Riluzole Induces Apoptotic Cell Death in Human Prostate Cancer Cells via Endoplasmic Reticulum Stress

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Abstract. Background: Ion channel modulators have been previously associated with cell proliferation and cell death in human cancer cell lines. Materials and Methods: The effects of riluzole, an ion channel modulator, on cell proliferation, apoptosis and the apoptotic pathway in the LNCaP and C4-2 prostate cancer cell lines were investigated. Results: Riluzole inhibited DNA synthesis and increased apoptotic cells in both cell lines. The activities of caspase-3, -8 and -9 were significantly increased, and caspase inhibitors for caspase-3, -8 and -9 significantly rescued the cell viability of both carcinoma cell lines treated with riluzole. However, a change in mitochondrial membrane potential, release of cytochrome c and cleavage of Bid were not observed in the riluzole-treated cells. Riluzole significantly induced elevation of caspase-4 activity, fluorescence indicating cytosolic calcium, and morphological changes in endoplasmic reticulum (ER) as observed by transmission electron microscopy. Conclusion: Riluzole induces inhibition of DNA synthesis and apoptotic cell death via ER stress in both the LNCaP and C4-2 prostate cancer cell lines.

Abbreviations: ALS, Amyotrophic lateral sclerosis; Apaf-1, apoptosis protease-activating factor-1; BrdU, 5-bromo-2’-deoxyuridine; ER, endoplasmic reticulum; PB, phosphate buffer; PI, propidium iodide; PKC, protein kinase c; TEM, transmission electron microscopy; TG, thapsigargin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling; VEGF, vascular endothelial growth factor.

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Prostate cancer is the most common tumor in males in the United States. Although the number of patients in Asian countries including Japan is currently low, it is expected that the number of Asian patients will increase rapidly as their average lifetime increases and with the westernization of their diet (1, 2).

Hormonal therapy, in which drugs, surgery or other hormones (androgens) are used to reduce the production of male hormones or prevent them from working, is usually selected to slow or stop the growth of the prostate cancer, as this cancer is sensitive to androgens (3). However, the cancer inevitably progresses from androgen-dependent to androgen-independent status, also known as hormone-refractory prostate cancer, which results in increased patient mortality (4). An effective therapy for androgen-independent prostate cancer is not currently available.

Riluzole is a benzothiazole that has neuroprotective actions and has been used to treat patients with amyotrophic lateral sclerosis (ALS) (5, 6). This compound has been reported to inhibit the glutamate release from nerve terminals in the central nervous system (7, 8), the binding of excitatory amino acids to glutamate receptors (9) and the activity of the voltage-gated Na+ channels. However, the mechanism of drug action of riluzole is still unclear, partially because the number of ALS patients is very few.

There is a lot of evidence that ion channels play important roles in regulating the tumor cell cycle, cell proliferation and apoptosis (10-12). K+ channels play important roles in cell volume regulation, proliferation, differentiation and cell death, and thus K+ channel agonists are expected to be useful drugs for autoimmune diseases and cancers (13-15). Riluzole is reported to activate K+ channels (16) as well as inhibit Na+ channels.

Riluzole has been shown to inhibit vascular endothelial growth factor (VEGF)-stimulated protein kinase c (PKC), β2 activation and cell proliferation in bovine retinal endothelial cell and human umbilical vein endothelial cell cultures (17). Systemic administration of riluzole also
substantially ameliorates abnormal new vessel formation in the rat retinopathy of prematurity model (17). These findings suggest that riluzole is able to control cell growth and abnormal cell production (17). Furthermore, the Na+ channel is known to participate in cell proliferation and control (18), and riluzole controls cell proliferation in various prostate cancer cells.

Although many studies of the relationships between ion channels and cell death have been performed, most were limited to acute physiological studies at a single cell level (10-12). Few studies have investigated the mechanisms of cell death, for example the involvement of apoptosis in long-term drug incubation. To develop an anticancer drug, it is important to observe the effects of the drug on a population of tumor cells and investigate the mechanism of the drug-induced cell death.

In this study, the effects of riluzole on cell death in the human prostate cancer cell lines LNCaP and C4-2 were investigated and it is demonstrated that the apoptotic pathway proceeds via endoplasmic reticulum (ER) stress.

