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ORIGINAL PAPER

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The role of superoxide anion in the regulation of epidermal growth factor or the expression and proliferation of its receptor in prostate cancer cell line PC3

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Abstract The purpose of this study was to investigate the role of superoxide anion(O_2^-) in the regulation of epidermal growth factor (EGF) or epidermal growth factor receptor (EGFR) expression and proliferation in the prostate cancer cell line PC3. Cell proliferation was tested by a 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay in the presence of O_2^-, EGF or their combination. Immunohistochemistry was carried out to assay the expression of EGF or EGFR. EGF or EGFR mRNA expression in the cells treated with O_2^- was examined by in situ hybridisation. The proliferation was significantly inhibited by O_2^- in a concentration-dependent manner ranging from 9 to 36 μmol/l nicotinamide adenine dinucleotide (NADH) combined with 2–8 μmol/l N-methylphenazonium methyl sulfate (PMS). The enhancement of proliferation induced by 5 ng/ml EGF was significantly overcome by O_2^-. Although O_2^- was not able to alter EGFR mRNA expression, O_2^- at the concentration of 18 μmol/l NADH and 4 μmol/l PMS reduced EGFR protein expression. O_2^- at the concentration of 18 μmol/l NADH and 4 μmol/l PMS can downregulate EGF and EGF mRNA expression.

Keywords Superoxide anion · Proliferation · Prostate cancer cell · Epidermal growth factor receptor · Epidermal growth factor

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Introduction

Reactive oxygen species (ROS) involved in the regulation of cellular proliferation and gene expression could act as biological messengers rather than as mediators of cellular damage [1]. Cell modulation by ROS depends on the relative concentration of exogenous superoxide or hydrogen peroxide in relation to the numbers of cells exposed as well as different the cell types involved [2]. The growth response to low concentrations of hydrogen peroxide is positive in BHK-21, 208F, HeLa and the mouse fibroblastic cells Balb 3T3 [16], but in certain situations they can be negative as in the mouse osteoblastic cells MC3t-E1 [13]. Tyrosine phosphorylation of three protein bands were stimulated in the presence of a nitric oxide generating system and epidermal growth factor (EGF) [14], indicating that ROS might modulate the action of cytokine. Engler found that O_2^- can inhibit DNA synthesis in Caco cells and EGF can restore DNA synthesis in oxidant-treated cells [4]. Whether ROS can modulate the expression of EGF and epidermal growth factor receptor (EGFR) is rarely reported.

In the early stages, prostate cancer growth is almost always androgen dependent, but eventually the tumour progresses to a more aggressive state in which growth is androgen independent [8]. This transition is a major obstacle to successful treatment not only in the prostate but also in many other tumours originating from hormone responsive tissues [11]. The role of EGF and its receptor as a mediator of prostate cancer cell growth has been intensely investigated in recent years. Many workers are now coming to recognise growth factors as playing a major role in the progression of androgen dependent to androgen independent prostate cancer growth [4]. The elucidation of the relation between ROS and prostate cancer would contribute to our understand of the mechanisms involved in prostate cancer and improve treatment. This study was undertaken to investigate the role of the superoxide anion (O_2^-) in the regulation of

EGF and EGFR in the prostate cancer cell line PC3. In addition, proliferation of PC3 cells was also assayed in the presence of O_2^-, EGF a combination of both.

Materials and methods

Chemicals

EGF was purchased from Biotin Biotechnology (China). Catalase (Cat) N-methylphenazonium methyl sulfate (PMS) and nicotinamide adenine dinucleotide(NADH) were obtained from Fluka (Switzerland). 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT), RPMI1640 and trypsin were purchased from Sigma. Fetal calf serum (FCS), sodium formate and diethylenetriaminepentaacetic acid (DETAPA) were obtained from Sino-American Biotechnology (China). RNA in situ hybridisation detection kit and immunohistochemistry kit of EGF and EGFR were provided by Boster Biotechnology (China).

Tumour cells

The human prostate cancer cell line PC3 was kindly provided by the Institute of Urology, Beijing Medical University. The cells were maintained in complete medium (CM), which consists of RPMI 1640, 100 U/ml penicillin, 100 U/ml streptomycin, 50 U/ml gentamycin, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and 10% heat-inactivated FCS at 37°C in a 5% CO_2-air environment. Tumour cells were harvested by overlaying the monolayer with a solution of 0.05% trypsin and 0.53 mmol/l EDTA and then resuspending them in CM. The cells were placed in 96-well plates at 3×10^3 cells per well in 150 µl CM for the proliferation assay.

