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Critical roles for Nitric oxide and ERK in the completion of prosurvival autophagy in 4OHTAM-treated estrogen receptor-positive breast cancer cells

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Abstract

Autophagy is a mechanism of tamoxifen (TAM) resistance in ER-positive (ER+) breast cancer cells. In this study, we showed in ER+ MCF7 cells that 4-hydroxytamoxifen (4OHTAM) induced cellular nitric oxide (NO) that negatively regulates cellular superoxide (O₂⁻) and cytotoxicity. 4OHTAM stimulated LC3 lipidation and formation of monodansylcadaverine (MDC)-labeled autophagic vesicles dependent on O₂⁻. Depletion of NO increased O₂⁻ and LC3 lipidation, yet reduced formation of MDC-labeled autophagic vesicles. Instead, NO-depleted cells formed remarkably large vacuoles with rims decorated by LC3. The vacuoles were not labeled by MDC or the acidic lysosome-specific fluorescence dye acridine orange (AO). The vacuoles were increased by the late stage autophagy inhibitor chloroquine, which also increased LC3 lipidation. These results suggest NO is required for proper autophagic vesicle formation or maturation at a step after LC3 lipidation. In addition, 4OHTAM induced O₂⁻-dependent activation of ERK, inhibition of which destabilized lysosomes/autolysosomes upon 4OHTAM treatment and together with depletion of NO led to necrotic cell death. These results suggest an essential role for endogenous NO and ERK activation in the completion of pro-survival autophagy.

Keywords

tamoxifen; autophagy; nitric oxide; reactive oxygen species; cell death; estrogen receptor; breast cancer

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1. Introduction

Approximately 70% of breast cancers express estrogen receptor (ER), so endocrine therapy with Tamoxifen (TAM) benefits patients by significantly reducing recurrence [1, 2]. Unfortunately, acquired TAM resistance is not uncommon [2, 3], emphasizing the challenge of emerging resistance [4]. TAM treatment changes different aspects of cell physiology, which is reflected in the complexity of TAM resistance mechanisms [2, 5, 6]. Besides inhibition of cell proliferation, *in vitro* TAM can also induce senescence and cell death [7-10]. Increased autophagy with a shift in balance between cell death and survival may be critical for *in vivo* response to TAM [11, 12]. Inhibition of autophagy enhances TAM-induced cell death in 4OHTAM-resistant cells [11-13], consistent with its role in cell survival [14, 15]. However, excessive autophagy in ER-positive (ER+) breast cancer cells treated with anti-estrogens can lead to type-II programmed cell death, autophagic death [8, 16], and necrosis [17]. Regulation of survival and apoptosis in response to TAM is poorly understood, which makes elucidation of appropriate mechanisms an important task for anti-estrogen therapy research.

TAM induces oxidative stress through reactive oxygen species (ROS) [18, 19]. Low levels of ROS activate stress signaling pathways and promote proliferation and survival while excessive ROS may cause irreversible damage to DNA, protein, and cell membranes leading to cell death [20, 21]. Increased transcription of antioxidant genes and activation of stress signaling pathways are associated with *in vivo* TAM-resistance in animal models [18, 22] and human breast cancers [23] suggesting adaptation to oxidative stress occurs in acquired TAM resistance. ROS stimulates autophagy by regulation of ATG4 and stress signaling pathways [24-26] suggesting autophagy may protect against ROS [26]. Active autophagy is observed in acquired TAM-resistance [11-13], implying that oxidative stress may function in both TAM-induced death and activation of pro-survival autophagy.

Nitric oxide (NO) is an integral part of ROS [27, 28] produced by nitric oxide synthases [29]. At low levels, NO is a scavenger of superoxide (O_2^-) [28]. However, excess NO can aggravate oxidative stress when converted to peroxynitrite [30]. NO regulates cellular signaling and is involved in tumorigenesis and cancer progression [31, 32]. Excessive NO production in mitochondria mediates TAM-induced cell death [33]. Lower expression of eNOS is associated with worse prognosis in ER+ breast cancer [34, 35] implying that NO regulates TAM response. Exogenous NO induces autophagy [36, 37]. However, the regulatory role of endogenous NO in TAM-induced oxidative stress, autophagy and cell death remains to be elucidated.

In this study, we investigated the role of NO in 4OHTAM-induced oxidative stress, autophagy, and cell death. We showed that endogenous NO was essential for completion of autophagy and protection of ER+ MCF7 breast cancer cells from 4OHTAM-induced cytotoxicity.

