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Original Article

Effects of liver depression and psychological stress on human uterine leiomyoma cells by an AR–cAMP–PKA signal transduction pathway

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ABSTRACT

Objective: Based on the emotional theory of Traditional Chinese Medicine, and combined with the modern medicine theory of psychological stress, a research model of human uterine leiomyoma cells (ULM) was cultured *in vitro* to determine the effectiveness of adrenergic receptor (AR) agonists in human ULM cell growth. In addition, we studied the functional influence of "liver depression and psychological stress theory" on fibroid formation by intervening in the AR–cAMP–PKA signaling pathway. The intention was to establish a new method to prevent and cure fibroids through "liver depression and psychological stress theory" and provide an experimental basis for the Traditional Chinese Medicine emotional theory.

Materials and methods: Primary human ULM cells were enriched by collagenase digestion. Immunohistochemistry and hematoxylin and eosin (HE) staining were used for cytological identification. Using this model, we studied intervention using specific AR agonists on ULM cells to observe the influence of "liver depression and psychological stress theory" on estrogen receptor (ER), progesterone receptor (PR), vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF).

Results: Norepinephrine (NE) and epinephrine (E) are adrenergic receptor agonists. They promoted ULM cell proliferation and increased the levels of ER, PR, VEGF and FGF. In contrast, isoproterenol (ISO) inhibited ULM cell proliferation and decreased the levels of ER, PR, VEGF and FGF. The protein expression of cAMP and PKA in ULM cells was reduced and the levels of ER, PR, VEGF and FGF were increased when co-treatment with the α-AR blocker (phentolamine). The β-AR blocker (metoprolol) displayed an opposite effect.

Conclusions: AR agonists modulated ER, PR, VEGF and FGF levels in ULM cells in an AR–cAMP–PKA-dependent signaling pathways to influence fibroid occurrence and development.

Introduction

Uterine leiomyoma is a very common benign gynecological tumor, which belongs to the abdominal mass in traditional Chinese medicine. Throughout the various opinions of many Chinese physicians, the pathogenesis of an abdominal mass includes internal causes and external causes. Often, when eating cold food or on exposure to exogenous wind-cold and damp-toxin during menstruation, or residuing the blood after delivery the human body can be potentially invaded by such external causes. The pathogenic qi is fighting with healthy qi and blood, which could obstruct the circulation of qi-blood and result in an abdominal mass. The internal cause, often provoked by a drastic and prolonged emotional stimulus that once exceeding a person's physiological regulatory ability, will cause qi stagnation to produce the blood-stasis setting.

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This will also lead to an abdominal mass. A person with "liver depression constitution" that easily succumbs to persistent anxiety and depression will be predisposed to blood stasis and abdominal mass, which is closely related to the onset of fibroid disease. As miraculous pivot record "if injured by anger, the qi will go upward and cause stagnation of qi and blood to result in abdominal mass", the Jing-yue's complete work also said "the sudden anger injure liver and cause regurgitation of qi and blood stasis result in abdominal mass; over-thinking and anxiety injury spleen and may make qi deficiency and blood stasis result in abdominal mass". So qi stagnation is the key pathogenesis of fibroid, for prolonged qi stagnation produce the blood-stasis and phlegm lead to fibroid.

Large sets of epidemiological data and clinical research indicate that psychological factors (including chronic stress, prolonged depression, social isolation, among others) [1], have a close relationship with the occurrence and development of tumors. Abnormal emotional activity leads to the occurrence and development of tumors by disturbing neuro-endocrine function and inhibiting immune function. However, positive emotional activity can prevent tumor formation and can improve the prognosis of tumor patients.

In the chronic psychological stress state, abnormal stress related neurotransmitter release stimulates the brain, pituitary gland, adrenal gland, sympathetic nerve endings and tumor cells to release multiple neurotransmitters and cytokines, which lead to prolonged production of those factors, and abnormally high levels in the blood and tissues. Thaker and Glaser's research indicated that chronic stress can improve the occurrence and development of tumors through interfering with neuro-endocrine and immune system functions [2,3].

Psychological stress may stimulate a neuro-endocrine-immunological regulatory network to modulate the body's response by generating the synthesis of cytokines and neurotransmitters. This function is similar to that of the liver in controlling dispersion (including regulating qi activity, smooth emotion, which is conducive to ejaculation in men, and promotes transformation and transportation in the spleen and stomach). At the same time, the traditional Chinese medicine emotional theory might cause disease, which could be reflected through a modern medicine psychological quantitative index.

Based on these observations, we hypothesized that liver depression and psychological stress might stimulate sympathetic nerve endings and the adrenal medulla to release catecholamine hormones, like NE, E, Dopamine and others, by excitation of the sympathetic nervous system. This will activate the α/β-AR–cAMP–PKA signaling pathway of ULM cells to mediate ER and PR and improve ULM cell growth by intervening in VEFG and FGF expression.

Materials and methods

Chemicals and reagents

E, NE (they are adrenergic receptor agonists, E is epinephrine, NE is norepinephrine), and metoprolol were obtained from Jin Yao Amino Acids, Co. Ltd, Tianjing, China. Isoproterenol was obtained from Hefeng Pharmaceutical Co., Ltd, Shanghai, China. Phentolamine was obtained from Haipu Pharmaceutical Co., Ltd, Xudong of Shanghai, China. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were both purchased from GIBCO, USA. Trypsin–EDTA and Collagenase I were purchased from Sigma, USA. The agents and cytokines PR, ER, VEGF, and FGF ELISA kits were obtained from R and B Co. USA. ^{125}I-cAMP free kits were obtained from Phoenix Pharmaceuticals, Inc, USA. The BCATM protein analysis kit was obtained from Pierce Co. USA.

Cell culture

Leiomyoma tissues were randomly obtained from patients undergoing Laparoscopic Uterine fibroids removal surgery at Nan Kai Hospital, Tianjin, China in May 2012 to December 2013, with the permission of patients' informed consent. This study included women with TCM syndrome of qi stagnation and blood stasis type caused by emotional stimulation, who did not receive any type of hormonal or drug therapy at least three months before the surgery and the pathological diagnosis showed uterine fibroids after the surgery.

