Altered expression of the tachykinins substance P/neurokinin A/hemokinin-1 and their preferred neurokinin 1/neurokinin 2 receptors in uterine leiomyomata

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Objective: To study the expression levels of tachykinins and tachykinin receptors in uterine leiomyomas and matched myometrium.
Design: Laboratory study.
Setting: University research laboratories and academic hospital.
Patient(s): Women undergoing hysterectomy for symptomatic leiomyomas.
Intervention(s): Quantitative polymerase chain reaction, immunohistochemistry and Western blot.
Main Outcome Measure(s): Expression and tissue immunostaining of substance P, neurokinin A, hemokinin-1, neurokinin 1 receptor full-length (NK1R-Fl) and truncated (NK1R-Tr) isoforms, and neurokinin 2 receptor (NK2R) in paired samples of leiomyoma and adjacent normal myometrium.
Result(s): *TAC1* messenger RNA (mRNA) was significantly up-regulated in leiomyomas, whereas intense immunoreaction for the three peptides was particularly abundant in connective tissue cells. Differential regulation of *TACR1* mRNA was observed, and at the protein level there was a significant increased expression of NK1R short isoform (NK1R-Tr). *TACR2* mRNA was significantly up-regulated in leiomyomas, although levels of NK2R protein were similar in normal and tumor cells.
Conclusion(s): These and our previous data demonstrate that the whole tachykinin system is differentially regulated in leiomyomas. The increased expression of NK1R-Tr might stimulate leiomyoma growth in a similar way to that observed in other steroid-dependent tumors. (Fertil Steril® 2016;106:1521–9. ©2016 by American Society for Reproductive Medicine.)
Key Words: Leiomyomas, myometrium, NK1 receptor short isoform, tachykinins, tachykinin receptors

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Mammalian tachykinins (TKs) are a family of biologically active and structurally related peptides derived from three different genes: *TAC1* encodes for substance P (SP) and neurokinin A (NKA), *TAC3* encodes for neurokinin B (NKB), and *TAC4* encodes for hemokinin-1 (HK-1) (1, 2). Tachykinins exert most of their actions by interacting with specific G protein–coupled membrane receptors: neurokinin 1 receptor (NK1R), NK2R, and NK3R encoded by

Received April 24, 2016; revised June 17, 2016; accepted July 6, 2016; published online July 25, 2016.
A.G.-S. has nothing to disclose. S.M.-H. has nothing to disclose. I.D. has nothing to disclose. M.H. has nothing to disclose. F.M.P. has nothing to disclose. D.B. has nothing to disclose. A.R.B. has nothing to disclose. L.C. has nothing to disclose. T.A.A. has nothing to disclose.
Supported in part by research grants from the Agencia Canaria de Innovación y Sociedad de la Información, ACIISI, (PI 2007/001), and from Ministerio de Economía y Competitividad (CTQ2011-25564 and RTC-2014-1431-1), Spain, in both cases with joint financing by FEDER funds from the European Union.
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Fertility and Sterility® Vol. 106, No. 6, November 2016 0015-0282/$36.00
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http://dx.doi.org/10.1016/j.fertnstert.2016.07.007

the *TACR1*, *TACR2*, and *TACR3* genes, respectively (3). Substance P and HK-1 bind preferentially to the NK1 receptor, NKA to the NK2 receptor, and NKB to the NK3 receptor. The genes encoding the three TK receptors have a similar structural organization, with five exons expanded by four introns allowing the generation of splice variants (1, 4). Two NK1R receptor isoforms have been reported in humans: a full-length receptor (NK1R-Fl) and a truncated receptor (NK1R-Tr) that lacks 96 amino acid residues at the C terminus (5, 6).

Tachykinins acting through TK receptors contribute to normal homeostasis of respiratory, cardiovascular, immune, endocrine, gastrointestinal, and urinary organ systems. There is also increasing evidence showing that TKs play an important role in the regulation of reproduction (1,7–10). Besides normal physiologic functions, TKs and their receptors have been involved in pathologic conditions, including neoplasia. In cancer cells expressing TK receptors, such as breast, pancreatic, gastric, and colon, TKs promote proliferation and survival (11–14). In addition, activation of NK1R by SP can directly stimulate the process of neovascularization through the induction of endothelial cell proliferation (15). In a large majority of tumors, SP and NK-1 receptors are found in the intra- and peritumoral blood vessels (16), and SP is involved in the growth of capillary vessels in vivo and in the proliferation of cultured endothelial cells in vitro. Recent findings point to NK1R-Tr as a good candidate to mediate malignant transformation in breast and colitis-associated colon cancer (17–19). In addition, a role for NK2R as a potential cell cycle regulator has been proposed (20). Neurokinin A coupled to NK2R activates p53, which in turn is able to bind to the *TACR2* promoter region increasing NK2R expression, leading to cell cycle quiescence of hematopoietic progenitors (20).