2. Materials and Methods

Antibodies and Chemicals

Rabbit anti-LC3 and anti-LAMP2 (H4B4) antibodies (Abcam). Mouse anti- β -actin antibody (Santa Cruz Biotechnology). Rabbit anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK, anti-phospho-p38 MAPK and anti-p38 MAPK antibodies (Cell Signaling). Alexa-Fluor 594-conjugated anti-mouse and Alexa-Fluor 488-conjugated anti-rabbit antibodies, 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) and Dihydroethidium (DHE) (Invitrogen). 4OHTAM, MTT, PD98059, Chloroquine, DEA NONOate, Acridine orange (AO) and Monodansylcadaverine (MDC) (Sigma-Aldrich).

Cells

MCF7 cells were described previously [38].

RNA isolation and reverse transcription

Total RNA was prepared using RNAqueous-4PCR Kit and the first DNA strand was synthesized using RETROscript kit (Ambion) using the manufacturer's protocols.

Quantitative PCR

Primer sequences for qPCR are listed in Table 1. SYBR green PCR kit (Applied Biosystems) and AB7500 system (in 9600 emulation mode) were used as follows: activation at 95°C; 2 minutes, 40 cycles of denaturation at 95°C; 15 seconds and annealing/extension at 60°C; 60 seconds, followed by melt analysis ramping from 60°C to 95°C. The standard curve method was used to determine relative gene expression.

Clonogenic Assay

500 cells were plated in a 60 mm plate. The medium was replaced with drug- or vehicle-containing medium for 14 days with medium replacement every two days; then recovery for two weeks, fixation with ethanol, staining with Methylene Blue, and colony count.

Propidium iodide measurement of plasma membrane integrity

10^5 cells were plated per 12-well culture dish, treated with 4OHTAM for specified time, stained with 10 μ g/ml propidium iodide (PI) (BD Bioscience) without fixation for 5 minutes at 37°C, and analyzed by fluorescence microscopy.

Quantitative measurement of intracellular NO and O₂–

Cells were loaded with DAM-FM diacetate (8 μ M in PBS) or DHE (2 μ M in PBS) for 30 minutes. For NO measurement, cells were kept in growth medium without DAM-FM at 37°C for 30 minutes before trypsinization. For ROS measurement, cells were trypsinized immediately. Collected cells were washed, transferred to a 96-well plate, and analyzed with a BioTekMx microplate reader (excitation/emission of 585/520 nm for NO and of 510/595 nm for ROS). Cell density was measured with light absorbance at 590 nm. The relative DAF-FM or DHE fluorescence was calculated by normalizing the fluorescence/cell density ratio to the control (vehicle-treated).

MDC sequestration assay and Picogreen staining of cellular DNA

The procedures have been previously described [38].

Immunostaining and confocal microscopy

Cells on glass coverslips were fixed in 4% formaldehyde/PBS, permeabilized by 1% Triton X-100/PBS, and immunostained as described [39] and analyzed by confocal microscopy.

Quantitative analysis of the volume and integrity of lysosomes

Cells in a 96-well plate were labeled with AO (2 µg/ml in PBS) for ten minutes, rinsed three times with PBS, re-fed with phenol-red free media, and then analyzed for fluorescence intensity using a BioTekMx microplate reader (excitation/emission of 475/520 nm for green fluorescence, 475/620 nm for red fluorescence). For analysis of lysosome stability, the fluorescence intensity of the labeled cells was tracked over a four hour period of time.

Analysis of cell viability by MTT assay

The procedures have previously described [38].

Immunoblot

The procedures have previously described [38].

Statistical analysis

Statistical analysis was done by one way ANOVA and two tailed t-test.

3. Results

4OHTAM induces NO while decreasing O₂⁻ in MCF7 cells. NO depletion increases O₂⁻ and 4OHTAM-induced cytotoxicity

To test induction of O₂⁻ and NO by 4OHTAM, we treated MCF7 cells with 4OHTAM (5 µM) and labeled intracellular O₂⁻ with DHE and NO with DAF-FM, respectively. An increase in NO and a decrease in O₂⁻, evidenced by increased DAF-FM and decreased DHE fluorescence, was observed (Fig. 1A). NO can counter O₂⁻ by functioning as an O₂⁻ scavenger. We therefore examined O₂⁻ and NO levels in cells co-treated with 4OHTAM and O₂⁻/NO scavengers. The O₂⁻ scavenger Tiron increased NO in vehicle and 4OHTAM treated cells, evidenced by increased DAF-FM fluorescence (Fig 1B, *left*). This was accompanied by decreased expression of eNOS and iNOS (Fig 1B, *middle and right*), indicating the increase in NO is not due to increased eNOS or iNOS expression. Conversely, O₂⁻ increased in cells treated with the NO scavenger c-PTIO or c-PTIO plus 4OHTAM (Fig 1C, *left*). The expression of heme oxygenase-1 (HO-1) gene, an indicator of oxidative stress, was also induced by c-PTIO or 4OHTAM, and further increased by c-PTIO plus 4OHTAM (Fig. 1C, *right*). These results suggest NO depletion increased oxidative stress by increasing intracellular O₂⁻.

