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Biochimie Open 3 (2016) 64–71

http://www.journals.elsevier.com/biochimie-open

Research paper

Inhibition of the MAPK pathway alone is insufficient to account for all of the cytotoxic effects of naringenin in MCF-7 breast cancer cells

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Received 23 September 2016; accepted 26 September 2016
Available online 22 November 2016

Abstract

Estrogen receptor (ER) antagonists such as tamoxifen (Tam) have been used successfully to treat ER+ breast cancers for more than 30 years. Unfortunately, long term use of Tam can result in resistance. Tam resistance is associated with the activation of growth factor signaling pathways that promote cell proliferation and survival. The mitogen-activated protein kinase (MAPK), is up-regulated in Tam resistant (Tam-R) cells. Previous studies have reported that the flavanone, naringenin (Nar) can inhibit cell proliferation and induce apoptosis in ER+ breast cancer cells. Furthermore, Nar has been shown to inhibit the MAPK signaling pathways in MCF-7 cells. In this report we investigated whether inhibition of MAPK alone is mediating the effects of Nar on cell proliferation and viability. These studies will determine the mechanism of action of Nar. Tam-R MCF-7 breast cancer cells were treated with Nar or U0126, a MAPK kinase inhibitor. Our studies show that while both U0126 and Nar impaired cell proliferation and viability the combination of U0126 and Nar resulted in greater inhibition of cell viability than either compound alone. It has been previously reported that Nar can bind the ER. Our lab has also shown that Nar localizes ERα to a peri-nuclear region of the cell. Confocal microscopy revealed that in U0126 treated cells ERα displayed an even distribution across the cytoplasm as seen in untreated Tam-R cells. These studies suggest that MAPK is not the only target of Nar.

Keywords: Naringenin; Tamoxifen resistance; MAPK signaling

1. Introduction

Since the majority of breast cancers are dependent on estrogen stimulated growth, anti-estrogen treatments such as tamoxifen (Tam) are successful [1]. Tam has been shown to be a safe and effective treatment for advanced breast cancer [2,3]. Tam binds the estrogen receptor (ER), and inhibits the expression of estrogen-regulated genes, thus impairing proliferation and viability [2,4]. Unfortunately, the therapeutic benefits of Tam are limited by acquired resistance [5,6]. Multiple signaling pathways, such as the MAPK pathway can activate the ER. Thus, Tam-resistant (Tam-R) cells have a heightened sensitivity to both growth factor and estradiol activation of MAPK [7–9]. The upregulation of MAPK signaling has been reported as a primary pathway by which ERα is activated in Tam-R cells. Therefore, inhibition of MAPK may be a likely means of inhibiting cell growth and survival of Tam-R breast cancer cells.

The ER is a hormone receptor and transcription factor. The ER is localized primarily within the nucleus, however it is present in the cytoplasm and at the membrane [10,11]. Activation of the ER can be achieved through ligand-dependent or independent pathways. Ligand-dependent activation of the ER is mediated by estrogen binding. Following estrogen binding, the ER forms homodimers that translocates to the nucleus and bind to estrogen-responsive element of target genes [12,13]. In contrast, the ER can also induce a non-genomic rapid response [1,11,12,14]. ERα can bind to the plasma membrane where the rapid, extra-nuclear response is initiated [11,14]. Once bound by estrogen, the ER is released from the

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http://dx.doi.org/10.1016/j.biopen.2016.09.004

membrane initiating the activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, both of which are pro-survival and growth [11,14,15]. ERα can also directly activate the epidermal growth factor receptor (EGFR), causing activation of the MAPK and PI3K pathways [15,16].

Ligand-independent activation of the ER is a result of phosphorylation of multiple serine and tyrosine residues [1,10,13]. Growth factor receptors can activate the ER through several signaling pathways including the MAPK (Ras-Raf-MEK-ERK1/2) and PI3K (AKT) pathways [6,10,16]. ERK (Extracellular signal-regulated kinases) 1 and 2 and AKT (protein kinase B) phosphorylate serine 118 of the ER resulting in its activation [10,16]. Ligand-independent activation of the ER has increased the need for treatments that target not only the ER but also these signaling pathways to block cell growth.

