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Induction of autophagy under nitrosative stress: A complex regulatory interplay between SIRT1 and AMPK in MCF7 cells

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ABSTRACT

Induction of nitrosative stress has been observed in various cancer types and in tumor environment. However, it is still unclear how cancer cells combat the effect of nitrosative stress. The main targets of nitrosative stress in cells are cellular lipids, proteins and DNA. Autophagy or self-cleaning generates energy for cell survival under stress conditions. In the present study we investigated the role of autophagy under nitrosative stress in MCF7, a breast cancer cell line. Interestingly, we observed induction of autophagy associated with cell death when MCF7 cells were treated with NO donor compound DETA-NONOate for eight hours. While investigating the mode of cell death under nitrosative stress in MCF7 cells, it was found that it was neither apoptotic nor necrotic. Moreover, nitrosative stress did not alter mitochondrial membrane potential and cellular redox status in MCF7 cells. But we observed an increase in NAD^+/NADH and a drop in NADH level in MCF7 cells following NO donor treatment. Sirtuins having NAD^+ dependent deacetylase activity, play an important role in cell survival mechanisms. So we further checked the status of SIRT1 under nitrosative stress in MCF7 cells. Surprisingly, we observed an induction of SIRT1, phospho-AMPK and p53 in MCF7 cells under nitrosative stress. Interestingly, autophagy markers were down regulated in MCF7 cells upon treatment with nicotinamide, an inhibitor of SIRT1 activity and dorsomorphin, a phospho-AMPK inhibitor when treated separately under nitrosative stress. To further confirm the role of SIRT1 in the induction of autophagy associated cell death, it was knocked down using si-RNA and nitrosative stress was applied. SIRT1 knock down led to increase in MCF7 cell viability along with down regulation of autophagic markers and phospho-AMPK as well as accumulation of acetylated p53. The increase in p53 controlled DRAM1 mRNA expression in MCF7 cells under nitrosative stress further confirmed a complex interplay between p53, SIRT1 and AMPK under nitrosative stress in MCF7 cells. Altogether our work for the first time suggests a complex inter-twined partnership between AMPK, SIRT1 and p53 in regulating autophagy in response to nitrosative stress in MCF7 cells.

1. Introduction

Nitric oxide is a short lived free radical that easily diffuses in tissues. There are three isoforms of nitric oxide synthases (i-NOS, e-NOS and n-NOS) that produce NO in cells under normal physiological condition by the conversion of L-Arginine to L- Citrulline [1]. NO functions in cell in a concentration and environment dependent manner. It functions as a signaling molecule at low concentrations whereas excess or deregulated production of NO causes stress which is termed as nitrosative stress [2,3]. It has been well documented that NO produces various reactive nitrogen species (RNS), e.g., nitrosonium ion NO^+, dinitrogen trioxide (N_2O_3), peroxynitrite ($-OONO$) due to its very short half-life [4]. The major targets of RNS are cellular DNA, protein and lipids [5–7]. All these RNS can exert their effect as potential nitrating, nitrosating and

Abbreviations: NO, nitric oxide; RNS, reactive nitrogen species; GSH, reduced glutathione; GSSG, oxidised glutathione; NAD^+, oxidised nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide; AMPK, 5'-AMP-activated protein kinase; SIRT1, silent mating type information regulation 2 homolog 1; DRAM1, DNA damage regulated autophagy modulator 1; NOS, nitric oxide synthase; i-NOS, inducible NOS; e-NOS, endothelial NOS; n-NOS, neuronal NOS; ATM, ataxia telangiectasia-mutated protein kinase; LKB1, live kinase B1; TSC2, Tuberous Sclerosis Complex 2; HIF-α, Hypoxia-inducible factor 1-alpha; DMEM, Dulbecco's modification of Eagle medium; ERK, extracellular signal-regulated kinases; CAMKK, Calcium/calmodulin-dependent protein kinase kinase; AMPKK, 5'-AMP-activated protein kinase kinase; FOXO, Forkhead box protein O1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Atg5, Autophagy related 5; LC3, Microtubule-associated proteins 1A/1B; p62, Sequestosome-1; MDC, monodansylcadaverine; 3-MA, 3-Methyladenine; PI3-K, Phosphoinositide 3-kinases; CR, Calorie restriction; si-RNA, small interfering RNA.

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oxidizing agents [8]. Controversial role of NO and RNS in both autophagy and apoptosis has been reported in different cell lines [9–13]. However, it is still unclear how cells combat nitrosative stress under pathophysiological conditions where NO induced pro death or pro survival mechanism(s) are operational.

Breast cancer is the most common cancer in women both in the developed and less developed world. MCF 7 or Michigan Cancer Foundation-7 cell line is a widely studied epithelial cancer cell line derived from breast adenocarcinoma, has characteristics of differentiated mammary epithelium. In previous reports it has been documented that treating breast cancer MCF7 cell line with low doses of NO (< 300 nM) induced proliferative events such as ERK phosphorylation and HIF-α accumulation [3], whereas p53 accumulation occurred at high doses of NO (> 300 nM) treatment. Cancer cells maintain a relatively elevated level of endogenous NO as it helps in tumorigenesis and also prevents apoptosis [14,15]. On the other hand, when high concentrations of NO crossed a threshold level, it leads to cytostatic or cytotoxic effect [16]. Thus the previously mentioned strategy of maintaining elevated levels of NO by cancer cells can be harmful and that can induce cell death on further influx of NO. Inhibition of ribonucleotide reductase, halting DNA synthesis in S-phase in response to nitrosative stress has previously been reported [17]. DNA damage in response to genotoxic agents can also activate p53 inducing apoptotic [18,19] or autophagic [20,21] response. Autophagy, an important regulator of cell physiology is a catabolic process for the degradation and recycling of macro-molecules and organelles. This intracellular degradation process is initiated by the formation of double-membraned vesicles called autophagosome, which transports cytoplasmic cargo to the lysosome for degradation. The formation of autophagosome is a three step process characterized by nucleation, elongation and completion of the isolation membrane or phagophore [22]. Any kind of stress which leads to metabolic emergency can be counteracted by cell through breaking down and recycling the impaired, long-lived or damaged cellular organelles by autophagy to maintain cellular homeostasis. But heightened or prolonged induction of autophagy can result in programmed cell death type II or cell death associated with autophagy [23]. Under energy restricted condition, drop in ATP/AMP ratio activates AMPK by phosphorylation which in turn inhibits mTORC1 thereby activating autophagy [24]. AMPK activation has been reported to be regulated by various proteins [25]. Apart from its upstream kinase activators like LKB1, CAMKKβ, AMPKK; SIRT1, an NAD-dependent histone de-acetylase known to regulate several transcription factors such as FOXO, tumor suppressor p53 and nuclear factor-kappa B (NF-κB) [26], has also been linked in regulating AMPK activation by de-acetylating LKB1 [27].

In recent years, NO has emerged as a significant regulator of autophagy in cancer cells. Both inhibitory [12] and promotional [11] role of nitric oxide on autophagy in cancer have been well documented. These contrasting reports arise due to the fact that biological effect of nitric oxide differs with cell lines, actual concentration of nitric oxide exposure and generation of reactive nitrogen species hence formed which causes difference in the cellular outcome in cancer cells [13].

In the present study, we investigated the nitrosative stress response mechanisms in MCF7 cells using NO donor compound DETA-NONOate. We demonstrated nitrosative stress induced autophagy with decreased cell survival. In this report, we first time provide a strong evidence that NO induces autophagy in a SIRT1-AMPK-p53 dependent manner in MCF7 cell line leading to loss of cell viability.

2. Materials and methods

2.1. Reagents and antibodies

DETA-NONOate, SIRT1 Activity assay kit, NAD/NADH cell based assay kit were purchased from Cayman chemicals (Ann Arbor, MI, USA). Etoposide, Cell Counting Kit-8, Annexin V-FITC Apoptosis Detection Kit, Carbonyl cyanide *p*-chlorophenylhydrazone (CCCP), monodansyl cadaverin (MDC), 3-methyl adenine, dorsomorphin, nicotinamide (NAM) and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA) while 5,5′,6,6′-Tetrachloro-1,1′,3,3′tetraethylbenzimidazoylcarbocyanine iodide (JC1), TRIzol was from Invitrogen/Molecular Probes (Carlsbad, CA, USA).

Primary antibodies to microtubule-associated protein 1 light chain 3 (LC3), Atg5, SIRT1, AMPK, p-AMPK, p53, acetylated p53 were from Cell Signaling Technology, Inc. (Danvers, MA, USA); PARP from Santa Cruz Biotechnology (Dallas, TX, USA) and β-actin from Abcam (Cambridge, UK) were used for Western blotting. The secondary antibodies, HRP-conjugated anti-rabbit and anti-mouse IgG (Sigma-Aldrich) (St. Louis, MO, USA) were used for detection.

2.2. Cell culture and treatment

The human breast cancer cell line MCF 7 was maintained in DMEM supplemented with 10% (*v*/v) FBS and antibiotics. Cultures were grown in a 95% air, 5% CO_2 atmosphere in a humidified incubator at 37 °C. 1×10^6 cells plated in 90 mm culture plate containing 10 ml of culture media were allowed to grow till the confluency reached 70%–75%. For NO donor treatment, cells were treated with NO pro drugs i.e. DETA-NONOate, freshly prepared in PBS (pH 7.4) (200 mM stock solution) before each experiment for requisite time point and concentration and finally harvested for protein extraction. Decomposed DETA-NONOate was used in control experiments. Autophagy inhibitor, 3-Methyladenine at a concentration of 5 mM was added in culture media containing MCF 7 cells in some experiments, 1 h before the DETA-NONOate treatment. To inhibit 5´adenosine monophosphate-activated protein kinase (AMPK) activation, dorsomorphin, an AMPK inhibitor at a concentration of 5 μM was added in culture media containing MCF 7 cells in some experiments, 2 h before the DETA-NONOate treatment. Nicotinamide was co-treated with DETA-NONOate to inhibit de-acetylation activity of SIRT1 at a concentration of 15 mM in some experiments.

