The role of 17 β -estradiol metabolites in chromium-induced oxidative stress

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Abstract

Background. The increasing incidence of estrogen-dependent breast cancer and the presence in the environment of a large number of factors that interact with estrogen receptors have sparked interest in chemical influences on estrogen-dependent processes. In a previous work, the authors examined the interaction of estradiol with chromium. In the present article the importance of estradiol biotransformation in these interactions is investigated. There is no information in the available literature about the role of metabolites in exposure to chromium. It seems important because estradiol metabolites have various carcinogenic abilities and their formation during biotransformation could be increased or decreased by environmental enzyme inducers or inhibitors. The metabolites could play a detoxifying role or create a toxic synergism in free radical processes induced by chromium VI (CrVI).

Objectives. The aim of this study was to evaluate the influence of 2 17 β -estradiol metabolites – 4-hydroxyestradiol (4-OHE2) and 16 α -hydroxyestrone (16 α -OHE1) – in conditions of oxidative stress caused by CrVI.

Material and methods. Human blood, erythrocytes or mitochondria isolated from human placentas after natural deliveries were used in the experiments. The influence of CrVI, 4-OHE2 and 16-OHE1 on thiobarbituric acid reactive substances (TBARS), the hydroxyl radical ($\cdot$ OH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), and the interactions of the metabolites exposed to chromium expressed by these factors were examined.

Results. 4-OHE2 reduced the level of TBARS induced by CrVI in mitochondria ($p < 0.05$) and in erythrocytes ($p < 0.05$), and increased SOD activity ($p < 0.05$). 16 α -OHE1 increased the activity of GST in erythrocytes exposed to CrVI ($p < 0.05$).

Conclusions. The metabolites do not have toxic interactions with CrVI. On the contrary, they exhibited a protective effect. The mechanism of protection varied: 4-OHE2 decreased TBARS and increased SOD activity, while 16 α -OHE1 increased GST activity.

Key words: oxidative stress, interactions, chromium VI, 4-hydroxyestradiol, 16 α -hydroxyestrone

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The increasing incidence of estrogen-dependent breast cancer has sparked researchers' interest in the influence of environmental factors on estrogen-dependent processes. The interaction of xenobiotics with estrogens has been widely studied in case of environmental toxins that interact with estrogen receptors (xenoestrogens). However, chromium is recognized as a metalloestrogen, and there are only a few reports in the available literature on chromium/estrogen interaction.^{1–3} There is no information about the role of estradiol metabolites in exposure to chromium. The problem seems important because the metabolites have various carcinogenic abilities, and their formation during estradiol biotransformation could be increased or decreased by environmental enzyme inducers or inhibitors. The main biotransformation pathway of estradiol is hydroxylation. The final products of C-4, C-16 or C-2 hydroxylation are 4-hydroxyestradiol (4-OHE₂), 16- α -hydroxyestrone (16 α -OHE₁) or 2-metoxyestradiol (2-MeOE₂) respectively. The role of these metabolites in carcinogenesis is unknown; however, there are some reports on the presence of 4-OHE₂-adducts in breast cancer tissue and on the anticancer activity of 2-MeOE₂.⁴

Chromium is widely present in the environment. Its toxicity depends on the valence. The International Agency for Research on Cancer (IARC) classifies chromium (VI) as a proven human carcinogen. Interactions between estrogens and carcinogenic factors need special attention because estrogen itself possesses carcinogenic activity, so synergistic effects with carcinogenic chromium could be very dangerous. Because exposure to metals is widespread, an elucidation of their roles in the etiology and development of hormone-related diseases such as breast cancer may have significant implications in risk reduction and disease prevention. Chromium compounds are able to generate free radicals, which have an indisputable role in carcinogenesis.⁵ Estrogens in general act as free-radical scavengers, but also demonstrate prooxidative activity. Not only chromium but also estrogens are present in environment. The metabolites of estrogen used in contraception or hormonal replacement therapy are found in ground water and wastewater. They also circulate in the environment via communal waste, increasing the risk of hormonally active compounds interacting with xenobiotics. A number of publications describe the presence of active hormonal compounds originating from waste or breeding farms and their influence on the environment.^{6,7}

The authors' previous work demonstrated the protective role of 17 β -estradiol (E₂) in chromium-induced lipid peroxidation in human erythrocytes, but in the mitochondrial membranes of human placenta, the opposite phenomenon was observed: 17 β -estradiol increased CrVI-induced lipid peroxidation in mitochondria, so a toxic interaction was noted.³ The mechanism of this interaction partially correlated with hydroxyl radical ($\bullet$ OH) formation, since E₂ increased $\bullet$ OH generation in mitochondria exposed to low (0.05 and 0.5 μ g/mL) CrVI concentrations. These

interactions were not observed for superoxide dismutase (SOD) activity.