The size of the tumors was about 2 cm^3 in diameter. All tissue samples used in this study were confirmed as histologically ordinary leiomyomas, with no cellular, epithelioid, bizarre, or plexiform variants present. Tissues were rinsed several times with saline and minced into about 1 × 1 cm^3 small pieces in a sterile petri-dish, then digested in Collagenase I solution (DMEM 20 mL, Collagenase I at 1 mg) in 50 mL Erlenmeyer flasks in which there was a 3 cm magnet. The flask was then incubated at 37 °C with a slowly rotating magnetic stirrer for 40–50 min. The digested tissue was passed through a 200 mesh sieve, and the uterine leiomyoma cells were collected by centrifugation (1000 rpm, for 10 min). The isolated cells were seeded in a 75 cm^2 culture flask in culture medium (DMEM) supplemented with 15% FBS and 1% antibiotics and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Cells were collected between passages three and four for the experiments, which were performed with 70–80% confluent cultures, and identified by hematoxylin–eosin (HE) staining and immunocytochemistry.

Cells (1 × 10^5 cells/mL) were seeded in 12-well plates in which there was culture medium (DMEM) that was supplemented with 15% FBS, following which they were cultured in a humidified atmosphere with 5% CO_2 in air. After adhering to the culture plates, cells were cultured in DMEM with 4% FBS for 24 h before starting the studies.

In the first experiment, the cells treated with E, NE or isoproterenol at 0, 0.1, 1 and 10 μM were harvested for the culture supernatants, which were removed at 3, 6, 12 and 24 h. According to the cell proliferation assays, we determined the optimal timepoints and the optimal drug concentration for subsequent experiments. In the second experiment, the cells were treated in three separate groups as follows: (i) control, (ii) NE and (iii) NE plus phentolamine. The cells were treated with 1 μM NE (except for the control group), and the supernatants were harvested at 12 h, centrifuged, and frozen at −80 °C until assayed. For the blocking experiments, phentolamine was added to the cell cultures for 1 h in the NE plus phentolamine group, before adding NE. In the third experiment, the cells were treated in three separate groups as follows: (i) control, (ii) iso, and (iii) iso plus metoprol, following which, the three groups of cells were treated with 1 μM iso, while the control group was left untreated. Supernatants were harvested at 24 h, centrifuged, and frozen at −80 °C until assayed. For the blocking experiments, metoprol was added to the cell cultures for 1 h in the iso plus metoprol group before adding the iso. Each experiment was repeated at least three times in duplicate with unstimulated/naïve to treatment cells.

Identification of uterine tumor cells

HE staining

Uterine leiomyoma cells were cultured in DMEM supplemented with 10% FBS on the cell monolayer cover-slides. The slides were treated with formalin for 30 min, and stained with the

hematoxylin solution at 1:20 dilution for 10 min, and then incubated in 1% $NaHCO_3$ solution to form a violet color, and then stained in aqueous eosin solution for 30 s–1 min. Slides were rapidly treated with acetone twice, and after washing with distilled water, slides were treated with acetone/xylene (2:1) twice, and 1:2 acetone/xylene (1:2) twice, through pure xylene for 5–10 min. Lastly, a drop of neutral gum was added to the specimen, which was then covered with a larger cover disc, and observed by light microscopy.

Immunohistochemistry

For immunohistochemistry, uterine leiomyoma cell samples were fixed in 4% paraformaldehyde or 10% formalin at −20 °C. Slides were then treated with 95% ethanol for 6–8 min to fix, air dried, and then treated with 3% H_2O_2 to block endogenous peroxidases, followed by treatment with 0.1% Triton x-100 and then blocked with normal goat serum. Slides were treated with the primary antibody, smooth muscle actin, at a 1:100 dilution. Secondary antibody (biotin labeling goat anti mouse IgG) was used at a 1:50 dilution. Finally, slides were incubated with the avidin horseradish enzyme marker chain SABS at 30 °C for 30 min, and then stained with DAB–H_2O_2 for 5–20 min and observed by microscopy.

Cell proliferation

Uterine leiomyoma cells were seeded in 96-well plates at a density of 1×10^5 cells/well in DMEM that was supplemented with 15% FBS, and cultured in a humidified atmosphere of 5% CO_2 in air. Once the cells achieved a 70%–80% confluence, they were treated with E, NE or isoproterenol at 0, 0.1, 1 and 10 μM, but not the control group. Next, 0.5% MTT (0.015 mL/well) reagent was added to the cells at 3,6,12 and 24 h time-points respectively, and cultured for 4 h in a fully humidified atmosphere of 5% CO_2 in air at 37 °C, following which the culture supernatants were removed, with PBS washing steps repeated twice. Finally, the dissolve the formalin crystals, DMSO was added to each well at 0.15 mL/well. Then, a typical spectrophotometer was used to detect the light absorption value of the wells. The increasing absorbance value of the dissolved formazan was proportional to the number of living cells. The experiment was repeated three times.

ELISA

Quantification of the levels of VEGF, FGF, PR, and ER in the cells was done by Enzyme Linked Immunosorbent Assay (ELISA) according to the manufacturer's kit protocol. The growth factors VEGF, and FGF were collected from the cell culture supernatants, and ER and PR were collected from the leiomyoma cells. Absorbance values were measured using a spectrophotometric plate reader set at a wavelength of 450 nm.

Western blot analyses

For the detection of PKA, uterine leiomyoma cells were harvested and lysed in lysis buffer consisting of 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 100 mmol/L Tris–HCl at pH 6.8, and 0.2% bromophenol blue. The protein lysates were separated by electrophoresis on an SDS-PAGE gel under reducing conditions, following which the lysates were transferred onto a nitrocellulose (NC) membrane. Blots was washed in PBS buffer containing 0.02% Tween 20 and then incubated with rabbit anti-human PKA primary antibody, and antibodies of rabbit anti-human GAPDH, overnight at 4 °C. Next, a secondary antibody of horseradish peroxidase conjugated goat anti-rabbit IgG antibody was added to the blots, which were incubated for 1 h, at room temperature, and then washed again. Finally, an ECL peroxidase substrate solution was used to immunologically detect the protein bands.

Radioimmunoassay

The content of cAMP was determined by a radioimmuoassay with a commercial ^{125}I-cAMP kit. Briefly, polyvinyl chloride (PVC) tubes were taken that included the total radioactive tube, the non-specific tube, the 0 standard tube, the standard tube and the sample tube. The total NSB tube contained 200 μL of acetic acid buffer, and the S0 tube had 100 μL of acetic acid buffer added. In addition, the standard tubes S1–S8 had respectively added into 100 μL of diluted standard solution, then joined in NSB tube and the sample tube 5 μL acetylation reagent, after that, respectively joined in S0 tube and the sample tube 100 μL antiserum; then an ^{125}I-cAMP labeled substance was added to all tubes and incubated at 4 °C for 24 h. Next, normal rabbit serum and sheep anti-rabbit IgG (secondary antibody) were added to all of the tubes, then mixed well and allowed to stand for 2–3 h at room temperature before being centrifuged at 3000 rpm for 15 min. The supernatants were then blotted to filter discs to permit measurement of the CPM values using a gamma counter.

