Gene regulation of neurokinin B and its receptor NK3 in late pregnancy and pre-eclampsia

N.M. Page¹, J. Dakour² and D.W. Morrish²

¹School of Life Sciences, Kingston University London, Penrhyn Road, Kingston-upon-Thames, Surrey, UK and ²Department of Medicine, The University of Alberta, Edmonton, Canada

Elevated circulating levels of the tachykinin, neurokinin B (NKB), have been observed in women with pre-eclampsia during the third trimester of pregnancy. Currently, the molecular mechanisms responsible for these increased levels remain unknown. To understand the molecular regulation, we have compared the differences in gene expression of the tachykinins and their receptors in control and pre-eclamptic placentae and the responses of the TAC3 gene encoding NKB to proposed physiological triggers of pre-eclampsia including hypoxia and oxidative stress using real-time quantitative PCR. We have determined the placenta to be the main site of TAC3 expression with levels 2.6-fold higher than the brain. TAC3 expression was found to be significantly higher in pre-eclamptic placenta (1.7-fold, \( P < 0.05 \)) than in normal controls. No evidence was found that hypoxia and oxidative stress were responsible for increases in TAC3 expression. In rat placenta, a longitudinal study in normal late pregnancy was associated with a significant down-regulation of the NKB/NK3 ligand–receptor pair (\( P < 0.05 \)). The present data suggest that the increased placental expression of TAC3 is part of the mechanism leading to the increased circulating levels of NKB in pre-eclampsia.

Key words: neurokinin B/pre-eclampsia/pregnancy

Introduction

The tachykinins are a family of peptides that comprise substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and the species-divergent endokinins including endokinin B (EKB) in humans (reviewed by Page, 2004, 2005). These tachykinins are encoded on three different genes, preprotachyhcin 1 (TAC1) encoding SP and NKA, TAC3 encoding NKB and TAC4 encoding EKB (reviewed by Page, 2004, 2005). Three tachykinin receptors have been identified, which interact with these tachykinins: NK1, NK2 and NK3, whereby SP and EKB show the greatest potency for NK1, NKA for NK2 and NKB for NK3 (Page et al., 2003).

Traditionally, these peptides have been classified as neurotransmitters being found in discrete neurons and immune cells (reviewed by Page, 2004, 2005). Recently, this conceivably godma was challenged when the placenta, a tissue devoid of nerves, was found to be a source of TAC3 gene expression (Page et al., 2000, 2001). Moreover, TAC1 expression was found to be absent from the placenta (Page et al., 2001, 2003). However, we now know that the placenta is an abundant source of the recently discovered SP-like endokinins, leading to the proposal that the endokinins are the peripheral SP-like endocrine/paracrine agonists where SP is not expressed (Page et al., 2003; Page, 2004). Moreover, pre-eclampsia has been associated with increased plasma levels of NKB (Page et al., 2000; D’Anna et al., 2004), where in the placenta it is expressed by the outer syncytiotrophoblast in an ideal position to be secreted into the maternal bloodstream (Page et al., 2000). Concentrations of NKB in the middle and late pregnancy have been found to be significantly higher than non-pregnant concentrations and decrease rapidly after delivery. This indicates that NKB secretion into both the fetal and the maternal circulation is derived mainly from the placenta (Page et al., 2000; D’Anna et al., 2002; Sakamoto et al., 2003; Schlembach et al., 2003; Tjoa et al., 2004).

A role for the tachykinins in the placenta has remained as yet undefined. Nevertheless, recent and consistent evidence suggests that they may play a role in utero-placental haemodynamic adaptation by inducing uterine and placental vasodilatation, thereby increasing placental blood flow (Page et al., 2000, 2001; Brownbill et al., 2003; D’Anna et al., 2004; Laliberte et al., 2004). In contrast, in the peripheral mammalian vasculature, NKB has been shown to have hypertensive effects at its preferred receptor, NK3 (Mastrangelo et al., 1987; D’Orléans-Juste et al., 1991; Thompson et al., 1998). Indeed, the placenta has been shown to play a major role in the pathogenesis of pre-eclampsia (reviewed in Scott, 1958; Shembrey and Noble, 1995; Palma Gamiz, 1998), where after poor placental perfusion (Pijnenborg et al., 1980, 1981), a process is initiated leading to placental hypoxia, ischaemia, ensuing oxidative stress and the symptoms of pre-eclampsia (reviewed in Page, 2002). To date, no study has yet analysed the molecular regulation of TAC3 in the placenta. In an attempt to clarify this issue, we have identified the placenta as the predominant site of TAC3 gene expression and determined the changes in tachykinin and tachykinin receptor expression between normal and pre-eclamptic placentae at term. We have also studied the effects of proposed pathophysiological triggers of pre-eclampsia, such as hypoxia and oxidative stress, on TAC3 and NK3 receptor expression.

