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Norepinephrine attenuates CXCR4 expression and the corresponding invasion of MDA-MB-231 breast cancer cells via β2-adrenergic receptors

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Abstract. – OBJECTIVE: The growing evidence from laboratory and clinical studies has shown that the stress hormone, norepinephrine, and chronic stress promote tumor progression in a variety of tumor types. Chemokines and chemokine receptors have been shown to play a pivotal role in tumor progression. Recently, norepinephrine was reported to have a significant effect on macrophage migration by altering the expression of the chemokine receptor CCR2.

MATERIALS AND METHODS: We investigated whether chemokines and their receptors are involved in the effects of norepinephrine on breast cancer. First, we used microarray analyses to detect the alteration of 128 chemotactically relevant genes after MDA-MB-231 cells were treated for 12 h with 100 μM norepinephrine. The CXCR4 gene demonstrated the greatest response to norepinephrine treatment, with a reduction of transcription of 95.7%, and was the focus of subsequent investigations. Real-time reverse transcription-PCR was used to determine the level of CXCR4 transcription after treatment with norepinephrine at various concentrations and for different durations.

RESULTS: The results revealed that norepinephrine reduced CXCR4 transcription in a dose-dependent manner. Norepinephrine was also found to exert a negative effect on CXCR4 translational expression, as evidenced by a 44 ± 1.7% reduction in expression after a 12-h treatment with 10 μM norepinephrine. A Matrigel assay demonstrated a 51.3 ± 9.1% reduction in the number of MDA-MB-231 cells driven to migrate by CXCR4. Finally, we found the specific β2-adrenergic antagonist, ICI 118,551, eliminated the impact of norepinephrine on CXCR4 expression.

CONCLUSIONS: Norepinephrine attenuates CXCR4 expression and the corresponding invasion of MDA-MB-231 tumor cells via the β2-adrenergic receptor. The complexity of the β2-adrenergic receptor signaling pathway might contribute to these unexpected observations in our research, and this justifies further investigation into the intricate mechanisms involved.

Key Words:
Adrenergic receptor, Breast cancer, Chemokine, CXCR4, Microarray, Norepinephrine.

Introduction

The catecholamine hormone, norepinephrine, has been found to promote tumor growth in a variety of tumor types, although there have been some conflicting reports[1-7]. Preclinical models suggest that norepinephrine induces tumor invasion, migration, and angiogenesis, and ultimately increases the potential for metastasis[6-12]. Interestingly, norepinephrine has also been reported to exert chemokinetic and chemotactic effects on various tumors[13-16]. Norepinephrine is a crucial neurotransmitter released by the sympathetic nervous system in response to physiological, psychological, or environmental threats; as over-excitation of this system is associated with increased cancer risk and poor treatment outcome, norepinephrine is considered a key epidemiological factor therein[17-21]. Families of adrenergic receptors mediate most of the biological effects of norepinephrine and are composed of the following four major subtypes: α1-, α2-, β1-, and β2-adrenergic receptors[22]. The growing evidence from laboratory research and clinical studies has demonstrated that β-adrenergic antagonists abrogate the facilitative impact of norepinephrine and chronic stress on the progression of cancer[7,8,12,14,23-25].

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Breast cancer is the most common malignancy in women worldwide, accounting for about 22% of all tumors in women. Similarly to other malignancies, breast cancer accelerates with chronic stress. The relationship between norepinephrine or chronic stress and breast cancer has been shown in an array of research studies[12,23,26-28]. Encouragingly, several recent epidemiological studies reported a link between the administration of β-adrenergic inhibitors and an improved prognosis for breast cancer patients, despite some divergence of opinion[24,25,29]. For instance, Powe et al[29] found that patients receiving β-adrenergic inhibitors for hypertension exhibited an obvious reduction in the development of metastasis and cancer-specific mortality. This result is analogous to the observation in orthotopic mouse models that norepinephrine plays an important role in the metastasis of breast cancer rather than tumor growth[28].

Cancer metastasis involves a chain of exceedingly complicated processes, the underlying mechanism of which is not yet well understood. Bernards[30] proposed that the ability to form a metastatic phenotype is established early in tumorigenesis through genetic changes, whereas activation and regulation of the phenotype depend on pro-metastatic regulators. A prerequisite for metastatic development is cell migration, and a prominent group of regulators of migration are ligands for the large family of G protein-coupled receptors (GPCRs), including chemokines[16].

