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Doxorubicin Inhibits the Production of Nitric Oxide by Colorectal Cancer Cells

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Doxorubicin (DOX) is an active and broad spectrum chemotherapeutic agent. Increased inducible nitric oxide synthase (NOS) expression and/or activity have been reported in several human tumors. While the relationship between DOX treatment and the enzymatic activity of endothelial NOS has been well characterized, little is known about the effects of DOX on the expression of iNOS in human cancer cells. In the present study, we characterized the effects of DOX on the nitric oxide (NO) production by colorectal cancer cells, DLD-1. IFN-γ/IL-1β (CM) increased the production of NO, whereas pretreatment of DOX inhibited the production of NO in response to CM in a dose dependent manner. The increased expressions of iNOS mRNA and protein by CM were completely blocked by DOX without affecting the iNOS mRNA stability. However, DOX activated nuclear factor-κB (NF-κB) in response to CM. Furthermore, the expression of inhibitor κBα was reduced by DOX in a dose dependent manner. Collectively, DOX inhibited the production of NO by DLD-1 cells, which is not linked to well known transcription factor, NF-κB. Therefore, further studies on the possible mechanisms of inhibitory effects of NO production by DOX would be worth pursuing.

Key words: Colorectal cancer, Doxorubicin, Nitric oxide, κBα, $I_kBα$

INTRODUCTION

Nitric oxide (NO) is a short lived free radical, synthesized from arginine, with extremely high reactivity and a variety of physiological activities (Ignao, 1989). NO is synthesized by a family of three distinctive nitric oxide synthase (NOS) isoforms named for the tissues in which they were originally described. Neuronal type NOS (nNOS) and endothelial type NOS (ecNOS) are calcium dependent, and constitutively produce relatively low levels of NO. An inducible calcium independent form, iNOS, may be found in macrophages, hepatocytes, neutrophils, endothelial cells, and astrocytes. NO causes damage to DNA and is a potential endogenous carcinogen (Ambs *et al.*, 1997), and its increased production may increase angiogenesis and contribute to tumor progression (Fukumura and Jain, 1998). However, the effects of NO on tumor cells are apparently production dependent, and cell type specific (Xie and Fidler, 1998). Overproduction of NO is cytotoxic, induces apoptosis, and suppresses tumor growth, whereas low NO production may protect cells from apoptosis and promote tumor growth (Brune *et al.*, 1998).

Doxorubicin (DOX) remains one of the most widely used antitumor agents (Singal and Iliskovic, 1998). The effects of DOX on eNOS are well established, and shows that an increased expression of eNOS is associated with DOX-induced apoptosis (Kalivendi *et al.*, 2001). However, the effects of DOX on the production of NO by iNOS from studies on rats are not well identified. While 4-epi-DOX inhibited the induction of iNOS in rats (Inagaki *et al.*, 1999), DOX was reported to increase the production of NO in macrophage of the L1210 leukemia model (Zagozdzon *et al.*, 1999). Furthermore, several anticancer agents, including DOX activated NF-κB, a critical transcription factor for the inducible expression of many genes involved in inflammation (Bian *et al.*, 2001; O'Neill, 1995), suggesting the increased creased expression of iNOS by DOX. Increased iNOS expression and/or activity have been reported in human gynecological (Thomsen *et al.*, 1994), breast (Thomsen

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et al., 1995), central nervous system (Cobbs *et al.*, 1995) tumors, and human colorectal adenomas (Ambs *et al.*, 1998). Furthermore, since NO is involved in tumor cell growth and invasion in the human colorectal adenocarcinoma cell line (Siegert *et al.*, 2002), it is important to identify the effects of DOX on the production of NO by colorectal cancer cells. In this study, we found that DOX inhibited the production of NO by colorectal cancer cells in response to IFN-γ/IL-1β (cytokine mixture; CM) without the destabilization of iNOS mRNA or the involvement of NF-κB.

