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Hydrogen Peroxide Activation of ERK5 Confers Resistance to Jurkat Cells Against Apoptosis Induced by the Extrinsic Pathway

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Abstract

Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) exhibit both pro-survival and pro-death signaling in leukemic cells. We examined the effect of exogenous H_2O_2 on Fas ligand (FasL) -induced apoptosis in Jurkat cells. H_2O_2 applied prior to (pre-conditioning) and during (post-conditioning) FasL stimulation attenuated early apoptosis through activation of EKR5. H_2O_2 increased the activated caspase-8 sequestered in the mitochondria thereby decreasing cell death through the extrinsic apoptotic pathway. In addition, inhibition of a protein tyrosine phosphatase likely explains the post-conditioning requirement for H_2O_2. Given that chemotherapeutic agents used for the treatment of acute lymphoblastic leukemia are thought to work partly through production of ROS, a simultaneous inhibition of the ERK5 pathway may abrogate the ROS-initiated pro-survival signaling for an enhanced cell kill.

Keywords

apoptosis; extrinsic pathway; hydrogen peroxide; Jurkat cell; FAS ligand

Introduction

The incidence of acute lymphoblastic leukemia (ALL) remains high with almost 4000 new patients diagnosed annually in the United States alone [1]. The rate of successful treatment has improved during the past three decades with the 5-year survival rate now exceeding 80% largely due to a better understanding of the immunobiology of ALL, establishment of central nervous system-directed therapy, and improvement of supportive care [1–3]. However, the state-of-art therapies fail in some patients and the outcome for these patients remains

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unsatisfactory. More effective treatment regimens are warranted to further improve the care of ALL patients.

ALL remission-induction therapy usually includes high-dose glucocorticoid, vincristine and additional drugs such as L-asparaginase and anthracyclines (typically daunomycin or doxorubicin) [3]. These drugs are thought to induce apoptosis of the leukemic cells partly secondary to the generation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) [4]. ROS-dependent increase in FasL expression and/or decrease in the FLICE-like Inhibitory Protein (FLIP)-decoy receptor that normally inhibits activation of caspase-8 are thought to enhance death through the extrinsic pathway. Decreasing the anti-apoptotic Bcl-2 expression sensitizes the cells to death through the intrinsic pathway as well. On the other hand, ROS regulates critical cellular functions including gene activation, proliferation, migration and differentiation [5–8]. Thus ROS initiates both pro-apoptotic and pro-survival signaling in T-cells.

We sought to better understand how ROS modulates leukemic cell apoptosis. Specifically, we wondered if exogenous ROS might contribute to leukemic T cell survival and focused on the effect of H_2O_2 on Jurkat leukemic T cell apoptosis induced by the extrinsic apoptosis pathway.

Materials and methods

Cell culture

Jurkat cells were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a humidified incubator at 37° C and 5% CO_2. The cells were passaged twice a week and used in between passages 5–20.

Jurkat cells (5×10^5 cells) were transiently transfected with 1 μg plasmid by electroporation (3 pulses of 1325 V x 10 ms, Neon™, Invitrogen, Carlsbad, CA, USA) resulting in approximately 10–15% transfection efficiency. cDNAs encoding a EGFP-tagged dominant negative (DN)-MEK1 and DN-MEK5 [9] subcloned in a pCIneo expression vector (Promega, Madison, WI, USA) were used for transfection. A cDNA for EGFP subcloned in the same vector served as a negative control.

Detection of apoptosis and measurement of caspase-like activity

After a 3 h incubation with 1 ng/ml FasL or 0.1 μg/ml TNFα and 0.5 μg/ml cycloheximide, cells were labeled with Alexa Fluor 488 dye-conjugated annexin V and propidium iodide (PI) (Invitrogen) following the kit instruction. Approximately 5,000 cells were counted from each sample using a flow cytometry (Guava Easycyte Plus, Millipore, Billerica, MA, USA) and the percentage of cells in early apoptosis was defined by the annexin V positive/PI negative population. Caspase-8-like and caspase-3-like activities were also evaluated by flow cytometry using a commercial kit (Millipore) following the manufacture's recommended protocol.

