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ORIGINAL ARTICLE

# p110α and p110β isoforms of PI3K are involved in protection against $H_2O_2$ induced oxidative stress in cancer cells

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## Abstract

**Purpose** Phosphatidylinositol-3 kinases (PI3Ks) are involved in regulating cell growth, proliferation, differentiation, apoptosis and survival. p110α and p110β, two ubiquitously expressed isoforms of PI3K signalling, are involved in growth factor mediated signaling and survival by generating second messengers. Earlier, we have generated GFP-fusion proteins of p110α and p110β and expressed them in normal and cancer cell-lines to investigate their subcellular localization and their role in various activities. Here, we sought to examine the role of p110α and p110β isoforms in protecting MCF-7 breast cancer cells against oxidative stress.

**Material methods** We performed cytotoxicity assays, DNA transfection, Plasmid DNA preparation, western blotting, flourscence microscopy and statistical analysis.

**Results** To know whether p110α and p110β are involved in protecting MCF-7 breast cancer cells against oxidative stress, we subjected MCF-7 cells to H2O2 treatment and observed a dose dependent decrease in cell viability and a marked increase in the levels of pro-apoptotic markers which include PARP, Bcl-2, Bax and procaspase-9. We then over-expressed recombinant GFP-fusion p110α and p110β proteins in MCF-7 cells and observed a significant decrease in apoptosis and a concomitant increase in pAkt levels.

**Conclusion** We report the involvement of p110α and p110β isoforms of Class 1A PI3K signalling in rescue from oxidative stress-induced apoptosis in MCF-7 cells in Akt dependent manner.

**Keywords** PI3K signalling · Akt · Apoptosis · Breast cancer

## Introduction

Based on their primary structure, regulation, and substrate specificity, PI3Ks are divided into various classes: Class I, II, III and IV [1]. The class I PI3Ks are further divided into class IA and class IB [2]. Class IA PI3Ks comprise of hetero-dimers of a regulatory subunit (p85α, p55α, p50α, p85β, p55γ) and a catalytic subunit (p110α, p110β, p110δ) [3]. Regulatory subunit p85α and catalytic subunits p110α and p110β are ubiquitously expressed proteins unlike p110δ which is expressed primarily in leukocytes [2, 4, 5]. The activation of class IA PI3K signaling starts with the binding of extracellular signalling molecules to plasma membrane-anchored receptors. The four major extracellular signals include: growth factors, cytokines, hormones, chemokines, and integrins. Upon this receptor activation, p110–p85 hetero-dimer of PI3K is recruited at the inner side of the plasma membrane. Thereupon, p110–p85 hetero-dimers phosphorylate a lipid moiety phosphatidylinositol (4,5)-bisphosphate (PIP2) to generate the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Upon PIP3 generation, Akt, also called protein kinase-B, interacts with PIP3 which then results in Akt activation via its phosphorylation at T308 and S473 positions [6–8]. Activated Akt then modulates function of different substrate proteins involved in the regulation of cell growth, proliferation, differentiation, survival, cell cycle progression, etc [9–11]. The class-IA PI3K pathway is negatively regulated by a lipid phosphatase

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tumor suppressor protein called PTEN through dephosphorylation of $PIP_3$ [12]. Although p110α and p110β catalytic isoforms are known to perform many redundant functions, unique and distinct roles have been associated with these isoforms as well. p110β is distinct among the class I PI3Ks in the diversity of its interacting partners and its subcellular localization in normal and cancer cells [13]. Both, p110α and p110β are shown to translocates into the nucleus [14]. Inside nucleus, p110β is seen to regulates DNA replication and repair [15].

Frequent genetic mutations in critical enzymes have made PI3K one of the most dysregulated pathways in cancer [16–19].Activating mutations in *PIK3CA (encoding for* p110α) [20], loss of function mutations in PTEN, AKT mutations and RTK amplification are the most common events causing tumorigenesis through upregulation of the PI3K/AKT signaling axis [20–23]. p110α is mutated in 30% of human cancers [24]; it is found to be mutated in breast, colon, endometrium and prostate cancers. 80% of p110α cancer causing mutations are constrained at 3 hotspots: E542K and E545K within the helical domain; and H1047R within the kinase domain [22, 23, 25]. p110β mediated oncogenic activities are caused by its gene amplification which results in the overexpression of p110β in many cancers.