MATERIALS AND METHODS

Chemicals

3-(4,5-Dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), IFN-γ, DOX and 5,6-dichlorobenzimidazole riboside (DRB) were purchased from Sigma; IL-1β was from Roche Diagnostics GmbH; TriZol was from Life Technologies, Inc.; Radioactive materials were from Amersham Corp.

Cell culture

Colorectal cancer cell line, DLD-1, was from Korean Cell Line Bank (Seoul, Korea) and grown at 37°C, 5% CO_2 in RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum. Colorectal cancer cells (5×10^6 cells/mL) were dispensed on 100-mm culture dishes and stimulated with CM in the presence or absence of DOX at concentrations and time points indicated. The production of NO was quantified by measuring nitrite plus nitrate (NO_2^- and NO_3^-) by an automated procedure based on the Griess assay (Green *et al.*, 1982).

Northern blot analysis

Total RNA was isolated using TriZol (Life Technology, Inc.) following the manufacturers suggested protocol. Using 20 μg of total RNA, northern blot analysis was performed as previously described (Lee *et al.*, 1999). To make specific probes of human iNOS and β-actin, RT-PCR reactions were performed using human iNOS primers 5-CCCGAGTCAGAGTCACCATCC-3, 5-TCAAACGTCTCACAGGCTGCC-3, and human β-actin primers, 5-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3, 5-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3. The band density was analyzed utilizing EagleSight Software v. 3.2 (Stratagene).

Electrophoretic Mobility Shift Assay (EMSA)

Adherent cells (5×10^6 per 100-mm tissue culture dishes) were stimulated with CM in the presence or the absence of DOX, washed, and scraped into 1 mL of cold phosphate-buffered saline. The cell suspensions were transferred to microcentrifuge tubes, pelleted, and the nuclear protein extracts were prepared as described (St-Denis *et al.*, 1998). The nuclear extracts were centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant was frozen at -70°C in aliquots until required for EMSA. Protein was quantified by Bradford assay (Bio-Rad). EMSA were performed by incubating ^{32}P-labeled NF-κB consensus oligonucleotide (5-AGC TTG GGG ACT TTC C-3) with 5 μg of nuclear extracts as described previously (St-Denis *et al.*, 1998).

Western blot analysis

Control and DOX treated DLD-1 cells were first lysed in buffer containing 30 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 5 μg/mL pepstatin, and 5 μg/mL trypsin inhibitor. Cell extracts were then fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed with polyclonal antibodies to iNOS (Transduction Laboratories, Lexington, KY), IκBα and p65 (Santa Cruz Biotechnology), and visualized by ECL-system (Amersham) using anti-rabbit horseradish peroxidase IgG (Sigma). The band density was analyzed utilizing EagleSight Software v. 3.2 (Stratagene).

Measurement of cell viability

Cell viability was measured by tetrazolium reduction by using MTT assay (Denizot and Lang, 1986). After each experiment, drug-treated samples showing higher than 85% of the nontreated control were used.

Statistical analysis

Results are expressed means ± s.d., and an analysis was done by one way Students *t*-test. *P*-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cytokine mixture increases the production of NO in colorectal cancer cells

Reactive oxygen and nitrogen species have been proposed to play an important role in human carcinogenesis. Many cancer cells, including cholangiocarcinoma and colorectal cancer cells, have a cytokine-induced NOS, and produce increased amounts of NO in the presence of CM (Chu *et al.*, 1998; Heitmeier *et al.*, 1997; Sherman *et al.*, 1993). To verify whether CM can stimulate the production of NO by colorectal cancer cells, DLD-1, we treated cancer cells with CM, and found that CM increased the production of NO in a dose and a time dependent manner (Fig. 1A and 1B), confirming that iNOS in human colorectal cancer cells is activated by CM.