ER+ Tam-R cell lines are reported to have constitutive activation of both the PI3K/AKT and MAPK pathways [15]. As mentioned above, this constitutive activation of these pathways results in activation of the ER independent of estrogen allowing growth to occur. The MAPK cascades are important regulatory pathways for cell proliferation, survival, and differentiation [17,18]. Various kinases in the MAPK pathway are often mutated in cancers, including Ras (the most frequently mutated oncogene) [18]. Abnormal activation of the MAPK pathway can result in alterations of proliferation as well as survival and migration, which is often associated with therapy resistance [19,20]. Thus the identification of kinase inhibitors is critical to impair Tam-R cell proliferation and survival.

Flavonoids have been implicated in the reduction and protection against the development of endocrine tumors by binding the ER in individuals consuming a diet rich in flavonoids [11]. Naringenin (Nar), a grapefruit flavanone has been reported to induce apoptosis in different cancer cell lines containing ERα or ERβ [11,14]. The exact mechanism of reduced proliferation and growth arrest of the cells is not understood. Our previous findings suggest that Nar affects the MAPK signaling pathway which can result in decreased proliferation and cell survival [14]. It has also been reported that Nar induces apoptosis in various cancer cells including MCF-7 breast cancer cells [14,21,22]. We have shown that the addition of Nar impaired proliferation of Tam-R cells. Furthermore, Tam-R cells exhibited an up-regulation of the MAPK pathway which was reversed by Nar. We also found that in Tam-R cells ERα exhibited a different localization pattern compared to the tamoxifen sensitive MCF-7 cells. ERα is distributed primarily in the nucleus of MCF-7 cells. Tam-R cells have an increased level of ERα in the cytoplasm that is evenly distributed. However, upon Nar treatment ERα localized to a peri-nuclear region of Tam-R cells. These known effects of naringenin can be used as markers to identify crucial targets and components of naringenin signaling.

The goal of this study was to identify the targets of Nar and to gain a greater understanding of the mechanism(s) involved in Tam-R MCF-7 cell proliferation and viability. Here we wanted to determine if inhibition of MAPK alone is mediating all the effects of Nar on cell proliferation and viability. Our studies show that while both Nar and inhibition of MAPK impaired viability, the combination resulted in greater inhibition than either compound alone. Furthermore, inhibition of MAPK is not involved in the peri-nuclear localization of ERα seen in Nar treated Tam-R cells.

2. Materials and methods

2.1. Materials

MCF-7 ER+ breast cancer cells (HTB-22) were purchased from ATCC. Dulbecco's Modified Eagle Medium was purchased from Gibco. Charcoal-stripped fetal bovine serum, naringenin and 4-OH-tamoxifen were from Sigma Aldrich. Antibodies for ERK1/2, p-ERK1/2, AKT, p-AKT, caspase 7, PARP and U0126 were obtained from Cell Signaling. Guava Via-Count Reagent was purchased from Millipore. Actin antibody was obtained from Abcam. Anti-ERα antibody (HC-20) was from Santa Cruz biotechnology. AlexaFluor 488 conjugated Goat anti-Rabbit secondary antibody was obtained from Jackson ImmunoResearch. Anti-mouse and anti-rabbit horseradish peroxidase conjugated secondary antibodies were purchased from Sigma Aldrich. The enhanced chemiluminescence (ECL) detection kit was from BioExpress.

2.2. Cell culture

Tam-R cells were cultured in Phenol-red free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% charcoal-stripped FBS, 0.01 mg/mL bovine insulin, and 100 U/mL penicillin/streptomycin. Tam-R cells were maintained by adding 100 nM of tamoxifen. Cells were maintained at 37 °C and 5% CO_2. Media was replaced every two days and cells were passaged at 80% confluency. Tam-R cells (2.45×10^4/plate) in growth phase were plated and either treated with DMSO (Cont) or 200 μM Nar or 10 μM U0126 or a combination of the two for 24, 48 or 96 h, as indicated.

