Downregulation of aromatase by siRNA decreases acetylcholinesterase mRNA and specific activity in SH-SY5Y cells

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ABSTRACT: Anti-hormone therapy mediated cognitive decline among cancer patients has been referred to as chemobrain. Current literature indicates that declined estrogen in the brain may be contributing to the genesis of this phenomenon. Aromatase is a key enzyme that converts C19 steroids to estrogen in the brain, although its involvement in cognitive function has remained obscure. This study evaluated the downregulation of aromatase by siRNA treatment and the effect of aromatase downregulation on mRNA expression and specific activity of acetylcholinesterase in SH-SY5Y cells. Short interfering RNA duplexes were employed to achieve aromatase gene downregulation in SH-SY5Y cells. The reduction of aromatase mRNA was analyzed by real-time PCR. Estradiol levels were determined to confirm the downregulation by a commercial ELISA kit. Acetylcholinesterase mRNA level of siRNA-treated SH-SY5Y cells was analyzed employing Real-time PCR. Specific acetylcholinesterase activity was determined using the Ellman method. Viability and caspase 3/7 activity was examined using ApoTox-GloTM Triplex Assay. zVad-Fmk was employed as a pan-caspase inhibitor in experimental groups. Real-time PCR analysis showed a significant decrease in aromatase mRNA. The downregulation data was also confirmed using an ELISA estradiol kit. Acetylcholinesterase mRNA level was determined significantly reduced in siRNA-treated SH-SY5Y cells. It was observed that the specific activity of acetylcholinesterase was also reduced. Aromatase downregulation didn’t alter viability or caspase 3/7 activity of SH-SY5Y cells. However, aromatase downregulation increased susceptibility to caspase-dependent apoptosis. Considering our findings, there may be interactions between aromatase and acetylcholinesterase that have not yet been elucidated and may contribute to the mechanism of chemobrain.

KEYWORDS: Aromatase inhibitor; Acetylcholinesterase; chemobrain; siRNA; estrogen.

1. INTRODUCTION

Anti hormone therapy is the main treatment regimen in estrogen-sensitive cancers like breast cancer. Aromatase inhibitors (AIs), and tamoxifen are the most commonly used drugs for anti-hormone therapy. The aromatase inhibitors decrease circulating estrogen dramatically, while tamoxifen does not change estrogen levels. Although the benefits of this drug regime are beyond doubt, cognitive decline is implicated many times among patients who use AIs. This phenomenon is called chemobrain and patients usually suffer from decreased estrogen levels [1-3]. Growing evidence supports the significance of estrogen in cognitive functions. Estrogen alters the brain’s both structure and function: plasticity, hippocampal neurogenesis, neurite growth, dendritic branching, neurotransmitter systems are altered, and cognition, memory, mood are affected. The major source of postmenopausal estrogen is aromatase (CYP19A1, ARO) that converts C19 steroids to estrogen. Significant levels of ARO activity is reported in different regions of the brain mostly produced by neurons [4].

The cholinergic hypothesis has been the major perspective for the cognitive decline since the 1960s and has been associated with dementia, Alzheimer’s disease, and Parkinson’s disease [5-8]. Growing evidence has indicated estrogen-mediated cholinergic effects in the cerebral cortex and hippocampus [9, 10]. Extensive studies have reported that estrogen interacts with the cholinergic system inducing cholinergic innervation, choline acetyltransferase (CHAT) level, and acetylcholine release [11]. Estradiol binding site in 30-kb region upstream from the human acetylcholinesterase (AChE) gene was reported [12]. Although estrogen and cholinergic system have many intersections in the brain, hormonal modulation of cognition is still not fully understood. Decreased AChE activity is a characteristic of cognitive decline, whereas...
increased AChE activity in the hypothalamus is documented after estrogen administration [13]. Furthermore, estradiol treatment was reported to increase acetylcholinesterase activity in lungs and ovaries[14-16].

Even though the cognitive decline was indicated many times with AI or antioestrogen treatment, the underlying mechanism has remained unclear[17-19]. Studies have shown escalated response to the AChE inhibitors tacrine and donepezil when combined with estrogen treatment, whereas cholinesterase inhibitor treatments didn’t change ARO mRNA level or activity[20, 21].

