ORIGINAL ARTICLE

Alternation of retinoic acid induced neural differentiation of P19 embryonal carcinoma cells by reduction of reactive oxygen species intracellular production

Roman Konopka [1], Lukáš Kubala [1], Antonín Lojek [1], Jiří Pacherník [2]

1. Institute of Biophysics Academy of Sciences of the Czech Republic, Brno, Czech Republic
2. Institute of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

Correspondence to: Lukas Kubala, Ph.D.
Institute of Biophysics, Academy of Sciences of the Czech Republic
Kralovopolska 135, CZ-612 65 Brno, Czech Republic
TEL.: +420-541 517 117, FAX: +420-541 211 293
E-MAIL: kubalal@ibp.cz

Submitted: 2008-06-26 Accepted: 2008-09-04

Key words: **embryonal carcinoma cells; neural differentiation; redox status; antioxidants**

Neuroendocrinol Lett 2008; **29**(5):770–774 **PMID:** 18987612 NEL290508A15

Abstract

OBJECTIVES: Intracellularly generated reactive oxygen species (ROS) are thought to modulate redox sensitive signaling pathways and thus regulate cell physiology including proliferation and differentiation. However, the role of ROS in neuronal differentiation of embryonic pluripotent cells is unknown. For this reason, the modification of retinoic acid (RA) induced neuronal differentiation of mouse embryonal carcinoma cells P19 by selected ROS scavengers and flavoprotein inhibitor was evaluated.

METHODS: Intracellular ROS was evaluated by flowcytometry. Cellular redox status was evaluated based on total levels of reduced thiol groups in cells. The activity of the RA responsive element (RARE) was evaluated by luciferase reporter assay. The RA-induced neuronal differentiation was determined based on changes in the expression of protein markers characteristic for undifferentiated (Oct-4) and neuron-like cell differentiated cells (N-cadherin and III-beta tubulin).

RESULTS: RA increased the intracellular ROS production that was accompanied by a decrease in thiol groups in cells. The ROS scavengers and flavoprotein inhibitor reduced RA-induced ROS production, RA-induced activity of RARE, and it decreased the RA-induced expression of N-cadherin and III-beta tubulin.

CONCLUSIONS: Our data outline a role of ROS as important molecules in the transduction of an intracellular signal during the neuronal differentiation of ES cells.

Abbreviations

Apo	- apocynin
Asc	- ascorbic acid
DHR-123	- Dihydrorhodamine-123
DMEM	- Dulbecco`s modified Eagle`s medium
DPI	- diphenyleneiodonium chloride
EC	- embryonal carcinoma cells
EDTA	- ethylene diamine tetraacetic acid
ES	- embryonic stem cells
Glu	- glutathion
NAC	- N-Acetyl-L-cysteine
NGF	- nerve growth factor
RA	- retinoic acid
RARE	- retinoic acid responsive elements
RFU	- relative fluorescence units
RLU	- relative luminescence units
ROS	- reactive oxygen species
SDS	- sodium dodecyl sulphate

INTRODUCTION

Differentiation of embryonic cells is highly sophisticated process orchestrated by various factors (Keller 2005). Murine embryonal carcinoma (EC) cell line P19 provides an excellent culture system to investigate this process of cellular determination given that these EC cells are pluripotent and can be maintained in an undifferentiated state *in vitro*. Nonetheless, they can be induced to differentiate into embryonic and extra-embryonic cell types through a variety of procedures, including aggregation and treatment with various drugs. Retinoic acid is widely used to study the commitment of P19 cells to neural lineage (Bain *et al*., 1994; Pachernik *et al*., 2005; Pachernik *et al*., 2007).

