Protection of cultured human hepatocytes from hydrogen peroxide-induced apoptosis by relaxin-3

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Abstract. Previous studies have suggested that hepatocyte apoptosis may be a fundamental underlying mechanism of liver injury and diseases, such as liver fibrosis. Relaxin-3 has been reported to have anti-fibrotic actions in the heart and to attenuate isoproterenol-induced myocardial injury; however, the beneficial role of relaxin-3 on hepatocyte apoptosis remains to be elucidated. The aim of the present study was to explore the role and possible mechanisms of relaxin-3 through hydrogen peroxide (H_2O_2)-induced apoptosis in primary human hepatocytes. Cells were treated with relaxin-3 and then cell viability, morphological features, the presence of cleaved caspases as well as the levels of endoplasmic reticulum stress (ERS) protein markers and autophagy markers were evaluated. The H_2O_2 group showed significantly decreased cell viability, increased apoptosis as well as upregulation of caspases (cleaved caspase-3, -8 and -9) and ERS protein markers compared with those of the control group. However, cells treated with relaxin-3 (10 ng/ml) demonstrated improved cell viability, reduced apoptosis and decreased expression of cleaved caspases and ERS markers. However, the expression of autophagy markers remained unchanged following H_2O_2-induced apoptosis and relaxin-3 treatment. In conclusion, relaxin-3 was shown to protect hepatocytes from H_2O_2-induced apoptosis via down-regulation of cleaved caspase-8 and -9, as well as inhibition of the ERS pathway.

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Introduction

Overproduction of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), may induce pathological hepatocyte apoptosis (1). Oxidant-induced apoptosis has a pivotal role in the development and progression of liver diseases, including alcoholic liver diseases (2), drug-induced liver injury (3), viral hepatitis (4,5), cholestatic liver diseases (6), non-alcoholic steatohepatitis (7) and ischemic/reperfusion injury, and also contributes to liver fibrogenesis (8). Therefore, the inhibition of hepatocyte apoptosis may be a promising novel therapeutic choice for the treatment of liver injury and fibrosis.

Apoptosis is initiated through two fundamental pathways: The extrinsic pathway, mediated by death receptors, and the intrinsic pathway, initiated by mitochondrial dysfunction (9). The cysteine aspartate protease (caspase) family of enzymes are key molecules whose activation may result in apoptosis; these include upstream initiator caspases (e.g. caspase-8 and -9) and downstream effector caspases (e.g. caspase-3). In addition, ROS-induced endoplasmic reticulum stress (ERS) may initiate pathways which lead to caspase activation and apoptosis (10). The primary ERS-associated pathways are activated by endoplasmic reticulum (ER) membrane-associated proteins, including protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1 and activating transcription factor 6. Each of these pathways upregulates the transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP), which results in decreased expression of anti-apoptotic B cell lymphoma (Bcl)-2 (11) and increased expression of pro-apoptotic Bcl-2 interacting mediator of cell death (12), therefore inducing apoptosis. The PERK pathway also activates caspase-12, which directly cleaves procaspase-9 and then activates caspase-3, resulting in apoptosis (13).

Relaxin-3, first identified in 2002, is the ancestral peptide of the human relaxin subclass of the insulin superfamily (14). The primary site of relaxin-3 messenger (m)RNA expression is the brain; however, relaxin-3 is also present in other tissues, such as the liver (14). At present, the roles and mechanism of action of relaxin-3 remain to be fully elucidated; one study suggested that relaxin-3 was involved in brain functions,

including the stress response and regulation of food intake, in combination with relaxin family peptide receptor 3 [RXFP-3/G protein-couple receptor (GPCR) 135] (15). In addition, relaxin-3 was also reported to bind to RXFP-1/leucine-rich repeat-containing GPCR 7 (LGR7; the primary receptor of relaxin-2) (16). In a previous study, LGR7 was expressed at a low level in normal rat livers; however, cirrhotic rat livers expressed significantly increased LGR7 levels in active hepatic stellate cells (17). Relaxin-3 treatment was reported to significantly increase the production of cyclic adenosine monophosphate, indicating the role of relaxin-3 in liver injury protection (18). Previous studies demonstrated that relaxin-2, in combination with LGR7, inhibited apoptosis in reproductive organ tissues during pregnancy (19) and in the heart (20). It was therefore hypothesized that relaxin-3 may attenuate hepatocyte apoptosis and protect against liver injury. The present study aimed to investigate the direct effect of relaxin-3 on hepatocyte apoptosis and its mechanism of action.

