acta medica

ORIGINAL ARTICLE

NFκB plays a role in basal levels of ROS in HCT116 cells

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Received 11 August 2016; accepted 5 October 2016; published online 20 October 2016

ABSTRACT

Objectives: Nuclear factor kappa B (NFκB), a transcription factor, controls expression of many genes to stimulate immune response, cellular growth and differentiation. It has been usually known as an anti-apoptotic factor and its low levels contribute to cytotoxicity of thymidylate synthase (TS)-directed cancer therapy. As TS inhibitors, fluoropyrimidines, fluorouracil (5-FU) and its nucleoside analog 5'-fluoro-2'-deoxyuridine (FdUrd) have been utilized in colorectal cancer therapy. One of their cytotoxic impacts on the therapy is elevating ROS levels in order to promote oxidative cell death. To understand whether NFκB is involved in TS inhibitors-mediated ROS generation, we investigated the effects of NFκB on drug-mediated ROS accumulation in HCT116 cells.

Materials and Methods: ROS measurement in response to TS inhibitors was done in HCT116 cell line and its NFκB deficient, HCT116-dnIkBα, and its revertent, HCT116-dnIkBα-R cells. Cells were treated with drugs in order to determine NADPH oxidase (NOX) activity and mRNA expression of p67phox.

Results: Loss of NFκB did not alter increase in ROS formation mediated by TS inhibitors and drug-induced mRNA expression of p67phox and NOX activity. Basal levels of ROS and p67phox mRNA were increased by NFκB deficiency.

Conclusion: Findings show that loss of NFκB has no effects on drug-induced ROS, p67phox mRNA expression and NOX activity. However, we have determined that NFκB is an important suppressor for basal ROS formation in correlation with the induction of p67phox mRNA.

Key words: NFκB, colon cancer, 5-FU, NADPH oxidase, ROS

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the disease rate is about 9% of the estimated cancer deaths in the US in 2015 [1]. Despite of advances in the therapy, survival rate (5 years) has not changed and chemotherapy still is the mainstay of treatment [2, 3]. In CRC therapy, fluoropyrimidines, in particular, 5-FU and its nucleoside analog 5'-fluoro-2'-deoxyuridine (FdUrd), are widely used agents [4, 5]. Investigating and understanding potential determinants of fluoropyrimidine metabolism could help to establish a strong rationale for improving therapeutic response.

Nuclear factor kappa B (NFκB) is a transcription factor that binds to specific sites on DNA and influences downstream expression of many genes involved in immune response, apoptosis, cell proliferation, adhesion and differentiation [6, 7]. Its function is inhibited by binding to the so-called Inhibitor of kappa-B-α (IκBα). Activation of NFκB follows the 26S proteasome-mediated degradation of IκBα that results from phosphorylation of IκBα by IκB kinase-β (IKKβ) [7]. NFκB signaling is a central coordinator of the innate and adaptive immune system. It is widely accepted that NFκB functions as an anti-apoptotic factor. Consistent with this, many investigations have shown that low levels of NFκB make cells vulnerable to the cytotoxic effects of thymidylate synthase (TS) inhibitors such as 5–FU, FdUrd [8-12].

In direct opposition to the generally accepted view, our laboratory has shown that NFκB promotes apoptosis during thymidylate deprivation, implicating a pro-apoptotic role in cells responding TS inhibitors [13, 14]. Other studies have also shown a pro-apoptotic function for NFκB, implicating its dual role in the regulation of apoptosis [15, 16].

The activation of NFκB is therefore a double-edged sword, and its function depends on context of signaling pathways.

To show that the apoptotic response to TS inhibitors is regulated by NFκB in human colon carcinoma cells, our lab utilized a NFκB deficient cell line, HCT116-dnIkBα, and its revertent, HCT116-dnIkBα-R [13]. HCT116-dnIkBα cell line is produced by stably transfecting HCT116 cells with a plasmid that expresses a dominant-negative mutant form of IkBα. To produce HCT116-dnIkBα-R cell line, HCT116-dnIkBα cells were passaged in non-selective media for 4 months and a revertent clone was obtained. It has been previously indicated that cell death mediated by TS inhibitors is ROS-dependent, and that NOX enzyme is involved [17]. Whether there is a link between ROS and transcription factor NFκB in apoptotic response to TS inhibitors is unknown. Here, we demonstrate that basal ROS levels were increased in HCT116-dnIkBα cells compared to HCT116 and HCT116-dnIkBα-R cells, however; the induced levels of ROS by TS inhibitors were not significantly changed in these cells. In addition to this finding, mRNA levels of p67phox were increased in HCT116-dnIkBα cells compared to HCT116 and HCT116-dnIkBα-R cells while the upregulation of p67phox mRNA by FdUrd was not significantly changed, indicating parallel changes with levels of ROS. We also demonstrate that drug-induced increases in NOX activity were similar in all three cells. Overall, these observations have led to the conclusion that NFκB acts on basal, but not drug-mediated ROS levels.

