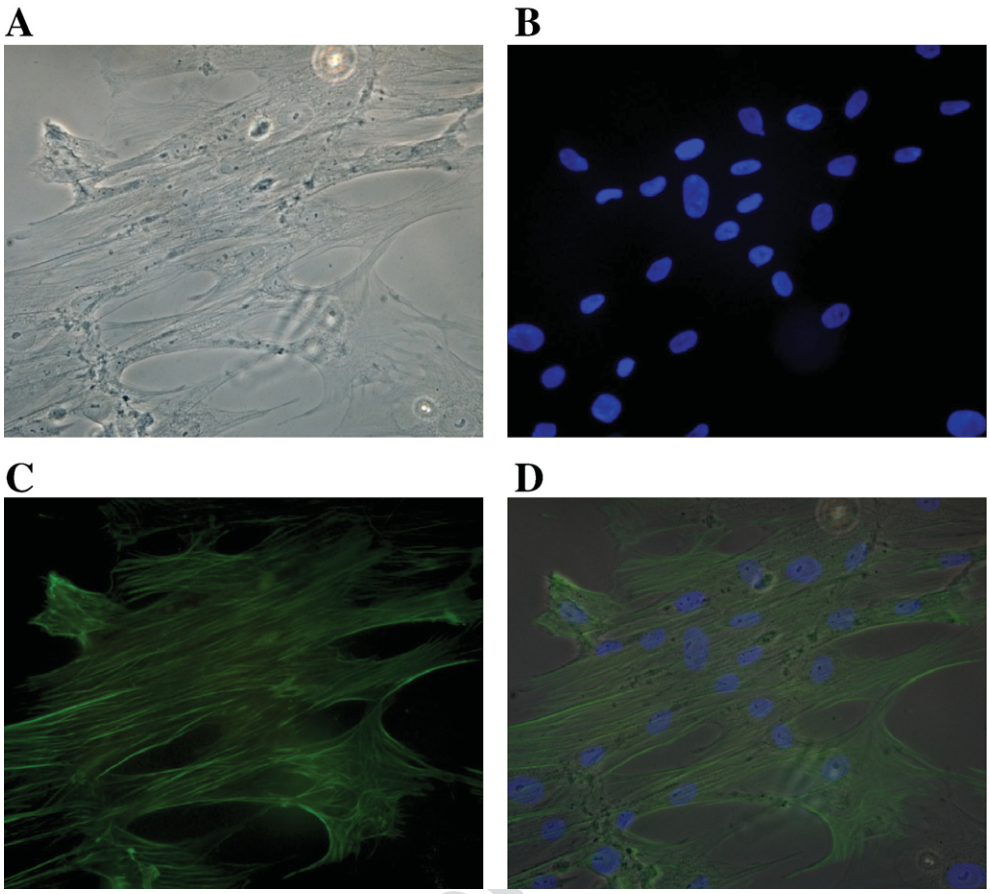

Fig. 2. Immunostaining of primary myometrial cells with smooth muscle α-actin, DAPI and fluorescence coupled antibody. A. Myometrial cells observed under 40-times magnification phase contrast. B. Same cells stained with DAPI. C. Same cells stained with anti-smooth muscle α-actin and fluorescent secondary antibody. D. Evaluation of myometrial smooth muscle cells on total cells.

used to perform semi-quantitative RT-PCR of OTR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Oligonucleotide primers for OTR RT-PCR developed by Takemura were used [36]. The sequences for the primers were OTR-F 5′CCTTCATCGTGTGCTGGACG3′ and OTR-R 5′ CTAGGAGCAGAGCACTTATG3′. These primers amplify a 391 bp (1215–1602) fragment of OTR cDNA. The primed region spans parts of exons 3 and 4 of the OTR gene [37].

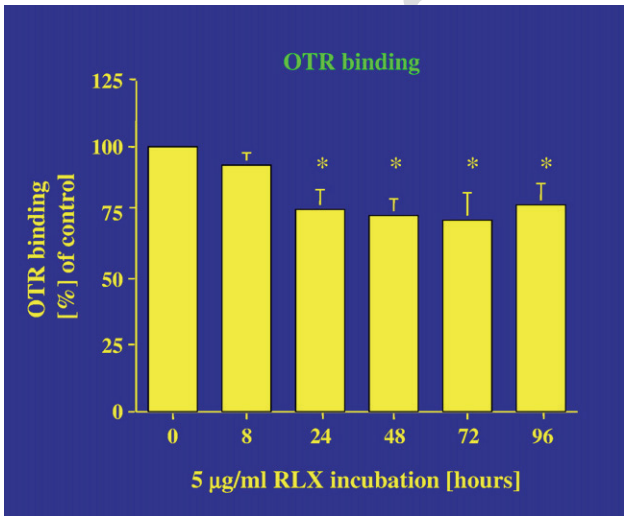

Fig. 3. Oxytocin receptor binding in human uterine smooth muscle cells after incubation with relaxin for different time periods (0–96 h). Data are represented as mean ± SEM (n = 5) and shown as percent of control (*p <0.05).