2.3. Immunoblotting

Protein extracts were subjected to 10% SDS-PAGE and then transferred to an Immobilon-P membrane. The membrane was incubated with the specific primary and secondary antibodies indicated and visualized using enhanced chemiluminescence (ECL) and a Bio-Rad ChemiDoc XRS. Protein bands were quantified using densitometric analysis using Quantity One analysis software.

2.4. Flow cytometry

MCF-7 cells were collected and centrifuged for 5 min at low speed (5), and the pellet was resuspended in 1×PBS. Guava Via-Count Reagent was added to cells at a 1:20 or 1:10 dilution and incubated for five min at room temperature in the dark. Guava easy-Cyte Flow Cytometry was used to determine the percentage of viable, mid-apoptotic, and dead cells. Values were analyzed by guavaSoft software.

2.5. Confocal microscopy

MCF-7 cells were grown on cover slips. Cells were washed with 1×PBS, fixed with 3.7% paraformaldehyde for 15 min and then permeabilized for 5 min in Triton-X (0.25% in 1×PBS). Cells were blocked in a 5% goat serum - 1% BSA - 1×PBS solution for 15 min. Cells were then incubated with anti-ERα antibody (1:100) for 1 h at room temperature, washed and incubated with secondary antibodies for 45 min at room temperature. Cells were then stained with DAPI for 5 min. Cells were viewed by confocal microscopy.

2.6. Quantification of ERα distribution

ERα levels were quantified by measuring the intensity of fluorescence in both the cytoplasm and the nucleus. The relative intensity was quantified using Image-Pro Plus software (Silver Spring, MD). Briefly, intensities of nuclear and cytoplasmic signals were measured and the ratio of the nuclear/cytoplasmic signal was averaged for individual cells ($n = 5$) for each treatment. A percent change formula, [(Treatment − Vehicle)/(Vehicle) x 100%], was used to determine change in protein localization.

2.7. Statistical analysis

Data presented as means ± SEM. The significance of comparing means was assessed by two-way analysis of Student's *t*-test (StatPlus, AnalystSoft).

3. Results

3.1. Naringenin does not alter ERα expression

Previous studies have shown that the optimal concentration of Nar required to significantly impair cell proliferation and cell viability is 100–200 μM in MCF-7 breast cancer cells [23]. Furthermore, it is known that MCF-7 cells are ERα positive, and require the ER for cell growth. To determine if the concentration of Nar administered in this study is altering the expression of ERα in Nar treated MCF-7 Tam-R cells, we treated cells with or without Nar for 24, 48, and 96 h. The protein levels of ERα were normalized to the levels present in vehicle control cells. Our findings revealed no significant difference in the expression of ERα in Nar treated cells compared to the vehicle control at all three time points (Fig. 1). Relatively low levels of the ER in Tam-R MCF-7 cells can be expected due to the decreased dependence on estrogen for growth as a result of increased signaling for alternative proliferation pathways.

3.2. Naringenin is a weak ERK1/2 inhibitor

Previous studies have shown that Nar inhibits cell proliferation as a result of decreased cell viability [24–26]. We have shown that Nar decreased ERK1/2 protein levels and relocalized ERα to a peri-nuclear region of the cell. These findings suggest that Nar's effects on cell proliferation, viability, and ERα localization could be a result of inhibition of ERK1/2. We wanted to determine if the inhibition of ERK1/2 could account for all of Nar's effects on Tam-R MCF-7 breast cancer cells. We compared the effects of Nar to that of U0126, a known inhibitor of MEK and thus phosphorylation of ERK1/2. First we wanted to determine if U0126 elicits the same effects as Nar on ERK1/2, so we treated Tam-R cells with Nar, U0126, and a combination of the two for 24, 48, and 96 h (Fig. 2). The protein levels (Fig. 2A and C) and phosphorylation status of ERK1/2 (Fig. 2A and B) were assayed using immunoblot analysis and normalized to vehicle treated Tam-R MCF-7 cells. Our findings confirmed that Nar is an inhibitor of ERK1/2 phosphorylation (Fig. 2A). However, when compared to the U0126 treatment, Nar appears to be a weak inhibitor of ERK1/2 phosphorylation (Fig. 2A). Phosphorylated ERK1/2 was either undetectable (24 h) or extremely low (48 and 96 h) in the U0126 alone and the combination treatment (Fig. 2A and B). In contrast, Nar treatment only resulted in a 50% inhibition of ERK1/2 phosphorylation. Lastly, all three treatments showed a decrease in the total levels of ERK after 96 h of treatment when compared to the vehicle control. However, the observed decrease is greater in the U0126 alone and

