The effect of the Substance P/NK1R system on thioredoxin and its target gene, miR-325-3p, in MCF-7 breast cancer cells

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Research Article

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Abstract

Purpose

Breast cancer (BC) is the most frequent malignancy with a high morbidity and mortality rate among women that can be initiated and be progressed through activating the neurokinin-1 receptor (NK1R) by substance P (SP), a highly conserved member of the tachykinin peptide family. The oxidative stress-mediated role of the SP/NK1R system results in BC pathogenesis is not entirely understood. Therefore, this study was designed to shed light on the link between SP/NK1R and cellular redox state in MCF-7 breast cancer cells.

Methods

Aprepitant IC50 was measured by resazurin assay. Reactive oxygen species were assessed utilizing DCFDA assay. Thioredoxin (Txn) and miR-325-3p genes expression were determined through Real-Time PCR. To evaluate the Txn protein expression, western blot analysis was performed.

Results

We found that SP elevated ROS production in these cells. furthermore, SP leads to a remarkable down-regulation of miR-325-3p and thioredoxin (Trx) target genes and protein expression of Trx in MCF-7 cells. In addition, aprepitant inhibited SP's effects; therefore, it decreased ROS accumulation, and up-regulated Trx and miR-325-3p genes, suggesting that aprepitant may render antioxidant properties through Trx.

Conclusion

Oxidative stress could have an essential role in BC pathogenesis via activating the NK1R by SP. SP can decrease the BC cell's antioxidative capacity by reducing the Trx gene and protein and miR-325-3p gene. Therefore, it causes an increase in ROS production and oxidative damage. the present investigation indicates that the SP/NK1R system might be an appealing and promising therapeutic target against BC.

1. Introduction

Breast cancer (BC) is the most frequently occurring cancer and the second cause of cancer-related death in women. The incidence of BC continues to rise by approximately 0.5% annually (1). Increasing evidence revealed that levels of aberrant ROS (reactive oxygen species) could mediate harmful cellular influences (2). Exceeding the generation of ROS, even more than the ability of the antioxidant defense system to eliminate them, can lead to Oxidative stress (3). An elevated level of ROS plays a crucial role in the initiation, promotion, and progression of cancers, including BC, through damaging cellular components,
including DNA, proteins, and lipids (4, 5). Antioxidant defense systems in the cells are responsible for controlling the excessive accumulation of ROS (6).

Moreover, the cancer cells adapt to their inherently elevated oxidative stress by activating these antioxidant defense systems (7). The Trx system is recognized as a critical modulator of multiple pathological conditions (8). The malfunction of this system is associated with the development and progression of tumor cells (9). In addition, Trx is a multifunctional protein involved in the regulation, modulation, and maintenance of the cellular redox balance (10). Trx and Trx reductase (TrxR) are essential components of the Trx system, which uses NADPH as the source of reducing equivalents to perform the protein-disulfide reduction. This system provides the electrons to thiol-dependent peroxidases to attenuate ROS and NOS-mediated damage with a fast reaction rate (11).

Recently, convincing evidence has introduced the role of the tachykinin family and their receptors as an essential player in the cancer initiation and progression by modifying the cellular redox state (12–14). Substance P (SP) is one of the primary Tachykinins (TKs) (15, 16), which plays an essential part in diverse pathophysiological processes by activation of its receptor, neurokinin 1 receptor (NK1R) (17, 18). Increased levels of ROS and decreased antioxidant defense efficiency affected by SP were recorded in several studies (12); therefore, SP can modulate the cell's redox equilibrium. The definite mechanism of NK1R and its ligand in redox equilibrium, along with antioxidant defense enzymes, has not been described adequately. Here, we assessed the effect of the SP/NK1R system and its selective antagonist, aprepitant, on the alteration of the ROS level and one of the significant regulating cellular redox in the body, the Trx system.

2. Materials And Methods

2.1. Cell culture and reagents

MCF-7 human BC cell line was provided by the national cell bank, Pasteur institute (Iran, Tehran). Examinations were conducted when the growth of cells was in the logarithmic changes with approximately 0.5-1 × 10^6 cells/ml to assess the effects of SP/NK1R. The cells were cultured in the DMEM medium, which was high glucose supplemented with 10% FBS (Fetal Bovine Serum) and 1% antibiotics (penicillin and streptomycin) (all products were from Cegrogen Biotech GmbH, Germany). Cultured MCF-7 cells were preserved under suitable moisture and in a 37°C incubator comprising 5% CO₂.