Recently, a wide range of data suggests the importance of the status of intracellular redox in cell differentiation (Li *et al*., 2006; Sauer *et al*., 2000; Sauer & Wartenberg, 2005; Schmelter *et al*., 2006; Suzukawa *et al*., 2000; Yang *et al*., 2005). At low concentrations ROS generated intracellularly can alternate redox sensitive signaling molecules that are involved in signal transduction cascades of numerous growth factor-, cytokine-, and hormone-mediated pathways, and regulate biological effects such as cell proliferation, differentiation and apoptosis (Chisu *et al*., 2006; Li *et al*., 2006; Sauer *et al*., 2005). Interestingly, the role of ROS in cardiogenesis and cardiovascular differentiation of mouse embryonic stem (ES) cells has already been shown (Li *et al*., 2006; Sauer *et al*., 2005; Schmelter *et al*., 2006). Furthermore, it was suggested that embryonic bodies formed from embryonic ES actively generated ROS presumably through activity of NADPH oxidases (Li *et al*., 2006; Sauer *et al*., 2000; Sauer *et al*., 2005). Conversely, the role of ROS in RA-induced neural differentiation of embryonic pluripotent cells is unknown. Thus, we investigated the modification of RA-induced neural differentiation of pluripotent mouse embryonal carcinoma cells P19 by modulating the cellular redox status. Selected ROS scavengers glutathione (Glu), N-Acetyl-L-cysteine (NAC), ascorbic acid (Asc), and apocynin (Apo) or flavoprotein inhibitor diphenyleneiodonium chloride (DPI) were tested to modulate intracellular redox state and differentiation of P19 to neural-like cells.

MATERIALS AND METHODS

Cell culture

EC P19 cells were purchased from the European Collection of Cell Culture, Wiltshire, UK. Embryonal carcinoma P19 cells were cultured and differentiated by RA as described previously (Pachernik *et al*., 2005; Pachernik *et al*., 2007). Briefly, cells were cultured on tissue culture dishes pre-treated for 5 minutes by a 0.1% aqueous solution of gelatin from porcine skin (Sigma-Aldrich, Germany), in Dulbecco`s modified Eagle`s medium (DMEM) containing 10% fetal calf serum, 0.05 mM β-mercaptoethanol, and 0.045 mg/ml gentamycin. Under serum-free conditions, P19 cells were cultured in DMEM/F12 (1:1) media supplemented with the Insulin Transferring Selenium supplement (Gibco, USA) and antibiotics as described above. For experiment, P19 cells (5×10^3 per cm^2) were seeded on the gelatinized dishes and cultured in complete medium for 24 hours. The medium was replaced for serum-free for over night incubation. Further, cells were treated by RA (0.2 μM) for 1 hour and consequently by NAC (5 mM), Glu (5 mM), Asc (2 mM), Apo (1 mM), or DPI (200 nM) (all Sigma-Aldrich, Germany) for 2 hours for ROS analysis, for 12 hours for -SH group analysis, and for 24 hours or 8 days for protein expression analysis. Selected concentrations of RA, ROS scavengers and DPI did not reveal significant toxicity as tested previously (data not shown).

Reporter gene assay

Transient transfections of P19 cells by luciferase reporter pRAREβ2-TK-luc plasmid (provided by Christopher Glass, University of California, San Diego, La Jolla, CA, USA) were performed by electroporation as described in (Pachernik *et al*., 2005). Twenty-four hours after transfection, the cells were treated with RA and selected ROS scavengers as well as DPI as described above. Thirty-six hours after transfection, the cells were assayed for luciferase activity according to the manufacturer's instructions – Luciferase Assay System (Promega, USA).

Western blot analysis

Stem-cell marker Oct-4, the neural cell-adhesion molecule N-cadherin, and III-β tubulin were quantified by Western blot analysis as described previously (Pachernik *et al*., 2002; Pachernik *et al*., 2005; Pachernik *et al*., 2007). The rabbit polyclonal anti-Oct-4 (Santa Cruz, USA), mouse monoclonal anti-N-cadherin (BD Biosciences, USA), mouse monoclonal anti-III-β tubulin (Exbio, Czech Republic), and goat anti-mouse or anti-rabbit IgG HRP labeled antibodies were employed. Protein equal loading was confirmed by determination of β-actin (data not shown).

