NOTE

Effects of Estrogen on PMCA 2 and 4 in Human Fibroblast-like Synovial Cells and Mouse Macrophage-like Cells

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Abstract. We investigated the possible roles of estrogen on plasma membrane Ca^{2+} -ATPase (PMCA) in human fibroblast-like synovial cells (HFLS) and mouse macrophage-like cells (RAW 264.7). Western blots revealed the expression of PMCA 2 and 4 in both cells. *In vitro* treatments with 17β -estradiol for 24 hours resulted in a concentration dependent decrease in PMCA expression. Moreover, Ca^{2+} -ATPase specific activity was similarly decreased with estrogen treatments. However, treatments for 1 hour in the presence or absence of cycloheximide demonstrated non-significant effects. These results suggest that estrogen has a modulatory role on Ca^{2+} homeostasis through decreasing PMCA expression and abating their activity.

Key words: PMCA, Estrogen, RAW 264.7 cells, HFLS cells, Ca^{2+} -ATPase activity

THE CLASSIC model of the estrogen action involves binding to intracellular steroid receptors. In the last decade, increasing body of evidence for the rapid non-genomic effects of the hormone has been demonstrated and its mechanisms of action were shown to include receptor and non-receptor-mediated pathways [1, 2]. Fast responses with unique steroid specificity have been reported to occur in purified plasma membrane preparations [3]. Possible mechanism has been attributed to interaction with membrane lipids that then altered the function of membrane proteins. It was reported that steroids may have a direct effect on the protein-protein interaction and may also bind directly to protein molecules [4]. In the past years, estradiol, a

lipophilic molecule that can easily cross plasma membranes and blood-brain barrier, has received considerably more attention. Numerous studies revealed that estrogens exert neuro-protective effect in the aged or injured brain, maintain intracellular calcium homeostasis and, at high hormone level, promote antioxidant activity [5-8].

Cytosolic Ca^{2+} regulates several cellular processes and its concentration is, in turn, finely regulated by various channels, pumps and exchangers. The plasma membrane Ca^{2+} -ATPase (PMCA) is an integral part of the Ca^{2+} regulatory system in eukaryotic cells playing a prominent role in restoring intracellular Ca^{2+} concentration to the basal level [9-12]. PMCA belongs to the P-type family of transport ATPases, which form a phosphorylated intermediate during the reaction cycle. Following PMCA cloning, four mammalian isoforms have been identified (PMCA1-4) [10].

An earlier study [13] demonstrated the ability of some neuroactive steroids to directly modulate the purified rat cortical Ca^{2+} -ATPase activity, but sur-

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prisingly the effect was dependent on the PMCA isoforms, as the synaptosomal enzyme was activated by the hormone, whereas others were insensitive to 17β -estradiol. Another study demonstrated the effect of 17β -estradiol on sarcoplasmic reticulum Ca^{2+} -ATPase in H9C2 cells [14].

Previously, we reported the effects of estrogen on RAW 264.7 and HFLS cells, regarding inflammation and bone resorption [15, 16]. In the current study we investigated the expression of PMCA isoforms in RAW 264.7 and HFLS cells, as well as, the modulatory effect of 17β -estradiol on the expression levels of PMCA isoforms. This study was conducted following thorough review of literature that revealed the lack of data concerning PMCA(s) expression in both cells. We further studied; whether 17β -estradiol could modify the Ca^{2+} -ATPase activity in HFLS and RAW 264.7 cells.

Materials and Methods

Cell culture

HFLS are primary human fibroblast-like synovial cells, from a 67 years Caucasian female; were purchased from (Cell Applications, Inc., San Diego, CA) and cultured in Synoviocyte Growth Medium (Cell Applications, Inc., San Diego, CA). RAW 264.7 (Macrophage-like cells) were cultured in complete medium α -MEM supplemented with 66.7 $\mu\text{g}/\text{mL}$ kanamycin and 10 % fetal calf serum (FCS). Cells were incubated in humidified 5 % CO_2 atmosphere at 37 °C.

Exogenous 17β -estradiol treatment

At confluence, (10^{-8} , 10^{-5} M) of 17β -estradiol (Sigma-Aldrich, St.Louis, MO) in ethanol were added to the medium for the period of 24 hours, while controls were treated with an equivalent volume of ethanol (estrogen vehicle). Cells were also treated with 17β -estradiol in the presence or absence of 10 μM cycloheximide (Sigma-Aldrich) for 1 hour.

Cell homogenate

For whole cell lysate extraction, at confluence, media were aspirated, cells were washed with Dulbecco's phosphate buffered saline (PBS) without calcium

and magnesium, and lysis buffer was added (10 mM HEPES-KOH at pH 7.5, 100 mM KCl, 0.1 % NP-40). Cells were lysed thoroughly and dislodged from plate by repetitive pipetting. Protein concentrations were assessed by Bio-Rad protein assay (Bio-Rad, Hercules, CA) employing bovine serum albumin (BSA) as standard according to manufacturer instructions.

