Comparative Medicine
Copyright 2015
by the American Association for Laboratory Animal Science

Vol 65, No 2
April 2015
Pages 140–143

Original Research

Comparative Immunohistochemistry of Placental Corticotropin-Releasing Hormone and the Transcription Factor RelB–NFκB2 Between Humans and Nonhuman Primates

Todd Rosen,[1,*] Jay Schulkin,[2] Michael Power,[3] Serkalem Tadesse,[4] Errol R Norwitz,[4] Zhaoqin Wen,[5] and Bingbing Wang[1,*]

The transcription factor RelB–NFκB2, activated by the noncanonical NFκB pathway, positively regulates corticotropin-releasing hormone (CRH) and prostaglandin production in the term human placenta and may play an important role in the timing of human parturition. Here we explored whether RelB–NFκB2 signaling plays a role in parturition in nonhuman anthropoid primates. We performed immunohistochemical staining to assess the correlation between CRH and nuclear activity of RelB–NFκB2 heterodimers in term placentas from humans, 3 catarrhine primate species, and a single platyrrhine primate species. Consistent with our previous studies, the human placenta showed cytoplasmic staining for CRH and nuclear staining for RelB–NFκB2. Similar staining patterns were noted in the 3 catarrhine primates (chimpanzee, baboon, and rhesus macaque). The platyrrhine (marmoset) placentas stained positively for CRH and RelB but not for NFκB2. Catarrhine (but not platyrrhine) nonhuman primate term placentas demonstrate the same CRH staining and nuclear localization patterns of RelB and NFκB2 as does human placenta. These results suggest that catarrhine primates, particularly rhesus macaques, may serve as useful animal models to study the biologic significance of the noncanonical NFκB pathway in human pregnancy.

Abbreviations: CRH, corticotropin-releasing hormone;

Whether human labor occurs at term or preterm, it is a complex physiologic process that has been the focus of intensive investigation for several decades. Several important regulators of uterine activity during pregnancy and labor have been discovered,[9] but the precise mechanisms that govern the onset of labor have remained elusive. Studies have been limited by a lack of appropriate animal models for human parturition.[13] Evidence points to a central role of the placenta in the timing of labor, but even investigations of the cellular and molecular mechanisms have been hampered due to the lack of a suitable cell line and the difficulties in working with primary human cell cultures.

Corticotropin-releasing hormone (CRH) produced by the placenta has been proposed to be part of a clock that governs the length of gestation.[7] CRH-like activity was identified in human placental extracts in 1982,[12] and subsequent studies have confirmed that the placenta is the source of CRH in maternal serum.[5] Maternal plasma CRH levels are undetectable until after the first trimester, when they increase exponentially as pregnancy advances, peaking at the time of delivery.[3,7] On average, women who deliver preterm have increased circulating levels of CRH early in pregnancy, whereas those who deliver postterm have lower CRH levels.[7,17]

Many tissues express CRH. In humans, CRH is the product of a single gene located on the long arm of chromosome 8.[2] Glucocorticoids regulate CRH expression in a tissue-dependent fashion. For example, glucocorticoids negatively regulate CRH in the hypothalamus and in skin but positively regulate CRH in the amygdala and placenta.[10] The positive regulation of placental CRH by glucocorticoid establishes a feed-forward loop between the fetus and placenta that appears to drive CRH production during pregnancy. The mechanism responsible for the differential regulation of the CRH in the placenta remains under investigation but may depend on epigenetic changes in chromatin,[1] among other factors.

A recent study has demonstrated that glucocorticoids stimulate CRH indirectly in the human placenta by activating RelB–NFκB2 heterodimers,[18] which bind to a previously undescribed κB response element in the CRH promoter.[20] Almost 20 y ago, progestins were shown to downregulate CRH in the placenta.[6] More recently, researchers found that this inhibition is mediated at least in part by the negative functional interaction of progester-

