Isofraxidin enhances hyperthermia-induced apoptosis via redox modification in acute monocytic leukemia U937 cells

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Abstract. The cell-killing potential of most chemotherapeutic agents is enhanced by a temperature elevation. Isofraxidin (IF) is a coumarin compound widely found in plants, such as the Umbelliferae or Chloranthaceae families. IF induces anticancer effects in lung and colorectal cancer. To the best of our knowledge, the combined effects of hyperthermia (HT) and IF on heat-induced apoptosis have not been reported. Acute monocytic leukemia U937 cells were exposed to HT with or without IF pre-treatment. Apoptosis was measured by Annexin V-FITC/PI double staining assay using flow cytometry and cell viability was observed by cell counting kit assay, DNA fragmentation. The mechanism involved in the combination was explored by measuring changes in the mitochondrial membrane potential, (MMP), intracellular ROS generation, expression of apoptosis related protein, and intracellular calcium ion level. It was demonstrated that IF enhanced HT-induced apoptosis in U937 cells. The results demonstrated that combined treatment enhanced mitochondrial membrane potential loss and transient superoxide generation increased protein expression levels of caspase-3, caspase-8 and phosphorylated-JNK and intracellular calcium levels. Moreover, the role of caspases and JNK was confirmed using a pan caspase inhibitor (zVAD-FMK) and JNK inhibitor (SP600125) in U937 cells. Collectively, the data demonstrated that IF enhanced HT-induced apoptosis via a reactive oxygen species mediated mitochondria/caspase-dependent pathway in U937 cells.

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Introduction

Isofraxidin (IF) is a coumarin compound widely found in plants, such as those in the Umbelliferae or Chloranthaceae families. It induces numerous biological effects including, anti-bacterial, antioxidant, anti-depressive and anti-inflammatory effects (1-4). However, studies have also reported that IF induces anticancer effects in lung and colorectal cancer cell lines via inhibition of the epidermal growth factor receptor and Akt signaling pathways (5,6). Moreover, IF is reported to exhibit anti-tumor effects on human hepatoma cell lines HuH-7 and HepG2 at a non-toxic concentration by inhibiting matrix metalopeptidase-7 expression and ERK1/2 phosphorylation (7).

Hyperthermia (HT) has been recognized as an effective tool to halt tumor growth, especially in combination with conventional therapies. The advantage of this type of combination therapy is the possibility of using lower doses of chemotherapy or radiation leading to more effective treatment with fewer unwanted side effects (8,9). HT induces apoptosis in cancer cells. Apoptosis is a tightly regulated cell suicide program that serves essential roles in development and maintenance of tissue homeostasis by eliminating unnecessary and harmful cells (10). Reactive oxygen species (ROS) serve an important role as intracellular mediators of HT-induced apoptosis. It has been previously reported that in acute monocytic leukemia U937 cells, HT-induced apoptosis is associated with generation of intracellular superoxide (11). Although cancer cells are sensitive to intracellular ROS, due to biological and technical difficulties, complete eradication of the tumor is usually not achieved with a single modality. Therefore, to overcome these challenges, a multimodal strategy is usually adopted in which a combination of chemotherapeutic drugs or chemotherapeutic drugs combined with a physical modality such as HT is utilized (12). An ideal chemical sensitizer for HT should be non-toxic at normal temperature but cytotoxic at hyperthermic temperatures. To the best of our knowledge, the anticancer effects of the IF + HT combination have not been reported before. Therefore, the present study evaluated the role of IF in U937 cells under HT conditions. In the present study

the ability of IF to enhance HT-induced apoptosis in U937 cells was assessed and its associated mechanism was investigated.

Material and methods

Chemicals. IF (7-hydroxy-6,8-dimethoxy-coumarin) was purchased from Wako Pure Chemical Industries, Ltd. Stock solutions were prepared using dimethyl sulfoxide (DMSO) as a solvent and further dissolved to make the desired concentrations for experimental use. The JNK inhibitor SP600125 was purchased from Calbiochem Biosciences, Inc. (Merck KGaA). The pan caspase-3 inhibitor Z-VAD-FMK was purchased from Promega Corporation.

Cell culture. The human acute monocytic leukemia U937 cell line was purchased from the Human Sciences Research Resource Bank (Japan Human Sciences Foundation) and was maintained in RPMI-1640 medium (Wako Pure Chemical Corporation) supplemented with 10% heat-inactivated fetal bovine serum (SIGMA Life Science) at 37˚C in humidified air with 5% CO_2.

Drug treatment and HT. A cell suspension containing $1x10^6$ cells/ml was pre-treated with IF for 1 h at 37˚C. For HT treatment, $1x10^6$/ml U937 cells were transferred to plastic tubes and exposed to 37 or 44˚C for 20 min by immersing tubes containing 1 ml cell suspension into a water bath. Following treatment, the cells were incubated at 37˚C for 6 h.

DNA fragmentation. The quantitative DNA fragmentation assay was performed according to the method reported by Sellins and Cohen (13). Briefly, U937 cells were lysed using a lysis buffer (Tris, 10 mM; EDTA, 1 mM; 0.2% Triton X-100; pH, 7.5) and centrifuged at 13,000 x g for 10 min at 4˚C. Subsequently, each DNA sample in the supernatant was precipitated by adding lysis buffer to the pellet and then added 25% TCA to both pellet and supernatant to attain 12.5% trichloroacetic acid (TCA) concentration. Incubate at 4˚C overnight in the dark and quantified using diphenylamine reagent following hydrolysis in 5% TCA at 90˚C for 20 min. The percentage of DNA fragmentation for each sample was calculated as follows: DNA fragmentation (%)=amount of DNA in the supernatant/total DNA in the sample x100.