Uterine leiomyomas or fibroids are the most common neoplasm of the female genital tract, being present in up to 70%–80% of white and black women by the age of menopause (21). This tumor is composed of various cell types, including smooth muscle cells (SMCs), vascular SMCs (VSMCs), and fibroblasts (FBs), usually surrounded by an enriched extracellular matrix (ECM) (22, 23). Although these tumors are benign, they are responsible for several symptoms, such as heavy or prolonged menstrual bleeding often leading to anemia, pressure symptoms involving increased urinary frequency and pelvic pain, and constipation. In addition, this tumor may interfere with reproduction, because submucosal and intramural leiomyomas that distort the uterine cavity decrease implantation and pregnancy (24). This fact becomes increasingly important becaues the number of infertile women with leiomyomas has increased owing to the delay in child-bearing (25). Moreover, complications during pregnancy and childbirth due to leiomyomas have also been reported (24, 26).

Like most reproductive tract tumors, leiomyomas are steroid hormone dependent, and growth factors, cytokines, chemokines, and ECM components are known factors involved in their pathogenesis (27). Tachykinin and TK receptors are abundantly expressed in the uterus of different mammalian species, including humans, rats, and mice, in which their expression varies with age, during the ovarian cycle, and during pregnancy, and is tightly controlled by ovarian steroids (28–39). In human uterus, TKs acting through NK2R induce myometrial contractions with different orders of potency, NKA $>$ SP $\geq$ NKB (33). We have previously found that NKB was overexpressed in uterine leiomyoma, it showed a subcellular location different from that found in normal myometrium, and the expression of its high-affinity NK3R receptor was also upregulated, supporting a role for this system in leiomyoma pathophysiology (40). To find out whether the other TKs members (SP, NKA, and HK-1) and their preferred receptors (NK1R and NK2R) could also be differentially regulated in leiomyomas, we have analyzed their expression pattern in leiomyomata at cellular and molecular levels and compared it with the adjacent normal appearing matched myometrium.

MATERIALS AND METHODS

Patients

Eighteen female patients aged 36–49 years, admitted to the Hospital Universitario de Canarias and the Hospital Quirón between 2006 and 2012 were enrolled in this study after giving informed consent. Ethical approval was granted by the Committee for Clinical Research Ethics of the Hospital Universitario de Canarias. Samples analyzed included 16 intramural, submucous, or subserous leiomyoma specimens from 16 women, as well as the matched myometrial tissue; two tumors (intramural and submucous) obtained from one woman, and her matched myometrial tissue; and two tumors (intramural and subserous) obtained from another woman, and her matched myometrial tissue. Myometrial samples were taken as far away as possible from leiomyomata. All patients underwent hysterectomy for menorrhagia without any previous treatment. Regarding the menstrual phase, participants enrolled in this study included 7 in the proliferative phase and 11 in the secretory phase. The proliferative and secretory phases were assigned according to the date of the last menstrual period and confirmed by histologic assessment.

TAC1, *TAC4*, *TACR1-Fl*, *TACR1-Tr*, and *TACR2* mRNA Quantification

RNA extraction and reverse transcription. Gene expression was analyzed in paired samples of leiomyomas and adjacent myometrial tissue (20 tumors and 18 myometrium). To avoid degradation, tissue sections were immersed in RNAlater (Sigma Aldrich) immediately after surgery, kept at 4°C overnight, and stored at −80°C until processed.

Total RNA was extracted using Tri-Reagent (Sigma) and the RNeasy Mini Kit (Qiagen). Fifty milligrams of tissue were homogenized in 1 mL of Tri-Reagent using TissueRuptor (Qiagen). After adding 200 μL of chloroform and mixing, samples were centrifuged at 12,000 $\times$ g for 15 minutes at 4°C. One volume of ethanol 100% was added to the supernatant, mixed, and then RNA was cleaned and eluted using the RNeasy Mini Kit according to the manufacturer's instructions. Residual genomic DNA was removed by incubating the RNA samples with RNase free DNase I and RNasin according to the manufacturer's instructions (Promega). The effectiveness of

the DNase treatment was assessed in samples with no reverse transcriptase added (RT-negative). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining (41). Finally, RNA was quantified by absorbance using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Retro-transcription was carried out using 2 μg of RNA, and first-strand complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase, RNase H Minus, Point Mutant (Promega), and a 1:1 mix of oligo(dT)$_{23}$ primer (Sigma Aldrich) and random hexamers (Roche) according to the manufacturer's instructions (Promega).

Real-time polymerase chain reaction. A Bio-Rad MyiQ real-time polymerase chain reaction (PCR) detection system apparatus was used to perform the quantification of all transcripts. Each sample was analyzed in triplicate in a total reaction volume of 20 μL consisting of a 100-fold dilution of complementary DNA, 10 μL of 2× SensiMix Plus SYBR Fluorescein Kit (Bioline, Ecogen, Barcelona, Spain) and 0.175 μM of each primer. The cycling conditions were 95°C for 10 minutes followed by 40–45 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. For each experiment, a nontemplate reaction was included as negative control. The specificity of the PCR reactions was confirmed by melting curves analysis of the products, as well as by size verification of the amplicon in a conventional agarose gel.