Received: 20 Mar 2014. Revision requested: 02 Sep 2014. Accepted: 01 Dec 2014.
[1]Department of Obstetrics, Gynecology, and Reproductive Sciences, Rutgers Robert Wood Johnson Medical School, New Brunswick, New Jersey, [2]Research Department, American College of Obstetricians and Gynecologists, Washington, District of Columbia, and Department of Neuroscience, Georgetown University, Washington, District of Columbia, [3]Nutrition Laboratory, Conservation Ecology Center, Smithsonian Conservation Biology Institute, National Zoological Park, Washington, District of Columbia, [4]Tufts University School of Medicine, Department of Obstetrics and Gynecology, Boston, Massachusetts, and [5]Department of Pathology, Saint Peter's University Hospital, New Brunswick, New Jersey.
**Corresponding author. Email: wangbi@rwjms.rutgers.edu and rosentj@rwjms.rutgers.edu*

one receptor A with RelB–NFκB2.[19] Of note, RelB–NFκB2 has also been shown to upregulate cyclooxygenase 2 (prostaglandin–endoperoxide synthase 2) activity in the placenta,[18] suggesting that the noncanonical NFκB pathway regulates several prolabor genes and plays a central integrative role in the clock that governs the length of human pregnancy.

Anthropoid nonhuman primates appear to be the only species whose placentas produce CRH[10] and are likely the only suitable animal model for studying the role of placental CRH in the timing of human parturition, that is, the putative placental clock. Studies using typical murine animal models have not been helpful in understanding the role of CRH in the human pregnancy clock. Indeed, the CRH knockout mouse does not have a prolonged gestation.[8] Patterns of CRH production in gorillas and chimpanzees are similar to those in humans, with an exponential increase in maternal blood levels as pregnancy progresses.[15] However, using gorilla pregnancies as a model for those in humans is impractical, and there are restrictive criteria for obtaining NIH funding and using chimpanzees for research in the United States. Other anthropoid primate placentas produce CRH, but the temporal pattern across pregnancy differs from that in apes and humans. Old World primates, such as baboons and rhesus monkeys, and New World species, for example, common marmosets, demonstrate a rapid increase in CRH in early pregnancy, followed by a decline to a plateau, with a possible rise again at the end of pregnancy.[10] As a first step toward evaluating whether nonhomininae primates would serve as useful animal models for evaluating the role of NFκB in regulating the human placental clock, we sought to assess whether NFκB is active in the placentas of these species and whether this signaling pathway may participate in regulating CRH production in this tissue. To this end, we performed immunohistochemistry for CRH, RelB, and NFκB2 on formalin-fixed paraffin-embedded placental tissues from these nonhuman primates after term deliveries and compared the staining patterns with those of term human placentas.

Materials and Methods

A single human full-term normal placenta was collected at Robert Wood Johnson University Hospital (New Brunswick, NJ) after informed consent was obtained. Paraffin blocks of normal chimpanzee (*Pan troglodytes*), baboon (*Papio hamadryas*), and common marmoset (*Callithrix jacchus*) placentas (n = 3 per species) were obtained from The Southwest National Primate Research Center (San Antonio, TX). Placentas from rhesus macaques (*Macaca mulatta*) were obtained from The Yerkes National Primate Research Center (Atlanta, GA). We obtained three independent specimens for each species. All placentas were obtained after vaginal deliveries.

Immunohistochemistry was performed as follows. Briefly, samples were fixed in buffered formalin for 24 h, dehydrated in 70% ethanol, paraffin-embedded, and sectioned. To improve antigen detection, we incubated slides with protease K (20 µg/mL) for 20 min. The primary antibodies used were RelB (1:50 dilution; Santa Cruz Biotechnology, Dallas, TX), NFκB2 (1:300 dilution; Cell Signaling Technology, Danvers, MA), and CRH (1:50 dilution: Abnova, Walnut, CA). For a positive control, e stained for p65 (Cell Signaling, Danvers, MA), which demonstrates cytoplasmic, but not nuclear, localization in the syncitiotrophoblast of human placenta.[20] CD45,[16] which is not produced by the syncitiotrophoblast, was used as a negative control. According to the manufacturers, all of these antibodies crossreact with humans and nonhuman primate species. After being incubated overnight at 4 °C in Tris-buffered saline (pH 7.4) with the appropriate antibody, the sections were washed with Tris-buffered saline containing 0.05% Tween 20 and incubated with biotinylated secondary antirabbit or antimouse antibody diluted in Tris-buffered saline. Sections were washed again with Tris-buffered saline containing 0.05% Tween 20, and secondary antibodies were detected by incubating tissue sections in ABC Reagent (Thermo Scientific, Rockford, IL) for 30 min. Two independent pathologists evaluated all stained slides. The research protocol was approved by the Institutional Review Board of Rutgers University.