Apoptosis assay. Apoptotic assay was determined by apoptotic cells percent in both early and late apoptosis. Apoptotic cells were assessed using Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Immunotech; Beckman Coulter, Inc.). Briefly, following IF pre-treatment (500 μM) for 1 h at 37˚C, the U937 cells were exposed to HT (44˚C; 20 min). Following 6 h incubation at 37˚C, cells were collected, washed with PBS and centrifuged at 160 x g for 3 min at 4˚C. The resulting pellet was mixed with binding buffer of Annexin V-FITC kit. Annexin V-FITC (5 μl) and PI (5 μl) were added to 490 μl each cell suspension, followed by gentle mixing. Following incubation at 4˚C for 30 min in the dark, the cells were analyzed using flow cytometry (Epics XL Flow Cytometer; Coulter Epics XL-MCL Flow Cytometer System II Software, version 3.0, Beckman-Coulter, Inc.

Cell counting assay. The cell counting assay was performed using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) assay according to the manufacturer's protocol. Briefly, following IF pre-treatment (500 μM) for 1 h at 37˚C, cells were exposed to HT (44˚C; 20 min). Following 6 or 24 h incubation at 37˚C, cells were incubated in 100 μl RPMI medium (containing 10 μl CCK-8 reagent) in a 96-well plate and incubated for 2 h at 37˚C in 5% CO_2. The absorbance at 450 nm was assessed using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.).

Cell cycle analysis. Following the drug alone or in combination treatment $1x10^6$ cells/ml were harvested and washed with Dulbecco's phosphate-buffered saline (PBS) and fixed in 10 ml 70% cold ethanol overnight at 4˚C. After washing with PBS, cells were incubated with RNase solution (0.25 mg/ml; Wako Pure Chemical Industries, Ltd.) containing PI (50 μg/ml; Wako Pure Chemical Industries, Ltd.) at 37˚C for 30 min in the dark. The percentage of subG1 cells was assessed using flow cytometry, as aforementioned. A total of 10,000 events was analyzed for each sample using an Epics XL Flow Cytometer, Coulter Epics XL-MCL Flow Cytomter System II Software, version 3.0, Beckman-Coulter, Inc.

Morphological changes of apoptosis. The morphological changes in the cells both treated and untreated were evaluated using Giemsa staining. The cells were harvested following 6 h incubation at 37˚C by centrifugation at 160 x g for 3 min at 4˚C and washed with PBS. Then cells were fixed with methanol and acetic acid (3:1) at 4˚C, 60 min and spread on glass slides. The slides were incubated for 60 min at room temperature. After drying, staining was performed using 5% Giemsa solution (pH 6.8) for 10 min at room temperature. Cells were observed using an Olympus IX70 light microscope (Olympus Corporation) at a magnification of x400. Cells that demonstrated shrinkage and nuclear changes (condensation, fragmentation and apoptotic bodies) were considered to be apoptotic cells (14).

Assessment of intracellular ROS production. To assess intracellular ROS production, both treated and untreated U937 cells were incubated at 37˚C for 15 min with 5 μM hydroxyphenyl fluorescein (HPF; Sekisui Medical Co., Ltd.) to assess intracellular hydroxyl radical and peroxynitrite levels and 5 μM hydroethidine (HE; Molecular Probes; Thermo Fisher Scientific, Inc.) to detect intracellular superoxide levels. The fluorescence emission of all probes was analyzed using flow cytometry as aforementioned. A total of 10,000 events was analyzed for each sample using an Epics XL Flow Cytometer, Coulter Epics XL-MCL Flow Cytomter System II Software, version 3.0, Beckman-Coulter, Inc.

Evaluation of mitochondrial membrane potential (MMP). To assess changes in MMP, acute monocytic leukemia U937 cells were stained with 10 nM tetramethylrhodamine methyl ester (TMRM; Molecular Probes; Thermo Fisher Scientific, Inc.) for 15 min at 37˚C in PBS. The fluorescence of red TMRM was analyzed using flow cytometry (excitation, 488 nm; emission, 575 nm), as aforementioned. The percentage of low-MMP cells was determined from cell counts in the 0.1-12.1 low

window of the TMRM log scale. A total of 10,000 events was analyzed for each sample using an Epics XL Flow Cytometer, Coulter Epics XL-MCL Flow Cytomter System II Software, version 3.0, Beckman-Coulter, Inc.

Western blotting. Both treated and untreated U937 cells were collected and lysed in lysis buffer [Tris-HCl, 1 M; NaCl, 5 M; 1% Nonidet P-40 (v/v); 1% sodium deoxycholate; 0.05% SDS; phenylmethylsulfonyl fluoride, 1 mM] for 20 min at 4˚C. After sonication at 20 khz for 5 sec, the lysates were centrifuged at 12,000 x g for10 min at 4˚C and the protein content in the supernatant was assessed using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.). The protein lysates were denatured at 96˚C for 5 min; after mixing with SDS-loading buffer, 20-30 μg protein/lane was loaded on a 5-20% SDS polyacrylamide gel (SuperStep™ Ace) for electrophoresis and transferred to nitrocellulose membranes. Blots were blocked using 5% skimmed milk for 1 hr at room temperature. Western blotting was performed using primary antibodies as follows: Anti-Bax polyclonal antibody (pAb) (1;1,000; cat. no. 2772; Cell Signaling Technology, Inc.), anti-caspase-3 pAb (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), anti-Bcl-xL pAb (1:1,000, cat. no. 2762; Cell Signaling Technology, Inc.), anti-JNK monoclonal antibody (mAb) (1:1,000, cat. no. 9255; Cell Signaling Technology, Inc.), anti-phospho-JNK mAb (1:1,000; cat. no. 9255; Cell Signaling Technology, Inc.), anti-phospho-p38 mAb (1:1,000; cat. no. 9211; Cell Signaling Technology, Inc.), anti-p38 pAb (1:1,000; cat. no. sc-728 Santa Cruz Biotechnology, Inc.). All primary antibodies were incubated overnight at 4˚C. Anti-β-actin mAb (1:1,000; cat. no. A5441; MilliporeSigma) was incubated for 1 hr at room temperature. Horseradish peroxidase (HRP) conjugated anti-rabbit IgG (1;1,000, cat. no. 7074 ; Cell Signaling Technology, Inc.) and HRP conjugated anti-mouse IgG (1:1,000; cat. no. 7076; Cell Signaling Technology, Inc.) were used as secondary antibodies, incubated for 1 hr at room temperature.

