The role of sodium hydrosulfide in the proliferation and apoptosis of exogenous SB203580 pre-treated human ovarian cancer cells

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Summary

Purpose: Explore the regulatory function of sodium hydrosulfide (NaHS) on the proliferation and apoptosis of exogenous SB203580 pre-treated human ovarian cancer SKOV3 cells. Methods: SKOV3 cells cultured in vitro were assigned to the control group, NaHS group, SB203580 group, and NaHS combined with SB203580 group. The effects and influences on cell proliferation were detected by MTT assay. Effects on the cell cycle and apoptosis were detected by flow cytometry. Western blot was used to detect the expression of P-p38MAPK. Results: Proliferation of SKOV3 cells was remarkably decreased in the NaHS, SB203580, and NaHS combined with SB203580 groups. Cell quantity in G1 phase and S phase increased and decreased remarkably, respectively. Obviously, cell apoptosis rate was increased in the combination group compared to single-drug treatment groups. P-p38MAPK expression in NaHS group was upregulated by comparing with that in control group, and that was markedly enhanced in the combination group compared to single-drug treatment group. Conclusions: NaHS inhibited proliferation and promoted apoptosis of SKOV3 cells by blocking the p38MAPK signaling pathway. Furthermore, coordination between NaHS and SB203580 regulated the proliferation and apoptosis of SKOV3 cells. Key words: Human ovarian cancer cells; Sodium hydrosulfide; p-P38MAPK; Cell proliferation; Cell apoptosis.

Introduction

Ovarian carcinoma causing death is one of female malignant cancers, posing a serious threat to female patients’ lives [1-3]. Ovarian cancer is usually diagnosed at a late stage and remains the most lethal gynecological malignancy because its development process is asymptomatic [4,5]. Therefore, the discovery of drugs or factors that can inhibit ovarian cancer cells’ proliferation and development has immense importance for the clinical treatment of ovarian carcinoma.

NaHS is the donor of Hydrogen sulfide (H₂S), which is broadly distributed in a variety of tissues including the cardiovascular system, internal organs, and nervous system [6-9]. Previous studies have shown that endogenous and exogenous H₂S could inhibit cancer cell proliferation and promote its apoptosis [10-12], but the mechanism of action remains unclear. SB203580, a pyridinyl imidazole (Figure 1), is a selective and ATP-competitive p38 mitogen-activated protein kinase (p38MAPK) inhibitor, which has been extensively used for assessing the role of p38MAPK in all kinds of biological systems [13,14]. Some reports have demonstrated that SB203580 has antiproliferative activity [15]. p38MAPK is an intracellular signal-transducing molecule, playing a key role during various biological processes. A growing number of evidence demonstrate that activation of the p38MAPK signaling pathway might facilitate cell proliferation and inhibit cell apoptosis [16-18].

During our experiments, we did research to explore whether NaHS will produce an influence on proliferation and apoptosis of SB203580 pre-treated human ovarian cancer, and at the same time, we examined the role of the p38MAPK signaling pathway for the sake of providing new directions for the clinical treatment of ovarian carcinoma.

Materials and Methods

Chemical reagents

SKOV3 human ovarian cancer cell line (purchased from Cell Bank of the Chinese Academy of Sciences). The human ovarian cancer SKOV3 cells were cultured in RPMI-1640 media containing 10% FBS, and incubated at 5% CO₂, 37 °C and saturated humidity. Cells were digested using 0.25% trypsin (includes 0.04% digested cells) and passaged at a ratio of 1:4 at 80-90% confluency. Cells were used once the passaging cycles became steady. PBS was obtained from Sangon Biotech (Shanghai, China). Propidium iodide, NaHS and blue tetrazolium MTT were purchased from Sigma. SB203580 was obtained from Merck (Germany). RPMI-1640 culture media and AnnexinV/PI (propidium iodide) double staining kit were obtained from Invitrogen. Mycoplasma-free FBS was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. Trypsin was purchased from Gibco. RNase was obtained from Sangon.
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Biotech. Experimental groups.

