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Ghrelin Impedes Oxidative Stress Induced Intestinal Epithelial Cell Apoptosis through Varying Signaling Pathways

Talha Asad, Usman Arshad, Saleem Arif

IMPORTANCE Ghrelin, a gut brain peptide, has been primarily studied in the regulation of body weight homeostasis. Recent studies indicate, however, that it has potent anti-apoptotic effects in various cell types and exogenous ghrelin prevents gastric mucosa from ethanol-induced ulcer formation. Given ghrelin is cytoprotective, here we hypothesized ghrelin's anti-apoptotic effect in intestinal epithelial cells when exposed to severe oxidative stress, which plays a key role in the pathogenesis of various intestinal disorders.

MATERIALS & METHODS Intestinal epithelial cells, FHs74Int, IEC-6 and Caco-2 were used to assess anti-apoptotic effects of ghrelin following H2O2 treatment by TUNEL technique and flowcytometric analyses. In addition, mechanisms responsible for these effects were explored in relation to relevant signaling pathways including PI3K/Akt pathway and cytochrome 'c' dependent caspase-3 activation. PI3K/Akt and mitochondrial cytochrome 'c' release was assessed by western analysis and caspase-3 activity was determined by ELISA and Immunoflorescence.

RESULTS H2O2 potently increases the intestinal epithelial apoptosis and necrosis. Ghrelin inhibits intestinal epithelial cell apoptosis through growth hormone secretagogue receptors (GHS-Rs). Further, ghrelin's anti-apoptotic effect against H2O2 –induced apoptosis is associated with activation of PI3K/Akt, inhibition of mitochondrial cytochrome-c release and likewise inhibition of caspase-3 activation.

CONCLUSION In aggregate, our findings suggest that ghrelin inhibits oxidative stress-induced apoptosis in intestinal epithelial cells and hence might serve as a therapeutic strategy in states associated with intestinal mucosal injury, where oxidative stress plays a central role.

KEYWORDS Ghrelin, Intestinal apoptosis, signaling pathways, Intestinal epithelial cells, Intestinal tract

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Ghrelin is a recently characterized short gastrointestinal peptide, which has been implicated in the physiological regulation of body-weight homeostasis [1-3]. It is mainly produced by gastrointestinal tract and is subsequently released into systemic circulation to perform numerous functions during set of physiological and pathological states[1-3]. The systemically available ghrelin binds to diversely expressed, known as growth hormone secretagogue receptors (GHS-Rs) to perform its molecular activities[4]. One of the relatively newer functions attributed to ghrelin is its role in cytoprotection and mitogenesis [5-15]. Few previous reports suggest that ghrelin has potent cytoprotective effects on various cell types when exposed to pro-apoptotic agents [5-15]. Baldanzi et al were the first to demonstrate ghrlein's anti-apoptotic effects on endothelial cells and cardiomyocytes when exposed to doxourubicin, a well-known anti-cancer drug that has potent pro-apoptotic effects [14]. Later Choi et al complemented this finding by revealing that ghrelin protects adipocytes from iso-proterenol-induced cell death [15]. Preliminary evidences indicate that ghrelin promotes cytoprotection by activating intracellular cell survival proteins such as PI3K/Akt and MAPK [21]. Sibilia et al have extended this information to in vivo models of gastric mucosal injury[8]. They have demonstrated that ghrelin prevents ethanol-induced ulcer formation in rats, however the mechanism of this cytoprotective effects remains largely uncharacterized.

Reactive oxygen species (ROS) -induced intestinal epithelial cell apoptosis contributes to intestinal mucosal barrier dysfunction in various intestinal states including ischemia-reperfusion injury, inflammatory bowel disease and

necrotizing enterocolitis [16-25]. The mechanism of ROS-mediated cellular injury is not completely understood however it is known that ROS alter the mitochondrial structure and function of the cell to promote the pro-apoptotic effects [16]. The reactive oxygen species alter the permeability of the mitochondrial membrane and release the mitochondrial cytochrome 'c' into the cytosolic compartment. In the cytosolic compartment, cytochrome 'c' binds to the Apaf-1 (apoptosis initiating factor-1) and initiates its ATP-dependent oligomerization prior to caspase-9 activation. Caspase-9 then further stimulates the activation of various other caspases to ultimately converge at caspase-3, which serves as an endpoint of caspase-dependent apoptosis [16-25].

