External hydrogen peroxide is not indispensable for experimental induction of lipid peroxidation *via* Fenton reaction in porcine ovary homogenates

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Abstract

OBJECTIVE: Substrates of Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+{}^{\bullet}OH+OH^-$) may be used to experimentally induce oxidative damage to macromolecules.
The study aimed at evaluating effects of Fe^{2+} and/or H_2O_2 on lipid peroxidation in porcine ovary homogenates.
MATERIALS AND METHODS: Ovary homogenates were incubated in the presence of either H_2O_2 (100, 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.25, 0.01, 0.001 mM) or $FeSO_4$ (Fe^{2+}) (300, 150, 75, 30, 15, 7.5, 3.0, 1.5, 0.75 μM), or of those two factors used together: Fe^{2+} (30 μM) plus H_2O_2 (concentrations as above), or H_2O_2 (0.5 mM) plus Fe^{2+} (concentrations as above). The concentration of malondialdehyde+4-hydroxyalkenals constituted the lipid peroxidation index.
RESULTS: H_2O_2 alone did not affect lipid peroxidation in porcine ovary homogenates at all, whereas Fe^{2+} (300, 150, 75, 30, and 15 μM) alone increased lipid peroxidation in a concentration dependent manner.
When Fe^{2+} and H_2O_2 were applied together, lipid peroxidation increased significantly without any concentration related effect of H_2O_2, but with a clear concentration dependent effect of Fe^{2+}; the damaging effect of Fe^{2+}, used together with H_2O_2, was the same as the one, obtained after Fe^{2+} was applied alone.
CONCLUSIONS: In conclusion, external H_2O_2 is not indispensable for experimental induction of lipid peroxidation by Fenton reaction in porcine ovary homogenates.

INTRODUCTION

Reactive oxygen species (ROS), free radicals included, participate in numerous metabolic processes. Under physiological conditions, biological cells produce low or moderate amounts of ROS that are required for life processes. Due to their highly reactive nature, which can damage DNA, proteins and lipids, the cells employ antioxidative defense systems to counteract these toxic products, thus maintaining the cells in redox balance status (Lau *et al.* 2008). In basal conditions, ROS are continuously detoxified by antioxidant systems and, therefore, they are not toxic. Ironically,

ORIGINAL ARTICLE

various ROS-mediated actions protect, in fact, cells against ROS-induced oxidative stress and reestablish or maintain redox balance, termed also redox homeostasis (Valko *et al.* 2007). However, any internal or external pathological factor may disrupt this balance, leading to conditions, referred to as oxidative stress and playing a significant role in the pathogenesis of several diseases (Dreher and Junod 1996; Valko *et al.* 2007).

The most basic reaction of oxidative stress is Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$; the most harmful free radical, hydroxyl radical (•OH), is produced during this reaction.

Iron is an essential element for normal metabolic processes, being a cofactor for many biological reactions. On the other hand, iron as a transition metal, has loosely bound electrons in the outer shell and is, therefore, potentially very reactive and toxic. It is known that increased iron stores in tissue result in cellular toxicity and are associated with increased risks of cellular damage, cancer initiation included (Dreher and Junod 1996; Eaton and Qian 2002; Letelier *et al.* 2007).

Hydrogen peroxide (H_2O_2) is one of the different forms of ROS. Under physiological conditions, different cells produce continuously low or moderate amounts of H_2O_2, which participate in numerous physiological processes. This ROS is mainly generated by the intracellular family of NADPH oxidases (NOX). At concentrations, higher than "physiological", H_2O_2 contributes to enhanced oxidative stress, with excessive DNA oxidative damage and consequent mutagenesis (Song *et al.* 2007).

Bivalent iron (Fe^{2+}) and H_2O_2, which initiate Fenton reaction under physiological conditions, may be used to experimentally induce oxidative damage to macromolecules, lipids and DNA included (Cabrera *et al.* 2001; Gitto *et al.* 2001; Horvathova *et al.* 2008; Karbownik *et al.* 2001a; Karbownik and Lewiński 2003a; Karbownik et al. 2001b; Karbownik *et al.* 2000; Reiter *et al.* 2008).