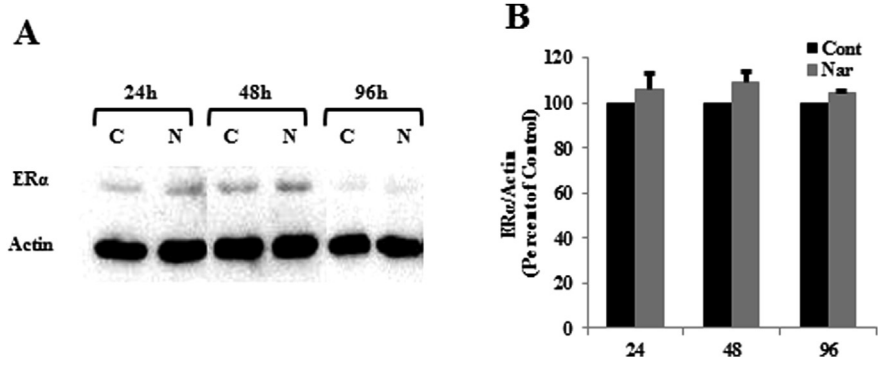

Fig. 1. Nar does not impair ERα levels. Tam-R MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence or absence of Nar (200 μM) for 24, 48, and 96 h. (A) Protein lysates were subjected to SDS-PAGE and immunoblotted using antibodies against ERα and actin. (B) ERα to actin was quantified using densitometric analysis by Quantity One Software and expressed as a percent of the control. The results are representative of 3 separate experiments. Results were the means ± SEM of three independent experiments. *$p < 0.05$.