Materials and methods

Reagents. Synthetic human relaxin-3 was obtained from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Rabbit anti-human CHOP polyclonal antibody as well as rabbit anti-human cleaved caspase 8 and 12 polyclonal antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-human Beclin 1 polyclonal antibody as well as rabbit anti-human cleaved caspase 9 and 3 antibodies were purchased from Cell Signaling Technologies, Inc. (Beverly, MA, USA). Rabbit anti-human microtubule associated protein 1 light chain 3 (LC3) polyclonal antibodies were purchased from Sigma (St. Louis, MO, USA). All chemicals and reagents used in this study were of analytical grade. The human normal liver cell line L02 was purchased from the cell bank of the Institute of Biochemistry and Cell Biology (Shanghai, China).

Hepatocyte culture and treatment. Human hepatocyte L02 cells were cultured at 37˚C in an incubator (5% CO_2, 95% air). RPMI-1640 medium (Hyclone, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (Sijiqing, Inc., Huzhou, China) was used for the cell cultures. A dose-response experiment was initially conducted on the cells, which were treated with 0, 20, 50, 100, 200, 400, 600, 800 and 1,000 μmol/l H_2O_2 (Sigma); 200 μmol/l was selected as the optimal dose for all subsequent experiments. At ~70-80% confluence, cells were divided into the following five groups: Control; H_2O_2, 200 μmol/l H_2O_2; R10, 200 μmol/l H_2O_2 + 10 ng/ml relaxin-3; R50, 200 μmol/l H_2O_2 + 50 ng/ml relaxin-3; and R100, 200 μmol/l H_2O_2 + 100 ng/ml relaxin-3. Following co-incubation for 24 h, cells from all five groups were collected for analysis.

Cell viability. Relative cell viability was determined using an MTT assay (Sigma). Cells were plated in 96-well microtiter plates (Corning, Inc., New York, NY, USA). Following cell treatment, MTT was added to the culture medium to yield a final MTT concentration of 0.5 mg/ml; cells were then incubated for 4 h at 37˚C. The dye was dissolved by adding dimethyl sulfoxide at room temperature for 10 min. The preparations were agitated thoroughly with the cells containing formazan crystals using a plate shaker. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Inc., Hercules, CA, USA).

Hoechst staining. Apoptotic cells were characterized due to a distinctive condensed nuclear structure following staining with Hoechst 33258 (Beyotime, Inc., Haimen, China) and visible chromosomal fragmentation. Treated cells were fixed with 4% paraformaldehyde (Westang, Inc., Shanghai, China) for 15 min at room temperature, washed in phosphate-buffered saline (PBS) and then stained with Hoechst dye for 20 min at room temperature in the dark. Following washing with PBS, blue fluorescent cells were examined under a confocal scanning laser microscope (Nikon, Inc., Tokyo, Japan).

Transmission electron microscopy. Cells were harvested and fixed using 3.0% glutaraldehyde and 1.5% paraldehyde. Cells were then washed in PBS, fixed in osmium tetroxide (Xiya, Inc., Chengdu, China) and then dehydrated in an ethanol series. Subsequently, the samples were embedded in epoxy resin and examined under a transmission electron microscope (Olympus, Inc., Tokyo, Japan).