SP was purchased from Abcam (Abcam, USA), and aprepitant was acquired from Sigma-Aldrich (St. Louis, MO, USA). The earliest, 70 µM and 74 mM concentration stock solutions of aprepitant and SP, were composed sequentially by solvating the compounds in 0.1% sterilized DMSO (dimethyl sulfoxide, Carlo Erba, Paris). These mixtures were segregated into aliquoted and retained frozen at −20°C.

2.2. Resazurin Cell viability assay
The reduction of the resazurin into a pink fluorescent resorufin final product is a property of live, metabolically active cells (19). The resazurin cell viability assessment was performed to quantify the IC50 aprepitant on the MCF-7 cell line. At the first step in a 96-well plate, $2.5 \times 10^4$ cells were cultivated in a total volume of 100 µL/cavity. Then, every cavity was treated by divers aprepitant concentrations of 0 (control), 5, 10, 20, 30, 40, 50, 60 µM and incubated for 24 hours. After that, each cavity was homogenized in a 10 µL solution of resazurin (solvated 0.01 mg/mL of resazurin in phosphate-buffered saline; Sigma-Aldrich). The cells were incubated for 3 hours in the dark at 37°C, under 5% CO2. Afterward, a colorimetric method using a microplate fluorimeter was accomplished to measure the fluorescence intensity under 600/570 nm (Excitation /Emission). Measured absorptions were converted to survival percentage rates by correlating treated cells’ absorbance regarded untreated control cells.

2.3. ROS content evaluation

2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) examination was applied to assess the ROS values. After passing the H2DCFDA through the cell membrane, cellular esterase cleaves it to produce non-fluorescent 2',7'-dichlorodihydrofluorescein (H2DCF). H2DCF oxidation by ROS eventually leads to produce 2',7'-dichlorofluorescein (DCF), which is significantly fluorescent. Briefly, $75 \times 10^4$ cells were cultured in the 6-well microtiter plate and then incubated at 37°C for 24 hours. Afterward, incubation was performed at 37°C for 45 minutes with 10 µM DCFHDA before treatment. Subsequently, to treatment, the MCF-7 BC cells were exposed to 100 nM and 400 nM concentrations of SP, solely or combined with 15 µM of aprepitant for 24 hours. TBHP (Tertbutyl hydrogen peroxide; Abcam, USA) was employed as the positive control. The fluorescence signal of DCF was evaluated using the Perkin-Elmer spectrophotometer (Norwalk, CT, USA) at the 495/529 nm wavelengths.

2.4. Total RNA extraction, cDNA synthesis, and thioredoxin qRT-PCR (quantitative real-time PCR)

The extraction of total RNA was exerted by an RNA extraction kit (Favorgene Biotech, Taiwan) conferring to the producer's instruction. Total RNA concentration and pureness were determined using a NanoDrop 1000™ spectrophotometer (USA). After that, RNA specimens were maintained at −80°C.

Total RNA was transcripted reversely to synthesize cDNA. Instruction was prosecuted with commercially available Iranian cDNA synthesis equipment (Pars Tous biotechnology, Iran). cDNA synthesis was performed applying Bio-Rad Thermal cycler (MyCycler, USA). Non-ROX Ampliqon SYBR Green supermix (Denmark) was utilized to accomplish qRT-PCR amplification in the Roche LightCycler 96 (Roche Diagnostics GmbH, Germany). The Ct comparative formula ($2^{−ΔΔCt}$) was applied to analyze the Trx gene’s expression compared with the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.5. mir-325-3p cDNA synthesis and qRT-PCR

According to the instruction, the reverse transcription of isolated total RNA to synthesize specific cDNA for miRNA was performed by BONmiR, Iranian commercial cDNA synthesis equipment (Stem Cell
technology, Tehran). The Bio-Rad Thermal cycler (MyCycler, USA) was applied for cDNA synthesis. Non-ROX SYBR Green master mix (Stem Cell technology, Tehran, Iran) was employed to perform qRT-PCR amplification in the Roche LightCycler 96 (Roche Diagnostics GmbH, Germany). For comparative miR-325-3p gene expression analysis, an internal control and housekeeping gene, SNORD47, was applied. Then gene expression value was assessed by the comparative formula \(2^{-\Delta\Delta Ct}\).

