Neferine treatment enhances the TRAIL-induced apoptosis of human prostate cancer cells via autophagic flux and the JNK pathway

UDDIN MD NAZIM*, HONGHUA YIN* and SANG-YOUEL PARK

Biosafety Research Institute, College of Veterinary Medicine, Jeonbuk National University, Iksan, Jeonbuk 54596, Republic of Korea

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Abstract. Prostate cancer (PCa) is a common type of cancer among males, with a relatively high mortality rate. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) family, initiates the apoptosis of certain cancer cells. Neferine, a primary ingredient of bisbenzylisoquinoline alkaloids, has various antitumor activities. The present study examined the effects of neferine treatment on human PCa cells. Human prostate cancer (DU145) cells were treated with neferine for 18 h, and subsequently treated with TRAIL for 2 h. Combined treatment with neferine and TRAIL significantly decreased cell viability compared to treatment with TRAIL alone. Furthermore, neferine treatment decreased the expression of p62 and increased LC3B-II expression, as assessed by western blot analysis and immunocytochemistry. It was also demonstrated that neferine and TRAIL act synergistically to trigger autophagy in PCa cells, as revealed by autophagosome formation, LC3B-II accumulation demonstrated by transmission electron microscopy (TEM) analysis and phosphorylated c-Jun N-terminal kinase (p-JNK) upregulation. When the autophagic flux was attenuated by the inhibitor, chloroquine, or by genetically modified ATG5 siRNA, the enhancement of TRAIL-induced autophagy by neferine-induced was also attenuated. Furthermore, treatment with the JNK inhibitor, SP600125, distinctly increased the viability of the cells treated with neferine and TRAIL. On the whole, the findings of the present study demonstrate that neferine treatment effectively promotes TRAIL-mediated cell death and this effect likely occurs via the autophagic flux and the JNK pathway.

Introduction

Prostate cancer (PCa) is one of the most frequently occurring malignant tumors among males, and is associated with high mortality and morbidity rates (1,2). To date, no standard regimen for PCa treatment has been established. Chemotherapy, immunotherapy and radiotherapy are the primary cancer treatments used following surgery (3). For patients with PCa, one goal of treatment is to initiate the apoptosis of cancer cells (4,5).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF protein family, is known to trigger the death of various cancer cells, but not normal cells (6,7), by binding the death receptors, DR-4 and DR-5, and by recruiting FADD and caspase-8 to create a death-inducing complex (8). TRAIL has been used as an apoptosis-inducing factor in various human cancers, and is thus a good candidate for use in novel cancer therapies (9). However, numerous cancer cells acquire resistance to TRAIL-induced apoptosis (5). Thus, for the treatment of PCa, considerable attention has recently been focused on overcoming the resistance of cancer cells to TRAIL.

Neferine is a major bisbenzylisoquinoline alkaloid present in *Nelumbo nucifera* Gaertn. green seed embryos (10). Recently, neferine has been demonstrated to exhibit efficient antitumor activities in HepG2 cells and human lung cancer cells (11,12), and to suppress the propagation of osteosarcoma cells (13). Further, neferine treatment has been shown to induce the release of reactive oxygen species (ROS) and trigger the mitochondrial apoptosis of liver and lung cancer cells (11,12).

The autophagic flux, which involves the degradation and recycling of damaged and harmful cellular components, is an important process for maintaining metabolism and energy homeostasis (14). Apoptosis leads to programmed cell death, whereas the autophagic flux can lead either to survival or death (15). During the induction of the autophagic flux, beclin-1 triggers the transformation of cytosolic microtubule-associated protein 1A/1B-light chain 3 (LC3-I) into LC3-phosphatidylethanolamine conjugate (LC3-II). The conversion of LC3-I to LC3-II and the recruitment of...
p62/SQSTM1 to the autophagosomal membrane are considered to be key features of the autophagic flux and are indicators that this process has been induced and activated (16-18), although the specific molecular pathways for this process in cancer cells remain unclear. c-Jun N-terminal kinase (JNK) is a stress-induced member of the mitogen-activated protein kinase (MAPK) family. JNK plays fundamental roles in cell growth, differentiation, attenuation and apoptosis (19). In the present study, the ability of neferine treatment to enhance the TRAIL-initiated apoptosis of PCa cells was assessed. The results indicate that combined treatment of PCa cells with and neferine and TRAIL is more effective than treatment with either substance alone.