We hypothesized ghrelin's cytoprotective role in intestinal epithelial cells on two previously published reasons. First, ghrelin has cytoprotective effects in variety of in vitro cell systems and second, ghrelin protects the gastric mucosa from ethanol-induced ulcer formation [8, 16-25]. Therefore we sought ghrelin's cytoprotective effect in ROS-mediated apoptosis in intestinal epithelial cells. In current set of experiments, we have demonstrated ghrelin's anti-apoptotic effect in the presence of H_2O_2, and subsequently have explored mechanisms in relation to PI3K/Akt pathway, mitochondrial cytochrome 'c' release and caspase cascade.

MATERIALS AND METHODS

Cell lines: Non-transformed human derived from normal human small bowel (FHs74Int), rat small intestinal crypt cell line (non-transformed, IEC-6) and transformed Caco-2 cell line derived from human colon cancer cells were obtained from ATCC (Rockville, MD). FHs74Int and IEC-6 cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) containing 10% fetal bovine serum (FBS) without or with insulin respectively. Eagle's Minimal Essential Medium (EMEM) containing 20% FBS in a humidified (37°C, 5% CO_2) incubator was used to maintain Caco-2 cell line. These cell lines were then sub-cultured before confluence and were seeded at a density of $5x10^4$ cells/ml to ensure harmony among cell lineage.

Preparation of total cell lysates and cytosolic fractions of cell lysates: The lineage cells were rinsed two times with phosphate buffered saline (PBS) with a pH 7.4. The cells were gently scrapped, suspended in PBS and were paletted with centrifugation at 3200 rpm. Cell lineage extracts were lysed with lysis buffer containing 20mM Tris, pH 7.5, 0.1% Triton X, 0.5% deoxycholate, 1mM phenylmethylsulfonyl fluoride, 10µg/ml aprotinin and 10µg/ml leupeptin. They were finally cleared by centrifugation at 12000g at 4°C. For obtaining cytosolic fractions of the total cell lysates for assessment of cytochrome c release, cells were lysed by three cycles of freeze-thawing in a lysis buffer containing 20 mM HEPES/KOH (pH 7.5), 1.5 mM MgCl2, 10 mM KCl, 1 mM NaEDTA, 1 mM EGTA, 1 mM DTT, 0.1 nM PMSF, and 250 mM sucrose. Cells were then spun at 100,000 x g (45,000 rpm) at 4°C for 30 min. The supernatant was collected and were subjected to western analysis.

Western blot analysis: BCA assay kit (Sigma, St Louis, MO) with bovine serum albumin as a standard was used to measure total protein concentration, according to the manufacturer's instructions. Cell extracts containing 30µg total proteins were subjected to 10% SDS/PAGE. The resolved proteins were transferred electrophoretically to PVDF membranes (Invitrogen, Carlsbad, CA). Membranes were incubated with primary antibodies in PBS containing 0.1% Tween 20 overnight at 4°C after blocking with PBS containing 0.2% casein for 1 hour at room temperature. Following secondary antibody incubation the Vecstain™ ABC kit and DAB liquid substrate were utilized for chromogenic detection. Equal protein loading was confirmed by probing with 3µg/ml anti-actin monoclonal antibody (Lab Vision, Freemont, CA). The primary antibodies used were rabbit pAkt 1/2/3 (473), rabbit Akt and rabbit cytochrome 'c' (Santa Cruz Biotechnologies, Santa Cruz, CA). The detailed protocols are described in legends of the data figure.

Assessment of Apoptosis by TUNEL staining: Cell Apoptosis was assessed by the Cell Death kit (Roche). The cells were grown in 96- well plates and at 80% confluency were subjected to overnight serum starvation and were treated with ghrelin with or without [D-lys-3]-GHRP-6. Apoptosis was induced by H_2O_2 and the anti-apoptotic effects of ghrelin were analyzed by TUNEL technique. Briefly, the cells were washed with PBS with maximum care to avoid the cell layer damage and were TUNEL-stained according to the manufacturer's instructions. The cells were counter-stained with DAPI to count the total number of the cells. The apoptotic cells were counted under the light and fluorescence microscope per field and were expressed in percentage of the total cell count.

Assessment of Apoptosis by FACS® following Annexin V and Propidium Iodide Staining: Flowcytometry was employed to analyze earlier even events of apoptosis and to confirm TUNEL assay findings; Briefly, Caco-2 cells were plated in 6 well plates to 90% confluency, subjected to H_2O_2 treatment or prolonged starvation in the presence or absence of ghrelin and were stained by Annexin V and

Propidium Iodide according to manufacturer's instructions (Vybrant™ Anexin V staining kit #3, Molecular Probes, Eugene, OR). The samples were run in FACS® (Becton-Dickinson, San Jose, CA) and the data was analyzed by using software CellQuest™ (BD Biosciences, San Jose, CA).