The PC3 cells were placed in 24-well plate with 7×10^4 cells per well and a slide was placed at the bottom of each well. After incubation for 48 h, CM was replaced with 1.5 ml CM supplemented with 18 µmol/l NADH and 4 µmol/l PMS for another 48 h incubation. The slides were then washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min. After washing with water, the slides were stored at –20 C until further use.

Generation of O_2^-

The O_2^- was generated in the CM supplemented with 2–36 µmol/l NADH and 0.5–8 µmol/l PMS plus 426 U Cat, 0.1 mmol/l DETAPA and 0.4 mmol/l sodium formate in order to generate $O\text{-}_2$ only [20].

Proliferation assays

The proliferation of the PC3 cell was determined using the MTT assay. The medium containing PC3 cells in 96-well plates for 48 h was replaced by 250 µl test medium which comprised CM supplemented with different concentrations of EGF (0–25 ng/ml), O_2^- (CM supplemented with 0–36 µmol/l NADH and 0–8 µmol/l PMS) and their combinations. After culturing for 48 h, the medium was removed and the plates were washed with RPMI 1640 containing 2% FCS. RPMI 1640 medium containing 0.5% MTT and 0.5% FCS was then added to each well. After incubation for 4 h, the medium was replaced by 100 µl DMSO (Sigma). The absorbance(A) at 570 nm in each well was determined by using Bioelisa reader ELx800(Biokit, USA). The proliferation assay was repeated four times for four wells.

Immunohistochemistry

Slides were incubated in methanol containing 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase activity. Normal rabbit serum diluted in PBS was then applied for a further 5 min at RT. The primary monoclonal antibodies of anti-EGF or anti-EGFR were used at the dilutions of 1:100 in PBS and incubated for 1 h at 37 C. The slides were incubated with biotinylated rabbit anti-rat IgG for 30 min at 37 C and then with avidin–biotin–peroxidase complex. Slides were washed three times with PBS between each antibody incubation. Immobilised peroxidase was visualised by incubation with 0.25 mg/ml of its substrate 3,3′-diaminobenzidine tetrahydrochloride in 0.05 mol/l Tris-HCL buffer, containing 0.03% hydrogen peroxide for 10 min. Finally the sections were counterstained with haematoxylin.

Negative and positive controls were used with each staining batch. Positive controls were tumour sections known to be positive for the antigen used. As negative controls we used normal rabbit cells, inappropriate antibodies instead of primary antibody, or similar slides treated in the same way except that primary antibody was omitted. Positive samples were defined as those showing immunostaining in the cytoplasm or membrane (Fig. 1). Immunostaining for EGF or EGFR was independently evaluated by two observers. For each slide 1,000 cells were randomly controlled to determine the percentage of positive cells.

RNA in situ hybridisation

RNA in situ hybridisation(ISH) procedures were performed according to the manufacture's instructions. Slides were first incubated for 30 min in 0.5% hydrogen peroxide, washed with water, and then digested in 3% pepsin-HCL at 37 C for 5 min followed by