The superfamily of chemokines, also known as chemoattractant cytokines, comprises small, secreted proteins, initially characterized by their ability to induce leukocyte migration[31-34]. Chemokine receptors guide cell migration to chemoattractant sources by inducing actin polymerization and cell filopodia. More than 50 chemokines and 20 chemokine receptors have been identified since the first member, interleukin (IL)-8, was discovered in 1987[35]. Chemokine receptors widely exist in epithelial and hematopoietic cells, but many types of tumors have progressively been shown to express chemokine receptors[36,37]. Tumor metastasis shares many similarities with leukocyte trafficking, such as non-randomicity and organ-selectivity. To date, a growing number of research studies have indicated that chemokines and their receptors play a pivotal role in tumor cell migration, invasion, and metastasis[31,32,36,38-40].

Previous studies established that the angiogenic pathway, triggered by norepinephrine, features increased production of various crucial pro-angiogenic regulators, such as vascular endothelial growth factor (VEGF), IL-6, and matrix metalloproteinases (MMPs)[6-11,41]. Consequently, the hypothesis that the critical pro-metastatic regulators of chemokines and their receptors are implicated in the norepinephrine-induced metastasis of cancer is worthy of verification. With this hypothesis in mind, we have investigated the effect of norepinephrine on the invasiveness of the MDA-MB-231 human breast cancer cell line.

Materials and Methods

Materials

The antibody against CXCR4 (affinity-purified goat anti-human CXCR4 [G-19] polyclonal antibody, No. sc-6279) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The HRP-labeled secondary antibody was purchased from Shanghai Kangcheng Biotechnology, Inc. (Shanghai, China). Norepinephrine (No. 74460), atenolol (No. A7655), and ICI 118,551 (No. I127) were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). CXCL12 (recombinant human CXCL12/SDF-1α, No. 350-NS/CF) was from R&D Systems, Inc. (Shanghai, China). The cell culture medium, DMEM/F12, and fetal bovine serum (FBS) supplement were purchased from Gibco (Grand Island, NY, USA).

Cell Culture

MDA-MB-231 human breast cancer cells were obtained from ATCC (Manassas, VA, USA) and maintained in DMEM/F12 medium, supplemented with 10% FBS, under normal culture conditions of 5% CO_2 in saturated air at 37°C. After digestion with 0.25% trypsin, the cells were passaged with a split ratio of 1:3. All experiments used approximately 80% confluent cultures.

Microarray

MDA-MB-231 cells at 80% confluence were incubated in medium supplemented with norepinephrine (100 μM) or in additive-free medium for 12 h. Next, cell samples were sent to Shanghai Kangcheng Biotechnology for microarray preparation and analysis, including RNA extraction, identification, marking, chip hybridization, chemiluminescence detection, and image acquisition. The microarray chips for the chemokines and chemokine receptors were purchased from SuperArray Biosciences (Frederick, MD, USA).

Real-time RT-PCR

Semi-quantitative real-time reverse transcription-PCR (RT-PCR) was used to assess the relative transcriptional production of *CXCR4* in the norepinephrine-treated MDA-MB-231 cells. Total RNA from cultured cells was isolated with TRIzol following the instructions from the manufacturer (Life Technologies, Waltham, MA, USA). Reverse transcription was performed using the Fermentas RT kit, according to the manufacturer's instructions (Fermentas, Waltham, MA, USA). The Real-time PCR Kit was purchased from Takara Bio, Inc. (Otsu, Shiga, Japan). The *CXCR4* mRNA and the internal positive control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were amplified with an Opticon 2 Real-time PCR thermocycler (MJ Research, Waltham, MA) and monitored with SYBR Green (Takara Bio, Otsu, Shiga, Japan). Reactions were carried out in 25-μL volumes, and each sample was run in triplicate. The cycling conditions used were according to the recommendations of the manufacturer. The level of expression of the *CXCR4* mRNA in each sample was normalized to that of the *GAPDH* mRNA. The relative expression of mRNA species was calculated using the comparative CT method. The PCR primers and probes were purchased from Shanghai Sangon Biotechnology, Inc. The primer sequences, product sizes, and experimental annealing temperatures were as follows:

CXCR4			
Fwd	5 -TTCTACCCCAATGACTTGTG-3	206 bp	56°C
Rev	5 -ATGTAGTAAGGCAGCCAACA-3		
GAPDH			
Fwd	5 -GGGAGCCAAAAGGGTCATCATCTC-3	353 bp	60°C
Rev	5 -CCATGCCAGTGAGCTTCCCGTTC-3		

In a time–concentration assay, MDA-MB-231 cells were treated with 1, 10, or 100 μM norepinephrine, or with additive-free medium, for 3, 6, or 12 h, after maintenance in serum-free medium for 24 h. For inhibition analysis, MDA-MB-231 cells were divided into six groups and then transferred into medium supplemented with 10 μM norepinephrine, atenolol (a β1-adrenergic antagonist), ICI 118,551 (a β2-adrenergic antagonist), atenolol + norepinephrine, ICI 118,551 + norepinephrine, or additive-fee medium for 12 h, respectively. Atenolol and ICI 118,551 were added to the cell culture medium 45 min prior to norepinephrine. All experiments were performed in triplicate and repeated once.