Detection of -SH groups

Cells were washed twice with PBS with 600 μM desferoxamine and 10 mM ethylene diamine tetraacetic acid (EDTA), lysed by 2% SDS with 10 mM EDTA and 600 μM desferoxamine and sonicated 2 times for 10 s on ice. 0.1 ml of 0.01 M dithionitrobenzoic acid was added to 9.9 ml 0.2 M Tris pH 8.2. 230 μl of this buffer was mixed with 20 μl of sample and incubated for 30 min at RT (Ondrejickova *et al*., 2006). Absorbance was measured at 412 nm on microplate reader Spectra Rainbow (Tecan, Austria). The amount of total -SH groups was adjusted to the level of protein level measured by BCA Protein Assay (Pierce, USA).

Flow-cytometry analysis of ROS

Cells were washed and incubated with 10 μM Dihydrorhodamine-123 (DHR-123) (Sigma-Aldrich, Ger-

many) (Stritesky *et al*., 2006) in serum free DMEM/F12 at 37 °C for 30 min. Then the cells were harvested and cell suspension placed on ice. At least ten thousand cells were analysed using a flow cytometer FACSCalibur (BD Bioscience) within 20 min. The geometric mean of relative fluorescence units (RFU) was quantified for each sample.

RESULTS

RA-induced ROS production and reduced thiol groups in P19 cells

The incubation of cells with RA induced significant induction of intracellular ROS production measured by DHR-123 (Figure 1). This effect was inhibited by NAC, Glu, Ask, and DPI. Apo did not reduce intracellular fluorescence of DHR-123, however, this could be a methodological artifact caused by nonspecific interaction of Apo with DHR-123 (Vejrazka *et al*., 2005). The RA-induced increase of intracellular oxidative state in P19 cells was confirmed by determining intracellular -SH groups as markers of intracellular redox status. Interestingly, RA significantly reduced the amount of -SH groups (control cells – 16.8 mmol/mg protein vs. RA treated cells – 9.1 mmol/mg protein) suggesting a RA-stimulated oxidation of intracellular pool of reduced glutathione.

Decrease of ROS production downregulated RARE activity

To further characterize the mechanism of modulation of RA-induced neural differentiation by a decrease in ROS production, the activity of RA-directed promoter was evaluated. In accordance with our hypothesis, the applied scavengers and flavoprotein inhibitor decreased the activity of RARE (Figure 2).

Decrease of ROS production reduced RA-induced neural differentiation

RA induces neural differentiation of P19 cells as was determined by an increased expression of N-cadherin and III-β tubulin and the downregulation of Oct-4 (Figure 3). All applied ROS scavengers (Glu, NAC, Apo) and the flavoprotein inhibitor (DPI) downregulated the RA-induced the expression of N-cadherin and III-β tubulin. Simultaneously, Glu, NAC, Apo, and DPI downregulated expression of Oct-4 (Figure 3).

DISCUSSION

For the first time this data showed a significant suppression of RA-induced neuronal differentiation of P19 cells by various ROS scavengers and the flavoprotein inhibitor DPI. RA-induced ROS production accompanied by a decrease of reduced thiol groups which were inhibited by ROS scavengers and DPI. The decrease of ROS production downregulated RARE activity as well as that of the expression of N-cadherin and III-β tubulin. However, impact of individual redox modulators on the N-cadherin expression was different with the most prolong effect of Asp, Apo and DPI, in contrast to NAC which effect diminished at the long time period. This could be connected with different effects of tested compounds on redox sensitive signaling pathways controlling N-cadherin expression. On the other hand, the decrease of intracellular ROS production did not prevent RA-induced decrease of Oct-4 expression suggesting a differentiation of P19 cells to non-neural lineages (Smith. *et al*., 2000; Pachernik *et al*., 2002).