To obtain some enlightenment about ARO and AChE interactions, short-interfering RNA (siRNAs) were used to specifically attenuate the aromatase gene in SH-SY5Y cells, and acetylcholinesterase (AChE) mRNA level, specific activity, and caspase-3/7 activity were determined.

2. RESULTS

2.1. Effect of ARO downregulation on AChE mRNA expression and specific activity

Although estrogen’s influence on cholinergic neurons is previously reported, interactions with AChE have remained enigmatic. Previous literature indicated that AChE inhibitor treatments didn’t change ARO mRNA level or activity, even though estrogen replacement therapy was found beneficial above a critical cholinergic threshold[21, 22]. On the other hand, a recent study in ovariectomized rats demonstrated that AChE activity was declined in the hippocampus. These reports raise the question of whether lack of estrogen is the underlying cause of the cholinergic deficit in cognitive declines. To tackle this issue, firstly down-regulation of ARO was confirmed by RT-PCR analysis and estradiol ELISA kit. ARO mRNA expression was decreased to 0.4% at 48 hours (Figure 1A). Estradiol level in collected media revealed considerable reduction (to 26.7%) at the time of 48 hours of post-transfection (Figure 1B).

After ARO suppression was confirmed, AChE mRNA expression (Figure 1C) and specific activity (Figure 1D) in SH-SY5Y cells were examined by RT-PCR analysis and Ellman assay. Downregulation of ARO decreased AChE mRNA expression and specific activity to %1.2 and 43.9% respectively.

Figure 1.A) Downregulation of ARO mRNA expression B) Estradiol levels C) AChE mRNA expression D) Specific AChE activity. Data were analyzed by one-way ANOVA and presented as means ± SEM of minimum three independent experiments (*p<0.05, **p<0.01, ***p<0.001) (mRNA expression experiments shown in A and C also include two technical replicates)
2.2. Effects of ARO downregulation on cell viability and caspase-3/7

Neuronal cell death is a common feature of cognitive decline. Neuroinflammation, amyloid β deposits, hyperphosphorylated tau protein, oxidative and nitrosative stress are inducers of neuron death. This process is mediated through various well-known mechanisms as apoptosis, necrosis, autophagy, and excitotoxicity.

Accumulating evidence suggests that estradiol and aromatase have neuroprotective roles including anti-apoptotic, anti-oxidant, and anti-inflammatory effects as well as regulation of calcium channels [23-25]. Regarding the apoptotic cascade, estradiol has been noted to decrease caspase-3 activation and to antagonize the increased levels of activated caspase-3 while aromatase inhibition didn’t activate caspase 3 [25, 26]. Viability (Figure 2A) and caspase 3/7 (Figure 2B) activity of ARO downregulated SHSY-5Y cells were not significantly changed. Viability results revealed that proliferation of the SHSY-5Y cells were not estrogen-dependent. Notably, viability was decreased when pan-caspase inhibitor zVad-Fmk was added to SHSY-5Y cells whereas the viability of SH-SY5Y cells transfected with ARO siRNA significantly increased to 160.8%. The following apoptosis analysis revealed that caspase 3/7 activity was significantly reduced after zVad-Fmk treatment in SH-SY5Y cells. However, the caspase inhibitory effect of zVad-Fmk was limited in the ARO siRNA group.

Figure 2. Viability and caspase-3/7 activity in ARO siRNA transfected SH-SY5Y cells at 48 hours. Data were analyzed by two-way ANOVA and presented as means ± SEM of minimum three independent experiments (*p<0.05).

3. DISCUSSION

Various studies show that estrogen has beneficial effects on cognitive functions. Estrogen therapy has been shown to improve memory and differential cognitive performances in ovariectomized rats dramatically [27, 28]. Similarly, studies in humans have also demonstrated the beneficial effects of estrogen on certain cognitive functions associated with verbal memory and executive functions in young menopausal or premenopausal women [29].

Brain ARO is the enzyme that converts androstenedione and testosterone to estrogen and is localized in many brain regions including the hippocampus and cortex. It exerts valuable effects on cognitive functions in these regions including neuroprotection through estrogen synthesis [30].