### 2.6. Western blot analysis

After the MCF-7 cell treatment with SP400 nM separately and combined with aprepitant (15 µM) for 24 hours, the supernatant culture medium was discarded, and the cavities were washed by chilled PBS. The cell lysate was collected after exposing the MCF-7 cells to chilled RIPA lysis buffer solution. Purified total protein concentration was quantified via the BCA kit (Pars Tous Biotechnology, Iran). Equivalent measures of protein were loaded on 15% SDS-PAGE. The separated proteins were electro-transferred to the German PVDF membrane (Roche Diagnostics GmbH). The blocking of the membrane was completed by overnight incubating in 5% non-fat skim milk at 4°C. Afterward, membrane incubation with monoclonal anti-Trx antibodies (1:500; Abnova, Taiwan) for four hours, three times soaking with PBS buffer, exposing to secondary antibody conjugated with HRP (1:2500; Abnova, Taiwan) for one hour, were performed at room temperature (RT), respectively. The membrane was rinsed four times with PBS, ultimately exposed to an Iranian commercial chemiluminescence detection equipment (Pars Tous Biotechnology), and incubated for one minute to strengthen the chemiluminescence emission (ECL). The intensity of detected Trx bands was assessed by comparing the β-actin reference protein.

### 2.7. Analytical assessment

All consequences were obtained from three independent experiments and exhibited as mean±SD (standard deviation). The analytical assessment was accomplished in GraphPad Prism® 7.0 (San Diego, CA, USA), applying the ANOVA test following Bonferroni’s post hoc t-test to compare different groups. The results with a P-value less than 0.05 are considered statistically significant.

### 3. Results

#### 3.1. Aprepitant decreased the cell viability of human breast cancer cells

The effect of gradually increasing aprepitant concentrations (5–60 µM) resulting from the cell viability resazurin test over 24 hours was manifested briefly in Fig. 1. According to Fig. 1, the viability of MCF-7 cells was diminished through the influence of aprepitant in a dose-dependent manner. Compared with untreated control cells, the 60 µM concentration of aprepitant significantly decreased the cell viability to approximately 25%. As the result of the resazurin assay, exposing the MCF-7 cells to the aprepitant with an evaluated IC50 quantity of 35.20 µM caused a distinct reduction in the cell’s viability. Half of the IC50 value, 15 µM concentration of aprepitant was conclusively taken to treat the MCF-7 cells for the following experiments.
3.2. Substance P elevated cellular ROS

To assess the influence of SP/NK1R on ROS production in MCF-7 cells, we applied the DCFH-DA fluorescent probe. As illustrated in Fig. 2, the DCF-positive measures of cells are an indicator of the high production of ROS in MCF-7 cells, which were significantly increased in the presence of exogenous SP400 nM. Although ROS generation levels were elevated in the MCF-7 cells treated with SP100 nM, it was not statistically remarkable. As shown in Fig. 2, ROS production diminished in exposure to aprepitant 15 µM, alone or combined with SP100 and SP400 nM for 24 hours. Moreover, aprepitant could cause a remarkable decline in the ROS of MCF-7 cells.

3.3. Substance P decreased the thioredoxin mRNA and miR-325-3p expression

To elucidate the pro-oxidative-related performance of SP/NK1R in MCF-7 cells, we assessed the Trx mRNA and miR-325-3p expression levels in response to both SP100 nM and SP400 nM, separately or in compound with apreptan at 15 µM concentration via realtime-PCR. As shown in Fig. 3a, the effect of 100 nM concentration of SP on the down-regulation of Trx mRNA was not remarkable; however, it is significantly down-regulated in the group treated with the 400 nM concentration of SP. At the same time, Trx mRNA level expression was considerably increased after 15 µM aprepitant treatment, separately or in a compound with SP100 nM or SP400 nM.

The result of miR-325-3p real-time-PCR shown in Fig. 3b implies that although the microRNA expression was elevated in the low concentration of SP (100 nM), it was diminished in a higher concentration of SP (400 nM). Besides that, as we observed, the miR-325-3p expression was considerably promoted in the cell treatment with aprepitant 15 µM separately or in combination with both SP100 nM and SP400 nM.