Materials and methods

Animals and human subjects

Adult female time-mated Crl : WI rats (Charles River, Margate, UK) were kept in standard laboratory conditions with a 12 h light and a 12 h dark cycle. All
procedures for the collection of tissues were followed according to the accepted standards of animal care. Human tissues were collected with approval from the local Research Ethics Committee (The University of Alberta) and in compliance with their guidelines. Term placenta from both normal (n = 12) and pre-eclamptic (n = 12) pregnancies were obtained. Samples for extraction were chosen randomly from cotyledons in areas without obvious infarcts or other pathology. A large portion of each placenta (~100–200 g) was used. All tissues were collected fresh and immediately frozen by immersing in isopentane with dry ice and transferred to –80 °C for storage. Pre-eclampsia patients: epilepsy (n = 1); smokers (n = 2), dexamethasone (n = 1), HELLP (hemolysis, elevated liver function tests, low platelet count) syndrome (n = 1). One patient had PIH without proteinuria.

**Quantitative PCR**

Total RNA was isolated from rat placenta at days 16, 19 and 21 of gestation and from human placental tissues at term using Tri Reagent (Sigma-Aldrich, Poole, UK). Contaminating genomic DNA was removed by DNase I treatment and the RNA then re-purified. Total human RNA from each of 24 tissues (brain, heart, kidney, liver, lung, colon, bone marrow, small intestine, spleen, stomach, thymus, prostate, skeletal muscle, testis, uterus, fetal brain, fetal liver, thyroid, placenta, adrenal gland, pancreas, salivary gland, trachea and mammary gland) was also obtained from a human RNA master panel (BD Biosciences, Oxford, UK). First-strand cDNA synthesis was performed using 1 μg of each RNA using the Powerscript™ reverse transcriptase kit (BD Biosciences) in the presence of 300 ng of random hexameric primers (Invitrogen, Paisley, UK) by following the manufacturer’s instructions. The resulting cDNA was diluted by adding 300 μl of nuclelease-free H2O. One microlitre of this cDNA was used in each quantitative PCR. Specific Taqman® probes and primer sets were designed using Primer Express™ 1.5 to span where possible an exon–exon junction (Applied Biosystems, Foster City, CA, USA). The sequences of the primers and probes used are listed in Table II. All probes and primers were synthesized by Sigma-Genosys (Pamisford, UK). Reactions were set up in triplicate in 25 μl using Absolute™ Quantitative PCR ROX master mix (ABgene, Epsom, UK) following the manufacturer’s instructions.

PCR cycling was performed in an ABI PRISM® 7700 sequence detector under the following conditions: initial denaturation/activation of the Thermo-Start® DNA polymerase at 95°C for 15 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The optimal concentrations of the primers (50–900 nM) and probe (25–225 nM) used to amplify each target gene were calculated using those combinations that gave the lowest threshold cycle (Ct) and highest normalized reporter (R) values. The derived optimal concentrations are summarized in Table II. The standard curve method for relative quantitation was used with normalization to the endogenous control, 18S rRNA. Separate standard curves were generated using a 10-fold serial dilution of template cDNA for each respective target gene and the endogenous control gene. The target amount was divided by the endogenous control reference to obtain a normalized target value to generate the relative expression levels; these levels were arbitrarily presented as percentages. The level of 18S rRNA in each tissue/sample was assessed using the Taqman® rRNA control reagents following the manufacturer’s instructions (Applied Biosystems). Controls containing no reverse transcriptase, no template and no probe were included.

**Statistical evaluation**

Statistical analysis was performed with one-way ANOVA used in conjunction with the post hoc Fisher’s protected least significant difference test for multiple comparisons and Student’s t-tests to compare the means of two groups. These analyses were undertaken using StatView (version 5.01) and plotted using GraphPad PRISM (version 3.0). *P* < 0.05 was considered to be significant. All values are expressed as mean ± SEM, where n represents the number in the group used.

**Results**

Quantitative PCR demonstrated the major site of expression of TAC3 to be the placenta (Figure 1) with levels 2.6-fold higher than those in the whole brain. Significant, but lower levels of TAC3 mRNA transcripts were distributed, in the fetal brain, with significant expression also in the endocrine/reproductive organs of the testis and mammary gland.