In this study, we have investigated protective role of p110α and p110β isoforms against $H_2O_2$ mediated oxidative stress in MCF-7 breast cancer cells. We subjected MCF-7 cells to $H_2O_2$ treatment and then over-expressed p110α and p110β proteins in these cells to examine the impact of p110α and p110β under oxidative stress. We observed a role for p110α and p110β in protecting MCF-7 cells against oxidative stress in Akt dependent manner.

## Materials and methods

### Antibodies and reagents

Anti-pAkt, anti-Akt, pGSK-3β, β-actin antibodies were procured from CST, anti-PARP, anti-GAPDH anti-GFP antibodies were purchased from Santa Cruz. RPMI media, BCA kit, paraformaldehyde and DAPI were purchased from Sigma.

### MTT assay

$H_2O_2$ mediated cytotoxicity was determined by MTT [3, 4, (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MCF-7 cells were seeded in a 96-well microtiter plate with a density of 5000 cells and final volume of 100 µl culture medium per well and allowed them to grow for 24 h at 37 °C with 5% $CO_2$ in a cell-culture incubator. At 24 h, cells were treated with different concentrations of $H_2O_2$ and put inside the incubator for another 24 h. Then 20 µl of MTT solution (2.5 mg/ml) prepared in PBS was added to each well and further incubated for 4 h. After 4 h media was discarded completely and 150 µl DMSO was added in each well so that formazen crystals get solubilized. Finally, the optical density (OD) was measured by microplate reader at wavelength of 490 nm.

### Plasmids

As described earlier [14], p110α-N-myc and pFast-p110β constructs were used as a backbone to amplify the DNA sequence of p110α using primers MJ-14 (5′-gcgctcgagctatgcctccacgaccatcatcaggtg-3′), MJ-15 (5′-gcgtcgacgttcaatgcatgctgtttaattgtgtggaagatc-3′) andp110β using primers MJ-61 (5′-gcaagcttcgatgtgcttcagtttcataatgcctcctgctatg-3′), MJ-62 (5′-cgcgtcgacagatctgtagtctttccgaactgtgtgggcccatccag-3′). The PCR products were then digested with restriction enzyme XhoI, SalI (NEB) for p110α-GFP and Hind-III, SalI (NEB) for p110β-GFP, respectively, and sub-cloned into pEGFP-N3 vector (Clontech, Palo Alto, CA).

### Cell lines and transfection

MCF-7 cells were cultured (37 °C, 5% $CO_2$) in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine, 10 mM HEPES, 100-U/ml penicillin G sodium, and 100-µg/ml streptomycin sulfate. For transfections, MCF-7 cells were seeded into 150-mm-diameter dishes ($4 \times 10^6$ cells per dish) and next day transfected with 3 µg of the following plasmids: empty vector (eGFPN3), p110α-GFP + p85α, p110β-GFP + p85β. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) using incomplete medium. At 4 h, incomplete medium was supplemented with 10% FBS and allowed cells to grow for 24 h.

### Western blotting

For western blot analysis, cells were rinsed with phosphate buffered saline and lysed using radioimmuno precipitation assay buffer supplemented with a cocktail of protease inhibitors (Sigma). Protein estimation was done using BCA kit (SIGMA). Equal amount of proteins was separated on 10% SDS–polyacrylamide gel and blotted onto a polyvinyl difluoride membrane (Bio-Rad). Membrane was then blocked in a Tris-buffered saline solution with 5% bovine serum albumin containing 0.1% Tween-20 and incubated with antibodies overnight at 4 °C. Immunoreactive signals were detected by incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h (CST) followed by chemiluminescent detection using SuperSignal substrate (Millipore).

### Microscopy and DAPI staining

For microscopic study, 6 well dish containing a glass coverslip in each well was used to seed the cells at a 70% confluency and then plasmids were transfected to analyze the expression of recombinant proteins. At 24 h of transfection, cells were washed with ice chilled PBS and 4% paraformaldehyde was used to fix the cells. Next, cells were permeabilized using 0.5% Triton X-100 and DNA was stained with DAPI at a final concentration of 1 µg/ml. Then samples were mounted with mountant (glycerol + PBS, 1:1) and sealed with nail polish. Finally, analysis of samples was performed using confocal microscope (Olympus Fluoview FV-1000).