For mitochondrial protein enrichment, cells were suspended in a mannitol-sucrose buffer (255 mM mannitol, 10 mM sucrose, 0.5 mM EGTA, 1 mM glutathione, 10 mM HEPES, pH 7.4), and lysed with a Dounce homogenizer using 40X strokes on ice. The first spin at 3000g for 10 min pelleted the unlysed cells and nuclei (P1). The supernatant from the first spin (S1) was re-spun at 6500g for 10 min and the pellet (P2) was collected as the fraction enriched in mitochondria. Caspase-8-like activity in the P2 fraction was measured using Ac-lle-Glu-Thr-Asp (Ac-IETD)-7-amino-4-methylcoumarin (AMC) as a substrate. The P2

fraction described above was lysed in a lysis buffer (10 mM Tris-CL, 10 mM NaH_2PO_4, 130 mM NaCl, 1% TX-100, 10 mM NaPPi, pH7.5) and 10 μg protein quantified by BCA protein assay was used for each assay. The assay buffer (40 mM PIPES, 200 mM KCl, 1 mM EDTA, 0.1% CHAPS, 20% sucrose, 10 mM DTT, pH7.2) contained 100 μM (final concentration) substrate. The rate of fluorescence increase (excitation: 360 nm, emission: 460 nm) was measured kinetically for 1 hour with a microplate reader (BioTek, Winooski, VT, USA) and the maximum relative-fluorescence unit/min obtained converted to μg AMC released μg protein^{-1} min^{-1} using an AMC calibration standard substrate.

Western blot analysis

Protein was extracted in a lysis buffer (1% NP40, 10 mM Tris pH7.6, 50 mM NaCl, 30 mM NaPPi, 50 mM NaF, 1% Triton, 0.5% deoxycholate and 0.1% sodium dodecyl sulfate) concentration determined by the BCA protein assay, and 20 μg of protein per sample was subjected to polyacrylamide gel electrophoresis and transfer to a nitrocellulose membrane. The antibodies used were: ERK1/2 (Promega, 1:10000), ERK5 (Millipore, 1:1000), phosphorylated-ERK1/2 (pERK1/2) and pERK5 (Cell Signaling Technology, 1:1000), cleaved caspase-8 (Cell Signaling Technology, 1:1000), VDAC (Abcam, 1:3200) and GAPDH (Advanced ImmunoChemical, 1:100000). Relative density of bands was quantified using Chemi Doc™ XRS+ (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data are presented as mean ± S.D. A statistical significance between two groups was determined by a two-tailed T-test. Comparison between multiple groups was by ANOVA followed by pair-wise comparisons using a post hoc procedure. The results were considered significant at P value < 0.05.

Results

H_2O_2 attenuated apoptosis induced by FasL

High concentration (>200 μM) long term (>3 hrs) exposure to H_2O_2 induced Jurkat cell necrosis indicated by a large increase in PI positive cells (data not shown) confirming earlier reports [10–11]. We explored the effects of lower physiological concentrations of H_2O_2 and discovered that a brief 3 minutes high concentration (200 μM) exposure before (pre-conditioning) followed by a lower concentration (5–20 μM) exposure during FasL stimulation (post-conditioning) dramatically reduced apoptosis through the extrinsic pathway (Figure 1A, B, C). Similar results were obtained when apoptosis was induced by TNF-α (0.1μg/ml) and cycloheximide (0.5 μg/ml) instead of FasL (data not shown), suggesting that the cyto-protective effect of H_2O_2 was on the extrinsic apoptotic pathway, and not specific to FasL. Exposure of cells only before or during FasL stimulation did not reduce apoptosis (Supp Figure 1). This requirement for H_2O_2 presence both before and during FasL stimulation to reduce apoptosis was confirmed by a co-administration of catalase that hydrolyzes H_2O_2 before or during FasL stimulation. Catalase co-administration with H_2O_2 before or during FasL stimulation abolished the cyto-protective effect (Figure 1D). The presence of catalase during the entire protocol not only eliminated the cyto-protective effect of H_2O_2 but increased the percentage of apoptotic cells, perhaps suggesting the presence of endogenous H_2O_2 conferring cyto-protection. The brief 3-minute exposure to H_2O_2 as high as 1 mM and/or a 3 hr exposure to 5–20 μM did not significantly induce apoptosis by themselves without the FasL stimulation (Supp Figure 1 and Figure 1C, D).