Table 1. — Effects on proliferation of human ovarian cancer cells after 48 h different treatments by MTT detection

<table>
<thead>
<tr>
<th>Group</th>
<th>A_{450}</th>
<th>IR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8423 ± 0.0269</td>
<td>0</td>
</tr>
<tr>
<td>NaHS (10^{-3}mol/L)</td>
<td>0.5073 ± 0.0460</td>
<td>▲ 39.77</td>
</tr>
<tr>
<td>SB203580 (0.1mol/L)</td>
<td>0.5357 ± 0.0389</td>
<td>▲□ 36.4</td>
</tr>
<tr>
<td>NaHS + SB203580</td>
<td>0.2953 ± 0.0358</td>
<td>▲■♦ 64.94</td>
</tr>
</tbody>
</table>

F value: 432.655

p value: < 0.01

▲ Difference was statistically significant by comparing with the control, p < 0.01; □ Difference was not statistically significant by comparing with NaHS group, p > 0.05.

■ Difference was statistically significant by comparing with NaHS group, p < 0.01; ● Difference was statistically significant by comparing with SB203580 group, p < 0.01.

Figure 1. — Chemical structure of SB203580

Cell culture

Human ovarian cancer SKOV3 cells in exponential phase were collected, counted, and plated in a petri dish. Once cells fully adhered, cell culture media was removed and cells were synchronized in serum-free RPMI-1640 for 24 h and assigned to the normal control, NaHS (10^{-3} mol/L), SB203580 (0.1 mol/L), and NaHS combined with SB203580 groups. An equal volume of RPMI-1640 culture media containing 10% FBS was added into the control group, and all treated groups were incubated under 5% CO2, 37 °C, and saturated humidity for 48 h.

MTT assay

Collect Human ovarian cancer SKOV3 cells in exponential phase and seed into a 96-well plate at 5 × 10^4 cells/mL, 200 μL/well. Once fully adhered, cells were synchronized in serum-free RPMI-1640 for 24 h, followed by 48 h of intervention according to their assigned groups (each group in triplicate). After adding 20 μL 0.5 mg/mL MTT 4 h before the end of treatment, cells were continued to be cultured for 4 h in the dark, after which removing supernatant and adding DMSO at 150 μL/well were executed. The plate was shaken in the darkness for 10 min at room temperature in order to dissolve the DMSO crystals and the absorbance values (A490 nm) were determined using a microplate reader. The experiment was repeated 3 times, each of which included a cell-free blank control for normalization. Cell inhibition rate was calculated as: Inhibition rate (IR) = (A_{control} - A_{treatment})/A_{control} × 100%.

Cell cycle analysis

Collect SKOV3 cells in exponential phase and seed them into 6-well plates at 5 × 10^5 cells/mL, 2 mL/well. Once fully adhered, cells were synchronized in serum-free RPMI-1640 for 24 h, followed by 48 h of intervention culture according to their assigned groups (each group in triplicate). Cells were digested and collected after 48 h, and then resuspended thoroughly in pre-chilled 70% ethanol and fixed at 4°C overnight. Ethanol was eliminated by centrifugation. When finishing washing cells with PBS for 3 times, cells were resuspended in 500 μL PBS, followed by the addition of RNase A to a ultimate concentration of 250 μg/mL. Incubating cells at 37°C for 30 min, and adding 5 μL propidium iodide dye were carried out, after which cells were incubated for another 30 min at 37 °C in the dark before detection by flow cytometer. Experiment was repeated 3 times, proliferation index (PI) being calculated through equation: PI = (S + G2 / M) / ( G0 / G1 + S + G2 / M).