Next, we examined the effects of NO and O₂⁻ on 4OHTAM-induced cytotoxicity. 4OHTAM reduced MTT absorbance and colony formation (Figs. 2A,B) which was further reduced by

c-PTIO. The c-PTIO-dose-dependent decrease in MTT absorbance appeared more pronounced in cells treated with combination c-PTIO and 4OHTAM, suggesting NO depletion enhanced 4OHTAM-induced cytotoxicity. Consistent with this, the NO donor DEA NoNoate blocked the 4OHTAM-induced decrease in MTT absorbance (Fig. 2C). Tiron abolished the cytotoxic response to 4OHTAM (Fig. 2C), indicating O₂⁻ contributes to cytotoxicity. Altogether, the results suggest NO negatively regulates O₂⁻ and 4OHTAM-induced cytotoxicity. The reduced MTT absorbance in 4OHTAM-treated cells appeared to result, in part, from a G1-phase cell cycle arrest and apoptosis, evidenced by a significant increase in G1 and subG1 populations in 4OHTAM-treated cells (Fig. 2D).

O₂⁻ mediates LC3 lipidation and formation of autophagic vesicles. NO depletion affects autophagic vesicle formation and lysosomal/autolysosomal integrity

To test if O₂⁻ and NO regulate autophagy, MCF7 cells were treated with 4OHTAM with or without Tiron and c-PTIO. LC3 lipidation is an early step in autophagy detected in immunoblots by the conversion of LC3-I to LC3-II. Formation of autophagosomes and autolysosomes, were monitored by accumulation of monodansylcadaverine (MDC) into autophagic vesicles. Acidic lysosomes and autolysosomes were labeled with acridine orange (AO) that emits green fluorescence in the cytosol and nucleus, but red fluorescence in acidic lysosomes/autolysosomes.[40]. 4OHTAM increased LC3-II expression and MDC fluorescence (Figs. 3A,B), indicating LC3 lipidation and formation of autophagic vesicles. 4OHTAM also increases the AO red/green ratio (Fig. 3C), indicating increased lysosome/autolysosome volume.

Lysosome/autolysosome permeability or loss of integrity can be detected by a rapid decrease in the AO red/green ratio [40-42]. Cells were pulse-labeled with AO and the red/green ratio monitored for increasing amounts of time up to 4 hrs. The AO red/green ratio in cells treated with 4OHTAM decreased rapidly, but remained relatively stable in vehicle-treated cells (Fig. 3D, left). This suggests that while 4OHTAM increased lysosomal/autolysosomal volume it also dampened the integrity and increased the permeability of lysosomes/autolysosomes.

Tiron reduced the 4OHTAM-dependent increase in LC3-II and MDC fluorescence (Figs. 3A,B), suggesting O₂⁻ is required for or promotes LC3 lipidation and autophagic vesicle formation. While Tiron had no effect on 4OHTAM-induced increase in the AO red/green ratio (Fig 3C), it blocked the effect of 4OHTAM on lysosome/autolysosome permeability (AO red/green ratio remained relatively stable, Fig 3D). This suggests O₂⁻ is responsible for the increased permeabilization and loss of lysosome/autolysosome integrity in 4OHTAM-treated cells. The NO scavenger c-PTIO increased basal and 4OHTAM-induced LC3-II expression (Fig. 3A). However, c-PTIO inhibited the 4OHTAM-induced increase in MDC fluorescence (Fig. 3B), suggesting autophagic vesicle formation was impaired. C-PTIO increased the basal AO red/green ratio, but abolished the 4OHTAM-induced increase (Fig. 3C). C-PTIO also induced a more rapid decrease in the AO red/green ratio in 4OHTAM-treated cells (Fig. 3D, middle), suggesting NO helps maintain lysosome/autolysosome integrity. In contrast to c-PTIO, the NO donor DEA NoNoate reduced basal and 4OHTAM-induced LC3 lipidation without affecting MDC fluorescence or the basal AO red/green ratio (Fig. 3A, B, C). Notably, however, the AO red/green ratio remained relatively stable in cells

co-treated with NoNoate and 4OHTAM, consistent with NoNoate increasing lysosome/autolysosome membrane integrity. In total, the results suggest NO inhibits LC3 lipidation, perhaps by scavenging or reducing O₂⁻, but is required for or contributes to formation of autophagosomes/autolysosomes and maintenance of lysosomal/autolysosomal integrity in 4OHTAM-treated cells.