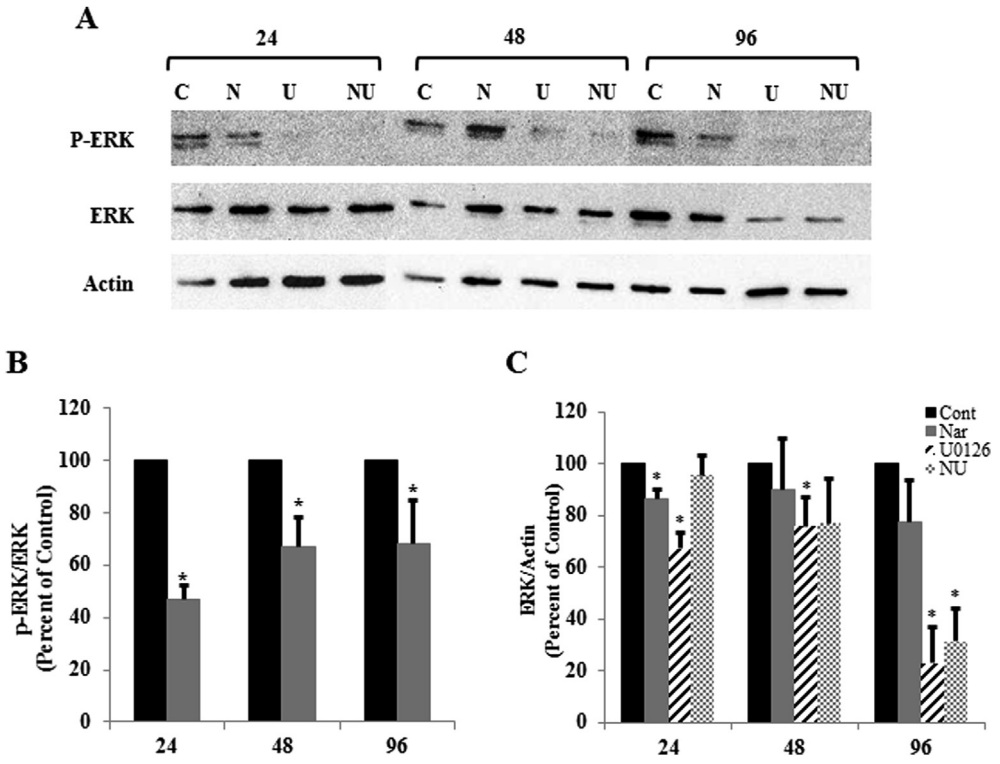

Fig. 2. Nar is a weak inhibitor of ERK1/2 phosphorylation. Tam-R MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24, 48, and 96 h. (A) Protein lysates were subjected to SDS-PAGE and immunoblotted using antibodies against phospho-ERK1/2, ERK1/2 and actin. (B) P-ERK to actin and (C) ERK to actin were quantified using densitometric analysis by Quantity One software and are expressed as a percent of the control. The results are representative of 3 separate experiments. *$p < 0.05$.

combination treatments (Fig. 2A and C). Thus while Nar treatment reduced the levels of ERK1/2, U0126 was more effective at lowering the levels.

3.3. Inhibition of ERK1/2 alone does not account for the decreased viability seen in Nar treated cells

Our previous studies have shown that Nar decreased cell proliferation [22,27,28]. This decrease in cell proliferation may be in part attributed to the observed inhibition on ERK1/2 levels. We wanted to determine if inhibition of ERK1/2 alone results in decreased cell proliferation to the same extent as Nar. We treated Tam-R cells as previously stated with Nar, U0126, or a combination of the two and assayed cell proliferation (Fig. 3). Cell densities (cells/mL) from each treatment were analyzed by flow cytometry (Fig. 3A). There was no significant difference in cell density in any of the treatment groups after 24 and 48 h when compared to the vehicle control. However, after 96 h of treatment all three groups showed a decrease in cell density. Both U0126 and Nar appear to elicit

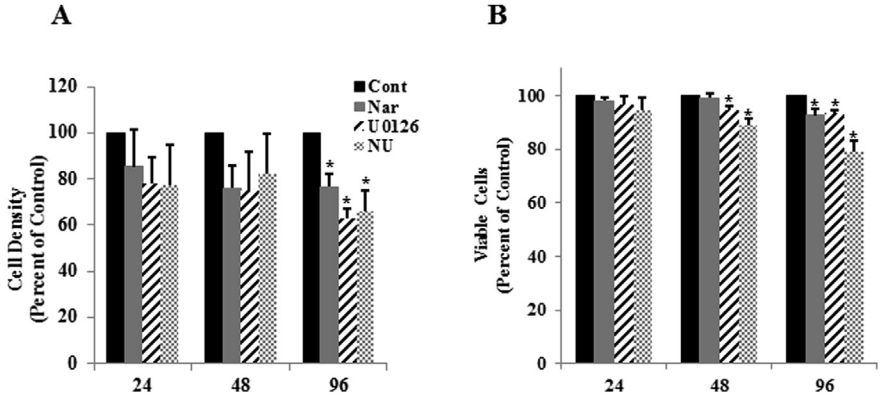

Fig. 3. Inhibition of ERK alone cannot explain Nar decreased cell viability. Tam-R MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24, 48, or 96 h. (A) Cell density (cells/mL) was determined by flow cytometry. Results are the means ± SEM of three separate experiments. Data were normalized to control. (B) Cell viability was determined by flow cytometry. Results are the means ± SEM of three independent experiments. Data were normalized to control. *$p < 0.05$.

a similar effect on cell proliferation (Fig. 3A). Since Nar has been shown to decrease cell proliferation as a result of decreased cell viability we wanted to determine if the effects on cell viability are a result of Nar targeting and inhibiting ERK1/2 (31). Cell viability analysis revealed that both Nar and U0126 reduced viability in 96 h to the same extent (Fig. 3B). However, when U0126 and Nar were used in combination there appears to be an additive effect resulting in a greater decrease in cell viability (Fig. 3B).