To normalize gene expression, we used two housekeeping genes, *GNB2L1* and *RPL32*, stably expressed (40). The sequence of the primer pairs used were as follows: *GNB2L1* forward (F): 5′-GAGTGTGGCCTTCTCCTCTG-3′ and reverse (R): 5′-GCTTGCAGTTAGCCAGGTTC-3′; *RPL32*-F: 5′-CATCTCCT TCTCGGCATCA-3′ and *RPL32*-R: 5′-AACCCTGTTGT-CAATGCCTC-3′; *TAC1*-F: 5′-ACTGTCCGTCGCAAAATCC-3′ and *TAC1*-R: 5′-ACTGCTGAGGCTTGGGTCTC-3′; *TAC4δ*-F: 5′-AGTGGGAGGCAGAGAGGA-3′ and *TAC4δ*-R: 5′-GCGAT-GAGGACAGGAGACACAG-3′; *TAC4γ*-F: 5′-AAGGAGAA AAAAAGGCAGAGAG-3′ and *TAC4γ*-R: 5′-ACTGCTGCTT GACACTGAGA-3′; *TACR1-Fl*-F: 5′-TCCTCCTGCCCTACAT CAAC-3′ and *TACR1-Fl*-R: 5′-TCATTTCCAGCCCCTCATAG-3′; *TACR1-Tr*-F: 5′-ACGAGCAAGTCTCTGCCAAG-3′ and *TACR1-Tr*-R: 5′-GAGAGCTCATGGGGTTGG-3′; *TACR2*-F: 5′-ATTGCTGCCGACAGGTACA-3′ and *TACR2*-R: 5′-ACGAGGT GGTACAGGAGGAG-3′. The individual efficiency values (E) were calculated by the DART (Data Analysis for Real-Time PCR) program, which determines E from the raw fluorescence data (42). The average E value obtained from all individual efficiencies was imported into qbase^{PLUS} data analysis software, which uses the classic $2^{-\Delta\Delta Ct}$ method with PCR efficiency correction and multiple reference gene normalization to calculate the relative expression ratio (43).

Histology

Leiomyoma and adjacent uterine myometrium samples were fixed in 10% buffered formalin, then embedded in paraffin and cut in 3-μm-thick sections. The sections were deparaffinized, hydrated, hematoxylin-eosin stained, and evaluated for histopathology.

Immunohistochemistry

Deparaffinized 3-μm sections were rehydrated in 0.05 M Tris-buffered saline (TBS; 0.05 M Trizma base containing 0.9% of NaCl, pH 7.4), which was used for all further incubations and washes. The sections were incubated overnight at room temperature with one of the following polyclonal anti-human antibodies: anti-SP (1:25; ABD Serotec), anti-NKA (1:50; Abcam), anti-HK-1 (1:50; Santa Cruz Biotechnology), anti-NK1R against N-terminal region (1:100; Abcam) or against C-terminal region (1:100; Santa Cruz Biotechnology), and anti-NK2R (1:20, Sigma) in TBS buffer containing 0.2% Triton X-100. After rinsing, the sections were incubated with a biotinylated goat anti-rabbit (1:1,000), followed by a streptavidin–peroxidase conjugate (1:1,000; Jackson ImmunoResearch), both for 60 minutes at room temperature. Peroxidase activity was detected using 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Aldrich) in TBS containing 0.01% hydrogen peroxide at room temperature. The specificity of the immunostaining was assessed by replacing the specific antisera by normal serum.

Western Blot

The protein expression of NK1R-Fl, NK1R-Tr, and NK2R in leiomyomas and matched myometrial samples was estimated by Western blot analysis. Fifty milligrams of tissue sections were homogenized in RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris, pH 8.0) containing protease inhibitors (Sigma) using TissueRuptor (Qiagen). Protein concentration was determined using the bicinchoninic acid assay, and 30 μg of protein was loaded on 10% TGX Stain-Free gels (Bio-Rad). After protein separation by electrophoresis, stain-free gels were activated according to the manufacturer's instructions using ChemiDoc MP (Bio-Rad). Proteins were then transferred using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) to polyvinyldifluoride membranes that subsequently were imaged for the total protein transferred using the stain-free application on the ChemiDoc MP imager (Bio-Rad). Membranes were blocked by incubating for 2 hours with 5% non-fat dry milk in TBS (100 mM Tris, 0.9% NaCl, pH 7.5) containing 0.1% Tween 20 (TTBS) and then incubated overnight either with rabbit anti-human NK1R antibody directed against the N-terminal region (1:5,000; Abcam) or rabbit anti-human NK2R (1:2,000; Santa Cruz Biotechnology) at 4°C–8°C. After three washes in TTBS, membranes were incubated with anti-rabbit horseradish peroxidase–conjugated secondary antibody (Sigma) diluted 1:80,000 for NK1R and 1:50,000 for NK2R for 1 hour at room temperature. After washing, protein bands were developed using an ECL chemiluminescence detection kit (Western Bright, Advansta), and detection and quantification of band intensities was conducted using Image Lab 5.0 software (Bio-Rad), which uses total protein for

normalization. This strategy has been shown to be more accurate than normalizing against a housekeeping gene (44).