It seems valid to assess the role of estradiol metabolites in the processes outlined above. Environmental toxins often change the biotransformation pathways, acting as inducers or inhibitors of particular enzymes. The aim of the present study was to evaluate the influence of estradiol metabolites 4-OHE₂ and 16 α -OHE₁ on mitochondria and erythrocytes when exposed to CrVI. There are no data on that topic at present. It is known that the biotransformation of estradiol plays important role in carcinogenesis, and the level of some metabolites, e.g. 16 α -OHE₁, is increased in women with breast cancer.⁸ In this work the influence of 16 α -OHE₁ and 4-OHE₂ on the lipid peroxidation level (TBARS) and hydroxyl radical generation ($\bullet$ OH level), elements of the enzymatic and non-enzymatic antioxidant barrier such as superoxide dismutase (SOD), and detoxifying capacity measured by GST activity was tested. All the experiments were conducted on human cells in vitro: erythrocytes of blood or mitochondria obtained from placenta.

Material and methods

The study was conducted on an in vitro model, using human cell erythrocytes and mitochondria. The study was approved by the Wrocław Medical University Ethics Committee (permit KB-292/2013). Mitochondria were isolated from human placenta, obtained after natural deliveries from the Clinic of Reproduction and Obstetrics, Wrocław Medical University (Poland), by a previously described method.⁹ The isolated mitochondrial pellet was suspended in 50 mM Tris-HCl buffer (pH 7.4), and the level of protein was measured using the Lowry method.¹⁰ The suspension was portioned into Eppendorf tubes (1 mL) and stored at -80°C , no longer than 3 months before use. The mitochondrial suspension was used for the evaluation of lipid peroxidation (TBARS), hydroxyl radical generation ($\bullet$ OH), superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities.

Fresh human blood taken on sodium citrate for SOD, GST and TBARS determination was obtained from healthy donors at Wrocław University Hospital. The erythrocytes were isolated by whole blood centrifugation for 10 min at room temperature (3000 rpm). The plasma and white blood cells were then removed and the resulting pellet of erythrocytes was washed 3 times in phosphate buffered saline (PBS). The erythrocytes were suspended in PBS at a 10% concentration for the SOD, GST and TBARS measurements.

Potassium dichromate (K₂Cr₂O₇) (POCH S.A., Gliwice, Poland) was used as the CrVI source. The CrVI concentrations used in the experiments were 0.05 μ g/mL, 0.5 μ g/mL and 1.0 μ g/mL.

Estradiol metabolites 16 α -hydroxyestrone (3,16 α -Dihydroxy-1,3,5(10)-estratrien-17-one) (Steraloids Inc., New-

port, USA) and 4-hydroxyestradiol (Estra-1,3,5(10)-triene-3,4,17 β -triol) (Sigma-Aldrich Corp., St. Louis, USA) were dissolved in 96% ethanol (P.P.H. POCh S.A.) to a concentration of 10 mM stock solution and used diluted in PBS for every experiment at concentrations of 1.0 μ M, 5.0 μ M and 10.0 μ M.

Appropriate controls were assayed in parallel to the samples. All the experiments were performed gradually, using different sources of red blood cells and mitochondria in each exposure scenario.

Determination of TBARS concentration

In mitochondria

The mitochondrial suspension (1 mL) was incubated in a water bath with a shaker at 37°C for 30 min with 30 μ L of CrVI, 16 α -OHE₁, 4-OHE₂ or a mixture (to assess interaction). The incubation was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA). Then 1.5 mL of 0.67% thiobarbituric acid (TBA) and 30 μ L of 1% butylhydroxytoluene (BHT) were added and the samples were heated at 95°C for 15 min. The cooled mixture was centrifuged to pellet the protein. The TBARS concentration was measured spectrophotometrically at 535 nm and expressed as nM per mg of mitochondrial protein, using the molar absorption coefficient $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.^{11,12}

The effects of metabolite interactions with chromium VI on TBARS concentration were examined in every concentration: 4-OHE₂ and 16 α -OHE₁ at 1.0 μ M, 5.0 μ M and 10.0 μ M; CrVI at 0.05 μ g/mL, 0.5 μ g/mL and 1.0 μ g/mL. First the mitochondria were incubated at 37°C for 30 min with 16 α -OH or 4-OHE₂ at the appropriate concentration, then chromium VI was added to the sample and the incubation was continued at 37°C for 30 min. Then the TBARS concentration was measured by the method described above.