The expression pattern of the tachykinin genes (TAC1, TAC3 and TAC4) and tachykinin receptor genes (TACR1, TACR2 and TACR3) was investigated by Northern analysis.
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placentae at term using real-time quantitative PCR. TAC1 mRNA expression levels in both control and pre-eclamptic placentae at term were found to be either absent (9 of 24 placenta) or present at extremely low levels, except for one pre-eclamptic placenta that was found to express much higher levels of TAC1 (Figure 2). A significantly higher level of TAC3 mRNA expression of 1.7-fold ($P < 0.05$, unpaired $t$-test, $n = 12$) was found in the pre-eclamptic placentae when compared with the control placentae ($n = 12$) (Figure 2). No significant alterations in TAC4 mRNA expression levels were observed between the pre-eclamptic and control groups (Figure 2). No significant alterations in any of the mRNA expression levels of the tachykinin receptors (TACR1, TACR2 or TACR3) were observed between the pre-eclamptic and control groups (Figure 2).

To determine whether proposed pathophysiological triggers of pre-eclampsia were responsible for the elevated TAC3 gene expression in the pre-eclamptic group, we analysed the effects of hypoxia and oxidative stress in term human placental cytotrophoblast and syncytiotrophoblast cultures. Significant decreases in TAC3 mRNA expression levels in differentiating placental cytotrophoblasts grown in hypoxic conditions (2% O$_2$) were found when compared with those grown in...
normoxic conditions (21% O\textsubscript{2}). These were represented by a 1.8-fold\(^a\) (\(P < 0.05\), paired \(t\)-test, \(n = 5\)) decrease in TAC3 expression levels as shown by quantitative PCR (Figure 3). There was a slight tendency for the increased expression of the NK3 receptor; however, this was not significant (Figure 3). In contrast, quantitative PCR of TAC3 mRNA expression levels in differentiated placental syncytiotrophoblasts (generated by 48 h exposure to EGF and 10% FBS) exposed to the conditions of oxidative stress (induced by the presence of peroxynitrite or xanthine/xanthine oxidase for 24 h) or of hypoxia (2% O\textsubscript{2}) for 24 h demonstrated no significant changes in the expression of TAC3 (Figure 3). Neither were significant changes in the expression of TAC3R found under the same conditions of oxidative stress and hypoxia (data not shown). Cultured mononuclear cytotrophoblast cells before experimental treatment are shown in Figure 4A. Cytokeratin staining confirms their trophoblast identity. Partly syncytialized cells at the end of the experimental period (72 h) are shown in Figure 4B. Approximately 50% of cytotrophoblasts have fused to form multinuclear syncytial units that are outlined by desmoplakin staining.

As we were unable for ethical reasons to obtain longitudinal placental samples throughout the course of the third trimester of human pregnancy, we investigated the expression pattern of TAC3 and TACR3 in the rat placenta at days 16, 19 and 21 of gestation using quantitative PCR. TAC3 mRNA levels were found to decline throughout the duration of late pregnancy by 2.0-fold at day 19 and 2.9-fold at day 21 when compared with day 16, respectively. This decline was found to be significant between days 16 and 21 (\(P < 0.05\), ANOVA, \(n = 4\)) (Figure 5). There was a significant decrease in TACR3 mRNA expression levels between days 16 and 19 of 90.6-fold (\(P < 0.05\), ANOVA, \(n = 4\)) and of 58.6-fold between days 16 and 21 (\(P < 0.05\), ANOVA, \(n = 4\)). The TACR3 gene appeared to be virtually switched off by day 19 (Figure 5).