2.3. Cell viability analysis

Cell viability was analyzed by WST-8 following the manufacturers' protocol (Sigma-Aldrich). Briefly, 2500 cells were seeded in 96 well-plates and kept for 24 h. The cells were then treated with DETA-NONOate at different concentrations. Just 2 h before the end point of incubation with DETA-NONOate, 10 μl WST-8 was added to each well and incubated for the remaining 2 h at 37 °C. The absorbance was measured using microplate reader (Molecular Device, Versa max) at 450 nm. Absorbance measured correlated directly with the number of metabolically active cells in culture and cell survival was expressed as a percentage of control viability.

2.4. Detection of apoptosis

For determination of rate of apoptosis, cells were cultured and given necessary treatment for required duration. Apoptosis was assessed by annexin-V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) apoptosis kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cells were collected, washed with PBS, resuspended in binding buffer provided, and stained with Annexin V (5 μl) and PI (10 μl), and fluorescence was measured on a FACS-Verse (Becton Dickinson).

2.5. Cell lysis and protein isolation

Cells were harvested to prepare cell lysates at various time intervals. In short, Cells were harvested and washed twice with ice cold phosphate buffer saline (PBS). Cell pellets were scraped in a RIPA buffer (NaCl-0.88 g, Tris buffer-0.605 g, Triton-X100–100 μl, SDS-0.1% in 100 ml) supplemented with protease inhibitor cocktail and 20 mM

sodium fluoride (Inhibitor of phosphatase) and were lysed by continuous up and down movement of pipette passing the cells through tip for 300 times placing the tubes on ice. Lysed cells were centrifuged twice at 10000 rpm, for 10 min at 4 °C. Protein concentrations were measured using Bradford assay.

2.6. Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as mentioned previously [13]. Fifty micrograms of proteins were separated in 10% or 15% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (250 mA, 1 h) using wet transfer apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA). Blocking of the PVDF membranes were done for 2 h at room temperature using blocking buffer (0.019 M Tris, 0.136 M NaCl, 0.1% *v*/v Tween 20 and 5% *w/v* nonfat dry milk). Respective primary antibodies at 1:2000 dilutions in tris-buffered saline with Tween 20 (TBST) (0.019 M Tris, 0.136 M NaCl, 0.1% v/v Tween 20) were used to probe the membranes overnight at 4 °C. The primary antibody probed membranes were washed three times in TBST for 10 min for each wash, and then reprobed with HRP-conjugated antimouse or antirabbit IgG antibody at 1:5000 dilutions for 1 h at room temperature. The membranes were then finally washed for six times in TBST and six times TBS (0.019 M Tris, 0.136 M NaCl) respectively (10 min for each wash). The immunopositive spots were visualized by using chemiluminescent reagent (Thermo Scientific Pierce, Rockford, IL, USA) as directed by manufacturer.

2.7. MDC staining

To detect the formation of acidic vacuoles during the process of autophagy monodansylcadaverin staining was performed. MCF 7 cells were counted and 20,000 cells were seeded in coverslips pretreated with poly-L-lysine. Cells were allowed to recover for 36 h after seeding. Coverslips containing control cells and cells treated with different concentrations of DETA-NONOate were harvested 8 h post treatment and washed twice with PBS and then stained with MDC (10 μM final concentration) for 15 min at 37 °C in dark. Coverslips were then washed twice with PBS in dark to remove excess stain and observed with confocal microscope (Olympus model IX 81) after placing the coverslips in glass slides. Light intensity and exposure time were constant for the different treatments and the modalities for MDC fluorescence (excitation 338 nm and emission 500 nm).

2.8. Mitochondrial membrane potential analysis

JC1, a lipophilic cationic fluorescent dye was used to measure the variation of mitochondrial membrane potential. MCF 7 cells were incubated in media containing 15 mM JC1 for 15 min at 37 °C. Cells were then washed twice with PBS and resuspended finally in 400 μl of PBS for flow cytometer analysis. The uncoupling agent carbonyl cyanide p-chloro phenylhydrazone (CCCP) at 50 μM concentration was used as a positive control. The green and red signals were detected at 530 nm (FL1 channel) and 575 nm (FL2 channel) for JC1 monomer and aggregates, respectively. Ten thousand cells for each sample were acquired and then analyzed using BD FACSuite software. Fluctuations in the ratio of red to green fluorescence indicated variations in $\Delta\Psi m$, which is dependent only on the membrane potential. The decrease in the ratio indicated mitochondrial depolarization.

2.9. Total glutathione and GSSG content

Total glutathione and oxidized glutathione were measured according to the method of Akerboom and Sies [28]. Equal volume of Solution-I (2 M $HClO_4$ containing 2 mM EDTA) was added to the crude cell free extract very slowly by tapping and incubated on ice for 20 min. It was centrifuged at 5000 *g* for 5 min at 4 °C. The supernatant was neutralized with Solution II (2 M KOH containing 0.3 M HEPES). After centrifugation at 5000 *g* for 5 min, the supernatant was used for estimation of total glutathione (glutathione (GSH + 2GSSG) by measuring glutathione reductase (GR)-dependent DTNB reduction spectrophotometrically at 412 nm. The same neutralized extract was incubated with 2-vinylpyridine (50:1, *v*/v) for 1 h at room temperature and then used for GSSG estimation using the above method.

2.10. Glutathione reductase (GR) and catalase activity measurement

Glutathione reductase activity was measured in the cell free extract of MCF 7 cells by measuring the decrease in absorbance at 340 nm due to utilization of NADPH. In this, NADPH oxidation was followed spectrophotometrically at 340 nm over 2 mins. The reaction mixture contained 1 mM EDTA, 1 mM GSSG, 160 μM NADPH (all final concentrations) apart from protein samples and 0.1 M potassium phosphate buffer (pH 7.5) in a final volume of 500 μl. Specific activity was calculated using extinction coefficient value of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ and the results were expressed in n mole $minute^{-1}\ mg^{-1}$ protein.

For determination of specific activity of catalase in MCF 7 cell free extract, degradation of hydrogen peroxide was followed spectrophotometrically at 240 nm. The reaction mixture contained 1 mM EDTA, H_2O_2 apart from protein samples and 0.1 M potassium phosphate buffer pH 7.5 in a final volume of 500 μl. Specific activity was calculated using extinction coefficient value of $43.6\ M^{-1}\ cm^{-1}$ and the results were expressed in n mole $minute^{-1}\ mg^{-1}$ protein.

2.11. Nicotinamide adenine dinucleotide assay

NAD assay was performed according to manufacturer's protocol; briefly 5000 cells were seeded in each well of 96 well plate and allowed to recover for 24 h. Cells were treated with different concentrations of DETA-NONOate for required time then the culture media was removed and cells were washed with assay buffer provided. Permeabilization buffer was added to the wells and incubated for 30 min in room temp in a shaker. 100 μl of buffer was aspirated followed by centrifugation and mixed with reaction buffer containing WST-1, Ethanol, alcohol dehydrogenase and diaphorase to determine total NAD and reaction buffer without ethanol and alcohol dehydrogenase was used for determination of NADH. Absorbance was measured at 450 nm after 2 h of incubation in gentle shaking condition.

2.12. SIRT1 activity assay

SIRT1 activity assay was done according the manufacturer's protocol; briefly 50 μg cell free extract was added in assay buffer provided. Assay was initiated by adding 125 μM of substrate solution (containing acetylated p53 sequence tagged with AMC) and 3 mM NAD^+. After incubating the mixture for 45 min in shaking condition at room temperature in dark, stop/ developer solution containing nicotinamide was added and kept in dark for 30 min at room temperature. The fluorescence was monitored using a fluorimeter (Excitation wavelength 355 nm and emission wavelength 345 nm).

2.13. SIRT 1 knock down by small interfering RNAs

SIRT1 knock down was performed according to manufacturer's protocol; briefly 4×10^5 cells were seeded in 60 mm plate the day before si-RNA treatment. On the day of transfection 20 nM was mixed with protocol mentioned amount of HiPerFect and serum free media to make a final volume of 100 μl transfection reagent mixture and incubated for 15 mins to form transfection complexes. Then the 100 μl solution was added dropwise to the cells and kept in incubator and the gene silencing was monitored.

2.14. Semi quantitative PCR

Total RNA from the control and treated cells was isolated by using TRIzol reagent (Invitrogen, CA, USA) according to manufacturer's protocol and was treated with DNase I (Thermo Scientific) as per manufacturer's protocol. cDNA was synthesized from 1 μg total RNA in 20 μl reaction volume using RevertAid reverse transcriptase (Thermos scientific) following manufacturer's protocol. Two microliters of cDNA products were used for PCR using Taq DNA polymerase (Thermo Scientific) in 10 μl total volume with gene-specific primers. PCR reactions were optimized to 95 °C for 5 min followed by 40 amplification cycles, which were programmed as follows: denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension of 10 min at 72 °C. Amplified products were resolved on 1% agarose gels and visualized by ethidium bromide staining. Primers for DRAM 1 were CTTGGATTGGTGGGATGT (forward), CCACTGGGG ACATGATTT (reverse) and for β-actin were ACGAGTCCGGCCCCTCC ATC (forward), TGGGGGATGCTCGCTCCAAC (reverse). Relative expression of target gene was compared after electrophoresis in agarose gel followed by ethidium bromide staining and expressions were analyzed with ImageJ software.

2.15. Densitometric analysis

In case of Western blot analysis fold change of protein expression was calculated compared to control set following normalization with respect to the housekeeping gene expression. In case of inhibitors and DETA-NONOate co-treatment, fold change of protein expressions was calculated with the corresponding control plus inhibitor set. In case of only DETANONOate treatment, fold change of protein expression was calculated with the corresponding control only.

