

Mimicking of glutathione peroxidase deficiency by exposition of JAR cells to increased level of synthetic hydroperoxide

Anna Hallmann¹, Ryszard Milczarek¹, Michał Szkatuła³, Michał Woźniak³,
Jan Henryk Spodnik², Jerzy Klimek¹

¹Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, Poland

²Department of Anatomy and Neurobiology, Medical University of Gdańsk, Poland

³Department of Medical Chemistry, Medical University of Gdańsk, Poland

[Received 15 July 2005; Revised 27 September 2005; Accepted 27 September 2005]

A short chain synthetic analogue of lipid hydroperoxides was used to overload glutathione peroxidase (GPx) in human choriocarcinoma cell line JAR cells. Cells exposed to 100 μM tBuOOH displayed a 40% reduction in ATP level and significantly increased in membrane permeability, visualised by the lactate dehydrogenase (LDH) release into the extracellular medium. The intracellular level of oxygen free radicals measured as an oxidation of the dichlorodihydro-fluorescein diacetate (H₂DCF-DA) significantly increased after 2 hours of cell exposition to 100 μM tBuOOH. Concomitantly MDA, 4-HNE level increased to 2 nmol/mg of cell protein after 2 hours. Mitochondria stained with MitoTracker Red CMXRos displayed a filamentous appearance in control cells but changed into granular less energised organelles after exposition to tBuOOH. Collectively, the above results indicate the importance of the contribution of oxidative stress in the development of pre-eclampsia.

Key words: mitochondria, choriocarcinoma, oxidative stress, pre-eclampsia

INTRODUCTION

Lipid hydroperoxides (LOOH) are ubiquitous components of biological membrane. Placental trophoblast cells are recognised as the main source of endogenous LOOH [16]. A deficiency of placental glutathione peroxidase (GPx) activity can possibly increase the level of tissue peroxides being stimulatory to the formation of placental thromboxane [6, 23] and LOOH [25] and ultimately contributing to the development of pre-eclampsia. Hydroperoxides appear to play a significant role in lipid peroxidation, being both a product of the initial phase of lipid peroxidation as well as a main substrate for its further propagation. The aim of the present study was to inspect the effect of a short chain synthetic per-

oxide, namely *tert*-butylhydroperoxide (tBuOOH), on the energetic state of *choriocarcinoma* cells, the generation of free radicals inside the cell and the morphology of mitochondria as investigated by confocal microscopy.

MATERIAL AND METHODS

Cell line

Human *choriocarcinoma* cells JAR (ATCC HTB-144) were routinely cultured in 75-cm² plastic flasks (Sarsted) in RPMI 1640 containing 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 4.5 g/L glucose, supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 IU/ml penicillin and

100 $\mu\text{g/ml}$ streptomycin. The cells were grown in a humidified atmosphere with 5% CO_2 at 37°C. They were then passaged weekly by trypsin 0.25% and EDTA 0.03%.

Reagents

RMPI 1640 and tBuOOH were purchased from Sigma Aldrich Chemical (USA). The foetal bovine serum was produced by Gibco BRL (Grand Island, NY, USA). Trypsin with EDTA and phosphate buffer saline (PBS) pH 7.4 were products of Sigma Chemicals (USA). Mito Tracker Red CMXRos (CMXRos) Molecular Probes, Inc. FITC — Phalloidin was produced by Sigma P5282 (USA). Formaldehyde and glutaraldehyde were purchased from Merck (Germany), Perma Fluor mounting solution from Immunon (Pittsburgh, PA, USA) and Triton X-100 from Sigma Aldrich Chemical (USA).

Treatment of cells with tert-butylhydroperoxide

Cells were plated in 6-well plates at a concentration of 5×10^5 – 1×10^6 cells/well (0.5–1 mg protein/ml/well). The cells were exposed to 100 μM tBuOOH at specific times (control = 0 h, 1 h, 2 h).