Material and Methods

Chemicals and Reagents

We obtained RPMI-1640 growth medium from Cellgro (Manassas, VA, USA), fetal bovine serum (FBS) from Atlanta Biologicals (Flowery Branch, GA, USA), cell permeable dyes, 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) and dihydroethidium (DHE) from Invitrogen/Molecular Probes Inc. (Eugene, OR, USA), paraformaldehyde from Alfa Aesar (Ward Hill, MA, USA), permount medium, G418, phosphate saline buffer (PBS) and bovine serum albumin (BSA) from Fisher Scientific (Fair Lawn, New Jersey, USA), Power SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA), EGTA from Research Organics (Cleveland, OH, USA), sucrose from Mallinckrodt Chemical (Paris, KY, USA), NADPH from Calbiochem/EMD Biosciences (San Diego, CA, USA), lucigenin from Enzo Life Sciences Inc. (Plymouth Meeting, PA, USA), 5-FU and FdUrd from Sigma-Aldrich Co. (St. Louis, MO, USA). Cell culture chamber slides were provided by VWR International LLC (Suwanee, GA, USA). Primers for p67phox were obtained from Integrated DNA Technologies Inc. (Coralville, IA, USA)

Cell culture

Human colon tumor cell line, HCT116 was kindly obtained from Dr. Michael G. Brattain. NFκB-deficient cell line, HCT116/dnIκB, and its revertent cell line, HCT116/dnIκB-R was previously described [13]. Cells were grown in RPMI-1640 medium supplemented with 10% heat-activated FBS at 37 °C in a humidified 5% CO_2 atmosphere. Additionally, HCT116/dnIκB cells were grown with 1mg/ml G418 to stabilize clone.

Fluorescence Microscopy

Intracellular ROS production was measured by oxidation of H_2DCFDA and DHE to fluorescent DCF by H_2O_2 and to fluorescent 2-hydroxyethidium (HE) by $O_2^{\cdot-}$ respectively. Cells were grown to 60% confluence in chamber slides. After incubation, they were washed twice with PBS and were fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were washed twice with PBS and separately stained with 5μM H_2DCFDA and DHE in dark followed incubation at 37°C for 30 min. Slides were kept in dark after this point. After incubation, cells were washed twice with PBS and chambers were separated from slides by slide separator. Coverslips were mounted on glass slides using permount medium. Images were acquired at room temperature using an inverted microscope (Axiovert 200, Carl Zeiss) equipped with a Plan-Apo 63×/1.40 objective and a charge-coupled camera (AxioCam HRm, Carl Zeiss) linked to AxioVision software (version 4.7, Carl Zeiss). To get the best visual reproduction, the image input levels were adjusted by stretching the histogram. In this point, image brightness and linearity were sustained. To analyze changes in DCF and HE fluorescence, ImageJ software (National Institutes of Health) was used to get fluorescence in numbers. Analysis of DCF and HE fluorescence was done by selecting cells with a pixel range for brightness and by subtracting background stained.

Flow Cytometry

Cells were grown to 80% confluence in 60 mm plates. After incubation, they were washed twice and suspended with fluorescence-activated cell-sorting (FACS) staining buffer (PBS with 1% BSA). 0.5 μM H_2DCFDA and DHE dyes were separately applied to cells for at 37 °C for 15min in dark. After this point, cells were kept in dark. They were washed twice, suspended with FACS buffer and analyzed by flow cytometer (FC 500 by Beckman Coulter Fort Collins Co). Histograms were analyzed in CXP software by obtaining X-mean value for each treatment. From 3 separate experiments, average X-mean was calculated for each treatment and data was normalized in fold increase of drug treatments to control.