2.16. Statistical analysis

The experimental results were expressed as mean ± SE of three independent experiments. Statistical evaluation was performed with ANOVA test followed by one-tailed paired Student's *t*-test using the GraphPad Prism six software; p value < .05 was considered as statistically significant like $*p < .05$, $** p < .01$, $***p < .001$.

3. Results

3.1. Exposure to nitric oxide caused decrease in MCF 7 cell survival

In DMEM media 1 mM DETA-NONOate releases 0.5 μM NO [29]. In an attempt to investigate the nitrosative stress response mechanisms in MCF 7 cells, we studied the effect of graded concentrations of DETA-NONOate treatment for 8 h to induce nitrosative stress. Exposure to DETA-NONOate for 8 h resulted in gradual decrease in cell viability as indicated by WST-8 cell viability assay. Percentage of cell survival was gradually decreased with increasing concentrations of DETA-NONOate, ranging from 86 to 24% compared to 100% in control cells where decomposed DETA-NONOate was used under similar experimental conditions [Fig. 1].

3.2. Exposure to Nitric oxide induced autophagy but not apoptosis in MCF7cells

To investigate the cause for the decrease in cell viability under nitrosative stress, we performed apoptosis-necrosis cell death assay using flow-cytometry with Annexin-V tagged with FITC and propidium iodide (PI). In control set, the total percentage of early and late apoptotic cells was 14.38% in the 10,000 events recorded in flow-cytometry analysis. Upon 100, 250 and 500 μM DETA-NONOate treatment the total apoptotic population was found to be ~11%, ~7% and ~9% respectively indicating mode of cell death was not associated with apoptosis as

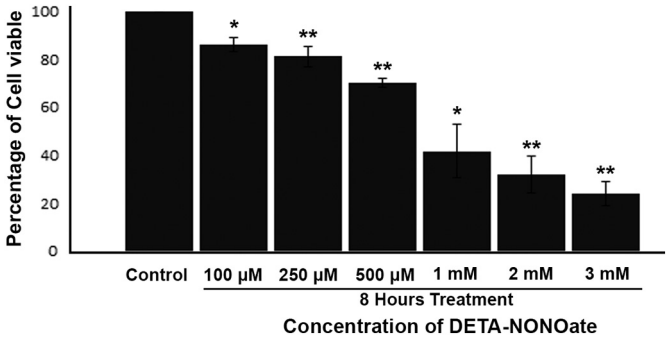

Fig. 1. Nitric oxide exposure for 8 h led to decrease in cell viability of MCF7 cells. Cell viability was estimated by WST-8 assay following treatment with graded DETA-NONOate concentrations of 100 μM, 250 μM, 500 μM,1 mM, 2 mM and 3 mM for 8 h which are presented in X axis while Y axis denotes the percentage of viable cells present. Results are expressed as mean ± SE, for $n = 3$ experiments. $*p < .05$, $** p < .01$.

represented in Fig. 2A. As a positive control, MCF7 cells were treated with 1 mM hydrogen peroxide (H_2O_2) for 3 h under similar experimental conditions where ~77% total apoptotic population was recorded. To further validate the result, we also performed immunoblot analysis with anti-PARP antibody as shown in the Fig. 2B. Absence of PARP cleavage at 89 kD implicated that there was no induction of apoptotic response supporting our Annexin V – PI staining data.

To further explore the other modes of cell death that may lead to this loss of cell viability, we performed confocal microscopic analysis with fluorescent stain monodansylcadaverine (MDC) to detect the presence of acidic vacuole resulting due to DETA-NONOate treatment in MCF7 cells. As shown in the Fig. 3A, a marked increase in the puncta formation was detected in MCF 7 cells upon 100 μM and 500 μM of post 8 h DETA-NONOate treatment indicating increase in the acidic vacuole formation. Control cells showed a basal level of fluorescence puncta due to basic cellular functions.

Increased acidic vacuole formation has been considered as an indicator of autophagy. Thus we further supported our microscopic finding with immunoblot analysis by studying the expression profile of biomarker proteins of autophagy, e.g., Atg5, LC-3b and p62. Control MCF 7 cells showed a basal level of Atg5, LC-3b and p62 expression which indicated a regular and normal cellular degradation process that eliminates cellular waste material. Interestingly, upon treatment with 100 μM and 500 μM DETA-NONOate we observed a gradual increase in Atg5 and LC-3b protein expression, a result of heightened autophagic response under nitrosative stress. Atg5 and LC-3b showed its maximum expression at 8 h followed by NO donor treatment [Fig. 3B]. p62 protein is a classical receptor of autophagic cargo that is degraded by autophagy itself [30]. p62 protein expression was gradually decreased upon DETA-NONOate treatment in MCF 7 cells with increasing time which strongly advocated for ongoing autophagy in MCF7 cells under nitrosative stress [Fig. 3B].

3.3. Inhibiting autophagy with 3-Methyladenine led to induction of apoptosis

Since our immunoblot analysis and confocal microscopy with MDC pointed towards augmentation of autophagy by up regulation of autophagic marker proteins and increase in acidic vacuole formation, we investigated the effect of inhibition of autophagy in MCF 7 cells under nitrosative stress. We used 3-Methyladenine (3-MA) as an inhibitor of autophagy, which selectively blocks of PI3-K, known to be essential for autophagy [31]. Control as well as 100 μM and 500 μM DETA-NONOate treated MCF 7 cells were pre-incubated with 5 mM 3-MA. 3-MA treatment resulted in decreased expression of autophagy marker protein Atg 5 and increase in p62 protein, confirming inhibition of autophagic

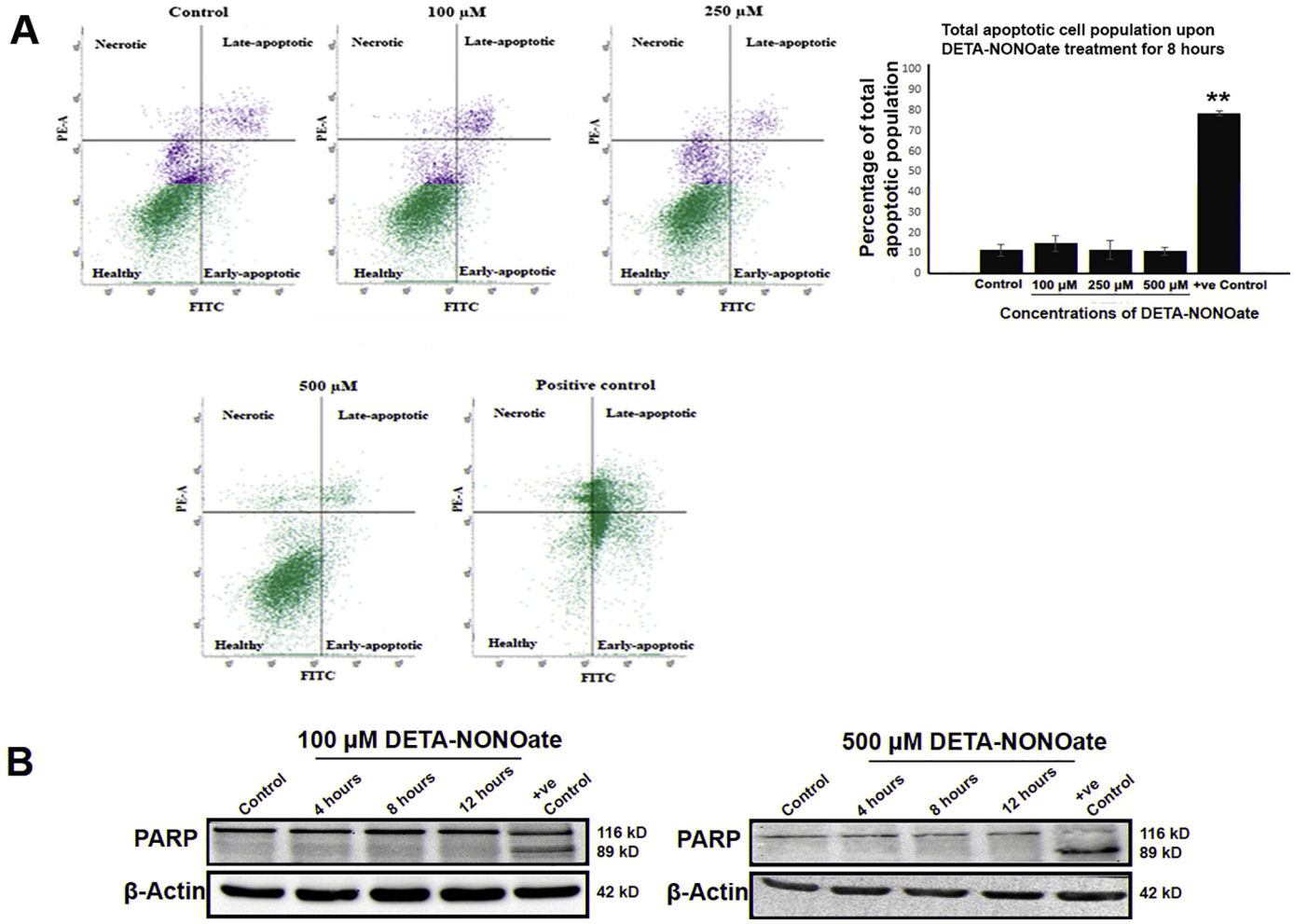

Fig. 2. Nitrosative stress didn't induce apoptosis or necrosis in MCF7 cells. A. MCF 7 cells were treated with 100 µM, 250 µM and 500 µM DETA-NONOate for 8 h. Cells were stained with annexin-V-FITC and propidium iodide. Populations of apoptotic or necrotic cells were estimated by Flow cytometry analysis. There was no significant increase in apoptotic / necrotic cell population compared to untreated cells. Hydrogen peroxide at a concentration of 1 mM was used as positive control. The data are represented as mean ± SE (n = 3). B. Western blot analysis with anti PARP antibody following treatment with 100 µM and 500 µM DETA-NONOate. Anti β-Actin antibody was used as loading control. Results are expressed as mean ± SE, for n = 3 experiments.

response [Fig. 4]. Interestingly, immunoblot analysis with anti-PARP antibody as presented in Fig. 4 showed distinct cleavage of PARP in 3-MA pre-treated samples suggesting activation of apoptosis upon autophagic inhibition.

