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Decrease in RelA phosphorylation by inhibiting protein kinase A induces cell death in NF-κB-expressing and drug-resistant tumor cells

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ABSTRACT

The RelA (p65) is a subunit of nuclear transcription factor kappa B (NF-κB) and actively participates in expression of NF-κB-dependent genes involved in inflammation and tumorigenesis. Hence, the regulation of p65 is an important strategy to regulate those responses. In this study, we provide data that the dichlorophenyl derivative of 1,2,4-thiadiazolidine (known as P_3-25) induced cell death in NF-κB-expressing and doxorubicin-resistant cells. P_3-25 inhibited NF-κB DNA binding activity partially, but inhibited NF-κB-dependent genes expression completely. It inhibited phosphorylation of Rel A (p65) by inhibiting activity of protein kinase A (PKA). The PKA inhibition was independent of adenylate cyclase activity or cAMP level. The PKA activity decreased due to inhibition of catalytic subunit of PKA. P_3-25 inhibited almost 80% PKA activity at 100 nM concentration, having an IC_{50} at 10.5 nM. P_3-25 potentiated different chemotherapeutic agents-mediated cell death. Our results suggest that P_3-25 inhibits PKA activity followed by decreased phosphorylation of p65 and transcriptional activity of NF-κB thereby decreasing antiapoptotic proteins resulting in induction of apoptosis in NF-κB-expressing and doxorubicin-resistant cells. The study might help to understand the mechanism of P_3-25-mediated apoptosis and to design it as new chemotherapeutic drug for tumor therapy.

1. Introduction

Nuclear transcription factor-kappaB (NF-κB)-dependent various genes expression plays critical role in apoptosis, viral replication, asthma and allergic diseases, tumorigenesis, various autoimmune diseases, and inflammation (Baichwal and Baeuerle, 1997; Karin and Greten, 2005). NF-κB is activated in response to various stimuli including cytokines, mitogens, bacterial products, viral proteins, and apoptosis-inducing agents (Karin and Greten, 2005; Baeuerle and Baichwal, 1997). NF-κB activation lies in the removal of inhibitory subunit of NF-κB (IκBα). The phosphorylation of p65 subunit of NF-κB is required to recruit the transcriptional apparatus and to stimulate expression of several genes including cyclin D1, adhesion molecules, cyclooxygenase (Cox)-2, inhibitor of apoptosis protein (IAP) 1, survivin, and matrix metalloproteinases. Phosphorylation of p65 at Ser^{276}, situated in the rel-homology domain (RHD), is mediated by protein kinase A (PKA). This phosphorylation is necessary for the recruitment of transcriptional apparatus and thereby expression of target genes (Zhong et al., 1997). IκBα keeps PKA inactive within the complex of NF-κB/IκB. When IκB is degraded, PKA becomes active and phosphorylates p65 at Ser^{276} residue (Zhong et al., 1998). Cyclic AMP (cAMP), the product of adenylate cyclase and ATP, is required for PKA activity. Forskolin, an activator of adenylate cyclase activates PKA through generation of cAMP (Lee et al., 2004). Stimulation of PKA by cAMP is also known to activate another transcription factor, cyclic AMP-responsive element binding-protein (CREB) directly or via p38 MAPK and MSK1 pathway (Delghandi et al., 2005). MSK1, a nuclear protein kinase that is itself activated by both ERK and p38 MAPK, phosphorylates p65 at Ser^{276} residue as shown by TNF treatment (Vermeulen et al., 2003).