Caspase-3 Profiling: Caspase-3 is an important endpoint of caspase-dependent cellular apoptosis. Caspase-3 activity was assessed by the TiterZyme Caspase-3 EIA kit (Assay Designs, Ann Arbor, Michigan) according to the manufacturer's instructions. Briefly, the Caco-2 cells were seeded and at 90% confluency were subjected to starvation in the presence or absence of various chemicals and peptides. Cell lysates were prepared as described in the western blot section and were analyzed for the caspase-3 activity according to the manufacturer's instructions.

Florescence Immunocytochemistry for Caspase-3 & 7: The role of ghrelin in modulation of caspase 3 & 7 was also confirmed by Magic RedTM detection kit (Immunochemistry Technologies, Bloomington, MN), which utilizes the fluorphore, cresyl violet coupled with MR-$(DEVD)_2$. This MR-Caspase photostable fluorogenic substrate easily penetrates the cell membrane and membrane of the internal organelles. In the presence of Caspase 3 and 7 within the cell, the caspase sequences are cleaved to yield a red fluorescent product visible under fluorescence microscope. Briefly the Caco-2 cells were grown in chamber slides and were subjected to H_2O_2 treatment for 24 hours. The cells were treated with MR-$(DEVD)_2$ for an hour and were counter stained by Hoechst 33342 for 10 minutes. The slides were analyzed by the Fluorescence microscopy using UV filter (excitation at 365 nm and emission at 480 nm) and green filter (excitation at 550 with a long pass >610nm emission/barrier filter pairing) respectively for the Hoechst staining and MR-$(DEVD)_2$.

Statistical Analysis: All of the data was expressed in terms of the means ± S.E for a series of 'n' number of the experiments. To assess the significant difference between the groups Non-parametric Mann-Whitney U t-test was employed to compare the groups. A p value < 0.05 was considered to be statistically significant. All of the statistics were performed using the program Statistica (StatSoft Inc. Tulsa, OK).

RESULTS

Ghrelin inhibits the H_2O_2 –induced apoptosis and necrosis in intestinal epithelial cells: Various reactive oxygen species (ROS) are produced on the inner mitochondrial membrane during physiological states. Hydrogen peroxide (H_2O_2) is one of the oxidants, which is known to be produced by the mitochondrial respiratory chain and induces apoptosis dose dependently in various cell types including intestinal epithelial cells [16,25]. Consistent with the previous findings, here we demonstrate that at concentrations consistent with the pathologic states like intestinal inflammation, ischemia or infection, H_2O_2 significantly induces the apoptosis in FHs74Int, IEC-6 and Caco-2 intestinal epithelial cells (*$p<0.05$ vs. untreated controls) (Fig.1 A&B). Our TUNEL analyses data suggest that ghrelin pretreatment inhibits H_2O_2-induced apoptosis in FHs74Int, IEC-6 and Caco-2 intestinal epithelial cells significantly compared to H_2O_2 treated cells in a dose dependent fashion (**$p<0.05$ vs. H_2O_2 treatment). We have previously shown that GHS-Rs are expressed in intestinal epithelial cells [26,27]. To address whether ghrelin mediates its anti-apoptotic effects specifically through GHS-Rs, we antagonized the GHS-R with a receptor specific antagonist (D[lys-3]-GHRP-6) and analyzed the apoptosis. Antagonism of GHS-Rs through a competitive antagonist (D[lys-3]-GHRP-6) leads to loss of ghrelin-mediated anti-apoptotic effect (Fig.1 A&B). In the next step, we confirmed our TUNEL assay findings with flowcytometric analysis following Annexin V and PI staining (Fig. 2 A&B). The flowcytometric experiments not only support the previous findings but also provide the assessment of oxidative stress-induced intestinal epithelial cell necrosis. Ghrelin not only inhibits the apoptosis (**$p<0.05$ vs. H_2O_2 treatment) but also significantly reduces the necrotic cell death in Caco-2 intestinal epithelial cells (¥, $p<0.05$ vs. H_2O_2 treatment) as compared to H_2O_2 treatment.

Ghrelin's anti-apoptotic effect in the presence of H_2O_2 is associated with activation of PI3K/Akt complex: In second set of experiments, we analyzed the signaling events associated with ghrelin's anti-apoptotic effects in the presence of H_2O_2. Phosphatidyl-inositol 3-kinase (PI3K) /Akt complex is an important intracellular moiety, which plays key role in cytoprotection during stress [27,29]. We analyzed the status of Akt phosphorylation in the presence of H_2O_2 and ghrelin.