Statistical Analysis

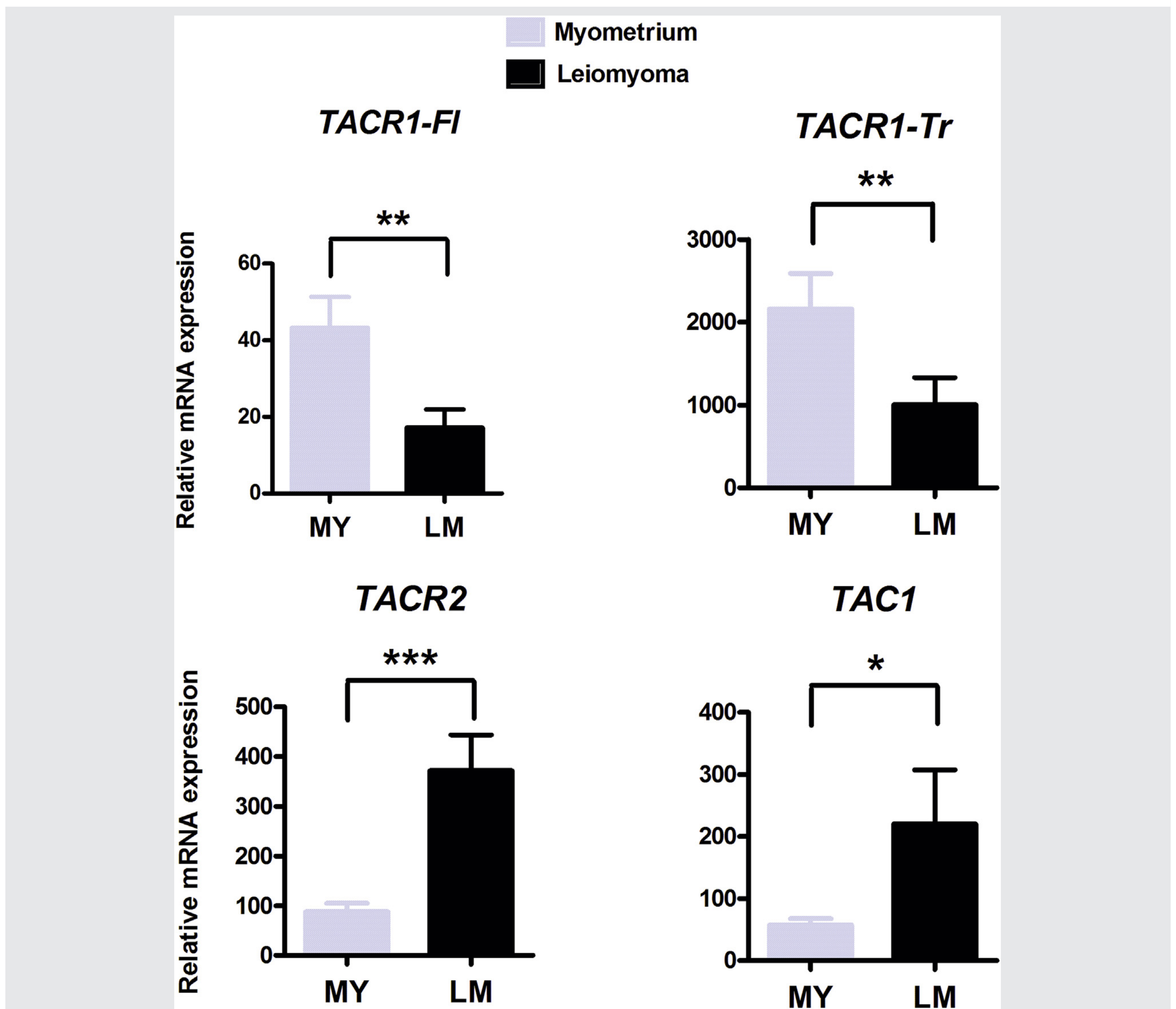
Normalized C_t values for all genes analyzed and normalized Western blot data for NK1R-Tr and NK2R did not pass the normality test, therefore a nonparametric Mann-Whitney *U* test (two-tailed) was carried out to compare leiomyoma and myometrium samples. In the case of NK1R-FL, sample groups were compared by two-tailed Student's *t* test. A P value $< .05$ was considered significant. Statistical analysis was performed using GraphPad Prism 6.

RESULTS

Detection and Quantification of TKs and TK Receptors Messenger RNA by Real-Time PCR

For each of the analyzed transcripts we compared the relative expression between leiomyomas and matched myometrium from 18 women using quantitative PCR. RT-negative samples showed no amplification. Melt curve analysis of positive samples displayed only a single peak corresponding to the analyzed transcript. Normalized data using *GNB2L1* and *RPL32*, two validated housekeeping genes, showed that *TAC1* expression was significantly up-regulated in leiomyomas, 3.8-fold on average, compared with matched

FIGURE 1



Relative quantification of *TACR1-Fl*, *TACR1-Tr*, *TACR2*, and *TAC1* mRNA in 20 leiomyomas (LM) and their matched myometrium (MY). Statistical analyses were performed using the Mann-Whitney test. *Bars* show the mean fold change, and *error bars* indicate SEM. *$P<.05$, ** $P<.01$, *** $P<.001$.