It is known that lipid peroxidation (which is mainly induced by •OH, produced during Fenton reaction) contributes to DNA damage and cancer initiation (Marnett *et al.* 1999). Accordingly, there is a clear evidence that oxidative stress is deeply involved in the process of carcinogenesis, playing an especially significant role in its first step – cancer initiation (Bolton and Thatcher 2008; Deo *et al.* 2008; Gesing and Karbownik-Lewińska 2008; Karbownik 2002; Lau *et al.* 2008; Valko *et al.* 2007; Valko *et al.* 2006).

As in other tissues and organs, oxidative reactions occur also in the ovary and, as such, are examined in experimental models (Orhan *et al.* 2006). However, the effects of Fe^{2+} and/or H_2O_2 on lipid peroxidation in the ovary have, up to now, been rather poorly identified, possibly because ovarian cancer, the most aggressive gynecologic neoplasm, is usually diagnosed at its advanced stages and, therefore, molecular alterations occurring at promotion, progression and metastasis stages are usually examined, but not those, associated directly with oxidative stress (Chien *et al.* 2007).

The aim of the present study was to evaluate the effect of ferrous iron (Fe^{2+}) and/or H_2O_2 on lipid peroxidation in porcine ovary homogenates. Our study is the first one, showing the influence of Fenton reaction substrates on lipid peroxidation in ovary tissue.

MATERIALS AND METHODS

Chemicals

Ferrous sulfate ($FeSO_4$) and hydrogen peroxide (H_2O_2) were purchased from Sigma (St. Louis, MO). The LPO-586 kit for lipid peroxidation was obtained from Calbiochem (La Jolla, CA). All the used chemicals were of analytical grade and came from commercial sources.

Animals

Porcine ovaries were collected from twenty animals at a slaughter-house, the organs after collection being immediately frozen on solid CO_2 and stored at –80 °C until assay.

Incubation of ovary homogenates

Ovary tissue was homogenized in ice cold 20 mM Tris-HCl buffer (pH 7.4) (10%, w/v), and then incubated for 30 min at 37°C in the presence of examined substances.

In Experiment I, ovary homogenates were incubated in the presence of H_2O_2 alone in the following concentrations: 100, 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.25, 0.01, 0.001 or 0.0 mM.

In Experiment II, ovary homogenates were incubated in the presence of $FeSO_4$ (Fe^{2+}) alone, used in different concentrations: 300, 150, 75, 30, 15, 7.5, 3.0, 1.5, 0.75 or 0.0 μM. On the basis of the concentration-dependent effect of $FeSO_4$, the concentration of 30 μM was selected for a subsequent study.

Thus, in Experiment III, ovary homogenates were incubated in the presence of those two factors used together, namely, in the presence of $FeSO_4$ (Fe^{2+}) (30 μM) plus H_2O_2 in different concentrations: 100, 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.25, 0.01, and 0.001 mM.

In Experiment IV, ovary homogenates were incubated also in the presence of those two factors, used together – in the presence of H_2O_2 in constant concentration (0.5 mM) plus $FeSO_4$ (Fe^{2+}) in the following concentrations: 300, 150, 75, 30, 15, 7.5, 3.0, 1.5, and 0.75 μM.

The reactions were stopped by cooling the samples on ice. Each experiment was run in duplicate and repeated three times.

Measurement of lipid peroxidation products

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA), as an index of lipid peroxidation, were measured in ovary homogenates. The homogenates were centrifuged at 3,000 × g for 10 min at 4 °C. The supernatant was mixed with 650 μl of a methanol:acetonitrile (1:3, v/v) solution, containing a chromogenic reagent, N-methyl-2-phenylindole, and vortexed. Following the addition of 150 μl of methane-

sulfonic acid (15.4 M), the incubation was carried out at 45 °C for 40 min. The reaction between MDA+4-HDA and N-methyl-2-phenylindole yields a chromophore, which is spectrophotometrically measured at the absorbance of 586 nm, using a solution of 10 mM 4-hydroxynonenal as the standard. The level of lipid peroxidation is expressed as the amount of MDA+4-HDA (nmol) per mg protein.