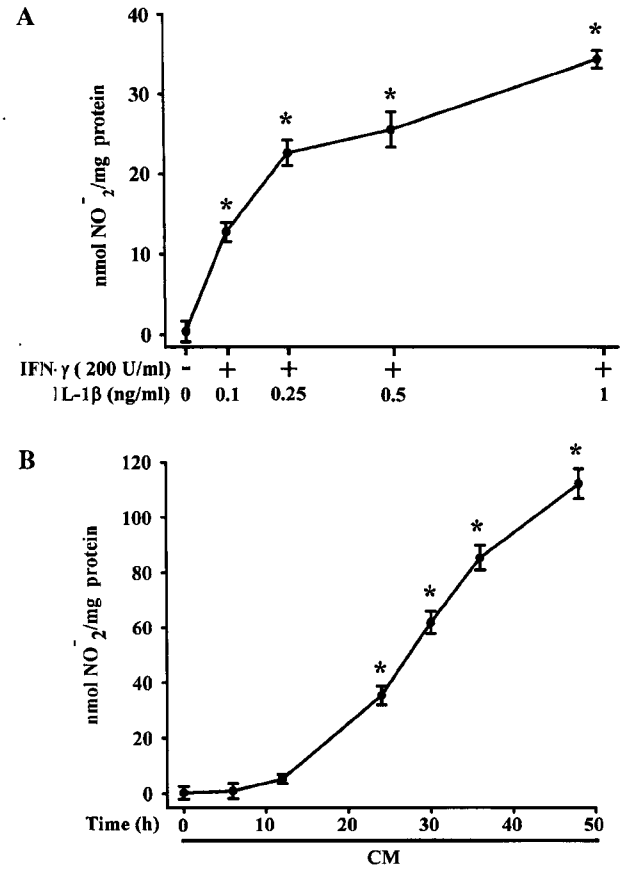

Fig. 1. Effects of IL-1β/IFN-γ on the production of NO by DLD-1 cells. DLD-1 colorectal cancer cells were treated with CM, and the nitric oxide production was quantified by measuring the accumulation of nitrite in the culture medium as indicated in "Materials and Methods." A, concentration-dependent increase in nitric oxide production 24 h after treatment with 200 U/mL IFN-γ and various amounts of IL-1β. B, time course of the nitrite accumulation with 200 U/mL IFN-γ and 1 ng/mL IL-1β. Data shown are the mean±S.D. from triplicate samples. *P < 0.05, significantly different from control.

DOX inhibits the production of NO in response to CM

We examined the effects of DOX on the production of nitric oxide by DLD-1 cells stimulated with CM. DOX, a broad-spectrum antitumor antibiotic, has been widely used in the treatment of many cancers (Singal and Iliskovic, 1998). Since increased iNOS expression and/or activity has been reported in several cancers, and the effects of DOX on the production of NO by human cancer cells are not well documented, we pretreated the colorectal cancer cells with DOX for 12 h, and further incubated them with CM for another 24 h. DOX was found to inhibit the production of NO by DLD-1 cells in a dose dependent manner, with an IC_{50} value 15 μM (Fig. 2A). DOX alone had no effect on the production of NO by the cells, and no cytotoxicity was observed at the tested concentrations (Fig 2A and 2B). We then compared the inhibition level of

