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All-Trans-Retinoic Acid Induces Manganese Superoxide Dismutase in Human Neuroblastoma Through NFκB

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Abstract

Retinoids are signaling molecules that are involved in proliferation, differentiation and apoptosis during development. Retinoids exert their effects, in part, by binding to nuclear receptors, thereby altering gene expression. Clinical use of retinoids in the treatment of neuroblastoma is of interest due to their success in management of acute promyelocytic leukemia. Using the SK-N-SH human neuroblastoma cell line we investigated the effect of the differentiation agent, all-*trans*-retinoic acid (ATRA) on manganese superoxide dismutase (MnSOD) expression, an enzyme previously shown to enhance differentiation in vitro. Manganese superoxide dismutase mRNA, protein and activity levels increased in a time dependent manner upon treatment with ATRA. Nuclear levels of the NFκB proteins, p50 and p65, increased within 24 h of ATRA administration. This increase paralleled the degradation of the cytoplasmic inhibitor, IκB-β. Furthermore an increase in DNA binding activity to a NFκB element occurred within a 342 base pair enhancer (I2E) of the *SOD2* gene with 10 μM ATRA treatment. Reporter analysis showed that ATRA-mediated I2E dependent luciferase expression was attenuated upon mutation of the NFκB element, suggesting a contribution of this transcription factor in retinoid-mediated upregulation of MnSOD. This study identifies *SOD2* to be a retinoid responsive gene and demonstrates activation of the NFκB pathway in response to ATRA treatment of SK-N-SH cells. These results suggest signaling events involving NFκB and *SOD2* may contribute to the effects of retinoids used in cancer therapy.

Keywords

Retinoids; reactive oxygen species; antioxidants; differentiation; chemoresistance

INTRODUCTION

Neuroblastoma, a solid tumor of early childhood, represents one of the most challenging malignancies for therapeutic intervention. Differentiation therapy, using pharmacological amounts of retinoids, has been used successfully in the treatment of acute promyelocytic

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leukemia (APL), a rare form of acute myelogenous leukemia [1,2]. Clinical trials are ongoing to determine the efficacy of retinoid therapy in the treatment of neuroblastoma and breast carcinoma however, one limitation of ATRA is the development of chemoresistance. In order to enhance the effectiveness of this drug in the treatment of tumors, a thorough understanding of signaling pathways involved in its use is needed. Dietary-derived ATRA is the main signaling retinoid in vivo and one of the most potent differentiation inducers for human neuroblastoma in vitro, mediating its actions in part through nuclear receptors [3]. Recently, retinoids have been reported to generate reactive oxygen species (ROS), particularly superoxide, in target tissues, an effect which is coupled with differentiation [4]. In addition, NFkB, a transcription factor activated in response to numerous stimuli, including ROS, has been reported to play an essential role in neuronal differentiation [5] as well as contribute to chemoresistance [6].

We have reported that NFkB is essential for induction of MnSOD by several agents including the differentiating agent, phorbol 12-myristate-13 acetate [7]. Studies suggest an association between ROS, MnSOD activity, and cellular differentiation however, nothing is known about the role, if any, of MnSOD in neuronal differentiation. Therefore, we wanted to determine if ATRA treatment of the SK-N-SH cell line would lead to induction of the *SOD2* gene and if so, did this occur through NFkB. Manganese superoxide dismutase is an essential, mitochondrial localized antioxidant which could promote an environment conducive to differentiation by protecting against oxidative damage and ensuring sufficient ATP production. Alternatively, MnSOD may promote differentiation by modulation of the intracellular redox state, which in turn alters gene expression. We have previously shown that overexpression of MnSOD in a murine fibrosarcoma cell line results in a decrease in the activity of Jun associated transcription factors resulting in a subsequent downregulation of target protooncogenes [8]. Additionally, our group and others have shown that MnSOD transgenic cell lines, relative to wild-type cells, have significantly slower growth rates, decreased colony formation and exhibit morphological changes consistent with a differentiated phenotype [9-12].