## Results

### Expression analysis of p110α-GFP and p110β-GFP proteins in MCF-7, HCT-116 and PC-3 cells

In our previous study we showed that MCF-7 cells have a relatively low level of endogenous p110α and p110β proteins in comparison to HCT-116 and PC-3 cells, on the contrary ectopic expression of p110αand p110β GFP-fusion proteins in MCF-7, HCT-116 and PC3 cells shows a reverse pattern (Fig. 1a). MCF-7 cells show a robust expression of p110α and p110β GFP-fusion proteins in MCF-7 in comparison to HCT-116 and PC3 cells (Fig. 1a). Furthermore, we examined the expression of GFP-fusion p110α and p110β proteins by confocal microscopy and observed a strong expression of these proteins in MCF-7 cells (Fig. 1b). To know whether the overexpression of GFP-fusion p110α and p110β results

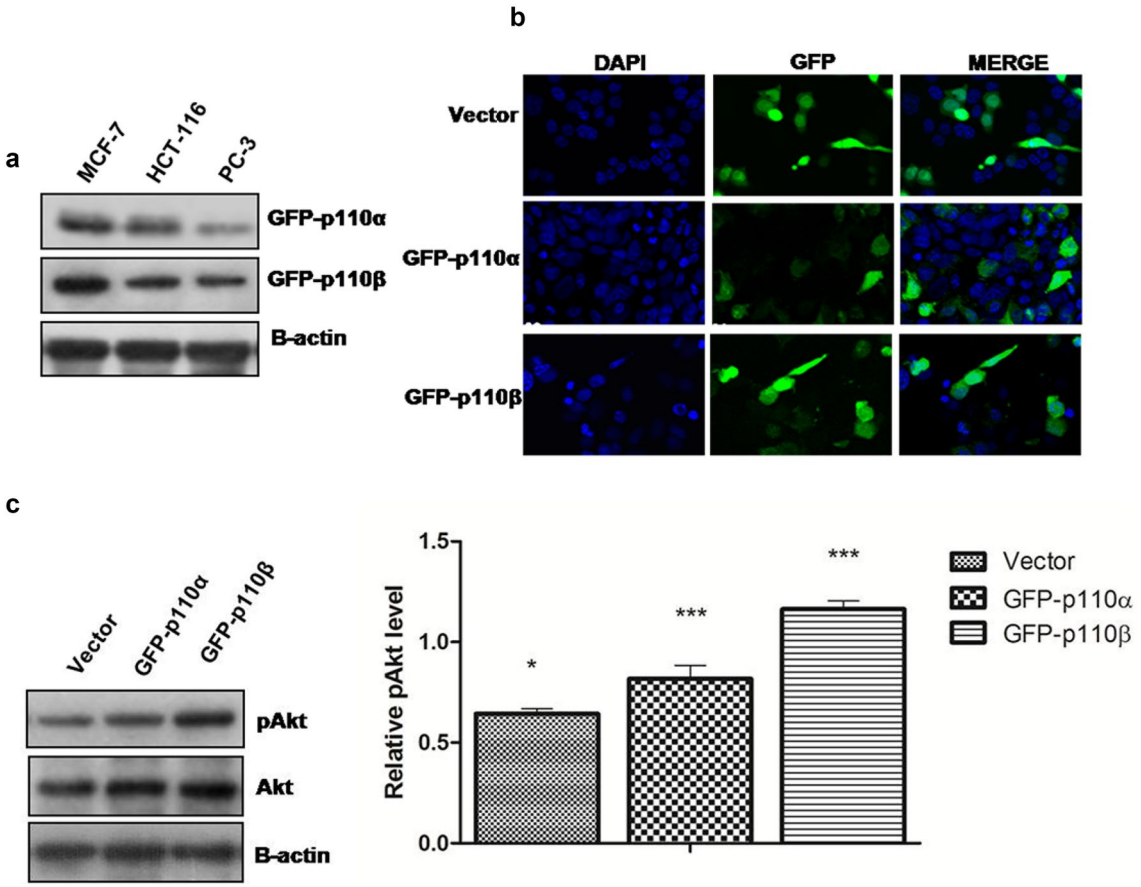


**Fig. 1** Expression analysis of recombinant p110α-GFP, p110β-GFP and endogenous pAkt proteins in MCF-7, HCT-116 and PC-3 cells. **a** MCF-7, HCT-116 and PC-3 cells were transfected with p110α-GFP and p110β-GFP plasmids and expression of recombinant proteins was quantified using anti-GFP antibody. **b** Transfected MCF-7 cells were fixed, DNA was stained with DAPI and expression was analyzed by confocal microscopy (40× magnification). **c** MCF-7 cells were lysed using RIPA buffer and expression of endogenous pAkt was probed with anti-pAkt antibody while β-actin was used as a loading control. On the right panel (**c**) is shown the densitometry analysis of western blot experiments ($n = 3$, mean ± SD) (***$P < 0.001$)

in the activation of PI3K signalling pathway in MCF-7 cells, we analysed the pAkt levels in these cells. We observed a significant increase in the pAkt levels in MCF-7 cells transfected with GFP-fusion p110α and p110β fusion proteins in comparison to vector control cells (Fig. 1c).

