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Research Article

The Role of Lipid Rafts and Membrane Androgen Receptors in Androgen's Neurotoxic Effects

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Abstract

Sex differences have been observed in multiple oxidative stress–associated neurodegenerative diseases. Androgens, such as testosterone, can exacerbate oxidative stress through a membrane androgen receptor (mAR), AR45, localized to lipid rafts in the plasma membrane. The goal of this study is to determine if interfering with mAR localization to cholesterol-rich lipid rafts decreases androgen induced neurotoxicity under oxidative stress environments. We hypothesize that cholesterol-rich caveolar lipid rafts are necessary for androgens to induce oxidative stress generation in neurons via the mAR localized within the plasma membrane. Nystatin was used to sequester cholesterol and thus decrease cholesterol-rich caveolar lipid rafts in a neuronal cell line (N27 cells). Nystatin was applied prior to testosterone exposure in oxidatively stressed N27 cells. Cell viability, endocytosis, and protein analysis of oxidative stress, apoptosis, and mAR localization were conducted. Our results show that the loss of lipid rafts via cholesterol sequestering blocked androgen-induced oxidative stress in cells by decreasing the localization of mAR to caveolar lipid rafts.

Key Words: AR45, oxidative stress, apoptosis, membrane androgen receptor, nystatin, caveolin-1, testosterone, lipid rafts, endocytosis, statin, aromatase

Abbreviations: BBB, blood-brain barrier; BSA, bovine serum albumin; CS-FBS, charcoal-stripped fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate conjugate; HMG-CoA, hydroxy-methyl-glutaryl-coenzyme A; mAR, membrane androgen receptor; MTT, thiazolyl blue tetrazolium bromide; OS, oxidative stress; PBS, phosphate buffer solution; PCR, polymerase chain reaction; PS, penicillin-streptomycin; RPMI, Roswell Park Memorial Institute medium; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; T-BSA, testosterone conjugated to bovine serum albumin.

Sex differences have been observed in neurodegenerative diseases, such as Alzheimer disease, Parkinson disease, Lewy body dementia, and vascular dementia. Men have a higher risk of vascular dementia, Parkinson disease, and Lewy body dementia than women [1, 2]. Common risk factors for these neurodegenerative diseases include cardiovascular diseases, such as hypertension [3]. Cardiovascular diseases can increase oxidative stress (OS) [4], which can increase cellular vulnerability. Interestingly, men have higher levels of cardiovascular disease relative to premenopausal women [5]. After menopause, cardiovascular diseases increase in women [6]. Therefore, men exhibit a higher OS load than premenopausal women, which could lead to increased cellular vulnerability in men. Furthermore, this increased OS load in men is chronic as it generally occurs in middle age and is sustained throughout aging [7], whereas the OS load is more acute in postmenopausal women. Interestingly, hypertension, which occurs at higher levels in men than women [5] has been associated with cognitive decline later in life [8]. Therefore, the chronic OS burden that is generally observed in men may increase the risk for neurodegenerative disorders, such as vascular dementia.

Vascular dementia, a heterogenous group of brain disorders, is a form of cognitive decline resulting from cerebrovascular disease in small or large vessels. Diseases such as hypertension, obesity, diabetes, and hyperlipidemia (high cholesterol), can increase OS levels that can lead to cerebrovascular impairment and consequently vascular dementia [9]. Vascular dementia is the second most common type of dementia [10], causing approximately 20% of known dementia cases [11]. According to the World Health Organization, an estimated 35.6 million people have dementia, and this number is expected to triple by 2050 [12]. Approximately 7.7 million new cases of dementia are diagnosed every year, therefore causing a great burden on families, caregivers, and society [13].

OS is a hallmark common to all these disorders [14]. It is possible that the effects of sex hormones on OS could mediate these sex differences. Estrogen, the major female sex hormone, is an antioxidant [15]. Many studies have shown that estrogen decreases OS by binding to estrogen receptors and upregulating the expression of antioxidant enzymes via intracellular signaling pathways [15]. Generally, estrogen has been shown to have protective effects in many neurodegenerative diseases [16]. Unlike estrogen, the major male sex hormone

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testosterone can have diverse effects on OS. Testosterone can be aromatized to an estrogen and protect cells from subsequent OS damage [17]. In contrast, testosterone can be reduced to dihydrotestosterone and thus increase OS [17]. How testosterone impacts the brain is dependent on the health of the brain, including basal OS levels. If OS levels are low, then androgens are protective against subsequent OS-related stressors [17]. If OS levels are high, then androgens can exacerbate OS damage [17].

In several cell types, such as dopaminergic cells, hippocampal cells, and cerebral cortical cells, the membrane androgen receptor (mAR) is localized to plasma membrane lipid rafts [18], which is a cholesterol-rich microdomain involved in cellular signaling (eg, endocytosis and intracellular cascades) [19]. This mAR present in the lipid rafts is an androgen receptor variant, missing the N-terminus domain, called AR45. AR45 has been observed to reside within caveolin-1 lipid rafts of neuronal N27 cells and brain tissue from young and middle-aged rats [18]. Within these lipid rafts, this mAR forms a complex with one of the major OS cellular generators, NADPH oxidase [20]. The involvement of the lipid rafts has been identified in several diseases, including Alzheimer disease, Parkinson disease, and vascular dementia [21].

Since lipid rafts are composed of cholesterol, lipid raft expression in plasma membranes can be decreased using statins. Statins are a class of drugs that blocks the conversion of hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) to mevalonic acid in the mevalonate pathway, thus they are referred to as HMG-CoA reductase inhibitors [22]. Statins are used for lowering serum cholesterol levels and other cardiovascular interventions [23]. There is evidence showing that individuals with hypertension and hypercholesterolemia resulting from high levels of cholesterol are at a higher risk for vascular dementia and Alzheimer disease [24, 25]. Since statins may decrease neurodegenerative disease risk and progression by lowering cholesterol levels [26, 27], recent studies have focused on understanding the efficacy of statins in the prevention of neurodegenerative diseases. Currently, there are no therapeutic drugs for many neurodegenerative diseases, such as vascular dementia. However, epidemiological studies indicate that statins are associated with decreased risk of these diseases [28]. In a meta-analysis that comprised observational studies and a randomized control study, it was observed that statins were associated with decreased cognitive impairment disorders [28]; thus, repurposing statins for neurodegenerative disease therapeutics could be advantageous.

The goal of this study is to determine if interfering with mAR-AR45 localization to cholesterol-rich lipid rafts decreased androgen induced neurotoxicity under OS environments. To decrease lipid rafts, we use nystatin as a cholesterol-sequestering agent [29].