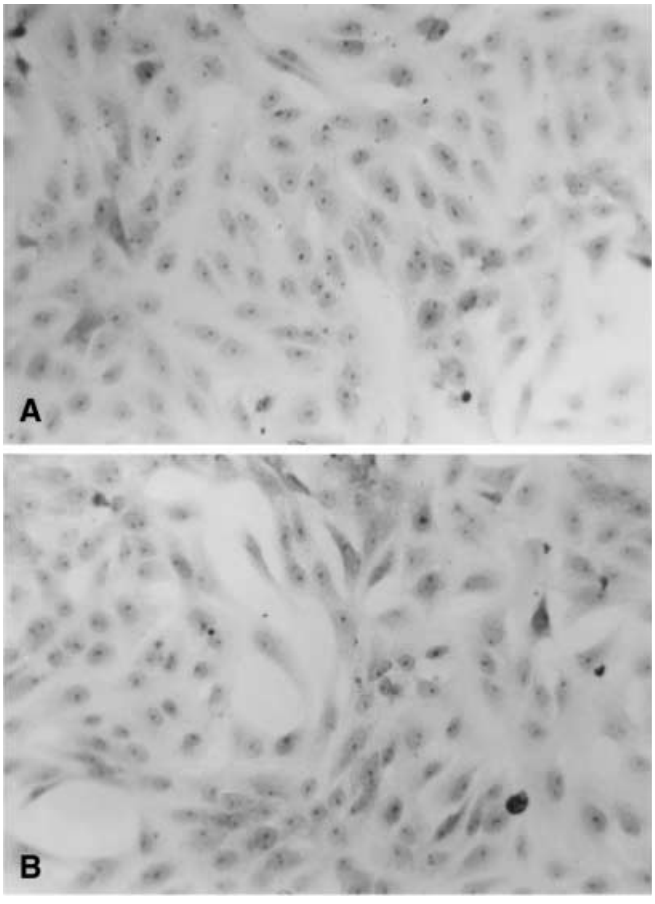

Fig. 1. A, B Immunohistochemical staining of EGF in PC3 cells. **A** EGF expression in PC3 cells treated with O_2^- at the concentrations of 18 µmol/l NADH and 4 µmol/l PMS. **B** control. Counterstained was done with haematoxylin and observations made with an Olympus BH-2 microscope at 400× magnification

washing with 0.5M PBS. Probes labelled with 20 μl digoxin, detecting EGF or EGFR, were added to slides, which were then incubated at 37 C overnight under coverslips before being sequentially washed three times in 2×SSC and 0.2×SSC at 30 C. After incubation with a protein blocker at RT for 20 min, 50 μl anti-digoxin antibody was pipetted onto the slides for 60 min incubation at 37 C followed by washing three times with 0.5M PBS. Fifty microlitres of biotinylated link antibody was added to the slides, which were further incubated at 37 C for 30 min and then washed three times with 0.5 M PBS. Finally, the slides were incubated first with a streptavidin–biotin–peroxidase complex at 37 C for 30 min, washed four times in 0.5 M PBS and then incubated in DAB/hydrogen peroxide for 10 min.

Negative and positive controls were used with each staining batch. Positive controls comprised rat sections known to be positive for the probe used. Negative controls were normal rabbit cells and similar slides treated in the same way except for omitting the probes. Positive samples were defined as showing immunostaining in the cytoplasm or membrane (Fig. 2). ISH for EGF mRNA or EGFR mRNA was independently evaluated by two observers. For each slide, 1,000 cells were randomly checked to determine the percentage of positive cells.

Statistical analysis

Statistical analysis of cell numbers was performed by analysis of variance. The statistical analysis of expression of EGF and EGFR or their mRNAs was carried out using a Wilcoxon test.