3.4. Substance P decreased the thioredoxin protein expression level

To evaluate the contribution of Trx protein associated with its anti-oxidative activity by SP/NK1R performance, the protein expression of Trx was assessed after MCF-7 cell treatment with aprepitant 15 µM independently or in the compound with SP400 nM by western blot. As shown in Fig. 4, Trx expression was considerably decreased in the treated group by SP400 nM. In contrast, it was raised in the treated group by the combination of SP400 nM and aprepitant 15 µM. Furthermore, we found that Trx expression was remarkably increased in the group treated by the aprepitant compared to the untreated control.

4. Discussion

Excessive production of ROS causes oxidative stress and damage to various cellular elements, like lipids, enzymes, proteins, cell membranes, and DNA in the living cells. It eventually promotes tumor development and progression by intervening in crucial cellular procedures involving apoptosis, proliferation, migration, and resistance (20). According to our results, exogenic SP, the natural ligand for the NK1 receptor, induced
ROS production in breast cancer cells, suggesting the SP/NK1R signaling is linked to BC pathogenesis by oxidative stress. In line with our results, some investigations reported that ROS production elevated in response to SP in the immune-inflammatory cells (21, 22), sensory neurons (23), and epithelial cells of the gastric and respiratory tract (24, 25). For example, SP-activated NK1R enhances the hemorrhagic lesion's development of the stomach barrier via increasing the cytotoxic ROS generation in the gastric epithelial cells (25). SP operates the neutrophil NADPH oxidase enzyme, one of the primary origins of intracellular ROS, related to NK1R to produce cytotoxic ROS, resulting in the hyperactive bladder (26–28).

Many investigations have been concentrated on the antitumoral influences of aprepitant on various cancer cells (29, 30). Our findings indicated that aprepitant exerts antitumoral effects, at least partly, by reversing the SP induced-ROS production.

In line with our results, a study has suggested that aprepitant could reduce cisplatin-induced ROS generation in PC12 of BC cells treated with aprepitant (15 µM) just 2 hours before cisplatin treatment (31). Another study that evaluated the aprepitant effect on ROS production proposed that the administration of aprepitant (35 µM) in U87 glioblastoma cells can significantly reduce ROS levels after 24 hours (12). In a similar investigation on U87 glioblastoma cells, ROS level remarkably was reduced as an effect of aprepitant (15 µM) treatment for 24 hours (32).

In contrast with these findings, there are some investigations reported that aprepitant positively regulates ROS production in K562 and HL60 myeloid leukemia tumor cells, treated with aprepitant (24 µM and 19µM, sequentially) for 3 hours (33) and TNBC (triple-negative BC) cell line, MDA-MB 231, pretreated with aprepitant for 2 hours (34). It seems aprepitant probably has the dual effect of regulating ROS production in tumor cells considering applied dose and exposure time. In addition, this contrast might be the differences in SP and aprepitant dosages used, oxidative capacity of the cancer cells, time of exposure to drugs, and the diverse redox conducting signaling pathways used via various cell types. These results, which accompany our previous findings, suggest that aprepitant might be able to promote antitumoral effects through suppressing ROS production (35). Although these studies have distinguished the role of SP on ROS generation, further verification in further studies is indispensable to clarify the exact molecular mechanism implied in the effects of SP in ROS induction in BC cells.

To evaluate the probable SP and aprepitant effects on cellular redox state, we tried to determine the gene and protein expression of Trx, one of the essential components of the enzymatic antioxidant defense system, upon aprepitant exposure of MCF-7 cells. Our findings revealed that the NK1R repression by aprepitant induced the gene and protein expression of Trx. In a study that operated on the U87 glioblastoma, similar to our findings, SP inhibited the expression of the Trx gene, and conversely, the aprepitant induced Trx gene expression significantly (32).

miRNAs participate in conducting and regulating their related target genes expression and play a crucial part in the occurrence and progression of several cancers (36–38). The microRNAs prediction database, TargetScan 7.2, anticipated that miR-325-3p could straight target Trx. MiR-325-3p is reported to promote cancer cell proliferation, metastasis, and EMT in vitro (39). For intense, it has been shown that an
increased miR-325-3p gene expression is linked to inhibition of invasion and proliferation of non-small cell lung cancer by differently targeting (39, 40). It is also reported miR-325-3p overexpression inhibits glioma (41) and bladder cancer cells growth and metastasis (42).