The effect of H_2O_2 on apoptosis was through ERK5 activation

We explored the signaling underlying this cyto-protective effect of H_2O_2 on FasL-induced apoptosis by administering various inhibitors of the signaling pathways potentially activated by H_2O_2. Concentrations of the various inhibitors were determined based on the published IC50 values [12–14]. The inhibitor administration was initiated 30 minutes before the H_2O_2 pre-conditioning to assure effective drug action. Although SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), LY294002 (PI3 inhibitor) and AZD6244 (specific MEK1/2 inhibitor) had little effects on the protective effect of H_2O_2 (Figure 2A), pretreatment with PD98059, which is a non-specific inhibitor of both MEK1/2 and MEK5 [15], abolished the cyto-protective effect of H_2O_2 in a dose-dependent manner (Figure 2B). Since MEK1/2 is the upstream kinase specific for ERK1/2 activation and MEK5 is the comparable upstream kinase responsible for ERK5 activation, these pharmacological results indicated that the cyto-protective effect of H_2O_2 signaled through ERK5. Although the lack of specificity of PD98059 and the more specific inhibition of ERK1/2 by AZD6244 have been reported [16], we confirmed this in Jurkat cells stimulated by H_2O_2. ERK1/2 and ERK5 were both activated by H_2O_2 and only AZD6244 demonstrated a potent and specific inhibition of pERK1/2 while preserving pERK5 at concentrations as high as 30 μM (Figure 2C, D).

To confirm that the H_2O_2 protection against FasL-induced apoptosis is through ERK5 activation, we used molecular reagents with greater specificity. The C-terminal epitope tagged dominant-negative (DN)-MEK1 and DN-MEK5 specifically inhibit the MEKs upstream of ERK1/2 or ERK5, respectively [9]. Jurkat cells were transfected with plasmids expressing EGFP, DN-MEK1-EGFP, or DN-MEK5-EGFP and subjected to the same H_2O_2 exposure protocol and FasL stimulation. We gated electroporated cells with the EGFP signal to allow simultaneous detection of apoptosis in transfected and non-transfected cells in the same experiment. As shown in Figure 2E, the protective effect of H_2O_2 was abolished only in cells transfected with DN-MEK5-EGFP confirming that ERK5 activation was necessary for the cyto-protective effect of H_2O_2 against FasL-induced apoptosis.

Mitochondrial sequestration of activated caspase-8 by H_2O_2

Ligation of Fas receptor with FasL initiates the recruitment of the adaptor protein Fas-Associated Death Domain (FADD) and procaspase-8 to form the death inducing signaling complex (DISC). The active caspase-8 which consists of each two 18 and 10 kDa oligomer, resulting from the cleavage of procaspase-8 in the DISC signals to the effector molecules executing the extrinsic apoptotic program [17]. To gain a better understanding of how H_2O_2 interferes with this process, we looked at what H_2O_2 does to the FasL- initiated extrinsic apoptotic program.

First, we asked whether the levels of active initiator caspase-8 and the executor caspase-3 were altered by H_2O_2. Flow cytometer analyses (Figure 3A, B) demonstrated the expected increase in both active caspase-8-like and -3-like activity by FasL stimulation which was deminished by H_2O_2. This H_2O_2-treatment dependent reduction in caspase-like activities was partially reversed by PD98059 consistent with our earlier apoptosis assay. We wondered whether the amount of cleaved caspase-8 was decreased by H_2O_2 and performed a Western blot for the cleaved caspase-8 (18k Da). FasL stimulation induced a time-dependent increase in the cleaved caspase-8 band intensity in whole cell lysates but was not affected by H_2O_2 (Figure 3C, D), suggesting that the decreased caspase-8-like activity was not due to generation of less cleaved caspase-8 by DISC.

Cells overexpressing Bcl-xL exhibit resistance to extrinsic apoptotic death due to mitochondrial sequestration of active cleaved caspase-8 [18]. We wondered whether a similar mitochondrial sequestration of activated cleaved caspase-8 could explain the H_2O_2-

dependent reduction of apoptosis. The mitochondrial-enriched P2 fraction was isolated by fractionation of cells subjected to the various treatments. The caspase-8-like activity in the P2 fraction assayed using a fluorescent substrate reporter was increased in the FasL with H_2O_2 treatment group compared to the FasL only group (Figure 3E). At the protein level, Western blot analysis confirmed an increase in the VDAC normalized cleaved caspase-8 (18k Da) band intensity in the P2 fraction upon H_2O_2 treatment (Figure 3F). These results suggested that a mitochondrial sequestration of the activated caspase-8 similar to that seen in Bcl-xL overexpressing cells was induced by H_2O_2 to reduce the amount of active cleaved caspase-8 available in the non-mitochondrial cellular compartment. This sequestration of active caspase-8 could explain the reduction of FasL-induced apoptosis.