Immunoblotting

For analysis of the production of the GAPDH and CXCR4 proteins, cell lysates were prepared from MDA-MB-231 cells which had been treated with 10 μM norepinephrine, ICI 118,551, ICI 118,551 + norepinephrine (ICI 118,551 was added 45 min prior to norepinephrine), or additive-free medium for 12 h. Total protein was extracted, and proteins (100 μg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. After blocking with 5% non-fat, dry milk in Tris-buffered saline-Tween 20 (TBST) for 1 h, the membranes were incubated with primary antibodies overnight at 4°C in TBST with 5% BSA. After antibody binding, the membranes were washed, incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature, and visualized using ECL. All the reagents used for immunoblotting were purchased from Amresco (Solon, OH, USA), except for the SDS-PAGE sample buffer, which was from Bio-Rad Laboratories (Hercules, CA, USA).

Matrigel Invasion Assay

MDA-MB-231 cells that had been treated with 10 μM norepinephrine or additive-free medium for 12 h, were suspended in serum- and phenol red-free DMEM/F12 culture medium. These cells were allocated (1×10^5) into the upper well of a Matrigel-coated Transwell chamber, with the lower well full of medium supplemented with 300 nM CXCL12, for a 24-h incubation. Next, the cells remaining in upper wells were removed and the ones at the bottom of the filter were fixed with 100% ethanol for 10 min and stained with 0.1% crystal violet solution for 30 min. The dye was eluted using 33% acetic acid, and the crystal violet absorbance was measured at 590 nm. All experiments were repeated three times. The Transwells (8 μm) and Matrigel were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Statistical Analysis

In this study, the related statistical analysis was performed using the SPSS 19.0 software (IBM SPSS Software, Chicago, IL, USA). The primary methods used were analysis of variance and a nonparametric test (Mann-Whitney test). Statistical significance ($p < 0.05$) was determined using the unpaired two-tailed Student's t test. To determine the mean values obtained from repeated experiments, the mean value obtained for control cells in each experiment was set to 100%.

Results

Norepinephrine Treatment of MDA-MB-231 Cells Most Markedly Affected the Production of The Chemokine, CXCR4

The microarray chips utilized in this study represented 128 chemotactically relevant genes, among which 24 genes exhibited a ≥ 2-fold increase in transcription over the control, after a 12-h treatment with 100 μM norepinephrine (Figures 1 and 2). The most pronounced change discovered from the array analysis was the diminished transcription of *CXCR4* in the norepinephrine-treated group, a mere 4.3% of the transcription in the control group. *CCL2* ranked second in amplitude of variation, with a reduction of 84.8%. In addition, norepinephrine dampened the transcription of *CXCL10* by 82.2%, *CMKLR1* by 74.7%, and *CKLFSF1* by 74.1%. Eight other genes with significantly reduced transcription included *EPO*, *CCR5*, *CCL28*, *CCRL2*, *CCL25*, *CCL13*, *CKLFSF2*, and *IL13*. In comparison, *TREM1* showed enhanced transcription of 449% above the control, *CXCL16* of 316%, *IL8* (associated with rheumatoid arthritis) of 309%, and *MMP7* of 238%. Moreover, seven other genes, which exhibited ≥ 2-fold transcriptional enhancement, consisted of *GPR2*, *GPR81*, *AS1R1*, *IL1A*, *TREM2*, *CKLFSF5*, and *AS1*. As *CXCR4* transcription was affected to the greatest extent, and CXCR4 plays a pivotal role in cancer invasion and metastasis, we chose to focus on this chemokine receptor and its regulation by norepinephrine in subsequent experiments.

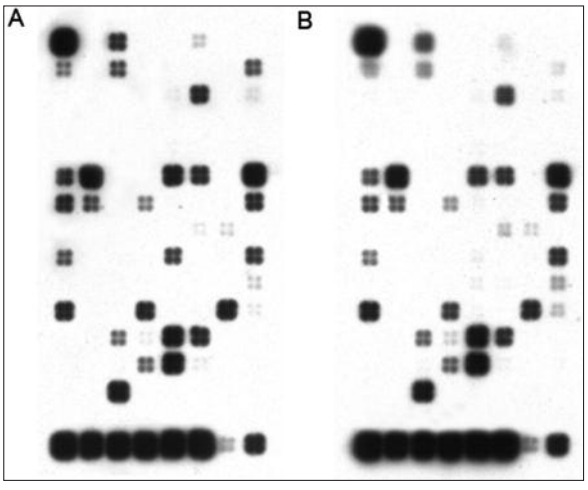

Figure 1. Microarray analysis was applied to detect 128 chemotactically relevant genes in MDA-MB-231 cells after 12 h treatment with norepinephrine. ***A,*** Cells in the control group were treated with ordinary culture medium without any additive. ***B,*** Cells in the norepinephrine group were treated with 100 μM norepinephrine.