In erythrocytes

The compounds being studied were added in 20 μ L quantities to 2 mL of erythrocyte suspension prepared as described above, and the samples were incubated at 37°C for 0.5 h in a water bath with shaker. The TBARS concentration was then measured as described by Stocks et al.¹³ The concentration of lipid peroxidation products was read from a standard curve, prepared with tetra ethoxypropane and expressed in nM/g Hb. Hemoglobin in the blood was determined using Drabkin's reagent.

Determination of hydroxyl radical generation

The mitochondrial suspension (0.5 mL) was incubated at 37°C (15 min) with 15 μ L of 1% t-BOOH, 0.5 mL of 20 mM deoxyribose and either 4-OHE₂, 16 α -OHE₁ or

CrVI; and to assess interactions, 4-OHE₂ combined with CrVI, or 16 α -OHE₁ combined with CrVI. After incubation, the samples were centrifuged and 0.8 mL of the supernatant was collected; then 0.5 mL of 2.8% TCA and 0.5 mL of 1% TBA in 0.1 M NaOH were added to the supernatant. The samples were incubated at 100°C for 20 min, then cooled and centrifuged. The level of $\bullet$ OH generation was measured spectrophotometrically at 532 nm and calculated using the molar coefficient of absorption $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.^{12,14}

Determination of superoxide dismutase activity

Superoxide dismutase activity was measured with a RANSOD kit (Randox Laboratories Ltd., Crumlin, UK) according to the manufacturer's instructions.^{15,16} The assay is based on the xanthine oxidase reaction, where superoxide anion product reacts with 2-(4-iodophenyl)-3-(4-nitro-phenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye measured at a wavelength of 492 nm. The strength of inhibition of formazan dye formation reflects the activity of SOD in the tested sample. The assay mixture, with a final volume of 0.2 mL, included xanthine oxidase, xanthine, INT and the sample. Absorbance was measured at 492 nm after the addition of xanthine oxidase. On the basis of the absorbance increase after 1 min and comparison with the calibration curve, the percentage of reaction inhibition and hence of SOD activity was calculated according to the kit manufacturer's instructions. The results were compared with a sample containing CrVI, prepared in the same way, but without estrogens.

Determination of glutathione-S-transferase activity

Glutathione S-transferase activity was assayed using 1-Chloro-2, 4-dinitrobenzene (CDNB), which is suitable for the broadest range of GST isoenzymes.¹⁷ Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in absorbance at 340 nm. Solutions of samples containing CrVI, 16 α -OHE₁, 4-OHE₂ or mixtures of them were mixed with the substrate on a shaker, and the absorbance was immediately measured at a wavelength of $\lambda = 340 \text{ nm}$ on a plate reader (Stat-Fax 2100 Awareness Technology, Block Scientific Inc., Bellport, USA). The increase in absorbance in the tested sample was directly proportional to the GST activity.

In order to evaluate the effect of estradiol metabolites on human placental mitochondria or erythrocytes stimulated with CrVI, the mitochondrial or erythrocyte suspension was preincubated at 37°C for 30 min with appropriate concentrations of the estradiol metabolites (16 α -OHE₁ or 4-OHE₂). Then a solution of CrVI was added to the sample and incubation was continued at 37°C for 30 min. Finally,

the level of the factor being tested was measured and compared with controls containing chromium in the appropriate concentration but without estradiol metabolites.

Statistical analyses

All statistical analyses were performed using Student's *t*-test or one-way ANOVA for repeated measurements. Differences between the groups were considered significant at $p < 0.05$. The variability of the distribution was checked with the Lilliefors test. Pearson's correlation coefficient was used to describe correlations. The statistical evaluation of the results was carried out using STATISTICA 10 software (StatSoft Inc., Tulsa, USA).

Results

The effect of CrVI compounds, 4-OHE₂ and 16- α OHE₁ on TBARS and •OH concentration, and on the activities of SOD and GST, both in mitochondria from human placenta and in erythrocytes from blood is presented. In order to better interpret the combined action of CrVI and the metabolites, the effect of the individual compounds (CrVI, 4-OHE₂ or 16- α OHE₁) on selected parameters in the mitochondria and erythrocytes was also presented (Table 1).

It was found that all the concentrations of chromium VI used in the tests (0.05, 0.5 and 1.0 $\mu\text{g/mL}$) increased the lipid peroxidation measured by TBARS concentration in the mitochondrial and erythrocyte suspension in comparison with equivalent controls (Table 1). The combined action of estrogens and CrVI showed that 4-OHE₂ at a concentration of 1.00 μM significantly ($p < 0.05$) reduced the level of chromium-induced (0.05 and 0.5 $\mu\text{g/mL}$ of CrVI) lipid peroxidation in erythrocytes. Decreases in

TBARS concentrations were not observed for the highest chromium concentrations (Table 2).