The protective effect of H_2O_2 required not only high micromolar preconditioning prior to but also low micromolar post-conditioning during FasL stimulation. Although we showed that ERK5 activation was necessary for this protective effect of H_2O_2, the appearance of pERK5 after a brief exposure of T-cells to 200 μM H_2O_2 was transient, as shown in Figure 4A. Therefore, the persistent ERK5 activation was not a likely mechanism for the low micromolar H_2O_2 requirement. The signaling downstream of ERK5 that ultimately manifests as reduction of FasL-induced apoptosis is unknown; however, ERK5 phosphorylation of a downstream substrate is most likely involved. Low micromolar H_2O_2 inhibits protein tyrosine phosphatase (PTP) through oxidation of the conserved cysteine in the active site of the enzyme [19, 20]. Inhibition of PTP could amplify the ERK5 signaling through prolongation of downstream signaling. We tested this idea by replacing low micromolar H_2O_2 with a cell-permeable PTP inhibitor benzylphosphonic acid (50 μM) or a specific PP2A (serine/threonine phosphatase) inhibitor okadaic acid (5 and 50 nM).

As shown in Figure 4B, replacement of low micromolar H_2O_2 post-conditioning with okadaic acid (5 and 50 nM) did not reduce apoptosis. However, benzylphosphonic acid (50 μM) post-conditioning after H_2O_2 pre-conditioning reduced apoptosis significantly, although benzylphosphonic acid by itself did not modulate apoptosis. Inhibition of PTP can mimic the cyto-protective effect of low micromolar H_2O_2 post-conditioning, albeit at a lower efficacy, suggesting that tyrosine phosphorylation down stream of ERK5 may be at work.

Discussion

Exogenous H_2O_2 conferred resistance to FasL-induced Jurkat cell apoptosis through the extrinsic pathway. Experiments with pharmacological and more specific molecular reagents indicated the role of ERK5 in this cyto-protection.

ERK5 has been reported to mediate proliferative signaling [21], and its activation is essential for survival of leukemic T cells through NF-KB activation [22]. ERK5 is a redox-sensitive MAPK activated by H_2O_2 signaling through upstream Src family kinases [23,24]. Its activation by exogenous H_2O_2 or possibly by chemotherapeutic drugs intended to kill leukemic cells triggering a pro-survival signaling opens up an interesting paradox that could reduce cell kill.

ERK1/2 suppression of death receptor-mediated apoptosis in Jurkat cells has been described [25–27]. This ERK1/2-mediated cyto-protection was inhibited by DN-MEK1 and, conversely, enhanced by expression of constitutively active-MEK1 through reduction in accumulation of cleaved caspase-8 with retention of the uncleaved procaspase-8. Our results suggested that H_2O_2 treatment reduced the activity of cleaved caspase-8 directly or indirectly without disrupting cleavage of procaspase-8, because H_2O_2 had little effects on the protein level of cleaved caspase-8 of whole cell lysate in cells stimulated with FasL. This

also implied that DISC formation was not disturbed since cleavage of procaspase-8 requires DISC [28]. The ERK5-mediated cyto-protection described in our present work appears distinct from the anti-apoptotic effect acting through ERK1/2. A recent report indicated that the palliative effect of phorbol ester on anti-Fas antibody-induced apoptosis was blocked by shRNA knockdown of ERK5 [29], suggesting a role of ERK5 in this cyto-protection as well.

Tyrosine phosphorylation is important in many cell-signaling pathways. Protein-tyrosine phosphatases (PTPs) regulate this phosphorylation negatively, and the final extent of protein phosphorylation is dependent on the balance between the action of protein tyrosine kinase and PTPs. Inhibition of the phosphatase activity is considered a potent mechanism for enhancing phosphorylation-dependent signaling [30]. ROS, including H_2O_2, are potent PTP inhibitors acting through oxidation of a critical cysteine residue in the active site of the enzyme [19, 20]. Our study of the low micromolar H_2O_2 requirement during FasL stimulation for the full manifestation of H_2O_2-induced apoptosis reduction revealed that a direct inhibition of PTP by benzylphosphonic acid could partially replace this post-conditioning requirement. Benzylphosphonic acid at the concentration used in our experiments did not exhibit an anti-apoptotic effect by itself, indicating that the effect of PTP inhibition was directly related to some downstream signaling involving tyrosine phosphorylation initiated by ERK5 activation. Further studies are necessary to identify the downstream targets phosphorylated by ERK5 and the phosphatases likely involved in the H_2O_2–modulation of anti-apoptotic ERK5 signaling.