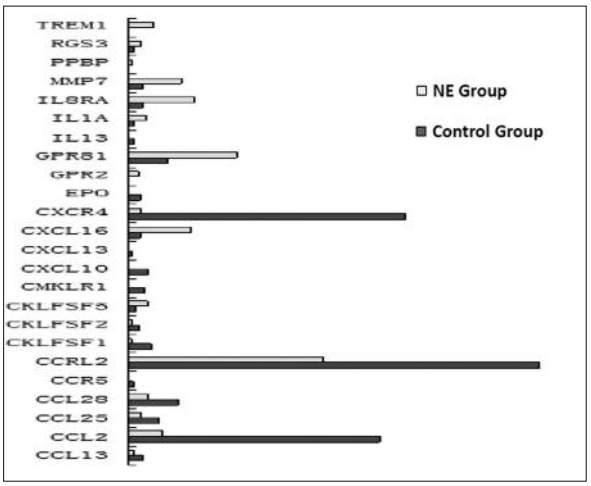

Figure 2. Chemotactically relevant genes that exhibited > 2-fold changes in mRNA transcription in the microarray analysis.

The Transcriptional Production of CXCR4 Was Attenuated in a Dose-Dependent Manner in Norepinephrine-treated MDA-MB-231 Cells

Semi-quantitative real-time RT-PCR was applied to determine the impact of norepinephrine on *CXCR4* transcription at three gradually increased concentrations of 1, 10, and 100 μM for a duration of 3, 6, or 12 h. The results revealed that the transcriptional production of *CXCR4* under any of the nine combinations of experimental conditions was attenuated to some degree (Figure 3). The greatest reduction of 96.2 ± 2.7% was reached after a 12-h treatment, in the presence of 100 μM norepinephrine, an amplitude highly consistent with that observed in the microarray assay. However, a 6-h treatment with 1 μM norepinephrine resulted in a mere 31.7 ± 4.5% transcriptional reduction of *CXCR4*. The variance in *CXCR4* mRNA levels corresponding to the concentration of norepinephrine indicates that norepinephrine regulates the transcriptional production of *CXCR4* in a dose-dependent manner in MDA-MB-231 cells. For instance, a 12-h treatment, with 1, 10, or 100 μM norepinephrine, resulted in a reduction of *CXCR4* transcription of 45.8 ± 1.9%, 63.9 ± 4.8%, and 96.2 ± 2.7%, respectively. In comparison, there was less association be-

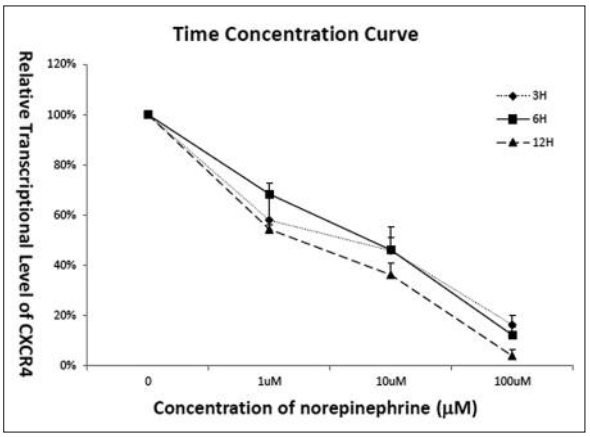

Figure 3. Time-concentration curve for the relative transcription of CXCR4 after incubation with various concentrations of norepinephrine for different durations. The decrease in CXCR4 transcription was less associated with incubation time than norepinephrine concentration.

tween treatment duration and *CXCR4* mRNA level. In the context of 1 μM norepinephrine, the *CXCR4* mRNA production after a 12-h treatment (45.8 ± 1.9%) was roughly equal to that for a 3-h treatment (42.1 ± 11.3%). In summary, the decreased *CXCR4* mRNA level after treatment with 100 μM norepinephrine ranged between 96.2 ± 2.7% and 83.8 ± 3.6%, 10 μM norepinephrine between 63.9 ± 4.8% and 54 ± 9.4%, and 1 μM norepinephrine between 45.8 ± 1.9% and 31.7 ± 4.5%. Finally, treatment with 10 μM norepinephrine for 12 h, conditions commonly used in previous studies, were designated as the experimental conditions for subsequent research.