Viability assay

Cell viability was evaluated by lactate dehydrogenase (LDH) release. After subtracting the relevant volume of medium from above the cells (to be used as reference in the following steps), the cells were lysed by the addition of Triton X-100 (final concentration in culture dish 1%). The aliquots of the lysed cells as well as the reference culture medium were centrifuged at $1500 \times g$ for 3 min and supernatants were used for the assay. The assay solution was added to a cuvette containing 0.05 M Tris-HCl buffer, pH 7.4, 1 mM sodium pyruvate and 0.15 mM NADH, and spectrophotometrical analysis was performed at 340 nm. LDH release was expressed as a percentage of total release, the release, that is, of LDH after cell lysis by 10% Triton X-100 [9, 13].

Measurement of intracellular ATP level

Sample preparation: Cells ($\sim 2 \times 10^6$ cells/ml) were collected by trypsinisation, centrifuged together with those floating in the culture medium and washed with PBS. The cells were then suspended in cold 1.4 M HClO_4 and incubated for 15 min on ice to extract cellular nucleotides. Acid extracts were centrifuged (14,000 RCF — 5 min), transferred to new tubes and neutralised to pH 6.5 by the addition of cold 3 M K_3PO_4 prior to centrifugation (14,000 RCF — 5 min). The

supernatants were subjected to further analysis by HPLC. The method employed for HPLC determination of the intracellular level of ATP was based on that described by Smoleński et al. [21]. Protein concentration was evaluated using the Bradford method [4] after dissolving the perchloric acid precipitates with 0.5 M NaOH.

Staining and Visualisation of Mitochondria by Confocal Microscopy

Cells growing on 24×24 mm-glass coverslips under various experimental conditions were incubated with 100 nM Mito Tracker Red CMXRos (CMXRos; Molecular Probes, Inc.) for 30 minutes at 37°C in an atmosphere of 5% CO_2 . The cells were fixed with a fixative containing 2% glutaraldehyde and 2% formaldehyde in PBS for 30 minutes at room temperature and then washed in PBS and permeabilised with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. This was followed by 4 washes in PBS. The coverslips with the cells were placed on glass slides in a Perma Fluor mounting solution (Immunon, Pittsburgh, PA) and analysed by a confocal system Radiance 2100 (Bio-Rad, UK), equipped with Krypton/Argon lasers and mounted on an Eclipse 600 (Nikon, Japan) microscope, using the software LaserSharp 2000 version 4.0 (Bio-Rad, UK) as described previously [11].

Flow cytometric determination of Reactive Oxygen Species and light scatter

Levels of Reactive Oxygen Species (ROS) were measured by flow cytometry as the fluorescence of DCF, which is the oxidation product of $\text{H}_2\text{DCF-DA}$ with a sensitivity to ROS (H_2O_2 , ROOH, NO). Cells were incubated for 30 min with the probe (20 μM) at 37°C. Cell samples were then fixed (1% paraformaldehyde), cooled (4°C), and protected from light for later analysis (cold-fixed cell samples). A FACSCAN (Coulter Elite) flow cytometer was applied to measure the ROS levels in the cells. Signals were obtained using a 530-nm bandpass filter (FL-1 channel) for DCF. Each determination was based on a mean fluorescence intensity of 7,000 cells [1, 20].

Measurement of lipid peroxidation products

Cells ($\sim 10 \times 10^6$ cells/ml) were collected by trypsinisation, centrifuged and washed with PBS. They were then suspended in PBS and the concentration of lipid peroxidation products measured. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured using a lipid peroxidation

assay kit (Calbiochem, San Diego, CA) [7]. Protein concentration was evaluated [4] and the lipid peroxidation products were described as MDA, 4-HNE/mg protein.

RESULTS

Effect of *t*BuOOH on intracellular levels of ATP and LDH release

Time-dependent changes of intracellular levels of the ATP in *t*BuOOH-treated cells are shown in Figure 1. Two hours after the treatment, ATP levels had decreased to about 60% of the control value (control \approx 12 nmol/mg protein) (Fig. 1). We noticed an increase in the permeability of the cell membrane to LDH, which was already distinct 2 hours after cell treatment (Fig. 1) and increased continuously in an extracellular medium thereafter to approximately 40% after 6 hours (result not shown).