## Effects of $H_2O_2$ induced toxicity on MCF-7 breast cancer cells

Since MCF-7 cells showed robust expression of GFP-fusion p110α and p110β proteins, we determined whether this was sufficient to prevent oxidative stress-induced cell death. To evaluate the effect of $H_2O_2$ on cell viability, we performed MTT assay. We seeded $5 \times 10^3$ MCF-7 cells per well in 96-well dishes and treated them with 50–500 µM of $H_2O_2$ for 24 h. MCF-7 cells treated with $H_2O_2$ showed cell death in a dose dependent manner (Fig. 2a, b). To prove this further, we analyzed nuclear morphology of MCF-7 cells with DAPI staining and found that indeed there is marked nuclear shrinking upon the treatment of these cells with the increasing concentrations of $H_2O_2$(Fig. 2c).These results suggested that $H_2O_2$mediates its antiproliferative role through apoptosis.

## Enhanced apoptosis observed in $H_2O_2$treated MCF-7 cells

To examine whether observed cell death was apoptosis mediated, we assessed the expression of PARP-1 protein, a well-known apoptosis marker by immunoblotting. We treated MCF-7 cells with $H_2O_2$in concentration dependent manner and incubated them at 37 °C for 24 h. At 24 h cell lysates were prepared and anti-PARP1 antibody was used to detect the cleavage of PARP-1 protein. During apoptosis PARP-1 is cleaved by caspases; cleaved PARP-1 is a hallmark of apoptosis [26, 27]. We observed a marked cleavage of PARP-1 in a concentration dependent manner in $H_2O_2$treated MCF-7 cells(Fig. 3a). Next, we confirmed apoptotic cell death by assessing the expression of Bcl-2 and Bax protein as representative of cell survival related proteins. We challenged the MCF-7 cells to $H_2O_2$ with 50–300 µM concentration for 24 h and expression of Bcl-2 and Bax was analyzed by immunoblotting using specific antibodies. Decreased expression of Bcl-2 (Fig. 3b) and increased expression of Bax(Fig. 3c) further ensured the$H_2O_2$ induced apoptotic cell death in MCF-7 cells.

Procaspase-9 has been exhibited to execute a significant role in $H_2O_2$induced apoptotic cell death. So, to evaluate the consequences of $H_2O_2$ induced oxidative stress on caspase-9 activation, we analyzed the pro-caspase-9 levels. We treated the cells with $H_2O_2$ of 50–300 µM concentration for 24 h. Decreased expression of procaspase-9 was observed