3.4. Nar induces apoptosis

Previous studies reported that Nar induced apoptosis through PARP and caspase activation in HeLa and MCF-7 cells [14,21]. We have shown that Nar can induce apoptosis through the activation of caspase 7, which may explain the observed decrease in cell viability. In order to determine if induced apoptosis in Nar treated cells is a result of ERK1/2 inhibition we examined the levels of apoptotic cells and the status of known apoptotic markers in U0126 treated cells. We treated Tam-R MCF-7 cells with Nar, U0126, or a combination of the two and determined the number of apoptotic cells to determine if the observed decrease in cell viability and apoptosis correlated and whether inhibition of ERK1/2 alone was responsible for the effects of Nar on viability. Our findings show that there was an increase in apoptotic cells in all treatment groups when compared to the control at each time point (Fig. 4A). Our data also indicated that there was no significant difference between the treatment groups over time. Our data for apoptotic cells correlated with the decrease in cell viability.

To determine the mechanism of apoptosis we assayed known apoptotic markers, caspase 7 and PARP, in Nar and U0126 treated Tam-R cells. Our results indicate that both Nar and U0126 lead to the activation of caspase 7 and the cleavage of PARP (Fig. 4B). After 24 h all three treatments showed a decrease in full-length caspase 7 expression. Nar maintains an approximate 20% decrease in caspase 7 at all three time points (Fig. 4C). The combination treatment and U0126 alone show an almost complete activation of caspase 7 at 96 h (Fig. 4B and C). The combination treatment shows a greater effect than Nar or U0126 alone at 48 h and 4 days (Fig. 4B and C).

Next we examined the expression of full-length PARP, a downstream target of caspase 7. Quantified data for PARP revealed that all three treatments decreased PARP expression (Fig. 4B and D). Nar resulted in a 10–30% decrease in PARP while U0126 resulted in an approximate 30% decrease at all three time points when compared to the control. The combination treatment shows an approximately 40–50% decrease across all time points when compared to the control. Similar to the flow cytometry findings, the caspase and PARP analysis