While the amount of ROS generated by anti-leukemic drugs is not fully understood, ERK5 activation and the pro-survival signaling elicited by H_2O_2 could attenuate their cancer cell-kill efficacy. Thus, co-inhibition of ERK5 signaling in conjunction with apoptosis-inducing chemotherapy might be a more effective combination therapy to maximize cell-kill of leukemic T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- H_2O_2 attenuates FasL-induced Jurkat cell apoptosis.
- ERK5 activation mediates this anti-apoptotic effect.
- Inhibition of ERK5 together with chemotherapeutic drugs should enhance cell kill.

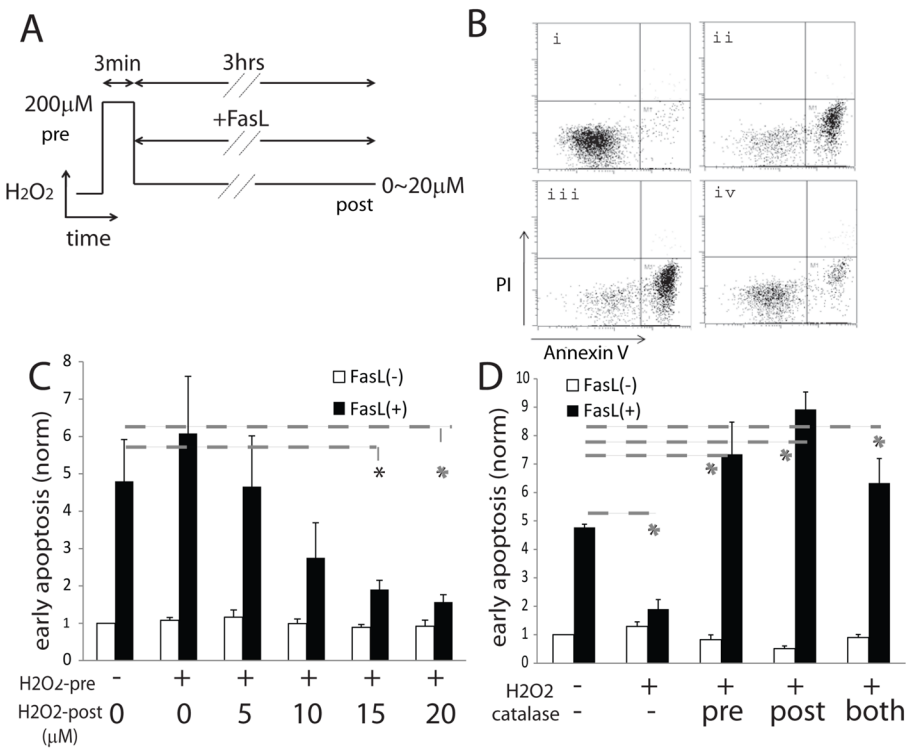

Fig. 1.

The effects of H_2O_2 treatment on apoptosis induced by FasL in Jurkat cells. (A) A diagram showing the H_2O_2 treatment protocol. After 3 minutes exposure to 200 μM H_2O_2 (pre-conditioning), apoptosis was induced by FasL (1 ng/ml) stimulation for 3 hours. Concurrently, cells were treated with various low concentrations of H_2O_2 (post-conditioning). (B) Scatter plots of flow cytometer read outs with annexin V (horizontal axis) and PI (vertical axis). The conditions were: i. non-stimulated control, ii. FasL stimulation only, iii. FasL with H_2O_2 pre-conditioning only, and iv. FasL with H_2O_2 pre-conditioning (200 μM, 3 minutes) and post-conditioning (20 μM) during FasL stimulation. (C) Summary bar graph of cells in early apoptosis (annexin positive, PI negative) normalized to a control population with no stimulation for the denoted post-conditioning H_2O_2 concentrations. Data from 3 independent experiments with triplicate measurements per experiment. (D) Catalase (2500 U/ml) was administered 30 minutes before H_2O_2 pre-conditioning (pre), only during FasL stimulation (post), or both. Data normalized to the no treatment control group. * $P < 0.05$ for the indicated comparisons for both bar plots.