**Discussion**

The mammalian tachykinins are a family of peptides that have been traditionally classified as neurotransmitters; however, we have found the placenta, a tissue devoid of nerves, to be the most abundant site of TAC3 mRNA expression. Furthermore, there is growing evidence to suggest that the tachykinins may play a significant role in the regulation of many different reproductive functions (Page et al., 2000, 2001; Pintado et al., 2003; Loffler et al., 2004). One such proposed reproductive function has come from the discovery of elevated levels of NKB detected in the plasma of third-trimester pre-eclamptic pregnancies (Page et al., 2000; D’Anna et al., 2004). It has been proposed that in response to defective trophoblast invasion, which is not rectified after the first trimester of pregnancy, the placenta will start secreting ever-increasing amounts of NKB into the maternal circulation (Page et al., 2001). We have found TAC3 mRNA expression levels to be significantly higher in pre-eclamptic placenta at term, a result consistent with the theory that TAC3 expression could be up-regulated in
response to poor placental perfusion after defective trophoblast invasion. We have believed, like many others (reviewed in Roberts, 1998; Taylor et al., 1998; Van Wijk et al., 2000), that a poorly diffused and ischaemic placenta releases an excess of such factor(s) including NKB. It has been suggested that the key physiological activators responsible for the production of these factors are hypoxia (Kingdom and Kaufmann, 1997) and oxidative stress (Roberts and Hubel, 1999). Nevertheless, the evaluation of these triggers demonstrated no evidence that they were responsible for increasing TAC3 mRNA expression. Paradoxically, the hypoxic conditions proposed to occur in pre-eclamptic placentae were found to significantly down-regulate TAC3 mRNA expression in cytotrophoblast cultures. These data do not support a role for placental hypoxia as the underlying cause of elevated NKB levels in pre-eclampsia, either in cytotrophoblast differentiating into syncytiotrophoblast in vitro (Figure 3). Regardless, this outcome is similar to that found for activin A in cytotrophoblasts, whose gene is likewise down-regulated by hypoxia, but associated with higher circulating levels of protein during pre-eclampsia (Blumenstein et al., 2002). Indeed, the concept of placental hypoxia in pre-eclampsia has been previously challenged, with some theories believing the converse that hyperoxic conditions develop during pre-eclampsia (Kingdom and Kaufmann, 1997). It is unlikely that serum deprivation accounted for either the decrease in TAC3 with hypoxia or prevented its increase, as the effect of hypoxia was selective for TAC3 and not seen for TAC4 or TACR1, TACR2 and TACR3 (data not shown). Similarly, short-term serum deprivation in past studies using this culture system has not been found to impair hormonal secretion from cyto- or syncytiotrophoblast (Morrish et al., 1987, 1997).

As we have previously found using semi-quantitative PCR, human placentae were either devoid of TAC1 expression (Page et al., 2001, 2003), or as in this study, some were found to express extremely low levels of TAC1. The latter observation perhaps arises owing to the more sensitive nature of the real-time quantitative PCR used in this study. Such low levels of TAC1 may originate from invading immune cells, as they have been previously shown to be a source of TAC1 expression (Weinstock et al., 1988; O’Connor et al., 2004). It is plausible that the much higher level of TAC1 expression seen in one of the pre-eclamptic placenta represents such an increased immune cell invasion. In regard to TAC4 expression, there was no overall difference in expression between the normal and pre-eclamptic groups, making TAC3 the only tachykinin gene significantly elevated during pre-eclampsia. Both Page et al. (2000) and D’Anna et al. (2004) have reported higher differences in circulating NKB peptide levels in pre-eclampsia (9.6- and 2.1-fold, respectively) than we report here in placental mRNA expression levels (1.7-fold). It is likely that circulating peptide levels will be determined by many other key factors including the manner of processing, turnover, storage, secretion and degradation, which are not reflective of mRNA levels. For example, in the case of corticotrophin releasing hormone (CRH), it has been demonstrated that most CRH in the human normotensive placenta exists as largely unprocessed/partially processed pro-CRH, with very little in the form of cleaved CRH except in the case of pre-eclampsia (Ahmed et al., 2000).

![Figure 3](image1.png)

**Figure 3.** Effects of hypoxia on the expression of TAC3. Term cytotrophoblasts were cultured in serum-free conditions in either normoxic (21% O2) (column 1) or hypoxic (2% O2) (column 2) culture conditions for a period of 24 h. *P < 0.05 showed a significant difference versus mRNA levels under normoxic conditions using paired Student’s t-test. Expression was also assessed in term cytotrophoblasts that had been differentiated into syncytiotrophoblasts using EGF and 10% fetal bovine serum for 48 h under normoxic (21% O2) conditions. Cells were then exposed for 24 h to normoxia (21% O2) (column 3), hypoxia (2% O2) (column 4), oxidative stress with 100 μM xanthine/5 μM/ml of xanthine oxidase (column 6) or 18 μM peroxynitrite (column 8) both under normoxic (21% O2) conditions. Control syncytiotrophoblasts exposed to normoxia are shown in columns 5 and 7. All expressions were assessed using quantitative PCR and normalized to those of 18S rRNA. Each bar represents the mean of six individual experiments for cytotrophoblasts and three for syncytiotrophoblasts, with SEM shown by vertical lines.

