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RESEARCH ARTICLE

The Importance of Protein Expression SOD2 in Response to Oxidative Stress for Different Cancer Cells [1]

Merve ALPAY [1] Kim WANJU [2]

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1 Ankara University, Faculty of Veterinary Medicine, Biochemistry Department, TR-06110 Ankara - TURKEY
2 University of Florida, Shands Cancer Center, Gainesville, FL 32611, USA

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Summary

As a causal relationship between DNA damage, repair mechanism disorders and cancer demonstrated by experimental and epidemiological data. Transforming process starts from DNA damage to oxidative stress is associated with mutational inactivation of suppressor genes, activation of oncogenes. Related to this aim, it is important to indicate how cancer cells react under oxidative stress through by SOD2 expression, besides presenting correlation between NF-kB mechanism. Cell lines were maintained and cultured as recommended by ATCC (American type culture collection) resource. Cells were grouped and after detecting optimum concentration by MTT test (cell viability assay), treated with DNA damage agent (MNNG) and antioxidant (tempol) for indicated time points. Western blot analysis revealed that cell lines with comparable levels of SOD2 (Superokside dismutase 2) protein expression. However, cells were collected to measure NF-kB (Nuclear Factor kappa B) enzymatic activity using luciferase expression by transfection way. We observed high constitutive NF-kB activity by using 20 µM MNNG although decreasing NF-kB during 30 µM tempol treatment. The results showed strong correlation between SOD2 expression and NF-kB activation.

Keywords: *Antioxidant, SOD2, DNA damage*

SOD2 Salınımının Farklı Kanser Türlerinde Oksidatif Strese Karşı Değişimi ve Önemi

Özet

DNA hasarı, onarım mekanizmasındaki bozukluklar ve kanser arasındaki nedensel ilişki deneysel ve epidemiyolojik veriler ile gösterilmiştir. DNA hasarının oksidatif strese dönüşüm aşamaları baskılayıcı genlerin mutasyonal inaktivasyonu ve onkogenlerin aktivasyonu ile ilişkilendirilir. Bu amaç doğrultusunda, kanser hücrelerinin SOD2 (Superoksit dismutaz 2) değişimine bağlı olarak oksidatif stres koşullarındaki etkileri ve bu mekanizmada NF-kB (Nükleer Faktör kappa B) transkripsiyon faktörünün önemi gösterilmiştir. Hücre hatları ATCC'nin (Amerikan tipi kültür kolleksiyonu) belirttiği prosedürlere uygun olarak kültüre edilerek saklandı. Hücreler gruplara ayrıldıktan sonra, MNNG ve tempol ajanlarının optimal konsantrasyonun hücre canlılık (MTT test) yöntemiyle belirlenmesiyle belirtilen zaman aralıklarında uygulandı. Hücre hatlarındaki farklı SOD2 protein düzeyleri western blot analizleri ile gösterilerek karşılaştırıldı. Bununla birlikte transfeksiyon yoluyla yapılan luciferase yöntemi kullanılarak NF-kB enzimatik aktivitesi belirlendi. Bunun sonucunda, 20 µM MNNG uygulandığında yüksek NF-kB aktivitesi gözlenirken, 30 µM tempol tedavisi ile NF-kB seviyesinin azaldığı görüldü. Sonuçlar SOD2 ekspresyonu ve NF-kB aktivasyonu arasında güçlü korelasyon gösterdi.

Anahtar sözcükler: *Antioksidan, SOD2, DNA hasarı*

INTRODUCTION

Oxidative stress is described as imbalance between production of free radicals and reactive metabolites, is called reactive oxygen species (ROS), and their eradication by protective mechanisms, referred as antioxidants [1]. It's also important from biomedical point of view which is related to human diseases such as neurodegenerative, cardiovascular, inflammatory, allergies, immune dysfunctions, diabetes, aging and cancer [2]. Oxidative stress has been

İletişim (Correspondence)

+90 505 7878799

mervealpay86@yahoo.com

implicated in both apoptosis and the pathogenesis of cancer providing contrived support for two notions: free radical reactions may be increased in malignant cells and oxidant scavenging systems may be useful in cancer therapy [3,4].