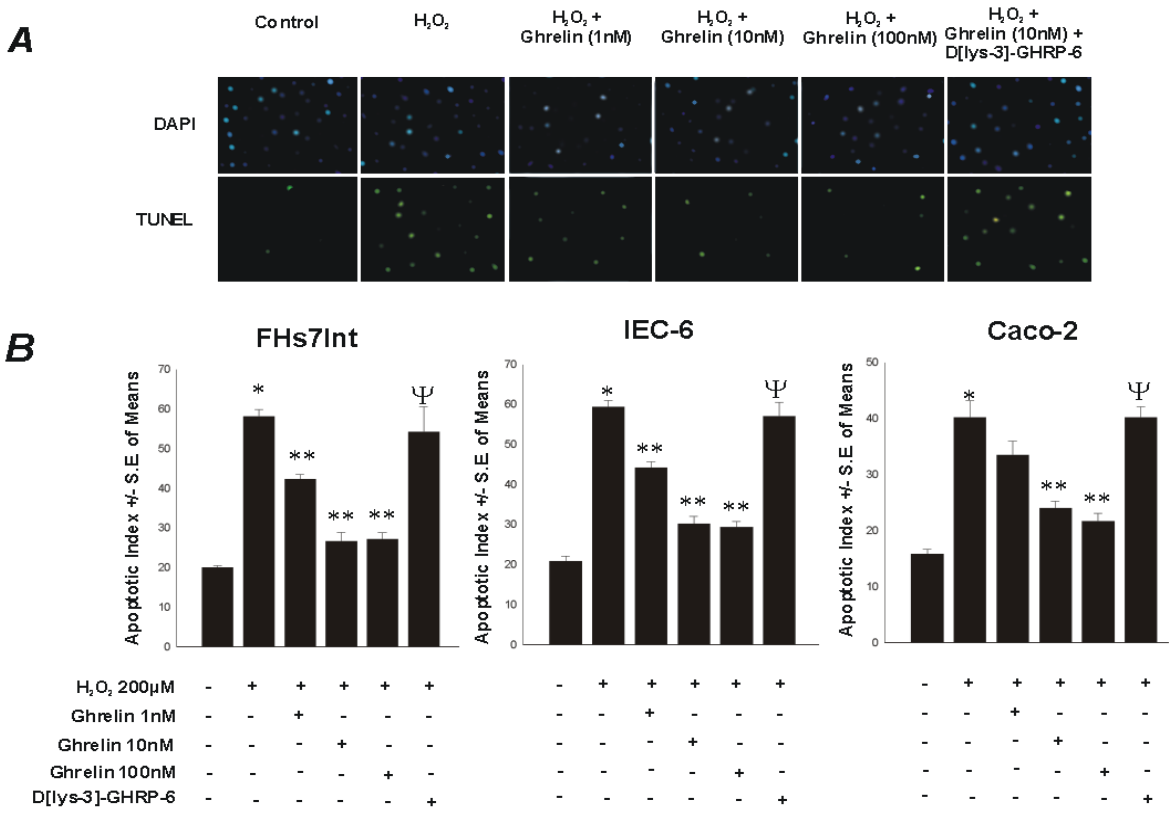
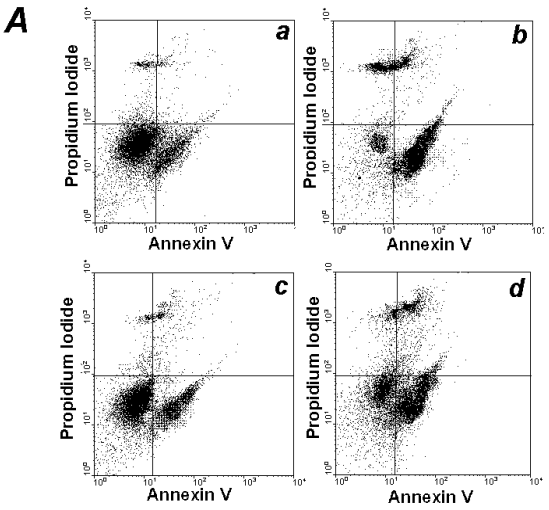