Both Protein Production and Chemotactic Function of CXCR4 Were Attenuated By Norepinephrine in MDA-MB-231 Cells

Western blotting revealed that norepinephrine exerted a similar effect on the translational level of CXCR4 expression. A 12-h treatment with 10 μM norepinephrine resulted in a reduction in CXCR4 protein production of 44 ± 1.7% (p = 0.046) (Figures 4 and 5), slightly less than that for the transcriptional level.

Generally, a decrease in the production of a protein that plays a series of crucial roles will inevitably result in the weakening of the corresponding protein functions. Matrigel invasion assays were used to assess the invasiveness of MDA-MB-231 cells chemotactically towards CXCL12, an endogenous ligand of CXCR4. As expected, after a 12-h treatment with 10 μM norepinephrine there was a 51.3 ± 9.1% (p = 0.046) reduction in the number of MDA-MB-231 cells driven by CXCR4 to penetrate through Matrigel in Transwells to the bottom of the upper well (Figure 6).

The Specific Antagonist of the β2-adrenergic Receptor, ICI 118,551, Eliminated the Effects of Norepinephrine on CXCR4 in MDA-MB-231 Cells

β-adrenergic receptors mediate the principal biological effects of norepinephrine and comprise two major subtypes, the β1- and β2-adrenergic receptors. We treated MDA-MB-231 cells with a 10 μM concentration of the specific β1-adrenergic antagonist, atenolol, or β2-adrenergic antagonist, ICI 118,551, for 12 h. However, nei-

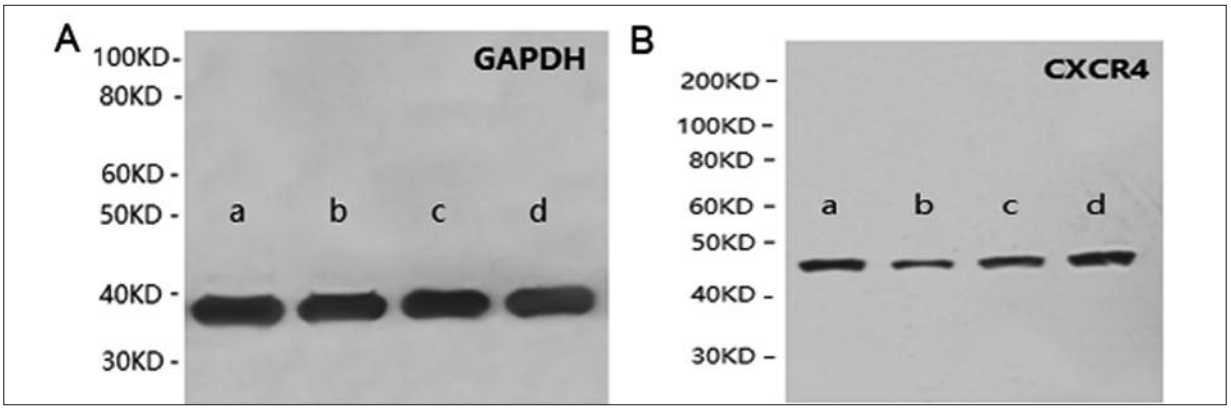

Figure 4. Immunoblotting was applied to assess translational production of the housekeeping protein, GAPDH ***(A)***, and CXCR4 ***(B)*** after treatment with various chemicals. "a" refers to the control group, "b" refers to the group in which MDA-MB-231 cells were treated with 10 μM norepinephrine for 12 h, "c" refers to the group in which the cells were treated with 10 μM ICI 118,551 for 12 h, and "d" refers to the group in which the cells were pretreated with 10 μM ICI 118,551, 45 min prior to 12-h treatment with equimolar norepinephrine.

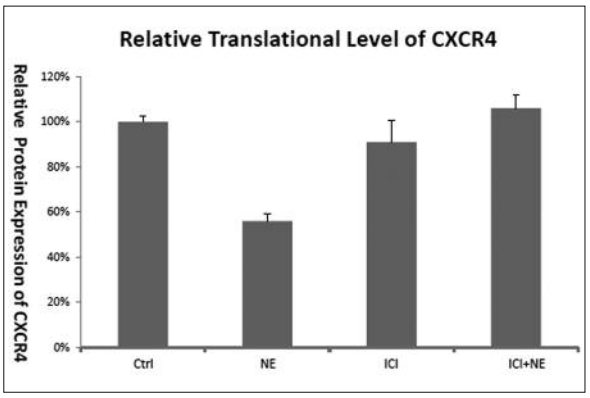

Figure 5. Relative translational expression of CXCR4 after treatment with various chemicals. "Ctrl" refers to the control group, "NE" refers to the group in which MDA-MB-231 cells were treated with 10 M norepinephrine for 12 h, "ICI" refers to the group in which the cells were treated with 10 μM ICI 118,551 for 12 h, and "ICI + NE" refers to the group in which the cells were pretreated with 10 μM ICI 118,551, 45 min prior to 12 h treatment with equimolar norepinephrine.