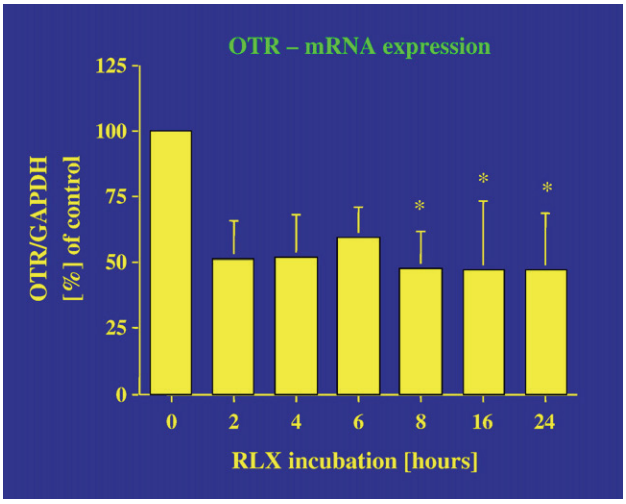

Fig. 4. Oxytocin receptor mRNA expression in human myometrial cells after short time incubation with relaxin (0–24 h). OTR mRNA was normalized to GAPDH mRNA. Data are represented as mean ± SEM (n = 4) and shown as percent of control (*p <0.05).

Theoretically the primers should not amplify any genomic DNA fragment, because Inoue et al. reported about a 12 kb intron at bp 1289 [37]. Oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplifying a 500 bp fragment of GAPDH cDNA were used as control (GAPDH 1 — 5′ GGCTGAGAACGGGAAGCTTG3′, GAPDH 2 — 5′TCTA GACGGCAGGTCAGGTC3′).

Briefly, cDNA was synthesized using total RNA, 20 U avian myeloblastoma virus reverse transcriptase (Promega, Madison, WI), 30 ng of oligo-dT, 5 mM $MgCl_2$, 1× RT-buffer and 200 μM dNTPs. One-fifth of the reverse transcription reaction was further amplified for 30 cycles with 1 U Taq polymerase (Promega), 100 pmol of the forward and reverse primers for OTR and GAPDH, 5 mM $MgCl_2$, 1× PCR-buffer and 200 μM dNTPs. The linearity of OTR RT-PCR products were established as described by Zingg et al. [38].

The amplified products of OTR and GAPDH were loaded on a 2% agarose gel (1:1 Standard/Nusieve GTG) and transferred to a nylon membrane (GeneScreen, New England Nuclear, Boston, MA) by overnight capillary transfer. The membranes were hybridized with randomly nick-linked OTR (kindly provided by Dr. Michael J. Brownstein, NIH, Bethesda, Maryland) and GAPDH (Clontech, Palo Alto, CA) cDNA probes labeled with ^{32}P-CTP (Amersham Pharmacia, Arlington Heights, IL) by random priming (Boehringer Mannheim). The filters were developed by autoradiography and the band density of OTR was normalized to the corresponding band for GAPDH by scanning densitometry with the Videk Harmony Program v. 4.03 (Videk Corporation, Rochester, NY). The experiments were performed with cells isolated from 4 different patients.

2.7. Flow cytometry for OTR

To confirm that the down regulation of OTR mRNA resulted in a down regulation of protein expression, myometrium cells were incubated with 5 μg/ml RLX for 24 h. The cells were trypsinized, centrifuged and the cell pellet was resuspended and diluted to 2×10^5 cells/200 μl phosphate buffered saline (PBS). To block non-specific binding the cells were incubated in 10% (w/v) normal rabbit serum in PBS with 0.05% (w/v) Tween 20 and 0.3% (w/v) Triton X-100. Afterwards, primary mouse monoclonal OTR antibody O-2F8 (Rohto Laboratories, Japan) at a dilution of 1:400 or a respective IgG isotype control (Becton Dickinson, Mountain View, CA) was added for 30 min on ice, and cells were washed in PBS containing 0.1% (w/v) BSA and 0.1% (w/v) NaN_3. Subsequently, a secondary rabbit anti-mouse FITC/Cy3 antibody (Jackson Immuno Research Inc., West Grove, PA) was added for another 30 min interval at a concentration of 1:1000. After two further washing steps, the cells were fixed with 1% (w/v) paraformaldehyde in PBS for 30 min at room temperature prior to flow cytometry. Flow cytometry analysis was performed as previously described by Hoffmann et al. [39], using a FACScan equipped with a single 488 nm argon ion laser (Becton Dickinson). At least 10,000 events were acquired for each sample and analysis was performed using the PC-Lysis program Version 1.0 from Becton Dickinson.

