Effect of Pharmaceutical Potential Endocrine Disruptor Compounds on Protein Disulfide Isomerase Reductase Activity Using Di-Eosin-Oxidized-Glutathion

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Abstract

Background: Protein Disulfide Isomerase (PDI) in the endoplasmic reticulum of all cells catalyzes the rearrangement of disulfide bridges during folding of membrane and secreted proteins. As PDI is also known to bind various molecules including hormones such as estradiol and thyroxin, we considered the hypothesis that adverse effects of endocrine-disrupter compounds (EDC) could be mediated through their interaction with PDI leading to defects in membrane or secreted proteins.

Methodology/Principal Findings: Taking advantage of the recent description of the fluorescence self quenched substrate di-eosin-oxidized-glutathion (DiE-GSSG), we determined kinetically the effects of various potential pharmaceutical EDCs on the in-vitro reductase activity of bovine liver PDI by measuring the fluorescence of the reaction product (E-GSH). Our data show that estrogens (ethynylestradiol and bisphenol-A) as well as indomethacin exert an inhibition whereas medroxyprogesteroneacetate and nortestosterone exert a potentiation of bovine PDI reductase activity.

Conclusions: The present data indicate that the tested EDCs could not only affect endocrine target cells through nuclear receptors as previously shown, but could also affect these and all other cells by positively or negatively affecting PDI activity. The substrate DiE-GSSG has been demonstrated to be a convenient substrate to measure PDI reductase activity in the presence of various potential EDCs. It will certainly be usefull for the screening of potential effect of all kinds of chemicals on PDI reductase activity.

Introduction

Endocrine-disrupting compounds are commonly considered as molecules acting either by mimicking or by blocking the transcriptional activation of hormone nuclear receptors such as those for estrogens, androgens, progestagens, thyroid hormones etc [1,2]. Nevertheless, other pathways are now taken into consideration because these hormones can affect nuclear receptors indirectly [3] or act through non-genomic pathways [4] or can bind to non-receptor cellular proteins [5].

In the frame of the european Food & Fecundity project (http://foodandfecundity.factlink.net/180095.1/), one of the main strategic objectives was to identify pharmaceutical products as potential endocrine disruptors of reproductive function and to determine their adverse effects and mechanisms of action in in-vitro and in-vivo systems. A prioritization list of seven pharmaceutical products bearing a potential of affecting human fecundity by entering the food chain has been created on the basis of production volume, recognized presence in environment and documented effect(s) on production [6]. The main drug of concern in this respect was 17β-ethinylestradiol that is the estrogenic component of most contraceptive pills.

Besides, it has been reported that Protein Disulfide Isomerase which catalyzes oxidative folding of proteins [7] in the endoplasmic reticulum has also been found to be a high-capacity binding protein for 17β-estradiol [5]. It was also found by the same authors that 17β-estradiol displayed an inhibitory effect on isomerase activity of PDI using scrambled RNA as substrate [5]. Taking this information into account, we considered the hypothesis that EDCs could exert part of their effects by affecting PDI properties. We have thus undertaken a study of the influence of estrogens as well as other pharmaceuticals of interest on another enzymatic function of PDI, i.e. its disulfide reductase activity.

Results

Preliminary control experiments were carried out in order to check whether reduction of DiE-GSSG by another enzyme than PDI or its cleavage by various proteases or conformational changes could interfere in the assay (see figure S1 in supplementary data). We found that on a molar basis, thioredoxin exhibited less than 0.1% reductase activity on DiE-GSSG compared to PDI. Likewise, Glutathion Reductase either in the presence of 1 mM
NADPH or 33 μM DTeT was unable to reduce DiE-GSSG. Several proteolytic enzymes (trypsin, thrombin, collagenase-dispase, leucine-aminopeptidase and carboxypeptidase) were tested and found to be without any effect on DiE-GSSG fluorescence. Moreover, neither 0.1% Tween nor 0.5 M concentrations of sodium chloride or ammonium sulfate had any direct effect on DiE-GSSG fluorescence. All these negative controls indicate that the reductase assay using DiE-GSSG as a substrate is highly specific for PDI (see supplementary material).

Figure 1A shows the kinetics of DiE-GSSG reduction catalyzed by bovine liver PDI. A dose-dependent inhibition of PDI by EE2 can be evidenced through the measurement of the initial rate of the reaction. Figure 1B shows that the kinetics of DiEGSSG reduction by PDI as followed by EGSH fluorescence is insensitive to the presence of ethanol up to 10% (v/v). In figure 1A, the maximum ethanol concentration (for EE2 1 μM) is less than 0.01%. The estrogens E2 and DES exhibited similar effects as EE2 as shown in figure 2. Indeed, figure 2 reports the effects of increasing concentrations of the various estrogenic EDCs under study (E2, EE2, DES) on the initial velocity of PDI activity using DiE-GSSG and DTcT as co-substrates. Interestingly, the three estrogenic molecules exhibit a significant inhibitory effect on PDI activity starting at concentrations as low as 10^{-8} M. BPA was also found to exert a partial (20%) inhibitory effect on PDI reductase activity but only at concentrations higher than 10^{-4} M (not shown).

Figure 3 shows the effects of three non-estrogenic potential EDCs, MPA, 19-NT and IMT, on PDI activity. Only IMT exhibits an inhibitory effect which is however much less important than that of estrogenic molecules (fig. 2). By contrast, the two non-estrogenic steroids MPA and 19-NT exhibit a potentiation effect on PDI reductase activity but only above 10^{-5} M for 19NT.

In addition we checked that 10^{-4} M bacitracin that is a known inhibitor of PDI activity [8,9] also exhibited such an inhibitory activity in this protocol (not shown).
Discussion

Protein Disulfide Isomerase (PDI) is a multifunctional enzyme mainly found in the endoplasmic reticulum of eukaryotes where its main function is to catalyze the rearrangement (isomerization) of disulfide bridges during folding of membrane and secreted proteins. Its concentration in the lumen of the endoplasmic reticulum is known to be very high [10] and it has been reported to act as a high capacity reservoir for various ligands including hormones such as estradiol (E2) and thyroxine (T3) [5,11]. This ability of PDI to bind a large number of different molecules prompted us to search whether potential endocrine disruptor compounds (EDCs) could exert an influence on its reductase activity. For this matter, we used the recently described fluorescence self-quenched substrate DiE-GSSG [12] that is easier to synthesize and exhibits a much higher signal than the previously described substrate di-aminobenzoyl-GSSG [13].

Since eosin fluorescence in DiE-GSSG is quenched because of the vicinity of eosin-groups at the N-termini of oxidized glutathion (GSSG), it was important to check that reduction by another enzyme than PDI or proteolysis or conformational change of GSSG could not interfere in the assay. Thioredoxin exerted a very high capacity reservoir for various ligands including hormones such as estradiol (E2) and thyroxine (T3) [5,11]. This ability of PDI to bind a large number of different molecules prompted us to search whether potential endocrine disruptor compounds (EDCs) could exert an influence on its reductase activity. For this matter, we used the recently described fluorescence self-quenched substrate DiE-GSSG [12] that is easier to synthesize and exhibits a much higher signal than the previously described substrate di-aminobenzoyl-GSSG [13].

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eventual effect on PDI activity. Since neither Thioredoxin nor Glutathion Reductase reduce DiE-GSSG in the presence of NADPH or DTeT, this substrate appears to be highly specific for PDI. This assay should thus be used as a screen to detect molecules that, individually or synergistically, would be potentially harmful for cell function by affecting protein folding.

Materials and Methods

17β-estradiol (E2), 17α-ethinylestradiol (EE2), diethylstilbestrol (DES), medroxyprogesterone acetate (MPA), 19-nor-testosterone (19NT), bacitracin, indomethacin (IMT), bisphenol A (BPA), dithioerythritol (DTeT), eosin 5-isothiocyanate, oxidized glutathion (GSSG), Protein Disulfide Isomerase (E.C. 5.3.4.1) from bovine liver (PDI), Thioredoxin (E.C 1.6.4.5) from spirulina and Glutathion Reductase (E.C 1.6.4.2) from S. cerevisiae as well as the protocytic enzymes trypsin, thrombin, collagenase-dispase, leucine-aminopeptidase and carboxypeptidase were all purchased from Sigma-Aldrich (Ile-de’Abeau, France) and were of the highest available grades. All steroids were initially dissolved in ethanol and serially diluted in the 100 mM phosphate buffer pH 8.0 used for the kinetics.

The PDI substrate, di-eosin-oxidized glutathion (DiE-GSSG), was synthesized and purified as previously described [12] with minor modifications. After final purification on sephadex G50 and freeze-drying, GSSG were freeze-dried and kept in the dark until use.

Supporting Information

File S1 Control experiments showing the effects of reductive enzymes and proteolytic enzymes on DiE-GSSG fluorescence (λex = 518 nm; λem = 545 nm)

Author Contributions

Conceived and designed the experiments: YC. Performed the experiments: DK MV YC. Analyzed the data: YC. Contributed reagents/materials/analysis tools: CC YC. Wrote the paper: YC.

References

1. Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray, et al. (1996) Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect 104 Suppl 6: 713–40.
2. Toppari J (2008) Environmental endocrine disrupters. Sex Dev 2: 260–7.
3. Tabb M, Blumberg B (2006) New models of action for endocrine-disrupting chemicals. Mol Endocrinol 20: 475–82.
4. Cress D, McLachlan JA (2006) Epigenetics, evolution, endocrine disruption, health, and disease. Endocrinology 147: 84–10.
5. Prüm TP, Gilbert HF (2003) Hormone binding by protein disulfide isomerase, a high capacity hormone reservoir of the endoplasmic reticulum. J Biol Chem 278: 281–6.
6. Piersma AH PV, Cagampang F, Luijten M, Tomonouko V (2005) Prioritization list of pharmaceutical products (PPs) bearing a potential of affecting human fecundity. Food and Fecundity contract n° 2004-519535.
7. Gruber CW, Cemazar M, Heras B, Martin JL, Craik DJ (2006) Protein disulfide isomerase: the structure of oxidative folding. Trends Biochem Sci 31: 431–8.
8. Polska AH, Konopka J (2004) Cross-linking protein disulfide isomerase: a new tool for the study of protein folding. J Mol Biol 336: 549–60.
9. Gutthapel R, Gueguen P, Quemener E (1996) Reexamination of hormone-binding properties of protein disulfide-isomerase. Eur J Biochem 242: 313–9.
10. Raturi A, Mutus B (2007) Characterization of redox state and reductase activity of protein disulfide isomerase under different redox environments using a sensitive fluorescent assay. Free Radic Biol Med 43: 62–70.
11. Raturi A, Vlachakis P, Zialva S, Lee L, Mutus B (2005) A direct, continuous, sensitive assay for protein disulfide-isomerase based on fluorescence self-quenching. Biochem J 391: 531–7.
12. Raturi A, Mutus B (2007) Characterization of redox state and reductase activity of protein disulfide isomerase under different redox environments using a sensitive fluorescent assay. Free Radic Biol Med 43: 62–70.
13. Hatahet F, Ruddock LW (2007) Substrate recognition by the protein disulfide isomerase. FEBS J 274: 5223–34.
14. Gilbert HF, Kruzel ML, Lyles MM, Harper JW (1991) Expression and purification of recombinant rat protein disulfide isomerase from Escherichia coli. Protein Expr Purif 2: 194–8.
15. Walker KW, Lyles MM, Gilbert HF (1996) Catalysis of oxidative protein folding by mutants of protein disulfide isomerase with a single active-site cysteine. Biochemistry 35: 1972–80.
16. Vong J, Brucker J, Cherbowmos W (2008) Long-term exposure of male rats to low-dose ethinylestradiol (EE2) in drinking water: effects on ponderal growth and on litter size of their progeny. Reprod Toxicol 25: 161–8.
17. Van Meewen JA, van den Berg M, Sanderson JT, Verhooft A, Peters AH (2007) Estrogenic effects of mixtures of phytos- and synthetic chemicals on uterine growth of prepubertal rats. Toxicol Lett 170: 165–76.
18. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM (2009) Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. Environ Res 30: 75–95.
19. Perdew M, Liu B (2008) Role of tissue factor disulfides and lipid rafts in signaling. Thromb Res 122 Suppl 1: S14–8.
20. Klappa P, Stremer T, Zimmermann R, Ruddock LW, Freedman RB (1998) A pancreas-specific glycosylated protein disulfide-isomerase binds to misfolded proteins and peptides with an interaction inhibited by oestrogens. Eur J Biochem 254: 63–9.
21. Fu XM, Zhu BT (2009) Human pancreas-specific protein disulfide isomerase homolog (PDHB) is an intracellular estrogen-binding protein that modulates estrogen levels and actions in target cells. J Steroid Biochem Mol Biol 115: 20–9.
22. Tsibris JC, hunt LT, Ballejo G, Barker WC, Toney LJ, et al. (1989) Selective inhibition of protein disulfide isomerase by estrogens. J Biol Chem 264: 13967–70.
23. Hiroi T, Okada K, Imaoka S, Osada M, Funae Y (2006) Bisphenol A binds to protein disulfide isomerase and inhibits its enzymatic and hormone-binding activities. Endocrinology 147: 2773–80.
24. Hashimoto S, Kuida K, Imaoka S, Osada M, Funae Y (2006) Bisphenol A binds to protein disulfide isomerase and inhibits its enzymatic and hormone-binding activities. Endocrinology 147: 2773–80.
25. Hashimoto S, Okada K, Imaoka S, Osada M, Funae Y (2006) Bisphenol A binds to protein disulfide isomerase and inhibits its enzymatic and hormone-binding activities. Endocrinology 147: 2773–80.