Materials and Methods

Reagents

Testosterone (A6950-000) was obtained from Steraloids. Goat anti-rabbit (sc-2004) secondary antibody, goat anti-mouse (sc-2005) secondary antibody, androgen receptor C-19 (sc-7305) primary antibody that targets the C-terminus domain, androgen receptor N-20 (sc-816) primary antibody that targets the N-terminus domain, and estrogen receptor alpha MC-20 (sc-542) primary antibody were obtained from Santa Cruz Biotechnology Inc. Caveolin-1 (3267) primary antibody was obtained from Cell Signaling Technology. Beta Actin (GTX629630) primary antibody was obtained from GeneTex. Aromatase antibody (ab18995) was purchased from Abcam. Spectrin antibody (MAB1622) primary antibody, testosterone 3-(O-carboxymethyl) oxime:bovine serum albumin–fluorescein isothiocyanate conjugate (T-BSA-FITC, #T5771), nystatin (#N6803), Millicell EZ Slide #PEZGS0816, and N27 rat dopaminergic neural cell line (#SCC048) were purchased from Millipore Sigma. DMSO, thiazolyl blue tetrazolium bromide (MTT) (#97062-380), 30% hydrogen peroxide (H_2O_2, EM1.07209.0250), Roswell Park Memorial Institute medium (RPMI) 1640 and penicillin-streptomycin (PS) were purchased from VWR Life Science. Fetal bovine serum (FBS) and phosphate buffer solution (PBS) were obtained from Corning. Charcoal-stripped fetal bovine serum (CS-FBS) was purchased from Atlanta Biologicals. Pierce BCA Protein Assay Kit (23225), Tris-buffered saline (TBS, BP2471), and Tween-20 (BP337), Nonidet P-40 lysis buffer, SuperSignal West Pico/Femto chemiluminescent substrates, dithiothreitol (DTT), and protease and phosphatase inhibitor cocktail (PI78442) were obtained from Thermo Fisher Scientific. Rat total cholesterol enzyme-linked immunosorbent assay (ELISA) (MBS846775) was purchased from MyBioSource. VECTASHIELD antifade mounting medium with DAPI (H-1200-10) from Vector Laboratories. Tris, Any KD polyacrylamide gel, Tris-glycine buffer, polyvinylidene difluoride (PVDF) membranes, *CAV1* primer (PrimePCR #10025636), GAPDH primer (PrimePCR #10025636), and iTaq SYBR Green PCR Master mix (#1725121) were purchased from BioRad. High-Capacity cDNA Reverse Transcriptase Kit (#010961510) was obtained from Applied Biosystems.

Testosterone was dissolved using an ethanol vehicle (final concentration of ethanol < 0.001%, 200 uM). Consistent with other studies showing that < 0.1% ethanol has minimal to no impact on cell OS generation and viability [30-32], our studies show that this concentration of ethanol has no impact on cell viability or androgen's actions [33-35]. Furthermore, this concentration of ethanol (17 uM or 17.17 mM/L) is 176-fold below the ethanol concentration (3-30 mM) needed for activation of GABA receptors [36-41]. Since little to no albumin is present in healthy brains [42], hormones in the brain are not protein bound and are considered free. In order to compensate for the albumin (2.1 g/dL) in the CS-FBS, we used the Vermeulen calculation to determine the appropriate hormone dosage to attain physiological brain hormone levels [43, 44]. Therefore, in CS-FBS 100 nM of testosterone is equivalent to 8 nM calculated free testosterone, which is consistent with liquid chromatography–tandem mass spectrometry (LC-MS/MS) brain testosterone levels in male rats (5-24 nM) [45-48].

Cell Culture

The immortalized neuronal cell line (N27; RRID:CVCL_D584, https://web.expasy.org/cellosaurus/CVCL_D584) was derived from female fetal rat mesencephalic tissue. The N27 cell line is positive for tyrosine hydroxylase (TH+) expression, an enzyme marker for dopamine neurons [49-52]. This cell line is also positive for the mAR-AR45, estrogen receptor alpha, and estrogen receptor beta but does not express full-length androgen receptors [17, 34]. N27 cells were

cultured and maintained at 37 °C in 5% CO2. Media used was RPMI 1640 medium supplemented with 10% FBS and 1% PS. N27 cells were only used within passages 13-19 to avoid changes in morphology.

Experimental Design

N27 cells were plated onto 96-well and 6-well plates with RPMI 1640 media supplemented with 10% FBS and 1% PS and incubated to proliferate for 24 hours at 37 °C, 5% CO2. Three hours prior to treatment for experimentation, the media was switched to RPMI 1640 with CS-FBS in all treatment and control groups to avoid confounding effects due to the presence of hormones in the serum. We have previously shown that CS-FBS does not impact cell viability or androgen actions [17, 34]. Maintaining cells in CS-FBS does not negatively impact cell viability, as the media still contains salt, glucose, amino acids, and other nutrients [33-35]. The use of CS-FBS impacts only nongenomic hormone action but not genomic or transcriptional hormone actions, which occur on a longer time frame.

Cells at 80% confluency were either exposed to vehicle (media alone) or hydrogen peroxide (H_2O_2; 18-30 μM) as an oxidative stressor for 2 hours prior to a 24-hour treatment period of androgen exposure (100 nM testosterone equivalent to 8 nM calculated free testosterone). This concentration of H_2O_2 was sufficient to induce 20% cell loss and achieve the OS threshold necessary to see androgen's negative effects on cells. Cells were exposed to nystatin (50-75 μM) 1 hour after H_2O_2 treatment and then treated with androgens or vehicle. Following treatments, cells were analyzed for cell viability or protein expression of various markers. For cell viability experiments, 50 μM nystatin was used, as this dose was sufficient to observe a functional effect. However, 75 μM nystatin was used for Western blot protein analysis experiments as this dose was sufficient to consistently decrease caveolin-1 protein expression. The difference in nystatin dose is due to the sensitivity of the assays, in which MTT is a functional assay and more sensitive to changes than structural protein assays.

Cell Viability

Cell viability was determined by MTT assay. Media was aspirated from all wells, replenished with 100 μL of RPMI-1640 phenol red–free medium, and supplemented with 10% CS-FBS, 1% PS, and 1% l-glutamine. This was followed by the addition of 20 μL of 5 mg/mL of MTT solution to each well. Experimental plates were then covered in foil to block additional light and incubated at 37 °C with 5% CO2 for 3 hours. Following incubation, MTT reagent was removed and replaced with 100 μL DMSO to solubilize the precipitate. Following 1 hour of agitation, plates were read at an absorbance of 595 nm. The colorimetric intensity is directly proportional to the number of viable cells in each well. Readings from different treatment groups were then normalized to the control group to determine cell viability. At least 3 different experiments were conducted; each n is a mean of 8 wells per treatment group on one plate.