3.4. Mitochondrial membrane potential remained unaltered in MCF 7 cells upon nitric oxide exposure

Mitochondrial membrane potential indicates health of mitochondria in a cell. Loss of mitochondrial membrane potential has been directly linked to apoptosis and unwanted loss of cell viability [32]. So we further checked the mitochondrial membrane potential of MCF 7 cells under nitrosative stress with potential sensitive fluorescent probe JC-1, which resulted in no significant increase in depolarized mitochondrial population. The percentage of the healthy polarized mitochondria remained unaltered upon graded concentration of DETA-NONOate treatment compared to control as shown in the Fig. 5. Thus absence of apoptotic MCF7 cell population in flow cytometry analysis under nitrosative stress can be correlated well with the data showing healthy mitochondrial population and unchanged mitochondrial membrane potential.

3.5. Cellular redox status remained unchanged in MCF7 cells upon nitric oxide exposure

Glutathione, an abundant low molecular weight thiol acts as a primary line of defense in protecting cells against any redox insult. Under oxidative stress condition, reduced form of glutathione (GSH) is converted to oxidized form of glutathione (GSSG), which is rapidly reduced back to GSH enzymatically by means of NADPH-dependent glutathione reductase (GR). To study the effect of nitrosative stress upon redox status of MCF 7 cells, we measured the levels of total glutathione (GSH + GSSG), oxidized (GSSG) and reduced (GSH) forms of glutathione and calculated the ratio of reduced to oxidized glutathione (GSH/GSSG) as a marker of cellular redox condition. Upon treatment with 100 µM and 500 µM DETA-NONOate for 8 h, ~14% and ~27% cell death were observed in MCF 7 cells respectively with a little increase in total thiol and reduced form of glutathione [Table 1]. We did not observe any change in GSSG content under nitrosative stress in MCF 7 cells. As a result, GSH/GSSG ratio did not change significantly upon nitrosative stress in MCF 7 cells upon 100 µM and 500 µM of DETA-NONOate treatment for 8 h. To investigate the reason behind the unchanged or little increase in GSH / GSSG ratio, we measured GR activity following the above mentioned treatments. It is important to note that GR activity was increased significantly (~27.8%) and catalase activity was also increased by ~14% in MCF7 cells upon 500 µM DETA-

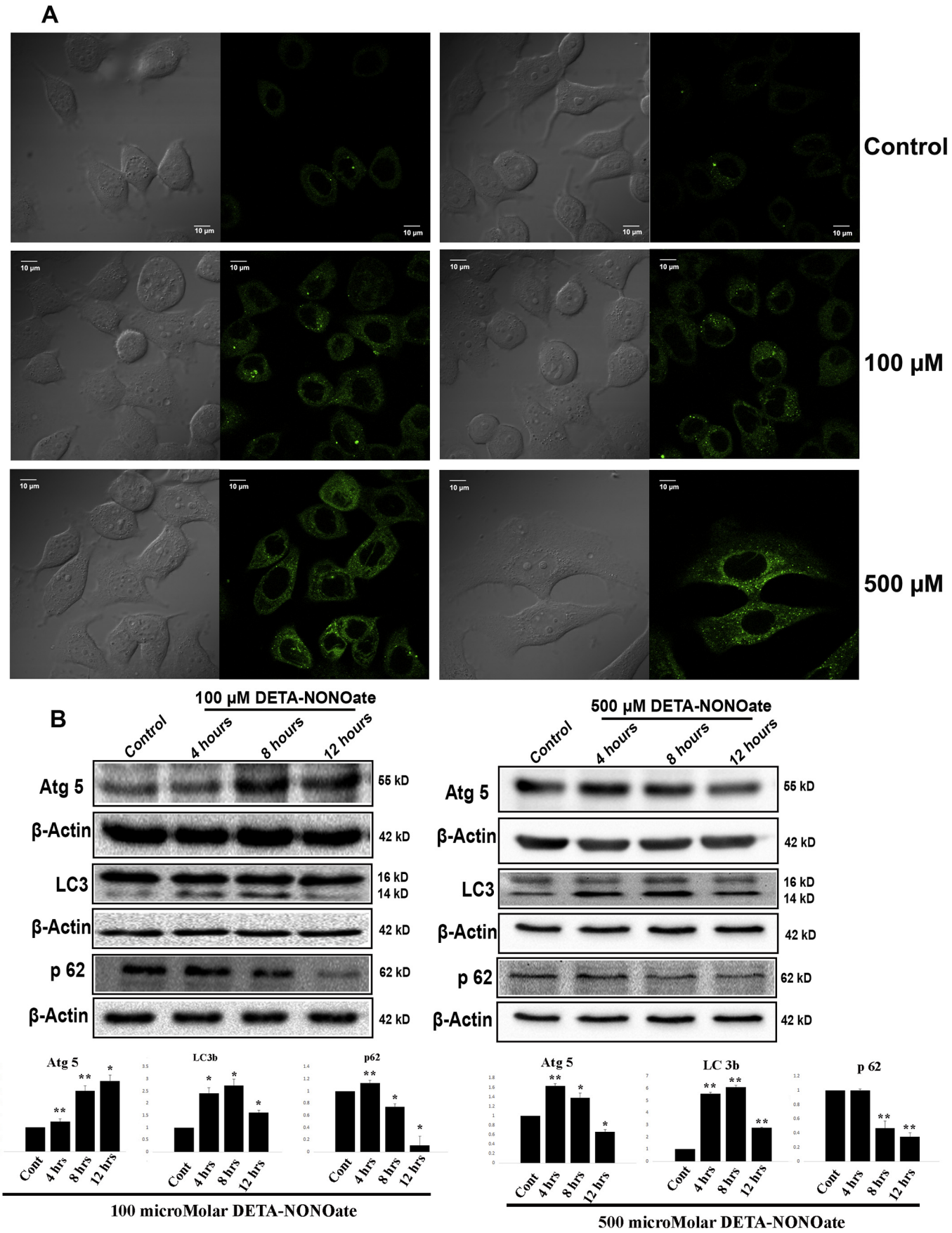

Fig. 3. Nitrosative stress induced autophagy in MCF7 cells. MCF7 cells were treated 100 μM and 500 μM DETA-NONOate for 8 h compared to control A. Confocal microscopy showed an increased autophagic vacuole formation when cells were stained with monodansyl cadaverine (MDC). Corresponding bright field images were also given. B. MCF 7 cells were exposed to 100 μM and 500 μM DETA-NONOate for 4, 8 and 12 h. Cells were lysed and whole cell protein extract were prepared. Western blot analyses were performed using anti Atg5, anti LC3, anti p62 antibodies. Anti β-Actin antibody was used as loading control.

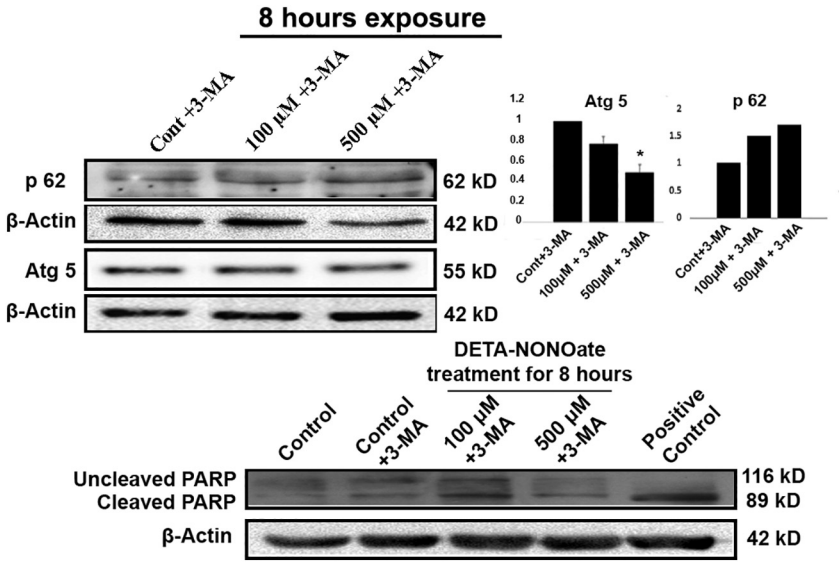

Fig. 4. Inhibition of autophagy by 3-methyl adenine resulted in apoptosis induction.
MCF7 cells were pre-treated with 5 µM 3-MA before 100 and 500 µM DETA-NONOate treatment for 8 h. Western blot analysis showed a decrease in the Atg 5 protein expression, increase in expression of p62 protein upon 3-MA treatment. However, analysis with anti-PARP antibody revealed cleavage of PARP in the 3-MA pre-treated cells. Western blot fold changes are expressed as mean ± SE, for $n = 3$ experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

NONOate treatment for 8 h compared to control cells [Fig. 6].