In this study we have evaluated the effects of ATRA on MnSOD mRNA, protein and activity levels in neuroblastoma and have examined the role of the NFkB pathway in regulation of this antioxidant. Taken together the results implicate that retinoids can alter gene expression through modulation of NFkB activity in the SK-N-SH cells. The contribution of other possible mediators in the ATRA-induced NFkB and MnSOD activation such as the retinoid receptors will be investigated in subsequent studies.

MATERIALS & METHODS

Cell Line

The human neuroblastoma cell line (SK-N-SH) obtained from ATCC was maintained in minimal essential medium supplemented with 10% heat-inactivated bovine serum, 1% antibiotics, 1% non-essential amino acids and 1 mM sodium pyruvate. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Nuclear Extract Preparation

SK-N-SH cells were plated at a density of 6.0×10^5 cells/100 mm dish. During the exponential growth phase, cells were treated and nuclear and cytoplasmic extracts were prepared according to the method by Dignam and Roeder [13] with the inclusion of 35% glycerol and protease inhibitors (pepstatin, leupeptin, aprotinin) at 1 µg/ml. Protein concentration was determined by a colorimetric assay from Bio-Rad Laboratories (Richmond, CA) using bovine serum albumin as a standard.

Electrophoretic Mobility Shift Assay (EMSA)

A consensus oligonucleotide corresponding to the NFkB element within the second intron of the human *SOD2* gene was synthesized by Life Technologies, annealed and radioactively end labeled with [γ - 32 P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) as previously described [7]. Previous experiments using a mutated construct or supershift analysis showed specificity of binding to the NFkB element within the *SOD2* gene [7].

Western Blot Analysis

Western blot analysis was performed as previously described [7]. Sources and dilutions of antibodies used were as follows: α -p65, α -p50, α -I κ B- β (Santa Cruz Biotechnology, Santa Cruz, CA 1:1000) α -MnSOD (Upstate, Lake Placid, NY 1:2500) α -NMDAR1 (Chemicon International, Temecula, CA 1:750) and α -GAPDH (Trevigen, Gaithersburg, MD 1:5000). All secondary antibodies were from Santa Cruz Biotechnology.

Superoxide Dismutase Activity Assay

SOD activity was measured in whole cell lysates using an indirect competition assay between SOD and the indicator compound, nitroblue tetrazolium (NBT), for superoxide produced by xanthine/xanthine oxidase according to the method of Spitz and Oberley [14]. The activity of MnSOD was measured in the presence of sodium cyanide, an inhibitor of copper, zinc superoxide dismutase. Activity units were determined by defining 1 unit of SOD activity as that amount of sample protein capable of inhibiting the reduction of NBT by 50% of maximum inhibition. The data were normalized to protein content as determined by the method of Lowry [15].

Luciferase Activity

To determine if NFkB was responsible for the ATRA mediated increase in MnSOD protein, SK-N-SH cells were transfected with a construct (I2E-p7pGL3) which contains a 342 base pair (bp) fragment corresponding to part of the second intron of the human *SOD2* gene as previously described [7,16]. The constructs were a generous gift of Dr. Daret St. Clair at the University of Kentucky. Twenty four hours after plating, cells were transfected with either 3 μ g of the empty vector (pGL3-Luc) or test vector using a modification of the calcium phosphate method [17]. After 24 h, the cells were trypsinized and plated in 24 well plates (1.5×10^5 cells/well) and treated after an additional 24 h with 10 μ M ATRA. Cells were washed in PBS, lysed in reporter passive lysis buffer, and analyzed for Luc activity using the Luciferase Assay System (Promega) in a TD-20/20 luminometer (Turner Designs).

Real time RTPCR

Cells were plated (2×10^6 /p150 plate) in routine growth medium and after 24 h treated with 10 μ M ATRA for 1-4 days. After the initial treatment, media was changed every 48 h with the addition of fresh ATRA. To isolate RNA, cells were washed with PBS prepared in 0.1% DEPC water followed by addition of 1.5 mL TriReagent. Cells were scraped into RNase free tubes and centrifuged at $12,000 \times g$ for 10 min at 4°C. The supernatant was transferred to a clean RNase free tube, and let stand for 5 min at room temperature. Chloroform (300 μ L) was added and mixed for 15 sec. After centrifugation the supernatant was mixed with 750 μ L 100% isopropanol and left for 10 min at room temperature. After centrifugation the pellets were washed with 75% ethanol in DEPC water, dried and reconstituted in RNase free water. The RNA concentration was determined spectrophotometrically and quality was determined using the Agilent 2100 Bioanalyzer utilizing the RNA 6000 NanoAssay Kit (Agilent Technologies, Palo Alto, CA).