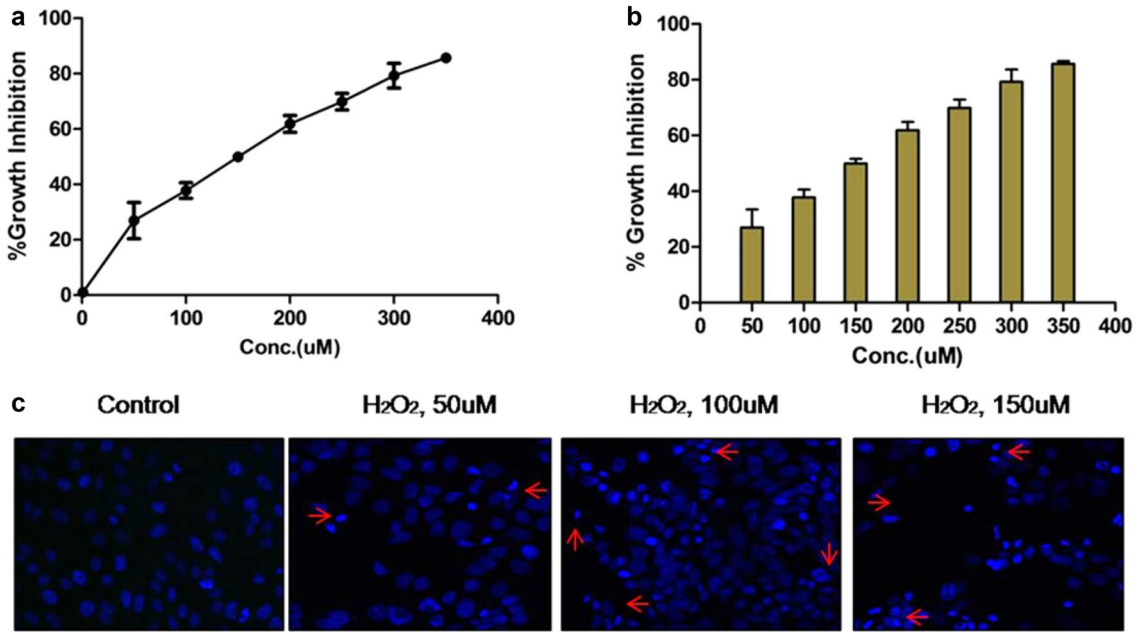


**Fig. 2** Effects of $H_2O_2$ induced toxicity on MCF-7 cells. **a** Dose–response curve from MCF-7 cells treated with various concentrations of $H_2O_2$ for 24 h. **b** Cell mortality was assessed by MTT assay. Data are mean of three individual experiments performed in triplicate. **c** DAPI staining of MCF-7 cell was done to examine the nuclear morphology and apoptotic bodies. Cells were incubated with indicated concentrations of $H_2O_2$ for 24 h. Condensed nuclei and the apoptotic bodies are indicated by arrows (20× magnification)