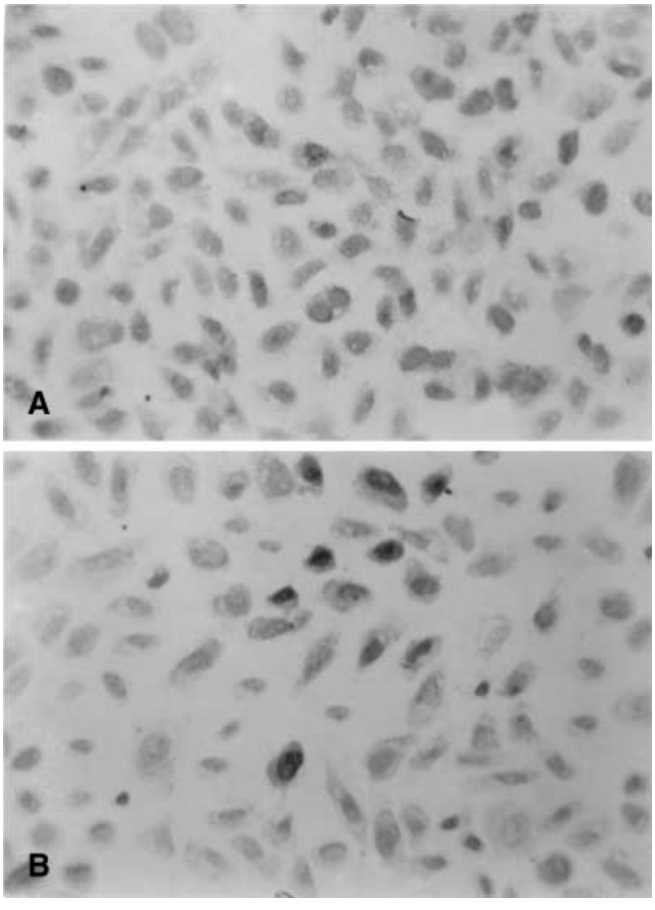

Fig. 2. A, B ISH staining of EGF mRNA in PC3 cells. **A** EGF mRNA expression in PC3 cells treated with O_2^- at the concentrations of 18 μmol/l NADH and 4 μmol/l PMS. **B** control. RNA in situ hybridisation observed using an Olympus BH-2 microscope at 400× magnification

Results

The effect of O_2^- on the proliferation of the prostate cancer cell line PC3 is shown in Table 1. O_2^- can inhibit the proliferation of the PC3 cell in a concentration-dependent manner, ranging from 9 to 36 μmol/l NADH and from 2 to 8 μmol/l PMS. Furthermore, the inhibition increased with the increase in the concentration of O_2^-.

EGF can induce the proliferation of PC3 cells in a concentration-dependent manner ranging from 0.2 to 25 ng/ml. The greatest enhancement occurred at the concentration of 5 ng/ml (Fig. 3, $P < 0.01$). The cell proliferation induced by EGF was inhibited by O_2^- in a concentration-dependent manner ranging from 9 to 36 μmol/l NADH and from 2 to 8 μmol/l PMS (Table 2).

O_2^- can downregulate the expression of EGF (Table 3, $P < 0.01$) and the mRNA expression of EGF in PC3 cell at the concentrations of 18 μmol/l NADH and 4 μmol/l PMS (Table 4, $P < 0.01$). Although O_2^- can downregulate the expression of EGFR (Table 3, $P < 0.01$) at the concentration of 18 μmol/l NADH and 4 μmol/l PMS, it does not affect the mRNA expression of EGFR (Table 4, $P > 0.05$).

Table 1. The inhibition of proliferation of PC3 cells by O_2^-. Each group contained four wells and the experiment was repeated four times

Concentration of NADH (μmol/l)	Concentration of PMS (μmol/l)	OD	*P*
0	0	0.702 ± 0.019	Control
2	0.5	0.698 ± 0.020	> 0.05
4	1	0.681 ± 0.021	< 0.05
9	2	0.582 ± 0.020	< 0.01
18	4	0.521 ± 0.023	< 0.01
36	8	0.504 ± 0.018	< 0.01

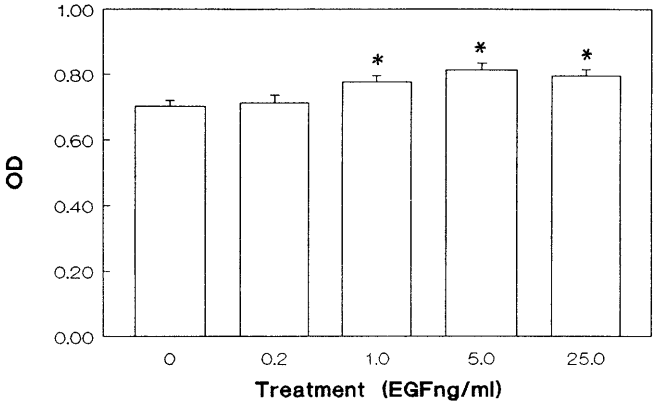

Fig. 3. Enhancement of proliferation of PC3 cells treated with EGF. Each group contained four wells and this experiment was repeated four times ($n = 4$, * indicates $P < 0.01$) compared with control (EGF = 0 ng/ml)

Table 2. Effect of superoxide anion on the proliferation of the PC3 cell line induced by EGF. Each concentration group contained four wells and the experiment was repeated four times

Concentration of NADH (μmol/l)	Concentration of PMS (μmol/l)	Concentration of EGF (ng/ml)	OD	*P*
0	0	0	0.702 ± 0.019	Control
0	0	5	0.813 ± 0.021	< 0.01
9	2	5	0.635 ± 0.018	< 0.01
18	4	5	0.620 ± 0.024	< 0.01
36	8	5	0.601 ± 0.019	< 0.01