Cell apoptosis analysis

Collect SKOV3 cells in exponential phase and seed them into 6-well at 5 × 10^5 cells/mL, 2 mL/well. Once fully ad-
Table 2. — Effects on cell cycle of human ovarian cancer SKOV3 cells after 48 h treatment by flow cytometry (%)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Cell cycle phase distribution</th>
<th>G_0/G_1 phase (±)</th>
<th>S phase (±)</th>
<th>G_2/M phase (±)</th>
<th>PI (%) (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>75.64 ± 1.46</td>
<td>20.27 ± 1.34</td>
<td>5.40 ± 2.46</td>
<td>25.67 ± 1.46</td>
</tr>
<tr>
<td>NaHS(10^{-3}mol/L)</td>
<td></td>
<td>81.71 ± 1.60</td>
<td>14.86 ± 1.06</td>
<td>3.69 ± 1.63</td>
<td>18.55 ± 1.60</td>
</tr>
<tr>
<td>SB203580 (0.1mol/L)</td>
<td></td>
<td>80.77 ± 0.42</td>
<td>14.18 ± 1.02</td>
<td>4.79 ± 1.71</td>
<td>18.97 ± 0.42</td>
</tr>
<tr>
<td>Combined NaHS and SB203580</td>
<td></td>
<td>89.40 ± 1.96</td>
<td>7.57 ± 2.58</td>
<td>3.03 ± 1.9</td>
<td>10.60 ± 1.96</td>
</tr>
<tr>
<td>F value</td>
<td></td>
<td>88.997</td>
<td>61.054</td>
<td>1.778</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>

▲ Difference was statistically significant by comparing with control, p < 0.01; □ Difference was not statistically significant by comparing with NaHS group, p > 0.05.
■ Difference was statistically significant by comparing with NaHS group, p < 0.01; ♦ Difference was statistically significant by comparing with SB203580 group, p < 0.01.

Figure 3. — Cell cycle of human ovarian cancer SKOV3 cells after 48 h treatment by flow cytometry (FL2, fluorescent channel 2; PI, propidium iodide).
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Figure 4. — Cell apoptosis rate of human ovarian cancer SKOV3 cells after 48 h treatment by flow cytometry

Figure 5. — The expression of P-p38 MAPK on SKOV3 cells after 48 h treatment. (1: NaHS (50 µmol/L) group. 2: SB203580 (75 µmol/L) group. 3: SB203580 (75 µmol/L) group. 4: NaHS+SB203580 group).

Figure 5. — The expression of P-p38 MAPK on SKOV3 cells after 48 h treatment. (1: NaHS (50 µmol/L) group. 2: SB203580 (75 µmol/L) group. 3: SB203580 (75 µmol/L) group. 4: NaHS+SB203580 group).

Western blot assay

Collect SKOV3 cells in exponential phase and seed them into 6-well at 5 × 10^5 cells/mL. After fully adhered, cells were synchronized in serum-free RPMI-1640 for 24 h, followed by 48 h of intervention according to their assigned groups (each group in triplicate). The uniform protein concentration was 20 g/L. Running SDS PAGE for equal protein extracts from each group was carried out, and then protein was transferred to a polyvinylidene fluoride membrane, followed by blocking with 5% skim milk. The immune reactions were detected with the anti-P-p38 MAPK (43KD) and anti-β-actin (43KD) primary antibodies.
by blocking with 5% BSA for 1 h under room temperature. Thereafter incubating membranes with primary antibody (1: 1000) in 4°C refrigerator overnight was executed. After washing in TBST, incubating the membranes with the horseradish peroxidase-labeled secondary antibody (1: 30000) for 2 h under room temperature was implemented. The protein bands of interest were visualized by ECL and analyzed by a gel imaging analysis.

Statistical analysis

Measurements are expressed as. ANOVA and LSD-t test were used for comparisons between groups. Analysis was carried out by using the SPSS 17.0 statistical analysis software and when $p < 0.05$, it was considered to be statistically significant.

Results

Effects on proliferation of human ovarian cancer cells after 48 h treatment by MTT detection

Compared to the control group, human ovarian cancer cells, proliferation was significantly inhibited in the NaHS ($10^{-3}$ mol/L), SB203580 (100 µmol/L), and NaHS combined with SB203580 groups ($p < 0.01$). However, compared to the NaHS ($10^{-3}$ mol/L) group, no significant enhancing or inhibitory effects on human ovarian cancer cells’ proliferation was observed in the SB203580 (100 µmol/L) group ($p > 0.05$), whereas cell proliferation was markedly inhibited in the NaHS combined with SB203580 group ($p < 0.01$). Similarly, human ovarian cancer cells’ proliferation was significantly inhibited in NaHS combined with SB203580 group compared to the SB203580 (100 µmol/L) group ($p < 0.01$) (Table 1 & Figure 2).