Western blot analysis. Following treatment for 24 h, cells were washed with PBS and resuspended in cold lysis buffer containing phenylmethylsulfonyl fluoride. Cell lysates were incubated on ice for 30 min and then centrifuged at 12,000 xg for 15 min at 4˚C. The protein content of the supernatant was determined using a bicinchoninic acid-200 protein assay kit (Beyotime, Inc.). Equal amounts of protein (20 μg) from each group were separated using 12% SDS-PAGE (Sanland, Inc., Xiamen, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (Gelman, Inc., Morgan Hill, CA, USA). The membranes were blocked using 5% skimmed milk (Yili, Inc., Neimenggu, China) for 1 h at room temperature with agitation and then incubated with the corresponding primary antibodies overnight at 4˚C. All of the following primary antibodies were diluted in Tris-buffered saline/Tween 20 (TBST) solution (anti-β-actin, 1:2,000; anti-CHOP, 1:500; anti-cleaved caspase-12, 1:500; anti-cleaved caspase-8, 1:1,000; anti-cleaved caspase-9, 1:1,000; anti-cleaved caspase-3, 1:1,000; anti-Beclin-1, 1:1,000; and anti-LC3, 1:1,000). Samples were then washed three times (10 min/wash) with TBST and a secondary antibody (1:2,000) conjugated with alkaline phosphatase (ZSGB-BIO, Inc., Beijing, China) was added to the membranes, which were then incubated at room temperature for 1 h with agitation. The membranes were then washed three times (10 min/wash) prior to visualization using Western Blue® stabilized substrate for alkaline phosphatase (Promega Corp., Madison, WI, USA). In order to quantify the band intensities, the western blot membranes were scanned using ImageJ software (National Institute of Health, Bethesda, MA, USA). The results were normalized based on the respective levels of β-actin.

Statistical analysis. GraphPad Prism software version 5.0 (La Jolla, CA, USA) was used for data analysis. Each experiment was repeated a minimum of three times and

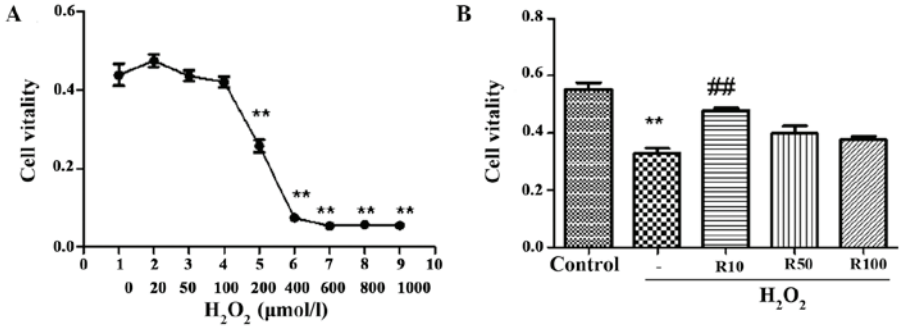

Figure 1. Relaxin-3 improves H_2O_2-induced decreased hepatocyte viability. (A) An MTT assay demonstrated the dose-dependent decrease in hepatocyte viability following treatment with concentrations of H_2O_2 for 24 h. (B) Hepatocyte viability following H_2O_2-induced apoptosis alone or in combination with relaxin-3 treatment (R10, R50 and R100) for 24 h. Values are expressed as the mean ± standard deviation (n=6). **P<0.01 vs. control, $^{\#\#}$P<0.01 vs. H_2O_2. H_2O_2, hydrogen peroxide-treatment; R10/50/100, treatment with 10, 50 or 100 ng/ml relaxin-3, respectively.