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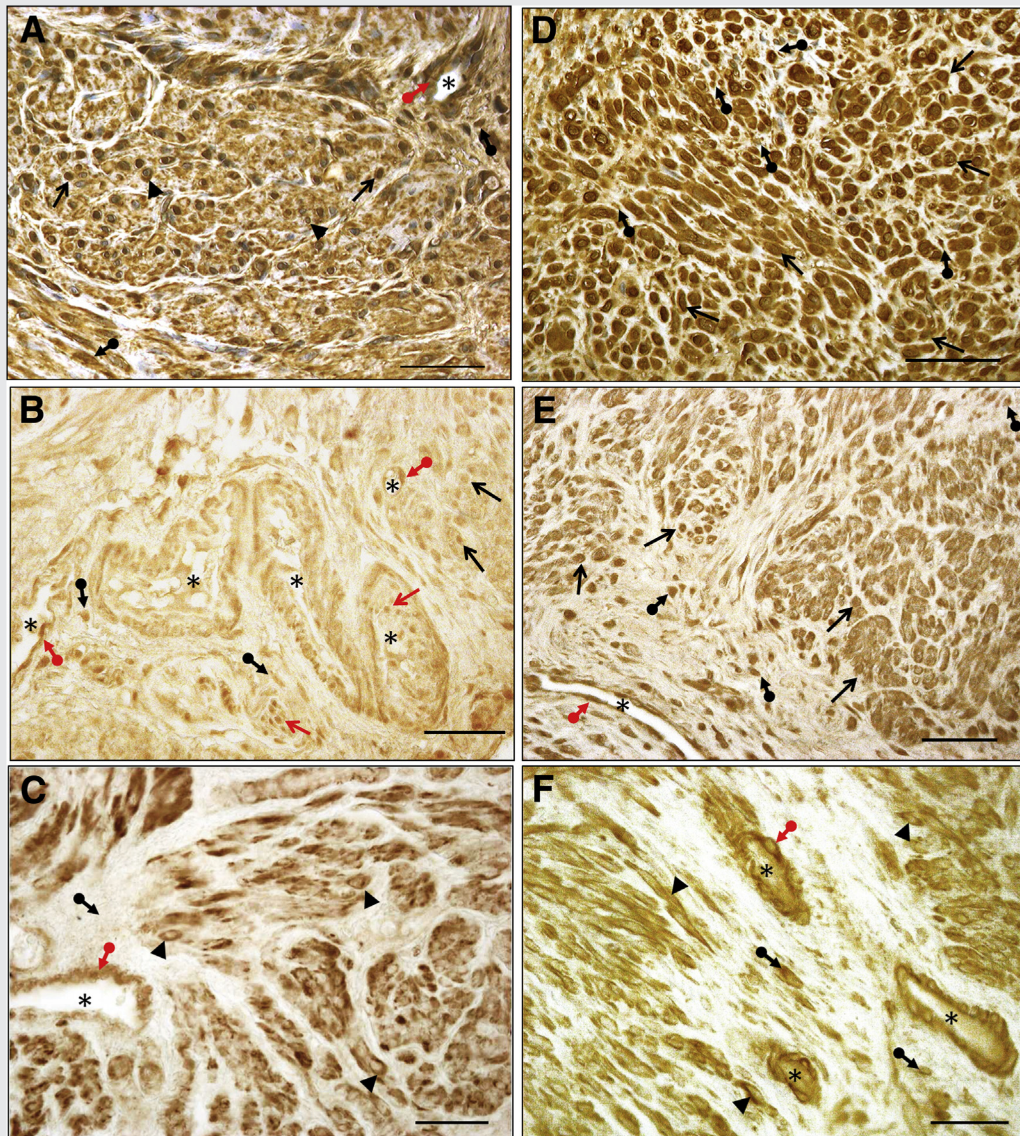
myometrium (P=.0193, n = 38; Fig. 1). In the case of *TAC4* mRNA, only variants δ-TAC4 and γ-TAC4 were detected, but no significant differences were observed among the two groups (data not shown). *TACR1* isoforms were significantly down-regulated in leiomyoma, with an average of 2.5-fold for *TACR1-Fl* (P=.0043) and 2.1-fold for *TACR1-Tr* (P=.009). Finally, *TACR2* mRNA showed a significant increase, 4.2-fold on average, in leiomyoma samples (P=.0002) compared with myometrium (Fig. 1).

Tachykinin Immunohistochemistry

In the human myometrium, numerous SMCs, VSMCs, and endothelial cells showed positive immunoreactivity (ir) for

FIGURE 2



Substance P, NKA, and HK-1 immunoreactivity in myometrium (**A**–**C**) and matched leiomyoma (**D**–**F**) tissues. (**A**) Substance P immunoreactivity in cytoplasm (*arrowheads*) and a few nuclei (*black arrow*) in SMCs, connective (*black arrow* with *solid circle* at base), and endothelial cells of myometrium (*red arrow* with *solid circle* at base. (**D**) Stronger immunoreaction in leiomyoma SMCs (*black arrows*) and connective cells (*black arrows* with *solid circle* at base). *Blue nuclei* (**A-D**) correspond to hematoxylin staining. (**B**) Neurokinin A immunoreactive nuclei in SMCs (*black arrows*), VSMCs (*red arrows*), endothelial (*red arrows* with *solid circle* at base), and connective cells (*black arrows* with *solid circle* at base) of myometrium. (**E**) Stronger immunoreaction in leiomyoma SMC (*black arrows*) and connective cells (*black arrows* with *solid circle* at base). Endothelial cells (*red arrow* with *solid circle* at base) are also shown. (**C, F**) Hemokinin-1 immunoreactive cytoplasm in SMCs (*arrowheads*) and endothelial cells (*red arrows* with *solid circle* at base) of myometrium and leiomyoma. In leiomyoma, connective HK-1 immunoreactive cells are present compared with myometrium (*black arrows* with *solid circle* at base). *Asterisks* denote the vessel lumen. No hematoxylin counterstain was applied for NKA and HK-1. Scale bars = 500 μm.