Cell Lysates and Homogenization

After treatments were carried out as stated in the experimental design, cells were placed on ice, washed with PBS, and lysed using Nonidet P-40 lysis buffer with a cocktail of dithiothreitol (1 μM), EDTA (1 mM), and phosphatase and protease inhibitor cocktail (1:200). The lysates were homogenized and centrifuged at 12 753 rpm at 4 °C for 20 minutes. The supernatant was then collected, and protein concentrations were measured using the Pierce BCA protein assay kit, according to the manufacturer's instructions.

Western Blot

Equal amounts of (30 μg protein) denatured whole cell lysates were loaded into a Bio-Rad Any KD polyacrylamide gel, electrophoresed in a Tris-glycine buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at room temperature at 25 mA. Next, proteins were transferred for 2 to 3 hours at 80 V onto a polyvinylidene difluoride (PVDF) membrane at 4 °C. Membranes were washed 3 times for 10 minutes each under agitation with Tris-buffered saline with Tween 20 and then membranes were blocked for 1 hour with 5% nonfat milk in TBS-Tween at room temperature. Following blocking, the membranes were incubated with specific primary antibodies (AR C-19 1:1000 to examine the mAR, AR N-20 1:1000 to examine full-length androgen receptor, aromatase 1:1000, ER MC-20 1:1000 to examine estrogen receptor alpha, Spectrin 1:1000 to examine caspase-3 activity, Beta Actin 1: 2000 for protein normalization, and caveolin-1 1:1000 to examine lipid rafts) in TBS-Tween with 1% nonfat milk overnight at 4 °C. Afterward, the membranes were washed three times for 10-minutes each for a total washing time of 30 minutes and then incubated with secondary antibodies (1:5000 dilution for goat anti-rabbit and goat anti-mouse) in TBS-Tween with 1% nonfat milk for 30 minutes at room temperature. After washing membranes 3 times for 30 minutes, protein bands on the membrane were visualized using an enhanced chemiluminescence detection assay (Thermo Fisher Scientific). Protein band intensities were imaged using GeneSys software corresponding with the Syngene G-Box Chemi XRQ system. Protein band densities were quantified by NIH Image J densitometer software and normalized to Beta Actin for whole cell lysates.

Immunocytochemistry

N27 cells were plated in 8-well glass slides (Millicell EZ Slide) at a density of 3.0×10^4 cells in each well. Cells were fixed after treatment exposures (testosterone-BSA-FITC). The cells on the slides were washed with cold 1× Phosphate Buffered Saline (PBS) twice for 1 minute each followed by 100% cold methanol fixation at –20 °C for 10 minutes. Afterwards, the slides were washed with 1× cold PBS, and then sealed with mounting VECTASHIELD medium containing DAPI. Once sealed with coverslips, the slides were stored at 4 °C. A fluorescence microscope (Olympus BX41, Olympus Corporation) equipped with wide UV and FITC filters was used to visualize fluorescence of the cells on the slides. Images of the cells at 40× magnification were captured using a digital camera (Olympus DP70, Olympus Corporation) and imaging software (DP Controller—version 3.3.1.292 and DP Manager—version 3.3.1.222, Olympus Corporation).

Cholesterol ELISA

Total cellular cholesterol levels were assayed by ELISA using a commercially available kit (MBS846775, MyBioSource) according to the manufacturer's instructions. The detection range was 0.3-28 nmol/mL. The intra-assay coefficient of variation is < 10% and the interassay coefficient of variation is < 12%. The sensitivity of this assay is < 0.1 nmol/mL. N27 cells were incubated with nystatin (30-75 μM) overnight for 18 hours prior to processing for ELISA.

Quantitative Real-Time Polymerase Chain Reaction

To examine caveolin-1 mRNA expression in our study, we used a relative quantitative real-time polymerase chain reaction (PCR) method to amplify *caveolin-1*. This method compares changes in gene expression between vehicle control and nystatin-treated samples. Total RNA was extracted from N27 cells via Trizol extraction (Invitrogen). RNA concentrations were measured with a NanoDrop-One Microvolume UV-Vis Spectrophotometer (ThermoFisher), and 1 µg was reverse transcribed using the High-Capacity cDNA Reverse Transcriptase Kit. The resulting cDNA was analyzed using the BioRad CFX Connect real-time PCR system. Each reaction included iTaq SYBR Green PCR Master mix, 30 ng of cDNA, Caveolin-1 primer, or GAPDH primer. Expression of *Caveolin-1* mRNA was normalized to GAPDH mRNA that was used as internal control. Quantification of PCR amplified mRNA specific cDNA was done by comparative cycle threshold (CT) method (2^-ΔΔCT).

Statistical Analysis

Analysis was performed using IBM SPSS Statistics version 21 software. Shapiro-Wilk test was used to test for normality of data, whereas Levene's test was used to examine homogeneity of variances. Control groups in cell viability and Western blot experiments were normalized to 100% and this caused normality to be affected only in this group. Since the majority of treatment groups in each experiment were normally distributed and all groups were homogenous, the use of parametric statistics was appropriate. Therefore, an analysis of variance (ANOVA), using independent factors of oxidative stress, testosterone, and nystatin was used. Tukey's honest significant difference (HSD) was used as a post hoc analysis in this study. Data were expressed as mean ± standard error of the mean (SEM). Significance was set at $P \leq 0.05$.

Results

Nystatin and Cholesterol Levels

In these experiments, we used nystatin to sequester cholesterol [29] in order to decrease plasma membrane lipid raft expression [53]. To ensure that nystatin does not impact cellular cholesterol levels, we quantified cholesterol levels following a nystatin dose response curve (30-75 µM nystatin). Our results showed that nystatin did not alter cellular cholesterol levels ($F_{3,8} = 0.010$, $P > 0.05$), regardless of the amount of nystatin used (Fig. 1A). Next, we conducted a dose-response curve (0-150 µM nystatin) to determine nystatin's effects on cell viability. Our results showed that cell viability was unaffected by nystatin ($F_{3,12} = 2.541$, $P > 0.05$ (Fig. 1B).