Figure 1. Representative TUNEL analysis showing ghrelin's anti-apoptotic effects on H2O2-induced apoptosis in intestinal epithelial cells. Intestinal epithelial cells were pretreated with ghrelin (1nM-100nM) for 4 hours along with or without ghrelin receptor antagonist, D[lys-3]-GHRP-6, apoptosis was stimulated by 24-hour treatment with 200µM H2O2 and was analyzed by TUNEL and DAPI staining. A, The upper panel shows DAPI staining of cellular nuclei in Caco-2 cells to mark the total number of cells; the lower panel is TUNEL staining. B, Quantitative representation of data; H2O2 potently induces apoptosis in FHs74Int, IEC-6 and Caco-2 intestinal epithelial cells (*p<0.05 vs. untreated controls), ghrelin pretreatment significantly inhibits the apoptosis (**p<0.05 vs. H2O2 200µM) and D[lys-3]-GHRP-6 pre-treatment abolishes ghrelin's anti-apoptotic effects (Ψ, p<0.05 vs. ghrelin10nM+ H2O2 200µM). Values have been presented in form of Means ± S.E. of at least three independent experiments; a p value <0.05 was considered to be statistically significant.



B	Agent / Peptide Treatment	No staining (% Living Cells Fraction)	Annexin V staining (% Apoptotic Cells Fraction)	PI staining (% Necrotic Cell Fraction)
	No treatment	75.88±3.7	15.85±3.38	8.27±0.89
	H_2O_2 treatment (200 µM)	15.94±1.9	58.60±4.80*	25.40±3.22$^{\Psi}$
	Ghrelin (10nM) + H_2O_2 (200 µM)	59.77±3.0	26.16±2.54**	14.05±4.41$^{¥}$
	D[lys-3]-GHRP-6 (1µM) + Ghrelin (10nM)+ H_2O_2 (200µM)	20.76±3.9	59.36±0.94§	19.87±3.46$^{\Phi}$

Figure 2. Representative flowcytometric analysis showing ghrelin's anti-apoptotic effects on H_2O_2-induced apoptosis in intestinal epithelial cells. Caco-2 intestinal epithelial cells were pretreated with 10nM ghrelin for 4 hours; apoptosis was stimulated by 24-hour

treatment of 200μM H_2O_2 and was analyzed by flowcytometric analysis followed by staining with Annexin V/ PI. A, Dot plot diagrams showing the induction of apoptosis by H_2O_2, and anti-apoptotic effects of ghrelin. A, The left lower quadrants in each dot plot panel represents living fraction of the cell (Annexin V-/PI-), right lower quadrant represents apoptotic fraction (Annexin V-/PI+) and upper right quadrant represents necrotic fraction of the cells (Annexin V+/PI-); A (a) un-stimulated control; A (b), H_2O_2 treatment; A (c), H_2O_2 + ghrelin (10nM); A (d), D[lys-3]-GHRP-6 + H_2O_2 + ghrelin. B, Table is quantitative representation of data; H_2O_2 potently induces apoptosis in Caco-2 intestinal epithelial cells (*p<0.05 vs. untreated controls), ghrelin significantly inhibits the apoptosis of epithelial cells (**p<0.05 vs. H_2O_2 200μM) and ghrelin anti-apoptotic effect is lost if we pretreat the cells with D[lys-3]-GHRP-6 (§p<0.05 vs. H_2O_2 200μM + 10nM ghrelin). Ghrelin also inhibits the necrosis of intestinal epithelial cells through GHS-Rs, significantly compared to H_2O_2 treatment (¥p<0.05 vs. H_2O_2 treatment). Values have been presented in form of Means ± SD of at least three independent experiments; a p value <0.05 was considered to be statistically significant.

Ghrelin stimulates the phosphorylation of Akt through GHS-Rs (Fig.3 A) and antagonism of GHS-Rs or PI3K by D[lys-3]-GHRP-6 or Wortmannin respectively leads to loss of ghrelin mediated Akt phosphorylation (Fig.3 A) and eventually loss of ghrelin's anti-apoptotic effects (**p<0.05 vs. H_2O_2 + ghrelin) (Fig.3 B).