3.6. Exposure to NO led to increase in NAD^+/NADH ratio in MCF7 cells

Next we evaluated the metabolic state of the MCF 7 cells upon DETA-NONOate treatment to understand cell death decision. Nicotinamide adenine di-nucleotide (NAD) in recent years has emerged as an indicator of cell metabolism and cellular energy state. NAD can either be in its oxidized state (NAD^+) or its reduced form (NADH) depending upon cellular metabolism. Being a vital cofactor in many enzymatic reaction, it can rewire metabolism [33], activate diverse survival fate deciding proteins [34] and change in NAD^+ or NAD^+ / NADH ratio can become crucial in determining cellular health [35]. The amount of total NAD dropped slightly upon DETA-NONOate treatment as shown in Table 2. A significant drop in reduced form (NADH) was observed in MCF 7 cells upon 100 µM and 500 µM DETA-NONOate treatment ranging from ~49% to ~72% decrease compared to the control. Similar condition may arise under caloric restricted condition. There was a significant increase in NAD^+ upon DETA-NONOate treatment in MCF 7 cells. This depletion in NADH and increase in NAD^+ led to a ~2 fold increase in NAD^+/NADH ratio.

3.7. Exposure to NO led to induction of p-AMPK, SIRT1 and p53 protein expression in MCF7 cells

It has been shown that nutrient depletion, DNA damage, oxidative and nitrosative stress can induce autophagic response in cell [36]. Caloric restricted (CR) condition can activate liver kinase B1 (LKB1) which in turn phosphorylate and activate AMPK, a major metabolic sensor of cellular energy state. AMPK phosphorylation is previously reported by several studies as a major regulator of autophagy [24,37]. As we observed depletion of NADH in MCF 7 cells under nitrosative

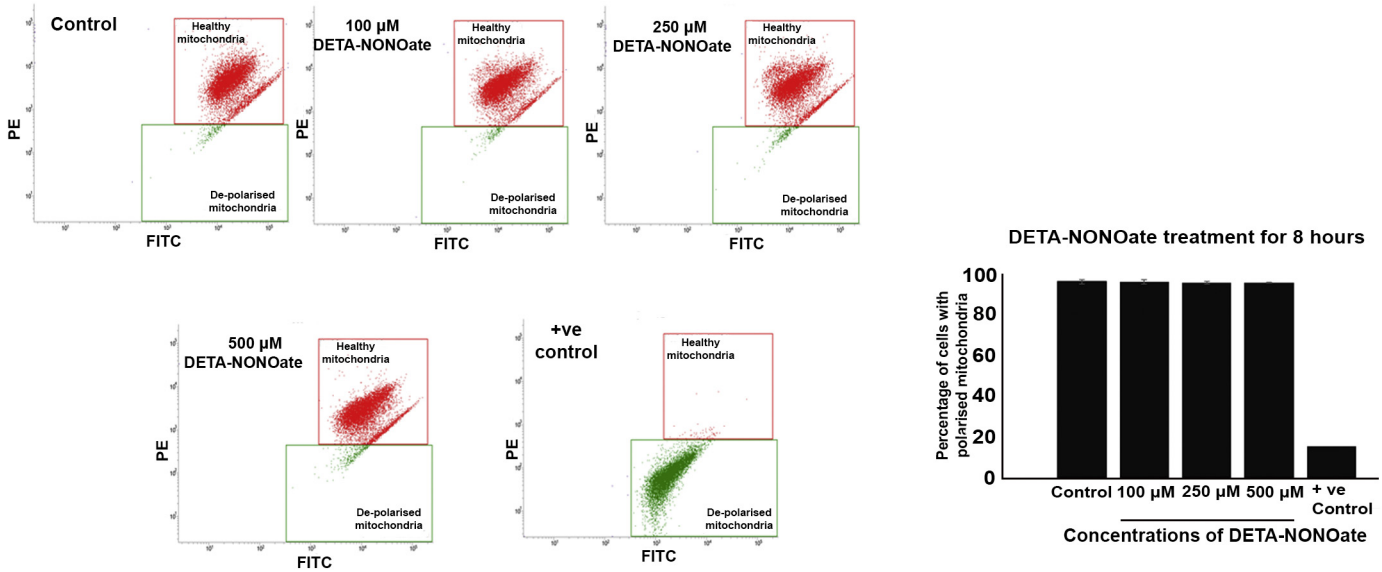

Fig. 5. Mitochondrial membrane potential remained unaltered upon nitrosative stress in MCF7 cells.
MCF 7 cells were treated with 100, 250 and 500 µM of DETA-NONOate for 8 h and potential sensitive JC1 fluorescent probe was used to estimate changes in mitochondrial membrane potential by flow cytometry. Un-coupler carbonyl cyanide p-chlorophenylhydrazone (CCCP) at a concentration of 50 µM concentration was used as positive control. Green fluorescent signal of JC1 monomer denotes cell population with depolarized mitochondria and red fluorescent signals JC1 oligomers depicting cell population containing healthy mitochondria. Results are expressed as mean ± SE, for $n = 3$ experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Estimation of cellular redox status of MCF7 cells under nitrosative stress for 8 h.

Samples	Total Glutathione (nmoles/mg protein)	GSSG (nmoles/mg protein)	GSH (nmoles/mg protein)	GSH/GSSG
Control	113.32 ± 1.87	2.52 ± 0.14	110.8 ± 1.75	44.17 ± 1.83
100 μM DETA-NONOate	119.73 ± 2.51	2.3 ± 0.07	117.44 ± 2.58	51.3 ± 2.65*
500 μM DETA-NONOate	129.38 ± 2.66	2.39 ± 0.042	126.99 ± 2.69	53.11 ± 1.82**

Results are expressed as mean ± SE, for $n = 3$ experiments. Results are expressed as mean ± SE, for $n = 3$ experiments.
* $p < .05$.
** $p < .01$.

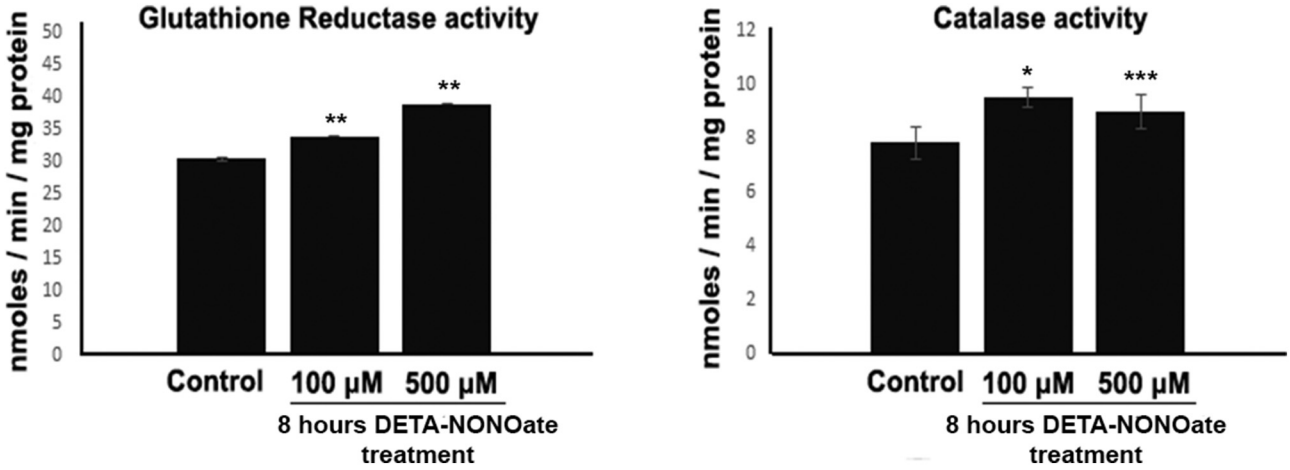

Fig. 6. Increase in Glutathione reductase (GR) and Catalase enzyme activity in MCF 7 cells in response to nitrosative stress. MCF7 cells were treated with 100 μM and 500 μM DETA-NONOate for 8 h. GR and catalase enzyme activity (represented in Y axis) were found to be upregulated compared to control cells. Results are expressed as mean ± SE, for $n = 3$ experiments. *$p < .05$, ** $p < .01$, ***$p < .001$.

stress which can create a condition similar to CR, we further checked the involvement of AMPK in this nitrosative stress induced autophagy in MCF 7 cells. Surprisingly, immunoblot analysis showed that there was an increase in the expression of phosphorylated form of AMPK (p-AMPK) followed by 100 and 500 μM DETA-NONOate treatment maximizing at 8 h and 4 h post treatment respectively [Fig. 7]. AMPK activation has previously been linked to another major regulator protein SIRT1 [38–40], a class III histone de-acetylase, capable of de-acetylating major cytoplasmic transcription factors like p53, NF-kB, FOXO, thus modifying gene expression and signaling cascade under their regulation. Moreover, NAD^+ acts as a cofactor of SIRT1 in its de-acetylating function and it has previously been reported that increase in NAD^+/NADH ratio activates SIRT1 [41–43]. As in our study we found significant increase in NAD^+/NADH ratio under nitrosative stress in MCF 7 cells, we proceeded further to investigate if in this autophagic induction, any part is being played by SIRT1. With the help of immunoblot analysis we found up-regulation in SIRT1 protein expression pattern in MCF 7 cells in response to 100 μM and 500 μM DETA-NONOate treatments maximizing at 8 h and 4 h respectively. We also checked another major master stress regulator protein p53, which was also found to be up-regulated in MCF 7 cells under nitrosative stress with increasing time point upon treatment.

3.8. Inhibition of AMPK activation caused down regulation of SIRT1 as well as autophagy marker proteins

To investigate whether induction of autophagy and increase in p-AMPK protein expression was mutually exclusive or had a causal correlation, we pre-treated the MCF 7 cells with dorsomorphin which is known to block the activation of AMPK. Pre-treating control and DETA-NONOate treated MCF7 cells with 5 μM dorsomorphin significantly reduced the p-AMPK protein level when compared with only DETA-NONOate treated cells showing inhibition of AMPK activation after 8 h of DETA-NONOate treatment. We checked the expression level of autophagy marker protein Atg 5 under similar experimental conditions and Atg5 protein expression was found to be increased upon NO donor treatment. However, the above-mentioned increased expression of Atg 5 was found to be abrogated by the dorsomorphin pre-treatment indicating importance of activation of p-AMPK in NO induced autophagy [Fig. 8]. Similarly, increased expression of SIRT1 protein under NO donor treatment was found to be decreased upon dorsomorphin pre-treatment indicating p-AMPK activation was crucial for SIRT1 activation. We also checked the protein expression of p62 under similar experimental conditions. p62 protein expression remained unaltered indicating inhibition of autophagy upon dorsomorphin treatment

Table 2
Estimation of cellular NAD pool of MCF7 cells under nitrosative stress for 8 h.