Sequestering Cholesterol Blocked Testosterone's Negative Effects in an Oxidative Stress Environment

Cholesterol sequestration can alter the composition of the caveolar lipid rafts, which can alter the function of receptors localized to lipid rafts [19, 54, 55]. Since the mAR AR45 (ie, 45 kDa MW), resides within a caveolin-1 lipid raft [18], we used nystatin to impact mAR function. We exposed N27 cells to the oxidative stressor H_2O_2 to induce 20% to 30% cell loss. An OS burden of 20% to 30% cell loss is necessary to observe testosterone's neurotoxic effects instead of testosterone's neuroprotective effects [17]. H_2O_2 decreased

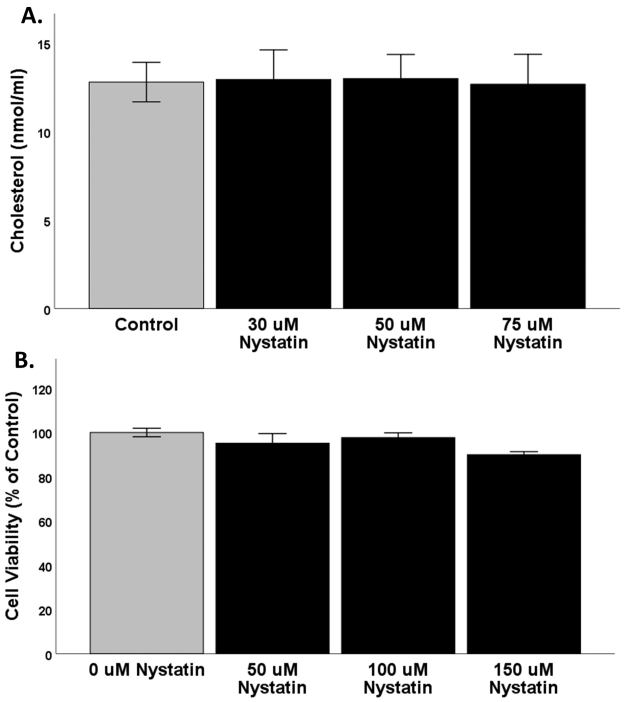

Figure 1. Nystatin and cholesterol concentration. Treatment with nystatin (18 hours overnight) does not alter cholesterol concentration, regardless of the concentration (A), nor does it impact cell viability (B). n = 3 in Panel A and n = 4 in Panel B.

cell viability ($F_{1,16} = 199.385$, $P \leq 0.05$), and this effect was exacerbated by testosterone exposure, as evidenced by a significant interaction between testosterone and OS ($F_{1,16} = 7.114$, $P \leq 0.05$) (Fig. 2). Nystatin (50 µM) blocked testosterone's negative effects on cell viability in an OS environment ($F_{5,16} = 5.175$, $P \leq 0.05$). Therefore, when cells are pretreated with nystatin to sequester cholesterol, testosterone was unable to exacerbate H_2O_2-induced cell loss. These results support the role of testosterone acting through a mAR localized within a cholesterol-rich lipid raft.

Nystatin Decreased Caveolin-1 Lipid Rafts and Membrane Androgen Receptors

To determine the role of caveolin-1 lipid rafts and mAR on OS, we quantified protein and mRNA expression of caveolin-1, which is a plasma membrane protein. Neither OS ($F_{1,40} = 0.416$, $P > 0.05$) nor testosterone ($F_{1,40} = 0.087$, $P > 0.05$) impacted caveolin-1 protein expression in N27 cells (Fig. 3A and 3C). As expected, nystatin (75 µM) decreased caveolin-1 expression ($F_{1,40} = 133.504$, $P \leq 0.05$). These results showed that nystatin decreased caveolin-1 lipid rafts, regardless of OS or hormone treatment. Since nystatin decreased caveolin-1 protein expression, we examined if cholesterol sequestering impacted caveolin-1 translation by quantifying caveolin-1 mRNA levels (Fig. 3B). Our results show that nystatin decreased caveolin-1 transcription in N27 cells ($F_{1,4} = 2007.35$, $P \leq 0.05$).

Since nystatin decreased caveolar lipid rafts, we examined the impact of nystatin on mAR-AR45 protein expression. Neither OS ($F_{1,40} = 0.195$, $P > 0.05$) nor testosterone ($F_{1,40} = 0.031$, $P > 0.05$) altered mAR expression in N27 cells, which is consistent with our previous studies [18, 20]. However, nystatin (75 µM) decreased mAR protein

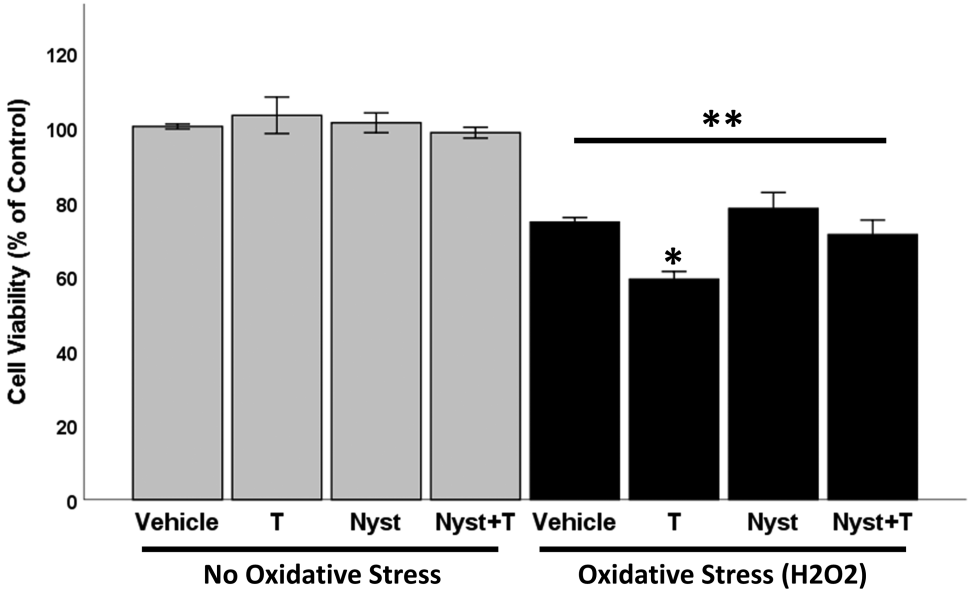

Figure 2. Impact of cholesterol sequestering on testosterone exacerbation of oxidative stress induced cell death. Neither testosterone (100 nM testosterone, equivalent to 8 nM calculated free testosterone) nor nystatin (50 μM) altered cell viability. In contrast, testosterone exacerbated H_2O_2-induced cell loss. Cholesterol sequestering via nystatin blocked testosterone exacerbation of H_2O_2-induced cell loss. Abbreviations: Nyst, nystatin; T, testosterone. ** $P \leq 0.05$: oxidative stress (H_2O_2 treated) vs no oxidative stress (not H_2O_2 treated); * $P \leq 0.05$ vs other treatments. n = 3.

