Effect of the O_2 pressure on antioxidant systems and the intracellular ROS production in tumoral cells

Iratxe Suárez Bilbao, Rosaura Navarro Lobato and Mª Begoña Ruiz Larrea
Department of Physiology, Medicine and Dentistry School, UPV/EHU. Leioa

INTRODUCTION

In aerobic organisms, the controlled delivery and utilization of oxygen is essential for the balance between energy generation and the avoidance of unwarranted oxidative reactions. During endogenous metabolic reactions, reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) are produced [1, 2]. High amounts of ROS are known to cause damage to proteins, DNA and lipids, resulting in oxidative stress [3]. Oxidative stress occurs when the amount of ROS exceeds the capacity of cellular antioxidant defenses [2]. The first line of defense against these harmful pro-oxidants is a complex system of enzymatic antioxidants, e.g., superoxide dismutase (SOD), glutathione peroxidase, glutathione S-transferase, or catalase, and non-enzymatic antioxidants (e.g., glutathione, vitamins C and E) [1].
One of the main causes involved in carcinogenesis is the modification of genetic material caused by oxidative damage, since the DNA alteration compromises transcription and signal transduction [4-6]. Solid tumors generally occur and progress in an atmosphere with a reduced O_2 partial pressure, since they are far away from the blood vessels, and do not receive the necessary O_2 and nutrients. Thus, the intracellular ROS production could be altered, affecting the progression, aggressiveness, invasiveness and recurrence probability of the tumor [7].

OBJECTIVE

The aim of this work was to determine the effects of O_2 pressure on the intracellular steady state levels of ROS and the antioxidant enzymatic defenses in different tumor cell lines. For this purpose, the human A375 melanoma cell line, the human HeLa cervix cell line, the human HepG2, SK-HEP-1, Huh-7, PLC/PRF/5, and CHL hepatoma cell lines, and the rat McA-RH7777 hepatoma cell line were used at two O_2 conditions:
a) high pO_2 of 21% (H) and b) low pO_2 of 8% (L).

Under these conditions, the following parameters were determined:

- Intracellular steady-state levels of ROS
- SOD activity

MATERIALS AND METHODS

Cell culture and maintenance

Human and rat cancer cell lines were maintained in the corresponding EMEM or DEMEM medium, supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Cells were grown in 75 cm^2 flasks at 37 ºC in humidified atmosphere with 5% CO_2. Medium was replaced every 2 to 3 days. When the cell monolayer reached 70% of confluence, cells were detached with a solution of 0.1% trypsin-0.04% EDTA and then harvested in culture medium with 10% FBS to perform subsequent experiments.

ROS determination

The dichlorofluorescein (DCF) fluorescent dye was used [8]. The dye in its reduced and esterified form 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is incubated with the cells. After hydrolysis of the diacetate groups by cytosolic esterases or base-catalyzed cleavage of the diacetate groups, DCFH is oxidized by ROS to the highly fluorescent product DCF.

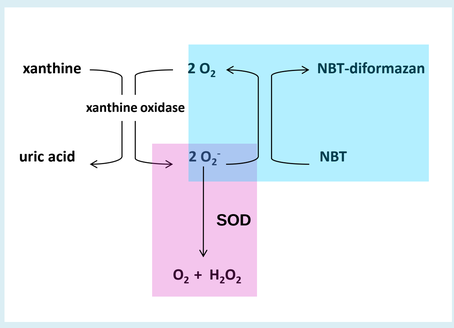
Cells were seeded in 6 well plates at a density of 150,000 (A375, HepG2 and McA-RH7777) or 200,000 (HeLa, SK-HEP-1, Huh-7, PLC/PRF/5, and CHL) cells and cultured under 21% (H) and 8% (L) pO_2 conditions with the corresponding culture medium at 37 ºC in humidified atmosphere with 5 % CO_2. Cell viability was determined by the Tripan blue exclusion test. When the cell monolayer reached 75% of confluence, the medium was removed and replaced with fresh medium without serum (1,5 ml per well). After 1 h, DCFH-DA (20 µM) was added and cells were incubated for 30 minutes. The cells were washed with PBS, then harvested in PBS supplemented with 10% FBS and put into tubes containing 0.05 µg DAPI. The formation of DCF was monitored (λem 525 nm, λex 488 nm) by flow cytometry (Beckman Coulter Gallios) in the General Research Services SGIker of the UPV/EHU (http://www.ikerkuntza.ehu.es/p273-sgikerhm/en/). Cells without DCFH-DA were used as controls (basal fluorescence).