Studies have reported adverse cognitive side effects, such as memory impairments and concentration difficulties, among patients on AI treatments [31, 32]. Previous studies have indicated possible interactions of ARO with the cholinergic system, showing that estrogen therapy supplements cholinergic tasks by boosting choline acetyltransferase, choline uptake, and acetylcholine release [33, 34]. However, it is still unclear how
the AI treatment alters cognitive functions. This study explored the in vitro effects of siRNA mediated ARO downregulation on acetylcholinesterase mRNA expression and specific activity, and caspase-3/7 activity.

AChE is the key enzyme of the brain cholinergic system that break downs the neurotransmitter acetylcholine in the synaptic cleft to choline and acetate. Mounting evidence has shown reduced activity of AChE in several brain disorders, including neurodegenerative disorders. Importantly, AChE deprivation is evident, as demonstrated by positon emission tomography and autopsy studies in the forebrain of Alzheimer's disease patients [35, 36]. In this study, downregulation of ARO decreased AChE mRNA expression and specific activity to 1.2% and 43.9% respectively. This observation is in accord with previous reports of decreased brain AChE activity in dementia patients and may explain cognitive decline which is observed with aromatase inhibitor treatment [11, 37, 38]. Remarkably, in a recent retrospective case-control study, it was also found that the probability of being diagnosed with Alzheimer's Disease was increased cumulatively with the period of aromatase inhibitor use [39].

Als anti-tumor effect has been reported to coming from suppressing estrogen's effects on proliferation and cell cycle, rather than inducing apoptosis [40, 41]. Als are usually combined with other adjunctive anti-cancer agents to enhance apoptosis [42]. In the current study, I observed that ARO siRNA transfection didn't alter viability of the SH-SY5Y cells pointing that proliferation of the SHSY-5Y cells were not estrogen-dependent. Treatment with pan-caspase inhibitor zVad-Fmk decreased the viability of SHSY-5Y cells which may be due to activation of caspase independent cell death mechanisms. Although zVad-Fmk is broadly used to elucidate cell death mechanisms, accumulating evidence have proposed that it would inhibit other enzymes such as peptide N-glycanase, lysosomal cysteine protease and cathepsin B [43, 44]. These caspase independent effects may be the reason of decreased proliferation as reported in numerous studies [45, 46]. Interestingly, the viability of SH-SY5Y cells transfected with ARO siRNA significantly increased to 160.8% with zVad-Fmk treatment which implied that lack of estrogen might counteracted this anti-proliferative effect via currently unknown mechanisms. Furthermore, caspase 3/7 activity was significantly reduced after zVad-Fmk treatment in SH-SY5Y cells whereas this caspase inhibitory effect was slighter in the ARO siRNA group. Consistent with my observations, earlier reports showed that antiestrogen or aromatase inhibitors may induce cell death without caspase -3/7 activation [26, 47].

A limitation of this study was that experiments were performed in the SH-SY5Y cell line rather than in vivo. In this context, in vivo and/or clinical studies focusing on the effect of aromatase enzyme on cholinergic transmission would be valuable. The significance of aromatase- acetylcholinesterase interactions will require further examination.

4. CONCLUSION

This study emphasizes that there may be intriguing interactions between AChE and ARO enzymes than is known, particularly affecting cognitive functions. The present data showed that siRNA-mediated ARO suppression decreased the mRNA level and specific activity of AChE and increased susceptibility of SH-SY5Y cells to caspase-dependent apoptosis.

5. MATERIALS AND METHODS

5.1. Chemicalsandcellculture

High-grade chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Merck (Quebec, Canada), and Calbiochem (Los Angeles, California). Cell culture consumables were obtained from ThermoScientific, Biowest, and Lonza.

The human SH-SY5Y neuroblastoma cells (CRL-2266, ATCC) were maintained in Dulbecco’s modified eagle’s medium (DMEM) containing 10% FBS, 1% L-glutamine, and 1% antibiotic in a humidified atmosphere in 5% CO2 at 37 °C. Cells were passaged at 80% confluency.