expression ($F_{1,40} = 96.451$, $P \leq 0.05$), regardless of OS environment (Fig. 3A and 3D). These results indicate that the loss of lipid rafts by nystatin also resulted in the loss of mAR that resides within lipid rafts. Consistent with our prior studies [17, 18], N27 cells do not express full-length androgen receptors (Fig. 3A), as evidenced by the lack of immunoreactivity at 110 kDa molecular weight using a C-terminus androgen receptor antibody. We also examined immunoreactivity to a N-terminus androgen receptor antibody (androgen receptor N-20 antibody), in which immunoreactivity should be observed if full-length androgen receptors (110 kDa) are present. No immunoreactivity was observed with a N-terminus androgen receptor antibody at either the 110 kDa or the 45 kDa molecular weight (Fig. 3A), further supporting the lack of full-length androgen receptors and only the presence of the mAR-AR45 that does not contain a N-terminus region.

Nystatin Does Not Impact Estrogen Receptor or Aromatase Expression

Our prior studies have shown that neither inhibition of cytosolic androgen receptors nor estrogen receptors blocked testosterone's negative actions on cell viability in an OS environment [17, 20, 33]. However, the role of nystatin on estrogen receptor expression is unknown. Similarly, the role of nystatin on aromatase, the enzyme that metabolizes testosterone to estradiol, is unknown. Our results in Fig. 4 show that nystatin (75 μM) does not impact estrogen receptor alpha expression ($F_{1,4} = 0.067$, $P > 0.05$) or aromatase expression ($F_{1,4} = 0.013$, $P > 0.05$), even though nystatin significantly decreased caveolin-1 lipid rafts ($F_{1,4} = 12.232$, $P \leq 0.05$).

Cholesterol Sequestering Decreased Endocytosis of Membrane Androgen Receptors

Caveolar lipid rafts are involved in multiple functions, such as intracellular signaling and endocytosis [19, 56]. In prostate cells and Sertoli cells, endocytosis or internalization of the mAR via a caveolin-1 mechanism has been observed [57-59]. Currently, only the mAR-AR45 has been found in caveolin lipid rafts in neuronal tissues in rats and humans [17, 18], but it is unknown if this mAR is involved in endocytosis. To examine if caveolin-1 lipid rafts internalize testosterone via the mAR, we used a FITC-tagged testosterone conjugated to cell-impermeable bovine serum albumin (T-BSA) to restrict its activity to the mAR [17, 33]. Within 30 minutes, T-BSA was internalized within N27 cells (Fig. 5). Nystatin (75 μM) blocked the internalization of T-BSA, indicating that mARs localized to caveolar lipid rafts in the plasma membrane of neuronal cells are involved in endocytosis.

Nystatin Decreased Apoptosis in Oxidatively Stressed N27 Cells Exposed to Testosterone

Our prior studies show that testosterone exacerbation of OS induced cell loss is via an apoptotic mechanism [33, 60]. Since nystatin decreased testosterone's negative effects on cell viability in an OS environment, we examined if nystatin (75 μM) decreases testosterone-induced caspase-3 mediated apoptosis in an OS environment, as shown in Fig. 6. OS (H_2O_2) significantly increased caspase-3 mediated apoptosis in N27 cells ($F_{1,40} = 209.50$, $P \leq 0.05$), which was exacerbated by testosterone exposure ($F_{1,40} = 13.15$, $P \leq 0.05$). However, this effect of testosterone in an OS environment was blocked by sequestering cholesterol in the cells with nystatin ($F_{1,40} = 3.121$, $P > 0.05$).

Discussion

The major findings of this study are (1) cholesterol sequestering blocks testosterone exacerbation of OS-induced apoptotic cell loss; (2) cholesterol sequestering decreases caveolin-1 lipid rafts, resulting in decreased mAR expression and endocytosis; and (3) cholesterol sequestering does not impact either estrogen receptor or aromatase protein expression, suggesting that cholesterol sequestering or altering lipid raft expression does not impact estrogen signaling. These findings