Statistical analysis

The SPSS version 19.0 software program was used for all of the analyses. Data were expressed as mean ± SE; the difference in multiple data sets were analyzed by repeated measures analysis of variance (ANOVA), when Mauchly spherically symmetric inspection results dissatisfied the spherically symmetric inspection, then use the Greenhouse–Geisser method to estimate the spherically symmetric coefficient, and take the spherically symmetric coefficient to correct the statistic values by the Bonferroni method for multiple comparisons. In addition, comparisons between groups were made by one-way ANOVA. Under conditions where the differences between groups was statistically significant, Fisher's least significant difference T test was used to compare the data. An alpha value of $P < 0.05$ signified data that was statistically significant.

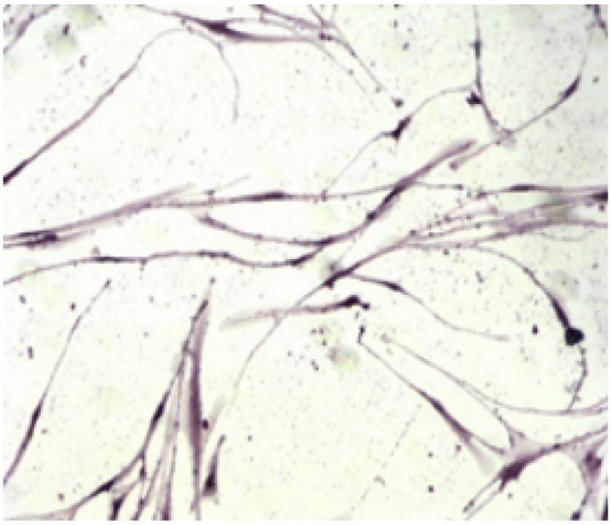

Fig. 1. The growing leiomyoma cells (HE staining, Magnification ×100).

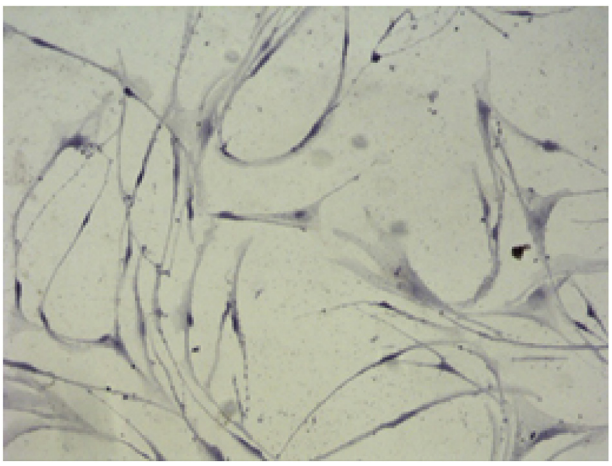

Fig. 2. The growing leiomyoma cells (Immunohistochemistry, Magnification × 100) (staining for alpha-smooth muscle actin).

Results

Identification of ULM cells

By light microscopy, the myofiber appeared like a spindle, was dyed red, and the cell nucleus was rod-shaped and blue in color. Profiles of the cells were clear, and the cytoplasm and nucleolus were clearly visible (as shown in Fig. 1). Cultured cells were stained by alpha-smooth muscle actin (α-actin, smooth muscle specific protein marker) monoclonal antibody by immunohistochemistry, the nucleus was stained blue, and the brownish-yellow filiform texture was actin, where the number of positive cells was more than 90%. Therefore, cultured cells by this method were highly pure, and this was in line with experimental requirements, and could be used to observe follow-up experiments (Figs. 1 and 2).

Effects of epinephrine, norepinephrine, epinephrine on uterine leiomyoma cells

It was seen that NE elicited the most proliferation of uterine leiomyoma cells, where maximum increases occurred by 12 h of incubation. As illustrated in Fig. 3, incubation of ULM cells with 1 μM NE for 12 h produced an approximately 4-fold increase in the proliferation of uterine leiomyoma cells ($P < 0.001$), and 10 μM NE produced a 2.8-fold increase in the proliferation of uterine leiomyoma cells ($P < 0.001$) as compared with the control. For E, the maximum increase (176% of control) occurred at 12 h with 1 μM of E. However, the overall proliferation of uterine leiomyoma cells that was induced by E in the 3- and 6-h model did not differ significantly from the control values. For isoproterenol, the maximum decrease in cell proliferation of uterine leiomyoma cells was seen after 12 h of incubation with 10 μM isoproterenol, which was 150% of the control population of cells ($P = 0.0001$). Induction of proliferation of uterine leiomyoma cells at other time points from isoproterenol treatment was not significant.

With the specified dose of NE, E and ISO treated uterine leiomyoma cells, the synthesis of VEGF and FGF was detected in the