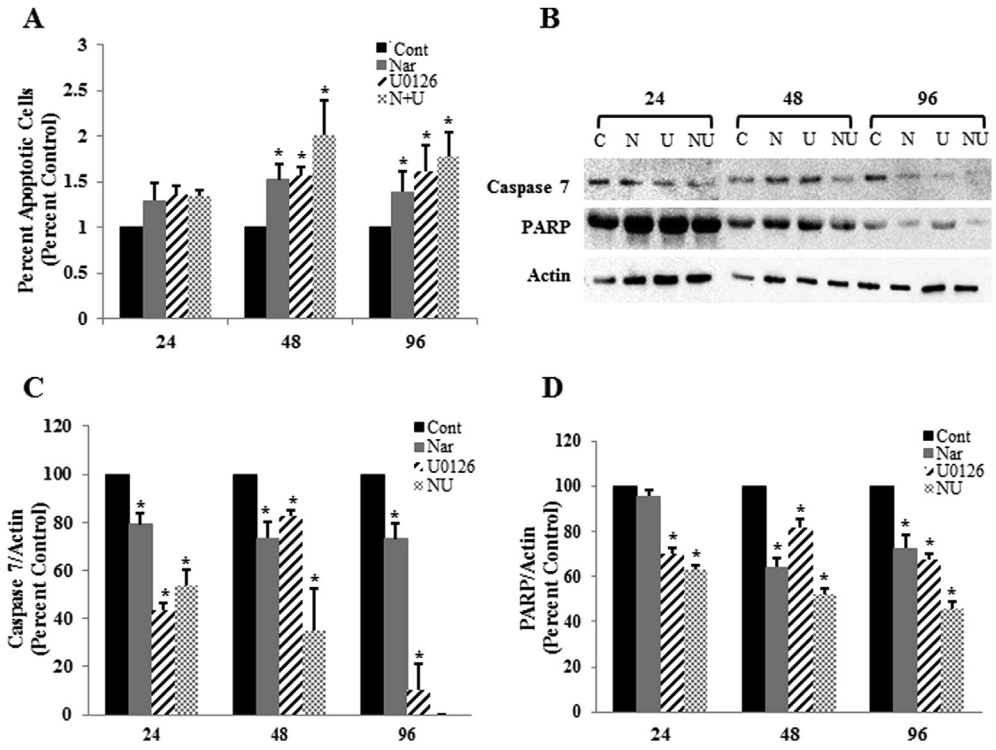

Fig. 4. Nar and U0126 induce apoptosis. Tam-R MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24, 48, or 96 h. (A) Apoptotic cells were determined by flow cytometry. Results are the means ± SEM of three independent experiments. Data were normalized to control. (B) Protein lysates were subjected to SDS-PAGE and immunoblotted using antibodies against Caspase 7, PARP, and actin. (C) Caspase 7 and (D) PARP were quantified using densitometric analysis using Quantity One software and are expressed as a percent of the control. Results are the means ± SEM of three separate experiments. Results are normalized to control. *$p < 0.05$.

showed that the combination treatment had a greater effect than either Nar or U0126 alone when compared to the control.

3.5. Nar alone influences ERα localization

Next we examined the localization pattern of ERα. ER is found in both the cytoplasm and nucleus [11,29–31]. ERα enters the nucleus after either estrogen binding or phosphorylation [10–13,16]. We have shown that Nar localizes ERα to a peri-nuclear region of the cell. To determine if the effect of Nar on ERα localization was a result of Nar inhibiting ERK1/2 we treated cells with Nar, U0126 or a combination of the two as previously stated and performed confocal microscopy as described in Material and Methods (Fig. 5A and B). Similar to previous studies, our results show that the vehicle treated Tam-R MCF-7 have an even distribution of ERα in the cytoplasm and nucleus in all three time points (Fig. 5B). ERα and DAPI were imaged at all time points and a representative image of the 96 h time point is shown in Fig. 5A, U0126 treated cells also show an even distribution of ERα at all the time points with no difference when compared to the control (Fig. 5A. g–i and B). In contrast, cells treated with Nar and the combination treatment exhibited only 20–30% of ERα within the nucleus. ERα was localized primarily to a peri-nuclear region of the cell at all three time points (Fig. 5A. d–f; j–l and B). These findings suggest that the inhibition of ERK1/2 by Nar is not responsible for the observed changes in ERα localization.

4. Discussion

Tam is the most widely used ER antagonist employed to treat ER+ breast cancers [2,4]. While ERα bound by Tam can still translocate into the nucleus it is unable to recruit the co-activators required for transcription. Unfortunately, the long term use of Tam, results in resistance [2,4]. Tam-R cells overcome the effects of Tam and achieve ERα activation through kinase signaling pathways such as the MAPK pathway [7,8]. It has been shown that Nar can inhibit the MAPK signaling pathway and interact with ER [30,31]. The effects of Nar and other flavanones have been studied in relation to glucose uptake, as endocrine disruptors as well as their use as a possible therapeutics in multiple cancers [11,22,24,30]. However, the specific mechanism(s) responsible for the effects of Nar are unknown.

Since Nar treatment has been shown to alter ERK and ERK is known to be involved in cell proliferation and survival, we wanted to determine if the cytotoxic effects observed with Nar could be attributed to the inhibition of ERK. Our studies suggest that all of the effects observed in Nar treated Tam-R cells cannot be fully explained by the inhibition of ERK1/2. Although our data show that both Nar and U0126 decrease cell viability and induce apoptosis to a similar extent, when used in combination there is a greater decrease that appears to be additive. The additive effect of Nar and U0126 on cell viability is present at both 48 and 96 h of treatment and correlates with the increased apoptosis seen at these time points in the combination treatments. These results suggest that while Nar does target ERK it also targets other proteins involved in cell viability and apoptosis. Our results do not show a significant change in cell density (approximate 50% decrease) at 30 h as shown in previous reports [14]. However, this difference could be a result of our studies using Tam-R MCF-7 cells compared to MCF-7 cells used in other previous studies.

Furthermore, our studies show that Nar is a weaker inhibitor of ERK1/2 phosphorylation and protein levels than U0126. Our results showed a 40–50% decrease in phosphorylated ERK1/2 in Nar treated cells which is comparable to previous studies using MCF-7 breast cancer cells [30]. Our data suggests that Nar and U0126 could be eliciting these effects through different mechanisms or that Nar may interact with different target proteins. Nar could be targeting proteins upstream of MEK or other proteins involved in the regulation of the MAPK pathway. This could explain our observed additive effect in the combination treatments.