Measurement of protein

Protein was measured, using Bradfords method (Bradford 1976), with bovine albumin as standard.

Statistical analyses

Results are expressed as means SE. The data were statistically analyzed, using a one-way analysis of variance (ANOVA), followed by the Student-Neuman-Keul's test. For verification, an unpaired Student's t-test was also used (experiment 4). Statistical significance was determined at the level of $p<0.05$.

RESULTS

The incubation of ovary homogenates for 30 min in the presence of H_2O_2 alone in different concentrations (100, 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.25, 0.01, 0.001, and 0.0 mM) did not cause any changes in the level of lipid peroxidation products (Figure 1).

In turn, Fe^{2+} alone increased the level of lipid peroxidation in concentration dependent manner with statistical significance for the highest used concentrations (300, 150, 75, 30, and 15 µM) (Figure 2). For a subsequent study, the concentration of 30 µM of $FeSO_4$ was selected.

When Fe^{2+} and H_2O_2 were applied together, the level of lipid peroxidation increased significantly without any concentration dependent effect of H_2O_2 (Figure 3), but with clear concentration dependent effect of Fe^{2+} (Figure 4); the damaging effect of Fe^{2+}, used together with H_2O_2, was the same as when Fe^{2+} was applied alone (Figures 2 and 4).

DISCUSSION

As mentioned before, free ionic iron (Fe^{2+}) and H_2O_2, were found to enhance – via initiation of Fenton reaction – lipid peroxidation in homogenates of different tissues, namely in rat (Gitto *et al.* 2001; Karbownik *et al.* 2000) and monkey (Cabrera *et al.* 2001) liver, in rat testes (Karbownik *et al.* 2001a), and in the thyroid gland (Karbownik and Lewiński 2003a). However, no reports exist, concerning that process in the ovary tissue. Additionally, it has never been examined before, if both Fenton reaction substrates are indispensable to experimentally induce lipid peroxidation under *in vitro* conditions. Our present study is the first attempt devoted to the subject in question.

We expected that external H_2O_2 alone would induce lipid peroxidation, as, for example, in rat brain homogenates (Sewerynek *et al.* 1995). At the same time, we assumed that external iron would require the presence of external H_2O_2 to increase lipid peroxidation. Our assumption was based on the fact that H_2O_2 does not play any particular role in ovarian physiology, at least, to such an extent as, for example, in the thyroid gland (Karbownik and Lewiński 2003b), thus probably occurring in ovary tissue in rather low concentrations. It should also be kept in mind that iron is normally avail-

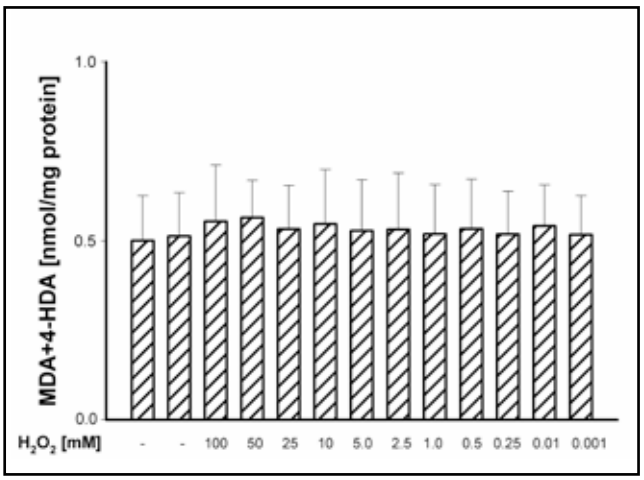

Fig. 1. Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in porcine ovary homogenates, incubated for 30 min in the presence of hydrogen peroxide (H_2O_2) alone, in different concentrations (100, 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.01 and 0.001 mM). Bars represent the mean ± SE of three independent experiments run in duplicates. No significant differences were found.