C-PTIO induces formation of giant vacuoles in 4OHTAM treated cells

Although c-PTIO reduced formation of autophagic vesicles, it induced giant vacuoles in a considerable proportion of cells, and this was enhanced by 4OHTAM (Figs. 4A,B). In contrast, Tiron diminished this effect (Fig. 4B), suggesting that NO prevented while O₂⁻ contributed to formation of these vacuoles. Cells treated with 4OHTAM and labeled with MDC and AO showed an increase in punctate vesicles co-labeled by MDC and red AO (Fig. 4C), indicating formation of autophagosomes and autolysosomes. In contrast, giant vacuoles formed in c-PTIO/4OHTAM treated cells did not stain with MDC or red AO (Fig 4C). Moreover, cells with giant vacuoles contained fewer and smaller sized MDC- and/or AO-labeled vesicles (Fig 4C), suggesting inefficient formation of autophagosomes/autolysosomes in c-PTIO/4OHTAM-treated cells. Immunostaining detected LC3 at the rims of giant vacuoles (Fig. 5A). LAMP2, a lysosomal membrane protein that regulates fusion of autophagosomes with lysosomes to form autolysosomes [43], was used as an autolysosome marker. LAMP2 colocalized with LC3 in autophagic vesicles in cells treated with 4OHTAM alone, but did not colocalize with LC3 in giant vacuoles produced in c-PTIO/4OHTAM treated cells (Fig 5B). Together, the results suggest the giant vacuoles represent disruption of proper autophagic vesicle formation or maturation at a step after LC3 lipidation.

Chloroquine increased LC3 lipidation and promoted giant vacuole formation in c-PTIO/4OHTAM-treated cells

Chloroquine is an inhibitor of lysosomal acidification that blocks autophagosome-lysosome fusion and causes a consequent increase in LC3-II expression and accumulation of autophagosomes [44, 45]. We used chloroquine to test if autophagy inhibition at a late stage could induce giant vacuole formation. Chloroquine increased LC3-II expression and MDC fluorescence (Figs. 6A,B), consistent previous reports [45]. However, chloroquine did not induce giant vacuoles (Fig. 6C and D), suggesting autophagy inhibition at a late stage could not, by itself, induce giant vacuoles. C-PTIO did not affect the chloroquine-induced increase in LC3-II expression, but blocked the increase in MDC fluorescence induced by chloroquine (Figs. 6A,B). Notably, the combination of chloroquine with c-PTIO or chloroquine with c-PTIO and 4OHTAM led to widespread formation of giant vacuoles (Figs. 6C,D). These results suggest that the giant vacuoles resulted from disruption of autophagy at a step between LC3 lipidation and formation of MDC-labeled autophagic vesicles.

4OHTAM induces O₂⁻-dependent ERK phosphorylation. ERK inhibition induces loss of lysosomal integrity and necrotic death in cooperation with 4OHTAM and c-PTIO

Stress signaling pathways are implicated in ROS-mediated autophagy, TAM-induced death, and TAM resistance [25, 46, 47]. The increase in O₂⁻ due to NO depletion could stimulate stress signaling, and we therefore examined the effect of NO and O₂⁻ on phosphorylation of ERK, p38 MAPK, and JNK. Phosphorylation of ERK (Figs. 7A,B), but not p38 MAPK and

JNK (Fig. 7A), was increased in 4OHTAM-treated cells. NO depletion by c-PTIO increased phosphor-ERK levels, which was further enhanced by 4OHTAM (Figs. 7A,B). In contrast, Tiron blocked the increase in pERK (Fig. 7A), suggesting increased O₂⁻ was responsible for the increase in ERK phosphorylation.