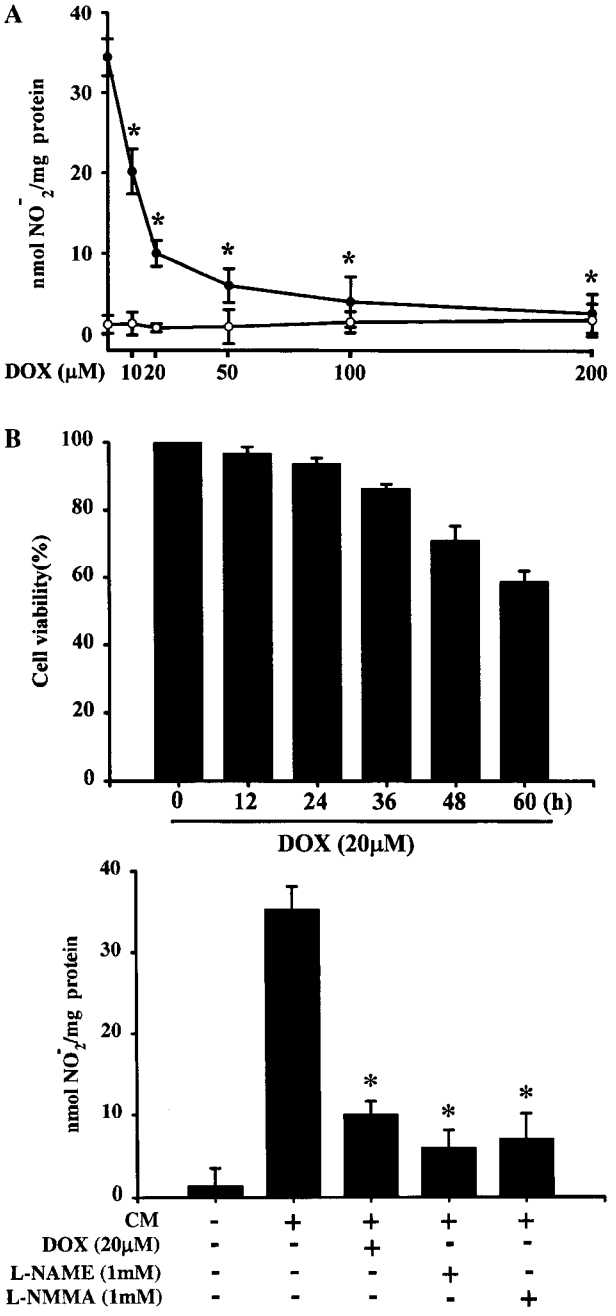

Fig. 2. Effects of DOX on the production of NO by DLD-1 cells. A, after incubation with various concentrations of DOX for 12 h, colon stomach cancer cells were treated with (●) or without (○) CM for another 12 h. B, cytotoxicity assay. The colorectal cancer cells were incubated for the indicated time with DOX (20 μM), and cell viability was measured by using MTT assay as described in "Materials and Methods". C, after incubation with DOX (20 μM) or nitric oxide synthase inhibitors (1 mM) for 12 h, the cancer cells were treated with CM for another 24 h, and nitric oxide production quantified by measuring the accumulation of nitrite in the culture medium. Data shown are the mean±S.D. from triplicate samples. *P < 0.05, significantly different from control.

DOX on the production of NO with the specific competitive NOS inhibitors, L-NAME and L-NMMA. DOX at a con-

centration of 20 μM inhibited the CM-induced production of NO to almost the same extent as 1 μM L-NAME or L-NMMA (Fig. 2C). These data strongly suggest that the increased NO production by CM could be blocked by the pretreatment with DOX, with no cytotoxicity.

5-FU inhibits the expression of NOS

Since NO is generated by the NADPH-dependent oxidation of arginine to citrulline by NOS in mammalian cells, we tested if the inhibition of NO production by DOX, in response to CM, is caused by its effect on the expression of iNOS. The colorectal cancer cells were pretreated with DOX for 12 h, and further incubated for 24 h with CM. Northern and western blot analyses showed significant reductions in NOS mRNA and protein expressions following pretreatment with DOX (Fig. 3A and 3B), suggesting that the effects of DOX, on the production of NO by colorectal cancer cells, are either on its interference on the transcription of iNOS or on its degrading of the iNOS mRNA. To test the effects of DOX on the stability of iNOS mRNA, colorectal cancer cells were treated with CM for 24 h, and further incubated with DRB (20 μM) for various times with or without DOX. Fig. 3C shows there was no change in the iNOS mRNA during the incubation with DRB up to 24 h, indicating DOX does not affect to the stability of iNOS mRNA (Fig. 3C).