ther antagonist altered the *CXCR4* mRNA level; the relative level, compared to the untreated control, in the atenolol-treated group was 89.6 ± 11.4% (p = 0.513) and 105.1 ± 5.4% (p = 0.127) in the ICI 118,551-treated group. As mentioned above, norepinephrine treatment was able to dampen *CXCR4* transcription. We, therefore, pretreated MDA-MB-231 cells with one of the two specific β-adrenergic antagonists, 45 min prior to the addition of equimolar norepinephrine, to determine whether β-adrenergic receptors were involved in the norepinephrine-induced reduction of *CXCR4* transcription. The results revealed that, after pretreatment with the β1- or β2-adrenergic antagonist, the relative transcriptional production of *CXCR4* was 47.3 ± 4.8% (p = 0.037) or 94.4 ± 6.3% (p = 0.275), respectively; this suggests that atenolol had little effect on the reduction of *CXCR4* transcription, whereas ICI 118,551 almost completely eliminated the impact of norepinephrine on *CXCR4* transcription (Fig. 7). At the translational level, immunoblotting confirmed that the norepinephrine-induced decrease in CXCR4 production was also abolished by ICI 118,551, with a relative protein production of 106.3 ± 6.1% (p = 0.127) in the pretreatment group compared to controls (Figures 4 and 5).

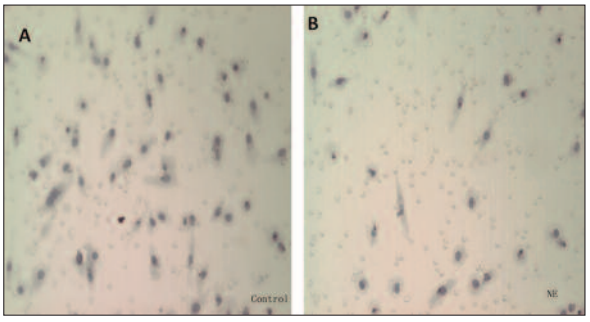

Figure 6. The Matrigel invasion assay was applied to assess the invasiveness of MDA-MB-231 cells chemotactically towards CXCL12 after a 12-h treatment in culture medium without any additive ***(A)*** or with 100 μM norepinephrine ***(B)***.

Discussion

Microarray analysis revealed that a considerable number of chemotactically relevant genes in MDA-MB-231 cells had altered expression in response to norepinephrine treatment, including 24 genes with ≥2-fold changes. There was a sharp decline for *CXCR4*, *CCL2*, *CXCL10,* and *CMKLR1,* while for *TREM1*, *CXCL16*, *IL8*, and *MMP7* it was significantly elevated. To our knowledge, this is the first study to apply microarray analysis to investigate the potential impact of norepinephrine on a superfamily of chemokines and their receptors, providing a great deal of information and many clues to further studies.

Chemokines and most neurotransmitters are ligands of GPCRs, which regulate most migration processes and, consequently, play an important role in tumor progression[16]. However, little attention has been given to possible crosstalk between these two families in the context of cancer. Several recent studies revealed that chemokines inhibit neurotransmitter release and the signal transduction of β-adrenergic receptors in neuronal and cardiac myocytes[42-46]. On the other hand, norepinephrine has been reported to have a significant effect on macrophage migration by altering the expression of the chemokine receptor, CCR2[47]. However, these experiments were not performed in tumor cells.

In our study, after treatment with norepinephrine, the transcriptional production of *CXCR4* mRNA was reduced by 95.7%. CXCR4 and its endogenous ligand, CXCL12 (SDF-1α), were first identified as a regulatory pair in lymphocyte trafficking to bone marrow[48]. Subsequently, the CXCL12–CXCR4 axis was demonstrated to regulate the trafficking of various tumor cells to