ROS (Reactive oxygen species) are induced by a variety of endogenous and exogenous sources [5]. At pathologically high levels, ROS cause damage to biological molecules, including DNA. It has been estimated that around 2×10^4 DNA damaging events occur in every cell of the human body everyday. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions (8-OH-G) have been noted in various tumours, strongly implicating such damage in the etiology of cancer [6]. Importantly, ROS does not always occur products of cellular metabolism sometimes they are generated by specific plasma membrane oxidases in response to growth factors and cytokines and serve as secondary messengers in specific signaling pathways [7,8]. ROS signaling is reversible, tightly controlled through a regulatory network. This network results from a concerted assembly of protein complexes, built through protein interactions mediated by interaction modules and posttranslational modifications in the binding partners [9]. Constitutive activation of cell survival signaling is a general mechanism underlying tumor development and resistance to therapy and constitutes a major clinical problem in cancer [8,9]. Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle. If the damage is determined to be beyond repair, the cell may undergo apoptosis to prevent mutations from being propagated [10]. An optimal cellular damage response requires both repair of damage and coordination of critical cellular processes such as transcription, translation, and cell cycle progression [11,12].

Antioxidant defences are built in a complex network of nonenzymatic and enzymatic components of the cell [13-15]. Enzymatic antioxidants include SOD, catalase, peroxiredoxin, and glutathione peroxidase (GPx) [16]. Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localised in the intermembrane space) and Mn-SOD (SOD2, localised in the matrix) [17-19].

Many conditions activating NF-kB are known to induce oxidative stress. In other words, these conditions increase the production of reactive oxygen species (ROS) such as superoxide (SOD), H_2O_2 and secondary reactive compounds. The reduction of SOD activity in neoplastic and transformed cells is regulated at the transcriptional level including NF-kB. Several studies have also shown different antioxidants inhibition of NF-kB in response to phosphorylation mechanism [20].

The aim of our study that cancer cells show upregulation SOD_2 under stress condition. We expect here to identify the ROS products and to gain more direct evidence for an involvement of ROS as messengers, also investigate whether changes in levels of enzymes that control intracellular ROS levels affect the activation of NF-kB.

MATERIAL and METHODS

Cell Experimental Procedures

Human cervical cancer (HeLa), human embryonic kidney cell (HEK293), human colorectal carcinoma (HCT116), human breast cancer (MDA-MB-231) cells were purchased from Dr. Brown lab (Shands cancer center FL/US) and cultured in 100 mm dish in DMEM (Invitrogen Co. USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (10.000 U/mL) at 37°C in constant atmosphere of 5% CO_2 in humidified air. All experiments were performed between passages 4 and 6.

Cell Treatments

Cells were grouped by several experimental applications. The plates were incubated for 24-48 h to allow complete 70-80% reattachment of the cells. Then detect subsequent optimum concentration on agent by MTT assay [21], cells were assigned to five groups and treated with 10 μM, 20 μM, 50 μM, 100 μM of MNNG (DNA damage agent) and control group. On the other side, cells were treated with 15 μM, 30 μM, 60 μM, 150 μM of tempol (antioxidant agent) and control group during 24 h, 48 hrs and 72 h. The absorbance of the plates was measured on ELISA microplate reader (Benchmark, BioRad) at a wavelength of 540 nm. After measurement, cell proliferation were also evaluated using inverted microscopy and then treated 20 μM MNNG for indicated time points of 48 h and 30 μM tempol for 7 days [22].

Western Blotting

Cells were washed with cold phosphate buffered saline (PBS) after treatment and lysed with RIPA buffer which contains 150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris. Then the cell suspension transferred into a precooled microfuge tube, incubated on ice at 10 min and centrifuged for 10 min at 4°C. The protein concentration of each sample were determined using BCA protein assay and bovine serum albumin (BSA, Roche applied science) was used as a protein standard. Equal amounts of protein were separated by 12.5% SDS-PAGE for SOD2. Then transferred electrophoretically onto nitrocellulose membranes [23].

Clarified membranes were blocked with 5% skim milk in TBS at room temperature for 1 h, the following stage subsequently incubation with primary antibodies dilutions anti-SOD2 (1:1000, Sigma) at a predetermined optimal concentration for 2-4 h at room temperature or overnight at 4°C. Membranes washed 3 times with rinsing buffer. Afterwards they incubated in proper secondary ab (rabbit) solution during 1 h at room temperature.