Recently, the role of ROS in neural differentiation was shown with model of pheochromocytoma PC12 cells. Suzukawa *et al*., observed with PC12 cells that a

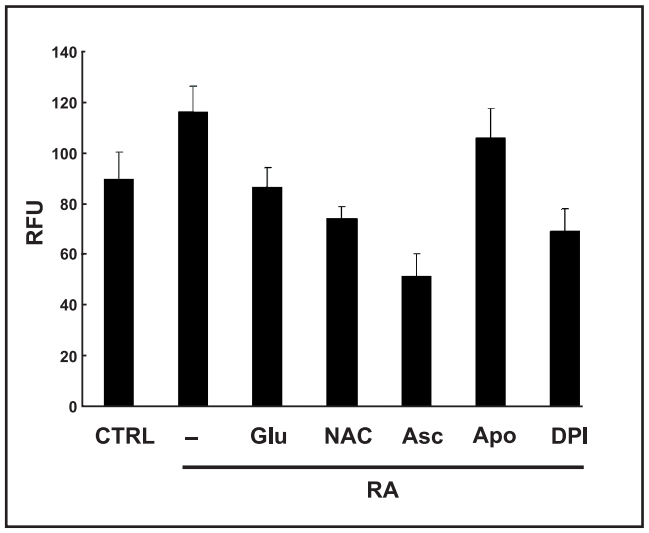

Figure 1. Flowcytometric analysis of ROS production measured by oxidation of DHR 123 in P19 cells treated by 0.2 μM RA and 5 mM Glu, 5 mM NAC, 1 mM Apo, 2 mM Asc and 0.2 μM DPI. The data represent mean ± standard error of mean (SEM) from at least three independent experiments.

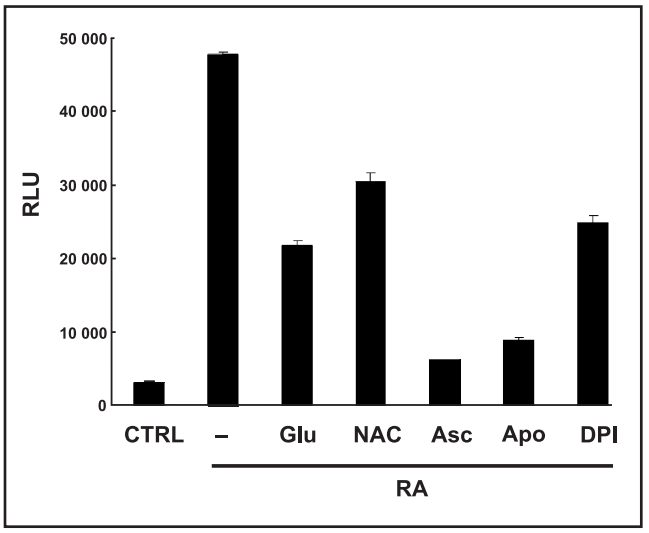

Figure 2. Analysis of RARE activity in P19 cells transiently expressing pRAREβ2-TK-Luc after treatment by 0.2 μM RA and 5 mM Glu, 5 mM NAC, 1 mM Apo, 2 mM Asc and 0.2 μM DPI for 12 h. The data represent mean ± SEM from at least three independent experiments.