Ca^{2+} -ATPase activity assay

The assay was performed as previously described [13]. In brief, 1 μL of RAW 264.7 cell homogenate or 3 μL of HFLS cell homogenate was added to the reaction mixture containing: 50 mM tris-HCl buffer pH 7.4, 25 mM Sucrose (0.1 mM EDTA), 1 mM EDTA and 2 mM CaCl_2 . The final volume of assay mixture prior to reaction initiation was 250 μL . The reaction was initiated by the addition of 5 mM ATP-tris, and stopped 2 hours following 37 °C incubation. Ca^{2+} -ATPase activity (Pi hydrolyzed from ATP) was measured by a spectrophotometer at 850 nm absorbance, employing Chifflet method [17]. The specific activity was calculated (nanomoles of Pi / mg of protein / minute). Presented data reflects the percentages of means $\pm$ standard deviation of 3 independent experiments. Data was statistically analyzed by one-way analysis of variance (ANOVA) with multiple comparative Dunnett test. Statistical significance was defined as ($P < 0.01$).

Western blotting

PMCA expression in HFLS and RAW 264.7 cells, as well as the effect of 17β -estradiol on those PMCA, was investigated using western blotting. Rat brain microsome was used as positive control for all PMCA subunits (1; 2 (a & b); 3; 4 (a & b)). Samples were separated on 10 % polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were blocked in 5 % skim milk. Primary antibodies PMCA 1-4 (Affinity BioReagents, Rockford, IL) were incubated overnight at 4 °C, on a shaker. Secondary antibody (IgG HRP conjugated) incubations for at least 1 hour at room temperature, were followed by luminol light signal detection using Supersignal Western detection (Pierce, Rockford, IL) reagents. The signal was recorded via blot incubations for 1-10 min

Fig. 1. Expression of PMCA isoforms.

Western blot of control “rat brain microsome” (left lane), RAW 264.7 “mouse macrophage-like cells” (middle lane) and HFLS “primary human fibroblast-like synovial cells” (right lane). Blots were probed for PMCA 1 (upper panel), PMCA 2 (a and b) (second panel from top), PMCA 3 (third panel from top) and PMCA 4 (a and b) (lowest panel). Both RAW 264.7 and HFLS expressed PMCA 2 and 4 (a and b), meanwhile, they both lacked PMCA 1 and 3.

with Kodak BioMax XAR films (Carestream Health, Rochester, NY) using intensifying screens.

Results and Discussion

Calcium is much more than a “bone nutrient”, as it is an electrolyte functioning as an intracellular messenger, regulator of cellular metabolism and replication. Cytosolic Ca^{2+} regulates several cellular processes and its concentration is finely regulated by various channels, pumps and exchangers including the PMCA [9-11]. In this study, we investigated the types of PMCA (1-4) expressed in the mouse RAW 264.7 (macrophage-like) cells and human HFLS (Human Fibroblast like Synovial) cells, that have otherwise, never been reported in literature. PMCA 2 (a & b) and 4 (a & b) were expressed in both cells, while PMCA 1 and 3 were not detected (Fig. 1). RAW 264.7 possessed stronger expression of PMCA 2 (a & b) than HFLS, while HFLS exhibited stronger expression of PMCA 4 (a & b) than RAW 264.7 cells. Expressions of PMCA 2 (a) and 4 (a) were stronger than (b) in both cells (Fig. 1).

Attention was drawn mainly to disruption of Ca^{2+} homeostasis due to its implication in different pathological conditions as ischemia, stroke, and cell death; in which excess intracellular Ca^{2+} can lead to free radicals [18, 19]. Meanwhile, the normal physiological process of aging reduces the functional capacities of Ca^{2+} -ATPase due to oxidative modifications of protein molecules [19-23]. Such progressive decline of Ca^{2+} -ATPase activity with age has been reported to be of approximately 35 % in aging rat brain synaptosomal membranes [24]. It was hypothesized that the highly sensitive PMCA pump to the inhibitory effects of the reactive oxygen and nitrogen species; might be a target of oxidative stress in the aging process [24].