Moreover, an investigation on hepatocellular carcinoma proved that miR-325-3p can prevent cell proliferation and lead to cell apoptosis induction (43). Recently, studies revealed that miR-325-3p increases in cancer, such as BC tissues and cell lines (44). To estimate the possible effect of SP/NK1R on miR-325-3p, we assessed its expression and found that miR-325-3p gene expression was elevated in the MCF-7 cell line. We also found that the miR-325-3p gene expression was following the Trx expression. In the current study, we observed that SP/NK1R could have a dual manner on the expression of miR-325-3p.

The current study explored the miR-325-3p and its target gene, Trx relation, and the SP and aprepitant effects on them in the BC for the first time. We found that the NK1R antagonism, aprepitant, remarkably prevents the Trx system and miR-325-3p alteration mediated by SP.

5. Conclusion

It is concluded that there is a crosstalk between BC initiation and progression and oxidative stress through NK1R signaling by SP. NK1R blockage by aprepitant leads to sufficiently diminished oxidative stress in BC, proposing the therapeutic importance of aprepitant in stress-related tumors, including BC. Nevertheless, further investigations of both in vivo and in vitro should be accomplished to clarify the influence of SP/NK1R signal transduction and their therapeutic importance related to modifications of redox states in BC.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated during and/or analysed during the current study are available if requested.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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**Authors' contributions**

Writing the original draft, writing review editing, software, validation, formal analysis, investigation, and visualization were performed by Amin Alaei. Writing review editing, data curation were performed by Arash Soltani. Resources, funding acquisition, and supervision were provided by Naser Mobarra. Conceptualization, methodology, project administration, and supervision were performed by Seyed Isaac Hashemy.

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**Compliance of interests**

The authors revealed there are no conflicts of interest related to the publication of the presented paper.

**References**


Figures
Aprepitant gave rise to growth prevention and decreased viability in MCF-7 cells after 24 hours. Aprepitant applies preventive influences on cell viability in a dose-dependent manner. The Aprepitant IC50 value in the MCF-7 cells was approximately 35.20 μM. Data from three independent experiments were presented as mean ± standard deviation (SD).
SP/NK1R and aprepitant effects on intracellular ROS generation in the MCF-7 cell line. The cells were treated with exogenic SP (100 and 400 nM) and aprepitant (15 μM) separately or in combination with SP (100 and 400 nM) for 24 hr. The results indicated that SP/NK1R elevated oxidative stress via promoting intracellular ROS. Coincidently, aprepitant (15 μM) separately or in combination reduced the ROS levels. (*P< 0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control; #P<0.05; ##P<0.01; ###P<0.001)
Figure 3

Results of the Trx and miR-325-3p target genes expression in the MCF-7 cells treated with both SP100 nM and SP400 nM separately or combined with aprepitant (15 μM) for 24 hours. a. The level of Trx expression evaluated in every group treatment after the Ct (cycle threshold) values normalized compared to the GAPDH housekeeping gene as an internal control. Our findings indicated that treatment by the aprepitant induces the Trx expression significantly. b. The miR-325-3p gene expression of each group was
evaluated after Ct normalizing to SNORD47 housekeeping gene as an internal control. Our consequences manifested that exposure to aprepitant promotes the miR-325-3p gene expression considerably. The data are acquired as the mean ± SD of two independent times examinations in both figures (P<0.05) (*P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control; #P<0.05; ##P<0.01; ###P<0.001).

Figure 4
The pharmacological influence of aprepitant on the Trx protein expression. After cells treatment by SP (400 nM) separately or combined with aprepitant (15 μM), the total cytoplasmic protein extraction was performed, and a western-blot examination was operated applying directed anti-Trx and anti-β-actin antibodies. The protein expression results were gained from two independent times examinations. Bands' severity was assessed using ImageJ software and compared. The western blot analysis demonstrated that the expression of the antioxidative Trx protein was significantly decreased in the cell group treated with SP (400 nM) compared to the β-actin control groups. (P<0.05)