Materials and methods

Cells and cell culture. Human PCa cells (DU145 and LNCaP; Korean Cell Line Bank) were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS). During experimentation, cells were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 1% FBS. Cells were grown at 37°C and 5% CO₂ in a humidified incubator.

Reagents. Neferine was acquired from Sigma-Aldrich; Merck KGaA, and TRAIL was acquired from Abfrontier.

Determination of cell cytotoxicity. Cytotoxicity is the amount of toxicity affecting cells. A cytotoxic agent can lead to a decrease in cell viability, the activation of apoptosis and the alteration of autophagy (20-22). Several methods for cytotoxicity assay, such as trypan blue stain, lactate dehydrogenase (LDH) assay, 3-(4,5-dimethyl-2-thiazoly)-2,5-dephenyl-2H-tetrazolium bromide (MTT) assay. In the present study, cell viability was assessed, and crystal violet and trypan blue staining was used to examine cells treated with a combination of neferine and TRAIL.

Cell viability assay. The DU145 and LNCaP cells seeded in 12-well plates were treated with 0, 5, 10, or 20 µM neferine for 18 h, cells were subsequently treated with 200 ng/ml TRAIL for 2 h and chloroquine (CQ; cat. no. c6628; Sigma-Aldrich; Merck KGaA) was used to treat the cells at 1 µM for 1 h prior to neferine or TRAIL treatment. In addition, the JNK inhibitor, SP600125 (cat. no. PAS-20839; Invitrogen; Thermo Fisher Scientific, Inc.) and anti-p-JNK (1:500; cat. no. c.s 9255s; Cell Signaling Technology; Inc.) antibodies for 2-3 h at room temperature. After washing with PBS, the cells were incubated in the dark at 20°C with secondary antibodies (Alexa Fluor® 488-conjugated donkey polyclonal anti-rabbit; 1:500; cat. no. A-21206; Thermo Fisher Scientific, Inc. and Alexa Fluor® 546 goat anti-mouse IgG (1:500; cat. no. A-21206; Thermo Fisher Scientific, Inc.) for 2 h. A fluorescence microscope (Nikon Corp.) was used to visualize immunostaining.

Transmission electron microscopy (TEM). Cells were bathed in a solution containing 2% glutaraldehyde, 2% paraformaldehyde and 0.05 M sodium cacodylate (pH 7.2) (all from Electron Microscopy Sciences) for 2 h at 4°C. The cells were then fixed by incubation with 1% osmium tetroxide (Electron Microscopy Sciences) for 1 h at 4°C, dehydrated with increasing concentrations of ethanol (25, 50, 70, 90 and 100%) for 5 min at each concentration, and embedded in epoxy resin (Embed 812; Electron Microscopy Sciences) for 48 h at 60°C following the manufacturers' instructions. Ultrathin sections (60-nm-thick) were sliced using an LKB-III ultratome (Leica Microsystems GmbH). The slices were stained with 0.5% uranyl acetate (Electron Microscopy Sciences) for 20 min and with 0.1% lead citrate (Electron Microscopy Sciences) for 7 min at room temperature. Fluorescent images were recorded using the Hitachi H7650 electron microscope (Hitachi, Ltd.; magnification, x10,000) located at the Center for University-Wide Research Facilities (CURF) at Jeonbuk National University.

Western blot analysis. Western blot analysis was performed as previously described (3). Total protein extraction was using immunoprecipitation assay buffer (Qiagen, Inc.). The supernatant was collected by centrifugation (11,200 x g); 4°C; 10 min; the protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific; Inc.). Proteins (30 µg) were separated on 10% SDS-PAGE gels and blotted onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dried milk at 25°C for 1 h antibodies against the flowing proteins were used: β-actin (1:10,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA), LC3A/B (1:1,000; cat. no. c.s 4108s; Cell Signaling Technology; Inc.), p62 (1:250; cat. no. PAS-20839; Invitrogen; Thermo Fisher Scientific, Inc.), ATG5 (1:1,000; cat. no. c.s 12994s; Cell Signaling Technology; Inc.), cleaved caspase-3 (1:1,000; cat. no. c.s 9661; Cell Signaling Technology; Inc.) (activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments, cleavage of caspase-3 requires aspartic acid at
thep1 position), cleaved caspase-8 (1:1,000; cat. no. 551242; BD Pharmingen) (caspase-8 is produced as a proenzyme (55/50 kDa, doublet) it is cleaved into smaller subunits (40/36 kDa, doublet). and p-JNK (1:500; cat. no. c.s 9255s; Cell Signaling Technology; Inc.). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. nos. ADI-SAB-100 and ADI-SAB-300; 1:1,000; Enzo Life Sciences, Inc.) at 25°C for 1 h. The immune reactive protein bands were visualized using enhanced chemiluminescence detection system (GE Healthcare Life Sciences) and detected with chemiluminescence imaging system (Fusion FX7; Viber Lourmat). The intensities of the protein bands were determined using Image J Java 1.8.0 software.xs.