Constitutive activity of NF-κB has recently been correlated with progression of different cancers, especially breast cancer, melanoma, and juvenile myelomonocytic leukemia (Nakshatri et al., 1997; Izban et al., 2000; Kochetkova et al., 1997) and also in chemoresistance (Arlt et al., 2003). Downregulation of constitutively expressed NF-κB is an important target for cancer therapy. Upon incubation with serum, lipopolysaccharide (LPS) forms LPS–LBP (LPS binding protein) complex (Hailman et al., 1994). Cells, stimulated with serum activated LPS (SA-LPS) prolong NF-κB

Abbreviations: IAP, inhibitor of apoptosis protein; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H-8, (methylamino)ethyl-5-isoquinolinesulfonamide; IKK, IκBα kinase; Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; MSK, mitogen activated stress kinase; MTT, 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide; NF-κB, nuclear transcription factor kappa B; P_3-25, 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine; Rp-cAMPS, adenosine cyclic 3′,5′-phosphorothioate triethylammonium salt; SA-LPS, serum activated lipopolysaccharide; SEAP, secretory alkaline phosphatase.

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DNA binding activity and are resistant to TNF-induced apoptosis (Manna and Aggarwal, 1999). Tumor cells, such as HuT-78, HT-29, PC3, have constitutive activity of NF-κB (Huang et al., 2005; Sreenivasan et al., 2003). Certain population of MCF-7 cells, when cultured with doxorubicin attained resistance to it (Devarajan et al., 2002). So, downregulation of high basal activity of NF-κB might be important to sensitize these cells for apoptosis.

The thiazolidones and thiadiazolie have drawn considerable attention for their anti-bacterial (Abdel-Halin et al., 1994), anti-fungal (Abdel-Halin et al., 1994), and anti-inflammatory (Geard et al., 2003) activities. Anti-fungal activity of some of N and S containing 1,2,4-thiadiazolidines and 3-oxo-1,2,4-thiadiazolidines (Choubey et al., 1998) prompted us to synthesize newer 1,2,4-thiadiazolidines by employing oxidative debenzylation technique (Manna et al., 2004) and study their biological activities. Previously we found that these derivatives inhibited TNF-induced NF-κB activation through IKK inactivation (Manna et al., 2005). As constitutively expressed NF-κB has shown resistance to cell death by different inducers of apoptosis and p65 subunit has been shown to transactivate NF-κB-dependent genes involved in cell progression and differentiation, we are interested in understanding the role of thiadiazolidine derivatives on the p65 level in NF-κB-expressing (HuT-78 or *p65* transfected U-937) and doxorubicin-resistant cells.

In this report we provide data that 5-(4-methoxyarylimino)2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (designated as P_3-25 hereafter) is a potent inhibitor of protein kinase A (PKA) involved in phosphorylation of p65 and thereby NF-κB-dependent genes transcription. The P_3-25 blocks PKA activity without altering cAMP level or adenylate cyclase activity. P_3-25 potently induces cell death in NF-κB-expressing and doxorubicin-resistant cells. For the first time, we provide data that P_3-25 blocks phosphorylation of p65 by inhibiting activity of PKA and thereby blocking expression of NF-κB-dependent genes in NF-κB-expressing cells. This observation may be helpful to design P_3-25 as a novel anti-inflammatory and/or anti-tumor drug in case of drug-resistant tumor cells where NF-κB-dependent genes expression is believed to be an important determinant.

2. Materials and methods

2.1. Materials

Lipopolysaccharide (*E. coli*, 055:B5), MTT [3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide], doxorubicin, etoposide, cis-platin, vincristine, adriamycin, taxol, 4-methyl umbelliferyl phosphate, propidium iodide (PI), forskolin, H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine], PKA catalytic subunit, and anti-tubulin antibody were obtained from Sigma–Aldrich Chemicals (St. Louis, MO). Penicillin, streptomycin, RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from Invitrogen Corporation (Carlsbad, CA). Dibutyryl cAMP (DB-cAMP), Adenosine cyclic 3′,5′-phosphorothioate triethylammonium salt (Rp-cAMPS), H-8 [(methylamino)ethyl-5-isoquinolinesulfonamide, hydrochloride], and cAMP assay kit were obtained from Calbiochem (San Diego, CA). The SuperFect transfection reagent was purchased from Qiagen (Hilden, Germany). The SignaTECT®cAMP-dependent PKA assay kit was purchased from Promega (Madison, USA). Antibodies against p65, IKKα, IKKβ, CRM1, MSK1, survivin, IAP 1, PKAα, Cox-2, Bcl-2, Bcl-xL, Cyclin D1, ICAM-1, goat-anti-rabbit IgG conjugated with HRP, ShRNA for PKA, and double stranded CREB oligonucleotide for gel shift assay were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against phospho-p65 was obtained from Cell Signaling Technologies (Danvers, MA). The 'Live & Dead' cells assay kit was purchased from Molecular Probe (Eugene, OR). Prof. B.B. Aggarwal of the University of Texas M.D. Anderson Cancer Center (Houston, TX) kindly gifted constructs for NF-κB-SEAP, p65, Cox-2-Luciferase, and green fluorescence protein (GFP). The 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine was prepared and provided by Prof. K.K. Narang, Dept. of Applied Chemistry, Banaras Hindu University, Varanasi, India.