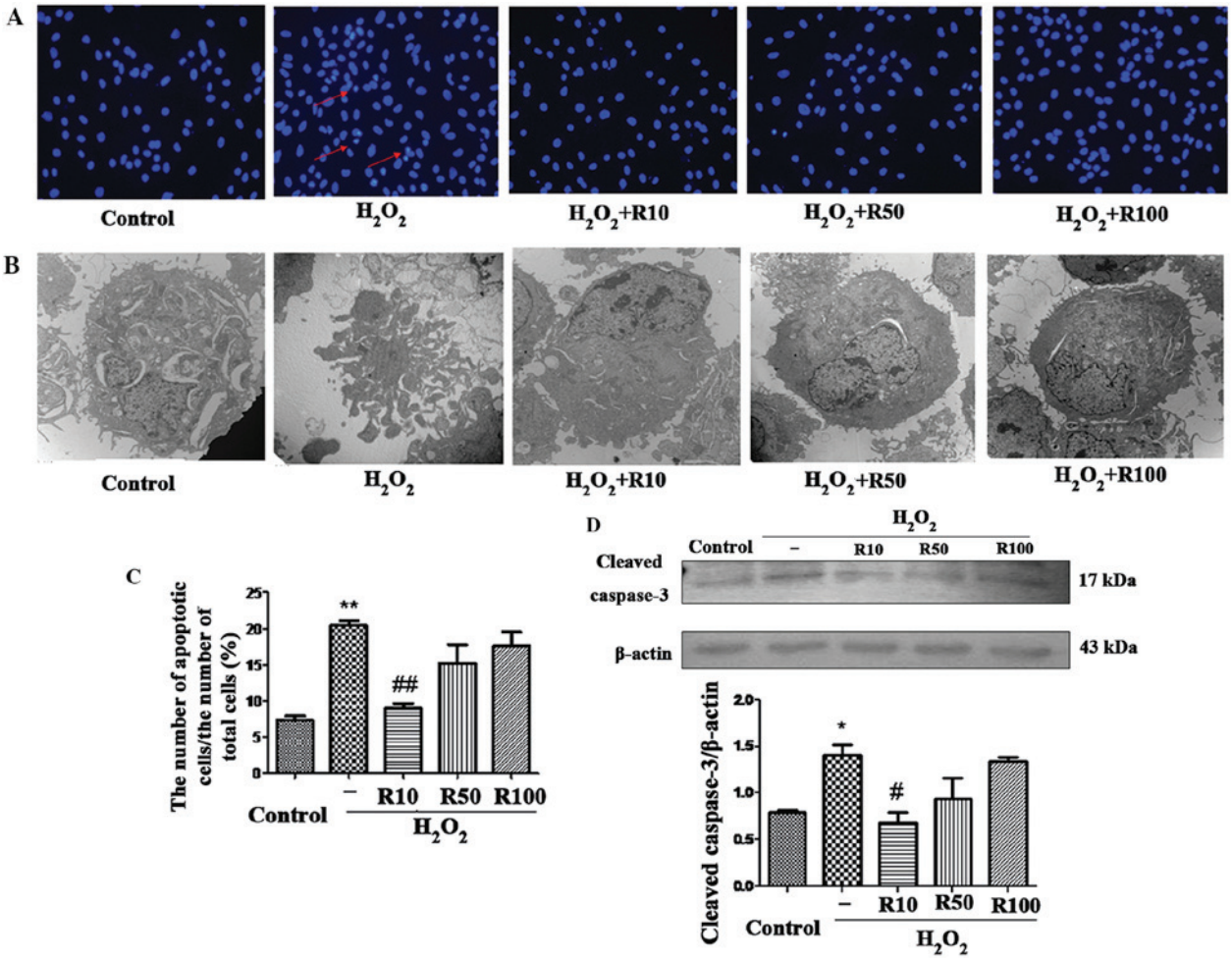

Figure 2. Relaxin-3 inhibits H_2O_2-induced hepatocyte apoptosis. (A) Hoechst staining reveals condensed and fragmented apoptotic nuclei of hepatocytes in the control, H_2O_2 or relaxin-3-treated (R10, R50 and R100 for 24 h) groups. Arrows indicate condensed nuclei (magnification, x100; n=5). (B) Transmission electron microscopy of hepatocyte apoptosis in each group (magnification, x10,000). (C) Quantification of the percentage of apoptotic cells counted in each group. (D) Western blot analysis and quantification of cleaved caspase-3 protein levels in each group. β-actin was used as the internal control. Values are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control, **P<0.01 vs. control, $^{\#}$P<0.05 vs. H_2O_2, $^{\#\#}$P<0.01 vs. H_2O_2. H_2O_2, hydrogen peroxide treatment; R10/50/100, treatment with 10, 50 or 100 ng/ml relaxin-3, respectively.

values are expressed as the mean ± standard deviation. The one-way analysis of variance followed by the Newman-Keuls multiple comparison test were used for comparisons among the five groups. P<0.05 was considered to indicate a statistically significant difference between values.