Effect of *t*BuOOH on morphological changes of the mitochondria in choriocarcinoma cells

The mitochondria in the control cells stained with fluorescent dye CMXRos showed thin filamentous structures (Fig. 2A) under a confocal laser microscope. JAR cells exposed to 100 μ M *t*BuOOH for 2 hours (Fig. 2B) showed a mixed population of granular mitochondria of different sizes, in many cases

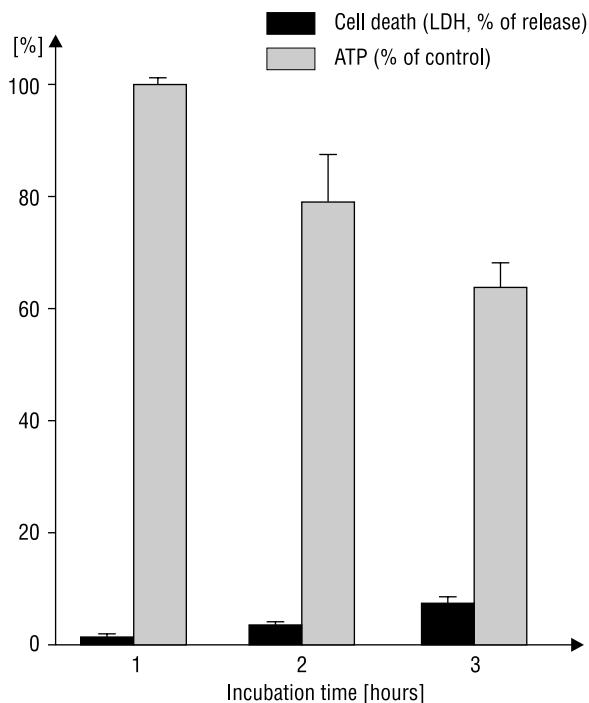


Figure 1. LDH release and the concentration of ATP in JAR cells after treatment with 100 μ M *t*BuOOH. The values represent means \pm SEM from 3 independent experiments.

distinctly enlarged. Exposition of cells to *t*BuOOH also resulted in notable changes in the shape of the cells as well as in perturbation of the plasma membrane integrity (Fig. 2B).

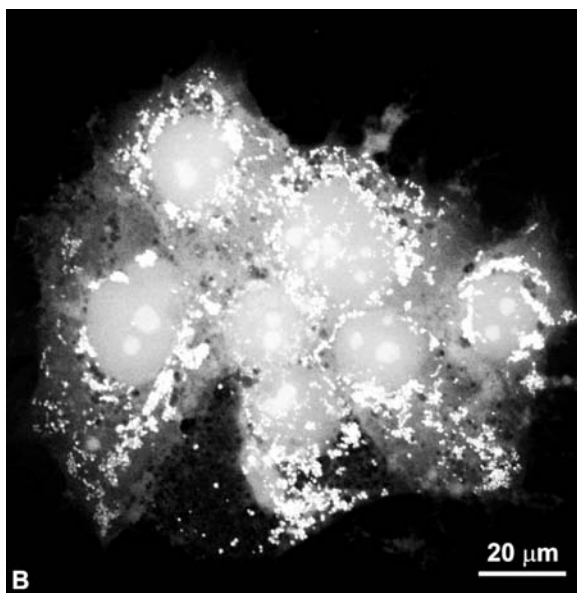
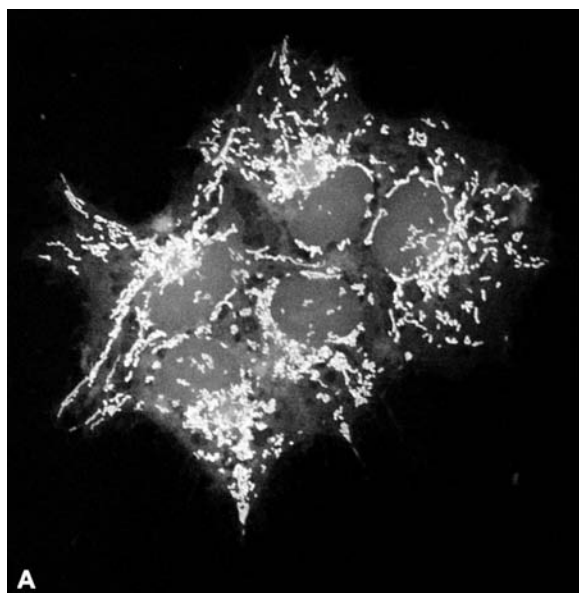