**Small interfering RNA transfection.** Media RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Atlas Biologicals) was seeded with DU145 cells, and 24 h later, the cells were transfected with silencer-select small interfering RNA (ATG5 siRNA; oligo ID HSS114103; Sequence GGU UUGGACGAUUCCCAUCUUUU; Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 as per the manufacturer's recommendations. Simultaneously, a negative control siRNA (GCU ACU UAG CUC GGC CAA G AUU C; Invitrogen; Thermo Fisher Scientific, Inc.) was used as a negative control. The transfection efficiency was confirmed using real-time PCR, and the relative expression of ATG5 was determined. The findings indicate that the knockdown of ATG5 significantly inhibited the growth of DU145 cells, suggesting that ATG5 plays a crucial role in the proliferation of prostate cancer cells. Therefore, the results support the hypothesis that the inhibition of ATG5 expression may be a potential therapeutic strategy for prostate cancer treatment.
control (Invitrogen; Thermo Fisher Scientific, Inc.) was trans-
fected with a non-targeting siRNA. The cells were incubated 
with ATG5 siRNA or negative control siRNA for 6 h and the 
medium was then changed to RPMI-1640 with 10% FBS for 
24 h. The cells were then treated with neferine or neferine in 
combination with TRAIL.

Statistical analysis. All experiments were performed in tripli-
cicate, and the data are reported as the means ± standard error. 
One-way factorial analysis of variance (ANOVA), followed by 
Duncan’s post-hoc test, was performed to evaluate the statis-
tical significance of the differences between the treatment and 
control groups.

Results

Effects of neferine treatment on the TRAIL-induced apoptosis 
of PCa cells. In the present study, in order to investigate the 
synergistic effects of combination treatment with neferine and 
TRAIL on PCa cells, the viability of neferine only-treated 
cells, TRAIL only-treated cells, and neferine and TRAIL 
combine-treated cells was compared. No significant changes 
were observed in the viability of the cells treated with neferine 
or TRAIL only; however, a significant decrease was detected 
in the viability of the cells treated with the combination of 
neferine and TRAIL (Fig. 1). To evaluate the effects of neferine 
treatment on PCa cell apoptosis, the DU145 and LNCaP cells 
were treated with various concentrations of neferine for 18 h 
and then exposed to TRAIL for a further 2 h. As shown in 
Fig. 1, treatment with TRAIL or neferine alone resulted in 
only marginal cell death and did not cause any novel morpho-
logical changes. By contrast, when cells were treated with both 
neferine and TRAIL, cell viability decreased significantly 
(Fig. 1). These data indicate that neferine treatment sensitizes 
human PCa cells to TRAIL-induced apoptosis.

Neferine treatment induces an autophagic flux and promotes 
TRAIL-mediated apoptosis. In response to neferine treat-
ment, the expression of LC3-II increased markedly in the 
DU145 cells, whereas p62 expression decreased significantly 
(Fig. 2A). The results obtained for p62 protein levels measured 
by immunofluorescence staining were consistent with those 
obtained by western blot analysis (Fig. 2B). TEM indicated 
that autophagic vacuoles were secreted by the neferine-treated 
cells (Fig. 2C). In addition, the cells treated with a combina-
tion of neferine and TRAIL exhibited higher expression 
levels of Ac-cas3 and Ac-cas8 (Fig. 2D). Caspase-8 activation 
also was detected in the cells treated with a combination of 
neferine and TRAIL, but not in the cells treated with neferine 
or TRAIL alone (Fig. 2D). Certain studies have compared the 
combination of two agents to either agent alone to investi-
gate the synergistic effects of the two agents (24-26). These
results suggest that neferine treatment can initiate autophagy in DU145 cells.