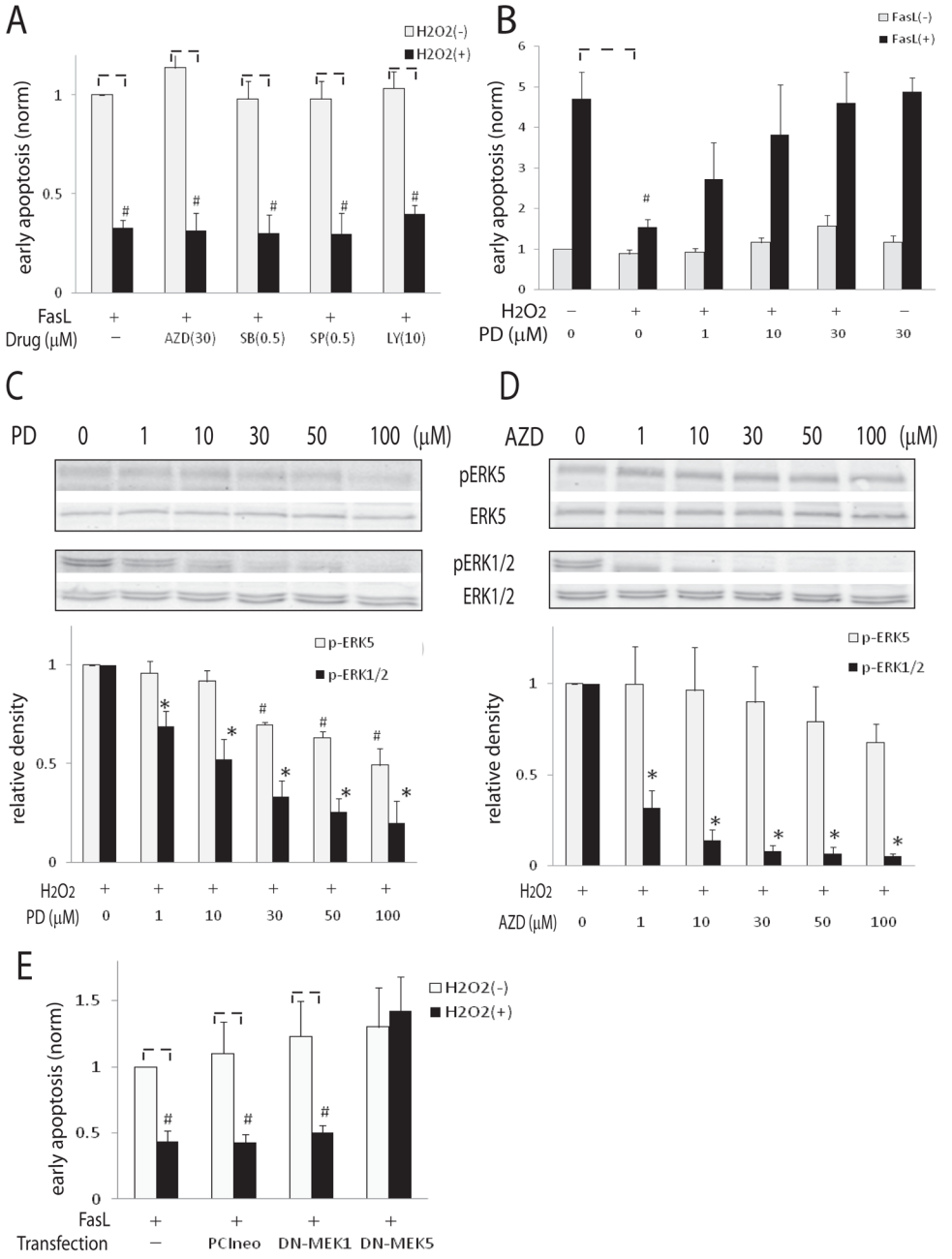

Fig. 2.

H_2O_2 provides cyto-protection through ERK5 activation. (A) Thirty minutes prior to the H_2O_2 pre-conditioning treatment, the cells were exposed to the following drugs: AZD6244 (specific MEK1/2 inhibitor, 30 μM), SB203580 (p38 inhibitor, 0.5 μM), SP600125 (JNK inhibitor, 0.5 μM), or LY294002 (PI3 kinase inhibitor, 10 μM). The drugs were present throughout the duration of FasL exposure. Bars represent the relative percentage of early apoptosis normalized to the FasL stimulation only group (data from 3 independent experiments each with triplicate measurements). # $P < 0.05$ for H_2O_2 (+) vs. H_2O_2 (−) comparison. (B) Similar experiment as above but with PD98059. # $P < 0.05$ for comparisons with no H_2O_2 no PD98059 group. PD98059 (C) or AZD6244 (D) were administered 30 minutes before a 3 minute 200 μM H_2O_2 exposure, cells were harvested immediately