Figure 2. Morphological changes of mitochondria in JAR cells after treatment with 100 μ M *t*BuOOH. **A.** Control cells; **B.** Treated with 100 μ M *t*BuOOH for 2 hours.

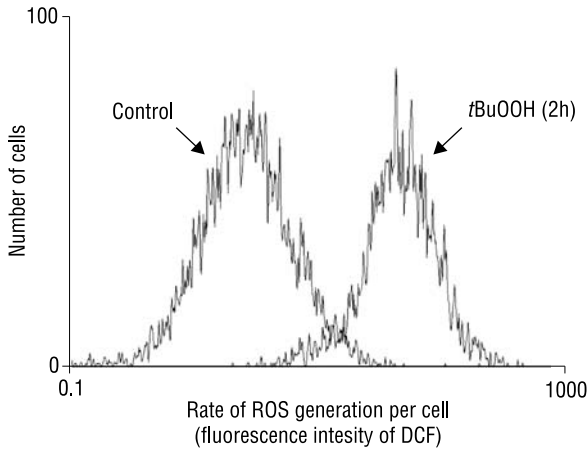


Figure 3. Changes in the intracellular levels of reactive oxygen species in JAR cells after treatment with 100 μ M *t*BuOOH.

Effect of *t*BuOOH on the rate of generation of ROS from choriocarcinoma cells

A typical example of a flow cytometric histogram obtained from untreated control and *t*BuOOH-treated JAR cells is shown in Figure 3. A remarkable shift in the peak intensity of DCF to the right was already detected at 2 hours of *t*BuOOH treatment, indicating that intracellular levels of ROS were distinctly increased by *t*BuOOH treatment. As a consequence

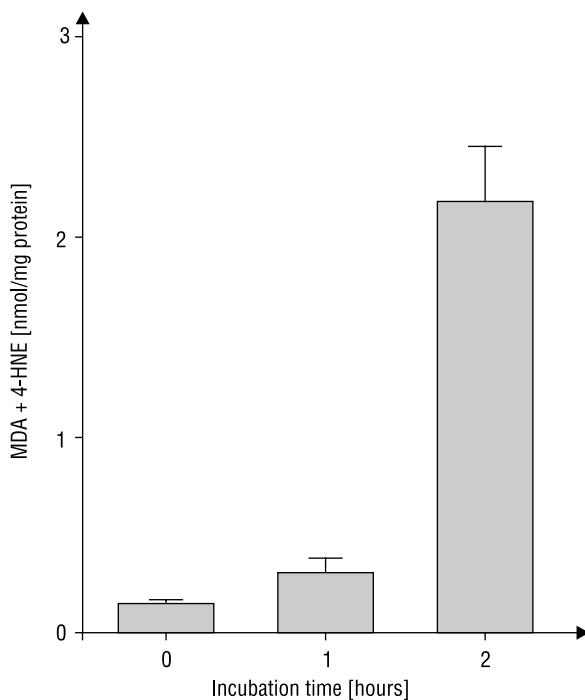


Figure 4. Changes in the intracellular levels of lipid peroxidation products (MDA, 4-HNE) in JAR cells after treatment with 100 μ M *t*BuOOH. The values represent means \pm SEM from 3 independent experiments.

of the significant generation of ROS, an elevated level of MDA and 4-HNE (10-fold) was observed after 2 hours of cells exposition to *t*BuOOH (Fig. 4).