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the peptides SP, NKA, and HK-1 (Fig. 2A–C). In addition, a weak immunoreaction occurred in connective tissue (Fig. 2A–C), HK-1 being the weakest (Fig. 2C). The subcellular location was cytoplasmic for HK-1 (Fig. 2C) and mostly cytoplasmic for SP, with some nuclei also showing SP-ir (Fig. 2A). In contrast, NKA immunoreactivity was mostly detected in the nuclei, alone or in combination with cytoplasmic staining (Fig. 2B).

In general, the same cellular distribution was observed for the three peptides in leiomyoma tissues. However, in all samples analyzed, immunostaining was more intense in SMCs and connective cells (Fig. 2D–F) and more predominantly in the latter, compared with matched myometrium from the same patients (Fig. 2D–F). No differences in staining pattern were observed between samples in the proliferative and secretory phases of normal or tumor tissue (data not shown).

Tachykinin Receptor Immunohistochemistry

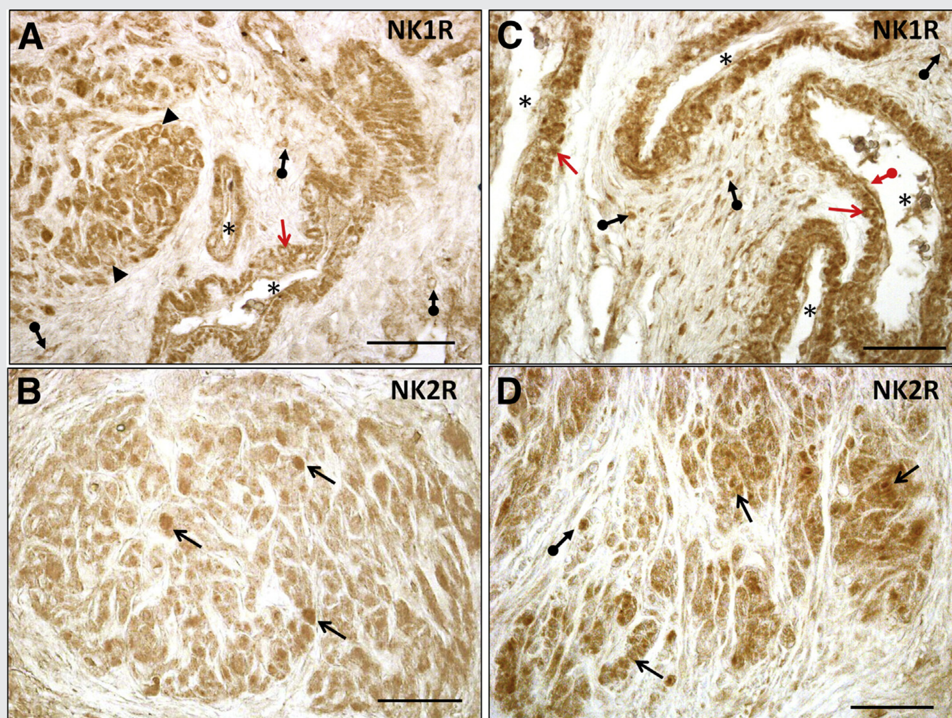
Two different antibodies were used to detect NK1R: one against the N-terminal region, which detected both isoforms, and another against the C-terminal domain, which only detected the full-length isoform. Very similar immunostaining patterns were observed with both antibodies (data not shown). Neurokinin 1R-ir was detected in SMCs and VSMCs from normal myometrium and tumor tissue (Fig. 3A and C) with a cytoplasmic location. A slight immunoreaction was observed in myometrial connective tissue (Fig. 3A). In comparison, the adjacent leiomyoma showed a much higher immunoreaction in connective tissue (Fig. 3C).

Both myometrium and leiomyoma showed NK2R immunostaining in SMCs (Fig. 3B and D) with nuclear and also cytoplasmic location. Some immunoreactive cells were also detected in the connective tissue, especially in the leiomyoma (Fig. 3D).

Western Blot

Neurokinin 1R-Fl and NK1R-Tr show different electrophoretic migration in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, therefore both can be detected and quantified using an N-terminal antibody (Fig. 4). The analysis of Western blot data normalized against total protein load showed decreased NK1R-FL expression in leiomyomas compared with myometrium, although differences were not statistically significant ($P=.1282$). Conversely, significant up-regulation of NK1R-Tr was observed in leiomyomas ($P=.0262$). Finally, there was no significant differences in NK2R expression between normal and tumor samples ($P=.7508$).