Samples	Total NAD (nM)	NADH (nM)	NAD^+ (nM)	NAD^+/NADH
Control	278.5 ± 8.95	137.65 ± 7.59	151 ± 8.19	1.01 ± 0.84
100 μM DETA-NONOate	253.8 ± 9.15	38 ± 1.5**	209 ± 2.42**	5.52 ± 0.26**
500 μM DETA-NONOate	236.9 ± 3.1	70.1 ± 3.6**	166.8 ± 0.58*	2.39 ± 0.13**

Results are expressed as mean ± SE, for $n = 3$ experiments. Results are expressed as mean ± SE, for $n = 3$ experiments.
* $p < .05$.
** $p < .01$.

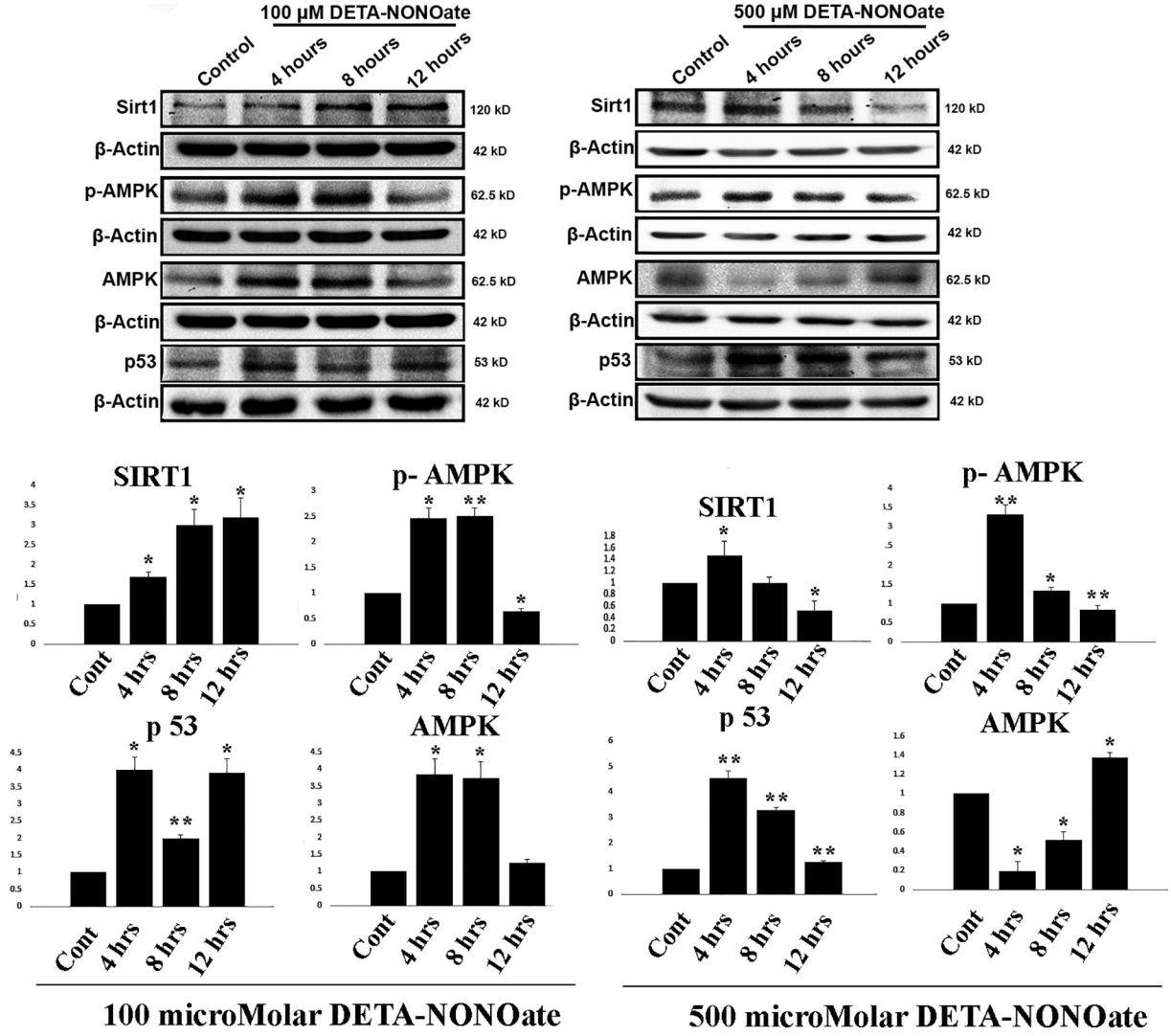

Fig. 7. Nitrosative stress induced three major stress responsive proteins. MCF7 cells were treated with 100 and 500 μM DETA-NONOate for different time points (4 h, 8 h and 12 h respectively) and cell lysates were prepared. Western blot analysis with anti-SIRT1, anti-p-AMPK and anti-p53 antibodies showed an increase in SIRT1, p-AMPK and p53 protein expression profile in response to nitrosative stress. Western blot fold changes are expressed as mean ± SE, for $n = 3$ experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

[Supplementary Fig. S1]. It has previously been documented that compound C or dorsomorphin greatly increases p53 accumulation inside cells which is independent of apoptosis [44], so we were more interested to see whether p53 acetylation profile changes upon dorsomorphin treatment in MCF cells or not. Interestingly, acetylated p53 protein expression was increased in MCF 7 cells pre-treated with dorsomorphin indicating AMPK activation affecting acetylation status of p53 upon DETA-NONOate treatment [Fig. 8].

3.9. Nicotinamide treatment resulted in down regulation of phospho AMPK and autophagy marker proteins

It has been reported that the de-acetylation activity carried out by SIRT1 is NAD^+ dependent and begins with nucleophilic attack of the carbonyl oxygen of acetyl lysine on the C 1′ of the nicotinamide ribose of NAD^+ resulting in release of nicotinamide, 3′-*O*-acetyl-ADPR and de-acetylated lysine. However, if nicotinamide binds to the enzyme in intermediate step in a process known as 'nicotinamide exchange' then it results in reformation of NAD^+ and acetyl-lysine. Thus nicotinamide (NAM) acts as naturally occurring feedback inhibitor of SIRT1. So we tested the effect of nicotinamide to inhibit de-acetylation activity of SIRT1. In our previous experiment, blocking of p-AMPK by dorsomorphin led to a decrease in SIRT1 and increase in acetylated p53 protein expression. So we further checked p-AMPK, p53 and Atg5 protein expression in nicotinamide and DETA-NONOate co-treated MCF7 cells. We also checked the protein expression of p62 under similar experimental conditions. p62 protein expression remained unaltered indicating inhibition of autophagy upon nicotinamide treatment [Supplementary Fig. S1].

As SIRT1 plays major regulatory roles in various important biological processes from life span extension to mitochondrial biogenesis to controlling gene expression by de-acetylating histones [38,45,46], complete inhibition of SIRT1 may affect several other pathways other than autophagy. Thus we used nicotinamide (NAM) at a concentration of 15 mM where ~20% inhibition of SIRT1's de-acetylase activity was recorded [Fig. 9A]. Co-treatment of MCF 7 cells with 15 mM NAM and DETA-NONOate resulted in [Fig. 9B] reduction in expression of autophagy marker protein Atg 5 when compared with expression level under similar concentrations of only DETA-NONOate. Phosphorylated form of AMPK protein level was also found to be decreased upon NAM treatment compared to only DETA-NONOate treatment, hinting towards the importance of SIRT1 de-acetylase activity in AMPK activation. Immunoblot analysis also indicated a significant amount of increase in acetylated p53 level in the MCF7 cells co-treated with 15 mM