Results

Relaxin-3 attenuates H_2O_2-induced hepatocyte apoptosis. Cell viability assays, Hoechst staining, electron microscopy and western blot analysis were used in order to determine the

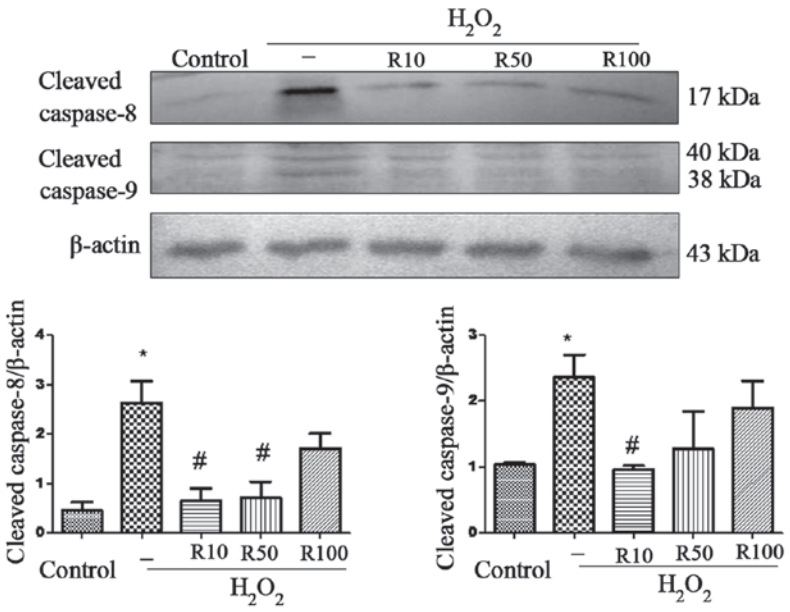

Figure 3. Relaxin-3 inhibits H_2O_2-induced hepatocyte apoptosis via suppression of cleaved caspase-8 and -9 expression. Western blot analyses of cleaved caspase-8 and -9 protein expression in hepatocytes of the control, H_2O_2 or relaxin-3-treated (R10, R50 and R100 for 24 h) groups. β-actin was used as the internal control. Values are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control, #P<0.05 vs. H_2O_2. H_2O_2, hydrogen peroxide treatment; R10/50/100, treatment with 10, 50 or 100 ng/ml relaxin-3, respectively.

effect of treatment with graded concentrations of relaxin-3 on H_2O_2-induced hepatocyte apoptosis.

As shown in Fig. 1A, exposure of human hepatocytes to graded concentrations of H_2O_2 (0-1,000 μmol/l) for 24 h remarkably decreased cell viability in a dose-dependent manner. Concentrations of H_2O_2 produced significant results at 200 μmol/l and reached maximum inhibition between 400-600 μmol. Administration of 10 ng/ml relaxin-3 to cells treated with 200 μmol H_2O_2 was found to significantly enhance cell viability; however, the protective effect of relaxin-3 diminished at higher doses (Fig. 1B).

Nuclear condensation, a hallmark characteristic of apoptotic cells, was observed in cells in the H_2O_2 only group following Hoechst staining. By contrast, cells treated with relaxin-3 were round and homogeneous, with a markedly decreased number of apoptotic nuclei, most notably in the R10 group (Fig. 2A). Cell morphological changes were also observed using electron microscopy (Fig. 2B). The later stages of apoptosis are characterized by nuclear pyknosis, mitochondrial and endoplasmic reticulum distension, as well as the formation of apoptotic bodies. These characteristics of later stage apoptosis were observed in cells in the H_2O_2 group; however, no obvious apoptotic changes were detected in the relaxin-3 treated cells. Quantification of the percentage of apoptotic cells counted in each group by Hoechst staining is shown in Fig. 2C. The results demonstrated that H_2O_2 treatment significantly increased the number of apoptotic cells compared with that of the control. By contrast, the R10 relaxin-3 treatment group (10 ng/ml) showed a significantly decreased number of apoptotic cells compared with that of the H_2O_2 group (P<0.05); however, the decrease in the number of apoptotic cells in the R50 and R100 treatment groups were not significant compared with levels in the H_2O_2 group (Fig. 2C).

Intrinsic and extrinsic apoptotic pathways activate the proenzyme form of caspase-3, which is then cleaved through self-proteolysis and other proteases (21). Therefore, endogenous cleaved (activated) caspase-3 is a common indicator of cellular apoptosis. In the present study, western blot analysis was used to determine the expression levels of cleaved caspase-3. The results demonstrated that H_2O_2 treatment significantly increased the expression of cleaved caspase-3 compared to that of the control. By contrast, the relaxin-3 R10 treatment group (10 ng/ml) showed significantly attenuated cleaved caspase-3 protein levels compared to those of the H_2O_2 group (P<0.05); however, the decrease in caspase-3 protein levels in the R50 and R100 treatment groups was not significant compared to levels in the H_2O_2 group (Fig. 2D).