Unexpectedly, in mitochondria treated with CrVI, the metabolite 4-OHE₂ also reduced the TBARS level ($p < 0.05$) with a clear negative statistically significant dose/response dependency (Pearson correlation coefficient $r = -0.972$ for 0.05 $\mu\text{g/mL}$ CrVI; $r = -0.9494$ for 0.5 $\mu\text{g/mL}$ CrVI; $r = -0.9856$ for 1.0 $\mu\text{g/mL}$ CrVI; $p < 0.001$) (Fig. 1). The statistically significant effect of 4-OHE₂ was observed on lipid peroxidation caused by CrVI at all 3 doses: 0.05 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$ and 1.0 $\mu\text{g/mL}$. The largest decrease in TBARS levels was at the highest concentration of 4-OHE₂ (10.0 μM). This is interesting, because the authors' previous report described 17- β -estradiol (E₂) having the opposite effect on erythrocytes than on mitochondria exposed to chromium.³

The other estradiol metabolite, 16 α -OHE₁, in contrast to 4-OHE₂, did not decrease chromium-induced lipid

Fig. 1. Effect of 4-OHE₂ on mitochondrial TBARS level upon Cr(VI) exposure
*Difference significant vs control (K) without 4-OHE₂ (containing CrVI in appropriate concentration) ($p < 0.05$) ** statistically significant correlation ($p < 0.05$)

Table 1. The mean levels of the examined parameters in mitochondria and erythrocytes after exposure to CrVI, 4-OHE₂ or 16- α -OHE₁

Compound – concentration	TBARS in mitochondria [nM/mg protein]	TBARS in erythrocytes [nM/g Hb]	•OH in mitochondria [nM/mg protein]	SOD in mitochondria [U/mg protein]	SOD in erythrocytes [U/g Hb]	GST in mitochondria [U/mg protein]	GST in erythrocytes [U/g Hb]
CrVI [$\mu\text{g/mL}$]							
Control (0)	2.69 $\pm$ 0.22	1.24 $\pm$ 0.11	5.52 $\pm$ 0.4	84.5 $\pm$ 8.5	79.5 $\pm$ 12.5	0.12 $\pm$ 0.02	0.095 $\pm$ 0.01
0.05	4.13 $\pm$ 0.19*	1.86 $\pm$ 0.13*	6.16 $\pm$ 0.16*	61.13 $\pm$ 11.2*	51.1 $\pm$ 6.2*	0.10 $\pm$ 0.03	0.080 $\pm$ 0.004*
0.5	4.13 $\pm$ 0.21*	1.92 $\pm$ 0.13*	5.85 $\pm$ 0.27	74.98 $\pm$ 15.2*	69.16 $\pm$ 9.8*	0.096 $\pm$ 0.02*	0.080 $\pm$ 0.003*
1.0	4.17 $\pm$ 0.11*	1.66 $\pm$ 0.09	5.59 $\pm$ 0.26	81.22 $\pm$ 20.1	76.43 $\pm$ 11.5	0.090 $\pm$ 0.004*	0.081 $\pm$ 0.002*
4-OHE ₂ [μM]							
Control (0)	2.58 $\pm$ 0.12	1.08 $\pm$ 0.16	4.82 $\pm$ 1.20	125.2 $\pm$ 20.3	106.9 $\pm$ 15.2	2.29 $\pm$ 0.05	0.85 $\pm$ 0.22
1.0	3.08 $\pm$ 0.40	1.02 $\pm$ 0.19	5.23 $\pm$ 1.55	160.0 $\pm$ 18.3	259.0 $\pm$ 17.3*	2.18 $\pm$ 0.11	0.47 $\pm$ 0.2
5.0	2.89 $\pm$ 0.35	1.15 $\pm$ 0.22	6.41 $\pm$ 1.28	285.0 $\pm$ 35.2*	212.2 $\pm$ 32.3*	1.88 $\pm$ 0.19*	0.49 $\pm$ 0.28
10.0	2.41 $\pm$ 0.39	1.48 $\pm$ 0.36	6.60 $\pm$ 2.15	350.0 $\pm$ 46.1*	317.6 $\pm$ 47.2*	1.50 $\pm$ 0.126*	0.56 $\pm$ 0.17
16- α -OHE ₁ [μM]							
Control (0)	3.32 $\pm$ 0.31	3.4 $\pm$ 0.23	8.80 $\pm$ 2.4	138.2 $\pm$ 18.0	124.0 $\pm$ 19.2	1.43 $\pm$ 0.15	1.38 $\pm$ 0.1
1.0	3.01 $\pm$ 0.29	2.8 $\pm$ 0.9	8.05 $\pm$ 1.9	168.0 $\pm$ 23.5	166.4 $\pm$ 22.3*	1.37 $\pm$ 0.2	1.73 $\pm$ 0.04*
5.0	3.72 $\pm$ 0.40	2.9 $\pm$ 0.8	8.95 $\pm$ 0.79	185.0 $\pm$ 42.8	125.0 $\pm$ 17.2	1.28 $\pm$ 0.11	1.71 $\pm$ 0.35*
10.0	3.94 $\pm$ 1.20	3.25 $\pm$ 0.9	8.5 $\pm$ 1.25	172.0 $\pm$ 79.2	130.0 $\pm$ 21.2	1.15 $\pm$ 0.12	1.81 $\pm$ 0.12*