After three washes with TBS, the resulting immunocomplexes were visualized by enhanced chemiluminescence (Supersignal west pico, Fisher, USA) and exposed to X-ray film (Fuji medical X-ray film, Tokyo, Japan). Differences in protein loading were monitored by reprobing stripped membranes with anti-β Tubulin antibodies [24].

Luciferase Reporter Assay

To specify NF-kB activity by Luciferase assay in addition to protein expression experiments, cells were seeded in to 12 well plates at a density of $2x10^4$ cells per well with 2 mL of FBS+DMEM and incubated for 24 h before the transfection study. NF-kB was constructed within the lentiviral plasmid vector (pLKO,1.9kb) followed by transformation into *Escherichia coli*. From consisting several colonies, picked most of them for mini-preparation of the plasmids. Then digested with restriction enzyme (EcoRI) to check for orientation of insertion. Digested plasmid was run with the uncut version and a DNA marker on an agarose gel to determine the size of plasmid. Furthermore, NF-kB concentration (266.9 ng) was measured from vector *E. coli* plasmid DNA by spectrophotometric method. Briefly, MDA-MB-231, HeLa, HEK293, HCT116 were 70% confluence transiently transfected in 12 well plates by turbofect reagent (Fisher, USA) in a 1 mL medium containing 2.0 µg NF-kB promoter with the control plasmid DNA (reference DNA, 171.29 ng). Between experimental group (NF-kB) concentration and control group (reference DNA) concentration should be 1:20 ratio in each well of total volume. Transfections were performed in triplicate. NF-kB transfected cells were either left untreated or treated with 20 µM MNNG, 30 µM tempol. Twenty four hours after treatment, the conditioned medium was removed and lysed with dual luciferase lysis buffer for 15 min.

After 48 h of transfection, cells were collected to measure NF-kB enzymatic activity using luciferase expression in the clones appeared to be constitutive. Adding luciferase detection reagent for 5 min to measure firefly activity. Additionally cells were incubated with second reagent during 5 min for renilla determination. According to luciferase assay protocol, analysis of luciferase activity driven by the synthetic along NF-kB dependent reporter using an automated bioluminescence reader [25].

Statistical Analysis

We analyzed all of the data using the graphpad prism 5 statistical programs, then evaluated the statistical significance using group comparable Tukey test [26]. We considered that our results $P<0.05$ to be statistically significant.

RESULTS

To delineate the possible mechanism by which SOD2 mediates NF-kB activation, we studied the protein change effect of SODs under stress and normal conditions. The activation of SOD2 by treatment with MNNG induced activation of NF-kB, in another experiment, we examined the effect of specific inhibitor antioxidant (tempol) on SOD2 production which in turn inhibits NF-kB transcription factor *(Fig. 1)*.

Our results shown that the most common cancer cells have increased expression of SOD2 protein level while treating with DNA damage agent correlated well with a parallel increase in the enzymatic activity. Next, we determined the influence of decreased SOD2 expression during tempol treatment *(Fig. 2, Fig. 3)*. Time course and dose response studies using cells revealed that treatment with 30 µM tempol for 10 days is optimal for suppressing stress-related protein levels. We observed high constitutive NF-kB activation by using 20 µM MNNG (DNA damage agent) while optimal period although decreasing activity of NF-kB during 30 µM tempol (antioxidant) treatment *(Fig. 4)*. The results obtain as a strong correlation between SOD2 repression and NF-kB inhibition which is given in *(Fig. 5)*.

Moreover, western blot analysis revealed for different

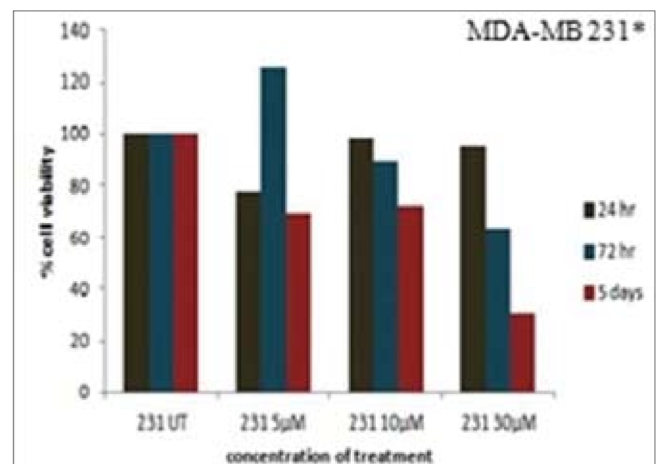

Fig 1. MTT test on MDA-MB 231 cells to detect optimal tempol concentration

Şekil 1. Optimal tempol konsantrasyonun MDA-MB 231 hücrelerinde MTT testi ile belirlenmesi