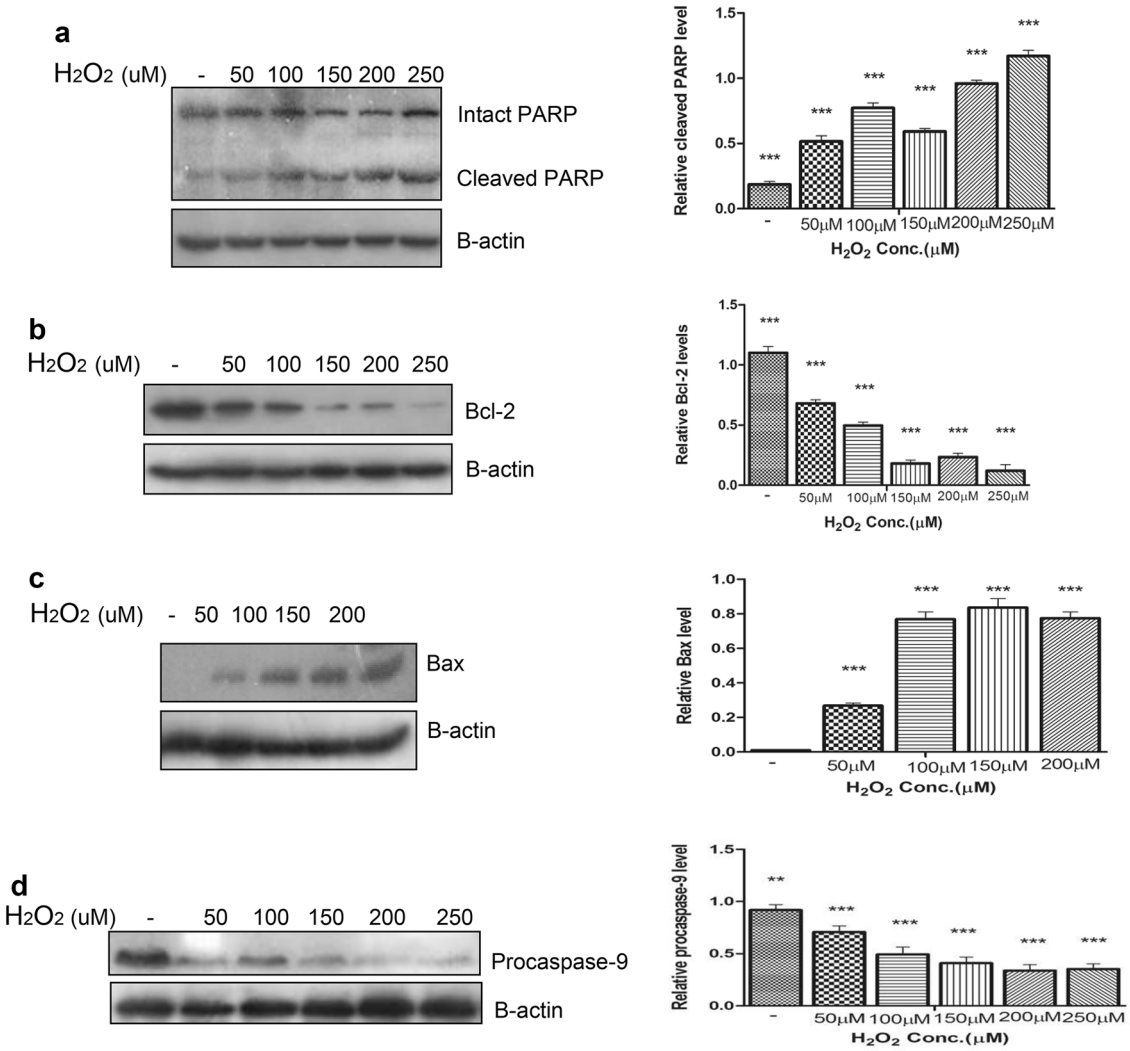


**Fig. 3** Validation of apoptotic cell death by examining various apoptotic marker proteins. **a** PARP-1 cleavage examined with anti-PARP antibody in cells treated with various concentrations of H2O2 for 24 h. **b** Assessment of Bcl-2 protein upon H2O2 treatment. **c** Western blot of Bax protein in H2O2 treated cells. **d** Cells were pretreated with different concentrations of H2O2 for 24 h and blot was examined with anti-procaspase-9 antibody. Densitometry analysis of western blot experiments ($n=3$, mean ± SD) (***$P<0.001$) is shown for each western blot

that confirms that procaspase-9 was activated upon $H_2O_2$ induction(Fig. 3d). The observed findings suggest that $H_2O_2$ stimulates apoptotic cell death in MCF-7 cells.

## Over-expression of p110α and p110β restores pro-survival effects against $H_2O_2$ induced apoptosis

To understand the role of PI3K isoforms p110α and p110β on $H_2O_2$ induced apoptosis, we transfected MCF-7 cells with plasmids expressing p110α and p110β GFP-fusion proteins and eGFPN3 vector (as a control). First, we measured the expression of recombinant protein p110α-GFP and p110β-GFP by immunoblotting. At 24 h after transfection cell lysates were probed with anti-GFP antibody. Immunoblot analysis showed the expression of recombinant p110α-GFP and p110β-GFP proteins when compared to vector control (Fig. 4a).

To determine impact of p110α and p110β on apoptosis, MCF-7 cells were transiently transfected with p110α-GFP and p110β-GFP plasmids along with vector control and after 4 h of post-transfection these cells were treated with $H_2O_2$ of 150 μM concentration. At 24 h after transfection cell lysates were prepared and effect on apoptosis was assessed by immunostaining using anti-PARP-1 antibody. Interestingly, we observed decreased cleavage of PARP-1 in p110α-GFP as well as in p110β-GFP transfected cells when compared to cells transfected with vector control (Fig. 4b) indicating that p110α and p110β attenuates $H_2O_2$ generated