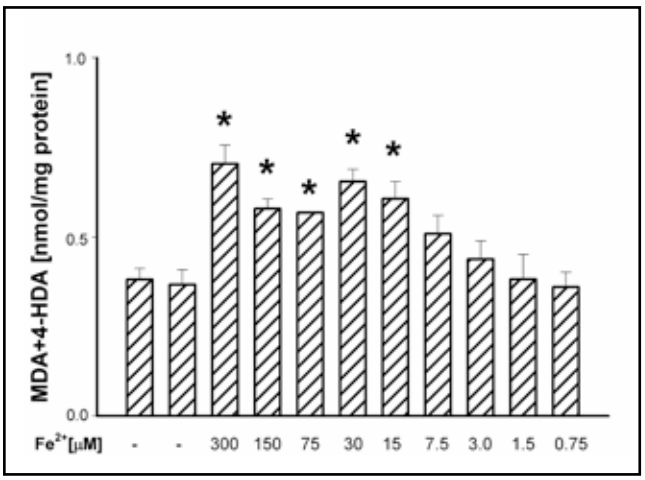

Fig. 2. Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in porcine ovary homogenates, incubated for 30 min in the presence of $FeSO_4$ (Fe^{2+}) alone, in different concentrations (300, 150, 75, 30, 15, 7.5, 3.0, 1.5 and 0.75 µM). Bars represent the mean ± SE of three independent experiments run in duplicates.
* $p<0.05$ vs. Control (in the absence of Fe^{2+}).

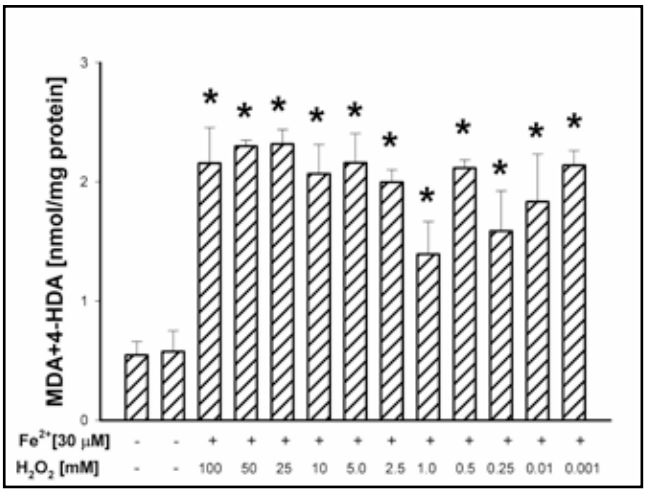

Fig. 3. Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in porcine ovary homogenates, incubated for 30 min in the presence of these two factors used together, namely in the presence of $FeSO_4$ (Fe^{2+}) (30 µM) plus H_2O_2 (100, 50, 25, 10, 5.0, 2.5, 1.0, 0.5, 0.25, 0.01 and 0.001 mM). Bars represent the mean ± SE of three independent experiments run in duplicates. * $p<0.05$ vs. Control (in the absence of either Fe^{2+} or H_2O_2).

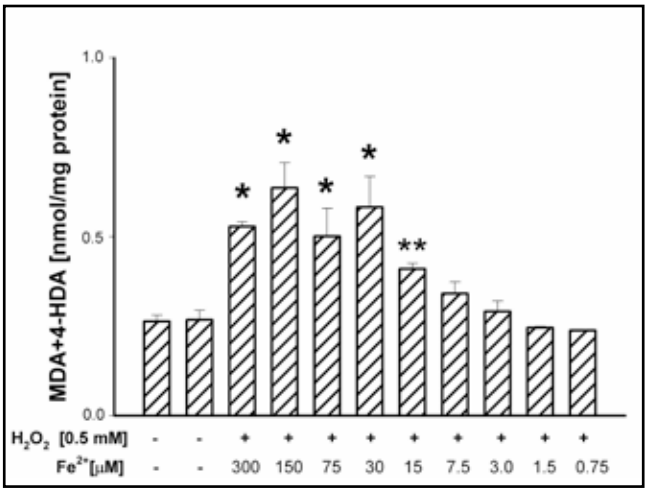

Fig. 4. Concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA) in porcine ovary homogenates, incubated for 30 min in the presence of these two factors used together, namely in the presence of H_2O_2 (0.5 mM) plus $FeSO_4$ (Fe^{2+}) (300, 150, 75, 30, 15, 7.5, 3.0, 1.5 and 0.75 µM). Bars represent the mean ± SE of three independent experiments run in duplicates. * $p<0.05$ vs. Control (in the absence of either Fe^{2+} or H_2O_2) (evaluated by ANOVA followed by a Student-Neuman-Keuls' test); ** $p<0.05$ vs. Control (in the absence of either Fe^{2+} or H_2O_2) (evaluated by an unpaired Student's t-test).

able in practically all tissues. However, our results were different from our expectations.