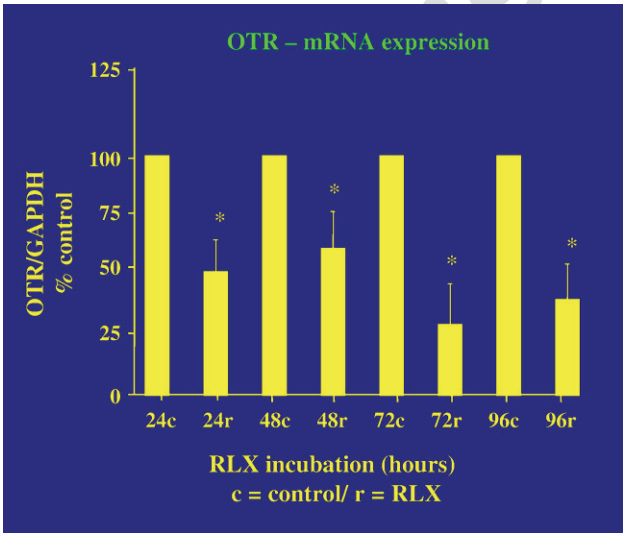

Fig. 5. Oxytocin receptor mRNA expression in human myometrium after long term incubation with relaxin for different time points (24–96 h). OTR mRNA was normalized to GAPDH mRNA. Data are represented as mean ± SEM (n = 4) and shown as percent of control (*p <0.05).

2.8. Statistical analysis

Repeated measures of time course experiments were analyzed using ANOVA (ANOVA Microsystems Inc., San Jose, CA) and differences among treatment groups were evaluated by Student's t-test. Significance was concluded for p <0.05.

3. Results

3.1. Immunocytochemistry

The isolated myometrial cells were observed under fluorescence microscope to evaluate the purity of the preparation procedure by immunocytochemical staining (Fig. 2A, B, C, D). More than 95% of cultured cells were identified as smooth muscle cells.

3.2. Radioligand-binding assay

Human myometrial cells were stimulated with RLX concentrations of 10 pg–20 μg/ml and a significant 30–40% reduction of OTR binding was found at concentrations from 1 ng to 20 μg RLX upon 24 h incubation. Thus, with further stimulation studies we decided to use a RLX concentration of 5 μg/ml medium similar to López Bernal et al. [40] using RLX concentrations of 4–8μg/ml medium to stimulate the PGE output in human amniotic discs.

Different time periods of RLX incubation significantly changed the OVT binding. While RLX incubation over 8 h did not markedly influence OTR binding, prolonged RLX incubation of myometrial cells led to a significant inhibition of receptor binding by 22–28% after 24 to 96 h (Fig. 3). As shown by scatchard blot analysis the binding affinity of the receptor remained unaffected with calculated K_d values of 0.42 nM for control and 0.37 nM for RLX treated cells, respectively (p-value: 0.72).

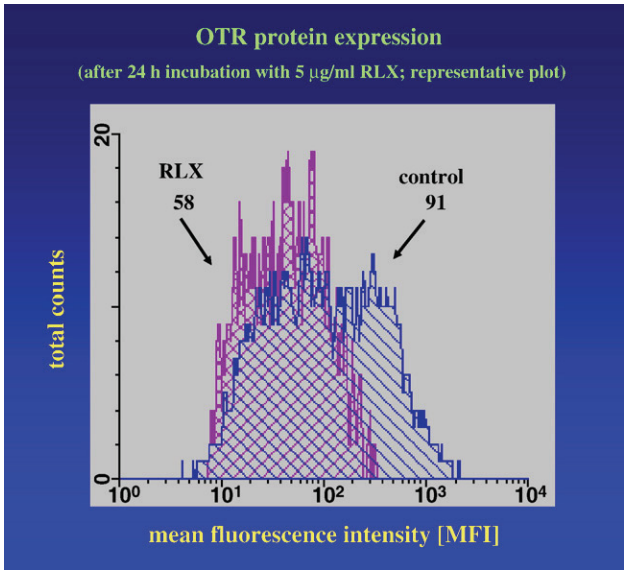

Fig. 6. Comparison of mean fluorescence intensity [MFI] indicating cell surface expression of oxytocin receptor protein in one representative human uterine smooth muscle cell culture after 24 h of relaxin stimulation (MFI 58) versus untreated control cells (MFI 91).