Band signals were visualized using a luminescent image analyzer (LAS4000, Fujifilm Co.) using ECL system (Amersham; Cytiva). Band density was semi-quantified using Image J software (ver 1.5.3; National Institutes of Health).

For the preparation of cytosolic and mitochondrial fractions, both untreated and treated U937 cells were collected, washed with PBS and suspended in 300 μl extraction buffer (D-mammitol, 210 mM; sucrose, 70 mM; EDTA, 5 mM; aprotinin, 1 μg/ml; pepstain, 1 μg/ml; leupeptin, 1 μg/ml; PMSF, 1 mM; HEPES-KOH, 10 mM; pH 7.4). Following 5 min incubation on ice, cells were homogenized and centrifuged at 1,500 x g for 5 min at 4˚C to remove nuclei and debris. The supernatant was centrifuged at 105,000 x g for 30 min at 4˚C. The resulting supernatant was used as the soluble cytosolic fraction (S100) and the pellet was resolved in mitochondrial buffer as the mitochondrial fraction. Blots were blocked using 5% skimmed milk for 1 hr at room temperature. Western blotting was performed to detect cytochrome c and Bax released to the cytosol and mitochondria using anti-cytochrome c pAb (1;1,000, cat. no. 4272; Cell Signaling Technology, Inc.), anti-Bax pAb (1:1,000, cat. no. 2762; Cell Signaling Technology, Inc.), anti-β-actin mAb (1:1,000; cat. no. A5441; MilliporeSigma) and anti-HSP60 mAb (1:1,000; cat. no. 611562: BD Biosciences) primary antibodies. All primary antibodies were incubated overnight at 4˚C except anti-β-actin mAb, incubated for 1 h at room temperature.

Evaluation of intercellular free calcium ions. To assess the effect of IF treatment on intracellular calcium homeostasis, intracellular free Ca^{2+} levels were assessed using Fluo-3/AM (Dojindo Laboratories Inc.) calcium probe. Cells were treated with IF (500 μM) and incubated for 1 h at 37˚C, and then exposed to 44˚C for 20 min. Following 6 h incubation at 37˚C, the cells were harvested and incubated with 5 μM Fluo-3/AM for 30 min at 37˚C. Excess Fluo-3/AM was removed by washing three times with PBS. The fluorescence intensity was assessed using Epics XL Flow Cytometer, Coulter Epics XL-MCL Flow Cytomter System II Software, version 3.0, Beckman-Coulter, Inc.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism version 5 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Effect of IF on HT-induced apoptosis. U937 cells were treated with HT exposure with or without IF pre-treatment. DNA fragmentation, cell survival and cell cycle assay were performed to evaluate the effects of IF on HT-induced apoptosis in U937 cells. The DNA fragmentation assay demonstrated that IF markedly enhanced HT-induced apoptosis in a dose-dependent manner (Fig. 1A). IF at a dose of 200 and 500 μM induced significant increase in DNA fragmentation % compared to control. However, 500 μM was used for subsequent experiments because it demonstrated the greatest significant difference in DNA fragmentation following HT exposure compared with 0 μM IF control. The cell survival assay demonstrated that IF in combination with HT significantly decreased cell survival percentage following 6 h incubation compared with the HT group (Fig. 1B). The fraction of cells in subG1 also significantly increased following combined treatment compared with the HT group (Fig. 1C). Similarly, a marked increase in typical apoptotic features, such as cytoplasmic aggregation, nuclear condensation and fragmentation were observed in the combined treatment cells than IF and HT alone treatment, as assessed using Giemsa staining (Fig. 1D). Taken together, the data indicated that IF markedly sensitized U937 cells to HT exposure.

Effect of IF on HT-induced Annexin V-FITC/PI double staining. To assess the effect of combined IF/HT treatment *in vitro*, U937 cells were incubated with or without IF for 1 h and then subjected to HT with subsequent recovery at 37˚C for 6 h. Cell death (early apoptosis and late apoptosis) was assessed using flow cytometry via annexin V-FITC/PI double staining. The IF treatment alone did not induce apoptosis compared with control. HT treatment alone markedly increased the proportion of apoptotic cells compared with control and IF treatment alone, however when cells were treated with IF + HT, the