MnSOD mRNA relative levels were determined by quantitative RTPCR using Taqman Universal PCR Master Mix and primers from Applied Biosystems (Assay ID#HS00167309 MnSOD ID#4326317E GAPDH). Normalization was performed using GAPDH primers. Optimal primer concentrations were determined to be 200 ng/μL. Total RNA concentration in the quantitative RTPCR reaction was 20 ng/μL, and the thermocycler was an ABI 7700 Sequence Detection System programmed as follows: 48° for 30 min; 95° for 10 min; then 40 cycles of 95° for 15 sec and 60° for 1 min.

RESULTS

ATRA Increases MnSOD Protein in a Time Dependent Manner

Previous reports suggest a potential role for MnSOD in the process of cellular differentiation therefore, we wanted to determine if the potent neuroblastoma differentiation agent, ATRA, could induce *SOD2* expression. Fig. 1 shows a time dependent increase in MnSOD protein as a result of ATRA treatment. It should be noted that cells were plated and after 24 h treated with the retinoid (0-10 μM). Media was removed every 48 h and replenished with fresh media containing 0-10 μM ATRA. We consistently see a 1.3-1.4 fold increase in MnSOD protein with retinoid treatment (10 μM) over control after 24 h. This continues through 96 h where the increase in MnSOD protein is approximately 4 times that of vehicle treated cells (Fig. 1).

ATRA Increases MnSOD Activity in a Time Dependent Manner

To determine if the increase in MnSOD protein level correlated with an increase in enzyme activity upon ATRA treatment, we used the NBT method previously described by Spitz and Oberley [14]. Although we consistently see an increase in MnSOD protein at 24 h after ATRA treatment, we did not see a statistically significant increase in activity until 72 h of continuous retinoid exposure (Fig. 2). With 72 h of continuous ATRA exposure, cells had a 1.5 fold increase in MnSOD activity compared to vehicle treated cells. The change in activity was increased by approximately 2 fold compared to control cells with an additional 24 h of ATRA treatment (Fig. 2).

ATRA Increases MnSOD mRNA Levels

Retinoids modulate gene expression primarily through activation of nuclear receptors. Since MnSOD is known to be an inducible enzyme we wanted to determine if the increase in protein and activity correlated with changes at the message level. We used RTPCR analysis to show a time dependent increase in MnSOD mRNA levels when normalized to GAPDH (Fig. 3). Although not statistically significant, we consistently find a 1.3-1.4 fold increase in MnSOD mRNA levels with 48 h of 10 μM ATRA treatment. A 72 h exposure to ATRA increases MnSOD mRNA levels approximately 2.6 fold compared to vehicle treated cells while a 96 h administration results in a 4.7 fold increase (Fig. 3). The rise in mRNA was consistent with the retinoid-mediated increases seen in both the protein and activity levels of MnSOD.

ATRA Enhances Degradation of IκB-β

We have previously reported the regulation of MnSOD in various cell lines in response to cytokines and phorbol esters to be mediated through a NFκB element within the second intron of the *SOD2* gene [7,16]; therefore we wanted to determine if ATRA could activate the NFκB pathway in SK-N-SH cells. We performed a time course to see if 10 μM ATRA would lead to the degradation of one or both of the two prominent NFκB inhibitors in the SK-N-SH cell line, IκB-α and/or β. We found that the retinoid did not cause degradation of IκB-α but that the beta isoform started to degrade after 8 h of 10 μM ATRA exposure (Fig. 4A).

ATRA Treatment Leads to p50 and p65 Nuclear Translocation

Upon degradation of the NF κ B cytoplasmic inhibitors by the 26S proteasome, release of the sequestered NF κ B occurs with unmasking of the nuclear localization sequence in the p50-p65 complex, allowing for nuclear translocation. Following ATRA treatment we wanted to determine if the protein levels of both p50 and p65 increased in the nucleus consistent with I κ B- β degradation. As seen in Fig. 4B both p50 and p65 levels increased in the nucleus 24 h following retinoid treatment.