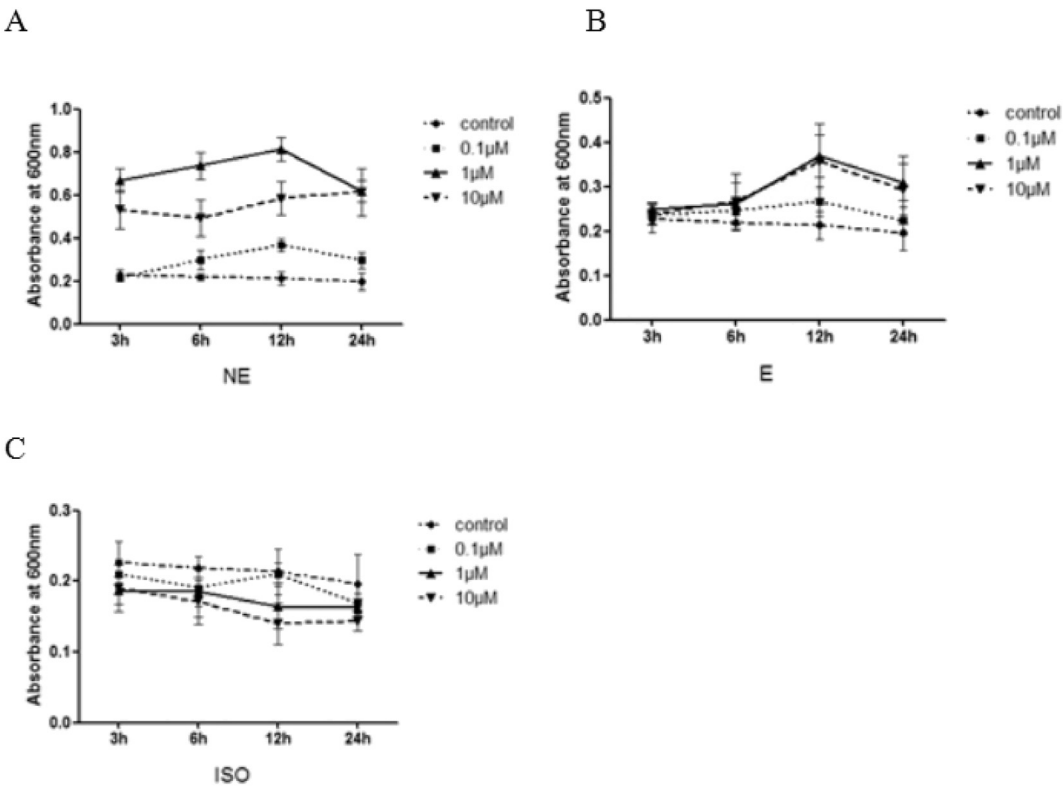

Fig. 3. The MTT mean values in different dose groups and measurement time-points are shown. Time and dose factors significantly affected cell proliferation as determined by the MTT assay in human uterine leiomyoma cells. By multiple analyses of different doses in the NE and control groups (A), the data suggested that OD values of different dose groups were higher than that found for the control group ($P = 0.001$). The OD value was highest in the 1 μM group, and was highest at 12 h than was found for 3 h, 6 h and 24 h ($P = 0.0001$, $P = 0.0001$, and $P = 0.029$ respectively). B. shows that the OD value in each dose of the E groups was higher than the control group ($P = 0.028$, $P = 0.0001$, and $P = 0.0001$), and the 1 μM group at 12 h was the highest. In Fig. C, the OD value for 1 μM and 10 μM for the ISO group was lower than the blank group ($P = 0.001$, and $P = 0.0001$), and at 24 h the OD value was the lowest.

culture supernatants (as shown in Fig. 4). The mean foundation value of VEGF was 16.41 pg/mL, and the average measurement in the expression of VEGF protein in all different dose groups of NE, and E was higher than that found in the control group. In the 1 μM NE group, the average level of VEGF protein expression was up to 42.8 pg/mL, and for the 10 μM E group was up to 46.34 pg/mL. Average measurements of NE, and E and subsequent VEGF protein expression were highest at the 6 h time point (i.e., 45.24 pg/mL, and 40.57 pg/mL respectively), which then decreased. The mean foundation value of FGF was 1.28 ng/mL, and the average measurement of the FGF protein expression in the 1 μM group and the 10 μM group of NE and E treatment groups were higher than was found for the control group. The average measurements of FGF protein expression of NE, and E were highest at 12 h, with value of 1.49 ng/mL and 1.46 ng/mL, respectively. Under the action of ISO, the average measurement for FGF and VEGF protein expression levels decreased concordantly with increasing dose and time points.

We made multiple analyses for the different dose groups and control groups and found that the levels of VEGF in the NE group for all doses were higher as compared to the control group ($P = 0.0001$; Fig. 4A). In addition, the 1 μM NE dose group had the maximal level of VEGF, and the VEGF levels seen in the 1 μM NE and 10 μM NE groups was not statistically significant ($P = 0.074$). The maximal level of VEGF was seen at 6 h with 10 μM of E (Fig. 4B), and then declined over time. For isoproterenol, the VEGF levels with the 1 μM and 10 μM NE treatments were lower as compared to the controls ($P = 0.0001$), and declined with time (Fig. 4C). As shown in Fig. 4D, the levels of E showed that FGF secretion in both the NE and E groups at 1 μM and 10 μM were higher as compared with the control group ($P = 0.003$, and $P = 0.001$ respectively). NE had the highest levels of FGF secretion at 12 h, and the maximal levels of