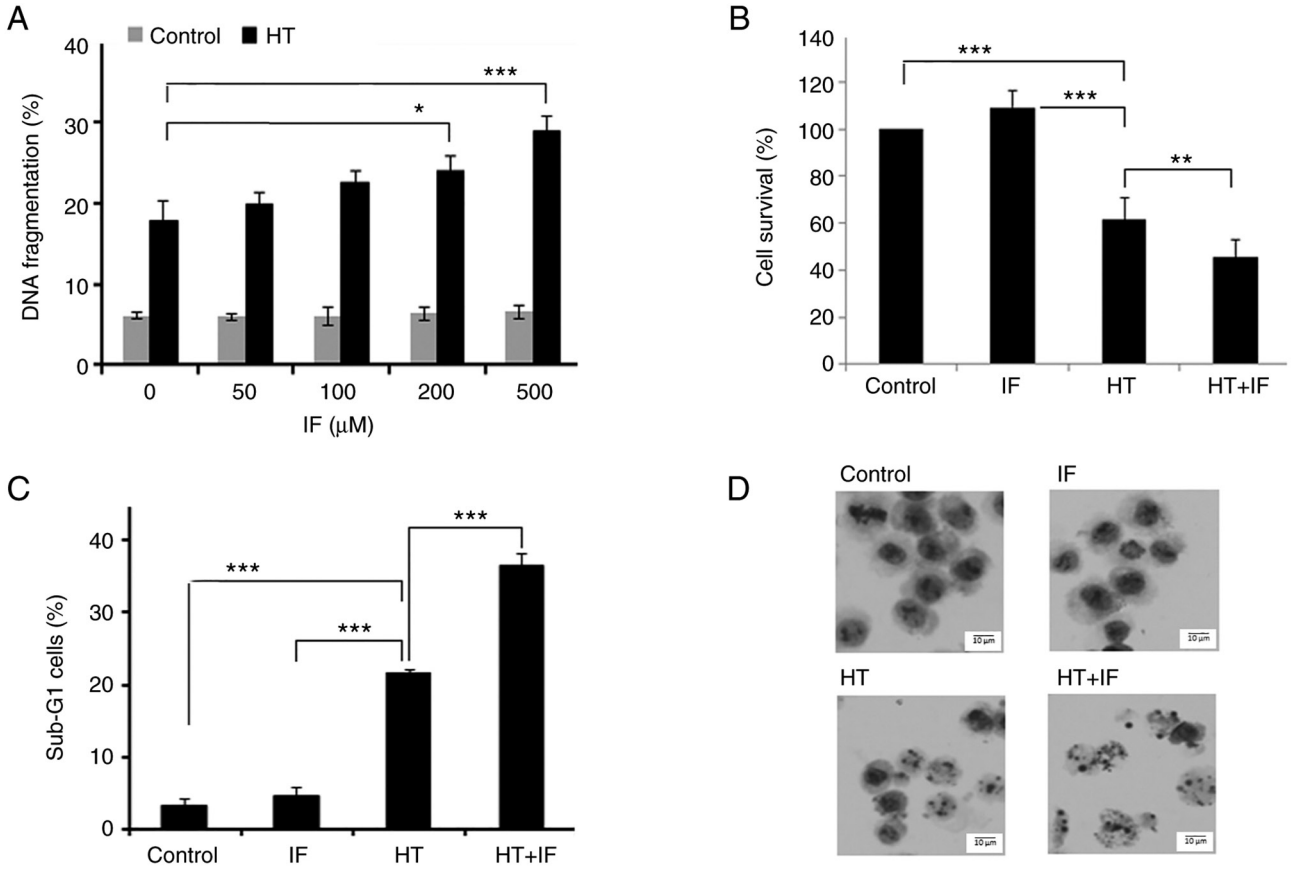

Figure 1. IF increases HT-induced apoptosis in U937 cells. (A) Cells were treated with HT (44˚C; 20 min) with or without IF (50, 100, 200 and 500 μM) pre-treatment for 1 h and incubated at 37˚C. DNA fragmentation assay was performed after 6 h. *P<0.05. (B) Cells were pre-treated with 500 μM IF for 1 h and then exposed to HT (44˚C; 20 min). Cell survival analysis was performed using Cell Counting Kit-8 assay after 6 h. **P<0.01. (C) Cells were collected after incubation for 6 h and incubated with RNase solution and propidium iodide at 37˚C for 30 min. The percentage of sub-G1 cells was determined using flow cytometry. The results are presented as mean ± SD (n=3); significance was assessed using one-way ANOVA with Tukey's multiple comparison test. ***P<0.001. (D) Apoptotic features of U937 cells in response to the combined treatment with HT and IF were assessed using Giemsa staining after 6 h incubation and evaluated under a microscope at x400 magnification. Scale bar 10 μm. IF, isofraxidin; HT, hyperthermia.

proportion of apoptotic U937 cells was significantly increased compared with either treatment alone (Fig. 2A and B).

Effect of IF on HT-induced MMP loss and ROS generation. It has been reported that apoptosis involves a disruption of mitochondrial membrane integrity, which is key for the cell death process (15,16). To determine whether MMP change was involved in IF-enhanced apoptosis, the change in the MMP was assessed 6 h after HT exposure. Loss of MMP was markedly induced by HT and was significantly elevated in the presence of IF at 6 h compared with the HT group (Fig. 3A).

Excess generation of intracellular ROS causes oxidative stress, which serves a key role in the induction of apoptosis (17,18). To assess whether IF increased oxidative stress induced by HT, intracellular ROS generation was evaluated using HE and HPF staining. The results demonstrated that the intensity of HE fluorescence increased markedly in HT-treated cells at 30 min than untreated control and IF treatment alone, which was further significantly enhanced in the combination-treated cells compared with the HT group (Fig. 3B). Furthermore, the intensity of HPF fluorescence was also markedly elevated at 30 min after HT exposure than untreated control and IF treatment, and was significantly enhanced following combination treatment with IF and HT compared with the HT group (Fig. 3C).

Effect of IF on intracellular Ca^{2+} levels. Calcium homeostasis is essential for cellular functions, such as protein folding, processing and transport and signal transduction (19). Increased levels of Ca^{2+} overexcite cells and cause the generation of harmful chemicals such as free radicals (20). Therefore, intracellular Ca^{2+} levels were assessed following treatment. It was demonstrated that the combined treatment significantly enhanced intracellular Ca^{2+} concentration compared with either treatment alone (Fig. 3D).

Effect of IF on HT-induced caspase activation. Caspases, a family of cysteine proteases, are activated in the execution phase of the apoptotic process. Once activated, caspases activate downstream caspases, leading to apoptosis (21). To assess protein expression levels of caspases western blotting was performed. Cells were pre-treated with IF for 1 h, followed by HT exposure and then collected for protein extraction. The results demonstrated that protein expression levels of cleaved caspase-3 and cleaved caspase-8 were markedly increased in the combined treatment cells than HT treatment alone (Fig. 4A). To verify the role of caspases, DNA fragmentation is performed using the pan caspase inhibitor Z-VAD-FMK. The results demonstrated that both HT and combined treatment-mediated apoptosis was significantly suppressed in