Materials and Methods

LNCaP cells were obtained from ATCC (Manassas, VA, USA) and C4-2 cells were purchased from UroCor, Inc. (Oklahoma City, OK, USA). The LNCaP cell line is an androgen-dependent human prostatic adenocarcinoma cell line established from a metastatic lesion (19). C4-2 is an androgen-independent cell line that originated from a lymph node metastasis (20). Androgen dependency was confirmed by preparatory experiments. Cells were cultured in RPMI-1640 containing 10% fetal bovine serum supplemented with 100 U/mL penicillin and 100 μg streptomycin. Cells were kept at 37°C in an humidified incubator in an air/CO2 5% atmosphere. Riluzole was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

At 24 and 48 h after the start of riluzole treatment (0-50 μM), cell viability was measured by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) using a spectrophotometer (VersaMax OY; Molecular Devices, Eugene, OR, USA) at 490 nm.

To measure DNA synthesis, cells were treated with 10 and 50 μM riluzole for 48 h, incubated in 100 μM 5-bromo-2′-deoxyuridine (BrDU) (Roche Diagnostics, Penzberg, Germany) for 60 min and cell proliferation was then measured using a cell proliferation ELISA BrDU kit (Roche Diagnostics). Absorbance at 450 nm was measured using a spectrophotometer (Molecular Devices) and the results were expressed as absorbance of BrDU incorporation divided by cell viability.

For terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling (TUNEL) staining, cells were treated with 10 μM riluzole for 48 h, embedded in OCT compound (Miles, Elkhart, IN, USA) and then cut into 10-μm sections using a cryomicrotome. Sections were fixed in 4% formaldehyde solution in phosphate buffer (PB) and stained using an in situ cell death kit with fluorescein (Roche Diagnostics). Propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) was used to stain nuclei. Fluorescent images were acquired with a fluorescence microscope (Nikon ECLIPSE E600; Nikon, Tokyo, Japan). To count the number of TUNEL-positive cells, sections were also stained using an in situ detection kit (Wako Pure Chemical Industries, Osaka, Japan). The number of TUNEL-positive cells per 1,000 cells was counted in various areas and expressed as a percentage of the total cells counted.

The activities of caspase-3, -8 and -9 were measured using a fluorometric protease assay kit (MBL, Inc., Nagoya, Japan). Cells were treated with 10 μM riluzole for 48 h and then suspended in lysis buffer; the protein concentration was adjusted to 20 μg/50 μL for each sample. Fluorescent intensity was read on a fluorometer (VersaFluor; Bio-Rad, Hercules, CA, USA) using a 390-nm excitation filter and a 510-nm emission filter.

To measure the mitochondrial membrane potential (Δψ), cells were treated with 10 μM riluzole for 48 h and then incubated in JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolocarbocyanin iodide) (DePsipher kit; Trevigen, Inc., Gaithersburg, MD, USA) for 60 min. The red fluorescent intensity was read on a fluorometer (Bio-Rad) using a 550-nm excitation filter and a 600-nm emission filter. The mitochondrial Δψ was expressed as the counted fluorescence of mitochondrial Δψ divided by cell viability.

To measure the release of cytochrome c, cells were treated with 10 μM riluzole for 48 h. The cytosol fraction was prepared using the ProteoExtract cytosol/mitochondria fraction kit (Calbiochem, San Diego, CA, USA). The release of cytochrome c was detected using the cytochrome c ELISA kit (Chemicon International, Inc., Temecula, CA, USA) and absorption was measured at 450 nm using a spectrophotometer (Molecular Devices).

To observe Bid cleavage, cells were treated with 10 μM riluzole for 48 h, lysed in Cellytic M (Sigma Chemical Co.) and homogenized. Aliquots of 40 μg protein were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane filter (0.45-μm pore; Invitrogen, Carlsbad, CA, USA). The primary antibody was the anti-Bid antibody FL-195 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the secondary antibody was the donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology). The bands were detected by Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Inc., Boston, MA, USA). As a control for equal loading of proteins, a β-actin antibody (Santa Cruz Biotechnology) was used.

For the caspase inhibitor experiments, 2 hours prior to 10 μM riluzole exposure, the cells were treated with the caspase inhibitors as follows: 10 μM and 100 μM z-DEVD-fmk against caspase-3, z-IETD-fmk against caspase-8 and z-LEHD-fmk against caspase-9 (MBL, Inc.) for 24 h and 48 h. Cell viability was measured using a spectrophotometer (Molecular Devices) at 490 nm. Data are expressed as percentage of the control values.

The activity of caspase-4 was measured using a fluorometric protease assay kit (Biovision, Palo, Alto, CA, USA). Cells were treated with 10 μM riluzole for 16 h and 24 h, suspended in lysis buffer; the protein concentration was adjusted to 50 μg/50 μL for each sample. Fluorescent intensity was read on a fluorometer (Bio-Rad) using a 390-nm excitation filter and a 510-nm emission filter.