**Inhibition of autophagy attenuates the neferine-mediated sensitization of TRAIL-induced apoptosis.** Cell morphological analysis revealed that treatment with chloroquine (CQ), an autophagy inhibitor, attenuated cellular apoptosis mediated by combined treatment with neferine and TRAIL (Fig. 3A). Co-treatment with neferine, TRAIL and CQ resulted in a distinct improvement in the viability of the DU145 cells (Fig. 3B-D). The autophagic flux activation by neferine was confirmed by the inspection of the autophagic flux following treatment with CQ as an autophagy inhibitor. CQ treatment led to the accumulation of membrane-bound LC3-II and an increase in p62 levels, these results indicated that CQ blocked neferine-induced autophagy (Fig. 4A). Immunofluorescence staining indicated that CQ treatment resulted in an increase in p62 protein levels (Fig. 4B). CQ also attenuated the upregulation of Ac-cas8 and Ac-cas3 that was observed following treatment with neferine and TRAIL (Fig. 4C). These results indicate that CQ modulates neferine-mediated, TRAIL-triggered apoptosis by inhibiting the autophagic flux.
Synergistic apoptosis mediated by neferine and TRAIL is blocked by the genetic inhibition of the autophagic flux. Cell morphological analysis indicated that transfection with ATG5 siRNA (Fig. 5A). Co-treatment with neferine, TRAIL and ATG5 siRNA significantly attenuated cell death and markedly increased the viability of the DU145 cells (Fig. 5B-D). In the cells in which ATG5 was knocked down, the LC3-II protein levels were markedly decreased and p62 protein levels were markedly increased (Fig. 6A). The protein levels of p62 measured by immunofluorescence staining were similar to the levels measured by western blot analysis (Fig. 6B). Co-treatment of the cells with neferine, ATG5 siRNA and TRAIL resulted in a decrease in Ac-cas3 and Ac-cas8 expression (Fig. 6C). These results suggested that ATG5 siRNA blocked the synergistic cell death induced by neferine and TRAIL treatment.

Neferine treatment induces JNK activation. As revealed by western blot analysis and immunofluorescence staining, neferine treatment of the DU145 cells resulted in a dose-dependent increase in the p-JNK protein expression levels (Fig. 7A and B). In the cells that were treated with 1 µM SP600125 for 1 h prior to neferine treatment, a decrease in neferine-induced p-JNK expression was observed. SP600125 treatment also increased the viability of the cells that had been treated with neferine and TRAIL (Fig. 7D and E). These results indicate that neferine treatment causes an increase in JNK expression that triggers TRAIL-mediated death of DU145 cells.

Discussion

TRAIL has been demonstrated to stimulate the apoptosis of cancer cells without harming normal cells; therefore, TRAIL administration is regarded as a prospective treatment strategy against cancer (27-29). However, TRAIL resistance has been observed in a number of different types of cancer (30). Neferine is a bisbenzyl isoquinoline alkaloid that has been shown to exert a number of biological effects, such as the inhibition of cancer cell proliferation (31). The autophagic flux, a process of lysosomal degradation of misfolded and unneeded proteins, plays a crucial role in maintaining homeostasis in healthy cells, and can also lead to the destruction of damaged or cancerous cells (32-34). JNK plays a critical role in inducing autophagy and triggering cellular apoptosis (35). Cell viability is the ratio of the initial cell number minus the dead cell number to the initial cell number. In the present study, cell viability assay was used to assess apoptotic cell death. Cleaved caspase-3 and caspase-8 were also detected for the assessment of apoptosis (Figs. 2D, 4C and 6C). TRAIL has received considerable attention as a novel anticancer agent. Although various types of tumor cells
are sensitive to TRAIL-initiated apoptosis, other cells, including PCa cells, are TRAIL-resistant (36). The data of the present study demonstrated that while TRAIL treatment alone did not trigger the apoptosis of DU145 cells, treatment of the cells with neferine prior to TRAIL treatment resulted in increased cell death (Fig. 1). Recent research has revealed that neferine attenuates cancer cell proliferation and inducetion and induces autophagy (12,31,37). The present study demonstrated that neferine initiated autophagy in PCa cells through the formation of autophagosomes and the conversion of LC3-I to LC3-II (Fig. 2), and that pharmacological (Figs. 3 and 4) and genetic (Figs. 5 and 6) autophagy inhibitors attenuated neferine-mediated, TRAIL-triggered apoptosis.