Results

Results of immunohistochemical staining for CRH, RelB, and NFκB2, p65, and CD45 are shown in Figure 1, with the relative intensity of staining among the various primate species described in Table 1. In term human placenta, the cytoplasm of cells in the syncytium and syncytial knots of the syncytiotrophoblast stained strongly for CRH. Both NFκB2 and RelB demonstrated both cytoplasmic and nuclear staining (20% to 25% of cross-sections of placental villi) in the human syncytiotrophoblast as well as cells morphologically resembling cytotrophoblast cells in the layer beneath the syncytium.

We found the same staining patterns for CRH, RelB, and NFκB2 in the placentas of chimpanzees, baboons, and rhesus monkeys as for humans. Like human placenta, marmoset placentas stained positively for CRH, but RelB nuclear staining was much less pronounced in marmoset tissue, and we were unable to demonstrate cytoplasmic or nuclear NFκB2 in marmoset samples.

As expected, CD45 staining was negative in the trophoblasts of all species evaluated. Conversely, we identified p65 in the cytoplasm, but not nucleus, of human as well as all nonhuman primate syncitiotrophoblast cells.

Discussion

Chimpanzees and humans are in the same subfamily of Hominidae and show similar patterns of placental CRH during pregnancy. As expected, we found that CRH and NFκB staining patterns were similar between humans and chimpanzees (Figure 1).

Baboons and rhesus macaques are Old World species that, unlike humans and chimpanzees, display a midgestational peak in placental CRH expression.[14,21] However, NFκB staining patterns of term placentas were similar among baboons, rhesus macaques, chimpanzees, and human. In addition, both RelB and NFκB2 nuclear staining were identified in all of these species. Therefore, if the RelB–NFκB2 heterodimer, which is generated via the noncanonical pathway,[20] has a role in the human placental clock, this function is likely to occur in chimpanzees, baboons, and rhesus monkeys as well.

Interestingly, although immunohistochemistry revealed CRH in the placenta of common marmosets, which also demonstrated RelB nuclear staining, we were unable to demonstrate nuclear staining of NFκB2. This New World species belongs to the platyrrhine subdivision of anthropoid primates. All of the other species in this study (including human) are members of the catarrhine subdivision. Given that the Platyrrhini and Catarrhini groups separated from each other 35 to 40 million years ago, it is perhaps