To measure cell recovery with riluzole following treatment with the caspase-4 inhibitor, 2 hours prior to 10 μM riluzole exposure, the cells were treated with 10 μM of the caspase-4-specific inhibitor z-LEVD-fmk (Biovision) for 24 and 48 h. Cell viability was measured (Promega) using a spectrophotometer (Molecular Devices) at 490 nm.
To observe ultrastructural changes using transmission electron microscopy (TEM), cells were treated with 10 μM riluzole for 24 and 48 h and then fixed in a 2.5% glutaraldehyde/2% paraformaldehyde solution in 0.1 M PB for 1 h, at 4°C. The cells were subsequently post-fixed in 1% OsO4 for 45 min at room temperature, dehydrated in a series of graded ethanol concentrations, cleared in propylene oxide, and embedded in an epoxy resin mixture. Ultrathin sections (60-70 nm) were prepared and mounted on grids and stained with uranyl acetate and lead citrate. Samples were then observed using TEM (Hitachi 7650; Hitachi Co., Tokyo, Japan).

To confirm the presence of cytosolic calcium, cells were treated with 10 μM riluzole and 0.5 μM thapsigargin (TG) (Sigma Chemical Co.) for 24 h. Fluo-3/AM (Dojin, Kumamoto, Japan) was used as the fluorescent probe. Fluorescent images were acquired using a fluorescence microscope (Nikon).

**Statistical analysis.** All data express the means±SD. To compare multiple groups, a one-way ANOVA was used. For paired data sets, a two-sided Student’s t-test was used.

**Results**

**Inhibition of cell viability and cell proliferation.** The cell viability of two types of cultured human prostate cells, LNCaP and C4-2, was inhibited by 5-10 μM riluzole for 24-48 h in a dose-dependent manner (Figure 1A, B). After 48 h treatment with 10 μM riluzole, the cell viability of LNCaP was 63% while that of C4-2 was 66%. Therefore, 10 μM riluzole for 48 h was chosen as the standard riluzole treatment in subsequent experiments. BrdU incorporation was also decreased in both cell lines by riluzole in a dose-dependent manner (Figure 1C).

**Apoptotic cell death.** Numerous TUNEL-positive cells were detected in the LNCaP and C4-2 cell lines treated with 10 μM riluzole for 48 h (Figure 2A-D), and the number of TUNEL-positive cells was significantly higher in these cell lines compared to corresponding control cells (Figure 2E).

**Apoptosis signaling pathway.** The activities of caspase-3, -8 and -9 were significantly increased in both the LNCaP and C4-2 cell lines treated with 10 μM riluzole for 48 h (Figure 3A). Mitochondrial Δψ was not changed upon exposure to riluzole in either cell line compared with the corresponding control (Figure 3B), and release of cytochrome c into the cytosolic fraction was also not seen in either cell line (Figure 3C). The cleavage of Bid was not observed in either the control or riluzole-treated LNCaP or C4-2 cells (Figure 4A). To determine whether the cell death induced by riluzole was caspase dependent, caspase inhibitor experiments were then conducted. The recovery of cell viability was observed in cells treated with all caspase-inhibitors and 10 μM riluzole as compared with riluzole alone for 24 h (Figure 4B). The recovery of cell viability by these inhibitors treated with riluzole for 48 h showed a similar tendency to a 24-h caspase inhibitor study (data not shown). The activity of caspase-4 was significantly increased in both cell lines treated with 10 μM riluzole for 48 h (**p<0.01).
µM riluzole for 24 h (Figure 5A) but caspase-4 activity was not increased in cells treated with riluzole for 16 h (data not shown). In the caspase-4 inhibitor (z-LEVD-fmk) study, cell viability was significantly increased in both cell lines treated with 10 µM riluzole (Figure 5B). The recovery of cell viability in the caspase-4 inhibitor study at 48 h was similar to the results from the 24-h treatment (data not shown). Morphologically, in both cell lines treated with 10 µM riluzole for 24 and 48 h changes in the ER were observed by TEM (Figure 6), and included atypical arrangement, e.g. a braided...
structure, ER luminal swelling, increased density of ER lumen and dissociation of ribosomes from rough ER. However, these changes were not seen in control cells. The TEM findings at 24 h were similar to the results from 48 h (data not shown).

Intense fluorescent staining of cytosolic calcium indicated large amount of calcium released from the ER into the cytoplasm in cells treated with riluzole or TG, compared to the control (Figure 7).

Discussion

In this study it was found that riluzole inhibited DNA synthesis and induced apoptosis via an endoplasmic reticulum stress in both androgen-dependent LNCaP and androgen-independent C4-2 prostate cancer cells.