Quantitative PCR

According to the manufacturer's suggestions, cDNA (1 μl) was amplified using Power SYBR Green PCR Master Mix. Reactions were run for one cycle at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 50 °C for 15s and 72 °C for 40s using an Applied Biosystems 7300 Real Time PCR System. All mRNA levels were quantified relative to GAPDH by measuring SYBR green incorporation during quantitative PCR using the relative standard curve method. Statistical analyses and calculations were done as described in the manufacturer's protocol. Relative changes in each gene levels between drug-treated and nontreated cells were expressed as fold induction compared with the basal level of expression in nontreated cells. Primers used for p67phox were; sense 5`ACC AGA AGC ATT AAC CGA GAC3`, antisense 5`TTC CCT CGA AGC TGA ATC AAG3` and for GAPDH, sense 5`TCC CTG AGC TGA ACG GGA AG3`, antisense 5`GGA GGA GTG GGT GTC GCT GT3`.

NADPH Oxidase Assay

Cells were washed twice with PBS after incubation. 10^5 cells for each cell line and treatment were suspended in 500 μl reaction buffer [50 mM phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose]. 100 μM NADPH was added as substrate. Light sensitive 5 μM lucigenin was added to reaction mixture in dark. Cells were kept in dark after this point and incubated at 37 °C for 10 min. Negative controls like adding only NADPH to the reaction mixture without cells and adding only cells to the mixture without NADPH were prepared. After incubation, NADPH oxidase activity was immediately detected by lucigenin-derived chemiluminescence using a luminometer (GloMax-20/20 luminometer, Promega, Madison, WI, USA). The levels of emitted chemiluminescence in every 15 s for 1 min of measurement were saved for each treatment. The signal reading was normalized by subtracting chemiluminescence obtained from negative controls and expressed as arbitrary light units per 10^5 cells.

Statistical Analysis

Experiments were independently repeated at least three times. Figures are representative of all the corresponding experiments performed. Results are presented as the mean ± SEM and a paired two-sided Student's t test was used to calculate statistical significance. Differences with p values of 0.05 or less were considered statistically significant.

Results

ROS formation mediated by TS inhibitors is not NFκB-dependent

NFκB regulates the expression of many genes for immune response, cell proliferation and differentiation, etc. Imbalance between NFκB and its inhibitor IκB has been associated with progress of many diseases, including cancer [7]. It is suggested that NFκB plays a pro-apoptotic role in apoptotic response to TS inhibitors [13, 14]. To determine whether it acts on oxidative or ROS-derived apoptotic response to TS inhibitors, we exposed parental line HCT116, NF-κB deficient cell line, HCT116-dnIkBα, and its revertent, HCT116-dnIkBα-R to TS inhibitors. Basal H_2O_2 and $O_2^{\cdot-}$ levels were higher in HCT116-dnIkBα cells as compared to HCT116, and were rescued in HCT116-dnIkBα-R cells. However, both H_2O_2 and $O_2^{\cdot-}$ levels induced by TS inhibitors were unchanged in all three lines (Figure 1).

Loss of NFκB does not alter mRNA levels of p67phox induced by FdUrd

As previously reported, p67phox induction is associated with in drug-mediated ROS generation in HCT116 cells [17]. In order to determine if NFκB plays a role in the induction of p67phox mRNA induced by TS inhibitors, we treated HCT116, HCT116-dnIkBα, and HCT116-dnIkBα-R cells with inhibitors and found that loss of NFκB increased basal levels of p67phox mRNA in HCT116-dnIkBα relative to HCT116 and HCT116-dnIkBα-R cells. However, NFκB deficiency did not significantly change mRNA levels of p67phox in drug-treated cells (Figure 2).