DOX activates NF-κB through IκBα degradation

NF-κB is critical for the inducible expression of many genes, including iNOS (Chu *et al.*, 1998; Dupraz *et al.*, 2000; O'Neill, 1995), and is important in the regulation of cell survival through the induction of antiapoptotic genes (Foo and Nolan, 1999). Since DOX reduced the production of NO by colorectal cancer cells in response to CM, but did not destabilize the mRNA of iNOS, we tested the effects of DOX on NF-κB by colorectal cancer cells in response to CM. After incubating with CM in the presence or absence of DOX, nuclear extracts of colorectal cancer cells were examined for NF-κB binding activity by EMSA using consensus NF-κB binding sequences. Increased NF-κB activity with CM treatment was not blocked by DOX pretreatment (Fig. 4A). Rather than blocking, the pretreatment with DOX increased the activity of NF-κB in colorectal cancer cells. This data is consistent to the effects of DOX on NF-κB activity in human lung adenocarcinoma cell line, A549, showing that antineoplastic drugs including DOX increased NF-κB activity (Das and White, 1997).

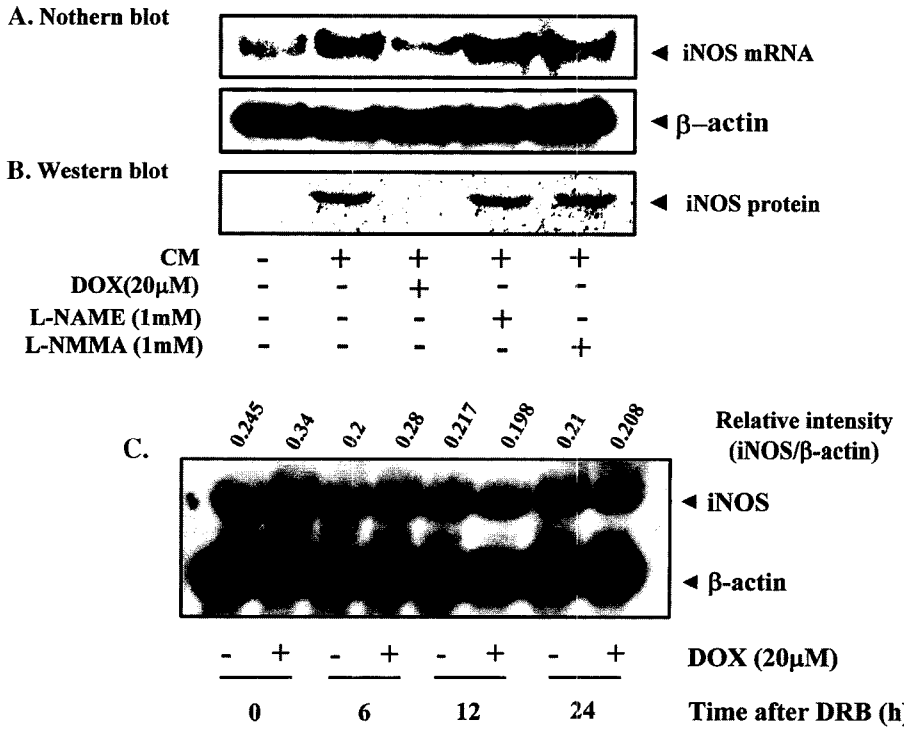

Fig. 3. Effects of DOX on the expression of iNOS in DLD-1 cells. A, Cells were treated with CM for the indicated time and iNOS mRNA expression was measured by Northern blot using specific iNOS primers. B, Western blot analysis. Cell extracts (25 μg) were fractionated on a SDS-polyacrylamide gel and analyzed with polyclonal antibodies to iNOS. The band intensity was analyzed utilizing EagleSight software v. 3.2 (Stratagene). Data shown are typically representative of four separate experiments with three replicates. C, iNOS mRNA stability. Cells were treated with CM for 24 h, and then with DRB (20 μM) in the presence or absence of DOX for various times. Isolation of RNA and Northern blot analysis were performed as described in "Materials and Methods".