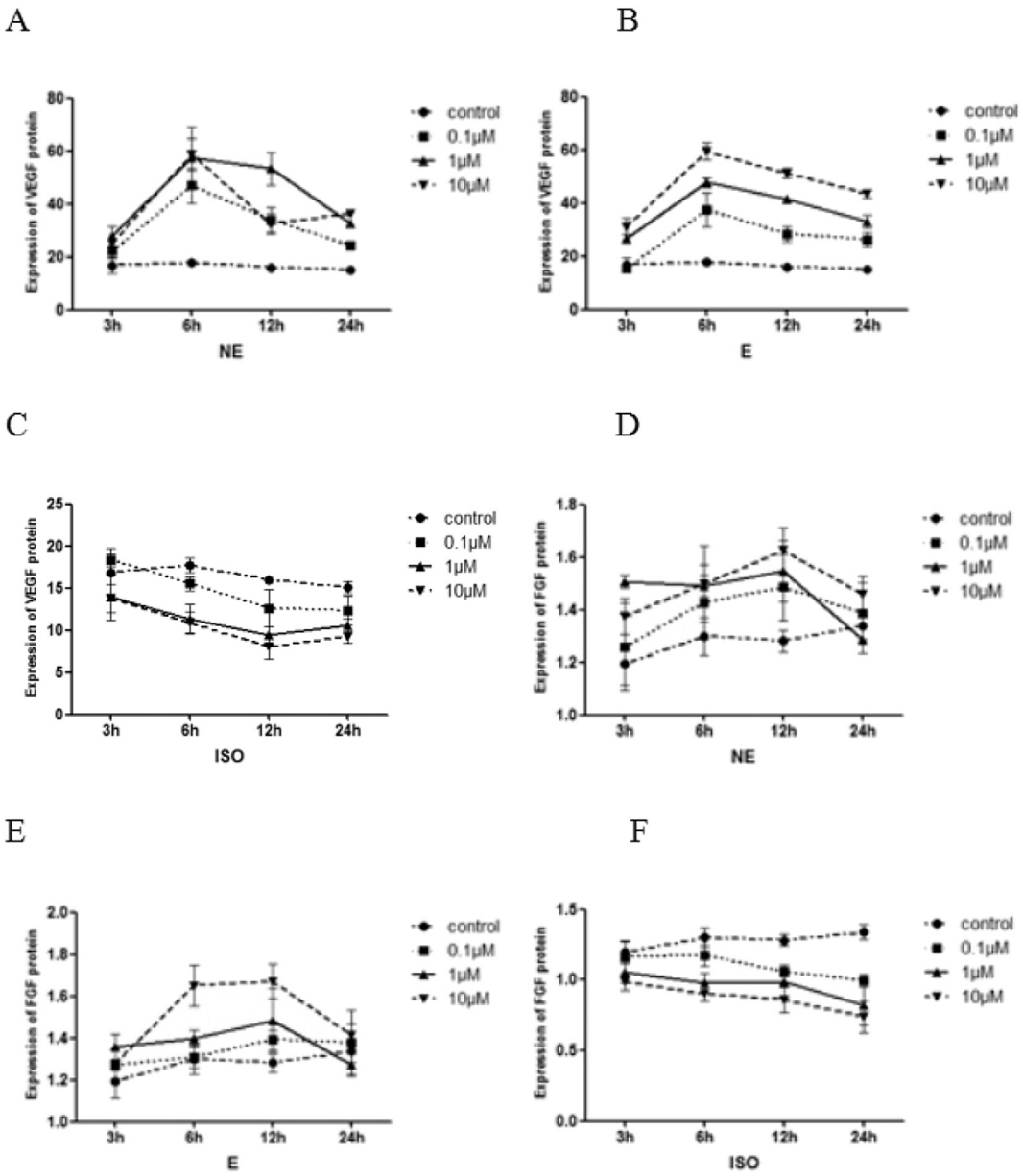

Fig. 4. Graphs demonstrating the mean VEGF and FGF protein expression values of different dose and time-point groups. The VEGF and FGF protein expression by ULM cells was statistically significant in terms of both dose and time-dependent factors.

FGF following treatment with E occurred at 6 h. For isoproterenol (Fig. 4F), the levels of FGF secretion at all doses of treatment were lower as compared with the control group (P = 0.0001), and thereafter declined with time.

Similarly, with the specified doses of NE, E and ISO treated uterine leiomyoma cells, the production of ER and PR was detected in the cells (as shown in Fig. 5). The basal mean value of ER was 85.98 pg/mL, and the average measurements for the expression of the ER protein in each dose group of NE and E were higher than that found in the control group. In addition, in the NE group, the levels of the ER protein increased with the increasing doses, which was also time-dependent. The average measurement for the expression of the ER protein in the E group at 12 h was the highest value of 93.36 pg/mL. The basal PR mean value was 2.50 ng/mL, and the NE, and E group increased with the increasing doses and time-points. Further, the highest average measured values of PR protein expression in the 1 μM group at 12 h were 3.60 ng/mL and 3.46 ng/mL respectively. For the ISO group, the expression of both ER, and the PR protein decreased with the increasing doses and time-points, which suggested that in the 1 μM group at 12 h the average measured expression of the ER protein had a minimal value of 59.03 pg/mL, while in 10 μM group at 12 h, the average measured expression level of the PR protein had a minimal value of 1.46 ng/mL.

The ER, and PR protein expression levels in ULM cells was statistically significant, and time and dose-dependent. The ER protein expression levels of the NE group in all doses were higher as compared with the control group (P = 0.0001 for all doses), and increased with time (Fig. 5A). The ER protein expression levels of the E group at 10 μM were higher as compared with the control group (P = 0.034), and the ER protein expression levels at 12 h was higher as compared with those at both 3 h and 6 h (Fig. 5B).

For isoproterenol (Fig. 5C), the ER expression levels at all doses were lower as compared with the control (P = 0.006, P = 0.0001, P = 0.0001), and the ER expression levels at 0.1 μM and 10 μM ISO were significant (P = 0.004). The ER expression levels of the ISO

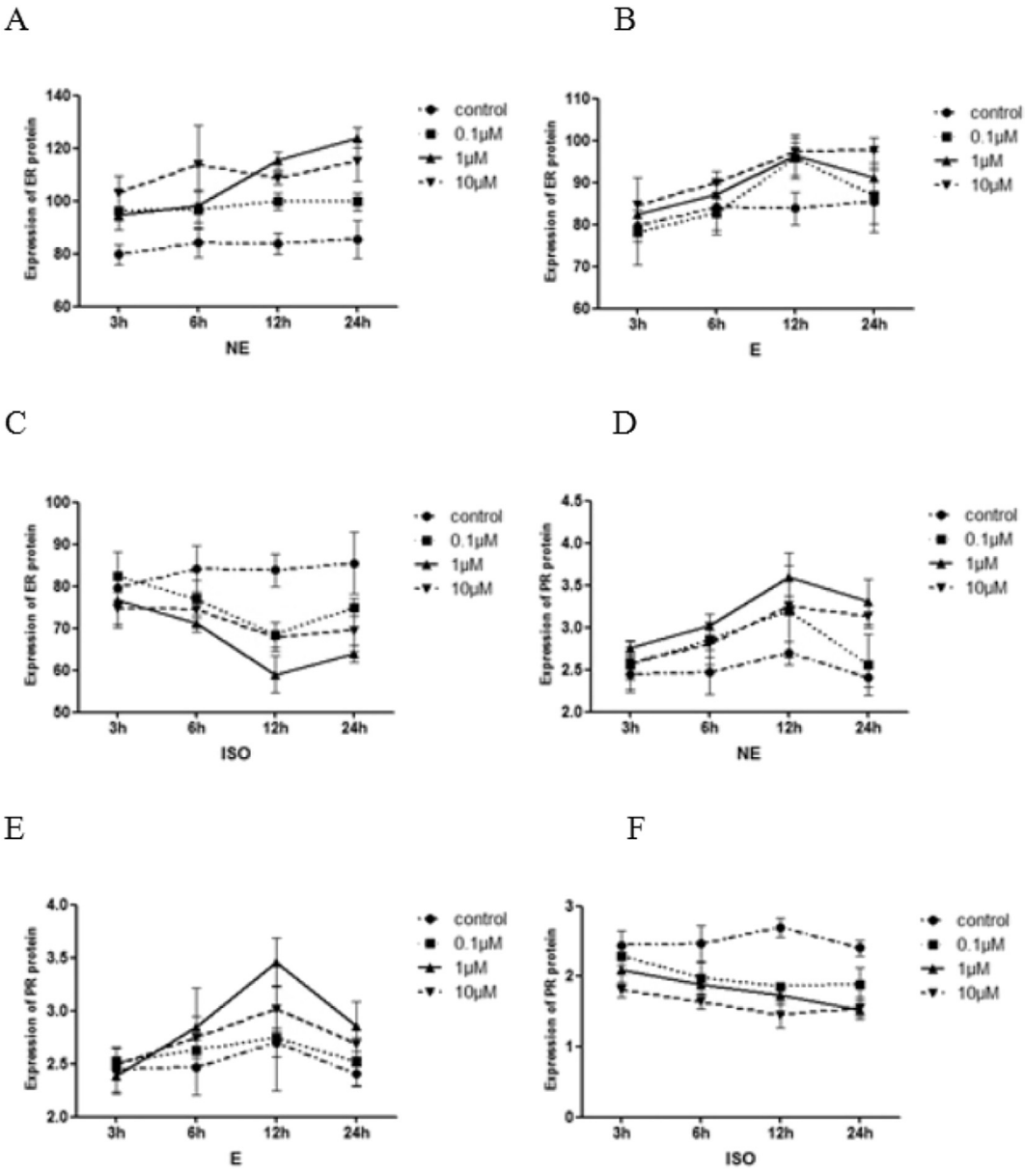

Fig. 5. Graphs demonstrating the mean ER and PR protein expression values of different dose and time-point groups.