The role of ERK in 4OHTAM-induced autophagy was tested using the ERK inhibitor PD98059. PD98059 prevented basal and 4OHTAM-induced ERK phosphorylation while having no effect on LC3-II expression (Fig. 7B) and a slight increase in 4OHTAM-induced MDC fluorescence (Fig. 7C), suggesting ERK is not involved in the early stages of autophagy. ERK was shown to promote autolysosomal maturation [25]. ERK inhibition by PD98059 increased the AO red/green ratio in vehicle and 4OHTAM-treated cells (Fig. 7D). However, PD98059 induced a more rapid reduction in the AO red/green ratio (Fig. 7E), and the combination of c-PTIO, PD98059, and 4OHTAM induced the most rapid reduction in the AO red/green ratio (Fig. 7E). These results, together with Fig. 3D, suggest NO and ERK activation are critical for lysosomal/autolysosomal integrity in 4OHTAM-treated cells. Importantly, ERK inhibition increased 4OHTAM- and/or c-PTIO induced cytotoxicity (Fig. 7F), suggesting NO and ERK promote survival. As suggested previously [48], disruption of lysosomes could be associated with necrotic cell death. We therefore used Propidium Iodide (PI) staining to score the percentage of cells undergoing necrotic death. Cells treated with 4OHTAM alone had few PI-positive cells, but c-PTIO and a combination of c-PTIO +PD98059 significantly increased their number (Fig. 8A-B), suggesting inhibition of ERK and NO increases necrotic cell death.

4. Discussion

Autophagy plays an intriguing role in 4OHTAM-induced cell death and survival. Initially, autophagy was suggested to promote cell death [8, 17], but recent studies by us and others indicate autophagy inhibition can enhance 4OHTAM-induced cytotoxicity while increased autophagy is associated with 4OHTAM-resistance [11-13, 38]. Although canonical regulators PI3K, beclin1 and ATGs are involved in 4OHTAM-induced autophagy [11-13], the exact mechanism(s) of pro-survival autophagy remains mostly unknown. ROS regulates autophagy [15, 49] and its induction by cancer drugs and radiation [50, 51] suggests ROS may facilitate/promote TAM-induced death of ER⁺ cells [18, 19, 33]. Physiological ROS levels can activate stress signaling pathways and promote cell survival [52], while excess ROS can cause irreversible damage [50, 51]. A role for oxidative stress in 4OHTAM-induced cytotoxicity is suggested by the prosurvival activity of antioxidant vitamin E, which blocks cell death induced by 4OHTAM [53]. Results of the current study suggest prosurvival autophagy in 4OHTAM-treated MCF7 cells is mediated by NO and activation of ERK. Specifically, our data suggest NO reduces the cytotoxic effects of ROS (O₂⁻) and together with ERK promotes the formation and/or integrity of lysosomes/autolysosomes, thus ensuring completion of pro-survival autophagy.

Oxidative stress is a reported TAM-induced response in ER⁺ cells [9, 19, 53]. Kallio et al showed that TAM induces a rapid (in 20 minutes) increase in ROS using DFH-DA as a general ROS indicator in MCF7 cells in the absence of serum [54]. In our study we focused on O₂⁻ by using DHE as a specific indicator. Surprisingly, our results showed a reduction

rather than an increase in O₂⁻ after 4OHTAM treatment in the presence of 10% serum, and a parallel increase in NO levels (Fig. 1A). The increase in NO was only detectable after 24 hours of 4OHTAM treatment which is different from the results reported by Kallio et al. The rapid induction of ROS and cell death by TAM in the experiments by Kallio et al may be attributable to the lack of serum in medium as TAM is known to bind to albumin which may delay its entry into cells and growth factors in serum may also counteract TAM drug effects. In our study the NO scavenger c-PTIO increased basal and 4OHTAM-induced O₂⁻ levels in MCF7 cells (Fig. 1C), suggesting NO is a negative regulator of O₂⁻ and consistent with its role as an O₂⁻ scavenger. C-PTIO increased 4OHTAM-induced cytotoxicity (Fig. 2A) while the NO donor Dea-NoNoate reduced 4OHTAM-induced cytotoxicity (Fig. 2C), indicating NO can alleviate oxidative stress and cell death.

Autophagy is a complex process involving formation of autophagosomes and their fusion with lysosomes to form autolysosomes [55]. Disruption of either step can cause cell death in 4OHTAM-treated cells [11-13] suggesting completion of pro-survival autophagy is essential. Our results showed that 4OHTAM-induced LC3-II expression and MDC fluorescence was appreciably blocked by the O₂⁻ scavenger Tiron, suggesting a critical role for O₂⁻ in the regulation of autophagy. Depletion of NO by c-PTIO increased O₂⁻ and O₂⁻-dependent LC3-II expression, suggesting NO counters some of the effects of O₂⁻. While c-PTIO increased LC3-II expression, it blocked the increase in MDC fluorescence (Figs. 3A,B). In contrast, the NO donor decreased LC3-II expression but had little effect on MDC fluorescence (Figs 3A,B). These results suggest that formation of autophagic vesicles required NO. It is possible that in addition to regulation of O₂⁻, NO mediated unspecified signaling pathway(s) that contributed to autophagic vesicle formation (Fig. 9). Consistent with this idea, a recent study showed exogenous NO can induce autophagy by suppressing mTORC1 [36].