Since previous studies have shown that Nar interacts with the ER, albeit weakly and that Nar changes the localization

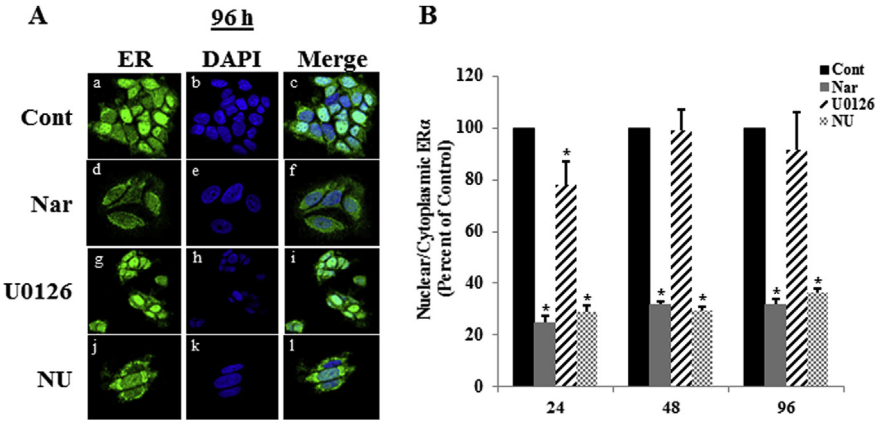

Fig. 5. ERK1/2 does not regulate ERα localization. Tam-R MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24, 48, and 96 h. (A) Cells (96 h time point) were fixed and stained with ERα antibody and DAPI and then subjected to confocal microscopy. (B) ERα localization data was quantified using intensity parameters as described in Method and Materials. Results are the means ± SEM of three separate experiments. Results are normalized to control. *$p < 0.05$.

pattern of the ER, we tested U0126 to determine if Nar elicited this change through direct inhibition of ERK. While neither Nar treatment nor U0126 altered the total protein levels of ERα in Tam-R MCF-7 breast cancer cells, only Nar altered the localization pattern of ERα when compared to untreated Tam-R cells. Cells treated with U0126 did not alter the ER localization pattern when compared to the untreated cells. These data suggest that Nar could be directly interacting with the ER to localize ER to a peri-nuclear region or it could be targeting proteins involved in ER localization and regulation. If Nar is binding to ERα then our studies would be the first to suggest that a Nar–ERα complex was either prevented from entering the nucleus or that the complex was actively transported out of the nucleus. Further studies are needed to determine the mechanism of action of Nar on ERα localization. This effect is specific to Nar and cannot be explained by inhibiting ERK1/2 protein levels and/or phosphorylation.

Furthermore, our studies show that even in the absence of estrogen, Nar is still able to inhibit ERK1/2 phosphorylation and change ERα localization. Previous studies suggested that the ER could activate the MAPK pathway and in turn ERK could activate the ER. By inhibiting ERK with U0126 or Nar the ER should not be activated by phosphorylation and thus remain inactive. Either the ER is activated by other signaling pathways or is bypassed altogether. This may suggest that Nar affects signaling pathways other than those dependent on estrogen and thus may be targeting multiple proteins.

5. Conclusion

While the use of natural compounds to treat various disorders is being explored, it is critical to determine the underlying molecular and cellular mechanisms of these compounds in order to determine efficacy. To investigate the mechanism of action of Nar, we examined the contribution of one of the main targets of Nar, ERK. More specifically, we wanted to determine if inhibition of ERK alone mediated all the effects of Nar on cell proliferation and viability in Tam-R MCF-7 breast cancer cells. Our studies demonstrated that Nar and ERK inhibition impaired proliferation and viability, but the combination resulted in greater inhibition than either compound alone suggesting that Nar may have other targets Surprisingly, ERK was not involved in the peri-nuclear re-localization of ERα seen in Nar treated Tam-R cells. Taken together, these studies suggest that Nar targets multiple proteins to elicit its effects on cell proliferation and survival.

Conflict of interest

The authors declare no conflict of interest.

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