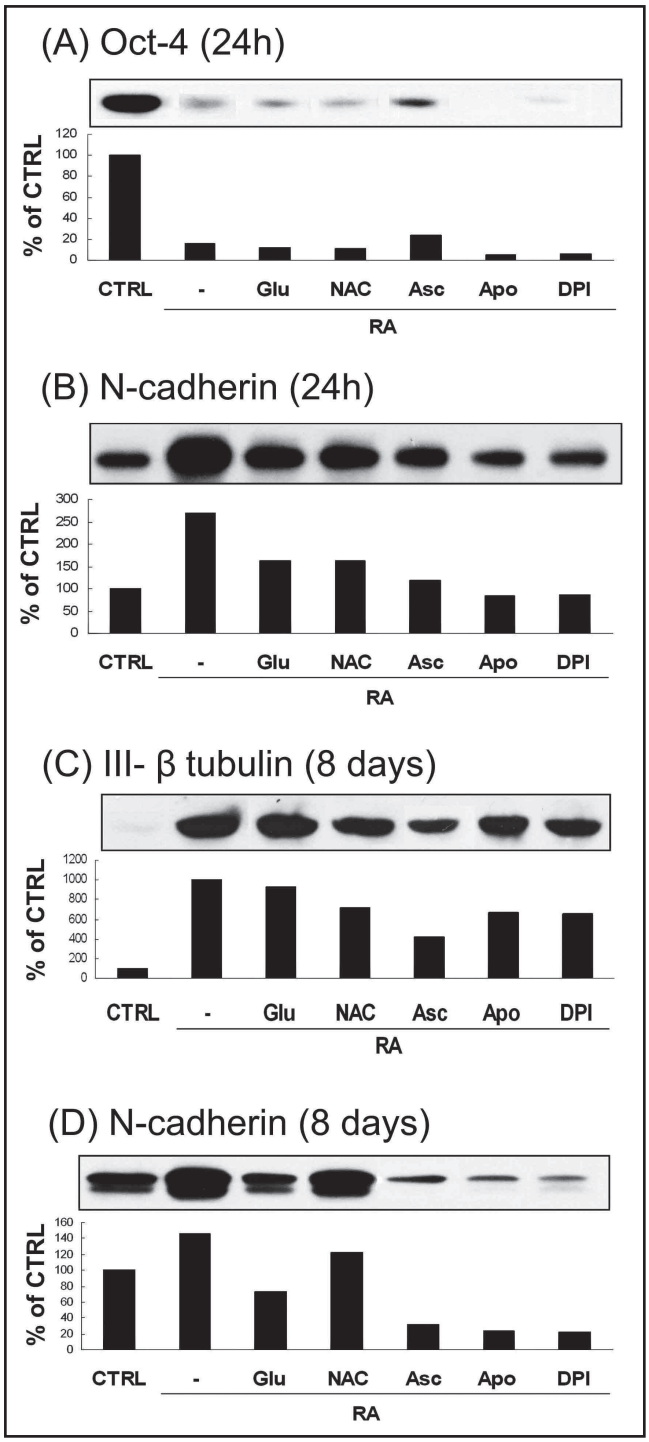

Figure 3. Western blot analysis of neural differentiation markers (**A**) Oct-4 (24 hours); (**B**) N-cadherin (24 hours); (**C**) III-beta tubulin (8 days); (**D**) N-cadherin (8 days) in P19 cells treated by 0.2 μM RA and 5 mM Glu, 5 mM NAC, 1 mM Apo, 2 mM Asc and 0.2 μM DPI for given periods of time. Representative western blot analysis with densitometric evaluation presented as percentage of control cells incubated in media with serum (CTRL) is shown.

neurite outgrowth induced by a nerve growth factor (NGF) was blocked significantly by NAC, DPI and also catalase (Suzukawa *et al*., 2000). These authors together with Yang *et al*., suggested that the intracellular ROS, particularly H_2O_2, acted as an intracellular signal mediator for NGF induced neuronal differentiation (Yang *et al*., 2005).

Further, a role of ROS in embryonic pluripotent ES cells differentiation to various lineages is suggested by supporting data of other researchers. It was shown that cardiomyogenesis and endothelial cell differentiation within embryoid bodies derived from embryonic stem cells was dependent on ROS (Li *et al*., 2006; Sauer *et al*., 2000; Schmelter *et al*., 2006). The cardiomyogenesis of mouse ES cells was accompanied by an increase in intracellular ROS production. Further, this ES cell differentiation was inhibited by DPI and a free radical scavengers and in contrast it was rescued by a pulse of low concentrations of H_2O_2 or menandione (Sauer *et al*., 2005; Schmelter *et al*., 2006).

Our data outline a role of ROS as important molecules in transduction of intracellular signals during neuronal differentiation of pluripotent embryonal cells. These data can contribute to our knowledge about the role of dysregulation of ROS production and the modulation of redox environments overall in pathogenesis of nervous system disorders including Parkinson's diseases and other brain defects (Ebadi *et al*., 1998; Ujhazy *et al*., 2006).

ACKNOWLEDGEMENTS

This work was supported by grants from Czech Science Foundation 524/06/1197, 301/08/0717 and research plans AV0Z50040507 and AV0Z50040702.

