NO donor and MEK inhibitor synergistically inhibit proliferation and invasion of cancer cells

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Abstract. Nitric oxide (NO) shows tumoricidal activity. We had previously reported that NO downregulates the phosphatidylinositol-3-kinase/Akt pathway, but upregulates the MEK/ERK pathway downstream of growth factor signaling. We hypothesized that NO donor and MEK inhibitor in combination synergistically inhibit the viability of cancer cells compared to either NO donor or MEK inhibitor alone. We determined the effects of S-nitrosoglutathione (GSNO, NO-donor) and U0126 (MEK inhibitor) on insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) signaling, proliferation and invasion in cancer cell lines. GSNO inhibits phosphorylation of IGF-I receptor (IGF-IR), EGF receptor (EGFR) and Akt, but upregulates ERK1/2 phosphorylation in MIAPaCa-2 and HCT-116 cells after stimulation by IGF-I and EGF. On the other hand, U0126 inhibits phosphorylation of ERK1/2, but upregulates phosphorylation of IGF-IR and EGFR in MIAPaCa-2 and HCT-116 cells. The combination of GSNO and U0126 downregulates phosphorylation of IGF-IR, EGFR, Akt and ERK1/2 after stimulation by IGF-I and EGF. GSNO as well as U0126, inhibits the proliferation of MIAPaCa-2, HCT-116, Panc-1, MCF-7, HT-29 and AGS cells in a dose-dependent manner. GSNO and U0126 in combination synergistically inhibit proliferation and invasion of cancer cells. These results indicate that the combined treatment of NO donor and MEK inhibitor may be promising in cancer therapy.

Introduction

Insulin-like growth factor (IGF) and epidermal growth factor (EGF) signaling play a key role in cancer proliferation and invasion (1-5). IGF-I and EGF bind to IGF-I receptor and EGF receptor and then phosphorylate the tyrosine of the cognate receptors. Insulin receptor substrate (IRS)-1, an adaptor protein, exists mainly in the cytosol, and binds to phosphorylated IGF-IR, resulting in phosphorylation and activation of IRS-1. IRS-1 and EGF receptor (EGFR) transduce phosphatidylinositol-3-kinase (PI3K), which in turn activates further downstream components, including Akt. Alternatively, phosphorylated and activated EGFR and IRS-1 can also bind to another adaptor protein, Grb-2, which activates the MEK/ERK pathway, another major IGF and EGF signaling cascade parallel to the PI3K/Akt pathway (6,7).

Recent studies have shown that nitric-oxide (NO) plays a role in the posttranslational modification of proteins (8-11). Controversial results have been reported regarding the roles of NO in cancer. Recent papers reported that endogenous NO promotes oncogenesis and angiogenesis in various cancers (12,13). In contrast, other studies have shown that NO inhibits cell proliferation and induces apoptosis in various cells including cancer cells, in vitro and in vivo (14-21). These studies suggest that NO can act either as a tumor suppressor or as a tumor enhancer depending on cell type and the level of NO in the cells. However, the molecular mechanism underlying the inhibitory effects of NO on cancer viability, remains unclear. We have reported that NO inhibits cancer cell proliferation and invasion through downregulation of IRS-1 protein, resulting in inhibition of the PI3K/Akt pathway (14). In contrast, NO enhances nitrosylation and activates N-Ras and H-Ras proteins (22). Members of the Ras family of small GTPase proteins including K-Ras, N-Ras, and H-Ras, play central roles in the transduction of growth factor signals (23). Thus, NO downregulates the PI3K/Akt pathway, but upregulates the MEK/ERK pathway. Therefore, we hypothesized that the combination of NO donor and MEK inhibitor synergistically inhibits the proliferation and invasion of cancer cells...
compared to either NO donor or MEK inhibitor alone. In the present study, we demonstrated that combination treatment of NO donor and MEK inhibitor shows greater inhibitory effects on the proliferation and invasion of cancer cells compared to either MEK inhibitor or NO donor alone, which are associated with the inhibition of both the PI3K/Akt and MEK/ERK pathways. These data provide new insight into the molecular basis underlying the regulation of cancer viability.