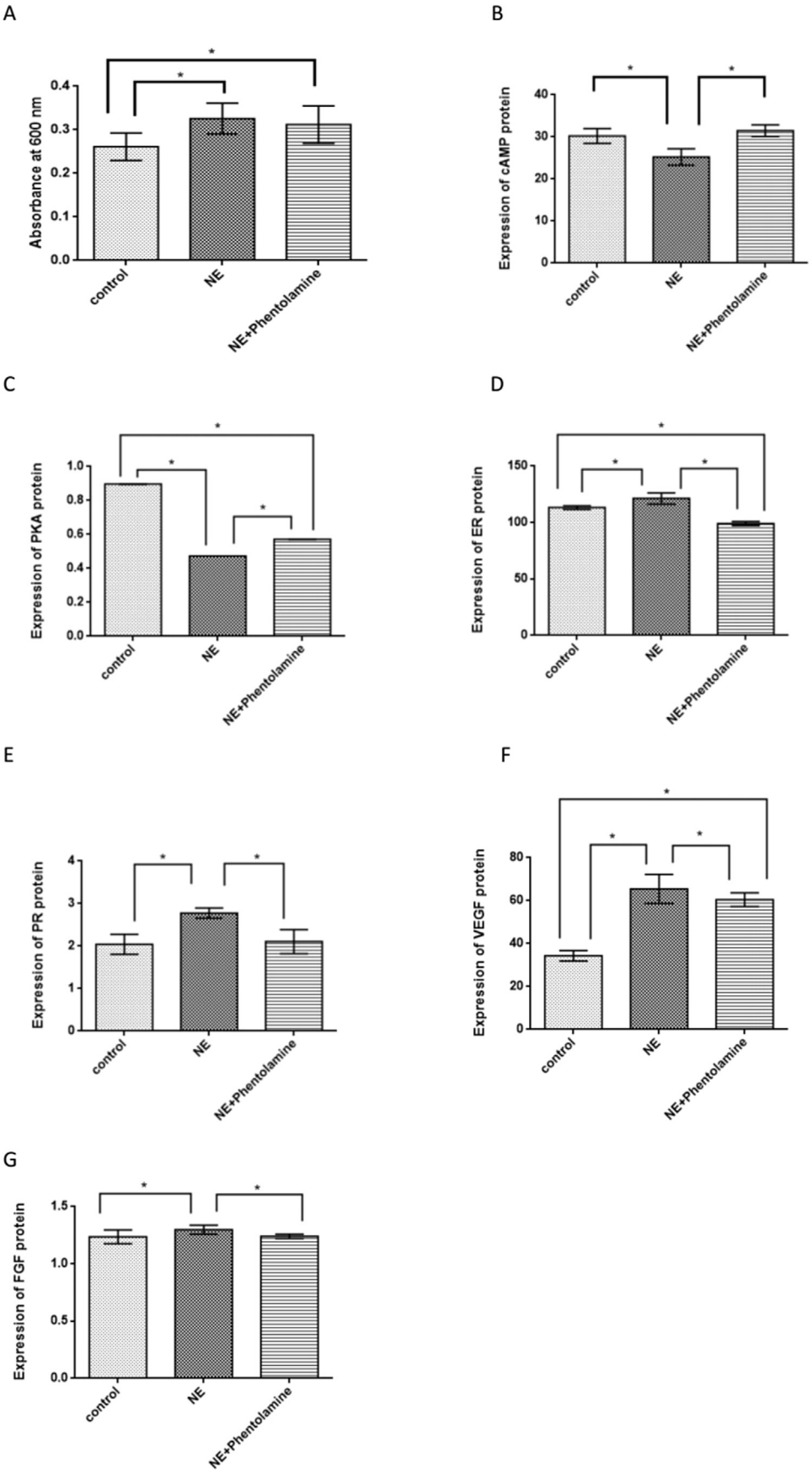

Fig. 6. Graphs demonstrating representative results from seven separate studies in uterine leiomyoma cells. The incubation was interrupted after 12 h of treatment. The data was represented as mean value ± SD. The differences between the drugs and controls were also significant ($P < 0.05$).

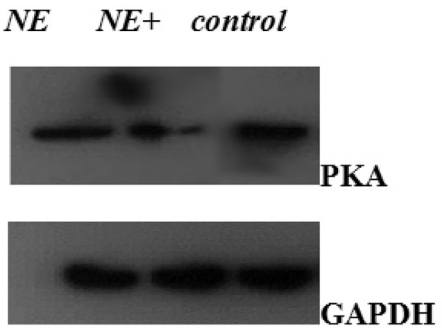

Fig. 7. Western blot analysis of PKA in uterine leiomyoma cells with NE and NE plus phentolamine (NE+).

group at 12 h was higher as compared with 3 h and 6 h ($P = 0.001$). Fig. 5D shows that the expression levels of PR at 1 μM and 10 μM NE were higher as compared with the control group ($P = 0.001$, and $P = 0.017$), and increased with time. The PR expression levels of the NE group at 6 h, 12 h and 24 h were significantly higher as compared with that at 3 h ($P = 0.043$, $P = 0.0001$, and $P = 0.008$), and the maximal increases in the levels of PR occurred at 12 h.

In Fig. 5E, 1 μM of E stimulated higher levels of PR protein as compared with the control ($P = 0.019$), and there was no other significant intergroup difference ($P > 0.05$). In addition, the levels of PR protein at 12 h were higher as compared with those at 3 h and 24 h ($P = 0.0001$, and $P = 0.001$). For isoproterenol (Fig. 5F), the levels of PR protein across all doses were lower as compared with control ($P = 0.0001$), and the PR levels with 10 μM of ISO were lower as compared with 0.1 μM and 1 μM ($P = 0.0001$, and $P = 0.043$). Finally, the levels of PR following treatment with 10 μM ISO were lower than that treated with 0.1 μM ISO ($P = 0.039$), and PR expression levels at both 12 h and 24 h was lower as compared with that at 3 h ($P = 0.024$, and $P = 0.0001$).