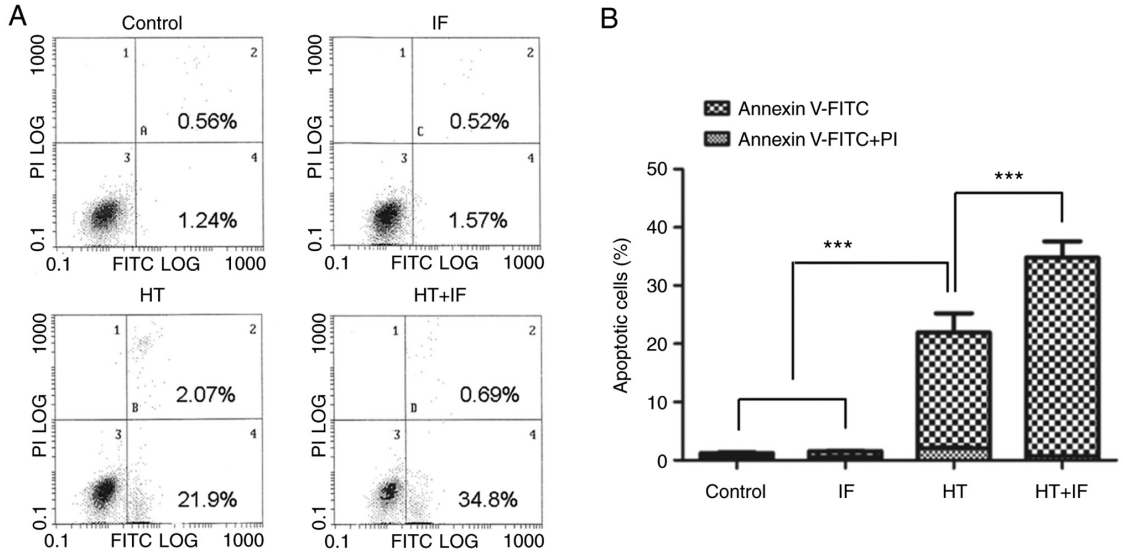

Figure 2. IF enhances HT-induced early apoptosis and secondary necrosis. (A) Representative flow cytometry dot plots of annexin V-FITC and PI-stained cells. (B) The cells were treated with HT with or without IF. The percentage of cells in early apoptosis and secondary necrosis were analyzed using flow cytometry 6 h after combined treatment. The results were presented as the mean ± SD (n=3), significance was assessed using one-way ANOVA with Tukey's multiple comparison test. ***P<0.001 control and IF vs HT. . IF, isofraxidin; HT, hyperthermia; PI, propidium iodide.

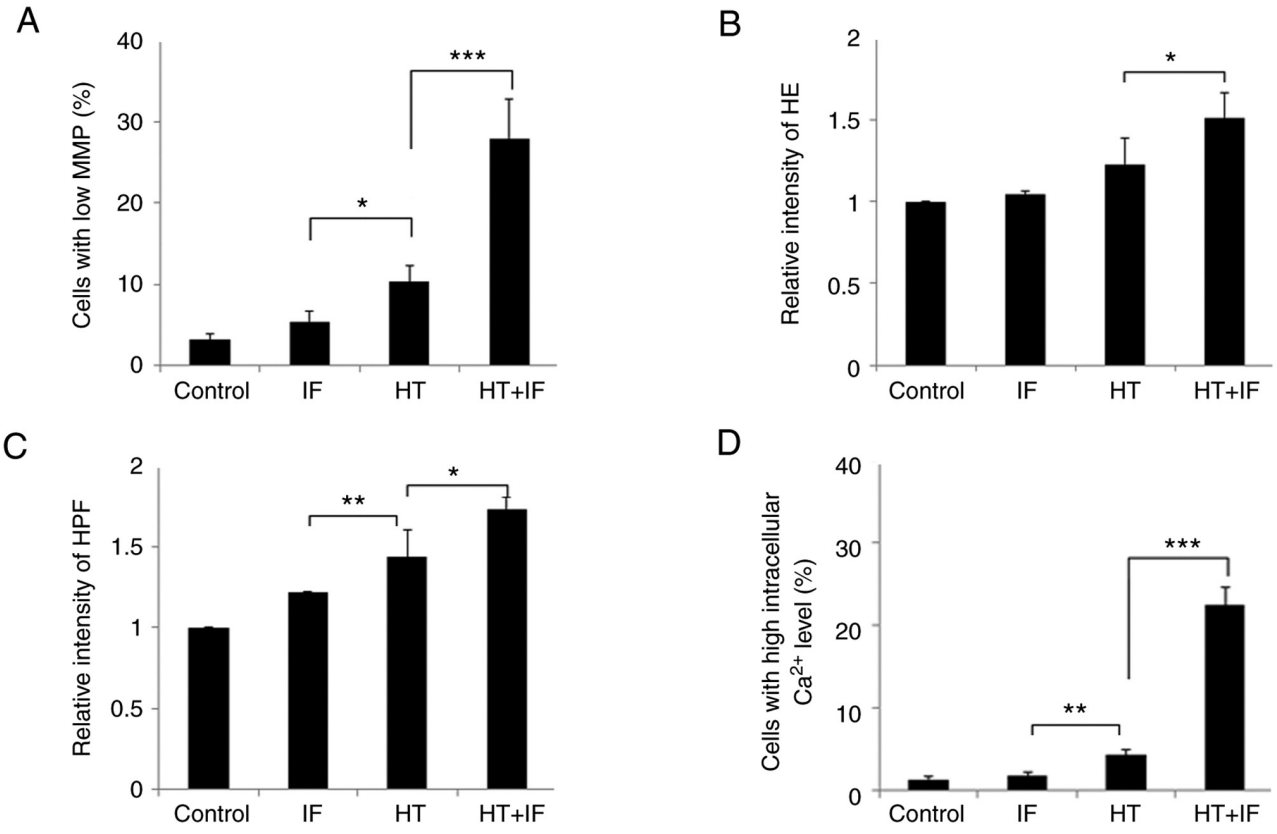

Figure 3. IF enhances HT-induced MMP loss and reactive oxygen species generation in U937 cells. Cells were pre-treated with 500 μM IF for 1 h and then treated with HT (44˚C; 20 min). (A) Following 6 h incubation, cells were collected and analyzed using flow cytometry using tetramethylrhodamine methyl ester staining. (B) Following 30 min incubation, cells were stained using HE for 15 min. Fluorescence intensity was detected using flow cytometry. (C) Cells were stained with HPF for 15 min. Fluorescence intensity was detected using flow cytometry. (D) IF promotes HT-elevated intracellular Ca^{2+} levels in U937 cells. Cells were harvested and stained using 5 μM Fluo-3/AM for 30 min and intracellular Ca^{2+} levels were assessed using flow cytometry. The results are presented as mean ± SD (n=3); significance was assessed using one-way ANOVA with Tukey's multiple comparison test. *P<0.05, ***P<0.001 HT vs. HT + IF. **P<0.01 IF vs. HT. HE, hydroethidine; HPF, hydroxyphenyl fluorescein; MMP, mitochondrial membrane potential; IF, isofraxidin; HT, hyperthermia.

the presence of the inhibitor compared with the uninhibited group, which suggested that caspase cascades were involved in IF-mediated apoptotic enhancement in response to HT exposure (Fig. 4B).