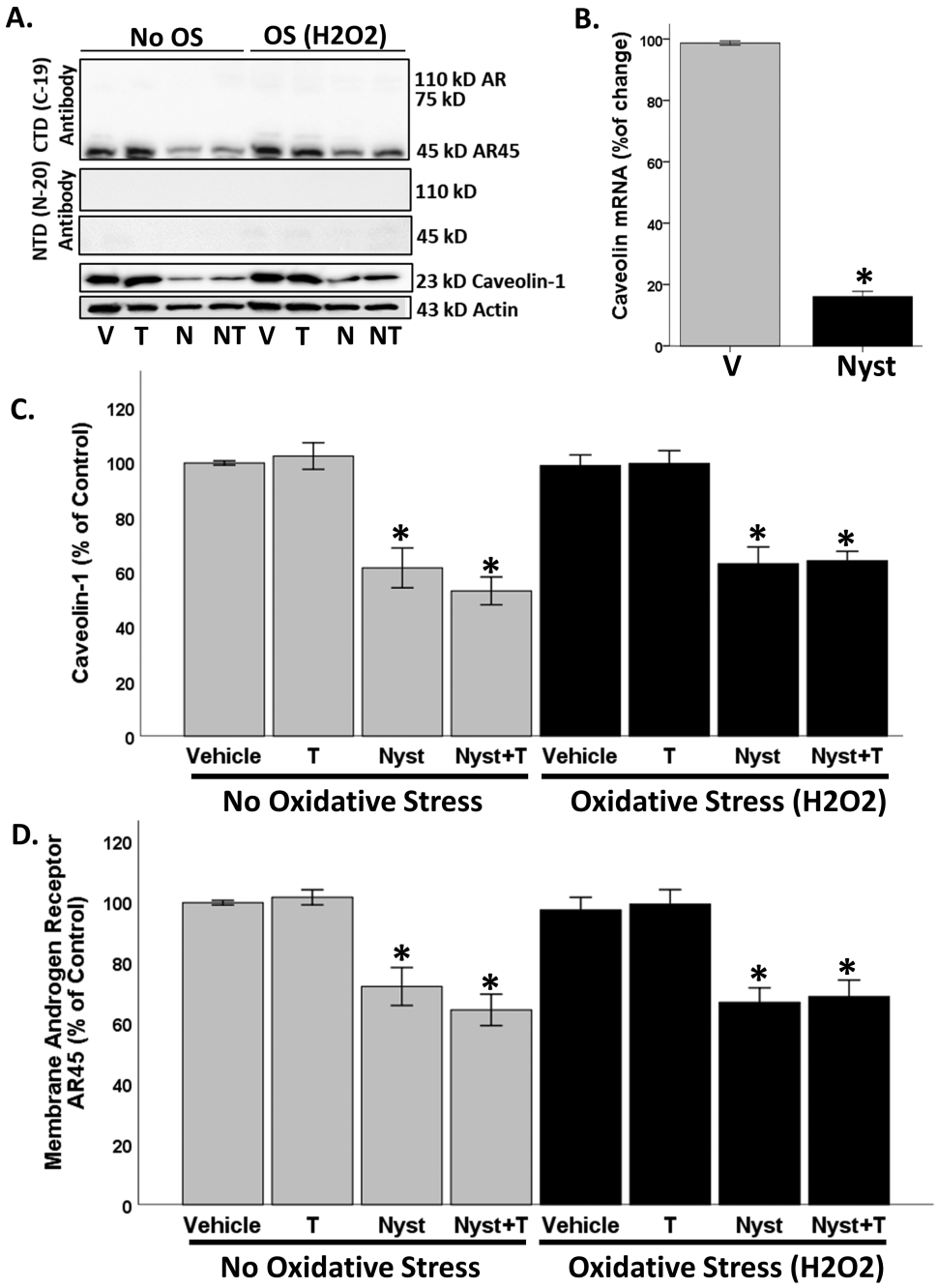

Figure 3. Cholesterol sequestering decreased caveolin-1 expression and mAR-AR45 expression. Neither H_2O_2 nor testosterone (100 nM) altered caveolin-1 lipid raft protein expression. Cholesterol sequestering by nystatin (75 μM) decreased caveolin-1 expression (A, C). Nystatin decreased transcription of caveolin-1 mRNA (B). Cholesterol sequestering by nystatin decreased mAR (AR45) expression (A, D). N27 cells do not express full-length androgen receptors, as indicated by the lack of immunoreactivity at 110 kD. Further, AR45 lacks a N-terminus domain (NTD), and this protein can only be observed with a C-terminus domain (CTD) androgen receptor antibody (C-19) and not a N-terminus domain androgen receptor antibody (N-20) (A). Abbreviations: Nyst/N, nystatin; T, testosterone; V, vehicle. * $P \leq 0.05$: vs non-nystatin treatment groups. N = 3 for Panel B and n = 6 for the other panels.

indicate that localization of the mAR-AR45 to cholesterol-rich lipid rafts is necessary for testosterone's neurotoxic effects in an OS environment. Thus, mAR-mediated endocytosis and apoptosis may be involved in neurodegenerative diseases associated with OS, which is consistent with our previous studies, which found that testosterone is associated with cognitive impairment in aged men with elevated OS [61].

This is the first study to show that lipid rafts are necessary for testosterone's neurotoxic effects in an OS environment. Our previous studies have shown that degrading the mAR-AR45 was sufficient to block testosterone's activation of OS signaling through the NADPH oxidase signaling cascade [20]. However, inhibition of androgen receptors may not be clinically appropriate, as androgens also have neuroprotective effects [62], and the loss of these neuroprotective actions of testosterone could negatively impact health. Therefore, the purpose of these experiments was to determine how to interfere with androgen's neurotoxic effects in an OS environment

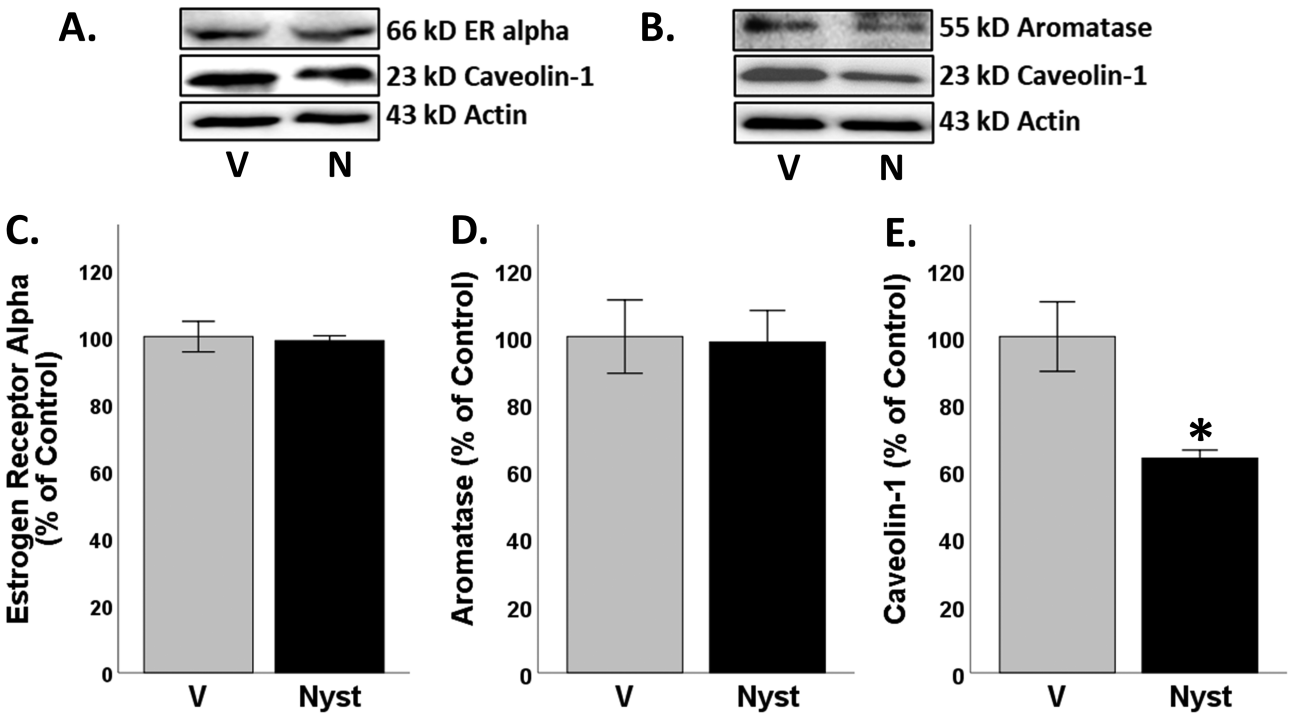

Figure 4. Cholesterol sequestering does not impact estrogen receptors or aromatase. Cholesterol sequestering by nystatin (75 μM) does not impact estrogen receptor alpha expression (A, C) or aromatase expression (B, D), even though it does decrease caveolin-1 expression (A, B, D). Abbreviations: Nyst, nystatin; V, vehicle. * $P \leq 0.05$: vs vehicle. n = 3.