ATRA Increases NF κ B DNA Binding Activity in SK-N-SH Cells

Daosukho et al. [18] reported that modulation of the DNA-binding activity in favor of the p50-p65 heterodimer, rather than the p50-p50 homodimer, enhances NF- κ B-mediated induction of MnSOD. Therefore we wanted to determine if the increase in nuclear levels of both p50 and p65 would lead to increased DNA binding activity to the intronic NF κ B element in the *SOD2* gene. Isolation of nuclear extracts 24 h after ATRA treatment showed an increase in DNA binding activity to the intronic NF κ B element in the *SOD2* gene compared to vehicle treated cells (Fig. 4C, arrow).

ATRA Mediates Inducibility of I2E-Dependent Luciferase Expression in a NF κ B Dependent Manner

We have previously described the construction and use of pGL3-Luc reporter vectors containing a 342 bp intron 2 fragment linked to the basal promoter of the *SOD2* gene [16]. Using this reporter in either transient or stable transfection studies we have reported responsiveness of the *SOD2* gene to cytokines and a phorbol ester [7,16,19]. In this study we performed transient transfections of the I2Ep7pGL3 construct and saw a statistically significant 1.3 fold increase compared to the empty vector after 10 μ M ATRA treatment (Fig. 4D, $p < 0.05$). We saw a decrease in luciferase expression when we transfected the p7pGL3 construct in the presence of ATRA when compared to the empty vector suggesting possible repressor activity upon retinoid stimulation. We have previously shown a decrease in p7pGL3 mediated luciferase expression in the presence of known *SOD2* inducing agents [16]; however, the mechanism of this repression is currently unknown. Since we were able to show an increase in DNA binding activity to the intronic NF κ B site upon ATRA stimulation we used a construct with a specific mutation in this element and compared responsiveness to the I2Ep7pGL3 construct. As can be seen in Fig. 4D, mutation of the NF κ B site blocked the increase in luciferase expression mediated by 10 μ M ATRA ($p < 0.05$ compared to I2Ep7pGL3). It should be noted that ATRA treatment of the empty vector resulted in increased luciferase expression over cells receiving only vehicle. This is an effect which has been observed in other cell lines including MCF-7 [20]. Nevertheless, cells receiving the vector containing the I2Ep7 insert consistently showed a greater inducibility in the presence of ATRA.

SN50 inhibition of NF κ B attenuates ATRA mediated expression of MnSOD

To further confirm the involvement of NF κ B in the ATRA dependent increase in MnSOD protein expression, we treated SK-N-SH cells with 10 μ M retinoid +/- 5 μ g/ml SN50. The inhibitor peptide SN50 contains the nuclear localization sequence of the NF κ B subunit p50, and concentration dependently inhibits translocation of the active transcription factor complex p50-p65 into the nucleus [21]. Cytoplasmic fractions were collected after 48 h of treatment and were then analyzed for MnSOD protein. The SN50 inhibitor peptide (5 μ g/ml) reduced the basal levels of the endogenous antioxidant in the SK-N-SH cells and attenuated the ATRA dependent increase in the expression of MnSOD (Lanes 3 and 4, Fig. 5). These results confirm the contribution of NF κ B in ATRA regulation of the primary antioxidant.

Inhibition of NFκB attenuates ATRA mediated expression of the differentiation marker N-methyl-D-aspartate receptor subunit 1 (NMDAR1)

Preliminary experiments indicated that ATRA increases the expression of the differentiation marker NMDAR1 with just 48 h of exposure. In order to study the contribution of NFκB to ATRA mediated NMDAR1 expression, we treated SK-N-SH cells with 10 μM retinoid +/- 5 μg/ml SN50. Cytoplasmic fractions collected after 48 h of exposure were then analyzed for NMDAR1 protein. The SK-N-SH cells exposed to ATRA (10 μM) exhibited detectable levels of NMDAR1 which was absent in the vehicle treated cells (Lanes 2 and 1, Fig. 6). The SN50 inhibitor peptide had no effect on the NMDAR1 level, but attenuated the ATRA dependent increase in the expression of the differentiation marker (Lanes 3 and 4, Fig. 6). Taken together these results demonstrate that NFκB contributes at least in part, to the retinoid dependent increase in NMDAR1 expression, thereby playing a previously unrecognized role in ATRA-mediated differentiation in the SK-N-SH cells.