3.3. OTR mRNA analysis

RT-PCR of GAPDH mRNA was performed as an internal control and for semi-quantitation. OTR mRNA expression was detected in all cell lines tested. Cells incubated with medium alone served as control. As seen in Fig. 4, RLX significantly reduced OTR mRNA expression in myometrial cells *in vitro* after 8–24 h of incubation. RLX reduced the OTR expression in primary cultures of human myometrial cells by more than 50%. Long term incubation of uterine smooth muscle cells with RLX also reduced OTR mRNA expression as shown in Fig. 5. mRNA expression measured by RT-PCR was inhibited by as much as 49%, 39%, 70% and 60% over a time period of 24, 48, 72 and 96 h of incubation with RLX, respectively (data not shown).

3.4. OTR protein analysis

OTR protein expression after treatment with RLX was measured by using flow cytometry. RLX led to a down regulation of OTR in a representative experiment with human myometrial cells (Fig. 6). RLX incubated cells showed a mean fluorescence intensity (MFI) of 58 compared to a MFI of 91 in untreated controls.

4. Discussion

RLX – one of the first human reproductive hormones discovered – plays an important role in growth and remodeling of various tissues during pregnancy. RLX is one of the key hormones in vascularization and differentiation of the endometrium preparing the inner uterine layer for implantation [41]. In addition, it promotes expansion of the birth canal by loosening the pubic symphysis during pregnancy and relaxation of the cervix at birth [42]. Its role in human uterine quiescence, however, is still unclear. Information about local RLX concentrations despite those of MacLennan et al. [43], who reported about RLX protein isolation from tissues collected after normal and Caesarian deliveries is lacking.

As we already mentioned in the introduction RLX seems to play a key role in controlling ion content of muscle cells and therewith, uterine cell contractility.

In the present study we focused our work on the effect of RLX in OTR signaling. By evaluating RLX's local effect on OTR regulation we found a marked down regulation of OTR binding capacity in human uterine smooth muscle cells *in vitro*. RLX incubation of the cells for 24 h to 96 h led to a reduced OTR binding capacity while the binding affinity was unaffected. Similarly, OTR mRNA and protein expression was shown to be reduced in corresponding time intervals, implying a reduction of OTR binding by lower OTR expression due to reduced mRNA transcription. One might speculate that one effect of RLX enhanced cAMP production is a down regulation of OTR expression leading to a secondary reduction of PIP_2 turnover inhibiting subsequent signal transduction pathways. This is supported by a study of Uenoyama et al. [44] demonstrating a marked down regulation of OTR expression through cAMP induction and the AC–PKA system in bovine granulosa cells. Furthermore, Bani et al. [45] reported a RLX induced increase in the expression of endothelial-type NOS in murine myometrium indicating an involvement of the L-arginine–NO pathway in smooth muscle relaxation [46].

Despite a clear down regulation of the OTR signal transduction pathway in human myometrium MacLennan and Grant [47] found a limited effect of RLX on human uterine muscle strips *in vitro* inhibiting the amplitude of estrogen primed contractions by just 5% and the frequency by 50%. Petersen et al. [48] showed no influence of RLX on OT or $PGF_{2\alpha}$ induced contractions in human myometrium suggesting RLX to be of minor importance for the regulation of myometrial activity in term pregnant women. A recent study of Siebel et al. [49] in RLX gene knockout mice as well failed to show evidence of a direct interaction between RLX and the OTR gene itself.

López Bernal et al. [40] demonstrated a significant reduction of prostaglandin secretion from amnion discs obtained from women delivered by Caesarean section before labor but increased prostaglandin secretion from amnion obtained from women after spontaneous labor after RLX stimulation. They propose a possible paracrine effect of RLX on the amnion, inhibiting prostaglandin E (PGE) production during pregnancy maintaining uterine quiescence but favoring its production during spontaneous labor. Summerlee et al. [50] showed different effects of RLX on the OT system in lactating rats. While RLX increases the basal levels of the hormone, there was an evident suppression of OT release during milk ejection.

In previous work of our study group we could show that the cytokine interleukin-1β (IL-1β) down regulates OTR mRNA and protein expression favoring uterine relaxation on the one hand but separately up-regulates cyclooxygenase II in these

cells increasing the production of counteracting contractile prostaglandins [34 and unpublished data].

Taken together, our study is the first one to show an effect of human RLX on the OTR in human myometrial cells. The presented results demonstrate a down regulation of OTR mRNA with a subsequent down regulation of OTR protein expression leading to a reduced receptor binding capacity without influencing its binding affinity. These results suggest that the peptide hormone RLX may have a role in the inhibition of human myometrial contractions and the maintenance of uterine quiescence of the human uterus during pregnancy.

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