Effects on cell cycle of human ovarian cancer cell SKOV3 after 48 h treatment by flow cytometry

Compared to the control group, cells quantity in the G0/G1 phase were remarkably increased while those in the S phase were markedly reduced in the NaHS ($10^{-3}$ mol/L), SB203580 (0.1 mol/L) and NaHS combined with SB203580 groups ($p < 0.01$). Compared to the NaHS ($10^{-3}$ mol/L) group, there is no significant difference in cell cycle phase distribution in the SB203580 (0.1 mol/L) group ($p > 0.05$), whereas cells quantity in the G0/G1 phase were markedly elevated and those in the S phase were noticeably decreased in the NaHS combined with SB203580 group ($p < 0.01$). Additionally, cells in the G0/G1 phase were markedly enhanced and those in the S phase were noticeably reduced in the NaHS combined with SB203580 group compared to the SB203580 (0.1 mol/L) group ($p < 0.01$). (Table 2 & Figure 3).

Effects on apoptosis of human ovarian cancer SKOV3 cells after 48 h treatment by flow cytometry

Through comparing with the HIV-negative control group, cell apoptosis rate was dramatically enhanced in the NaHS ($10^{-3}$ mol/L), SB203580 (0.1 mol/L) and NaHS combined with SB203580 groups ($p < 0.01$). Compared to the NaHS ($10^{-3}$ mol/L) group, there is no significant increase or decrease in cell apoptosis rate in the SB203580 (0.1 mol/L) group ($p > 0.05$), while NaHS combined with SB203580 group showed marked increase in cell apoptosis ($p < 0.01$). Moreover, Compared with SB203580 (0.1 mol/L) group, cell apoptosis rate was significantly elevated in NaHS combined with SB203580 group ($p < 0.01$) (Table 3 & Figure 4).

Effects on the expression of P-p38MAPK on SKOV3 cells after 48 h treatment

As shown in Figure 5 and Table 4, by comparing with the control group, P-p38MAPK protein’s proliferation in NaHS (50 mol/L) group was noticeably increased, while P-p38MAPK protein’s proliferation was remarkably decreased in SB203580 (75 µmol/L) group and NaHS+SB203580 group; Compared with NaHS (50 mol/L) group, P-p38MAPK protein’s proliferation in SB203580 (75 µmol/L) group and NaHS+SB203580 group was remarkably reduced, while the expression of P-p38MAPK protein in NaHS+SB203580 group was dramatically higher than that in SB203580 (75 µmol/L) group.

Table 3. — Effects on apoptosis of human ovarian cancer SKOV3 cells after 48 h treatment by flow cytometry (%)

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Cell apoptosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.77 ± 0.75</td>
</tr>
<tr>
<td>NaHS (10⁻³ mol/L)</td>
<td>7.14 ± 0.56</td>
</tr>
<tr>
<td>SB203580 (0.1 mol/L)</td>
<td>6.52 ± 0.63</td>
</tr>
<tr>
<td>Combined NaHS and SB203580</td>
<td>10.54 ± 2.08</td>
</tr>
</tbody>
</table>

F value: 43.469
p value: < 0.01

▲ Difference was statistically significant by comparing with control, $p < 0.01$; □ Difference was not statistically significant by comparing with NaHS group, $p > 0.05$.

■ Difference was statistically significant by comparing with NaHS group, $p < 0.01$; ● Difference was statistically significant by comparing with SB203580 group, $p < 0.01$.