Cell protein extraction and quantification

Cells were harvested, lysed by freeze-thaw in liquid N_2 and centrifugated for 2 minutes at 200 g at 4ºC. Cells corresponding to 4 independent harvests were pooled and protein concentration was determined spectrophotometrically at 595 nm by Coomassie Blue staining (Bradford) [9], using bovine serum albumin as standard .

SOD activity

SOD activity was determined by the method that utilizes nitroblue tetrazolium (NBT).
Xanthine-xanthine oxidase system was used to generate superoxide anion that in turn, reduces NBT to its corresponding diformazan absorbing at 570 nm. In presence of SOD, O_2^- undergoes a dismutation into O_2 and H_2O_2, which decreases the diformazan formation. Hence, this competing assay yields to the indirect measurement of SOD activity [10]. The reaction took place in a final volume of 275 µl and started with the addition of NBT. The increase in absorbance was monitored at 570 nm every 60 seconds for 15 minutes in a 96-well plate reader at 37 ºC. Increasing concentrations of the sample protein were assayed and the IC_{50} (50% inhibition) was determined. An inhibition curve was prepared using commercially available SOD to transform the IC_{50} value into SOD units. Results are expressed as U/mg of protein.



Data analysis

Data are expressed as mean ± standard error (SE) of the mean for at least 3 independent experiments. Statistical significance was estimated by Student´s *t*-test for paired and unpaired data (software package SPSS 19.0). The results were considered significant at $p < 0.05$.

CONCLUSIONS

- The steady-state levels of ROS vary depending on the tumor cell type. Thus, human SK-HEP-1 and PLC/PRF/5 hepatocarcinoma cell lines showed the highest ROS values, while the human HeLa cervix tumor cell line showed the lowest basal ROS levels. The rat hepatoma McA-RH7777 showed diminished levels of ROS, compared to those found in HepG2.
- The intracellular ROS levels in A375, SK-HEP-1, Huh-7, and McA-RH7777 decreased significantly under low pO_2.
- The O_2 pressure affects SOD activity depending on the cell type. Thus, a low pO_2 increased SOD activity in HepG2, while decreased it in PLC/PRF/5 and CHL.

FURTHER RESEARCH

- To better understand this work about the effect of pO_2 on ROS levels, we have to take into account other results obtained in our research group in order to get global conclusions. Since the steady-state levels of ROS are the result of their production and elimination, and other antioxidant systems different from SOD also contribute to the final ROS balance, the antioxidant activities of catalase, glutathione peroxidase, and intracellular levels of glutathione are crucial to understand these results.
- During this period, I have also extracted RNA from the cell cultures in order to analyze the expression of antioxidant enzymes by RT-qPCR, and relate it with the total amount of these proteins determined in our laboratory by Western blot.

RESULTS

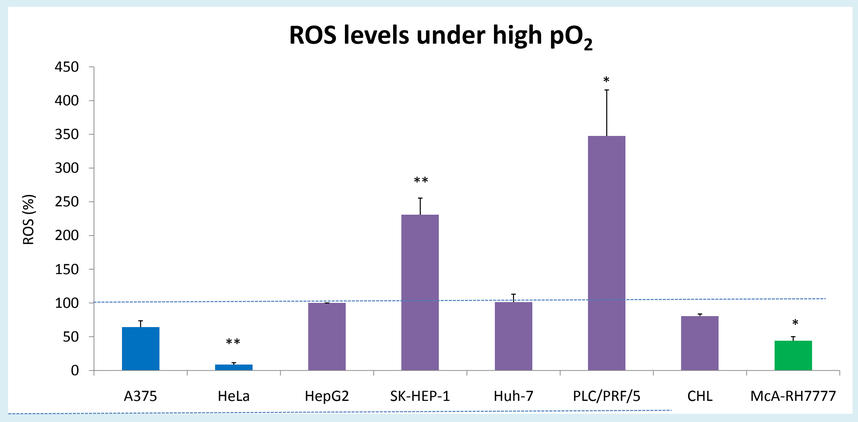


Fig 1. Intracellular steady-state ROS values in the different human and rat tumor cell lines. ROS were detected under high pO_2 conditions in all the cell lines. Values were normalized using the ROS levels in HepG2 as control (100%). Results are expressed as the mean ± SE of 4-7 independent experiments (*$p < 0.05$, **$p < 0.01$).