Estrogen is a crucial sex hormone that has been involved in various physiologic processes. Decline in estrogen levels has been reported as a factor in the etiology/progression of age associated neurodegenerative states [5]. Moreover, estrogens in the micromolar range possess intrinsic antioxidant activity, and may serve as scavengers for free radicals [6]. This led to the speculation that such neuro-protective function of 17β -estradiol could also be exerted at the membrane level, through direct regulation of Ca^{2+} -ATPase activity, acting as the first line of defense against calcium overload. A report stated the modulation of PMCA in rat cortex synaptosomes by 17β -estradiol and testosterone [13]; but surprisingly the effect was dependent on the PMCA isoforms. Another reported the effect of 17β -estradiol on sarcoplasmic reticulum Ca^{2+} -ATPase in H9C2 cells [14]; neither report assessed the direct effects of 17β -estradiol on PMCA, nor the relevantly affected PMCA isoform. We investigated the effects of our previously reported optimal concentration of 10^{-8} M 17β -estradiol [15, 16] on HFLS and RAW 264.7 cells. The specific Ca^{2+} -ATPase activity in RAW 264.7 and HFLS treated for 24 hours decreased to 62% and 94% respectively, compared to that of their control (100%) (Fig. 2). Further increase in estrogen concentrations to 10^{-5} M, led to further decline in activity in to 45% and 67% in RAW 264.7 and HFLS cells respectively, compared to their untreated control replicates, denoting a statistically significant ($P < 0.01$) concentration-dependent decrease in specific Ca^{2+} -ATPase activity with increasing estrogen concentrations (Fig. 2). Hence, we concluded that increasing estrogen concentration, led to a decrease in the Ca^{2+} -ATPase activity that in turn leads to an increase in intracellular Ca^{2+} . While this finding per se might sug-

Fig. 2. Effect of estrogen on specific Ca^{2+} -ATPase activity.

HFLS (solid bars) and RAW 264.7 (open bars) were treated with different concentrations (0, 10^{-8} and 10^{-5} M) of estrogen (17β-estradiol). The specific Ca^{2+} -ATPase activity in terms of liberated Pi from ATP hydrolysis was spectrophotometrically recorded at 850 nm, and the specific activity calculated (nmol/mg/min). Data presented as (%) of specific activity from control, and representative of the mean of 3 independent experiments $\pm$ standard deviation. One-way analysis of variance (ANOVA) carried out, with multiple comparative Dunnett test where (*) denotes statistical significance ($P < 0.01$).

Fig. 3. Effect of different estrogen concentrations on PMCA 2 and 4.

Western blots of RAW 264.7 (left panels), and HFLS (right panels), treated with different concentrations (0, 10^{-8} and 10^{-5} M) of estrogen (17β-estradiol) for up to 24 hours. Blots were probed for PMCA 2 (a and b) (upper panels), and PMCA 4 (a and b) (lower panels). Both PMCA isoforms exhibited reduced expression in RAW 264.7 cells, while similar effects were only observed at the highest estrogen concentration in HFLS cells.

gest a disturbance in cellular function due to elevated intracellular Ca^{2+} as mentioned [18, 19], we tend to consider the process is more complex, based on the reported intrinsic antioxidant activity of estrogen [6]. Meanwhile, 1 hour treatments of 17β-estradiol demonstrated non-significant effects on Ca^{2+} -ATPase activity, both in the presence or absence of cycloheximide (data not shown), denoting that such effects of 17β-estradiol are rather genomic in nature.

Estrogen (10^{-8} M 17β-estradiol) treatments of cells for up to 24 hours in culture, demonstrated a marked decrease in the expression of PMCA 2 (a & b) and 4 (a & b) in RAW 264.7 cells (Fig. 3). On the other hand, HFLS expression of PMCA 2 (a & b) and 4 (a & b) showed slight to no change at concentration of 10^{-8} M 17β-estradiol. PMCA isoforms in both RAW 264.7 and HFLS cells were of further diminished expression with further increase in estrogen concentration to 10^{-5}

M (Fig. 3). Concordant with our above reported estrogen effects on Ca^{2+} -ATPase activity, we here demonstrate a further modulatory role of estrogen on PMCA expression. Short (1 hour) treatments of 17β-estradiol in the presence or absence of cycloheximide, demonstrated no obvious effects on both PMCA 2 and 4 expression (data not shown), also denoting that such effects of 17β-estradiol are rather genomic in nature.

In summary, we demonstrate a dose-dependent inhibitory role of estrogen (17β-estradiol) on both the expression of PMCA 2 (a & b) and 4 (a & b), and the specific Ca^{2+} -ATPase activity in HFLS and RAW 264.7 cells. Since, estrogen affected all detectable PMCA isoforms in both studied cells; we decline to conclude any specific PMCA isoform specificity, and rather suggest that the effects of estrogen are generalized, awaiting further investigations.