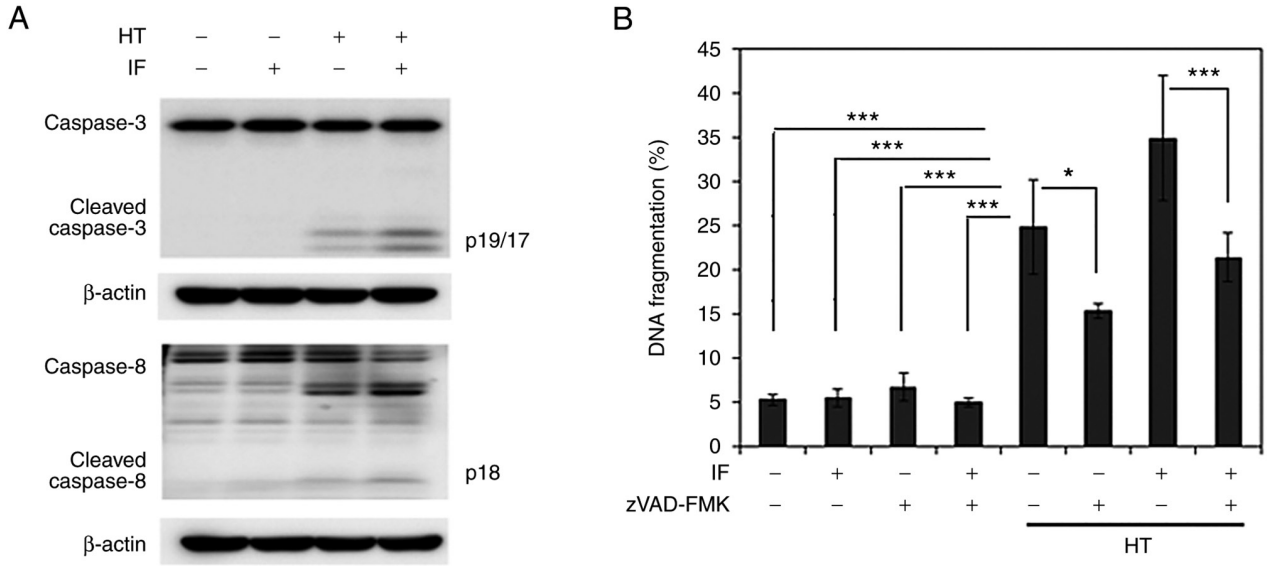

Figure 4. IF promotes HT-induced apoptosis via a caspase-dependent pathway in U937 cells. (A) Cells were pre-treated with 500 μM IF for 1 h and then treated with HT (44˚C; 20 min). Following 6 h incubation, protein was extracted from cells and western blotting was performed to assess protein expression levels of caspase-3 and caspase-8. (B) Cells were pre-treated with 10 μM zVAD-FMK, a pan-caspase inhibitor, for 1 h and exposed to HT (44˚C; 20 min) in the presence of IF (500 μM). DNA fragmentation was performed following 6 h incubation. The results are presented as mean ± SD (n=3); significance was assessed using one-way ANOVA with Tukey's multiple comparison test. *P<0.05 HT vs. HT + zVAD-FMK, ***P<0.001 IF + HT vs. IF +HT + zVAD-FMK. IF, isofraxidin; HT, hyperthermia.

Effect of IF treatment on apoptosis-associated protein. To assess the molecular mechanism of IF in response to HT treatment, western blotting was performed. Bcl-2 family proteins with anti- or pro-apoptotic functions control release of mitochondrial apoptosis factors including cytochrome c and apoptosis-inducing factor (22). Markedly decreased Bcl-xL protein expression levels were demonstrated 6 h following combined treatment compared to either treatment alone (Fig. 5A). No changes in protein expression levels of Bax were demonstrated in whole cell lysate 6 h after HT or combined treatment, however translocation of Bax from cytosol to mitochondria was observed in HT-treated cells and further augmented in combined treatment cells (Fig. 5B). Western blotting also demonstrated that IF markedly enhanced release of cytochrome c from mitochondria to the cytosol compared to control and IF treatment alone, which was induced by HT treatment. Furthermore, induction of heat shock protein is the most well-characterized heat shock response. HSP 70 has been reported to serve a critical role in cell survival and thermotolerance, potentially by preventing recruitment of procaspase-9 to the apoptosome by binding to apoptotic protein activation factor-1 (Apaf-1), thereby inhibiting activation of caspase-3 (23). Therefore, expression of HSP 70 was also assessed using western blotting. HT-induced HSP70 expression was not significantly affected by combined treatment. These results indicated that a mitochondria-dependent pathway was involved in IF-mediated enhancement of apoptosis induced by HT treatment.

Effect of IF on MAPK-associated pathway. Activation of MAPKs serves a key role in apoptosis induced by cellular stresses and oxidative stress is known to activate MAPK family members by protein phosphorylation (24). To assess whether the MAPK signaling pathway was involved in IF-mediated enhancement of apoptosis, protein expression levels of JNK and p38 were assessed using western blotting at 1 h after HT or combined IF + HT treatment. The results demonstrated that JNK phosphorylation was markedly induced at 1 h following HT treatment compared with the untreated control and IF, and substantially further, markedly elevated by IF + HT compared with the HT group (Fig. 6A). P38 phosphorylation was also markedly induced at 1 h after HT treatment; however, IF did not significantly promote phosphorylation of P38. The protein expression levels of JNK and p38 remained unchanged in cells with HT and/or IF treatment. To evaluate the role of JNK signaling pathway in IF-mediated enhancement of apoptosis induced by HT, a JNK inhibitor, SP10025, was used. The inhibitor did not suppress HT-induced apoptosis; however, it partially suppressed HT + IF-induced apoptosis, which indicated that JNK was, in part, involved in IF-mediated apoptotic enhancement following HT treatment (Fig. 6B).