DISCUSSION

Although the role of MnSOD has not been defined in SK-N-SH cells treated with ATRA, there is a correlation between the enzyme's activity and the process of differentiation. Total SOD activity has been shown to increase as human monocytes differentiate in culture [22] and more specifically, while remaining low in nondifferentiating cells the activity of MnSOD increases as rat embryo fibroblasts differentiate into muscle or adipose cells [23]. Transfection with subsequent overexpression of human MnSOD into C3H10T1/2 and murine fibrosarcoma (FSa) cells enhanced differentiation into myoblasts upon treatment with 5-azacytidine [24-25].

To our knowledge this is the first study to identify MnSOD as a retinoid responsive gene in neuroblastoma and to show an increase in the mRNA, protein and activity level following ATRA administration. Although enzyme activity wasn't measured, Mantymaa et al. [4] reported a time dependent increase in MnSOD protein level upon ATRA treatment in acute myeloblastic leukemia (AML) cells. While we consistently find a 1.3-1.4 fold increase in MnSOD protein after 24 h of ATRA exposure, 72 h was required in the AML cell line. As can be seen in Fig. 1, MnSOD protein increases up through 96 h to approximately 4 fold over control with 10 μM ATRA. Interestingly, ATRA mediated changes in proliferation in the SK-N-SH cells does not occur until 72 h post treatment, suggesting upregulation of MnSOD protein, but not activity, prior to a decrease in the growth rate (data not shown). Upon ATRA treatment the mRNA, protein and activity of MnSOD increased prior to the formation of neurites, a hallmark of neuronal differentiation which occurs after 12 days of continuous retinoid stimulation. Pizzi et al. [26] reported that the neuronal differentiation markers NMDAR, NeuN, Tau and MAP2 increased in SK-N-SH cells upon 10 μM ATRA treatment 15 days post administration, well beyond the time of the increase in MnSOD protein and activity.

The mechanism responsible for retinoid-mediated MnSOD upregulation has not been identified, but the *SOD2* gene is known to be induced in response to superoxide production. Retinoids have been reported to increase ROS including superoxide and peroxides in AML cell lines [4,27-28]. The specific source of ROS in response to ATRA is yet to be determined however, studies by Suzuki et al. [29] using the synthetic retinoid, N-(4-hydroxyphenyl) retinamide, showed mitochondrial-mediated ROS generation in cervical carcinoma cells. Also, recent reports have suggested that ROS can modulate signal transduction and activate the NFκB pathway, thereby regulating the process of cell survival among others [30].

To our knowledge this is the first report to suggest that ATRA administration can regulate MnSOD expression through activation of the NFκB pathway. Activation of NFκB is a multistep process that involves phosphorylation of the IκB inhibitors, followed by ubiquitination and

degradation by the proteasome. In the SK-N-SH cells we saw a slow degradation of the I κ B- β isoform but no effects on the stability of the I κ B- α inhibitor (Fig. 4A), suggesting specificity in the activation of the NF κ B pathway mediated by ATRA. Previously we have reported inducer specific effects on the kinetics of degradation of the I κ B isoforms with I κ B- β having a more sustained decrease [31]. This may be explained by the fact that transcription of the I κ B- β gene, unlike the alpha isoform, is not increased by NF κ B activation [32]. In numerous models the NF κ B pathway and/or upregulation or overexpression of MnSOD has conferred resistance to a variety of chemotherapeutic drugs. Zhao et al. [25] showed that in murine fibrosarcoma cells over-expressing MnSOD, NF κ B was increased and if that signaling pathway was inhibited then differentiation markers such as MyoD decreased and the cells were more susceptible to 5-azacytidine mediated apoptosis. In another study Ahlemeyer et al. [33] reported a preservation of both copper, zinc and manganese-containing SOD protein levels in neonatal rat hippocampal cultures pretreated with retinoic acid (10 nM) prior to staurosporine treatment. This increase in protein correlated with an increase in total SOD activity and was thought to prevent staurosporine-induced apoptosis in the primary culture.