FIGURE 3



NK1R and NK2R immunoreactivity in myometrium (**A, B**) and matched leiomyoma (**C, D**) tissues. (**A**) Neurokinin 1R-ir in cytoplasm of SMCs (*arrowheads*), VSMCs (*red arrow*), and weaker in connective cells (*arrows* with *solid circle* at base) of myometrium. (**C**) Intense NK1R-ir connective cells (*arrows* with *solid circle* at base) in leiomyoma. Leiomyoma VSMCs (*red arrows*) and endothelial cells (*red arrow* with *solid circle* at base) are also shown. (**B, D**) NK2R-ir in SMCs (*black arrows*), with predominantly nuclear localization, in myometrium and leiomyoma. NK2R-ir in leiomyoma connective cells (*black arrow* with *solid circle* at base (d). *Asterisks* denote the vessel lumen. Scale bars = 500 μm.

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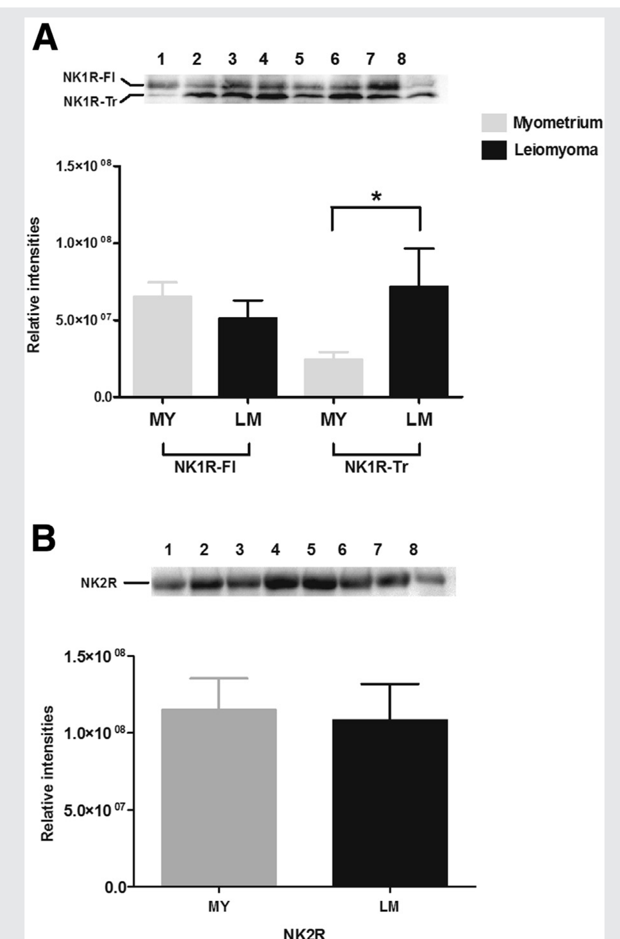
FIGURE 4



Comparative expression of tachykinin NK1R isoforms and NK2R proteins, in leiomyomas and their matched myometrium. Lanes 1–2, 3–4, 5–6, and 7–8 represent bands detected in myometrium-matched leiomyoma of four patients, respectively. (**A**) Western blot analysis with a specific N-terminal antibody against human NK1R showed two bands corresponding to NK1R-Fl and NK1R-Tr. Relative expression of NK1R-Tr was higher in leiomyomas compared with myometrium, but no differences were observed for NK1R-Fl. (**B**) Western blot analysis with a specific anti-human NK2R antibody shows similar expression levels in both tissues. Each *bar* represents the mean with SEM of eight different samples. *$P<.05$.

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DISCUSSION

Uterine leiomyomas affect approximately 80% women during their lifetime (24–27), but despite this high prevalence, little is still known about their etiology. In the present study we have found dysregulated expression of the TK members SP, NKA, and HK-1 and their preferred NK1R and NK2R in leiomyoma compared with normal myometrium. A previous study carried out by our group also found altered expression patterns of the NKB/NK3R system in leiomyomata (40). Together these data suggest that TKs and their receptors may play an important physiologic role in the uterus and that their altered expression may contribute to leiomyoma development.

It is increasingly accepted that the TK family plays an important role in the neuroendocrine control of mammalian reproductive function, acting at both central and peripheral levels (1,7–10,33). This strongly argues for a participation of the TK system in reproductive disorders, as has already been demonstrated for NKB/NK3R in human normosmic hypogonadotropic hypogonadism (8) and pre-eclampsia (7). In this study we assessed whether the dysregulated expression previously observed for the NKB/NK3R system in uterine leiomyomas (40) could be extrapolated to the other members of the TK family.