Discussion

Heat stress induces formation of ROS, such as superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$) by cells. Several studies have reported a link between oxidative stress and heat stress and synergistic augmentation of cell death as increased ROS generation is observed in HT-exposed cells (25-27). $O_2^{\bullet-}$ is the precursor of most ROS, a mediator in oxidative chain reactions and react with other radicals, including $^{\bullet}NO$ (28) to form $ONOO^-$, which is also a potent oxidant (29). HE indicates the presence of superoxide anions and HPF indicates the presence of $^{\bullet}OH$ and $ONOO^-$. Therefore, in the present study, both HE and HPF staining were used to assess the effect of IF on HT-induced ROS formation. The intensity of HE and HPF fluorescence was significantly increased in the presence

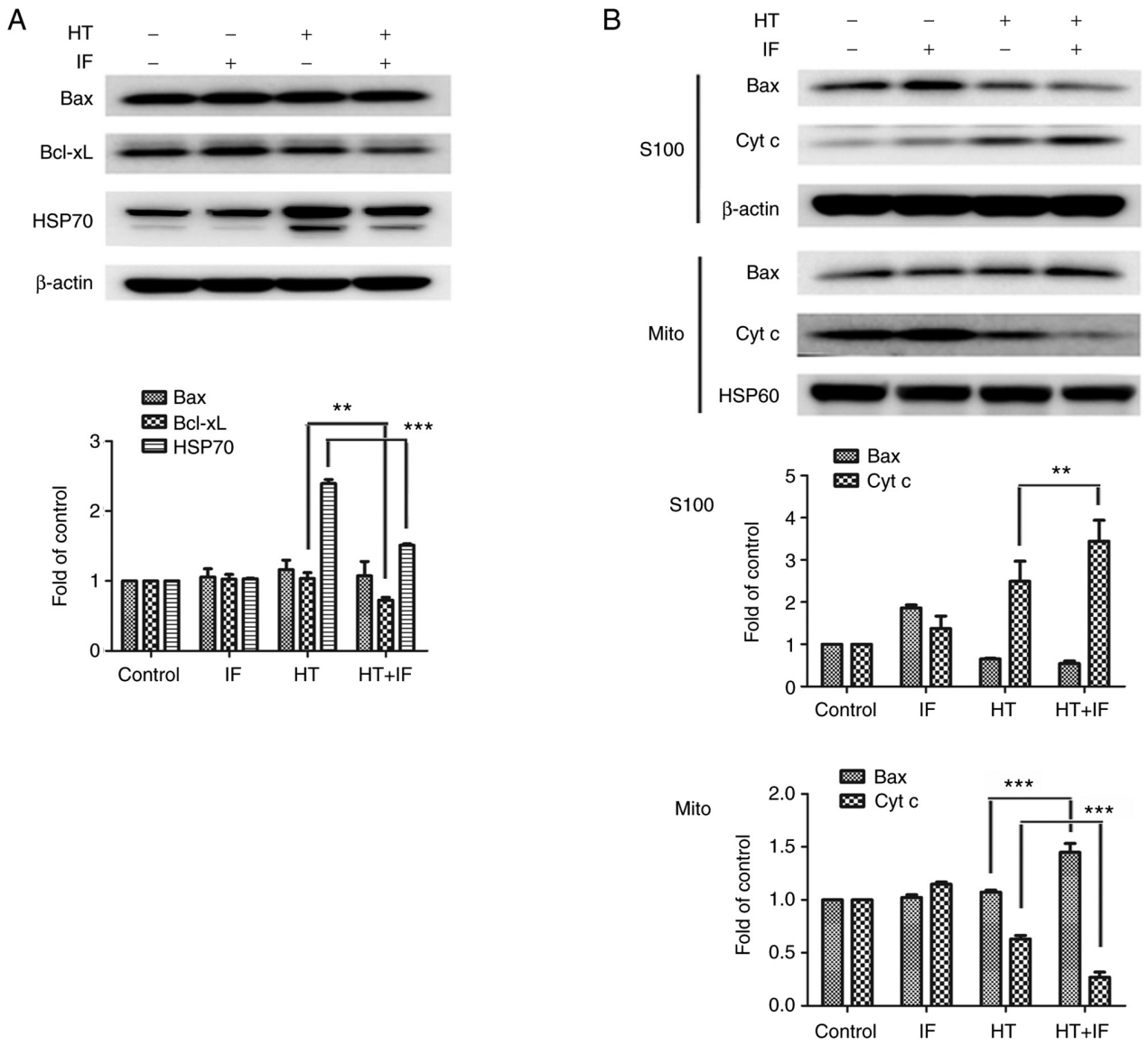

Figure 5. IF elevates HT-induced apoptosis in U937 cells via the mitochondrial pathway. Cells were pre-treated with 500 μM IF for 1 h and exposed to HT (44˚C; 20 min). Following 6 h incubation, cells were collected for use in experiments. (A) Western blotting demonstrated changes in expression levels of cell death-associated proteins Bax, Bcl-xL and HSP 70. (B) Changes in protein expression levels of cytochrome c and Bax in the Mito and S100 fractions. Band density was semi-quantified using Image J software (ver 1.5.3) and presented as fold-change vs. control (untreated). Semi-quantified data are presented as mean ± SD (n=3); significance was assessed using one-way ANOVA with Tukey's multiple comparison test. **P<0.01 HT vs. HT + IF (Bcl-XL, Cyto c) and ***P<0.001 HT vs. HT + IF (HSP70, Cyto c and Bax). HSP70, heath shock protein 70; Mito, mitochondrial; S100, cytosolic; IF, isofraxidin; HT, hyperthermia; Cyt c, Cytochrome c.