While c-PTIO inhibited formation of autophagic vesicles, it induced giant vacuoles which were increased by 4OHTAM, but partially blocked by Tiron (Fig. 4). Although the rims of these giant vacuoles were decorated by LC3, they did not contain LAMP2 and were not labeled by MDC or AO (Fig.4). Chloroquine, by blocking lysosome-autophagosome fusion [44, 45], increased LC3-II expression and MDC-labeled autophagic vesicles without inducing giant vacuoles by itself. NO depletion blocked the increase in MDC-labeled autophagic vesicle formation in chloroquine-treated cells and enhanced giant vacuole formation (Fig 6A, B). These results suggest the giant vacuoles result from disruption of proper autophagic vesicle formation or maturation, and are driven by O₂⁻-activated autophagy signaling and/or damage. We speculate chloroquine blocked autophagosome-lysosome fusion which led to accumulation of LC3 on membranes that require NO signaling for further formation of competent autophagic vesicles.

Oxidative stress can activate stress signaling pathways including ERK, p38 MAPK, and JNK [25, 46, 56]. ERK regulates TAM-induced death in the absence of estrogen [47] and is found to regulate autophagy in other systems [46, 57]. We show that 4OHTAM induced an O₂⁻-dependent phosphorylation of ERK (Fig. 7B). Interestingly, while ERK inhibition did not alter LC3-II expression or formation of MDC-labeled autophagic vesicles, it worsened the 4OHTAM-induced loss of lysosomal/autolysosomal integrity (Fig. 7). The damage to

lysosomal/autolysosomal integrity by 4OHTAM is most likely due to O₂⁻ that is neutralized by Tiron and NO (Fig. 3D). We noticed that depletion of NO increased AO red/green ratio (Fig. 3C) while inducing a rapid decline in the AO red/green ratio (Fig. 3D, middle). It is likely that depletion of NO increases lysosome/autolysosome volume while inducing leakage by oxidative damage. It appeared that ERK was activated by O₂⁻ and appears to promote lysosomal/autolysosomal integrity (Fig. 7). ERK can regulate lysosome-associated proteins and autophagosome-lysosome fusion [25, 46] and elucidation of how ERK regulates lysosomal/autolysosomal integrity is important for future studies. Depletion of NO and inhibition of ERK may disrupt autophagic process and induce damage to lysosomes/autolysosomes, leading ultimately to the release of lysosomal proteases into the cytosol and cell death. This is supported by the finding that a fraction of 4OHTAM/c-PTIO/PD98059 treated cells become PI permeable, symptomatic of necrotic death (Fig 8A-B). This result is consistent with the finding of Ono et. al. [48] that lysosome rupture induces destruction of the plasma membrane and necrotic cell death.

In conclusion, we found NO is a critical regulator of pro-survival autophagy in response to 4OHTAM. We propose NO regulates autophagy in response to 4OHTAM-induced O₂⁻ production, and together with ERK maintains lysosomal/autolysosomal integrity and/or function (Fig. 9). Synergistic blockage of NO and ERK disrupts pro-survival autophagic process, leading to cell death.

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Abbreviations

4OHTAM	4-hydroxytamoxifen
AO	acridine orange
DAF-FAM	4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate
DHE	dihydroethidium
ERK	Extracellular Signal-Regulated Kinase
ER	estrogen receptor
HO-1	heme oxygenase-1
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
JNK	Jun N-terminal Kinase
LC3	microtubule-associated protein 1 light chain 3
MDC	Monodansylcadaverine
NO	nitric oxide
P38	MAPK mitogen-activated kinase p38
ROS	reactive oxygen species
TAM	Tamoxifen. C-PTIO (2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, monopotassium salt), Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt)

Highlights for [CAN_11954] to be published in Cancer Letters

1. Tamoxifen induces breast cancer cell production of superoxide and nitric oxide.
2. Nitric oxide is critical for formation of functional autolysosomes.
3. Superoxide activates ERK, which is critical for autolysosomal integrity.
4. Inhibition of ERK and nitric oxide destroys autolysosomes, leading to necrosis.