Relaxin-3 attenuates the H_2O_2-induced increase in cleaved caspase-8 and -9 levels. Caspase-8 and -9 are upstream mediators of the extrinsic and intrinsic apoptotic pathways, respectively; cleavage of these caspases results in the activation of downstream executioner caspases, such as caspase-3 (21). Therefore, in the present study, western blot analysis was used to determine the levels of cleaved caspase-8 and -9 using western blot anlaysis and investigate whether these pathways were involved in the anti-apoptotic effect of relaxin-3 (Fig. 3). The results revealed that the protein expression of cleaved caspase-8 and -9 was significantly increased in the H_2O_2 group compared to that of the control group. However, the R10 and R50 relaxin-3 treatment groups demonstrated significantly attenuated cleaved caspase-8 levels compared to those of the H_2O_2 group; in addition, cleaved caspase-9 protein expression was significantly reduced in the R10 group (P<0.05) .

Relaxin-3 inhibits H_2O_2-induced ERS. Sustained or severe ERS leads to apoptosis through several pathways, including the CHOP and caspase-12 pathways (22). Therefore, the present study used western blot analysis to detect protein

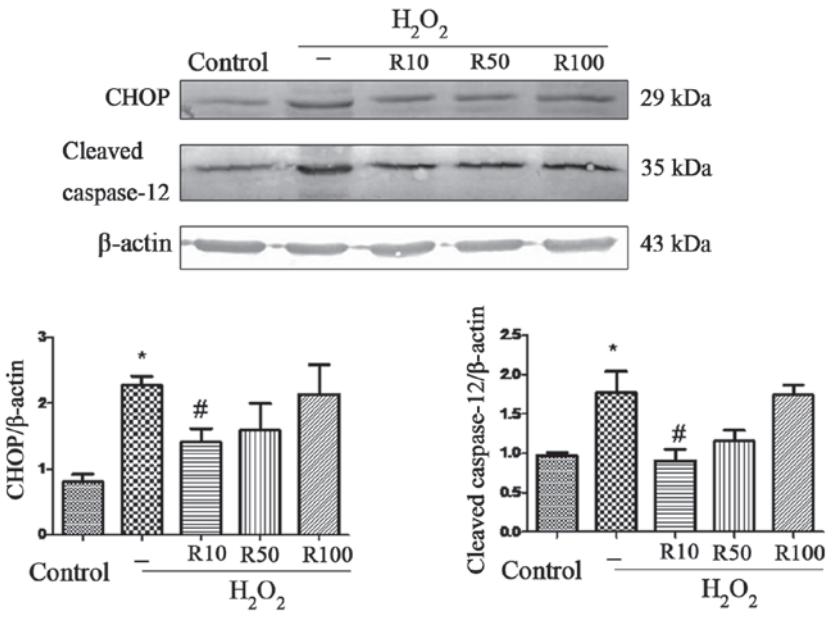

Figure 4. Relaxin-3 inhibits H_2O_2-induced hepatocyte apoptosis via suppressing endoplasmic reticular stress. Western blot analyses of CHOP and cleaved caspase-12 protein expression in hepatocytes of the control, H_2O_2 or relaxin-3-treated (R10, R50 and R100 for 24 h) groups. β-actin was used as the internal control. Values are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control, #P<0.05 vs. H_2O_2. H_2O_2, hydrogen peroxide treatment; CHOP, CCAAT-enhancer-binding protein homologous protein; R10/50/100, treatment with 10, 50 or 100 ng/ml relaxin-3, respectively.