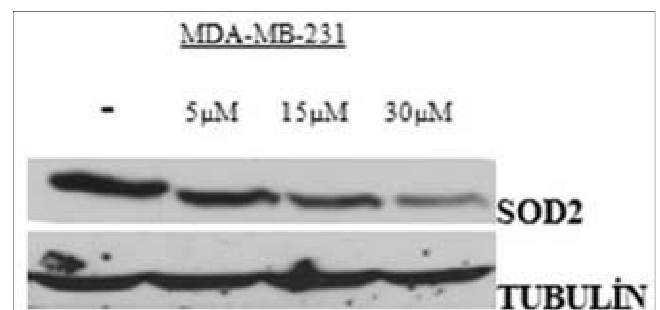

Fig 2. Different concentration of tempol effect on MDA-MB 231 cell with indicated time point

Şekil 2. Değişik zaman aralıklarında uygulanan farklı konsantrasyonlardaki tempol'ün MDA-MB 231 hücreleri üzerine etkileri

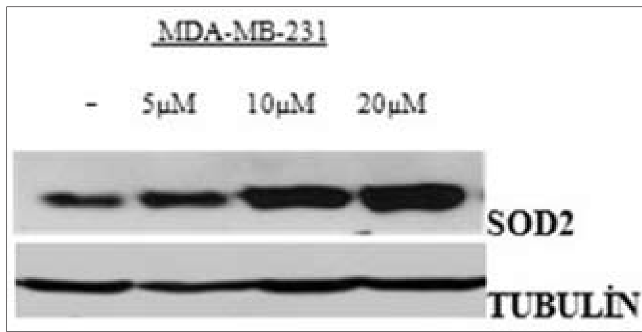

Fig 3. MNNG application on MDA-MB-231 cells to detect optimal concentration

Şekil 3. MDA-MB-231 hücrelerinde uygun MNNG konsantrasyonunun belirlenmesi

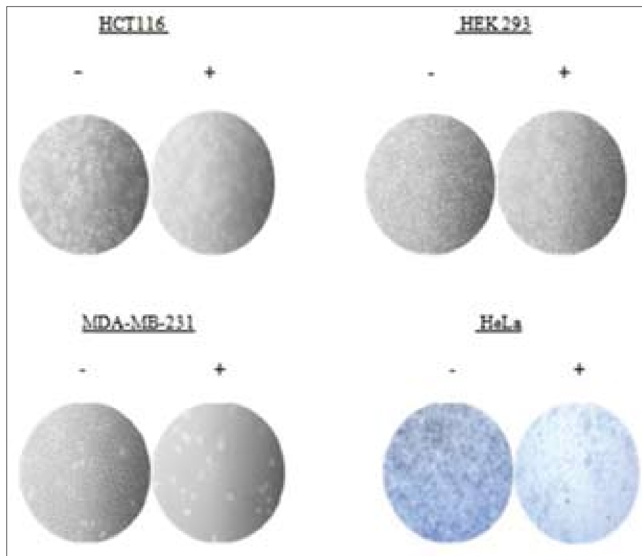

Fig 4. Cell morphology varying on antioxidant applications (30 µM tempol-48 h)

Şekil 4. Antioksidan uygulama koşullarında hücre morfolojisinin gösterilmesi (30 µM tempol- 48 saat)

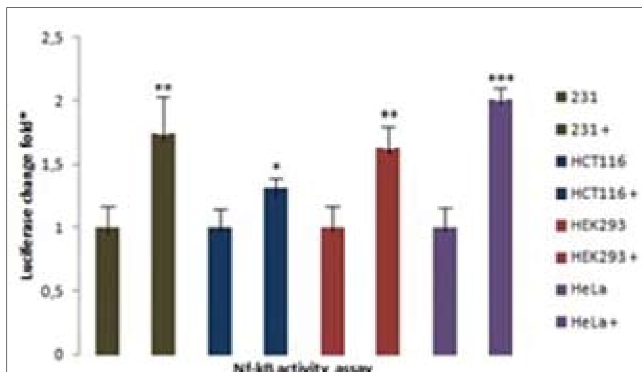

Fig 5. NF-kB transcription factor activity on different cancer cells (average fold for MDA-MB 231 cells while treat with MNNG have shown 1.7390±0.0877; for HCT116 cell 1.3111±0.0863; for HEK293 cell 1.6219±0.0845; for HeLa cell 2.0013±0.1439)