5.2. ARO siRNAtransfection

SH-SY5Y cells were transiently transfected with a pool of small interfering RNAs (siRNA) designed against ARO (GenBank Accession number: D29757) using Lipofectamine™ RNAiMAXreagent (Invitrogen, CA, USA). Cells were seeded in a six-well cell culture plate (6 x 105 cells/well) in antibiotic-free DMEM supplemented with 10% FBS. After siRNA transfection (1 μg/100 μl), plates were incubated for 48 hours before RNA isolation, cDNA synthesis, and RT-PCR.
5.3. Total RNA isolation, cDNA synthesis, and RT-PCR

RNA was isolated using RNAGEM Tissue (ZyGEM New Zealand), as claimed by the manufacturer's recommendations. A Nano drop spectrophotometer was employed to determine the purity and concentration of RNAs. The Proto Script First Strand cDNA Synthesis Kit (BioLabs New England) was used to convert total RNA to cDNA on a Veriti 96 well thermocycler (AppliedBiosystems). cDNA synthesis reaction occurred for an hour at 42 °C including inactivation of the enzyme at 80 °C for 5 min.

Evaluation of gene expression was conducted in a ViiA 7 by Life Technologies Real-Time PCR system and software (AppliedBiosystems) as claimed by the manufacturer's instructions. RT conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and 1 min at 72 °C.

All data were analyzed using the ΔΔCt method. Sample Ct values were normalized by Ct values of the GAPDH. Primers for AChE, ARO, and GAPDH (housekeeping gene) employed in quantitative real-time PCR experiments are listed in Table 1.

| Primer Name | Primer sequence (5’-3’) |
|-------------|------------------------|
| AChE (F)    | CCTGTCTCGTCTGGATCTATG  |
| AChE (R)    | AAGAAGGGGCCATCGTACAC   |
| ARO (F)     | GACGCAGGATTTCCACAGAAGAG|
| ARO (R)     | ATGGTGTCAGGAGCTGCGATCA |
| GAPDH (F)   | GTCGTATTGGGCGCCTGGTCAC |
| GAPDH (R)   | GCCAGCATCGCCCCCACCTTGATT|

5.4. Estradiol Assay

SH-SY5Y cells were transfected with siRNAs targeting ARO and the medium was collected 48 h later. Estradiol levels were determined using human estradiol ELISA kit (CusabioBiotech) according to the manufacturer's instructions.

5.5. AChE specific activity

Acetylcholinesterases eactivity was determined by the colorimetric Ellman method at 412 nm. [48, 49] Cells were trypsinized and centrifuged at 3700xg for 5 minutes at 4 °C. After the addition of 1 drop of glycerol onto the pellet, the pellet was sonicated with 10x5 sec 21% amplitude and waited for 50 seconds between each treatment. The pellet was diluted 7 fold with lysis buffer pH 7.4 containing 50 mMTris-HCl, 0.5% Triton X 100, 1% Nonidet P40, 5 mM EDTA, 150 mM NaCl, protease inhibitor and incubated for 30 minutes on ice in an orbital shaker. After this period, it was centrifuged again at 14,000xg for 5 minutes at 4 °C. The supernatant was assayed with a reaction mixture containing 20 mM KP pH 7.4, 5 mM acetylthiocholine (ATC) as a substrate, and 1.25 mM DTNB. The reaction was monitored spectrophotometrically at 412 nm by BiotekPowerWave XS microplatereader. Calchem (SantaCruz) BCA protein assay kit was used to calculate the
amount of protein in the samples. Tetraisopropylpyrophosphoramide (iso-OMPA; 100 μM) was added into the reaction mixture for specific inhibiting the BChE activity in samples.

5.6. Viability and apoptosis analysis

ApoTox-GloTMTriplexAssay (Promega) was used to estimate the levels of viability, cytotoxicity, and apoptosis in a 96-well assay plate as a triplex assay by FLUOstar® Omega multi-modemicroplaterreader. [49] Human neuroblastoma SH-SY5Y cells (5x 10^4 cells/well) were plated in 96-well plates in highglucose DMEM including 10% FBS and 1% antibiotic. After 48 h of siRNA transfection, cell viability (wavelength 400/505 nm) and cytotoxicity (wavelength 485/520 nm) was measured fluorometrically. Apoptosis was determined by the luminogenic caspase-3/7 substrate as claimed by the manufacturer’s instructions. The pan-caspaseinhibitor zVad-Fmk (50 μM) was used as a negative control.

5.7. Statistical analysis

Statistical analysis was performed using the GraphPadPrism 8.0 software (GraphPad Software Inc. USA). Data were given as the mean ± SEM. Comparisons between the different groups were performed by the one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Differences were considered statistically significant at p < 0.05.

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