Figure 1. NO negatively regulates O₂⁻ in 4OHTAM-treated MCF7 cells

(A) Cells were stimulated with vehicle (ethanol) or 4OHTAM (5 μ M), labeled with DAF-FAM (NO) or DHE (O₂⁻), and their fluorescence was analyzed. The average fluorescence of triplicate was normalized to the vehicle-treated condition and presented as a graph with standard deviation indicated. (B) Cells treated with vehicle or 4OHTAM (5 μ M) in combination with Tiron (2 mM) for 48 hours were analyzed for DAF-FAM (NO) and for eNOS3 and iNOS gene expression by qPCR. (C) Cells treated with vehicle or 4OHTAM (5 μ M) in combination with c-PTIO (100 μ M) and were analyzed for DHE (O₂⁻) and HO-1 gene expression. The average from triplicate was presented with standard deviation indicated. There is significant difference for DHE between vehicle and c-PTIO (C, $p < 0.01$) and for DAF-FM between vehicle and Tiron (D, $p < 0.01$).

Figure 2. NO negatively regulates 4OHTAM-induced cytotoxicity MCF7 cells

A. The cells were treated with 4OHTAM (5 μ M) in combination with the indicated doses of c-PTIO for four days and then analyzed with MTT assay, the average MTT absorbance from octuplicate was normalized to the vehicle-treated condition and presented as a graph with standard deviation indicated. There is significant difference between vehicle and c-PTIO ($p < 0.05$, $p < 0.05$, $p < 0.01$ for three different doses respectively) and between 4OHTAM alone and 4OHTAM plus c-PTIO ($p < 0.01$, $p < 0.01$, $p < 0.001$ for three different doses respectively). B. The cells were treated with 4OHTAM (5 μ M) in combination with vehicle or c-PTIO (100 μ M) for four days and recovered to form colonies in media without drugs. The average numbers of colonies in triplicate were presented with standard deviation indicated. C. The cells were treated with 4OHTAM (5 μ M) in combination with vehicle or Tiron (2 mM) or

Dea NoNoate (100 μ M) and then analyzed with MTT assay, the average MTT absorbance from octuplicate was normalized to the vehicle-treated condition and presented as a graph with standard deviation indicated. There is significant difference between vehicle and 4OHTAM ($p < 0.01$). There is no significant difference ($p > 0.05$) between Tiron alone and Tiron in combination with 4OHTAM, and between Dea NoNoate alone and Dea NoNoate in combination with 4OHTAM. D. Cells were treated with vehicle or 4OHTAM for three days and then analyzed for cell cycle by PI staining. Average relative subG1, G1, S, and G2/M population from triplicate was presented as a table with standard deviation indicated. There is significant difference in subG1, G1, and S phase between vehicle and 4OHTAM-treated cells ($p = 0.01$) (Representatives of three independent experiments)

Figure 3. O₂⁻ and NO regulate formation of autophagic vesicles and lysosomal/autolysosomal integrity in MCF7 cells

(A) The cells were treated with the 4OHTAM (5 μ M) in combination with vehicle or Tiron (2 mM) or c-PTIO (100 μ M) or Dea NoNoate (100 μ M) for 48 hours. Whole cell lysates were immunoblotted for LC3 with β -actin as loading control. The cells in triplicate were also analyzed for MDC sequestration (B) and AO red/green ratio (C). The average was normalized to the vehicle condition and presented with standard deviation indicated. In B, There is significant difference between 4OHTAM alone and 4OHTAM plus Tiron ($p=0.001$) or plus c-PTIO ($p<0.05$) while there is no difference between 4OHTAM alone and

4OHTAM plus NoNoate ($p>0.05$). In C, there is significant difference ($p<0.01$) between vehicle and 4OHTAM in vehicle, Tiron, and NoNoate-treated cells while no significant difference ($p>0.05$) in c-PTIO-treated cells. In addition, there is significant difference ($p<0.01$) between vehicle and c-PTIO. D. The cells were treated with the indicated drugs for 3 days, labeled with AO and measured the cellular fluorescence at the indicated time points, and the average red/green ratio normalized to the non-treatment condition was presented with standard deviation indicated. (Representatives of at least three independent experiments)

Figure 4. C-PTIO induces formation of giant vacuoles that are not labeled by MDC and AO in MCF7 cells

(A). The cells were treated with the indicated drugs for three days. Cell morphology was analyzed by a phase contrast microscope and average percentage (triplicate) of the cells with giant vacuoles was presented. There is significant difference between vehicle and c-PTIO ($p < 0.01$) and between c-PTIO alone and c-PTIO plus 4OHTAM ($p < 0.01$). There is no significant difference between vehicle and 4OHTAM ($p > 0.05$). (C) Cells treated with the indicated drugs were labeled with MDC or AO for 30 minutes and analyzed by a confocal microscope. (Representatives of three independent experiments)