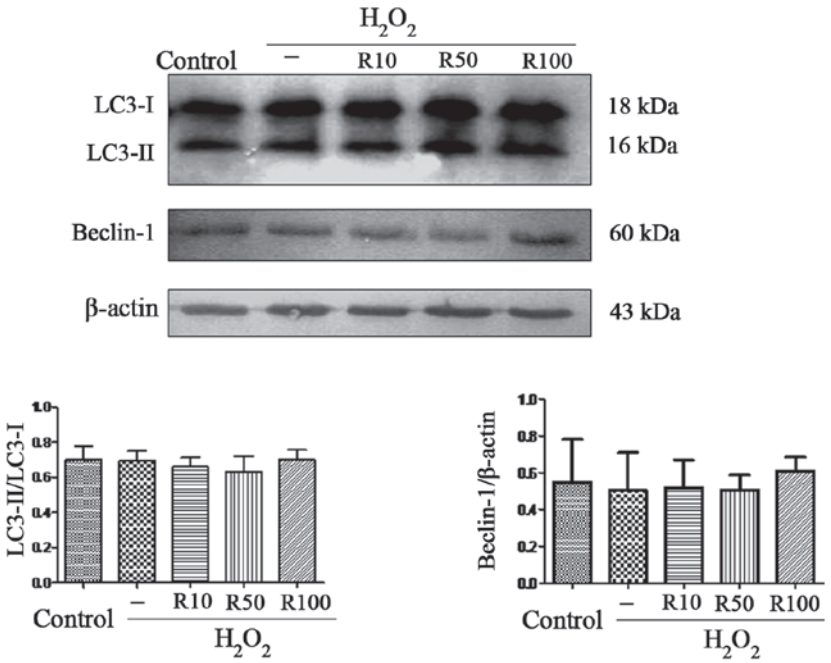

Figure 5. Expression of autophagy markers is unchanged following H_2O_2 treatment alone or in combination with relaxin-3. Western blot analysis of LC3-II/LC3-I and Beclin-1 protein expression in hepatocytes of the control, H_2O_2 or relaxin-3-treated (R10, R50 and R100 for 24 h) groups. β-actin was used as the internal control. Values are presented as the mean ± standard deviation (n=3). H_2O_2, hydrogen peroxide treatment; LC3, microtubule-associated protein 1 light chain 3; R10/50/100, treatment with 10, 50 or 100 ng/ml relaxin-3, respectively.

expression levels of CHOP and cleaved caspase-12 in order to directly examine the role of relaxin-3 in ERS-associated apoptosis (Fig. 4). The results revealed elevated levels of CHOP and cleaved caspase-12 in the H_2O_2 group. However, 10 μg/ml relaxin-3 treatment significantly inhibited the H_2O_2-induced overexpression of CHOP and cleaved caspase-12 (P<0.05), while this effect was not observed at higher doses of relaxin-3.

Relaxin-3 has no effect on markers of autophagy in apoptotic hepatocytes. Autophagy is the process by which cells recycle cytoplasm and degrade surplus or dysfunctional organelles (23). It has been reported that autophagy was the cell survival mechanism of primary human hepatocytes during oxidative stress (24). In order to examine the effect of relaxin-3 on autophagy in H_2O_2-induced hepatocyte apoptosis, the present study aimed to determine the expression levels of

LC3-II/LC3-I and Beclin-1. The results showed that the expression of these autophagy markers was unchanged following H_2O_2 treatment alone or in combination with relaxin-3 treatment (Fig. 5).

Discussion

Relaxin-3, the 'ancestral' member of the relaxin peptide family, activates RXFP3 which is involved in stress and anxiety responses (25) as well as appetite regulation (26,27), and whose expression is highest in the brain (28). However, the effect of relaxin-3 on other organs and tissues is has not been studied in detail (29). To the best of our knowledge, the present study was the first to demonstrate the impact of relaxin-3 on H_2O_2-induced hepatocyte apoptosis.

H_2O_2 is widely used as a model of oxidative stress in order to induce apoptosis. H_2O_2 stimulation induces enhances expression of ROS, which may exhaust hepatocellular antioxidant defenses, resulting in oxidative stress and hepatocyte apoptosis (30). In the present study, hepatocytes were incubated with 200 μmol/l H_2O_2 for 24 h, as previously reported (31). Following treatment with relaxin-3 (10 ng/ml), a significant increase in cell viability was observed as well as a marked decrease in cleaved caspase-3 expression compared with levels in the untreated H_2O_2 group. In addition, Hoechst staining and electron microscopy revealed a decrease in the number of apoptotic hepatocytes. The results therefore indicated that relaxin-3 was involved in the protection of hepatocytes from pathological apoptosis.