Materials and methods

Materials. S-nitrosogluthathione (GSNO) and U0126 were purchased from Calbiochem (San Diego, CA). Recombinant IGF-I and EGF were purchased from Peprotech (London, UK). Antibodies against phospho-Tyr Tyr1135/1136 IGF-I Rβ, phospho-Tyr1068EGFR, phospho-Ser 473 Akt, phospho-ERK1/2, IGF-IR, EGFR, Akt, and ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture. MIAPaCa-2, HCT-116, Panc-1, MCF-7, HT-29 and AGS cells were obtained from the American Type Culture Collection (Manassas, VA), and were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO2.

Cell lysis. Cells were lysed with cell lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM DTT, and 1% NP-40]. Following incubation on ice for 30 min, lysate samples were centrifuged at 13,000 g for 30 min. Aliquots of the supernatant containing equal amounts of protein, determined using Lowry assay, were subjected to immunoblotting followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting. A total of 20 µg of each protein sample was mixed with 5X sample buffer including 10% β-mercaptoethanol and the mixture was boiled for 5 min. The total cellular protein extracts were separated on 10% SDS polyacrylamide gels. The extracts were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), the membranes were blocked with 5% nonfat dried milk for 2 h at room temperature, and incubated with primary antibody for either 2 h at room temperature or overnight at 4°C. This was followed by incubation with secondary antibody conjugated with rabbit or mouse IgG antibody peroxidase for 2 h at room temperature. Detection was done with an enhanced chemiluminescence (ECL) reagent (GE Healthcare, Piscataway, NJ). Bands of interest were scanned by using Colorio GT-X970 (Epson, Tokyo, Japan) and were quantified with NIH Image 1.62 software (NTIS, Springfield, VA).

Cell proliferation assay. The mixture of GSNO (NO donor), U0126 (MEK inhibitor) and 100 µl of medium containing 5x105 cancer cells was added to each well (of a 96-well plate). Cell proliferation assay was performed using Cell Counting kit-8 containing 2-(2methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) (Dojin Laboratories, Kumamoto, Japan) after the incubation for 72 h. At the end of each experiment, the cell proliferation reagent WST-8 was added to each well and the plates were incubated at 37°C for 2 h. Optical density (OD) (A450 nm) was measured using an automatic microplate reader (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate.

Invasion assay. The invasive activities of cultured MIAPaCa-2 and HCT-116 cells were assayed using BD BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA). MIAPaCa-2 cells were initially seeded on a 24-well plate at a density of 5x104 cells/well and cultured in DMEM/10% FBS, in the presence of 1% glutamine and antibiotics (1% penicillin and streptomycin sulfate) at 37°C in a humidified atmosphere of 5% CO2. Medium containing NIH3T3 fibroblasts in the lower chamber served as a chemotacticant. The cells were then exposed to NO donor (GSNO 200 µM) and MEK inhibitor (U0126 10 µM). The chambers were incubated for 24 h, to determine invasion efficiency. HCT-116 cells were initially seeded on a 24-well plate at a density of 1x104 cells/well and cultured in RPMI-1640/10% FBS, in the presence of 1% glutamine and antibiotics (1% penicillin and streptomycin sulfate) at 37°C in a humidified atmosphere of 5% CO2. Medium containing NIH3T3 fibroblasts in the lower chamber served as a chemotacticant. The cells were then exposed to NO donor (GSNO 200 µM) and MEK inhibitor (U0126 10 µM). The chambers were incubated for 48 h, to determine invasion efficiency. MIAPaCa-2 and HCT-116 cells on the upper surface of the filter were removed using a cotton-wool swab, and the cells that had invaded the lower surface were stained with 1% toluidine blue after fixation in 100% methanol. The number of invading cells was counted in 5 randomly selected fields under a light microscope. Each experiment was performed in triplicate.

Statistical analysis. Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts Inc., Berkeley, CA). Comparisons were carried out using Student’s t-test, and P<0.05 were considered statistically significant. ANOVA followed by Tukey-Kramer post hoc test was used to compare multiple samples, with a significance level of 0.05.