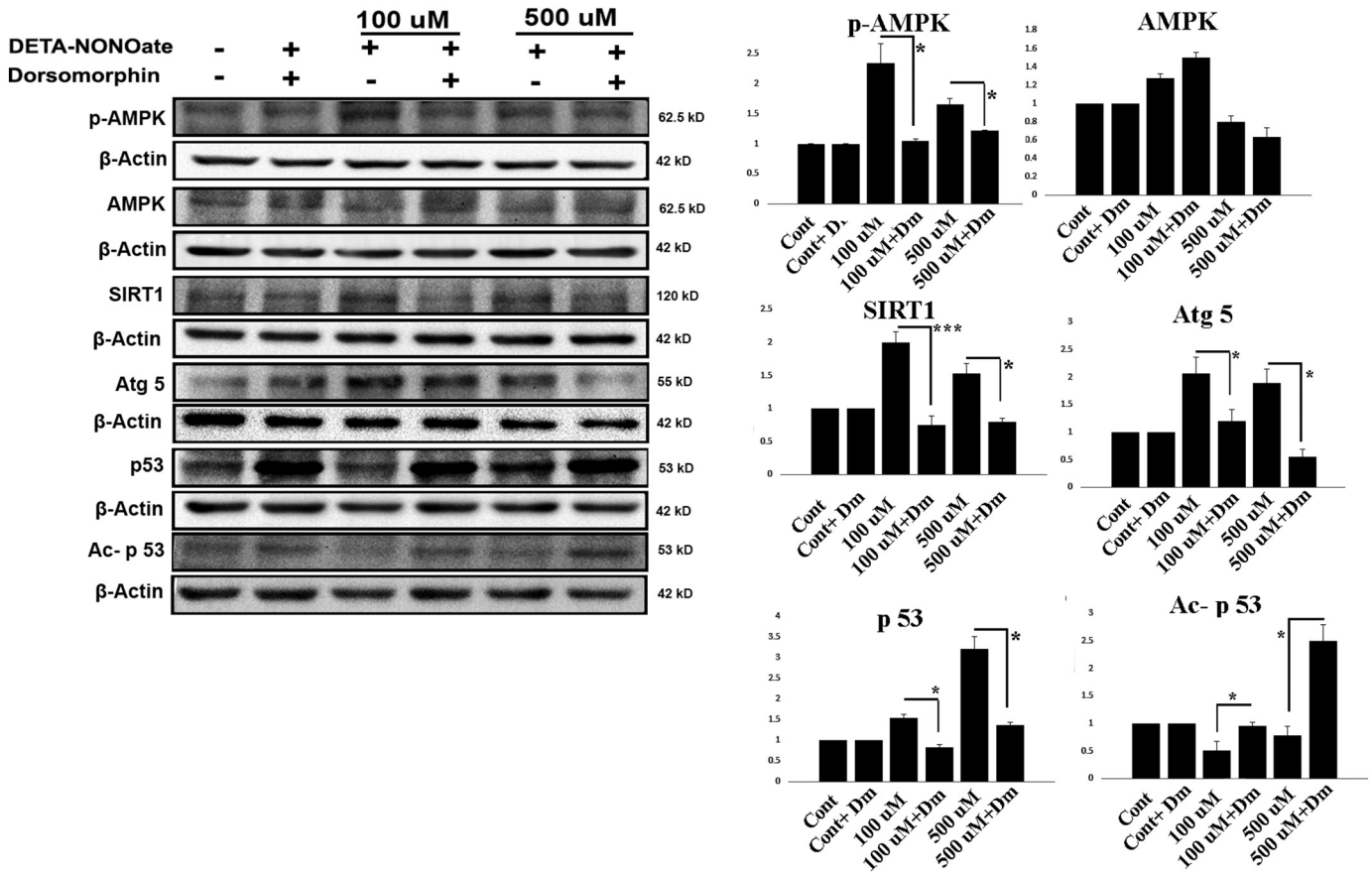

Fig. 8. AMPK inhibition led to decrease in autophagy. MCF7 cells were pre-treated with 5 μM dorsomorphin before 100 and 500 μM DETA-NONOate treatment for 8 h and cell lysates were prepared. Anti-p-AMPK, anti-AMPK. anti-SIRT1, anti-Atg5, anti-p53 and anti-acetylated p53 antibodies were used in Western blot analysis to study expression patterns of the proteins. Western blot fold changes are expressed as mean ± SE, for $n = 3$ experiments. *$p < .05$, ** $p < .01$, ***$p < .001$.

NAM and 500 μM DETA-NONOate. Thus 20% decrease in de-acetylation activity of SIRT1 could lower not only autophagy regulator p-AMPK and autophagy marker Atg 5 protein expression but also changed the acetylation state of p53 protein indicating towards a probable crucial role of SIRT1 in post-translational modification of p53 in NO induced autophagy in MCF 7 cells.

3.10. Knocking down SIRT 1 by si-RNA treatment down-regulated autophagic response and increased cell viability

To further validate the regulation of SIRT1 in controlling phosphorylation of AMPK and autophagy we knocked down SIRT1 with specific si-RNA. We checked the expression of SIRT1 following si-RNA treatment after 48 h and there was a significant decrease in SIRT1 expression (75%) in MCF 7 cells compared to the untransfected cells or

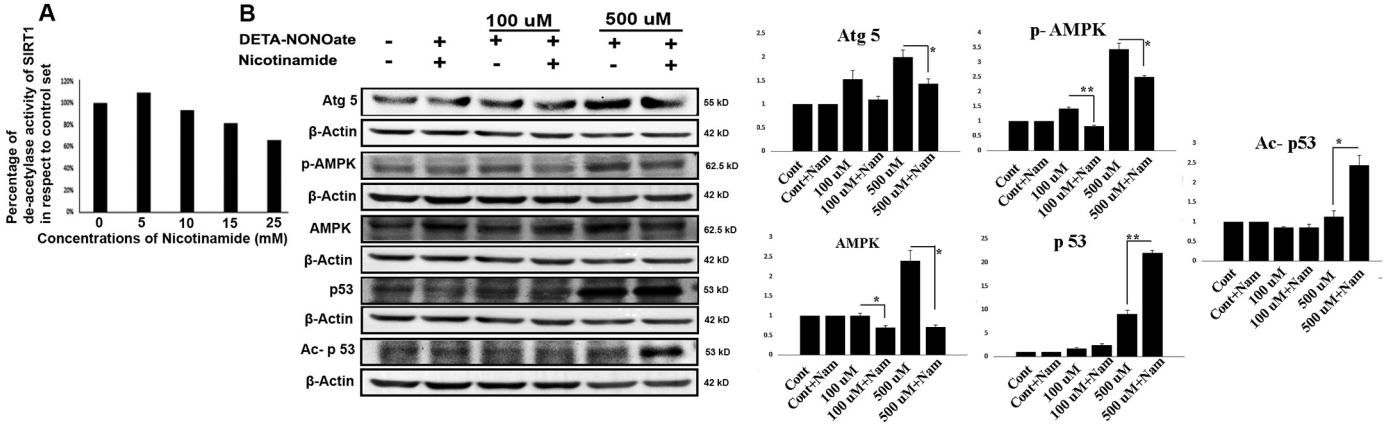

Fig. 9. Inhibition of SIRT1 led to a decrease in AMPK phosphorylation and autophagy. A. MCF7 cells were treated with graded concentrations of nicotinamide (presented in X axis) for 8 h. De-acetylase activity of SIRT1 (represented as percentage of activity in respect to control in Y axis) was found to be decreased compared to control cells. B. MCF7 cells were co-treated with 15 mM nicotinamide and DETA-NONOate for 8 h and immunoblot analysis for Atg5, p-AMPK, AMPK, p53 and acetylated-p53 proteins were done with respective antibodies from the cell lysate. Western blot fold changes are expressed as mean ± SE, for $n = 3$ experiments. *$p < .05$, ** $p < .01$, ***$p < .001$.

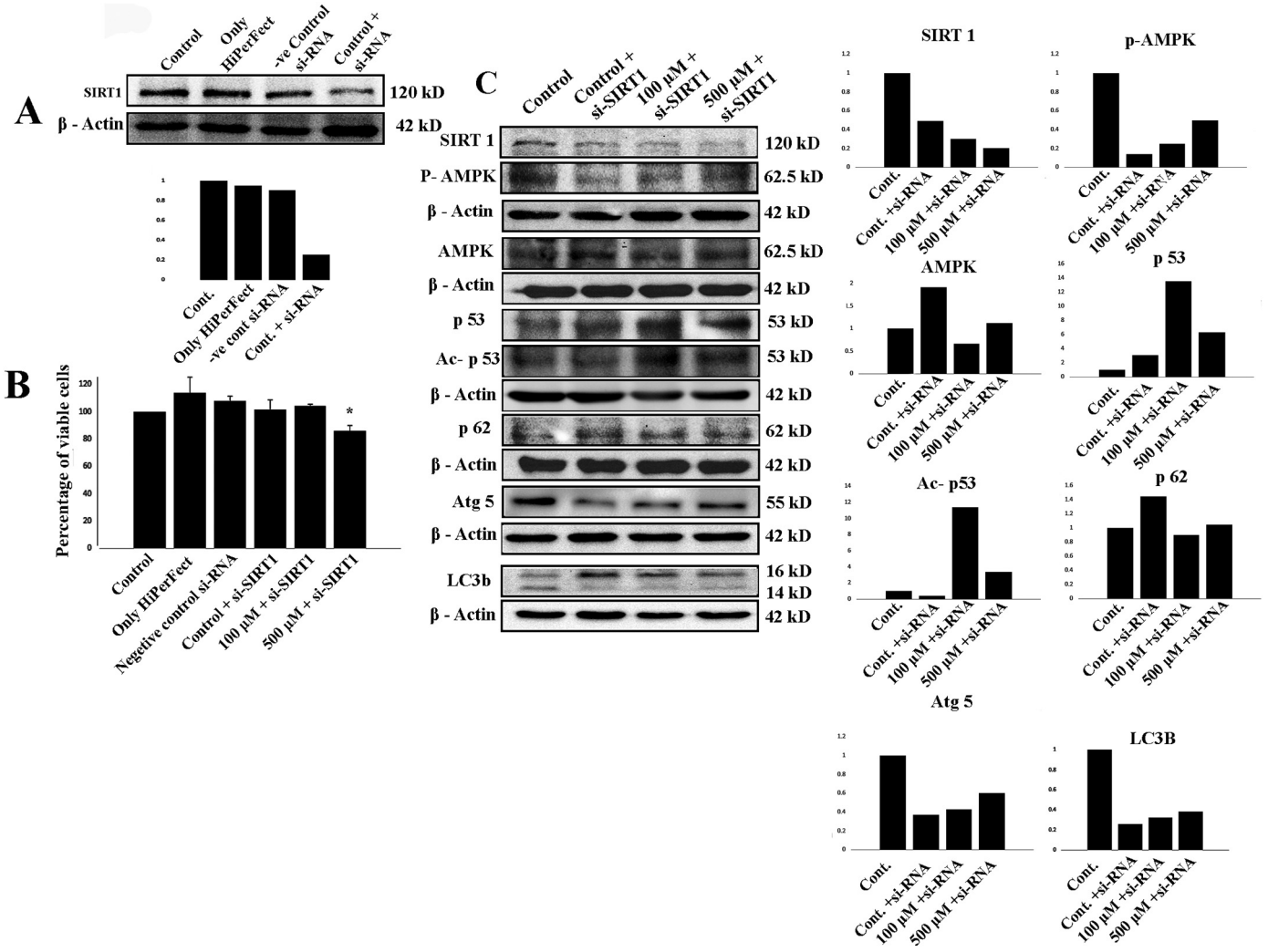

Fig. 10. Knocking down of SIRT1 with si-RNA resulted in a decrease AMPK phosphorylation and autophagy. A. Expression of SIRT1 protein was analyzed of the MCF7 cells treated with 20 nM of si-RNA specific for SIRT1 along with un-transfected cells, only HiPerFect treated cells and cells treated with negative control si-RNA. B. MCF 7 cells were treated with si-RNA for 48 h to knock down SIRT1 followed by 100 µM, 500 µM concentrations of DETA-NONOate exposure for 8 h led to an increase in cell viability compared to only NO donor treatment. Cell viability was estimated by WST-8 assay after treating the cells with DETA-NONOate. C. MCF7 cells were pre-treated with SIRT1 specific si-RNA for the required time as mentioned above followed by DETA-NONOate treatment for 8 h and immunoblot analysis was performed for p-AMPK, AMPK, p53, acetylated-p53, Atg5, p62 and LC 3B proteins with respective antibodies from the cell lysates. Results are expressed as mean, for $n = 2$ experiments.

negative control si-RNA treated cells [Fig. 10 A].