Regarding the peptides analyzed (SP, NKA, and HK-1), all three showed increased immunostaining in leiomyoma SMCs and VSMCs compared with myometrium. However, the main differences when comparing normal and tumor tissues were observed in connective cells; thus, in myometrium, immunoreactivity was slight and in some cases not detected, whereas leiomyomas showed an intense immunoreaction in all cases. In the case of SP and NKA, the increased immunoreaction observed for these peptides was accompanied by a significant upregulation of *TAC1* mRNA. Fibroblasts are the principal components of connective tissue, and they are capable of producing different components of extracellular matrix. Fibroblasts and ECM are parts of the tumor microenvironment that it is increasingly clear can directly influence tumor development (22, 23, 45, 46). In fact, FBs isolated from uterine fibroids stimulate leiomyoma SMC cell proliferation in culture (22, 45). In addition, leiomyoma-derived FBs express higher levels of estrogen receptors than SMCs, and after estrogen stimulation, stromal FBs promote the expression of ECM components, cytokines, and growth factors, inducing cell proliferation (45). Recent data supporting the role of leiomyoma microenvironment come from the differential expression pattern observed in FBs and VSMCs derived from fibroids, compared with those obtained from myometrium (23). Taking into account that TKs are involved in a myriad of processes, including proliferation, angiogenesis, inflammation, and blood vessels dilatation, and that TKs and their receptors are present in fibroid SMCs, VSMCs, and FBs, it is tempting to speculate that TKs synthetized and secreted from connective and tumor cells may act in a paracrine and/or autocrine manner, thus contributing to leiomyoma pathophysiology.

We also observed differential expression of the two isoforms of the NK1R, NK1R-Tr and NK1R-Fl, when comparing leiomyoma with matched myometrium. In leiomyomas there was a decrease in *TACR1-Fl* mRNA and a lower expression of NK1R-Fl, although these differences only reach statistical significance at the mRNA level. Similar to our results, NK1R-Fl is expressed in normal breast cells, and its expression decreased gradually with cancer progression (19). On the contrary, although *TACR1-Tr* mRNA decreased in leiomyomas, the NK1R-Tr receptor protein showed higher expression levels in tumor cells compared with normal myometrium. This correlation at the protein level has been previously detected in different cancer types. Thus, in colitis-associated colon cancer, in hepatoblastoma, and in breast cancer primary tumors, NK1R-Tr levels increase progressively with cancer progression (18, 19, 47). In addition, breast cancer cell lines showed

increased expression of NK1R-Tr compared with the normal epithelium. Recently Berger et al. (48) detected high levels of NK1R-Tr in hepatoblastoma cell lines, whereas levels were almost undetectable in normal FBs and in the embrionary HEK-293 cell line. In vitro experiments have been carried out to elucidate the role of both isoforms in cancer. In this sense, stable transfection of NK1R-Tr in nontumorigenic breast cells led to the production of SP, which in turn acts autocrinally to increase cell proliferation, a fact that was not observed in NK1R-Fl transfectants and untransfected cells (17). In addition, injection of HBL-100 cells transfected with NK1R-Tr into nude mice resulted in increased numbers of lung metastases than in controls, whereas injection of MDA-MB-231 cells transfected with NK1R-Fl showed an opposite effect (19). Together these data suggest that the two isoforms of NK1R may exert opposite functions in the regulation of many cellular aspects of tumorigenesis, including proliferation, adhesion, infiltration, and metastasis (19). In leiomyomas the observed decreased expression of NK1R-Fl concomitantly with the increased expression of NK1R-Tr may also be involved in tumor proliferation. Given that NK1R-Tr may play a key role in malignant transformation (17–19), it would be interesting to analyze the expression pattern of both isoforms in uterine leiomyosarcoma, a highly aggressive malignant cancer that, although very rarely, may arise from uterine leiomyoma and for which no prognostic marker exist (49–52).

We found a clear up-regulation of *TACR2* mRNA in leiomyomas, confirming the results obtained in a recent genome-wide DNA methylation analysis that detected hypomethylation and increased mRNA expression of *TACR2* (53). Although the SP/NK1R system has been extensively studied in different cancers, little is known about the role of the other TK family members in neoplasia, although it has been proposed that NK2R may act as a potential cell cycle regulator (20, 54). Neurokinin 1R and NK2R exhibit a "ying-yang" relationship with respect to their expression and function in bone marrow stroma (54, 55), and whereas NK2R is expressed at high levels, NK1R expression is down-regulated, and vice versa. Interactions between NK2R and its preferred ligand, NKA, lead to inhibitory effects, whereas binding of SP to NK1R mediates an increase in the proliferation (20, 54, 55). In leiomyomas the up-regulation of *TACR2* mRNA concomitantly with down-regulation of *TACR1* mRNA suggests that the internal cross-talk regulating the transcription of these receptors may be operating. In addition, the low proliferation rate and benign nature of these tumors probably reflect that some of the mechanisms controlling cell cycle arrest are still functioning. The similar NK2R protein levels detected in normal and tumor cells suggest that this receptor may contribute to cell cycle arrest in leiomyomas. However, further studies are needed to determine whether the dysregulated expression of *TACR2* and *TACR1* mRNA may contribute to leiomyoma pathophysiology.

In conclusion, this work presents a series of experiments showing the dysregulated expression of SP/HK-1/NK1R and NKA/NK2R systems in leiomyomata, and a pattern of expression of NK1R isoforms similar to that found in different neoplasias, such as colon, liver, and breast cancer. These and our previous data, which evidenced dysregulation of the NKB/NK3R system, contribute to increase our understanding of changes occurring, at the mRNA and protein levels, in this reproductive disorder.

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