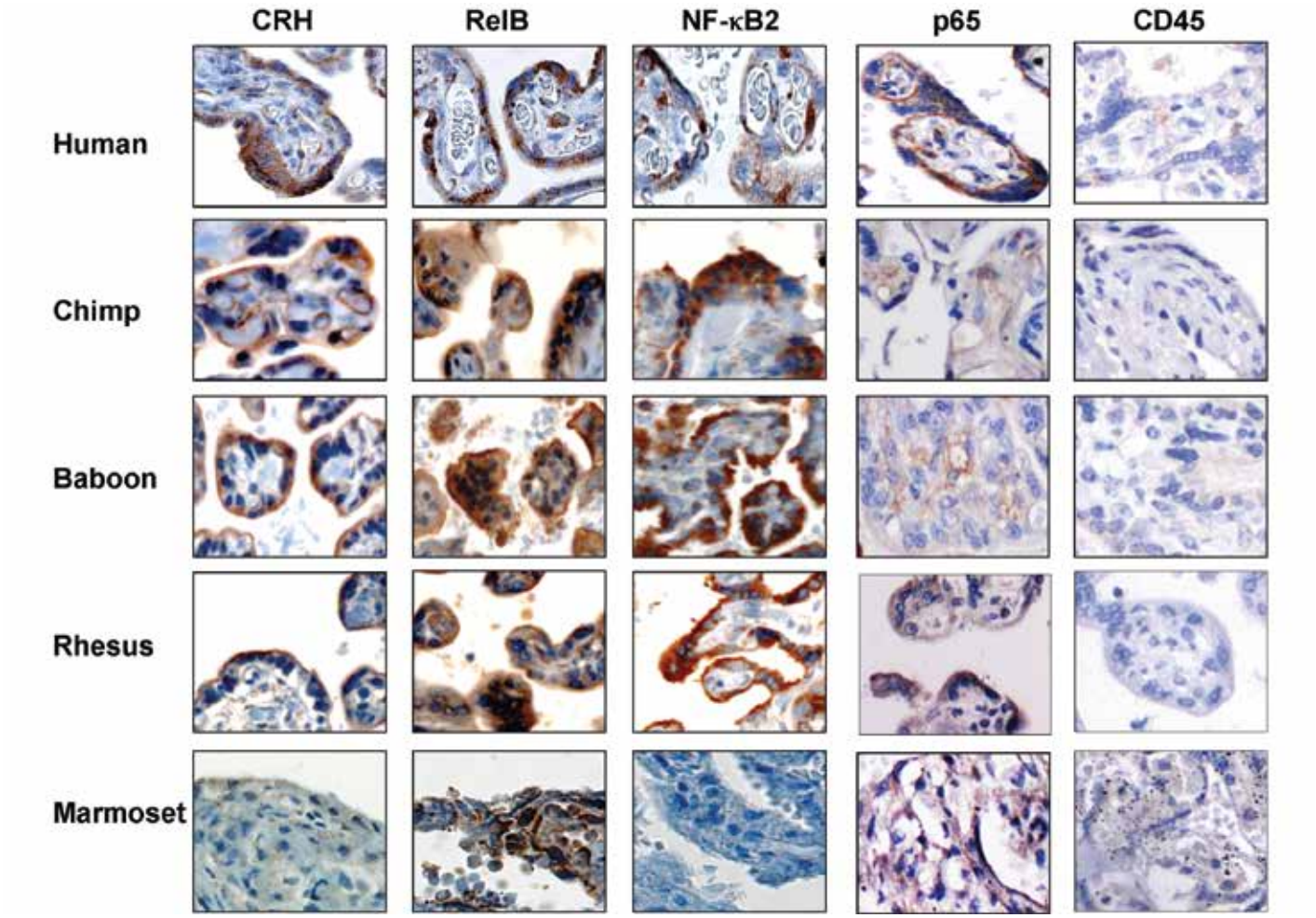

Figure 1. Representative immunohistochemical staining of CRH, RelB, and NFκB2 in 3 independent placentas from each species studied. When inactive, p65, a subunit in the canonical NFκB signaling pathway, is sequestered in the cytoplasm; its identification in the cytoplasm served as a positive control. CD45, a receptor-linked protein tyrosine phosphatase that is expressed in leukocytes but not the placental trophoblast, was used as the negative control. Original magnification, ×200.

Table 1. Summary of relative expression of CRH, RelB, and NFκB2 in 3 independent normal placentas by use of immunohistochemical staining

	Intensity of staining		
	CRH	RelB	NFκB2
Human	3	3	3
Chimpanzee	3	3	3
Baboon	2	3	3
Rhesus macaque	2	3	3
Marmoset	1	2	0

Two independent pathologists evaluated intensity of cytoplasmic and nuclear staining on a scale of 0 (none) to 3 (high).

not surprising that regulatory mechanisms for CRH in marmosets might differ from the other, more closely related species studied. Other members of the primate platyrrhine subdivision in which placental CRH production has been characterized are squirrel monkeys and owl monkeys, and their temporal patterns of maternal circulating CRH in pregnancy are similar to those of common marmosets as well as baboons.[11] It would be worthwhile to assess placental specimens from these other platyrrhine species to determine whether their patterns of NFκB nuclear localization are similar to those of marmosets. Similar data from all the platyrrhine species would suggest differences in CRH regulation between platyrrhine and catarrhine primates during pregnancy. Of note, we did not study prosimian primates because their placentas (at least those of lemurs) do not produce CRH.[10]

Perhaps the most important implication of the finding that nuclear RelB and NFκB2 were identified in term placentas from both chimpanzees and rhesus macaques is the ability to study the biologic effects of the noncanonical NFκB pathway on the timing of parturition in rhesus macaques. Although CRH may play different roles in different primate species, combining the central role of prostaglandins in primate parturition[4] with our previous finding that RelB–NFκB2 regulates prostaglandin–endoperoxide synthase 2 in the human placenta[17,18] suggests that the noncanonical NFκB pathway may play a role in determining the length of pregnancy in humans and various nonhuman primates. Studies in rhesus macaques have the potential to determine the biologic significance of the noncanonical NFκB pathway in primate preg-

nancy and could help to resolve the mechanism that governs the length of pregnancy in humans.

Acknowledgments

We thank L Cong for technical support in immunohistochemistry and Dr C Duzyj-Buniak for reading the manuscript.