The role of relaxin-3 in stress/anxiety and appetite regulation is currently defined by the regional distribution of RXFP3 in the brain, and relaxin-3 was also reported to bind to RXFP1, a relaxin-2 receptor, which is expressed primarily in the liver (16,32). A previous study reported that relaxin-3 had an anti-fibrotic effect on cirrhotic liver tissue, which resulted in RXFR1 upregulation (18). In addition, the anti-apoptotic action of relaxin-2 in combination with RXFP1 was previously described in cardiac (20) and reproductive tissues (19,33). Based on evidence provided by these previous studies, it was hypothesized that relaxin-3 may attenuate hepatocyte apoptosis via the activation of RXFP1; however, further studies are required in order to characterize the role of RXFPs in apoptotic hepatocytes and determine the signaling properties of relaxin-3.

Of note, in the present study, the anti-apoptotic action of relaxin-3 did not proceed in a dose-dependent manner; by contrast, the low dose of relaxin-3 (10 ng/ml) produced significant results, indicative of its effectiveness in protecting hepatocytes from apoptosis, while higher concentrations of relaxin-3 (50 and 100 ng/ml) were less effective and produced non-significant results in the majority of experiments performed. Further studies are required in order to elucidate the mechanisms underlying the effectiveness of low-dose relaxin-3 compared to that of higher doses.

Numerous studies have confirmed that caspases have a pivotal role in H_2O_2-induced apoptosis. There are two major pathways by which hepatocyte apoptosis is induced (34): The extrinsic (death receptor-mediated) pathway, which is initiated through the activation of caspase-8 by death receptors followed by the caspase-8 induced activation of an effector caspase (caspase-3); the intrinsic (mitochondrial apoptosis) pathway, which is initiated by the activation of caspase-9 following cytochrome c release from mitochondria, which in turn activates caspase-3. Previous studies have associated H_2O_2-mediated apoptosis with the intrinsic pathway of hepatocyte apoptosis (30,35); however, the results of the present study demonstrated the significantly increased expression of cleaved caspase-8 as well as caspase-9 following hepatocyte treatment with H_2O_2. These results may be explained by the close interlinking of the two pathways, as the mitochondrial pathway may amplify the relatively weak death receptor-induced apoptotic signal in hepatocytes (36). Furthermore, relaxin-3 treatment was found to significantly decrease the expression of cleaved caspase-8 and -9, indicating that relaxin-3 prevented hepatocyte apoptosis via the inhibition of caspase-8 and -9 activation.

The ER, analogous to mitochondria, directly initiates caspase activation and apoptotic pathways (10). A previous study reported that ERS was involved in H_2O_2-induced apoptosis in hepatocytes (37), which is consistent with the results of the present study, as levels of cleaved caspase-12 and CHOP were markedly increased following incubation with H_2O_2. In addition, relaxin-3 significantly reduced the overexpression of cleaved caspase-12 and CHOP, providing evidence for the attenuation of H_2O_2-induced hepatocyte apoptosis following relaxin-3 treatment via the inhibition of ERS.

Autophagy is an important mechanism by which cells maintain homeostasis; however, the precise role of autophagy within hepatocytes during liver disease and injury remains to be fully elucidated. A recent study reported that hepatocyte autophagy served as a cell survival mechanism for primary human hepatocytes under hypoxic stress (24). However, the results of the present study revealed that H_2O_2 and relaxin-3 did not induce any obvious changes in the expression of hepatocyte autophagy markers. This therefore indicated that autophagy was not involved in the late stages of severe hepatocyte injury, which contradicts the results of a previous study, which reported that mild ischemia led to the induction of autophagy and apoptosis, while moderate/severe ischemia induced apoptotic and necrotic cell death without increasing autophagy (38). The morphological observations of the present study revealed that the H_2O_2-treated cells showed charateristics of the late stages of apoptosis; therefore suggesting that autophagy may not be the major mechanism mediating late-stage cell death in hepatocytes.

In conclusion, to the best of our knowledge, the present study provided the first evidence for the protective effect of relaxin-3 in hepatocytes following H_2O_2-induced apoptosis. Furthermore, the anti-apoptotic role of relaxin-3 was demonstrated to be mediated via decreased activation of upstream initiator caspases (caspase-8 and -9) and the inhibition of the ERS pathway.

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