![Figure 4](image2.png)

**Figure 4.** Trophoblast culture. (A) Cytotrophoblast after attachment at T = 0. Dark mononuclear cytotrophoblast cells are stained with cytokeratin. (B) Syncytialized cells at 72 h culture (end of experimental period) stained with desmoplakin, outlining cell groups. Many cells have fused to form syncytial units (S) with some mononuclear cytotrophoblasts (C) remaining. Magnification, ×250.
NKB plasma levels equivalent to those found during pre-eclampsia are shown to be vasodilatory in the normal placental vasculature (Brownbill et al., 2003; Laliberte et al., 2004), where they cause their effects by solely activating the NK1 receptor subtype (Brownbill et al., 2003). Consequently, as there are no differences in the expression of the three tachykinin receptors during pre-eclampsia, NKB is also expected to induce placental vasodilatation in the pre-eclamptic placenta and would therefore not contribute to enhancing any placental vasoconstrictor response. In addition, evidence has been presented that the vasoconstrictor NK3 receptor is either absent or expressed at extremely low levels in the human placenta at term compared with those of NK1 and NK2 receptors (Brownbill et al., 2003). This would certainly advocate a mechanism in the human placenta, whereby high NKB levels induce placental vasodilatation predominantly via the NK1 receptor. The rat model consolidates such a mechanism where during late pregnancy there is a significant decrease in placental TACR3 mRNA expression as term approaches without any significant change in NK1 receptor expression (data not shown). Similar declines in TAC3 and TACR3 gene expression have also been observed in the rat uterus, where there is a significant reduction in their expression throughout gestation (Candenas et al., 2001; Patak et al., 2005). Furthermore, we have also observed a significant decline in TAC3 expression during late pregnancy in the rat placenta. In essence, we conclude that normal late pregnancy, at least in the rat, is associated with a down-regulation of the NKB/NK3 ligand–receptor pair in both the placenta and the uterus.

Estrogen is a prime candidate responsible for the down-regulation of the NKB/NK3 ligand–receptor pair in late pregnancy. The placenta is the major site of estrogen production during pregnancy where its synthesis occurs exponentially during gestation with the highest serum levels obtained in late pregnancy (Candenas et al., 2001). There is consistent evidence to show that TAC3 and TACR3 expressions are strongly down-regulated by estrogen in many different biological systems (Rance and Young, 1991; Pinto et al., 1999; Candenas et al., 2001; Cintado et al., 2001; Pillon et al., 2003). Cintado et al. (2001) have tentatively hypothesized that the NKB/NK3 ligand–receptor pair could be involved or, at least, be an indicator of estrogen-related pathophysiology. In this context, low circulating levels of estrogens have been associated with pre-eclampsia (Innes and Byers, 1999). In the case of pre-eclampsia, possible mechanisms of high NKB expression include disruption/failure to down-regulate the NKB/NK3 system during the third trimester as observed in the rat, or an unknown stimulus inducing NKB secretion. Current data in humans, which lack early pregnancy expression patterns in the third trimester, do not allow differentiation of these possibilities for ethical reasons.

Our data support previous findings that argue for a role for NKB and the NK3 receptor in the placenta and during pregnancy. Indeed, female rats treated with the NK3 receptor antagonist SR142801 before mating exhibited a tendency towards decreased fertility with a significant reduction in their litter sizes (Pinton et al., 2003). In addition, the highest levels of uterine NK3 receptor expression in the pregnant rat (peaking at day 3) were detected before and at the time of implantation (Candenas et al., 2001). NKB could play a key role in early pregnancy at the time of implantation. In humans, elevated plasma levels of NKB have also been associated with IUGR that likewise occurs with impaired placental implantation (D’Anna et al., 2004). In our studies, none of the placenta used came from patients with IUGR. Nonetheless, such increases in NKB production may represent sustained synthesis because of the early failure of trophoblast invasion into the uterus although the precise mechanism remains unknown.

Figure 5. Real-time quantitative PCR analysis of the expression of TAC3 and TACR3 during late rat pregnancy. Expression was normalized to that of 18S rRNA. Each bar represents the mean tissue expression from four different animals at days 16, 19 and 21 of gestation, with SEM shown by vertical lines. *P < 0.05, significant difference versus mRNA levels from day 16; **P < 0.05, significant difference versus mRNA levels from day 16; ***P < 0.05, significant difference versus mRNA levels from day 16, using unpaired Student’s t-test.

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References