Table 3. Regulation of EGF and EGFR expression in PC3 cells by superoxide anion. The PC3 cells treated with O_2^- (CM supplemented with 18 μmol/l NADH and 4 μmol/l PMS) for 48 h. The percentages of EGF and EGFR positive cells were determined. Each group contained four slides and the experiments were performed four times

Group	Control	O_2^-	*P*
EGF	43.8 ± 2.68	34.6 ± 2.63	< 0.01
EGFR	37.6 ± 2.83	30.2 ± 2.79	< 0.01

Table 4. Regulation of EGF and EGFR mRNA in PC3 cells by superoxide anion. The PC3 cells treated with O_2^- (CM supplemented with 18μmol/l NADH and 4 μmol/l PMS) for 48 h. The percentages of EGF mRNA and EGFR mRNA positive cells were determined. Each group contained four slides and the experiments were performed four times

Group	Control	O_2^-	*P*
EGF mRNA	36.8 ± 2.61	29.3 ± 2.55	< 0.01
GFR mRNA	32.1 ± 2.86	31.9 ± 2.79	> 0.05

Discussion

Superoxide and hydrogen superoxide are the main kinds of ROS derived from activated neutrophils and monocytes and play an important role in immunocyte-mediated tumour cell damage [19]. In addition, high levels of hydrogen peroxide are constitutively released from a wide range of human tumour cells [17]. Cells' modulation by ROS depends on the relative concentration of ROS exogenous superoxide or hydrogen superoxide in relation to the number of cells exposed, as well as the different types of cells. Thus it is important to assess how this modulation may be modified biologically in different cell types. In BHK-21 cells, the symptoms of programmed cell death are evident when the cells are exposed to 10–100 μmol/l hydrogen peroxide [3]. The study presented here also demonstrates that O_2^- significantly inhibits the proliferation of PC3 cells in a concentration-dependent manner. Nose et al. [13] suggested that hydrogen peroxide can act as a negative signal in mouse osteoblast cells late in the G1 phase. In addition, there have been a number of observations that indicate an apparent inverse relationship between levels of cellular lipid peroxidation, resulting from ROS, and rates of cell proliferation [2]. Lipid peroxides can break down non-enzymatically to yield a variety of carbonyls such as 4-hydroxynonenal which may play a central role in the downregulation of cell proliferation [2, 5].

EGF is a 53-amino-acid protein of 6045 MV originally found in rodents and humans [3, 7]. It has been shown to be a potent mitogen for a variety of cell types both in vitro and in vivo [3, 7]. Monteiro and Stern found that ROS could regulate the proliferation of cells by affecting EGF [12]. The study presented here demonstrated that EGF at 5 ng/ml stimulated the growth of PC3 cells and the proliferation of PC3 cells induced by EGF was significantly reduced by certain concentrations of O_2^-. Our data also demonstrated that O_2^- at the concentrations of 18 mol/l NADH and 4 μmol/l PMS can downregulate the expression of EGF and EGF mRNA. This may explain why O_2^- can inhibit the proliferation of PC3 cells.

EGFR is a 170 kDa cell surface glycoprotein which can bind with EGF and activate a tyrosine-specific protein kinase [10, 15]. The observation that $O\text{-}_2$ can downregulate the protein expression of EGFR but does not alter the mRNA expression of EGFR of PC3 cells raises the question of possible molecular mechanisms. ROS or lipid peroxidation reduces membrane fluidity, which is known to be essential for the proper biological functioning of membranes [18]. Most of the proteins that play key roles in proliferative signal transduction actually function in a membrane environment, or in close association with membranes. The activity of integral membrane protein which is modulated by ROS is also affected [9]. As O_2^- affected the function of the membrane and many enzymes which are essential for the translation of EGFR, the protein expression of EGFR was downregulated and perhaps the activity of EGFR was also affected. This is in agreement with the results of Goldkorn et al., who reported that ROS can downregulate the expression of EGFR, which is due to the activation of protein tyrosine kinase, and decrease the activity of protein kinase C-alpha by causing its translocation from the membrane to the cytoplasm [6].

It is only through binding with EGFR that EGF can enhance the proliferation of prostate cancer cells. Because the protein expression of EGFR was downregulated by O_2^-, the cell proliferation induced by EGF was inhibited.

This may demonstrate that O_2^- can regulate the cell proliferation of PC3 cells by affecting the expression of EGF and EGFR.

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