Şekil 5. Farklı kanser türlerinde NF-kB transkripsiyon faktör aktiviteleri (MNNG uygulanan MDA-MB 231 hücrelerinde ortalama değer gösterimi 1.7390±0.0877; HCT116 hücreleri için 1.3111±0.0863; HEK293 için 1.6219±0.0845; HeLa için 2.0013±0.1439)

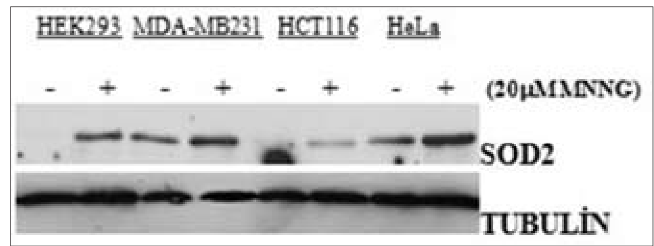

Fig 6. SOD2 expression of different cancer cell line under oxidative stress condition

Şekil 6. Oksidatif stres koşullarındaki farklı kanser türlerinde SOD2 protein ekspresyonları

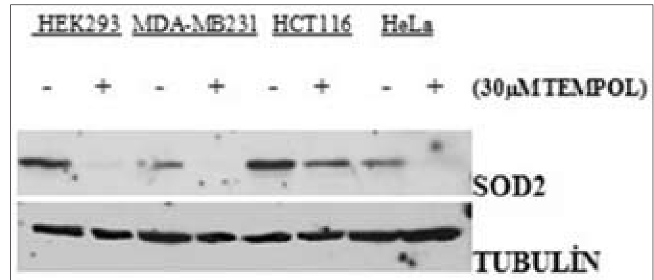

Fig 7. SOD2 expression of different cancer cell lines during antioxidant treatment

Şekil 7. Antioksidant uygulanan farklı kanser türlerinde SOD2 protein ekspresyonları

cancer cell lines have high basal levels of SOD2 protein expression. It is reasonable evidence that under oxidative stress condition, cells activate NF-kB loop and acting upregulation of SOD2 proteins *(Fig. 6, Fig. 7)*.

DISCUSSION

Our aim supports the ROS induced oxidative stress is involved in multistage process of carcinogenesis by genetic and epigenetic mechanisms [27,28]. Our study shows that when different type of cells treated by DNA damage agent, they all response to upregulation of oxidant related proteins.

The opposite affect for antioxidant treatment, we see downregulation that proteins. Evidence of our result, we detect NF-kB activity of different cancer types. The homeostasis mechanism for controlling ROS levels presented here is controlled by signalling pathways that can provide both negative and positive inputs on NF-kB [29,30]. Also have shown morphology and consistency on cells under different conditions.

Many cancer cells and cell lines have constitutive NF-kB activation, which enables malignant cells to escape apoptosis [31]. In contrast, activation of NF-kB in normal cells is transient, which prevents abnormal cell growth and survival [32].

Other studies show previously that, irrespective of their source or type, cancer cells selected for resistance to antioxidants exhibit high levels of SOD2 expression.

The data presented here suggest that SOD2 expression contributes to the development of antioxidant and metastatic phenotypes by inducing constitutive activation of NF-kB [33].

Recent research indicates that cells within a field defect characteristically have an increased frequency of epigenetic alterations and these may be fundamentally important as underlying factors in progression to cancer. Inherited germ line mutations in DNA repair genes similarly cause an increase in DNA damages due to a deficiency in repair capability, and these also cause increases in cancer risk. At least 34 inherited human DNA repair gene mutations increase cancer risk, including, for example, germ line mutations in the *BRCA1, XPC* and *MLH1* genes [34].

Controlling the cellular redox state is highly complex process governed by cellular factors and systems [35,36]. Oxidant signal, chronic inflammation, and cancer are closely linked. Antioxidants will be more useful for regulated oncogenesis and understanding pathway in future cancer research [37].

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