In Fig. 5, we demonstrate that the SN50 inhibitor peptide prevents upregulation of MnSOD in SK-N-SH cells exposed to ATRA, confirming NF κ B dependent regulation of the antioxidant by the retinoid. Studies suggest that SN50 (210 μ g/ml) can inhibit transcription factors other than NF κ B such as AP-1 [34]. In our experimental system 10 μ M ATRA does not alter transcriptional activity of AP-1 as determined by luciferase dependent reporter assays (data not shown). In addition the concentration of SN50 (5 μ g/ml) which was used in these experiments is reported to selectively inhibit NF κ B translocation to the nucleus [35]. The observed increase in NF κ B activity in response to ATRA may or may not be retinoid receptor dependent and will be investigated in future studies.

In the present study we show that ATRA increases NMDAR1 as early as 48 h post retinoid administration consistent with changes observed in the NF κ B pathway in the SK-N-SH cells. In addition we show that inhibition of NF κ B by SN50 can attenuate the ATRA dependent increase in NMDAR1, a well known marker of neuronal differentiation. Previous reports suggest that NMDAR1 is regulated by NF κ B, AP-1, CREB, and SP-1 elements in the promoter region of the human gene [36-39]. Taken together these results allow speculation that ATRA mediated differentiation involves pathways which are sensitive to cellular redox status. Future studies will be directed at determining whether MnSOD is essential to ATRA-mediated differentiation and whether this is dependent entirely on activation of the NF κ B pathway or is contingent on retinoid receptor activity.

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Figure 1.

Western blot analysis of MnSOD after ATRA (0-10 μ M) administration. 100 μ g of protein isolated from cytoplasmic fractions were analyzed over the course of 96 h. GAPDH was used as a loading control.

* $p < 0.05$; + $p < 0.001$

Figure 2.

MnSOD activity analysis in cell lysates treated with 0 or 10 μ M ATRA for 24, 72 or 96 h. Results are expressed as means $\pm$ S.E.M. for three independent experiments; * $p < 0.05$; + $p < 0.001$, significant difference between ATRA and control.

Figure 3. RTPCR analysis of MnSOD mRNA levels from cells treated with 0 or 10 μM ATRA for 24-96 h. Results are from three independent experiments; *p<0.05; +p<0.001, significant difference between ATRA and control.

A **B**

Figure 4.

(A) Western blot analysis of I κ B- β in cytoplasmic fractions of cells treated with 0-10 μ M ATRA. 100 μ g of protein was analyzed over the course of 24 h. GAPDH was the loading control; (B) Western blot analysis of either p50 or p65 in nuclear extracts (50 μ g) from cells treated with 0 or 10 μ M ATRA; (C) EMSA of 10 μ g of nuclear extracts isolated from cells treated with 0 or 10 μ M ATRA. Samples were collected after 24 h of ATRA treatment and incubated with a radiolabelled NF κ B oligonucleotide; (D) The effects of 10 μ M ATRA on the expression of the I2E/p7 dependent luciferase reporter gene are shown. Results are means $\pm$ S.E.M. for three independent experiments. Bars indicate respective comparisons with p values displayed.

Figure 5. Western blot analysis of MnSOD in cells receiving either ATRA (0-10 μM) or SN50 (5 μg/ml) or a combination of both. 50 μg of protein isolated from cytoplasmic fractions collected after 48 h of treatment was analyzed with GAPDH as a loading control. Results are from three independent experiments; *p<0.05 vs. control; #p<0.05 vs. ATRA.

Figure 6.

Western blot analysis of cells exposed to either 0-10 μ M ATRA or 5 μ g/ml SN50 or a combination of both. Protein (150 μ g) isolated from cytoplasmic fractions collected after 48 h of treatment was examined for NMDAR1 with GAPDH as a loading control. Results are from three independent experiments. *p<0.001 vs. control; #p<0.05 vs. ATRA.