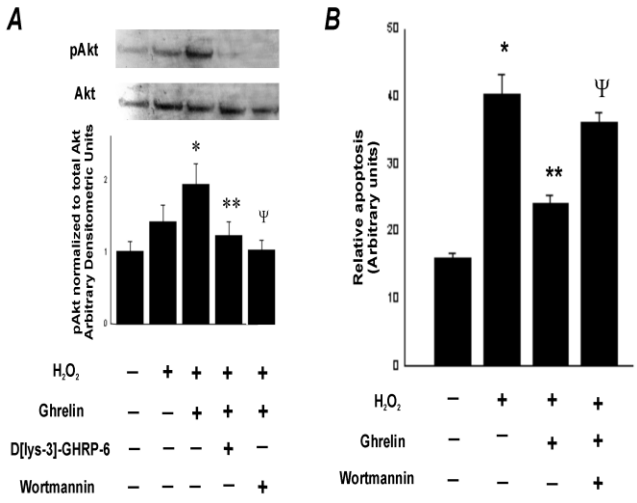


Figure 3. Ghrelin prevents Caco-2 intestinal epithelial cell apoptosis through PI3K/Akt dependent mechanism. A, Caco-2 intestinal epithelial cells were treated with H_2O_2 with or without ghrelin in the presence or absence of Wortmannin (1μM) and D[lys-3]-GHRP-6 (1μM). Ghrelin stimulates the phosphorylation of Akt in Caco-2 intestinal epithelial cells in the presence of H_2O_2, which is abolished following PI3K/ Akt and D[lys-3]-GHRP-6 pretreatment. Densitometric analysis complements the blot; ghrelin significantly stimulates phosphorylation of Akt in Caco-2 intestinal epithelial cells (*p<0.05 vs. untreated controls) and ghrelin-mediated Akt phosphorylation is lost if we pretreat the cells with D[lys-3]-GHRP-6 (**p<0.05 vs. H_2O_2 200μM + 10nM ghrelin) or Wortmannin (Ψ, p vs. H_2O_2 200μM + 10nM ghrelin). B, TUNEL analysis; Wortmannin pretreatment (60 minutes prior to ghrelin treatment) inhibits the ghrelin-mediated anti-apoptotic effects in Caco-2 intestinal epithelial cells. Values have been presented in form of Means ± SE of at least three independent experiments; a p value <0.05 was considered to be statistically significant.

Ghrelin's anti-apoptotic effect in the presence of H_2O_2 is associated with inhibition of mitochondrial cytochrome 'c' release into the cytosol: ROS structurally and functionally disable the mitochondria, which are the main sources of energy to the cells. Various oxidative agents including H_2O_2 are produced in the mitochondrial membrane and disrupt it to release cytochrome 'c' into the cytosolic compartment [30]. Cytochrome 'c' subsequently initiates the caspase cascade to promote the cellular apoptosis [30]. Therefore, we analyzed the release of cytochrome 'c' into cytosolic compartment through western blot analysis of cytosolic extracts of the cell lysates. Our data (Fig 4& B) reveal that H_2O_2 potently stimulates the release of cytochrome 'c' into the cytosolic compartment (Fig 4B; *p<0.05 vs. untreated controls). Ghrelin pretreatment inhibits the release of cytochrome 'c' into the cytosolic compartment (Fig.4 A&B), hence might prevent the initiation of caspase dependent apoptosis.

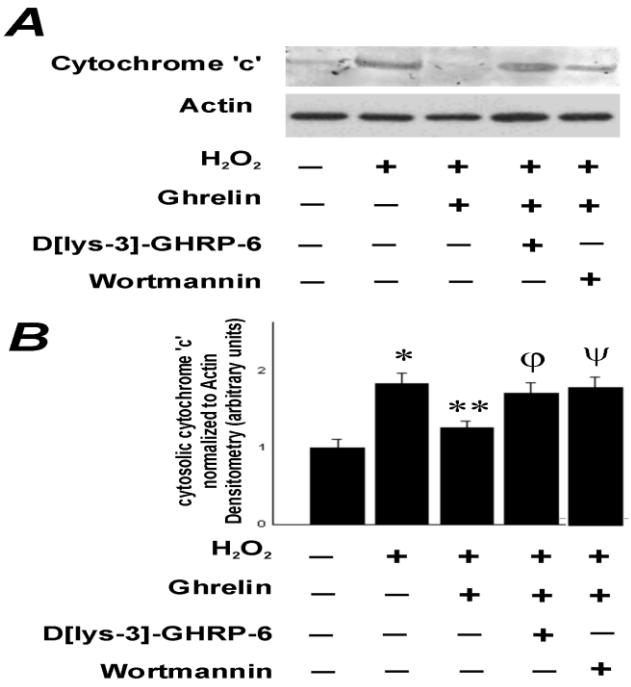


Figure 4. Ghrelin's anti-apoptotic effects are associated with inhibition of mitochondrial cytochrome-c release into cytosolic

compartment in Caco-2 intestinal epithelial cells. A, Caco-2 intestinal epithelial cells were treated with H_2O_2 with or without ghrelin (10nM) in the presence or absence D[lys-3]-GHRP-6 (1μM) and western analysis was performed with cytosolic fractions of the cell lysates. Ghrelin inhibits H_2O_2induced mitochondrial cytochrome-c release into cytosolic compartment, which is abolished following D[lys-3]-GHRP-6 pretreatment. B, representative densitometric analysis of three independent experiments normalized to Actin levels. H_2O_2 induces the release of mitochondrial cytochrome-c (*p<0.05 vs. no treatment); ghrelin inhibits the release of mitochondrial cytochrome-c release (**p<0.05 vs. H_2O_2). Values have been presented in form of Means ± SE; a p value <0.05 was considered to be statistically significant.