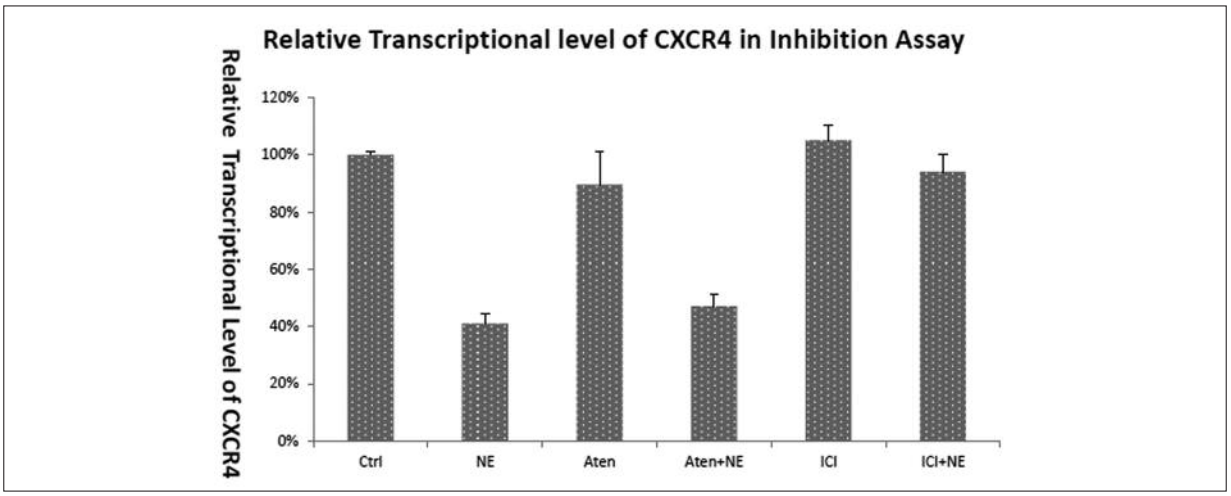

Figure 7. Real-time RT-PCR was applied to detect the relative transcriptional levels of CXCR4 in the inhibition assay. "Ctrl" refers to the control group, "NE" refers to the group in which MDA-MB-231 cells were treated with 10 μM norepinephrine for 12 h, "Aten" refers to the atenolol-treated group, "Aten + NE" refers to the group in which the cells were pretreated with 10 μM atenolol, 45 min prior to a 12-h treatment with equimolar norepinephrine, "ICI" refers to the ICI 118,551-treated group, and "ICI + NE" refers to the group in which the cells were pretreated with 10 μM ICI 118,551, 45 min prior to 12 h treatment with equimolar norepinephrine.

sites of metastasis[33,37,49-53]. It has been established that CXCR4 plays a central role in tumor cell invasion and dissemination in the majority of malignant diseases[34,35]. Therefore, the norepinephrine-induced decrease in *CXCR4* expression observed in breast cancer cells warrants further investigation. In addition, other chemotactically relevant genes implicated in tumor progression, such as *MMP7*, *CXCL10*, *CCR5*, *CCL25*, and *CCL2*, are also noteworthy[54-59].

Real-time RT-PCR confirmed the attenuation of *CXCR4* transcription in a dose-dependent manner, with a maximum decrease of 96.2 ± 2.7% after a 12-h treatment with 100 μM norepinephrine and a minimum reduction of 31.7 ± 4.5% after a 6-h treatment with 1 μM norepinephrine. There was no significant difference between different time points at set concentrations of norepinephrine, suggesting the attenuation was not correlated with the duration of treatment over relatively short periods (3-12 h). These results led to the decision to use a treatment regimen of 10 μM norepinephrine for 12 h for subsequent experiments, which was in agreement with previous studies[60-62].

As evidenced by immunoblotting assays, norepinephrine treatment dampened protein production of CXCR4 by a similar percentage as transcription of the *CXCR4* gene, validating the hypothesis of changes at the translational level. Subsequently, Matrigel invasion assays provided strong evidence of the functional regulation of CXCR4 by norepinephrine. As a crucial mediator in tumor progression, CXCR4 is modulated by a diverse array of tumor-relevant molecules[63-67], indicating it might be integral to the typical regulatory pathway of tumor metastasis. To our knowledge, this is the first account of evidence regarding norepinephrine regulation of CXCR4 expression in cancer cells.

As an endogenous ligand to adrenergic receptors, norepinephrine has a mixed affinity for both α- and β-adrenergic receptors. The principal biological effects of norepinephrine on tumor progression are mediated by β-adrenergic receptors[7,9,12-15,25,28,68,69]. The major subtypes of β-adrenergic receptors, β1 and β2, are present at many sites of tumor growth and metastasis. However, pharmacologic dissection of preclinical models of a variety of tumor types revealed that, unlike β1, the β2-adrenergic receptor plays a predominant role in the processes of tumor progression, such as the regulation of VEGF and MMPs[7,9,12-15,25,28,68,69]. Despite some divergence of opinion, the accumulating body of epidemiological research provides evidence in humans to support preclinical observations, which suggest that inhibiting β-, especially β2-, adrenergic signaling pathways could impede cancer progression and mortality[24,25,29,70-74]. Consistent with this, we found that in MDA-MB-231 cells, the specific β2-adrenergic antagonist, ICI 118,551, almost

completely eliminated the norepinephrine-induced negative effect on the transcriptional and translational production of CXCR4. In contrast, real-time RT-PCR revealed that the specific β1-adrenergic antagonist, atenolol, elicited little effect on the norepinephrine-induced decrease in CXCR4 expression. In summary, we demonstrated in this study that norepinephrine attenuated CXCR4 expression in MDA-MB-231 cells via the β2-adrenergic receptor.