* $p < 0.05$ as compared with control (0) [CrVI = 0 $\mu\text{g/mL}$; 4-OHE₂ = 0 μM ; 16- α -OHE₁ = 0 μM].

Table 2. Effects of 4-OHE₂ (1.0; 5.0 and 10 μ M) on TBARS levels in erythrocytes exposed to CrVI (0.05, 0.5 and 1.0 μ g/mL)

Cr (VI) concentration [μ g/mL]	TBARS concentration [nM/gHb] $\pm$ SD			
	control	4-OHE ₂ concentration [μ M]		
		1.0	5.0	10.0
0.05	1.86 $\pm$ 0.13	1.42* $\pm$ 0.12	1.74 $\pm$ 0.08	1.99 $\pm$ 0.11
0.5	1.92 $\pm$ 0.12	1.66* $\pm$ 0.11	1.12* $\pm$ 0.12	1.76 $\pm$ 0.21
1.0	1.66 $\pm$ 0.09	1.60 $\pm$ 0.23	1.61 $\pm$ 0.19	1.62 $\pm$ 0.10

* difference significant as compared with the control without 4-OHE₂ (containing only CrVI) ($p < 0.05$).

peroxidation in erythrocytes and mitochondria (data not shown). It could therefore be concluded that 16 α -OHE₁ did not participate in the decrease in lipid peroxidation shown by E₂ exposed to CrVI in the authors' previous study.³ It appears that the positive effect of E₂ could be increased by its biotransformation to 4-OHE₂, not to 16 α -OHE₁.

The studies of the joint effects of 4-OHE₂ or 16- α OHE₁ and CrVI in mitochondria revealed that neither of the metabolites in combination with CrVI influenced $\bullet$ OH generation (data not shown). These results suggest that the effect of the examined compounds on free radical processes engages a mechanism that is not dependent on the $\bullet$ OH level.

As to the enzyme activity studies, CrVI in doses of 0.05 μ g/mL and 0.5 μ g/mL decreases SOD in a statistically significant way in comparison to equivalent controls, in both erythrocytes and in mitochondria ($p < 0.05$) (Table 1). The joint influence of metabolites and CrVI on SOD revealed the clear stimulative effects of all concentrations of 4-OHE₂ on SOD activity in erythrocytes exposed to CrVI; dose/response dependency was observed for CrVI in concentrations of 1.0 μ g/mL and 0.5 μ g/mL ($r = 0.5908$ and $r = 0.6041$ respectively; $p < 0.05$).

Most of the data showed a statistically significant increase in SOD activity treated with 4-OHE₂ and CrVI in comparison to controls where erythrocytes were exposed to chromium alone (Fig. 2). As in erythrocytes, 4-OHE₂ significantly increased SOD activity in mitochondria exposed to CrVI (Table 3), but without clear dose/response dependency. No effects of 16 α -OHE₁ on SOD activity in erythrocytes or mitochondria were observed (data not shown).