2.2. Cell lines

The U-937 (human histiocytic lymphoma), HuT-78 (cutaneous T-cell lymphoma) and MCF-7 (human breast carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA, USA). Doxorubicin resistant and revertant MCF-7 cells were obtained from Prof. Kapil Mehta, M.D. Anderson Cancer Center, Houston, USA. Doxorubicin resistant cells were cultured in the presence of 2 μM doxorubicin (Devarajan et al., 2002). Cells were cultured in RPMI-1640 (for U-937) or MEM (for MCF-7, doxorubicin-resistant, and -revertant) medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.3. NF-κB, Sp1, and CREB activation assays

To determine NF-κB, Sp1, and CREB DNA binding activities, EMSA was conducted essentially as described (Manna and Ramesh, 2005). Briefly, 8 μg nuclear extract proteins were incubated with ^{32}P end-labeled double-stranded NF-κB, Sp1, or CREB oligonucleotides for 30 min at 37 °C, and the DNA–protein complex was separated from free oligonucleotide on 6.6% native polyacrylamide gels.

2.4. NF-κB-dependent reporter gene expression assay

The NF-κB-dependent reporter gene expression was measured as previously described (Manna and Ramesh, 2005). Briefly, cells were transfected with Qiagen SuperFect reagent with 1 ml medium containing *p65* plasmid (2.5 μg), reporter gene of NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (SEAP) (0.5 μg) or GFP (0.5 μg) constructs as described by Darnay et al. (1999). After 3 h of transfection, cells were washed and cultured for 12 h. Cells were visualized under fluorescent microscope and 27–30% cells were GFP positive for different combinations. After different treatments, cell culture-conditioned media were harvested, and 25 μl of each was analyzed for alkaline phosphatase activity essentially as per the CLONTECH protocol (Palo Alto, CA).

2.5. IKK, MSK1, and PKAα assays

The IKK was assayed by a method described previously (Sarkar et al., 2004). Briefly, IKK complex from whole-cell extract (300 μg) was precipitated with anti-IKKα and -IKKβ antibodies (1 μg each), followed by incubation with protein A/G-Sepharose beads (Pierce, Rockford, IL). After 2 h incubation, the beads were washed with lysis buffer and then assayed IKK activity using 2 μg of substrate GST-IκBα. Similarly, MSK1 or PKAα activity was assayed using GST-p65 full-length fusion protein. The samples were then analyzed in 8% SDS-PAGE. The gel was dried and exposed to PhosphorImager screen and scanned using Image Reader software (Fuji, Japan). PKA was also assayed with PKA assay kit (Promega).

2.6. Detection of Cox-2 by RT-PCR

After treatment, total RNA was extracted using TRIzol (Gibco BRL) and 1 μg of total RNA was reverse-transcribed using poly-T oligonucleotide and M-MuLV reverse transcriptase (Invitrogen). The PCR was performed using primers for Cox-2 (5′- TTCAAATGAGATTGTGGGAAAAT-3′, sense primer and 5′-AGATCATCTCTGCCTGAGTATCTT-3′, antisense primer) (Wu et al.,