References

1. Losel R M, Falkenstein E, Feuring M, Schultz A, Tillmann H C, Rossol-Haseroth K, Wehling M (2003) Nongenomic steroid action: controversies, questions and answers. *Physiol Rev* 83: 965-1016.
2. Moore F L, Evans S J (1999) Steroid hormones use nongenomic mechanisms to control brain functions and behaviors: a review of evidence. *Brain Behav* 54: 41-50.
3. Whiting K P, Restall C J, Brain P F (2000) Steroid hormone-induced effects on membrane fluidity and their potential roles in non-genomic mechanisms. *Life Sci* 67: 743-757.
4. Woolley C S (1999) Effects of estrogen in the CNS. *Curr Opin Neurol* 9: 349-354.
5. Behl C (2002) Estrogen as a neuroprotective hormone. *Nat Rev Neurosci* 6: 433-442.
6. Lee S J, McEwen B S (2001) Neurotrophic and neuroprotective actions of estrogens and their therapeutic implications. *Annu Rev Pharmacol Toxicol* 41: 569-591.
7. Rupprecht R, Holsboer F (1999) Neuropsychopharmacological properties of neuroactive steroids. *Steroids* 64: 83-91.
8. Zinder O, Dar D E (1999) Neuroactive steroids: their mechanism of action and their function in the stress response. *Acta Physiol Scand* 167: 181-188.
9. Carafoli E (1994) Biogenesis: plasma membrane calcium ATPase: 15 years of work on the purified enzyme. *FASEB J* 8: 993-1002.
10. Prasad V, Okunade G W, Miller M L, Shull G E (2004) Phenotypes of SERCA and PMCA knockout mice. *Biochem Biophys Res Commun* 322: 1192-1203.
11. Strehler E E, Treiman M (2004) Calcium pumps of plasma membrane and cell interior. *Curr Mol Med* 4: 323-335.
12. Strehler E E, Zacharias D A (2001) Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. *Physiol Rev* 81: 21-50.
13. Zylinska L, Gromadzińska E, Lachowicz L (1999) Short time effects of neuroactive steroids on rat cortical Ca-ATPase activity. *Biochim Biophys Acta* 1437: 257-264.
14. Chen-Geng L, Ke-Qian X, Xia X, Jian-Jun H, Jing-Chuan X, Jie-Ping Z, Hui-Ping S (2007) 17 β -estradiol regulates the expression of Na⁺/K⁺-ATPase β 1-subunit, sarcoplasmic reticulum Ca²⁺-ATPase and carbonic anhydrase IV in H9C2 cells. *Clin Exp Pharmacol Physiol* 34: 998-1004.
15. Galal N, El-Beialy W R, Deyama Y, Yoshimura Y, Suzuki K, Totsuka Y (2007) Novel effect of estrogen on RANK and c-fms in RAW 264.7 cells. *Int J Mol Med* 20: 97-101.
16. Galal N, El-Beialy W R, Deyama Y, Yoshimura Y, Yoshikawa T, Suzuki K, Totsuka Y (2008) Effect of estrogen on bone resorption and inflammation in temporomandibular joint cellular elements. *Int J Mol Med* 21: 785-790.
17. Chifflet S, Torriglia A, Chiesa R, Tolosa S (1988) A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPase. *Anal Biochem* 168: 1-4.
18. Lehotsky J, Kaplan P, Matejovicova M, Murin R, Racay P, Raeymaekers L (2002) Ion transport systems as targets of free radicals during ischemia reperfusion injury. *Gen Physiol Biophys* 21: 31-37.
19. Lehotsky J, Kaplan P, Murin R, Raeymaekers L (2002) The role of plasma membrane Ca²⁺ pumps (PMCA) in pathologies of mammalian cells. *Front Biosci* 7: 53-84.
20. Hanahisa Y, Yamaguchi M (2001) Decrease in Ca²⁺ATPase activity in the brain plasma membrane of rats with increasing age: involvement of brain calcium accumulation. *Int J Mol Med* 7: 407-411.
21. Michaelis M L, Bigelow D J, Schoneich C, Williams T D, Ramonda L, Yin D, Huhmer A F, Yao Y, Gao J, Squier T C (1996) Decreased plasma membrane calcium transport activity in aging brain. *Life Sci* 59: 405-412.
22. Squier T C, Bigelow D J (2000) Protein oxidation and age dependent alterations in calcium homeostasis. *Front Biosci* 5: 504-526.
23. Zaidi A, Gao J, Squier T C, Michaelis M L (1998) Age related decrease in brain synaptic membrane Ca²⁺ATPase in F344/BNF1 rats. *Neurobiol Aging* 19: 487-495.
24. Qin Z, Zaidi A, Gao J, Krainev A G, Michaelis M L, Squier T C, Bigelow D J (1998) Decrease in Ca²⁺ATPase activity in aged synaptosomal membranes is not associated with changes in fatty acyl chain dynamics. *Mech Age Dev* 105: 291-300.