Figure 5. Neferine mediates TRAIL-triggered apoptosis by the genetic attenuation of autophagy. DU145 cells were transfected with ATG5 siRNA or negative control siRNA for 24 h prior to exposure to 20 µM neferine for 18 h and to TRAIL for 2 h. (A) The morphology of the cells was assessed by light microscopy. (B) Cell viability was determined by crystal violet assay. (C) Bar graph presents the average densities of dyed crystal violet. (D) Cell viability was also determined using a trypan blue exclusion assay. *P<0.01, indicates significance for comparisons between the untreated control and treatment groups. Nef, neferine; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand.
Figure 6. Neferin mediates TRAIL-triggered apoptosis by activating the autophagic flux. DU145 cells were transfected with ATG5 siRNA (siATG5) or negative control siRNA (siNC) for 24 h prior to exposure to 20 µM neferine for 18 h, and to TRAIL for 1 h. (A and C) p62, LC3-II, ATG5, Ac-cas3 and Ac-cas8 levels were evaluated by western blot analysis and (B) cells were immunostained with p62 antibody (green) and counterstained with DAPI (blue). Nef, neferine; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand.

Figure 7. Neferine activates the JNK pathway. DU145 cells were treated with neferine (0, 5, 10 and 20 µM) for 18 h. (A and B) p-JNK protein levels were evaluated by western blot analysis and immunofluorescence assay. (C) DU145 cells were treated with SP600125 for 1 h prior to exposure to 20 µM neferine for 18 h. (D and E) and to 200 ng/ml TRAIL for 1 h, after which cell viability was assessed using a crystal violet assay. Nef, neferine; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand.
Autophagy and apoptosis are self-destructive processes in response to cell stress. These two processes are activated by different signaling pathways, but also interact with each other. They finally lead to cell death and decrease cell viability, particularly that of cancer cells; however, these two processes have different biomarkers for identification. Apoptosis is programmed cell death. There are certain biomarkers for monitoring apoptosis, such as caspase, mitochondrial potential, the sub G1 population, DNA fragmentation and nuclear condensation (38). Western blot analysis is a powerful method for the detection of apoptosis. During apoptosis the levels of a number of proteins are altered. The levels of caspasases, such as caspase-3, caspase-8 and caspase-9 are significantly increased during apoptosis (39). In addition, the levels of Bcl family proteins are altered during apoptosis (40). The depolarization of mitochondrial potential is the central mechanism of apoptosis (41). The induction of apoptosis induces DNA fragmentation and nuclear condensation, leading to nuclear morphological changes, and this increases the Sub G1 cell population (42). These methods can identify apoptotic cell death. Autophagy selects and tags cytoplasmic components and organelles into the autophagosome and which are then degraded by the lysosome. There are some available methods with which to detect autophagy structures and monitor the autophagic flux (43). In the present study, LC-3 and p62 protein expression was examined by western blot analysis and immunocytochemistry. LC-3 and p62 are typical protein markers of the autophagic flux (43). LC-3 as an autophagosome marker, is presented in Figs. 2A, 4A and 6A. LC-3 is a microtubule-associated protein 1A/1B-light chain, and during autophagy the cytosolic form of LC-3 (LC-3I) is conjugated to phosphatidyl- ethanolamine (PE) to form LC-3-PE (LC-3II). Subsequently, LC-3II contributes to autophagosome formation. LC-3 has been used as a maker of autophagosome formation (44,45). The present study detected p62 as a maker of the autophagic flux (Figs. 2A and B, 4A and B, and 6A and B). p62 is sequestosome-1, and is an ubiquitin-binding protein. It delivers cytosolic protein to the autophagosome and directly binds to LC-3II. p62 allows the cytosolic protein to locate into the autophagosome and be degraded. A decrease in p62 expression can represent an increase in the autophagic flux. In several studies, the expression of p62 has been monitored for the investigation of the autophagic flux (46,47). The present study also detected autophagosome structure using TEM (Fig. 2C). The autophagosome is a double membrane vesicle, substrates some cellular organelles and aggregated proteins. TEM is a conventional method used to identify the autophagosome and monitor the morphology of the autophagosome (48).

The present study also demonstrated that neferine treatment increased p-JNK protein expression in a dose-dependent manner (Fig. 7A and B). The effects of neferine treatment were suppressed in the presence of SP600125, which inhibits p-JNK activity (Fig. 7C and D). In conclusion, the findings of the present study demonstrate that neferine treatment mediates TRAIL-induced death of DU145 tumor cells via the autophagic flux and JNK pathway. These results indicate that combined treatment with neferine and TRAIL may be effective against TRAIL-resistant cancers.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors’ contributions
UMDN, HY and SYP designed, executed the study and analyzed data. UMDN and HY wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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