Ghrelin's anti-apoptotic effect in the presence of H_2O_2 is associated with inhibition of caspase-3 activation: Additionally, we analyzed the status of activation of caspase-3 in H_2O_2 activated Caco-2 cells by ELISA assay and by immunostaining the caspase-3/7 by a newly introduced technique (see the details in Materials and Methods section). Ghrelin inhibits the activation of caspase-3 (**p<0.05 vs. H_2O_2)(Fig.5 A&B), which is central effecter caspase molecule in caspase dependent apoptosis. Ghrelin mediates this caspase-3 activation through GHS-R because GHS-R antagonism leads to loss of ghrelin's cytoprotective role. Additionally, caspase-3 inhibitor also abolishes the ghrelin-mediated caspase-3 activation further confirming the involvement of caspase pathway in ghrelin-mediated anti-apoptotic mechanisms.

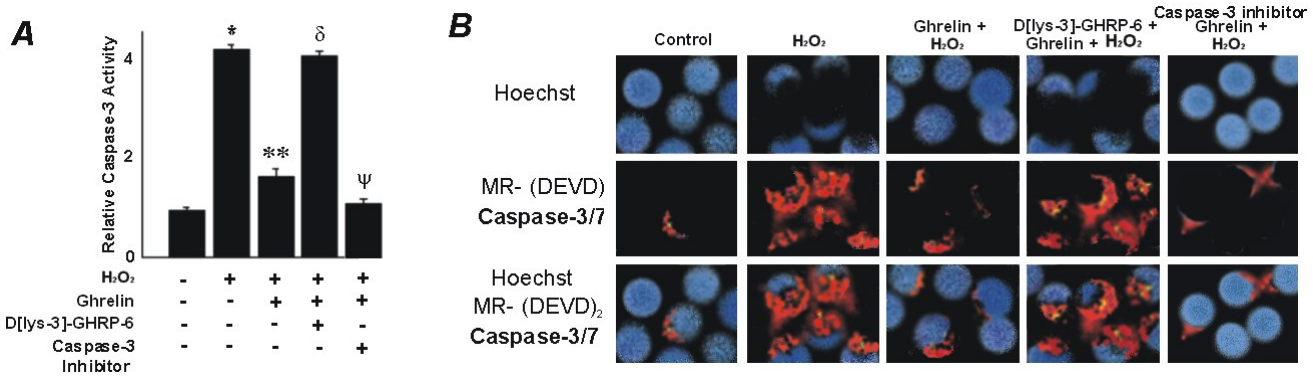


Figure 5. Ghrelin's anti-apoptotic effects are associated with inhibition of Caspase-3 activation in Caco-2 intestinal epithelial cells. A, Caco-2 intestinal epithelial cells were treated with H_2O_2 with or without ghrelin (10nM) in the presence or absence of D[lys-3]-GHRP-6 (1μM) and Caspase-3 levels were analyzed by ELISA. H_2O_2 stimulates the activation of caspase-3 significantly above the untreated controls (*p<0.05 vs. no treatment). Ghrelin pretreatment inhibits H2O2-induced caspase-3 activation (**p<0.05 vs. H_2O_2), and ghrelin mediated caspase-3 activation is abolished D[lys-3]-GHRP-6 pretreatment (δ, p<0.05 vs. H_2O_2+ ghrelin) or caspase-3 inhibitor (1μM) pretreatment (Ψ, p<0.5 vs. H_2O_2 + ghrelin). Values have been presented in form of Means ± SE of at least three independent experiments; a p value <0.05 was considered to be statistically significant. B, representative fluorescence staining of caspase-3/7 following various treatments; Upper panel represents the Hoechst staining of cellular nuclei, Middle panel represents the caspase-3/7 staining of the cells and in Lower panel, the Hoechst and caspase-3/7 stainings have been merged. H_2O_2induces the activation of caspse-3/7 in Caco-2 cells following H_2O_2 treatment; ghrelin inhibits the activation of caspase-3/7.