The results observed in our study were unexpected. In fact, Gruber-Olipitz et al[75] reported that CXCR4 expression in the spleen of normal BALB/c mice was significantly increased after administration of norepinephrine. Therefore, we initially postulated that the enhanced aggressiveness in response to norepinephrine treatment reported in previous research might partially result from increased CXCR4 expression. Remarkably, our research findings led to the opposite conclusion, and were similar to the observation that norepinephrine inhibited macrophage migration by decreasing expression of the chemokine receptor, CCR2[47]. However, the conclusion that norepinephrine has a negative impact on CXCR4 expression should not be generalized to include other breast cancer cell lines or tumor types, or *in vivo* models. First, a noticeable variation in the expression levels and signaling activity of β-adrenergic receptors between breast cancer subtypes has been observed in preclinical studies[25,76]. Second, compared to the other three subtypes, MDA-MB-231 cells represent a "triple-negative" phenotype and exhibit much higher expression of β-adrenergic receptors[76]. In addition, the estrogen receptors expressed in other subtypes might modulate the responses to β-adrenergic stimulation[24].

The discrepancies between previously reported studies might provide us with some possible explanations for our unexpected data. Classically, ligation of β-adrenergic receptors by norepinephrine stimulates adenylyl cyclase-catalyzed synthesis of cyclic AMP (cAMP), and the subsequent transient cAMP flux regulates a diverse array of cellular processes via two major downstream effectors, protein kinase A (PKA) and exchange protein directly activated by cAMP[77]. Madden et al[76] reported that β2-adrenergic stimulation regulated VEGF production through the classical β-adrenergic receptor-cAMP-PKA pathway, whereas this pathway could elicit directionally opposite outcomes. Norepinephrine and a specific β2-adrenergic agonist, terbutaline, reduced VEGF production in MDA-MB-231 cells, but enhanced VEGF production in a variant cell line, MDA-MB-231BR, despite similar β2-adrenergic receptor densities. Further investigation revealed that an impaired feedback mechanism prolonged the duration and increased the amplitude of β-adrenergic receptor-induced cAMP flux in MDA-MB-231 cells. Meanwhile, the downstream mediator, PKA, was able to phosphorylate multiple substrates with the capacity to either facilitate or inhibit VEGF production. Eventually, excessively elevated cAMP resulted in an unexpected inhibitory regulation of VEGF expression in MDA-MB-231 cells. In contrast, the variant MDA-MB-231BR cells seemed to successfully repair the feedback abnormality. Another interesting phenomenon occurred in pancreatic cancer cells, which exhibited reduced migratory activity upon norepinephrine treatment[61]. A possible explanation was that the pancreatic cancer cells possessed unusually high spontaneous migratory activity, and the constitutive activation of the phospholipase C-γ pathway led to no further response of this pathway to norepinephrine. Finally, the imbalance between the phospholipase C-γ and cAMP pathways elicited an inhibitory effect. Taking these data together, the heterogeneity in β-adrenergic receptor signaling capacity might contribute to the norepinephrine-induced decrease in CXCR4 expression observed in MDA-MB-231 cells.

Conclusions

We found that norepinephrine attenuated CXCR4 expression and the corresponding invasion of MDA-MB-231 cells via the β2-adrenergic receptor. Our study adds to the accumulation of conflicting observations in this field, suggesting that further evidence is urgently needed before the clinical application of β-adrenergic receptor inhibitors in patients with breast cancer, due to the complexity of the somatic impact of norepinephrine and chronic stress. Both norepinephrine and CXCR4 play an important role in tumor progression; therefore, the intracellular signaling pathway involved in the norepinephrine-induced decrease in CXCR4 expression observed in MDA-MB-231 cells deserves further investigation.

Notably, binding to the I B protein of β-arrestin 2, the latter being involved in the β2-adrenergic receptor signaling pathway, was reported to

result in the inhibition of nuclear factor B activation, which has been demonstrated to attenuate CXCR4 production in breast cancer cells[78-80]. In future studies, our group aims to explore this potentially relevant pathway and extend our experiments to other breast cancer cell lines and tumor types, and to *in vivo* models. Furthermore, a considerable number of intriguing changes in the expression of other chemotactically relevant genes induced by norepinephrine are worthy of attention. For instance, the observations that the level of MMP-7, with the capacity to facilitate tumor invasion, was elevated in response to norepinephrine treatment and that of CXCL10, competent to inhibit neovascularization, was decreased requires confirmation[54,55].

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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