DISCUSSION

We have shown in the present study that the *t*BuOOH-substance with well defined pro-oxidant properties induces mitochondrial stress in human *choriocarcinoma* cells. Generation of free radical species from *t*BuOOH was noticed after a 2-hour exposition of cells to the pro-oxidant. This indicates an imbalance in antioxidant defence, in which glutathione peroxidase plays a significant role, and the generation of ROS. Mitochondria are the main intracellular source of ATP and ROS [10, 15]. It has been demonstrated that mitochondria are mainly responsible for the biotransformation of *t*BuOOH into free radical products [12, 17]. As the result of an imbalance between the generation of ROS and antioxidant defence, cellular energetic catastrophe is created and the intracellular ATP level decreases abruptly, reaching 60% as early as 2 hours after the treatment. As a consequence of the depletion of ATP, *choriocarcinoma* cells start to lose the integrity of the plasma membrane and LDH starts to escape from the cell. In our experimental conditions, the increased level of the hydroperoxides definitively overwhelms the capacity of placenta glutathione peroxidase, the enzyme which is mainly responsible for the "safe" two-electron reduction of hydroperoxides. Under these circumstances one-electron oxidation or a reduction in *t*BuOOH may create either the peroxy *t*BuOO \cdot or alkoxyl *t*BuO \cdot radical respectively [5]. The mechanism for the reaction of cytochrome c with *t*BuOOH was investigated by Barr and Mason by ESR spin trapping using DMPO [3]. From these analyses it was concluded that the alkoxyl radical of the hydroperoxide was the initial radical product. Lipid peroxidation is thought to be involved in the aetiologies of various diseases including pre-eclampsia [18, 22, 24]. So far, phospholipid hydroperoxides have been thought to be cytotoxic because phospholipase A2 specifically releases fatty acid hydroperoxides from phospholipids [14, 19]. Transgenic mice over-expressing GPx1 have been found to be partially protected from the lethal effect of anti CD95, underlining the importance of GPx1 and elevated peroxide formation in apoptotic signalling [2, 8]. According to Walsh and Wang, pre-eclamptic trophoblast cells produce about 900 pmol more peroxides than normal cells [23]. We noticed an increased level of MDA and 4-HNE as early 2 hours from the incubation of the *choriocarcinoma* cells with 100 μ M

tBuOOH. The above results raise the possibility of the participation of endogenous lipid peroxides in the disturbance of hydroperoxide metabolism. On the basis of the results obtained we propose that a naturally occurring rise in hydroperoxides may contribute to the development of pre-eclampsia.