In all the doses used, chromium VI decreased GST activity in erythrocytes and in mitochondria ($p < 0.05$) in comparison to equivalent controls (0) (Table 1). In erythrocytes exposed to 0.05 μ g/mL, 0.5 μ g/mL and 1.0 μ g/mL of CrVI, the metabolite 16 α -OHE₁ caused a clear increase in GST activity (Fig. 3). The most significant effect was observed when erythrocytes were exposed to the lowest dose of CrVI (0.05 μ g/mL). The effect on mitochondria

Fig. 2. Effect of 4-OHE₂ on erythrocyte SOD activity upon Cr(VI) exposure *difference significant vs control (K) without 4-OHE₂ (containing CrVI in appropriate concentration) ($p < 0.05$) ** statistically significant correlation ($p < 0.05$) **Fig. 3.** Influence of 16 α -OHE₁ on erythrocyte GST activity upon Cr(VI) exposure *difference significant vs control (K) without 16 α -OHE₁ (containing CrVI in appropriate concentration) ($p < 0.05$)**Table 3.** Effects of 4-OHE₂ (1.0, 5.0 and 10 μ M) on mitochondrial SOD activity in exposure to CrVI (0.05, 0.5 and 1.0 μ g/mL)

CrVI concentration [μ g/mL]	SOD activity [U/mg protein] $\pm$ SD			
	control	4-OHE ₂ concentration [μ M]		
		1.0	5.0	10.0
0.05	61.13 $\pm$ 11.2	126.37* $\pm$ 24.96	93.07 $\pm$ 13.02	99.05* $\pm$ 558
0.5	74.98 $\pm$ 15.2	218.87 $\pm$ 102.19	221.96* $\pm$ 76.32	138.42* $\pm$ 19.37
1.0	81.22 $\pm$ 20.1	129.48* $\pm$ 14.32	152.62* $\pm$ 15.13	150.19* $\pm$ 36.32

* difference significant as compared with the control without 4-OHE₂ (containing only CrVI) ($p < 0.05$).

was not significant (data not shown). No influence of 4-OHE₂ on GST activity was observed in erythrocytes or mitochondria exposed to CrVI.

To summarize, it was observed that neither 4-OHE₂ and 16 α -OHE has toxic reactions to CrVI; on the contrary, they even show positive, protective effects in mitochondria and in erythrocytes exposed to CrVI: 4-OHE₂ decreases lipid peroxidation in mitochondria and erythrocytes, increasing SOD activity. The positive role of

16 α -OHE₁ involves a different mechanism, influencing phase-II detoxification by stimulating glutathione transferase activity.

Discussion

The role of estradiol in oxidative stress is prominent because it demonstrates free radical scavenging activities. This activity is enabled by the presence of a hydroxyl phenolic group that acts as a proton donor in chemical reactions.¹⁸ The estrogenic radical formed in this deprotonation is stabilized by electron delocalization inside the aromatic ring. On the basis of this mechanism, it can be concluded that the estradiol metabolites 4-OHE₂ and 16 α -OHE₁ should demonstrate similar properties, since both these compounds contain an aromatic ring substituted with a hydroxyl group. It follows that 4-OHE₂ should be an even stronger free-radical scavenger in cells than in the substrate, since it contains a second additional phenolic group typical of catecholestrogens. The catecholic structure favors transformation to o-quinone and semi-quinone radicals, which are less toxic than the hydroxyl radical. The catecholic structure also has the ability to neutralize other free radicals. The quinons are reduced with quinone reductase or CYP reductase and removed by conjugation with reduced glutathione.⁴

Some authors have demonstrated that 4-OHE₂ reduces the toxic effects of oxidative stress in cells and oxidative DNA-damage.^{19–21} The current study also confirms the ability of 4-OHE₂ to decrease chromium VI-induced lipid peroxidation. The metabolism of estradiol is based mainly on hydroxylation reactions induced or blocked by endo- or exogenous compounds that induce or inhibit cytochrome P450. C-4 hydroxylation leads to 4-OHE₂ formation, but C-16 hydroxylation leads to 16 α -OHE₁.

The influence of estradiol and its metabolites on oxidative stress has been widely discussed in the literature, however there is little information about the role of estradiol in xenobiotic-induced oxidative stress.²² Sowers et al. hypothesized that physiological levels of estradiol and its metabolites 2-hydroxyestrone and 16 α -hydroxyestrone decrease oxidative stress measured as isoprostane levels in women's plasma; unfortunately, a test on 1647 smoking and non-smoking women at the age of 47–57 did not confirm this hypothesis.²³ On the other hand, Tang et al. showed that 4-hydroxyestrone acted as an inhibitor of lipid peroxidation in tissue cultures, while Seeger et al. demonstrated that 2-metoxysterone and 2-hydroxyestrone blocked lipid peroxidation more efficiently than estradiol and 16 α -hydroxyestrone in the serum of healthy subjects.^{24,25}