Results

NO donor and MEK inhibitor influence IGF-I signaling in MIAPaCa-2 cells. Stimulation of IGF-I resulted in marked phosphorylation of IGF-IR, Akt and ERK1/2 in MIAPaCa-2 cells. GSNO, a NO donor, inhibited IGF-I-stimulated phosphorylation of IGF-IR and Akt. On the other hand, GSNO induced phosphorylation of ERK1/2 without stimulation of IGF-I, and enhanced IGF-I-stimulated phosphorylation of ERK1/2. However, GSNO did not influence IGF-IR, Akt, and ERK1/2 protein expression in MIAPaCa-2 cells. U0126, a MEK inhibitor, inhibited IGF-I-stimulated phosphorylation of ERK1/2. On the other hand, surprisingly, U0126 enhanced IGF-I-stimulated tyrosine phosphorylation of IGF-IR, without the influence of IGF-IR protein expression. In addition, U0126 upregulates IGF-I-stimulated phosphorylation of Akt. The combination of GSNO and MEK inhibitor inhibited IGF-I-stimulated phosphorylation of IGF-IR, Akt, and ERK1/2 without the influence of IGF-IR, Akt, and ERK1/2 protein expression (Fig. 1).
NO donor and MEK inhibitor influence EGF signaling in HCT-116 cells. Stimulation of EGF resulted in marked tyrosine phosphorylation of EGFR, Akt and ERK1/2 in HCT-116 cells. GSNO inhibited EGF-stimulated phosphorylation of EGFR and Akt. However, GSNO induced phosphorylation of ERK1/2 without stimulation of EGF, and enhanced EGF-stimulated phosphorylation of ERK1/2. U0126 inhibited EGF-stimulated phosphorylation of ERK1/2. On the other hand, surprisingly, U0126 enhanced EGF-stimulated tyrosine phosphorylation of EGFR without the influence of EGFR protein expression. In addition, U0126 upregulates EGF-stimulated phosphorylation of Akt. The combination of GSNO and U0126 inhibited EGF-stimulated phosphorylation of EGFR, Akt and ERK1/2 without the influence of EGFR, Akt, and ERK1/2 protein expression (Fig. 2).
Figure 4. NO donor alone and MEK inhibitor alone significantly inhibit the proliferation of cancer cells in a dose-dependent manner, when the cells were incubated in medium containing with FBS. The combination of NO donor and MEK inhibitor inhibit the proliferation of MIAPaCa-2 cells compared to either NO donor or MEK inhibitor alone.
The combination of NO donor and MEK inhibitor inhibits the proliferation of cancer cell lines compared to either NO donor or MEK inhibitor alone. IGF-IR protein was expressed in MIAPaCa-2, HCT-116, Panc-1, MCF-7, HT-29 and AGS cells, and EGFR protein was expressed in MIAPaCa-2, HCT-116, Panc-1, HT-29 and AGS cells (Fig. 3). To evaluate the effects of the combination of NO donor and MEK inhibitor on MIAPaCa-2, HCT-116, Panc-1, MCF-7, HT-29 and AGS cells, we analyzed cell proliferation using an MTT assay after the cells were treated with NO donor and MEK inhibitor for 72 h. As shown in Fig. 4A, GSNO alone and MEK inhibitor alone significantly inhibited the proliferation of MIAPaCa-2 cells in a dose-dependent manner when the cells were incubated with FBS. The combination of NO donor (200 µM, 500 µM) and MEK inhibitor (10 µM) decreased the proliferation of MIAPaCa-2 cells compared to either NO donor or MEK inhibitor alone when the cells were incubated with FBS (Fig. 4B). Similar results were found in HCT-116, Panc-1, MCF-7, HT-29 and AGS cells (Fig. 4C-H).

GSNO alone and MEK inhibitor alone significantly inhibited the proliferation of MIAPaCa-2 cells in a dose-dependent manner when the cells were incubated with IGF-I (Fig. 5A and B). The combination of NO donor (100 µM) and MEK inhibitor (10 µM) decreased the proliferation of MIAPaCa-2 cells compared to either NO donor or MEK inhibitor alone when the cells were incubated with IGF-I (Fig. 5C).