Adrenergic receptor-α agonists promote ER, PR, VEGF and FGF expression of uterine leiomyoma cells by cAMP–PKA signaling pathway

Through the above experiments we concluded that NE at 1 μM, and for a period of 12 h maximally stimulated the proliferation of uterine leiomyoma cells. Thus, we selected 1 μM NE and its blocker to observe its effects on the cells. The MTT assay indicated that treatment with NE significantly promoted the proliferation of uterine leiomyoma cells, and co-treatment of phentolamine inhibited the proliferation of uterine leiomyoma cells (Fig. 6A). The levels of cAMP and PKA were significantly reduced following treatment with NE. In contrast, co-treatment of phentolamine promoted the levels of cAMP and PKA (Figs. 6B, C, 7). Moreover, the levels of ER, PR, VEGF and FGF were measured by ELISA to evaluate the effect of NE on their expression in uterine leiomyoma cells (Fig. 6D–G). We found that NE increased ER, PR, VEGF and FGF expression significantly, whereas co-treatment of phentolamine had the opposite effect.

Adrenergic receptor-β agonists inhibit ER, PR, VEGF and FGF expression of uterine leiomyoma cells by cAMP–PKA signaling pathway

Through the above experiments, we concluded that 1 μM ISO for 24 h decreased the proliferation of ULM cells to the lowest levels. Simultaneously, we also chose metoprolol to study its effects on ULM cells. The MTT assay indicated that ISO significantly decreased the proliferation of uterine leiomyoma cells, while co-treatment of metoprolol promoted the proliferation of ULM cells (Fig. 8A). Under the effect of ISO, the levels of cAMP and PKA were significantly increased (Fig. 8B, C). In contrast, co-treatment of metoprolol reduced the levels of cAMP and PKA (Fig. 9). Using commercial ELISA, we measured the protein levels of ER, PR, VEGF and FGF, and found that ISO markedly decreased the expression of ER, PR, VEGF and FGF, while metoprolol promoted the level of expression of ER, PR, VEGF and FGF (Fig. 8D–G).

Discussion

There is no exactly corresponding disease of fibroid in TCM ancient books, but fibroid belongs to abdominal mass in TCM according to its symptoms. Most TCM experts consider internal stagnation of blood stasis as the key pathogenesis of fibroid in recent researches. For “The female take the liver as congenital”, so the negatively emotional stimulation, depression and sudden anger will cause liver-Qi stagnation to produce the blood-stasis constitution, which accumulate in uterus will lead to fibroid. So modern TCM experts consider qi stagnation as key pathogenesis of fibroid, for prolonged qi stagnation produces the blood-stasis and phlegm lead to fibroid.

Uterine leiomyomata is the most prevalent indication for hysterectomy [4], which constitutes the most common surgery on adult women [5]. In the USA, 1.7 million women underwent hysterectomy from 1988 to 1990 [6]. Psychological stress is closely related to tumor development. Some researches have indicated that psychological stress can affect women's estrogen and progesterone levels, and play an important role in fibroid development, but the exact mechanism is not clear yet [7,8]. Psychological stress can activate the neuroendocrine system, and induce the secretion of catecholamines, which plays a key role in malignant transformation through the adrenal receptor cAMP–PKA signaling pathway. Thaker et al. showed that chronic stress exposure increased VEGF production in vivo in MB-231 tumors through a β-AR-mediated mechanism. It was further showed that the β2-adrenergic receptor (β2-AR) is a critical mediator for the stress effects in a mouse model of ovarian carcinoma using gene specific siRNA [9]. Shan et al. revealed that behavioral stressors can enhance the pathogenesis of pancreatic cancer in vivo via elevated levels of stress hormones [10]. In addition, *in vitro* studies indicated that activation of the β2-AR signaling pathway enhanced pancreatic cancer progression [11].

VEGF is one of the most important angiogenic growth factors and is expressed in malignant tumors. VEGF is also an important mediator of chronic stress-induced exacerbation of tumor growth [9]. In this research study, it was found that there were increased levels of VEGF within the tumor microenvironment of high-anxiety mice, which might mediate the effects of anxiety-related behavioral phenotypes on SCC number and progression [12]. Polymorphisms in growth factor genes might play a role in the complex pathogenesis of leiomyoma [13]. It is known that VEGF mRNA and protein expression levels have been identified in the smooth muscle cells of both normal myometrium and leiomyoma. A higher level of VEGF expression was found in leiomyomas than in corresponding adjacent myometrium, which indicated that local angiogenesis may be important for the development and growth of these tumors [14].

The hypo-estrogenic environment leads to atrophy of certain smooth muscle cells, including leiomyomas and myometrium [15], which are to some extent, regulated by growth factors, including bFGF [16,17], which is a potent mitogen for smooth muscle cells *in vitro* [18]. In addition, bFGF regulates angiogenesis [19]. Although bFGF is mitogenic for both human uterine myometrial and leiomyoma cells, it was found that leiomyoma cells were less responsive [20]. Xuxia Wu and Dixon also found that there was no