REFERENCES

1 Bain G, Ray WJ, Yao M, Gottlieb DI (1994). From embryonal carcinoma cells to neurons: the P19 pathway. Bioessays. **16**: 343–348.
2 Ebadi M, Rodriguez-Sierra J, Norton N (1998). Glutathione and Metallothionein in Neurodegeneration-Neuroprotection of Parkinson's Disease. Neuroendocrinol Lett. **18**: 111–122.
3 Chisu V, Manca P, Zedda M, Lepore G, Gadau S, Farina V (2006). Effects of testosterone on differentiation and oxidative stress resistance in C1300 neuroblastoma cells. Neuroendocrinol Lett. **27**: 807–812.
4 Keller G (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev. **19**: 1129–1155.
5 Li J, Stouffs M, Serrander L, Banfi B, Bettiol E, Charnay Y, Steger K, Krause K-H, Jaconi ME (2006). The NADPH Oxidase NOX4 Drives Cardiac Differentiation: Role in Regulating Cardiac Transcription Factors and MAP Kinase Activation. Mol Biol Cell. **17**: 3978–3988.
6 Ondrejickova O, Rapkova M, Snirc V, Dubovicky M, Jariabka P, Zacharova S, Stolc S (2006). Content of protein carbonyl groups in gerbil brain after reversible bilateral carotid occlusion: effect of 2,3-dihydromelatonin. Neuroendocrinol Lett. **27 Suppl 2**: 156–159.
7 Pachernik J, Esner M, Bryja V, Dvorak P, Hampl A (2002). Neural differentiation of mouse embryonic stem cells grown in monolayer. Reprod Nutr Dev. **42**: 317–326.
8 Pachernik J, Bryja V, Esner M, Kubala L, Dvorak P, Hampl A (2005). Neural differentiation of pluripotent mouse embryonal carcinoma cells by retinoic acid: inhibitory effect of serum. Physiol Res. **54**: 115–122.

9 Pachernik J, Horvath V, Kubala L, Dvorak P, Kozubik A, Hampl A (2007). Neural differentiation potentiated by the leukaemia inhibitory factor through STAT3 signalling in mouse embryonal carcinoma cells. Folia Biol (Praha). **53**: 157–163.
10 Sauer H, Rahimi G, Hescheler J, Wartenberg M (2000). Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cells. FEBS Lett. **476**: 218–223.
11 Sauer H, Wartenberg M (2005). Reactive oxygen species as signaling molecules in cardiovascular differentiation of embryonic stem cells and tumor-induced angiogenesis. Antioxid Redox Signal. **7**: 1423–1434.
12 Schmelter M, Ateghang B, Helmig S, Wartenberg M, Sauer H (2006). Embryonic stem cells utilize reactive oxygen species as transducers of mechanical strain-induced cardiovascular differentiation. FASEB J. **20**: 1182–1184.
13 Smith, J., E. Ladi, M. Mayer-Proschel & M. Noble. 2000. Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. *Proc Natl Acad Sci U S A* **97**, 10032–7.
14 Stritesky Larssen K, Lyberg T (2006). Oxidative status – Age- and circadian variations?– A study in leukocytes/plasma. Neuroendocrinol Lett. **27**: 445–452.
15 Suzukawa K, Miura K, Mitsushita J, Resau J, Hirose K, Crystal R, Kamata T (2000). Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. J Biol Chem. **275**: 13175–13178.
16 Ujhazy E, Schmidtova M, Dubovicky M, Navarova J, Bruckner-ova I, Mach M (2006). Neurobehavioural changes in rats after neonatal anoxia: effect of antioxidant stobadine pretreatment. Neuroendocrinol Lett. **27 Suppl 2**: 82–85.
17 Vejrazka M, Micek R, Stipek S (2005). Apocynin inhibits NADPH oxidase in phagocytes but stimulates ROS production in non-phagocytic cells. Biochim Biophys Acta. **1722**: 143–147.
18 Yang LY, Ko WC, Lin CM, Lin JW, Wu JC, Lin CJ, Cheng HH, Shih CM (2005). Antioxidant N-acetylcysteine blocks nerve growth factor-induced H2O2/ERK signaling in PC12 cells. Ann N Y Acad Sci. **1042**: 325–337.