thereafter, and lysates were subjected to Western blots. The membranes were probed with pERK5, pan ERK5, pERK1/2 and pan-ERK1/2. Representative blots shown on the top with a summary bar graph of a densitometric quantitation of 3 independent blots of the respective bands normalized to the no drug control condition on the bottom. # and * indicate $P < 0.05$ for pERK5 and pERK1/2, respectively, vs. no drug. (E) Cells were electroporated with plasmids encoding EGFP alone, DN (dominant negative)-MEK1, or DN-MEK5 both tagged with EGFP, and subjected to flow cytometry gated by EGFP fluorescence for detection of early apoptotic cells. The non-transfected group (left bars) was from cells without EGFP fluorescence in the same experiments thus controlling for potential cell damage from electroporation. The relative percentages of early apoptosis were normalized to the non-transfected control group stimulated with FasL but without H_2O_2. # $P < 0.05$ for H_2O_2 (+) vs. H_2O_2 (−) comparisons.

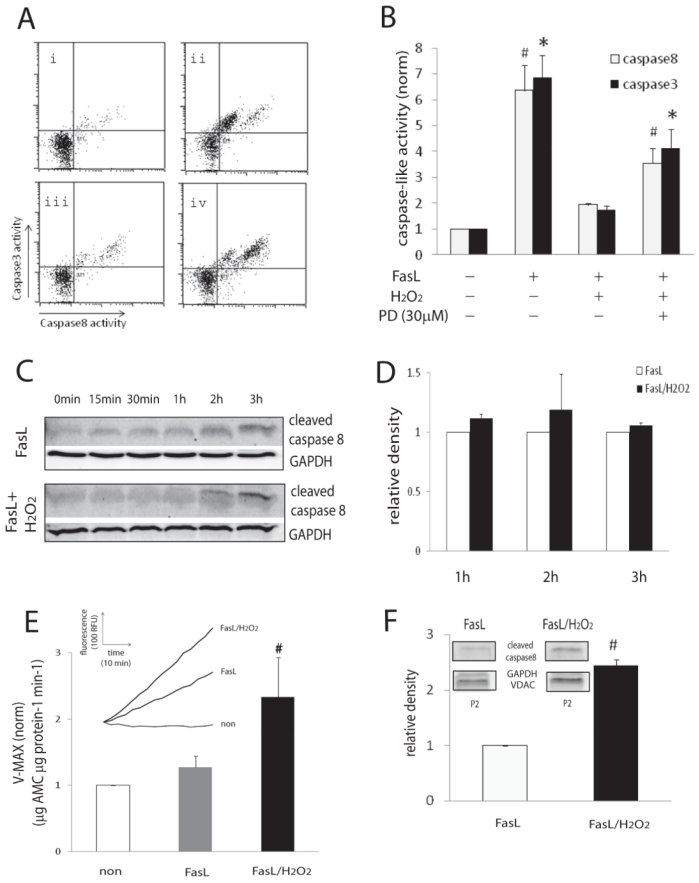

Fig. 3.

H_2O_2 decreases FasL-induction of active caspase-8 through increased mitochondrial sequestration. (A) Flow cytometer scatter grams depicting caspase-8 (horizontal) and -3 (vertical) activities in: i. non-stimulated, ii. FasL only, iii. FasL with H_2O_2, and iv. FasL with H_2O_2 but with PD98059. (B) A bar diagram summary (mean ± S.D. of 3 independent experiments) of caspase-8 and -3-like activities normalized to the non-stimulated group. PD98059 was initiated 30 minutes before H_2O_2 pre-conditioning. # and * $P < 0.05$ vs. non-stimulated group for the respective caspase. (C) Lysates harvested from cells at the indicated time points after FasL stimulation were subjected to a Western blot analysis and the membrane probed with anti-cleaved caspase antibody. GAPDH served as a loading control. Upper: FasL only group, Lower: FasL with H_2O_2 treatment group. (D) Densitometric quantitation of cleaved caspase-8 normalized to GAPDH. No difference in the protein amount of cleaved caspase-8 was seen between the FasL only and FasL with H_2O_2 treatment groups. (E) The caspase-8-like activity was measured using Ac-IETD-AMC as a substrate in lysates prepared from the mitochondrial protein enriched (P2 fraction). The inset shows the fluorescence signal over time in non-stimulated (non), FasL stimulated (FasL), and FasL stimulated with H_2O_2 treatment (FasL/H_2O_2) cells. Similar results were obtained in 3 independent experiments. V-MAX, the highest rate of fluorescence increase during kinetic measurement, was obtained and presented as μg AMC released μg protein^{-1} min^{-1} based on an AMC calibration standard defining fluorescence/AMC substrate. The bar graph shows the relative V-MAX normalized to the non-stimulated group. Data from 3 independent experiments. # $P < 0.05$ vs. non-stimulated group. (F) The protein amount of cleaved caspase-8 (18k Da) in the P2 fraction was evaluated in FasL stimulated (FasL) and FasL stimulated with H_2O_2 treatment (FasL/H_2O_2) groups by a Western blot (inset). Cells were