We demonstrated that H_2O_2 alone did not affect the basal levels of lipid peroxidation in the ovary tissue, although it was used in concentrations, exceeding – several orders of magnitude – those which could be reached physiologically in cells (they vary from 0.001 µM to 15 µM (Schröder and Eaton 2008; Song *et al.* 2007)). It is possible that H_2O_2, normally present in the ovary, although probably in low concentrations, contributes quite effectively to the "physiological" level of lipid peroxidation and external H_2O_2 does not introduce here any additional effect, even after addition of exogenous iron. However, the observation that H_2O_2 alone did not reveal any ability to induce lipid peroxidation in porcine ovary homogenates, does not rule out its potential to increase oxidative changes in this tissue. Clear evidence for such an action was obtained from experimental studies. For example, treatments of human ovary homogenates with H_2O_2 (10, 50 and 100 µM; the concentrations being in the range of those used in the present study) resulted in a decreased aromatase activity, the effect being prevented by glutathione peroxidase (an effective antioxidative enzyme) (Okatani *et al.* 1993).

Unexpectedly, Fe^{2+}, when used alone, significantly increased lipid peroxidation in porcine ovary exactly to the same extent as when the metal was used together with H_2O_2. Thus, it appeared that external H_2O_2 was not indispensable to experimentally enhance lipid peroxidation in porcine ovary homogenates. Instead, the amount of iron present ("physiologically") in ovary tissue was not sufficient to enhance lipid peroxidation in the presence of external H_2O_2. This could be possibly explained by defense mechanisms evolved against iron toxicity, especially that mammals do not possess any machinery for iron excess elimination (Kabat and Rohan 2007). Under normal conditions, iron is bound up with proteins (such as transferrin, ferritin) and, as such, is neither highly reactive nor toxic for cells.

It may be concluded from the present results that enhanced lipid peroxidation, caused by the addition of iron alone to the incubation medium, resulted actually from Fenton reaction between that external iron and H_2O_2, being already present in the ovary tissue at physiological concentrations. It is obvious that Fenton reaction occurs also under physiological conditions, as it has been documented for human extracellular fluids, namely both Fenton reaction substrates, being at physiological concentrations (20 µM concentrations of H_2O_2 and 9.8 µM concentration of free bivalent iron), caused macromolecule oxidation (Waugh 2008). However, it is still puzzling, why H_2O_2, being present in the ovary tissue at physiological concentrations, could have contributed to the enhanced oxidative lipid damage. One can speculate that the ovary tissue is not specialized to compartmentalize H_2O_2, just because H_2O_2 – as a species being present in low physiological concentrations – it is not potentially dangerous in this tissue by itself, while it may be continuously available for any adverse reaction.

A question arises as to clinical applications of the present finding. It is known that obese women with polycystic ovary syndrome (PCOS) are characterized by increased body iron stores (Luque-Ramirez *et al.* 2007). At the same time, PCOS constitutes a well known risk factor for ovary cancer. Taking the above observation

into account, iron overload could contribute to the process of cancer development in the ovary via the mechanism – at least in part – of Fenton reaction, in which H_2O_2 participates, being always present in the tissue, although in rather low concentrations.

Thus, when the ovary tissue is exposed to excessive amount of Fe^{2+}, Fenton reaction occurs more intensively, producing highly toxic •OH in high amounts and, therefore, creating favorable conditions for cancer initiation. An experimental model of such a process was demonstrated in the reported study.

In conclusion, whereas both Fenton reaction substrates – Fe^{2+} and H_2O_2 – are indispensable for oxidative damage to lipids in living organisms, external H_2O_2 is not indispensable for experimental induction of lipid peroxidation via the mechanism of Fenton reaction in porcine ovary homogenates.

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