Estrogens may act as pro-oxidants or antioxidants depend on their concentration.²⁶ The present study shows that in contrast to 4-OHE₂, 16 α -OHE₁ does not decrease CrVI-induced lipid peroxidation. The advantageous effect

of 4-OHE₂ on lipid peroxidation in mitochondria suggests that the increase in TBARS in mitochondria exposed to chromium treated with 17 β -estradiol described in the authors' earlier publication is in fact a result of substrate not metabolite interaction between E2 and CrVI; 4-OHE₂ is not involved in this interaction.³ The mechanism of the antioxidative action of 4-OHE₂ in exposure to CrVI seems to be connected with stimulation of SOD activity, especially in erythrocytes exposed to CrVI. While previous studies have shown that CrVI compounds strongly inhibit SOD activity, the present work demonstrates that this effect can be repaired by 4-OHE₂.²⁷ It can be concluded that increased formation of 4-OHE₂ in estradiol biotransformation appears beneficial for the inhibition of oxidative stress in mitochondria and erythrocytes exposed to CrVI compounds.

Human erythrocytes seem to be an appropriate model to study the estrogen-chromium effect on the antioxidative barrier. They contain antioxidative enzymes (SOD, catalase, GPx) and non-enzymatic antioxidants (vitamins C and E, beta-carotene and uric acid). Additionally, CrVI has the ability to accumulate in human erythrocytes. It has been shown that increased levels of estrogens in erythrocytes in female rats protect them from induced trauma/hemorrhagic shock (T/HS). In comparison to erythrocytes in male rats, a positive action in lowering red blood cells' deformability was observed.^{28,29} The current study compared 2 cell models. Both mitochondria and erythrocytes were sensitive to the influence of chromium- and estrogen-caused oxidative disturbances. In most cell types mitochondria are the predominant source of reactive oxygen species (ROS), including superoxide anion, H₂O₂ and •OH. Although estrogen synthesis occurs in mitochondria, exogenously added estrogens are also transported to this organelle; their lipophilic properties allow them to diffuse through the lipid bilayer of cell membranes.³⁰ Mitochondria have a unique characteristic that allows them to participate in growth signal transduction. They are a regulatable source of ROS in response to external stimuli, for example metalloestrogens or estrogens.³¹

Mitochondria are a major target of estrogens. It appears that through their interaction with the cytoskeleton, export of cleaved signaling peptides, and/or generation of ROS, mitochondria may transduce signals to the nucleus for the activation of transcription factors involved in the cell cycle progression of estrogen-dependent cells. These interactions between estrogens and mitochondria merit future investigation, which could shed new light on the role of mitochondria in cell growth. In the present study these organelles were used to examine the interactions between estrogens and metalloestrogen in reaction to oxidative stress.^{25,30,32}

Considering the results obtained in relation to CrVI-induced carcinogenesis, the 4-OHE₂ metabolite demonstrated a positive effect on the free radical reaction, while

16 α -OHE₁ did not. These results support the general assumption that the introduction of another phenol group to the aromatic ring of estradiol (4-OHE₂) increases the free radical scavenging properties of the metabolite-formed. The 16 α -OHE₁ metabolite also shows a positive effect, but by a different mechanism. It influences the second phase of detoxification, thus increasing GST activity. It can be postulated that the biotransformation of estradiol to 4-OHE₂ and 16 α -OHE₁ in exposure to CrVI has advantageous results.

Conclusions

4-hydroxyestradiol reduces the level of lipid peroxidation induced by CrVI compounds in mitochondria and in erythrocytes, increasing SOD activity. 16 α -hydroxyestrone increases the activity of GST in erythrocytes exposed to CrVI.