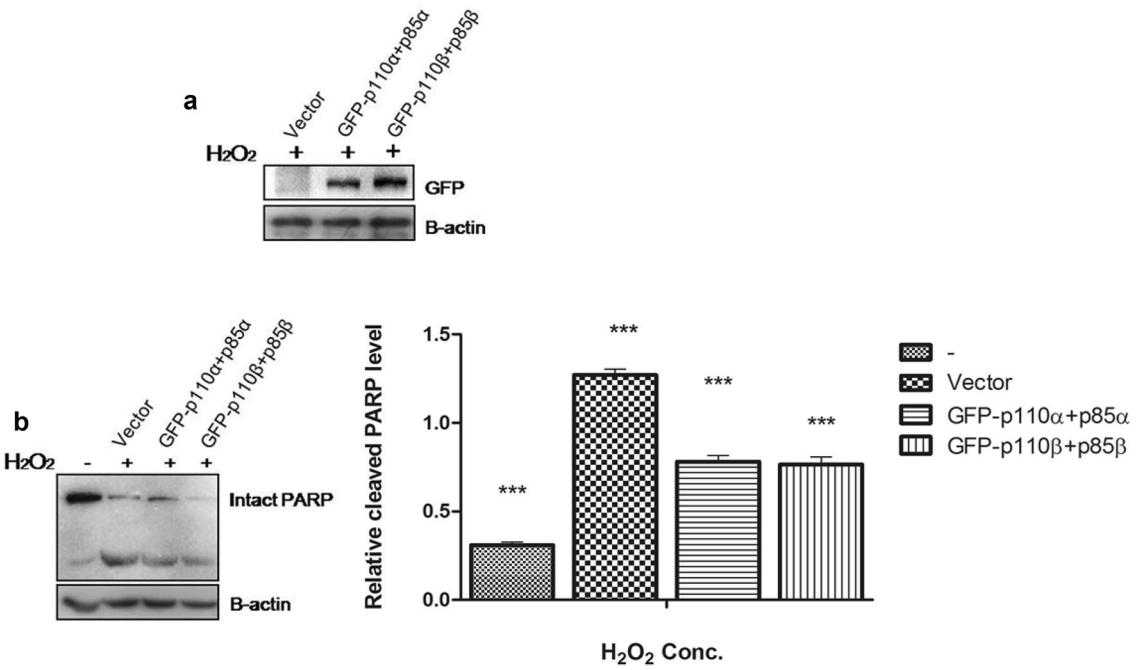


**Fig. 4** Over-expression of p110α and p110β restore pro-survival effects against $H_2O_2$ induced apoptosis. **a** Expression of exogenous p110α-GFP and p110β-GFP proteins was analyzed by immunoblotting with anti-GFP antibody and β-actin was used as a loading control. **b** Cells were transfected with vector control, GFP-p110α, GFP-p110β and treated with $H_2O_2$,blot was then examined with anti-PARP antibody

apoptotic effects and suggesting anti-apoptotic role of p110α and p110β in $H_2O_2$ induced MCF-7cells.

### pAkt mediates protective effects of p110α and p110β against oxidative stress

Further to understand the mechanism underlying the PI3K induced rescue, we carried out immunoblotting to evaluate the activation of Akt as this biomolecule is considered to be involved in $H_2O_2$stimulated oxidative stress. We treated MCF-7 cells with 50–300 µM of $H_2O_2$ for 24 h and protein extract was analyzed by immunoblotting using pAkt specific antibody. Our results show that $H_2O_2$ treatment causes a decrease in Akt phosphorylation in a concentration dependent manner (Fig. 5a). To inquire the potential of p110α and p110β to modulate $H_2O_2$ induced apoptotic effect, further investigation was done by overexpressing p110α and p110β in MCF-7 Cells. We transiently transfected MCF-7 cells with empty vector, p110α-GFP and p110β-GFP constructs then treated these cells with $H_2O_2$. After 24 h of $H_2O_2$ treatment lysates were examined by immunoblotting. The results revealed that Akt phosphorylation was increased in p110α-GFP as well as p110β-GFP transfected cells when compared to cells transfected with vector alone suggesting that p110α and p110β attenuate the $H_2O_2$ induced apoptotic effects by increasing the level of pAkt (Fig. 5b). These findings indicate that p110α and p110β exert their protective effects by restoring the intact PARP-1, and these protective effects are mediated by pAkt.

## Discussion

There are many signal transduction cascades activated against oxidative stress but PI3K/Akt signalling pathway, also called cell survival pathway, is very well known to protect cells from various types of stress. Role played by individual p110α and p110β isoforms in differentially regulating cell growth, survival and Akt activation was summarized by Singh et al. [13]. There are reports of p110α and p110β involvement in $H_2O_2$ mediated oxidative stress in various cancer cells. Recently, p110α was found to be involved in protection of various cells against $H_2O_2$ mediated oxidative stress. Moreover, p110α and p110β are reported to have differential effects on Akt activation and protection against $H_2O_2$ mediated oxidative stress in myoblasts. Pertinently, p110α is reported to be involved in oxidative stress-induced apoptosis in Akt and MEK independent manner [28, 29].