Two different pathways, the death receptor pathway and the mitochondrial pathway are currently proposed to play major roles in regulating apoptosis in mammalian cells (21, 22). The caspase family includes critical mediators of apoptosis. The death receptor pathway and the mitochondrial pathway initially start by activation of caspase-8 and -9, respectively. Both the activated caspase-8 and -9 activate caspase-3 and finally induce apoptosis. Most of the apoptosis signals are transduced to the mitochondria and increase the permeability of the mitochondrial membranes, which leads to the dissociation of cytochrome c from the mitochondria into the cytoplasm. The dissociated cytochrome c binds to apoptosis protease-activating factor-1 (Apaf-1) and the complex activates caspase-9 (intrinsic pathway) (23). Activated caspase-8 is suggested to activate the mitochondrial pathway through cleavage of Bid (24).
In the present study, it was found that caspase-3 and -9 were activated in two prostate cancer cell lines (androgen-dependent and androgen-independent) treated with riluzole. However, neither a change in mitochondrial membrane potential nor cytochrome c release was detected. Treatment with caspase-3- and caspase-9-specific inhibitors significantly reduced the riluzole-induced cell death. A previous study found that cytochrome c knock-out in mice induce apoptosis in thymus cells, however, knocking out Apaf-1 made the thymus cells resistant to apoptotic stimuli (25). This suggests the existence of an Apaf-1-dependent and cytochrome c-independent apoptosis signaling pathway diversity of the mitochondrial intrinsic pathway. Based on these findings, it is suggested that riluzole might induce apoptosis via a pathway that is mediated by caspase-9, but not mediated by mitochondria, and is cytochrome c independent.
In the riluzole-induced apoptosis of the present study, Bid cleavage was not observed, whereas caspase-8 activity increased. The caspase-8 specific inhibitor rescued 10-20% of the prostate cancer cells from the riluzole-induced cell death. In previous studies of mammary cancer cells or endometrial cancer cells treated with lovastatin or raloxifene, caspase-8-specific inhibitor failed to prevent cell death and Bid cleavage was not detected whereas the caspase-8 activity was increased (26, 27). It has also been reported that caspase-8 induces apoptosis downstream of caspase-3 and -9 without involving the death receptor (28, 29). Therefore, the results suggest that the apoptosis signaling pathway in cells treated with riluzole is other than the mitochondrial pathway including Bid, because neither a change in the mitochondrial Δψ nor release of cytochrome c was observed, whereas caspase-3, -8 and -9 were activated. It is commonly recognized that the major apoptotic signaling pathway is the one mediated by leaking of cytochrome c from the mitochondria. However, a recent study found that the intracellular organelles, including the ER, promote the apoptosis signal. Therefore the possibility that riluzole induces apoptosis via ER stress was hereby examined.

ER stress is defined as the accumulation of abnormal proteins in the ER induced by ischemia, heat shock, etc (30). Caspase-12 is known to mediate the pathway for cell death induced by ER stress in mice. Calcium release from the ER induces the activation of calpain (cytosolic calcium-activated neutral cysteine endopeptidases) and calpain-activated
caspase-12, which leads to apoptosis (31). The expression of caspase-12 and its functional contribution to ER stress-induced apoptosis have been confirmed in mice and rats, but not in humans. Human caspase-12 shares 68% homology with mouse caspase-12 but is reportedly not functional due to a one base insertion (32). In humans, caspase-4 and -5, which are “ER-localized caspases” with about 40% homology to the mouse caspase-12 and caspase-4, have been suggested to mediate cell death in the ER stress pathway, similar to caspase-12 in mice and rats (33). Therefore, the activity of caspase-4 was measured after 24 h of riluzole treatment and it was found that riluzole increased the activity of caspase-4. Moreover, TEM showed significant morphological changes in the ER, and riluzole-induced release of calcium from the ER into the cytoplasm was observed.

It has been reported that in mice the activated caspase-12 activates caspase-9, Apaf-1 and cytochrome c independently, and finally caspase-3 (34, 35). It is therefore suggested here that a novel, cytochrome c-independent, caspase-3-, caspase-8- and caspase-9-dependent, ER stress-mediated pathway might underlie riluzole-induced apoptosis.

In conclusion, riluzole induced inhibition of cell proliferation and apoptotic cell death via ER stress in both androgen-dependent and androgen-independent prostate cancer cells. Since it has been hypothesized that anticancer drugs induce formation of free radicals damaging the ER and increasing the cytoplasmic Ca^{2+} levels thereby leading to apoptosis (36), the present experiment also suggests that riluzole may be a useful adjuvant to current chemotherapy for androgen-dependent and androgen-independent prostatic cancers.
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References


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