References

- Byrne C, Divekar SD, Storch GB, Parodi DA, Martin MB. Metals and breast cancer. *J Mammary Gland Biol Neoplasia*. 2013;18:63–73.
- Mishra AK, Mohanty B. Effect of sublethal hexavalent chromium exposure on the pituitary-ovarian axis of a teleost, *channapunctatus* (bloch). *Environ Toxicol*. 2010;27:415–422.
- Rembacz K, Sawicka E, Długosz A. Role of estradiol in chromium-induced oxidative stress. *Acta Pol Pharm*. 2012; 69:1372–1379.
- Licznarska B, Baer-Dubowska W. Estrogen intracrinology: Therapy and chemoprevention of breast cancer. *Post Hig Med Dosw*. 2010;64:220–230.
- Martin BM, Reiter R, Pham T, et al. Estrogen-like activity of metals in MCF-7 breast cancer cells. *Endocrinology*. 2003;144:2425–2436.
- Soto AM, Calabro JM, Prechti NV, et al. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska USA. *Environ Health Persp*. 2004;112:346–352.
- Matejczyk M, Zalewski P. Związki endokrynnie aktywne i ich aktywność biologiczna. *Kosmos*. 2011;60:17–32.
- Belous AR, Hachey DL, Dawling S, Roodi N, Parl FF. Cytochrome P450 1B1-mediated estrogen metabolism results in estrogen-deoxyribonucleoside adduct formation. *Cancer Res*. 2007;67:812–817.
- Długosz A, Sawicka E, Marchewka Z. Styrene and ethylene glycol have a synergistic effect on lipids peroxidation, that is better protected than repaired by CoQ₁₀. *Toxicol In Vitro*. 2005;19:581–589.
- Lowry OH, Rosbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem*. 1951;193:265–275.
- Długosz A, Piotrowska D. Lipid peroxidation stimulated by solvents, bawanol and methanol and its counteraction by antioxidants in human placental mitochondria. *Toxicol In Vitro*. 2002;16:649–656.
- Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods in Enzymology*. 1978;52:302–310.
- Stocks J, Offerman EL, Model CB, Dormandy TL. The susceptibility to autooxidation of human red cell lipids in health and disease. *Br J Haematol*. 1972;23:713–724.
- Rice-Evans CA, Diplock AT, Symons MCR. Techniques in free radical research. Amsterdam, London, New York, Tokyo, Elsevier; 1991.
- Mistra HP, Fridovich I. Superoxide dismutase: Photo-chemical augmentation assay. *Arch Biochem Biophys*. 1985;181:308–312.
- Woolliams JA, Wiener G, Anderson PH, McMurray CH. Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. *Res Vet Sci*. 1983;34(3):253–256.
- Tamaki H, Yamamoto K, Kumagai H. Expression of two glutathione S-transferase genes in the yeast *issatchenkia orientalis* is induced by o-dinitrobenzene during cell growth arrest. *J Bacteriol*. 1999;181:2958–2962.
- Asatiani N, Sapojnikova N, Abuladze M, et al. Effects of CrVI long-term and low-dose action on mammalian antioxidant enzymes (an in vitro study). *J Inorg Biochem*. 2004;98:490–496.
- Stachowiak G, Połać I, Jędrzejczyk S, Pertyński T, Stetkiewicz T. Antyoksydacyjne działanie estrogenów. *Prz Menopauz*. 2003;2:19–22.
- Rogan E. Xenoestrogens, biotransformation and differential risks for breast cancer. *Altern Ther Health Med*. 2007;13:112–121.
- Muñoz-Castañeda JR, Muntané J, Muñoz MC, Bujalance I, Montilla P, Túnez I. Estradiol and catecholestrogens protect against adriamycin-induced oxidative stress in erythrocytes of ovariectomized rats. *Toxicol Lett*. 2005;160:196–203.
- Długosz A, Roszkowska A, Zimmer M. Oestradiol protects against the harmful effects of fluoride more by increasing thiol group levels than scavenging hydroxyl radicals. *Basic Clin Pharmacol Toxicol*. 2009;105:366–373.
- Sowers MF, Mc Connell D, Jannausch ML, et al. Oestrogen metabolites in relation to isoprostanes as a measure of oxidative stress. *Clin Endocrinol*. 2008;68:806–813.
- Tang M, Abplanalp W, Ayres S, Subbiah MT. Superior and distinct antioxidant effects of selected estrogen metabolites on lipid peroxidation. *Metab Clin Exp*. 1996;45:411–414.
- Seeger H, Mueck AO, Lippert TH. Effect of estradiol metabolites on the susceptibility of low density lipoprotein to oxidation. *Life Sci*. 1997;61:865–868.
- Światalska M, Strządała L. Non-genomic action of estrogens. *Post Hig Med Dosw*. 2007;61:541–547.
- Długosz A, Rembacz KP, Pruss A, Durlak M, Lembas-Bogaczyk J. Influence of chromium on the natural antioxidant barrier. *Pol J Environ Stud*. 2012;21:331–335.
- Doucet DR, Bonitz RP, Feinman R, Colorado I, Ramanathan M, Feke-teova E. Principles and practice of medicine. In: Churchill Livingstone, ed. New York, 2002:889–956.
- Machiedo GW, Zaets S, Berezina T, Xu DZ, Spolarics Z, Deitch EA. Red blood cell damage after trauma-hemorrhage is modulated by gender. *J Trauma*. 2004;56:837–844.
- Felty Q, Roy D. Estrogen, mitochondria and growth of cancer and non-cancer cells. *J Carcinog*. 2005;4:1–18.
- Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci*. 1995;15:6377–6388.
- Kabat GC, O'Leary ES, Gammon MD, et al. Estrogen metabolism and breast cancer. *Epidemiology*. 2006;17:80–88.