DISCUSSION

The intestinal epithelium functions as a highly selective barrier that allows the absorption of dietary nutrients yet restricts entry of the pathologic antigens [31]. The precision and balance in the absorptive function of this barrier function entails critical role in maintaining the mucosal immune homeostasis and epithelial integrity [32,33]. During the intestinal states associated with inflammation, ischemia and infection the intestinal barrier function is significantly compromised leading to the structural and functional abnormalities in the intestinal tract [32,33]. Reactive oxygen species (ROS) are one of the important pathologic products of the mucosal leukocytes during ischemia, infection and inflammation that lead to intestinal epithelial cell apoptosis and eventually intestinal barrier dysfunction [32,33].

Ghrelin has recently gained attention in relation to cytoprotection and mitogenesis in variety of in vitro cell systems [5,8,26]. It prevents apoptosis in endothelial cells and cardiomyocytes and cell death in adipose cells [21]. Though the detailed mechanism of ghrelin's cytoprotective activities has not been well characterized, however the preliminary studies indicate that ghrelin prevents cellular apoptosis through induction of various cytoprotective intracellular proteins including Akt and MAPK [13]. Here, we have examined the role of ghrelin in H_2O_2-induced

apoptosis in intestinal epithelial cells and subsequently have explored subcellular signaling events.

Hydrogen peroxide (H_2O_2) is also one of the oxidative agents, which promote both apoptosis and necrosis in intestinal epithelial cells [33,34]. It is believed to induce the intestinal epithelial cell necrosis and apoptosis through either the exogenous sources or through autocrine mechanisms of ROS overproduction triggered by the pro-inflammatory cytokines [33]. In current set of experiments, we have used the dose of H_2O_2 that is equivalent to the amount produced by the macrophages during intestinal ischemia-reperfusion injury or inflammation. Consistent with previous literature, exogenous H_2O_2 induces significant apoptosis and necrotic cell death following a 24-hour treatment. Our TUNEL analyses show that exogenous ghrelin treatment prevents H_2O_2-induced apoptosis in FHs74Int, IEC-6 and Caco-2 intestinal epithelial cells. This anti-apoptotic effect is mediated in a dose dependent manner through GHS-Rs, because ghrelin-mediated cytoprotective effect is lost in the presence of GHS-R antagonist, D[lys-3]-GHRP-6. In addition, our flowcytometric data corroborate our TUNEL assay findings. Ghrelin significantly reduces the apoptotic cell fraction from ~60% to ~25%. Flowcytometric analyses also indicate that ghrelin reduces intestinal epithelial necrosis significantly suggesting that ghrelin prevents apoptosis as well as necrosis in intestinal epithelial cells under oxidative stress.

Phosphatidylinositol 3-kinase (PI 3-kinase), a ubiquitous lipid kinase, along with Akt is involved in cell survival. Promotion of cell survival by the activation of PI 3-kinase/Akt occurs by the inhibition of proapoptotic signals and the induction of survival signals [35-37]. PI3K/ Akt also inhibits the production of endogenous ROS to limit the apoptosis by maintaining the mitochondrial integrity. Baldanzi et al have previously shown that ghrelin prevents doxourubicin-induced cell death in cardiomyocytes through activation of Akt [13]. Consistent with these previous findings our data indicate that ghrelin stimulates the phosphorylation of Akt in intestinal epithelial cells in the presence of H_2O_2 through GHS-Rs and chemical inhibition of PI3K-Akt signaling results in failure of the ghrelin to inhibit the oxidative stress-induced apoptosis.

Another mechanism of H_2O_2-induced apoptosis in intestinal epithelial cells is mediated through disruption of mitochondrial membrane. Ruptured mitochondrial membrane releases cytochrome 'c' into cytosolic compartment, which in turn activates caspase-9, an initiator caspase and caspase-3, an effecter endpoint caspase to promote apoptosis in intestinal epithelial cells [34]. Here we show that ghrelin inhibits the H_2O_2-mediated release of cytochrome 'c' from intestinal epithelial cells. The ghrelin-mediated inhibition of mitochondrial cytochrome 'c' release also affects the downstream caspase-pathway. Our data clearly shows that ghrelin potently inhibits the activation of caspases in the presence of H_2O_2 to prevent intestinal epithelial apoptosis.

In summary, we have clearly demonstrated that ghrelin has potent anti-apoptotic effects on intestinal epithelial cells under oxidative stress. Further, ghrelin's anti-apoptotic effects on intestinal epithelial cells under oxidative stress are associated with activation of PI3K/Akt and inhibition of release of mitochondrial cytochrome 'c' release and caspase-3 activity. Given, ghrelin has been found to be mitogenic, anti-inflammatory and anti-apoptotic, the therapeutic utility of ghrelin in intestinal conditions associated with mucosal barrier dysfunction warrants further investigation [38,39].

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