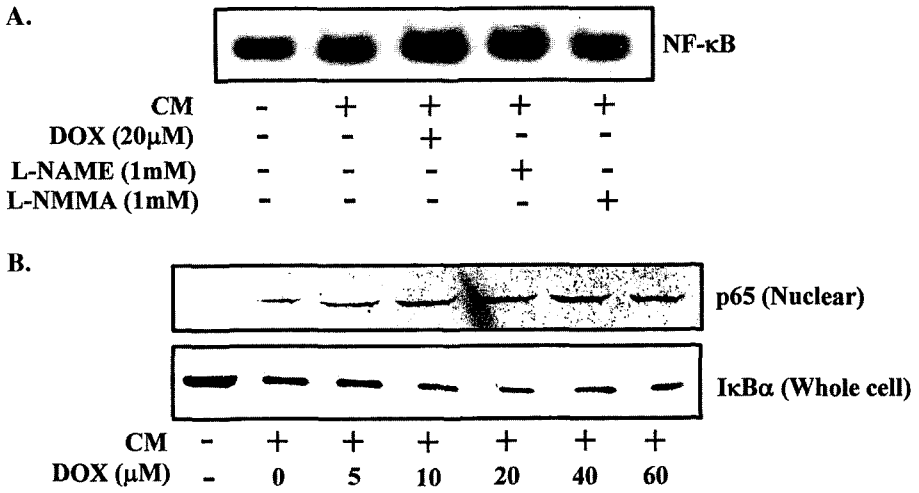

Fig. 4. Effects of DOX on NF-κB and IκBα. A. EMSA analysis for the activation of NF-κB. Cancer cells were pretreated with various concentrations of DOX for 12 h, then further incubated with CM for 1 h. Isolation of nuclear extracts and EMSA analysis were carried out as described in "Materials and Methods". B. The effects of DOX on p65 translocation and IκBα. Cancer cells were treated with DOX for 12 h, then further incubated with CM for 20 min. Nuclear (p65) and whole cell (IκBα) extracts were prepared and analyzed in 10% SDS-PAGE. Data shown are typically representative of three separate experiments.

NF-κB dimers are held in the cytoplasm in an inactive state by inhibitory proteins, the IκBs. which preferentially associate with various Rel family protein dimers (Thanos and Maniatis, 1995). Since DOX increases the activation of NF-κB by CM in colorectal cancer cells, we investigated whether DOX destabilizes IκBα and increases the translocation of p65 to the nucleus. Pretreatment of DOX increased p65 translocation into the nucleus, as well as decreasing the stability of IκBα in a dose dependent manner, suggesting the effects of DOX on NF-κB activation are caused by the degradation of IκBα and the accumulation of p65 in the nucleus (Fig. 4B).

Our study is the first report as far as we know showing the whole effects of DOX, from IκBα stability to the production of NO by colorectal cancer cells. While DOX increased the production of NO by peritoneal macrophages (Zagozdzon *et al.*, 1999), it inhibited the induction of NOS in rats *in vivo* (Inagaki *et al.*, 1999). DOX inhibited the production of NO and the expression of iNOS in colorectal cancer cells in response to CM, without affecting the stability of iNOS mRNA. However, DOX increased the activity of the major transcription factor, NF-κB through dose dependent degradation of IκBα. At present, we do not know how DOX inhibits the production of NO by colorectal cancer cells. However, increased iNOS expression and/or activity in several human tumors (Cobbs *et al.* 1995; Thomsen *et al.*, 1994; Thomsen *et al.*, 1995) and the involvement of NO in tumor cell growth and invasion in human colorectal adenocarcinoma cell line (Jenkins *et al.*, 1995; Siegert *et al.*, 2002), suggests the importance of NO on tumor proliferation and metastasis. Furthermore, activation of iNOS and the excessive production of NO in response to inflammatory cytokines cause DNA damage and inhibit DNA repair proteins (Jaiswal *et al.*, 2000). Therefore, further studies on the possible mechanisms of the inhibitory effects of NO production by DOX would be worth pursuing.

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