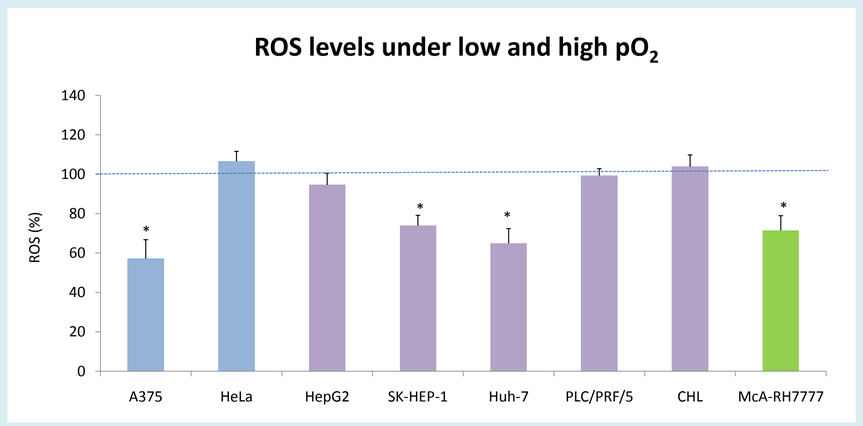


Fig 2. Intracellular steady-state ROS values under low pO_2 in human and rat tumor cell lines. The relative ROS values are expressed as the percentage of the intracelular ROS levels detected under high pO_2 conditions in each cell type (100%). Results are expressed as the mean ± SE of 4-7 independent experiments (*$p < 0.05$).

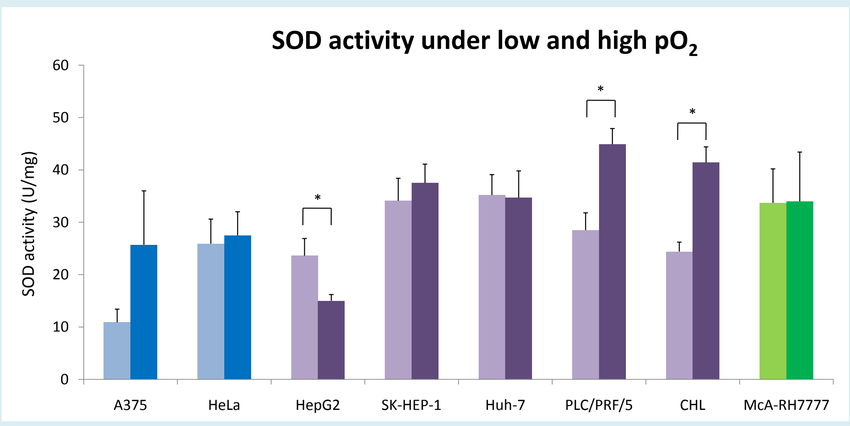


Fig 3. SOD activity under low (light colour) and high (dark colour) pO_2 conditions in human and rat tumor cell lines. Results are expressed as the mean ± SE of 4 independent experiments (*$p < 0.05$).

ACKNOWLEDGEMENTS

José Ignacio Ruiz-Sanz, Jenifer Trepiana, Susana Meijide, Adrián Castillo and Oihana Lopez de Vergara.

REFERENCES

[1] **Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB.** Oxidative stress, inflammation, and cancer: How are they linked*? Free Radic Biol Med* 49:1603–1616 (2010).

[2] **Limón-Pacheco J, Gonsebatt ME.** The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress*. Mutat Res* 674:137–147 (2009).

[3] **Kietzmann T, Görlach A.** Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. Semin Cell Devel Biol 16:474–486 (2005).

[4] **Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M.** Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 160:1-40 (2006).

[5] **Cooke MS, Evans MD, Dizdaroglu M, Lunec J.** Oxidative DNA damage: mechanisms, mutation and disease. *FASEB J* 17:1195-214 (2003).

[6] **Marnett LJ.** Oxyradicals and DNA damage. *Carcinogenesis* 21**:** 361-70 (2000).

[7] **Hanahan D, Weinberg RA**. The hallmarks of cancer. *Cell* 100**:** 57–70 (2000).

[8] **Giordo R, Cossu A, Pasciu V, Hoa PT, Posadino AM, Pintus G.** Different Redox Response Elicited by Naturally Occurring Antioxidants in Human Endothelial Cells*. The Open Biochem journ,* 7, 44-53 (2013).

[9] **Bradford Mn.** A rapid and sensitive metod for the quantitaion of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal Biochem*; 72:248-254 (1976).

[10] **Zhou JY, Prognon P**. Raw material enzymatic activity determination: a specific case for validation and comparison of analytical methods – the example of superoxide dismutase (SOD). *J Pharm Biomed Anal* 40(5): 1143-8 (2006).