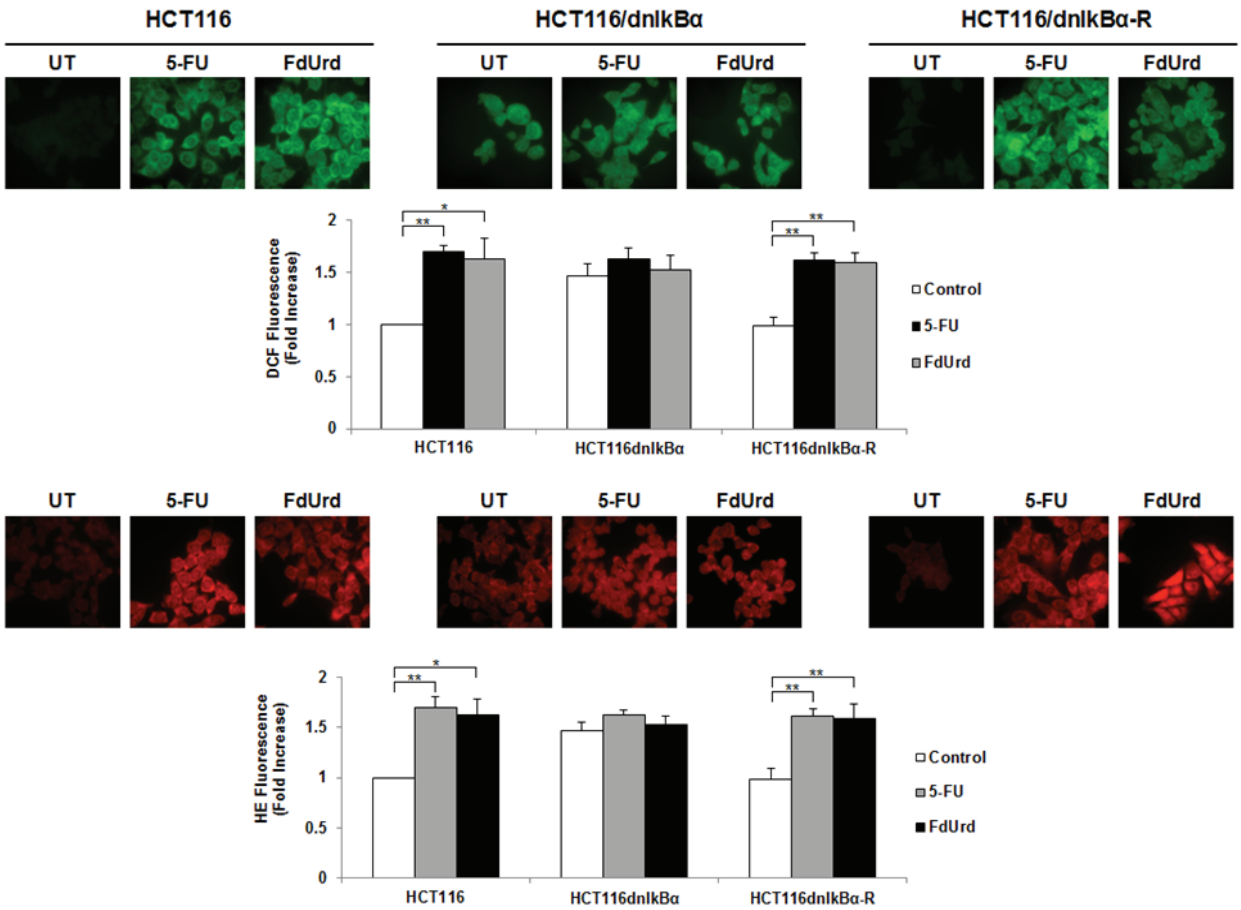

Figure 1. Loss of NFκB increases basal ROS levels. HCT116, HCT116/dnIkBα and HCT116/dnIkBα-R cells were treated with 10 μM 5-FU, and 10 μM FdUrd for 24 h and then stained by 5 μM H_2DCF-DA and 5 μM DHE. ROS detection was done in both fluorescence microscopy and flow cytometry. Pictures from fluorescence microscopy represent H_2O_2 formation (green) and $O_2^{\cdot-}$ (red). Bars from flow cytometry represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot-}$ (HE fluorescence) formation ± SEM from 3 experiments (UT: untreated, 5-FU: fluorouracil, FdUrd: 5'-fluoro-2'-deoxyuridine; * $p<0.05$, ** $p<0.01$).

NFκB deficiency does not affect increases in NOX activity mediated by TS inhibitors

In order to examine whether drug-mediated increases in NOX activity are altered by NFκB, we exposed HCT116, HCT116-dnIkBα, and HCT116-dnIkBα-R cells to TS inhibitors and measured NOX activity. We determined that loss of NFκB did not alter increases in NOX activity mediated by TS inhibitors in any of the three cells (Figure 3).

Discussion

NFκB is a multipotent transcription factor that regulates cell proliferation and differentiation, immune response, apoptosis and other integral cellular functions [6, 7]. Although several studies have reported it as having a pro-apoptotic role [13, 14], most investigations have indicated it to be an anti-apoptotic factor [8, 9, 18], showing its dual functions in the control of cell functions. We have found that NFκB deficiency increases basal levels of ROS and p67phox mRNA. However, it has no effect on drug-mediated ROS production and p67phox expression.