harvested at 3h after FasL stimulation. VDAC (mitochondrial marker) and GAPDH (cytosolic marker) served as markers to document P2 fraction enrichment of the mitochondria. Summary bar plot of densitometric quantitation of cleaved caspase-8 normalized to VDAC. The relative density in the FasL stimulated group was defined as 1.0. # $P < 0.05$.

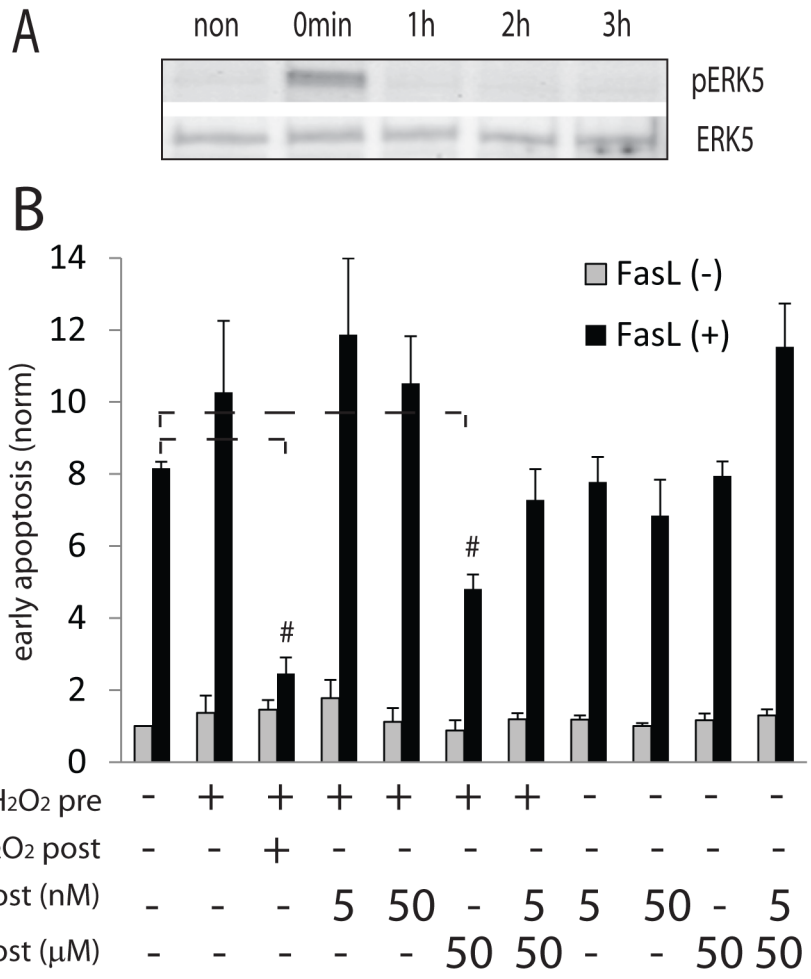

Fig. 4.

Benzylphosphonic acid mimics H_2O_2 post-conditioning. (A) Time course of pERK5 during H_2O_2 treatment examined by Western blot. Cells subjected to H_2O_2 pre- and post-conditioning were harvested at the indicated times. Note the transient increase in pERK5 completely gone by 1h. (B) H_2O_2 post-conditioning (H_2O_2 post, 20 μM) was replaced with the serine/threonine phosphatase inhibitor okadaic acid (OA post; 5 and 50 nM) or the protein tyrosine phosphatase inhibitor benzylphosphonic acid (BA post; 50 μM), and early apoptosis was evaluated 3 hours after FasL stimulation. Data from 3 independent experiments. # $P < 0.05$ for the indicated comparisons.