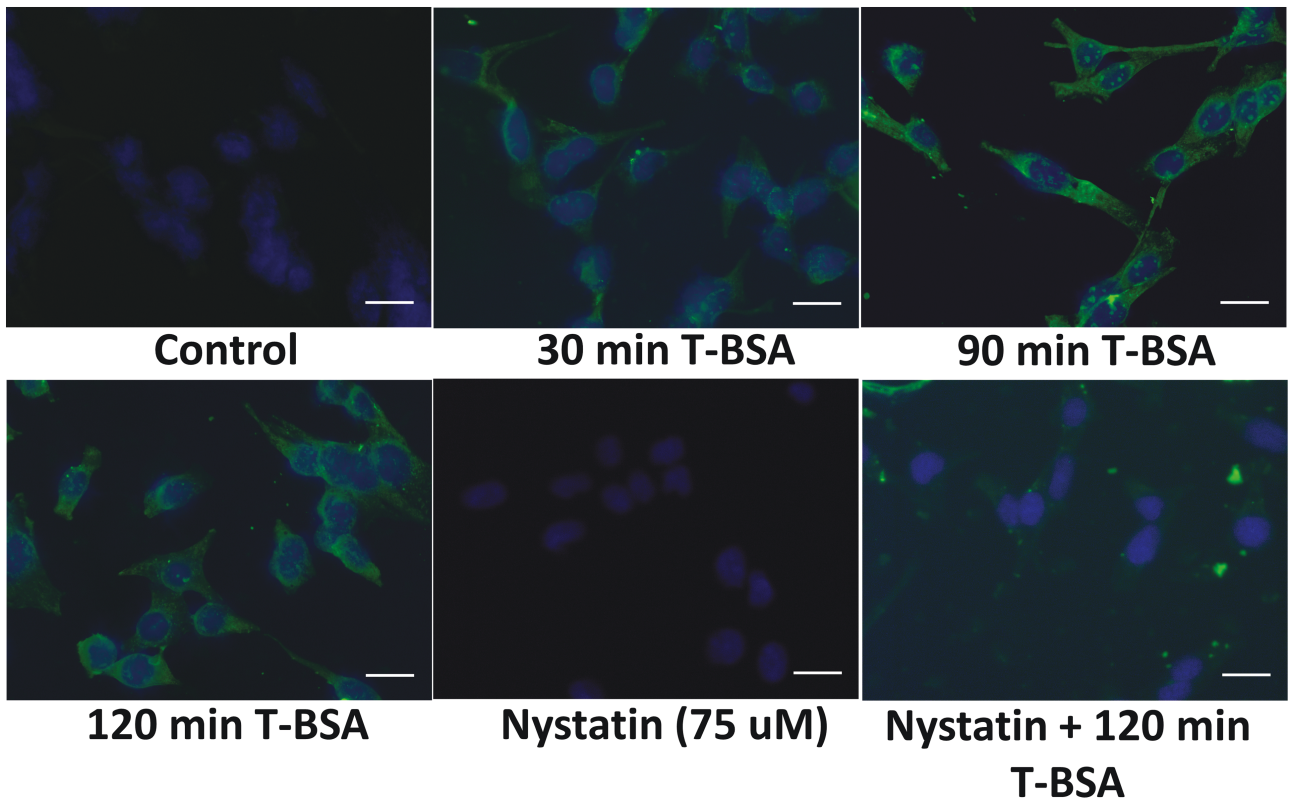

Figure 5. Cholesterol sequestering blocked membrane androgen receptor mediated endocytosis. N27 cells were exposed to either 0 μM (negative control) or 5 μM testosterone-BSA-FITC (T-BSA) to examine internalization of a cell impermeable androgen that is restricted to membrane androgen receptor activation. Within 30 minutes of T-BSA exposure, internalization of T-BSA via membrane androgen receptors occurs. This effect is sustained for at least 2 hours (120 minutes T-BSA). Pretreatment with nystatin (75 μM overnight) blocked T-BSA internalization (endocytosis). DAPI-label nuclear staining (blue) and T-BSA-FITC fluorescence (green). n = 3. Scale bar = 50 μm.