It is known that NFκB promotes cell death due to thymidylate deprivation resulting from TS inhibition. However, the mechanism underlying NFκB-mediated cell death is not well understood. Our data suggests that this does not occur via alterations in ROS induction mediated by TS inhibitors. We have shown that HCT116-dnIkBα, NFκB deficient, cells had high basal levels of ROS compared to HCT116 and HCT116-dnIkBα-R cells. This might suggest that absence of NFκB results in abnormalities in cells because it affects expressions of many genes in cellular metabolism, giving rise to oxidative stress. Due to loss of NFκB, TS inhibitors do not further induce ROS generation in HCT116-dnIkBα cells compared to HCT116 and HCT116-dnIkBα-R

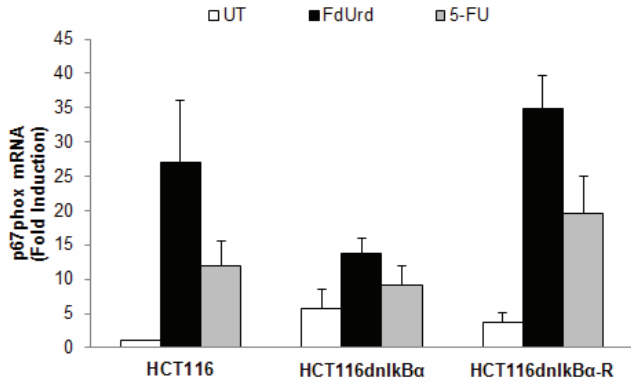

Figure 2. Basal levels of p67phox mRNA is increased by loss of NFκB. qPCR was used to measure mRNA levels of p67phox in total RNA isolated from HCT116, HCT116-dnIkBα and HCT116-dnIkBα-R cell lines cultured for 24 h ± 10 μM 5-FU and 10 μM FdUrd. GAPDH was tested as a loading control. Bars represent an average of fold increase ± SEM from 2 experiments.

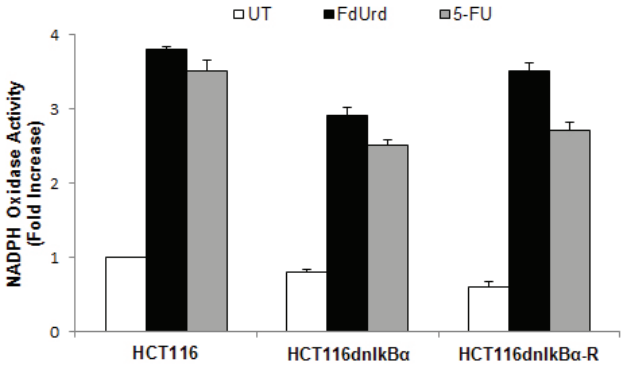

Figure 3. Drug-increased NOX activity is unchanged in NFκB deficiency. HCT116, HCT116-dnIkBα and HCT116-dnIkBα-R cell lines were grown ± 10 μM 5-FU and 10 μM FdUrd for 24 h and then were subjected to NOX activity assay. Lucigenin derived chemiluminescence was measured by luminometer. Bars represent fold increase of chemiluminescence ± SEM from 3 experiments.

cells, indicating the effect of NFκB on drug-mediated ROS generation.

We have also shown that basal levels of p67phox mRNA were increased in HCT116-dnIkBα cells relative to HCT116 and HCT116-dnIkBα-R cells, but induced mRNA levels by TS inhibitors is similar between these 3 cells. Additionally, increases in NOX activity are also similar in these cells, indicating that under the conditions of our experiments, NFκB has no role in NOX activity in response to TS-directed agents.

Under certain conditions, it is suggested that pro-apoptotic role of NFκB in thymidylate deficiency might be ROS-dependent, but it is not due to p67phox or NOX enzyme. It will be important to reveal the mechanism by which it promotes apoptosis in future work and to determine how the anti-apoptotic responses were silenced.

Acknowledgements

We acknowledge Dr. Franklin G. Berger for guidance with experiments. This work was supported by the National Institute of General Medical Sciences [Grant GM103336] and the National Cancer Institute [Grant CA44013].

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