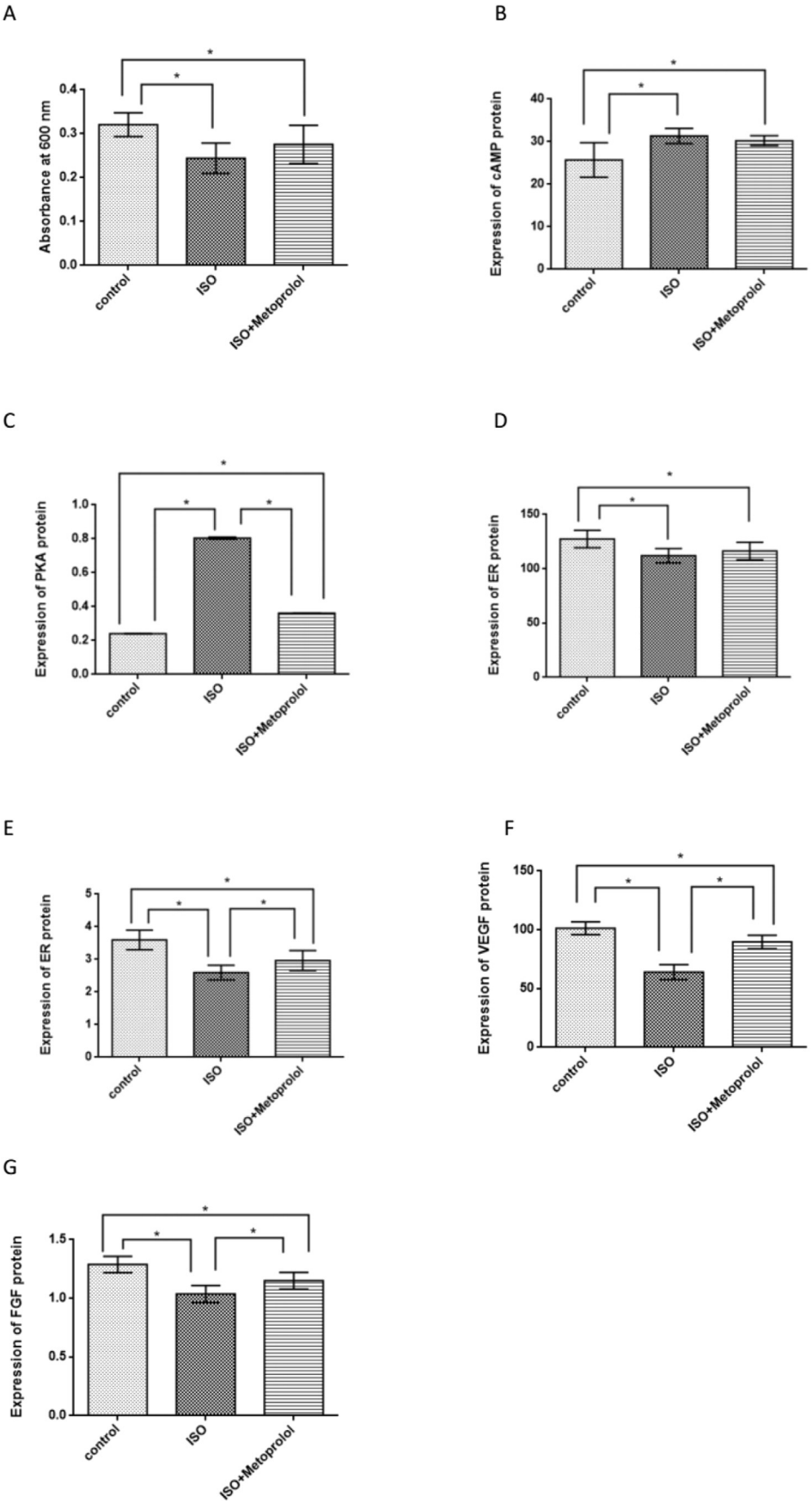

Fig. 8. Graphs demonstrating representative results from seven separate studies in uterine leiomyoma cells. The incubation was interrupted after 24 h. The data was represented as mean value ± SD. The differences between the drugs and controls were significant ($P < 0.05$).

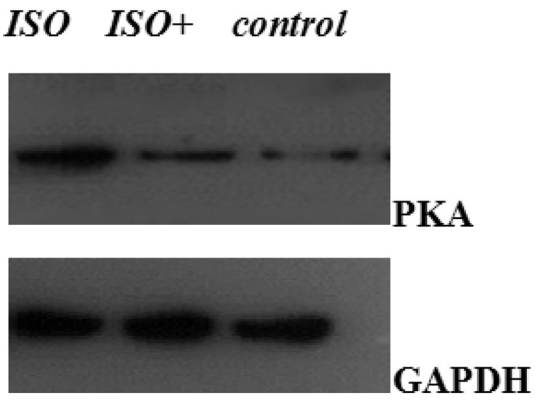

Fig. 9. Western blot analysis of PKA in uterine leiomyoma cells with ISO and ISO plus metoprolol (ISO+).

difference in expression of bFGF between leiomyomas and matched myometrial samples [21,22]. However, several researchers reported the expression of bFGF and its receptors FGFR-1 and FGFR-2 in both leiomyoma and myometrial cells, with more distinct expression of FGFR-1 in tumors as compared with myometrium [23,24].

In our study, we showed that VEGF and bFGF were expressed in uterine fibroid cells. We further showed that there were differences in the response to VEGF, and FGF exposure after stimulation by the adrenal agonists and blockers. We observed that the level of VEGF, and FGF was increased following stimulation with NE and metoprolol, and yet both ISO and phentolamine had the opposite effect. At the same time, the levels of cAMP and PKA were significantly reduced by stimulation with NE. In contrast, phentolamine promoted cAMP and PKA synthesis. Under the effect of ISO, the levels of both cAMP and PKA were significantly improved. By contrast, metoprolol reduced the levels of cAMP and PKA. Our data demonstrated that adrenergic agonists or blockers stimulate via the α- and β-AR on ULM cells that provokes the increased cAMP-PKA levels, activates the adrenergic receptor-cAMP–PKA pathway, and increases the expression of its downstream target genes that are related to angiogenesis (e.g., VEGF and FGF), which promote uterine fibroid growth and angiogenesis.

Stress-related mediators (i.e., NE and isoproterenol) can influence ER and PR from uterine leiomyoma cell-lines. These effects are mediated through α-AR and β-AR (i.e., α-AR and β-AR mRNAs and proteins were expressed in uterine leiomyoma cell-lines). Recent research found that ERα and bFGF increased tumor volume [25]. Evidence supporting the role of these receptors in the NE-dependent effect is provided by our results showing that phentolamine inhibited the NE-dependent up-regulation of ER and PR expression, as well as metoprolol inhibited the isoproterenol-dependent down-regulation of ER and PR expression. Phentolamine and metoprolol, which are α-AR and β-AR antagonists respectively, eliminated the effect induced by these adrenergic agents. It is well known that leiomyoma growth is mostly dependent on estrogen and progesterone. The interaction between the two hormones and their respective receptor levels is of interest with regard to the promotion of fibroid growth.

Angiogenesis is an important component of tumor development. ER and PR might also influence angiogenesis of uterine leiomyomas by promoting the expression of VEGF and FGF, which has been shown in ovarian cancer [26]. Our studies suggested that stress-related activation of the sympathetic-adrenal-medullary axis may have a role in the progression of uterine leiomyomas in that NE could promote the levels of ER and PR, and isoproterenol inhibited the expression of ER and PR by uterine leiomyomas cell-lines.

When treated with phentolamine and metoprolol, we observed that the expression of ER and PR was influenced. Stress reduction has also been associated with decreased levels of these neuroendocrine hormones [27,28]. These data support the clinical significance of increases in sympathetic-adrenal-medullary stress hormones as they relate to tumor progression. Previous studies have supported the relationship between biobehavioral and resilience factors, such as social support and distress, and cancer progression [29]. Although the effects of stress on immunological, neurochemical and endocrinological functions are well known, this is the first report to demonstrate that the neuroendocrine stress response can also directly affect the growth and activity of uterine leiomyomas tissue via hormone receptors on leiomyoma cells.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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