Surprisingly, knocking down SIRT1 following si-RNA augmented MCF 7 cells less sensitive towards nitrosative stress. There was no decrease in MCF 7 cell viability following 100 µM DETA-NONOate treatment compared to the control cells and 500 µM DETA-NONOate treatment resulted in only 14% cell death [Fig. 10 B]. Immunoblot analysis showed decreased expression of phosphorylated AMPK protein upon DETA-NONOate treatment when SIRT1 was knocked down in MCF 7 cells [Fig. 10 C]. We observed similar results when SIRT1 activity was inhibited with nicotinamide. There was a significant increase in both p53 and acetylated form p53 protein expression upon treatment with SIRT1 specific si-RNA and DETA-NONOate. Knocking down SIRT1 also abrogated the autophagic response as evidenced by decrease in expressions of autophagy marker proteins Atg 5 and LC-3b. Another autophagy marker p62 remained unchanged upon DETA-NONOate treatment when SIRT1 was knocked down indicating that SIRT1 protein plays an important role in nitrosative stress induced autophagy.

3.11. Exposure to nitric oxide induced DRAM 1 in MCF7 cells

Post-translational modification plays a critical role in modulation of p53 activity. In recent years special emphasis has been given to the effect of acetylation and de-acetylation of p53 and the consequences associated with that in modulating cell survival and cell death [47]. Induction of p53 occurred upon DETA-NONOate treatment and inhibition of AMPK and/or de-acetylase activity of SIRT1 also increased the acetylation of p53. Considering these results we further investigated the probable involvement of p53 in this autophagic but non-apoptotic response. We performed semi-quantitative PCR using primers against DNA damage regulated autophagy modulator 1 (DRAM 1) to estimate the m-RNA level of the gene upon DETA-NONOate treatment. DRAM 1 encodes a lysosomal membrane protein in autophagy. Interestingly, there was > 7-fold increase in m-RNA level of DRAM-1 gene upon 100 and 500 µM of DETA-NONOate treated MCF 7 cells compared to control cells suggesting involvement of DRAM 1 [Fig. 11].

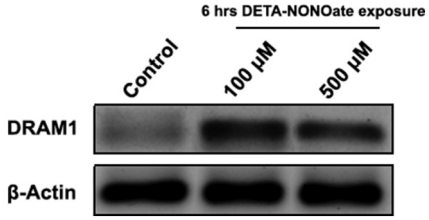

Fig. 11. Transcriptional upregulation of DNA damage regulated autophagic modulator DRAM1. cDNA was prepared from the extracted RNA obtained from control cells, 100 and 500 μM DETA-NONOate treated MCF 7 cells. Semiquantitative PCR with DRAM 1 primer revealed upregulation of DRAM 1 m-RNA upon nitrosative stress.

4. Discussion

Effect of nitric oxide varies in-vivo from cell to cell depending upon its genetic makeup, concentration of NO and cellular redox environment. In a previous study in MCF7 cell line, Tripathi et al. showed that steady state exposure to 11 μM NO for 24 h led to decrease in cell viability. They also showed that this loss of cell viability occurred through the induction of autophagy upon steady state NO exposure in MCF 7 cells by mTORC1 repression via ATM / LKB1 / AMPK / TSC2 signaling cascade [11]. In our previous study in leukemic cell line K562, we reported the role of cellular redox balance in inducing different modes of cancer cell death under nitrosative stress [13]. In this study, we attempted to unravel the factors involved in taking the death decision. We also determined the redox scenario involved in this death response induced by nitrosative stress in MCF7 cell line. Surprisingly, we identified the involvement of three stress responsive proteins namely AMPK, SIRT1 and p53 in MCF7 cells under nitrosative stress. Under nitrosative stress, phosphorylation of AMPK led to increase in SIRT1 and p53 protein expression which indicated that energy sensor protein AMPK could modulate the signaling of the two above-mentioned stress responsive proteins in MCF7 cells. In fact this is the first report of its kind where the interplay between energy sensor protein AMPK with HDAC- class III SIRT1 and their inter-dependency in initiating autophagy has been established in response to external NO donor treatment in MCF7 cells. Under nitrosative stress in MCF7 cells, we also report an increase in the protein expression of the transcription factor p53 and its control over the DRAM 1 at the transcription level which could induce autophagic condition. This points towards a crucial role of previously un-appreciated axis AMPK / SIRT1 / p53 in augmenting autophagy.

AMPK works as a fuel sensing enzyme that is activated by an increase in AMP / ATP ratio that induces autophagy by suppressing mTORC1. The partnership and a positive feed-back loop between AMPK and a class III HDAC, SIRT1 is also being documented in recent reports. phospho-AMPK can either activate SIRT1 directly or indirectly by interacting and phosphorylating DBC1, an inhibitory interacting partner of SIRT1 [48,49]. Reports show that phospho-AMPK can also lead to an increase in nicotinamide phosphoribosyltransferase (NAMPT) enzyme activity and thereby increasing NAD^+/NADH ratio which in turn induces SIRT1 [38,40]. On the other hand, there are reports showing decrease in SIRT1 activity was accompanied by downregulation of AMPK phosphorylation [50,51]. Lan et al. [27], demonstrated that SIRT1 can modulate AMPK activation by de-acetylating LKB1, an immediate upstream kinase of AMPK. Apart from induction of autophagy by regulating AMPK, Lee et al., provided evidences of SIRT1 is responsible for directly interacting and de-acetylating several essential components of autophagy proteins and thereby playing a positive regulatory role in autophagic induction [52]. Interestingly, we found a drop in NADH level upon NO donor treatment that actually mimic energy depleted condition. Nonetheless, we provide evidences for time-dependent activation of AMPK and increase in SIRT1 expression level in MCF7 cells, which correlates with increase in NAD^+/NADH ratio. Experiments carried out using AMPK inhibitor dorsomorphin, SIRT1 inhibitor nicotinamide and si-RNA against SIRT1 also provided evidences for the regulatory interaction between AMPK and SIRT1. p53, a stress responsive protein which is mutated in several cancers, is known to play a crucial role in tumor suppression and regulation of antioxidant and redox proteins [53].

Post translational modifications of p53 is critical for its transcriptional activity [54–57]. p53 is one of the first protein which was identified as SIRT1's non-histone substrate for de-acetylation. It is important to note that p53 expression was increased in MCF7 cells under nitrosative stress which was more prominent in higher dose of NO treatment. Immunoblot analysis revealed that p53 remained in acetylated state when AMPK activation was blocked by dorsomorphin or SIRT1 activity was blocked by NAM or SIRT1 was knocked down using si-RNA treatment which is definitely indicating the role of SIRT1 in maintaining p53 in its deacetylated state. This de-acetylation of p53 might have repressed apoptotic induction in the present instance, as acetylation of p53 enhances pro-apoptotic gene transcription to facilitate apoptosis in response to stress [56,57]. In the last decade, several reports indicated a positive role of p53 in autophagy. Contreras et al., found de-acetylation of p53 that induces autophagy by suppressing Bmf expression [58]. Induction of a lysosomal protein DRAM1 which is a transcription target of p53 has also been well documented in autophagic induction by the work of Crighton et al. [21]. Interestingly, a dose dependent increase in mRNA level of DRAM1 in MCF 7 cells under nitrosative stress also opens up scope for further investigation about its role in inducing / increasing autophagic flux upon nitrosative stress. It is interesting to note that in line with the findings of other studies linking AMPK and SIRT1, results from our present study identified them as key molecular players involved in an intertwined regulatory loop which played a crucial role in augmenting nitrosative stress mediated autophagy in MCF 7 cells. In this report, our data also suggests that p53 must be interconnected with the AMPK-SIRT1 partnership that ultimately affects its downstream transcriptional activity to induce autophagy but not apoptosis.

Autophagy promotes cell survival by providing building blocks of the macromolecules and it has been reported that cancer cells frequently use autophagy under nutrient deprived conditions. In the present study, we discovered a novel signaling pathway under nitrosative stress in MCF7 cells involving autophagy associated with a complex network of SIRT1, phospho-AMPK and p53, which ultimately led to cell death. Breast cancers cells show resistance to apoptosis. This study might provide an avenue to kill various types cancer cells and opens up a therapeutic strategy using NO donor compounds.

5. Conclusion

Our study discovered a complex regulatory network of three master regulators namely, p-AMPK, SIRT1 and p53 that are involved in autophagic induction in MCF7 cells in response to nitrosative stress.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109411.

Statement of author contributions

Subhamoy Chakraborty performed the experiments and wrote the manuscript. Sampurna Datta performed few experiments and analyzed the data. Sanjay Ghosh and Subhamoy Chakraborty designed, analyzed and revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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