Figure 5. Giant vacuoles are decorated by LC3 and do not colocalize with LAMP2
 (A) Cells were immunostained for LC3 and analyzed by a confocal microscope. (B) The cells were and co-immunostained for LC3 and LAMP2 and analyzed by a confocal microscopy. (Representatives of three independent experiments)

Figure 6. C-PTIO inhibits chloroquine-induced formation of autophagic vesicles without affecting LC3 expression in MCF7 cells

The cells were treated with vehicle or 4OHTAM (5 μ M) in combination with c-PTIO (5 μ M), chloroquine (10 μ M), or c-PTIO/chloroquine. Whole cell lysates were immunoblotted for LC-3 and β -actin (A). The cells were analyzed for MDC sequestration (B). There is significant difference ($p < 0.01$) between vehicle and chloroquine. Cells morphology analyzed by a phase contrast microscope (C). Average percentage of cells with giant vacuoles from triplicate was presented with standard deviation indicated (D). There is significant difference ($p < 0.01$) between c-PTIO and c-PTIO plus chloroquine with or without 4OHTAM. There is

no difference between vehicle and chloroquine. (Representatives of three independent experiments)

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Figure 7. O2- mediates 4OHTAM-induced activation of ERK which is enhanced by c-PTIO critical for lysosomal stability and survival in MCF7 cells

The cells were treated with the indicated drugs (5 μ M of 4OHTAM, 100 μ M c-PTIO, 2 mM of Tiron and 20 μ M of PD98059) for two days. Whole cell lysates were immunoblotted for phosphorylated and total protein of ERK, JNK, and p38 MAPK (A) and ERK and LC3 (B). C. Cells treated with 4OHTAM (5 μ M) and PD98059 (20 μ M) and analyzed for MDC sequestration. D. The cells were treated with the indicated drugs for 3 days, labeled with AO and measured the cellular fluorescence. Relative AO red/green ratio was presented as a graph with standard deviation indicated. E. The cells were treated for 3 days, labeled with

AO and measured at the indicated time points, and the average red/green ratio normalized to the non-treatment condition was presented with standard deviation indicated. F. Cell viability was analyzed by MTT assay. There are significant differences between 4OHTAM and combination of 4OHTAM with c-PTIO or PD98059 ($p<0.01$), there is significant difference between combination of 4OHTAM with c-PTIO or PD98059 and combination of all three ($p<0.05$) (Representatives of three independent experiments)

Figure 8. Depletion of NO and inhibition of ERK lead to necrotic cell death

MCF7 cells were treated with 4OHTAM (5 μ M) alone or in combination with c-PTIO (100 μ M), PD98059 (20 μ M), or c-PTIO/PD98059 for 4 days. Live cells were stained with propidium iodide without fixation and analyzed by a fluorescence microscope (A). (B) The average percentage of cells positive for PI in triplicate was presented as a graph with standard deviation indicated. There is no significant difference ($p>0.05$) between vehicle and 4OHTAM in PD98059-treated cells. There is significant difference between vehicle and 4OHTAM in c-PTIO-treated cells ($p<0.01$) and c-PTIO/PD98059-treated cells ($p<0.001$).

There is significant difference between c-PTIO- treated cells and c-PTIO/PD98059-treated cells in the presence of vehicle ($p<0.01$) or 4OHTAM ($p<0.001$). (Representatives of three independent experiments)

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Figure 9. Proposed model

4OHTAM induces NO and O₂⁻. NO is upregulated by O₂⁻-mediated NOS gene expression and negatively feedback to suppress O₂⁻. O₂⁻ initiates autophagy by inducing LC3 lipidation. NO regulates unspecified signaling pathways that converge with the lipidated LC3 to control formation of autophagic vesicles. O₂⁻ also activates ERK that plays a critical role in lysosome stabilization. Completion of the pro-survival autophagy requires formation of competent autophagic vesicles that fuse with lysosomes that are inhibited by chloroquine (Chlor). Depletion of NO increases O₂⁻ that subsequently activates ERK as a compensatory mechanism to stabilize lysosomes for cell survival. Concurrent inhibition of NO and ERK results in destruction of the autophagic process leading to cell death.

Table 1

Genes	Forward Sequence	Reverse Sequence
HO-1	GTCTTCGCCCTGTCTACTTC	CTGGGCAATCTTTTGAGCAC
iNOS	TCTGTTCAAGACCAAATCCACC	CGGGGACTCATCTGCTGC
eNOS	TGGTACATGAGCACTGAGATCG	CCACGTTGAGGGCCACTGCTG
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT