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Participation of the endoplasmic reticulum protein chaperone thio-oxidoreductase in gonadotropin-releasing hormone receptor expression at the plasma membrane

W. Lucca-Junior, J.A. Janovick and P.M. Conn

Division of Neuroscience and Reproductive Sciences, Oregon National Primate Research Center, Oregon Health and Science University, ONPRC/OHSU, Beaverton, Oregon, USA

Correspondence to: W. Lucca-Junior, Rua Pedro Pacheco, 3, Cohab II, 14750-000 Pitangueiras, SP, Brasil
Fax: +55-16-3633-0017. E-mail: wlucca1@hotmail.com

Chaperone members of the protein disulfide isomerase family can catalyze the thiol-disulfide exchange reaction with pairs of cysteines. There are 14 protein disulfide isomerase family members, but the ability to catalyze a thiol disulfide exchange reaction has not been demonstrated for all of them. Human endoplasmic reticulum protein chaperone thio-oxidoreductase (ERp18) shows partial oxidative activity as a protein disulfide isomerase. The aim of the present study was to evaluate the participation of ERp18 in gonadotropin-releasing hormone receptor (GnRHR) expression at the plasma membrane. Cos-7 cells were cultured, plated, and transfected with 25 ng (unless indicated) wild-type human GnRHR (hGnRHR) or mutant GnRHR ($Cys^{14}Ala$ and $Cys^{200}Ala$) and pcDNA3.1 without insert (empty vector) or ERp18 cDNA (75 ng/well), pre-loaded for 18 h with 1 µCi myo-[2-3H(N)]-inositol in 0.25 mL DMEM and treated for 2 h with buserelin. We observed a decrease in maximal inositol phosphate (IP) production in response to buserelin in the cells co-transfected with hGnRHR, and a decrease from 20 to 75 ng of ERp18 compared with cells co-transfected with hGnRHR and empty vector. The decrease in maximal IP was proportional to the amount of ERp18 DNA over the range examined. Mutants ($Cys^{14}Ala$ and $Cys^{200}Ala$) that could not form the Cys^{14}-Cys^{200} bridge essential for plasma membrane routing of the hGnRHR did not modify maximal IP production when they were co-transfected with ERp18. These results suggest that ERp18 has a reduction role on disulfide bonds in wild-type hGnRHR folding.

Key words: Protein chaperones; Gonadotropin-releasing hormone receptor; Calnexin; Calreticulin; ERp18

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After synthesis in the endoplasmic reticulum (ER), G protein-coupled receptors (GPCRs) are often folded and assembled to be packaged into the ER-derived COPII-coated vesicles and transported through the Golgi apparatus and the trans-Golgi network in order to arrive at the plasma membrane. During transportation through the ER and Golgi structures, GPCRs are submitted to post-translational modifications to acquire the mature conformation (1). These events - folding and trafficking of newly synthesized proteins - are highly regulated processes that likely require a number of different chaperone molecules belonging to the cell's quality control system (QCS). These QCS chaperones recognize non-native conformations of newly synthesized proteins and prevent their aggregation and export of the incompletely folded chains from the ER (2). When the maturation of a newly synthesized protein is aborted or inefficiently performed, chaperones catalyze a covalent bond between ubiquitin and the unfolded protein.

This reaction targets misfolded proteins to proteosomal degradation by the ER-associated degradation process (3-5). A large number of diseases are associated with degradation of misfolded proteins such as Parkinson's, Alzheimer's, hypogonadotropic hypogonadism, diabetes insipidus, and others (6). When the human gonadotropin-releasing hormone receptor (hGnRHR), the smallest representatives of this GPCR superfamily of receptors, is not expressed in the cell's plasma membrane because it was retained in the ER or eventually degraded in the cytosol, its normal function (activation of luteinizing hormone release) is not performed and this results in the disease hypogonadotropic hypogonadism (1,7). Hypogonadotropic hypogonadism is characterized by 1) complete or partial absence of any endogenous GnRH-evoked luteinizing hormone pulsations, 2) delayed sexual development, and 3) normalization of pituitary and gonadal function in response to physiological regiments of exogenous GnRH replacement (8).

Chaperones are an interesting potential therapeutic target because of their role in the cellular QCS, regulating the folding and assembly of newly synthesized proteins, including hGnRHR (1). They are present in the ER, mitochondria and cytoplasm, and comprise a wide class of proteins that may be categorized into five groups: the heat shock protein family, lectins, substrate-specific proteins, protein disulfide isomerases (PDI) and peptidyl prolyl isomerases (6). Each group of chaperones has a different chemical means to retain, refold and assemble misfolded proteins or promote their eventual degradation. One of these groups is the PDI family of chaperones, which recognize and catalyze the formation and isomerization of non-native disulfide bonds (9).

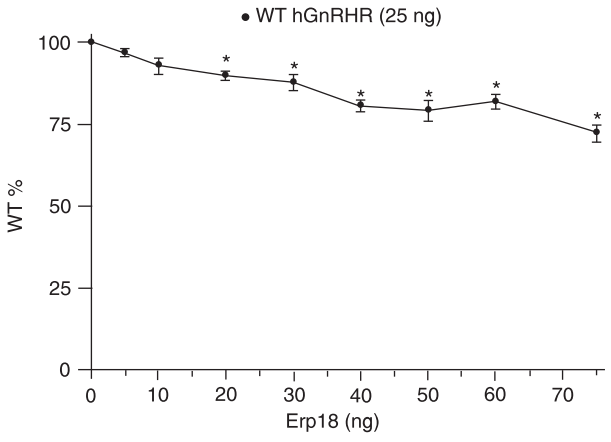

Figure 1. Wild type percent (WT%) indicates the inositol phosphate production upon buserelin stimulation of WT human gonadotropin-releasing hormone receptor (hGnRHR) co-transfected with the ERp18 chaperone. Data are reported as mean ± SEM of at least three independent experiments in triplicate incubations. *P < 0.05 compared with WT hGnRHR (25 ng) without ERp18 co-transfected (one-way ANOVA followed by the Bonferroni post-test).

PDI family members can catalyze the thiol-disulfide exchange reaction with a pair of cysteines that are frequently arranged in a Cys-X-X-Cys motif (where X is any amino acid). There are 14 PDI family members, but the ability to catalyze a thiol disulfide exchange reaction has not been shown for all members. Some members like PDIr, ERp72, P5, PDIp, ERp28, and ERp18 have the same or partial oxidative activity as PDI, while only ERp57 has the ability to reduce disulfide bonds (10,11).

The aim of the present study was to evaluate the participation of the human endoplasmic reticulum protein chaperone thio-oxidoreductase (ERp18) in gonadotropin-releasing hormone receptor expression at the plasma membrane.

Cos-7 cells were cultured, plated, and transfected, as previously reported (12), with 25 ng (unless indicated) WT hGnRHR, or mutant GnRHR (C14A and C200A) and pcDNA3.1 without insert (empty vector) or ERp18 cDNA (75 ng/well), as indicated, and 1 µL lipofectamine in 0.125 mL OPTI-MEM (room temperature), according to manufacturer instructions. Empty vector (pcDNA3.1, without insert) was included to bring the total cDNA to a final concentration of 100 ng/well, which provides optimal transfection efficiency (12,13). Five hours after transfection, 0.125 mL DMEM containing 20% fetal bovine serum and 20 µg/mL gentamicin were added to the wells. Twenty-three hours after transfection, cells were washed, then preloaded for 18 h with 1 µCi myo-[2-3H(N)]-inositol in 0.25 mL DMEM (prepared without inositol (12)). The cells were then washed twice with 0.25 mL DMEM containing 5 mM LiCl (without inositol), treated for 2 h with 0.25 mL of the indicated buserelin concentration in the same medium (LiCl prevents inositol phosphate (IP) degradation). Total IPs were determined as described previously (12,14). Data are averaged from at least three independent experiments (15).

We observed a decrease in maximal IP production in response to buserelin in the cells co-transfected with hGnRHR and from 20 to 75 ng ERp18 compared with cells co-transfected with hGnRHR and empty vector (Figure 1). The decrease in maximal IP was proportional to the amount of ERp18 DNA over the range examined. These results suggest the participation of ERp18 in GnRHR folding and assembly.

Wild-type hGnRHR has two disulfide-bond bridges - Cys^{114}-Cys^{196} and Cys^{14}-Cys^{200}. All mutants without the

disulfide bridge (Cys^{114}-Cys^{196}) remain in the ER and can never be rescued by IN3 (a pharmacological chaperone with the ability to rescue unfolded proteins by correcting their routing) (7,16,17). However, the Cys^{14}-Cys^{200} bridge can be rescued by IN3 (17). In our experiments, mutants that could not form the Cys^{14}- Cys^{200} bridge (i.e., Cys^{14}Ala and Cys^{200}Ala) did not change the maximal IP production when they were co-transfected with ERp18 (Figure 2), but when this bridge was present (wild-type hGnRHR), the co-transfection with ERp18 decreased maximal IP production. These results suggest that ERp18 has a reduction effect on the disulfide bond in wild-type hGnRHR folding. Conversely, *in vitro* studies (18) have demonstrated that the reduced form of ERp18 is more stable than the oxidized form, suggesting that it is involved in disulfide bond formation. Moreover, they also have demonstrated that ERp18 is relatively inefficient in catalyzing oxidoreductase reactions (15% of a domain of PDI) (18), probably because it lacks the glutamic acid proton acceptor (11). However, inside the ER there are many different oxidoreductases with a range of abilities to catalyze oxidoreductase reactions. Then, even with a relatively inefficient power to catalyze an oxidoreductase reaction there are many possibilities of interaction between ERp18 and other oxidoreductase proteins to reduce the disulfide bond in wild-type hGnRHR.

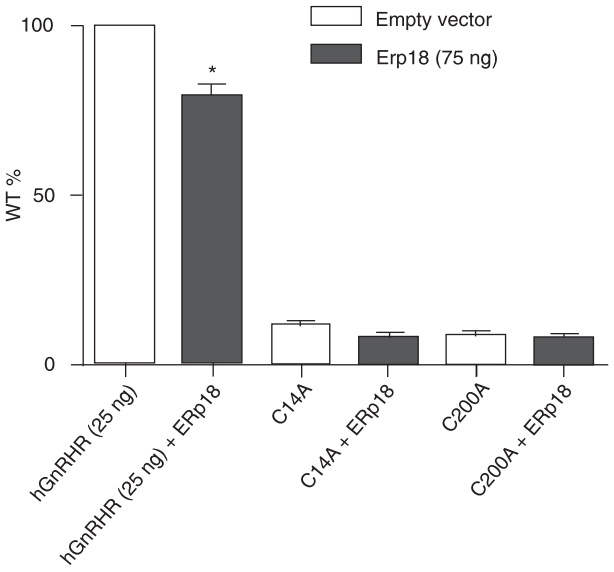

Figure 2. Wild type percent (WT%) indicates the inositol phosphate production upon buserelin stimulation of WT human gonadotropin-releasing hormone receptor (hGnRHR) and mutant GnRHRs (C14A and C200A) co-transfected with the ERp18 chaperone. Data are reported as mean ± SEM of at least three independent experiments in triplicate incubations. *P < 0.05 compared with WT hGnRHR (25 ng) without ERp18 co-transfected (one-way ANOVA followed by the Bonferroni post-test).

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