Since there are no reports regarding role of PI3K signalling pathway during oxidative stress in MCF-7 cells, we analyzed the role of p110α and p110β isoforms in these cells using DNA constructs overexpressing GFP-fusion

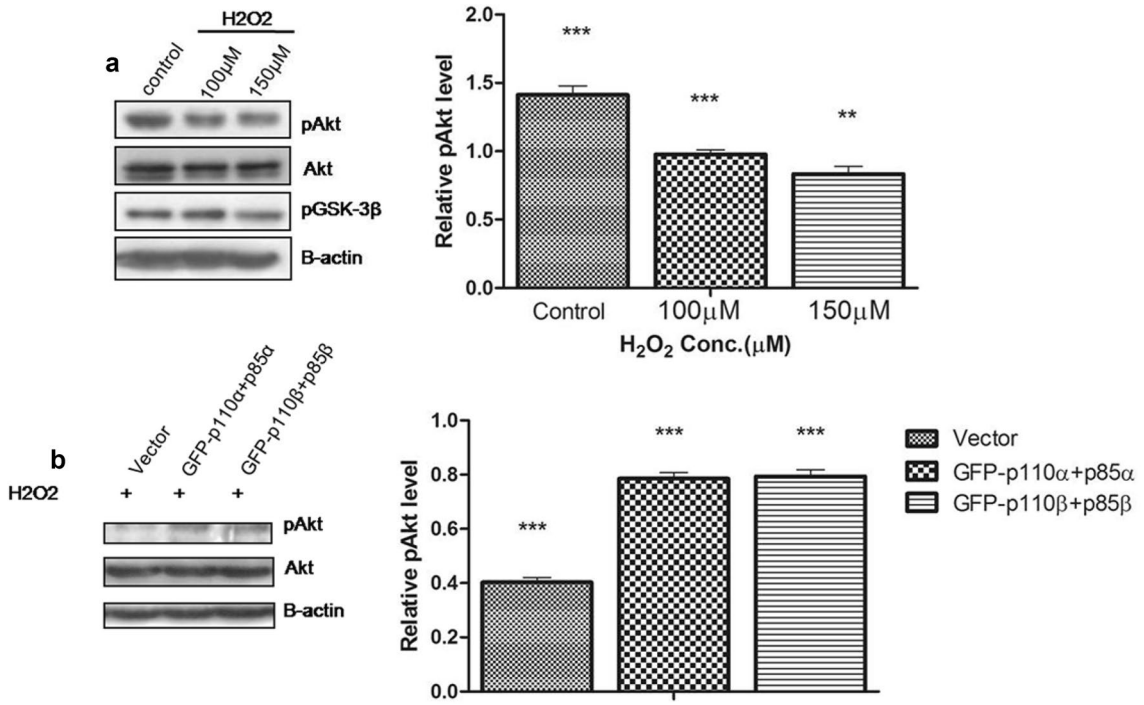


**Fig. 5** pAkt mediates protective effects of p110α and p110β against oxidative stress. **a** MCF-7cells were exposed to different concentrations of $H_2O_2$ for 24 h. pAkt, Akt, pGSK-3β levels were examined with specific antibodies and β-actin was used as a loading control. **b** pAkt level was measured using anti-pAkt antibody from MCF-7 cells transfected with p110α-GFP, p110β-GFP, vector control and pretreated with $H_2O_2$ for 24 h. Densitometry analysis of western blot experiments ($n = 3$, mean ± SD) (***$P < 0.001$)

proteins of these isoforms. We used GFP-fusion proteins to visualize the cells in real time under microscope for monitoring their characteristics upon $H_2O_2$ treatment, and observed that overexpression of p110α as well as p110β significantly suppressed the apoptosis as assessed by the quantification of pro-apoptotic markers which include PARP, Bcl-2, Bax, procaspase-9. Further, we explored the mechanism underlying p110α and p110β mediated protection against $H_2O_2$ induced toxicity in MCF-7 cells. Akt has been established as an endogenous protective factor against cell death in many kinds of cell insults and can inhibit apoptosis in many ways. Therefore, we sought to determine the role of Akt in MCF-7 cells against $H_2O_2$ mediated cell injury. We found decreased pAkt levels upon $H_2O_2$ treatment in a concentration dependent manner. Then, we assessed the effect of p110α and p110β overexpression on pAkt in MCF-7 cells treated with $H_2O_2$ and observed that level of pAkt is increasing upon p110α and p110β overexpression which coincides with decreasing level of cleaved PARP-1. These, findings suggest the involvement of pAkt in p110α and p110β mediated rescue of $H_2O_2$ induced oxidative stress.

Thus, we observed functional consequences of p110α and p110β overexpression proteins in MCF-7 cells upon $H_2O_2$ mediated oxidative stress. We report that p110α as well as p110β are involved in protecting MCF-7 cells against oxidative stress induced apoptosis in Akt dependent manner.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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