GSNO alone and MEK inhibitor alone significantly inhibited the proliferation of HCT-116 cells in a dose-dependent manner when the cells were incubated with EGF.
Figure 5. NO donor alone and MEK inhibitor alone significantly inhibit the proliferation of MIAPaCa-2 cells in a dose-dependent manner, when the cells were incubated with IGF-I (A and B). The combination of NO donor and MEK inhibitor inhibits the proliferation of MIAPaCa-2 cells compared to either NO donor or MEK inhibitor alone (C). MIAPaCa-2 cells were incubated with the combination of GSNO and U0126 in medium containing IGF-I (100 nM) for 72 h and then cell proliferation was determined. *P<0.05, compared to control; †P<0.01, compared to GSNO alone; ‡P<0.01, compared to U0126 alone. NO donor alone and MEK inhibitor alone significantly inhibit the proliferation of HCT-116 cells in a dose-dependent manner, when the cells were incubated with EGF (D and E). The combination of NO donor and MEK inhibitor inhibits the proliferation of HCT-116 cells compared to either NO donor or MEK inhibitor alone (F). HCT-116 cells were incubated with the combination of GSNO and U0126 in medium containing EGF (100 ng/ml) for 72 h and then cell proliferation was determined. *P<0.05, compared to control; †P<0.01, compared to GSNO alone; ‡P<0.01, compared to U0126 alone.

Figure 6. The combination of NO donor and MEK inhibitor inhibits the invasion of MIAPaCa-2 and HCT-116 cells compared to either NO donor or MEK inhibitor alone. Cell invasion was determined using BioCoat Matrigel invasion chambers. MIAPaCa-2 cells (A and B), or HCT-116 cells (C and D) were placed in the upper chambers. Conditioned medium from NIH 3T3 cells was used as a chemoattractant. A and C show stained cancer cells, that migrated. Data shown are the results of triplicate experiments (B and D). Error bars indicate the standard errors of the means. *P<0.01, compared to GSNO alone; †P<0.01, compared to U0126 alone.
reported that NO directly modifies Akt by 47.5% in mice inoculated with PTC cells bearing a RET/PTC1 rearrangement (39). CI-1040 has been tested in other cancers, including colon cancer, breast cancer, non-small cell lung cancer (NSCLC), and melanoma, and was well tolerated by patients in phase I-II trials (40,41). NO donor upregulates phosphorylation of ERK1/2, although NO donor downregulates the PI3K/Akt pathway. In the present study, we showed that the phosphorylation of ERK1/2 is upregulated by NO donor without the stimulating of growth factors in MIAPaCa-2 and HCT-116 cells. These results are in accordance with previous studies reporting that NO directly modifies H-Ras and N-Ras through S-nitrosylation, resulting in the activation of their signaling (22,42). The present study showed that U0126 completely inhibits phosphorylation of ERK1/2, although U0126 upregulates IGF-I- and EGF-stimulated Akt phosphorylation (Figs. 1 and 2). These results are in accordance with previous reports (43,44). Furthermore, we showed that U0126 upregulates IGF-I and EGF-stimulated phosphorylation of IGF-IR and EGFR. The U0126-induced upregulation of Akt phosphorylation may be explained by the upregulated phosphorylation of IGF-IR and EGFR. We surmise that a negative feedback mechanism from the inhibition of ERK activity may regulate this phenomenon. As described above, NO inhibits U0126-induced upregulated IGF-IR, EGFR, and Akt phosphorylation. These results suggest that the combination of NO donor and MEK inhibitor may be more effective in cancer therapy.

The usefulness of cancer therapy using NO, including inducible NO synthase gene therapy and administration of NO donor, was recently confirmed in animal models (45-47). Consequently, NO therapy has received considerable attention and is currently undergoing clinical evaluation for cancer prevention (48). On the other hand, MEK inhibitor is also feasible for cancer therapy (40,41). We believe that combined treatment of NO donor and MEK inhibitor may be an effective and promising strategy for cancer treatment.

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References