References

1. **Abou-Seif C, Shipman KL, Allars M, Norris MH, Chen YX, Smith R, Nicholson RC.** 2012. Tissue specific epigenetic differences in CRH gene expression. Front Biosci (Landmark Ed) **17:**713–725.
2. **Arbiser JL, Morton CC, Bruns GA, Majzoub JA.** 1988. Human corticotropin-releasing hormone gene is located on the long arm of chromosome 8. Cytogenet Cell Genet **47:**113–116.
3. **Campbell EA, Linton EA, Wolfe CD, Scraggs PR, Jones MT, Lowry PJ.** 1987. Plasma corticotropin-releasing hormone concentrations during pregnancy and parturition. J Clin Endocrinol Metab **64:**1054–1059.
4. **Giannoulias D, Haluska GJ, Gravett MG, Sadowsky DW, Challis JR, Novy MJ.** 2005. Localization of prostaglandin H synthase, prostaglandin dehydrogenase, corticotropin-releasing hormone. and glucocorticoid receptor in rhesus monkey fetal membranes with labor and in the presence of infection. Placenta **26:**289–297.
5. **Grino M, Chrousos GP, Margioris AN.** 1987. The corticotropin-releasing hormone gene is expressed in human placenta. Biochem Biophys Res Commun **148:**1208–1214.
6. **Karalis K, Goodwin G, Majzoub JA.** 1996. Cortisol blockade of progesterone: a possible molecular mechanism involved in the initiation of human labor. Nat Med **2:**556–560.
7. **McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R.** 1995. A placental clock controlling the length of human pregnancy. Nat Med **1:**460–463.
8. **Muglia L, Jacobson L, Dikkes P, Majzoub JA.** 1995. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature **373:**427–432.
9. **Norwitz ER, Robinson JN, Challis JR.** 1999. The control of labor. N Engl J Med **341:**660–666.
10. **Power ML, Schulkin J**. 2006. Functions of corticotropin-releasing hormone in anthropoid primates: from brain to placenta. Am J Hum Biol **18**:431–447.
11. **Power ML, Williams LE, Gibson SV, Schulkin J, Helfers J, Zorrilla EP.** 2010. Pattern of maternal circulating CRH in laboratory-housed squirrel and owl monkeys. Am J Primatol **72:**1004–1012.
12. **Shibasaki T, Odagiri E, Shizume K, Ling N.** 1982. Corticotropin-releasing factor-like activity in human placental extracts. J Clin Endocrinol Metab **55:**384–386.
13. **Smith R.** 2007. Parturition. N Engl J Med **356:**271–283.
14. **Smith R, Chan EC, Bowman ME, Harewood WJ, Phippard AF.** 1993. Corticotropin-releasing hormone in baboon pregnancy. J Clin Endocrinol Metab **76:**1063–1068.
15. **Smith R, Wickings EJ, Bowman ME, Belleoud A, Dubreuil G, Davies JJ, Madsen G.** 1999. Corticotropin-releasing hormone in chimpanzee and gorilla pregnancies. J Clin Endocrinol Metab **84:**2820–2825.
16. **Tang Z, Tadesse S, Norwitz E, Mor G, Abrahams VM, Guller S.** 2011. Isolation of Hofbauer cells from human term placentas with high yield and purity. Am J Reprod Immunol **66:**336–348.
17. **Wadhwa PD, Porto M, Garite TJ, Chicz-DeMet A, Sandman CA.** 1998. Maternal corticotropin-releasing hormone levels in the early 3rd trimester predict length of gestation in human pregnancy. Am J Obstet Gynecol **179:**1079–1085.
18. **Wang B, Palomares K, Parobchak N, Cece J, Rosen M, Nguyen A, Rosen T.** 2013. Glucocorticoid receptor signaling contributes to constitutive activation of the noncanonical NFκB pathway in term human placenta. Mol Endocrinol **27:**203–211.
19. **Wang B, Parobchak N, Rosen M, Roche N, Rosen T.** 2014. Negative effects of progesterone receptor isoform A on human placental activity of the noncanonical NFκB signaling. J Clin Endocrinol Metab **99:**E320–E328.
20. **Wang B, Parobchak N, Rosen T.** 2012. RelB–NFκB2 regulates corticotropin-releasing hormone in the human placenta. Mol Endocrinol **26:**1356–1369.
21. **Wu WX, Unno S, Giussani DA, Mecenas CA, McDonald TJ, Nathanielsz PW.** 1995. Corticotropin-releasing hormone and its receptor distribution in fetal membranes and placenta of the rhesus monkey in late gestation and labor. Endocrinology **136:**4621–4628.