Table 4. — The statistical Analysis of the expression of P-p38MAPK

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>P-p38MAPK/β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.342 ± 0.112</td>
</tr>
<tr>
<td>NaHS (50 µmol/L)</td>
<td>1.919 ± 0.185</td>
</tr>
<tr>
<td>SB203580 (75 µmol/L)</td>
<td>0.610 ± 0.061</td>
</tr>
<tr>
<td>NaHS+SB203580 group</td>
<td>0.854 ± 0.042</td>
</tr>
</tbody>
</table>

F value: 59.314
p value: < 0.01

▲ Difference was statistically significant by comparing with control, $p < 0.01$; □ Difference was statistically significant by comparing with NaHS group, $p < 0.01$; ● Difference was statistically significant by comparing with SB203580 group, $p < 0.01$. 
Discussion

Of all gynecological malignancies, ovarian carcinoma has the highest fatality rate due to its undetectable nature and hence is difficult to be treated [19-21]. Although conventional treatment methods such as surgery and chemotherapy are continuously improving, ovarian cancers’ 5-year overall survival rate still keep less than 30% [22]. Therefore, discovery of drugs or factors that can inhibit cancer cells’ proliferation and development has substantial importance in the clinical treatment of ovarian carcinoma.

In mammalian cells, NaHS is the donor of H$_2$S, and it generates H$_2$S through the catalytic reactions of cystathionine lyase, cystathionine-$\beta$-synthase, and aminotransferase, and exists in the forms of H$_2$S gas and NaHS in vivo [23-25]. A study by Yan et al. found that low doses of NaHS (30-50 $\mu$mol/L) could reduce intracellular free radical generation and enhance cell survival, while high dose NaHS (1 mmol/L) was able to inhibit cell proliferation and induce apoptosis [26]. In addition, Abrahamsen H. et al. also showed that H$_2$S could induce cell death by activating the apoptotic mechanism [27].

In this study, human ovarian cancer cells cultured in vitro were treated with NaHS and their proliferation was examined using MTT. Our results indicated that cell proliferation was significantly inhibited in the NaHS group, with an inhibition rate of 39.77%. Further analysis from flow cytometry revealed that cell numbers of the NaHS group were markedly reduced, along with a significant increase in cell apoptosis rate, compared to the control, implicating that NaHS can induce human ovarian cancer cells apoptosis.

Activation of the p38MAPK signaling pathway is able to inhibit the apoptosis of various types of cells and promote the cell cycle and proliferation, thus playing a key role in cell proliferation and apoptosis [28-30]. Keuling et al. found that specific blocking of the p38MAPK pathway by SB203580 could enhance the ABT-737-induced apoptosis of melanoma [31]. In our study, human ovarian cancer cells cultured in vitro were treated with the p38MAPK signaling pathway inhibitor SB203580 for 48 h and cell proliferation was examined using MTT. The results demonstrated that proliferation of the human ovarian cancer cells was markedly inhibited. Furthermore, flow cytometry revealed that human ovarian cancer cells cycle in G$_1$ phase was blocked, indicating that proliferation of human ovarian cancer cells was significantly inhibited. These results demonstrated that human ovarian cancer cell proliferation maybe was regulated by the p38MAPK signaling pathway, and blocking this pathway markedly inhibited human ovarian cancer cells’ proliferation. When simultaneously treated with NaHS and SB203580 for 48 h, human ovarian cancer cells’ inhibition rate was markedly enhanced, cell numbers in the G$_1$ phase remarkably increased and those in the S phase were markedly reduced, along with a significant increase in cell apoptosis rate, compared to the NaHS and SB203580 groups. These results indicated that SB203580 could synergize with NaHS to inhibit the proliferation of ovarian cancer cells. Therefore, we speculate that the specific blocking of the p38MAPK signaling pathway by SB203580 activates the NaHS-mediated inhibition of ovarian cancer cell proliferation, which then synergizes with the latter’s ability to promote ovarian cancer cell apoptosis to delay the development of ovarian carcinoma.

Conclusions

NaHS may work in synergy with SB203580 to inhibit cell proliferation and promote the human ovarian cancer cells apoptosis through the p38MAPK signaling pathway. However, further experiments and studies are still imperative to reveal the underlying mechanism and provide new insights into treating ovarian carcinoma.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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