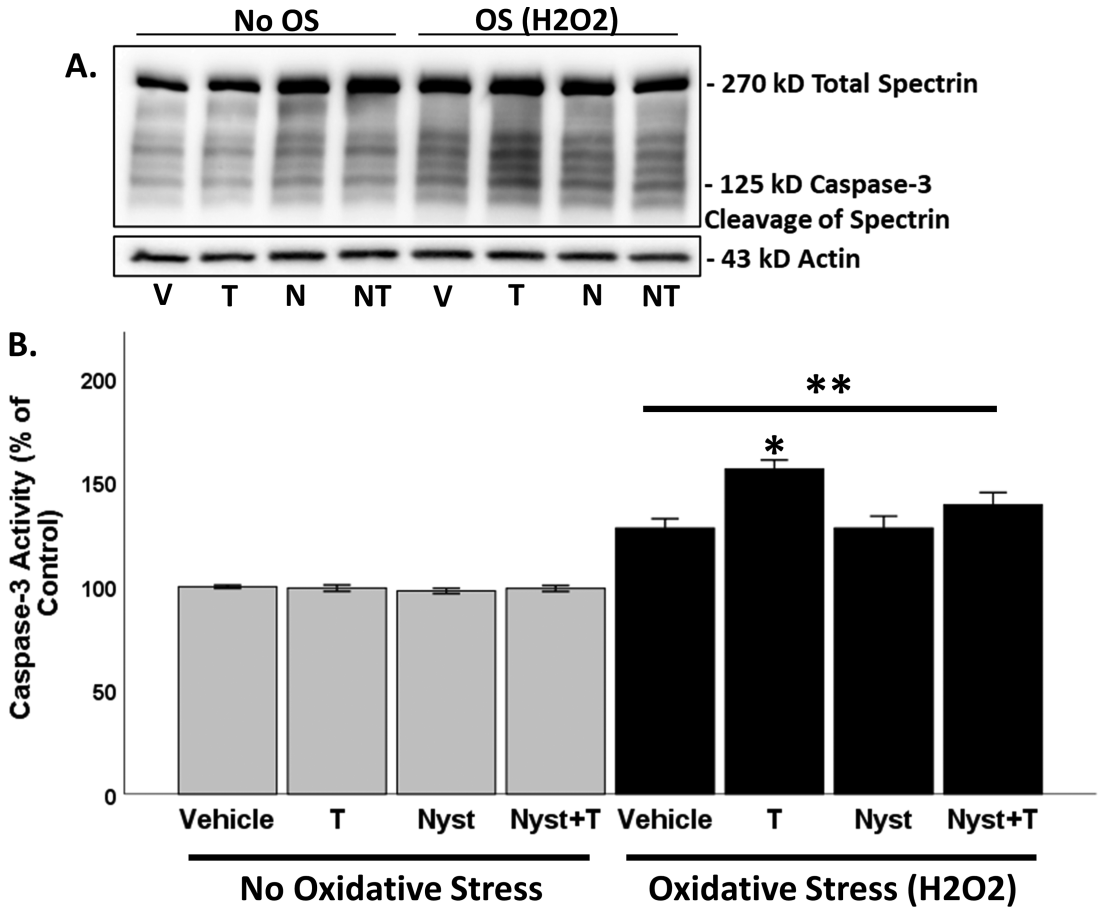

Figure 6. Cholesterol sequestering blocked testosterone exacerbation of oxidative stress induced apoptosis. Caspase-3 is the primary executor of apoptotic cell death. Caspase-3 activity can be quantified by measure cleavage of the cytoskeletal protein Spectrin at 125 kD. In the absence of oxidative stress, neither testosterone nor nystatin (75 μM) increased caspase-3 activity compared to vehicle control. As expected, H_2O_2 oxidative stress increased caspase-3 activity. Testosterone exacerbated H_2O_2 induced caspase-3 activity. Sequestering of cholesterol by nystatin blocked testosterone exacerbation of H_2O_2 induced caspase-3 activity. Abbreviations: Nyst/N, nystatin; T, testosterone; V, vehicle. ** $P \leq 0.05$: oxidative stress vs no oxidative stress; * $P \leq 0.05$: vs other treatments. n = 6.

without impacting overall androgen signaling. Our results show that decreasing mARs by decreasing cholesterol-rich lipid rafts blocked the neurotoxic effects of androgens in an OS environment. These results suggest that cholesterol sequestering by statins may have a beneficial effect in individuals that exhibit elevated OS and androgens.

Even though the mechanisms of statin-associated neuroprotection are not well understood [63], lipophilic statins can impact brain function. Currently, there are no studies published on the uptake, distribution, and metabolism of statins in the brain, but lipophilic statins can cross the blood-brain barrier (BBB) more freely than hydrophilic statins by passive diffusion [26, 64]. Indeed, studies using the in situ rat brain perfusion technique observed increased radiolabeled lipophilic statins (lovastatin and simvastatin) in rat cerebral cortical brain tissue, indicating that lipophilic statins can cross the BBB [63]. However, this effect on the BBB was not observed with the radiolabeled hydrophilic statin pravastatin [63]. Therefore, one potential mechanism of action for statin-associated neuroprotection could be the ability of statins to cross the BBB.

The impact of lipophilicity of statins on neuroprotection was observed in both large retrospective cohort studies and cross-sectional studies, which found an association between the lipophilic statins (eg, lovastatin, simvastatin, atorvastatin) and decreased incidence of dementia [28, 65]. Interestingly, race and sex may play a role in lipophilic statin-associated decreased dementia. Studies using lipophilic statins found an association with decreased dementia risk in Caucasian and Hispanic men and women, along with Black women [26, 66]. In contrast, statins have not been associated with a reduction of dementia risk in Black men, regardless of lipophilicity [26]. More studies need to be conducted to examine the influence of sex and race on statin neuroprotection, as statins could have different effects on dementia risk in diverse patient populations. Generally, statin concentrations range between 100 nM and 1 μM in studies showing a neuroprotective benefit [63].

This study provided evidence that cholesterol is essential for mAR-AR45 cell signaling. Nystatin, a cholesterol sequestering agent [29], was used to determine whether sequestering of cholesterol to decrease lipid rafts can alter cell signaling through the mAR and improve cell survival. Indeed, nystatin sequestered the cholesterol and decreased caveolar lipid rafts. This loss of caveolar lipid rafts blocked the negative effects of testosterone on cell viability in an OS environment, indicating that the cholesterol-rich lipid rafts are necessary for mARs for androgen's neurotoxic effects. Notably, only mAR-AR45 expression, and not estrogen receptor or aromatase expression, was impacted by loss of caveolin-1 lipid rafts, further

supporting the role of lipid raft–associated mARs mediating testosterone's negative effects in an OS environment.

Cholesterol is involved in the steroidogenesis of steroid hormones, including androgens [67]. Although statins can lower plasma cholesterol levels [68, 69], prior studies have found that statins do not impact androgen production and serum androgen levels [23]. This was shown in a population-based, cross-sectional epidemiologic study which was carried out among 5506 men and women between ages 30 to 79 who were diagnosed with cardiovascular diseases [23]. Each participant was treated with statins, which included atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin, and simvastatin. Before and after the study, testosterone, sex hormone–binding globulin (SHBG), and dehydroepiandrosterone sulfate (DHEAS) levels were measured, and results showed that therapeutic concentrations of statins did not impact androgen synthesis and androgen serum levels [23]. Similarly, statins have been shown to not impact estrogen (ie, 17β-estradiol and estrone) serum levels [70, 71]. It has been suggested that the mechanisms by which prostatic tissue maintains tissue androgen levels may include metabolism of androgens in the adrenal glands or by de novo synthesis from cholesterol [23].

Our study has many strengths, which include using in vitro models to study the effects of cholesterol on androgen's neurotoxic effects in a high OS environment. A major limitation in this study is that we did not include a male (XY) cell line to compare against the findings in the N27 female (XX) cell line. However, we previously published that testosterone and its metabolite dihydrotestosterone in an OS environment negatively impacts cell viability through the mAR, regardless of the sex (XX vs XY) of the cells using N27 cells and PC12 cells that are derived from rats [17].

A few epidemiological studies have examined the efficacy of statins in treating dementia [72]. While some studies have shown that statins decreased the risk of dementia (73), others have not [74, 75]. Epidemiological studies may be more successful in demonstrating decreased risk for dementia after statin use if sex and gonadal hormone status are independently examined. Our results suggest that cholesterol-lowering agents, such as statins, may be a useful therapeutic agent for OS-associated neurodegenerative diseases.

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Author Contributions

O.F. conducted experiments, analyzed the data, interpreted the findings, and was a major contributor in writing the manuscript. N.R., S.M., and D.H.N. conducted experiments and analyzed the data. R.L.C. designed the study, interpreted the findings, and was a major contributor to the writing of the manuscript. R.L.C. has full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosures

The authors have nothing to disclose.

Data Availability

All data generated or analyzed during this study are included in this published article.

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