REFERENCES

- Afri M, Frimer AA, Cohen Y (2004) Active oxygen chemistry within the liposomal bilayer. Part IV: Locating 2',7'-dichlorofluorescein (DCF), 2',7'-dichlorodihydrofluorescein (DCFH) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the lipid bilayer. *Chem Phys Lipids*, 131: 123–133.
- Bajt ML, Ho YS, Vonderfecht SL, Jaeschke H (2002) Reactive oxygen as modulator of TNF and fas receptor-mediated apoptosis in vivo: studies with glutathione peroxidase-deficient mice. *Antioxid Redox Signal*, 4: 733–740.
- Barr DP, Mason RP (1995) Mechanism of radical production from the reaction of cytochrome c with organic hydroperoxides. An ESR spin trapping investigation. *J Biol Chem*, 270: 12709–12716.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72: 248–254.
- Davies MJ (1989) Detection of peroxy and alkoxy radicals produced by reaction of hydroperoxides with rat liver microsomal fractions. *Biochem J*, 257: 603–606.
- Eisenmann CJ, Miller RK (1995) The effect of selenium compounds (selenite, selenate, ebselen) on the production of thromboxane and prostacyclin by the human term placenta in vitro. *Toxicol Appl Pharmacol*, 135: 18–24.
- Esterbauer H, Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol*, 186: 407–421.
- Gouaze V, Andrieu-Abadie N, Cu villier O, Malagarie-Cazenave S, Frisach MF, Mirault ME, Levade T (2002) Glutathione peroxidase-1 protects from CD95-induced apoptosis. *J Biol Chem*, 277: 42867–42874.
- Gretzer C, Thomsen P (2000) Secretion of IL-1 and H₂O₂ by human mononuclear cells in vitro. *Biomaterials*, 21: 1047–1055.
- Karbowski M, Kurono C, Wozniak M, Ostrowski M, Teranishi M, Nishizawa Y, Usukura J, Soji T, Wakabayashi T (1999) Free radical-induced megamitochondria formation and apoptosis. *Free Radic Biol Med*, 26: 396–409.
- Karbowski M, Spodnik JH, Teranishi M, Wozniak M, Nishizawa Y, Usukura J, Wakabayashi T (2001) Opposite effects of microtubule-stabilizing and microtubule-destabilizing drugs on biogenesis of mitochondria in mammalian cells. *J Cell Sci*, 114: 281–291.
- Kennedy CH, Church DF, Winston GW, Pryor WA (1992) tert-Butyl hydroperoxide-induced radical production in rat liver mitochondria. *Free Radic Biol Med*, 12: 381–387.
- Kim JS, Southard JH (1999) Membrane stabilizing effects of calcium and taxol during the cold storage of isolated rat hepatocytes. *Transplantation*, 68: 938–943.
- van Kuijk FJ, Handelman GJ, Dratz EA (1985) Consecutive action of phospholipase A₂ and glutathione peroxidase is required for reduction of phospholipid hydroperoxides and provides a convenient method to determine peroxide values in membranes. *J Free Radic Biol Med*, 1: 421–427.
- Lee HC, Wei YH (2000) Mitochondrial role in life and death of the cell. *J Biomed Sci*, 7: 2–15.
- Maseki M, Nishigaki I, Hagihara M, Tomoda Y, Yagi K (1981) Lipid peroxide levels and lipids content of serum lipoprotein fractions of pregnant subjects with or without pre-eclampsia. *Clin Chim Acta*, 115: 155–161.
- O'Donnell V, Burkitt MJ (1994) Mitochondrial metabolism of hydroperoxide to free radicals in human endothelial cells: an electron spin resonance spin-trapping investigation. *Biochem J*, 304: 707–713.
- Poranen AK, Ekblad U, Uotila P, Ahotupa M (1996) Lipid peroxidation and antioxidants in normal and pre-eclamptic pregnancies. *Placenta*, 17: 401–405.
- Sevanian A, Wratten ML, McLeod LL, Kim E (1988) Lipid peroxidation and phospholipase A₂ activity in liposomes composed of unsaturated phospholipids: a structural basis for enzyme activation. *Biochim Biophys Acta*, 961: 316–327.
- Sharikabad MN, Ostbye KM, Lyberg T, Brors O (2001) Effect of extracellular Mg²⁺ on ROS and Ca²⁺ accumulation during reoxygenation of rat cardiomyocytes. *Am J Physiol Heart Circ Physiol*, 280: 344–353.
- Smoleński RT, Lachno DR, Ledingham SJ, Yacoub MH (1990) Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. *J Chromatogr*, 527: 414–420.
- Walsh SW (1994) The role of fatty acid peroxidation and antioxidant status in normal pregnancy and in pregnancy complicated by preeclampsia. *World Rev Nutr Diet*, 76: 114–118.
- Walsh SW, Wang Y (1995) Trophoblast and placental villous core production of lipid peroxides, thromboxane, and prostacyclin in preeclampsia. *J Clin Endocrinol Metab*, 80: 1888–1893.
- Walsh SW (1998) Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. *Semin Reprod Endocrinol*, 16: 93–104.
- Wang YP, Walsh SW, Guo JD, Zhang JY (1991) The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. *Am J Obstet Gynecol*, 165: 1695–1700.