of IF following HT treatment compared with the HT-alone group. IF was previously reported to protect U937 cells against radiation-induced apoptosis via scavenging of $^{\bullet}$OH generation (3). The reason for this discrepancy in IF effects may be because in irradiated cells, $^{\bullet}$OH radicals are generated directly and transiently and IF quenches radiation-induced $^{\bullet}$OH production, immediately suppressing radiation-induced apoptosis. However, HT generates $O_2^{\bullet-}$ heterogeneously and chronically and does not directly generate $^{\bullet}$OH production within cells (30). SOD-mimic nitroxides have been reported to cause dismutation of $O_2^{\bullet-}$ in the presence of H^+ and produce H_2O_2 and O_2 by the subsequent chemical reaction *in vitro* (30). Moreover, H_2O_2 participates in the Fenton reaction in the presence of transition metals, particularly Fe^{2+}, to release $^{\bullet}$OH (31). In the present study, IF transiently increased the intensity of HE and HPF fluorescence 30 min after HT treatment. This transient elevation of ROS by IF may have resulted in apoptotic enhancement in response to HT. Furthermore, HT has an advantage to sensitize cells because cells in late S phase, which are resistant to X-rays, are most heat sensitive, cells deficient in nutrients are heat sensitive and cells subject to chronic hypoxia demonstrate slightly enhanced heat sensitivity (12). HT also changes cellular status, such as causing increases in membrane fluidity and permeability, alteration of protein structure and function and, increase in cellular metabolism. HT is also selective towards cancer cells; our previous studies demonstrated that HT is well-tolerated by non-tumor cells (12,32,33). Therefore, the cell killing potential of most chemotherapeutic agents may be substantially enhanced by temperature elevation.

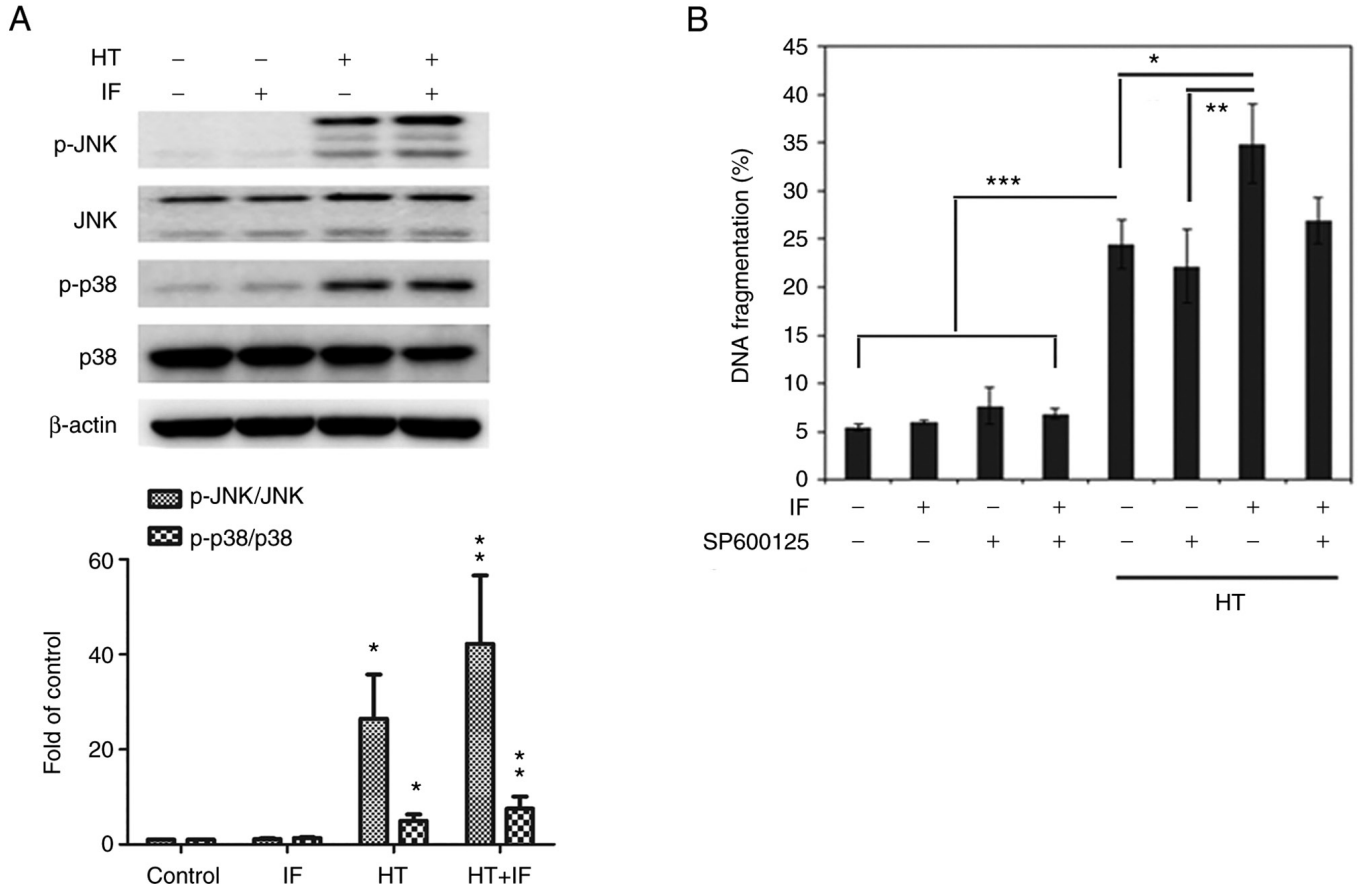

Figure 6. Role of IF in the MAPK signaling pathway in HT-treated U937 cells. (A) Cells were pre-treated with 500 μM IF for 1 h and exposed to HT (44˚C; 20 min). Following 1 h incubation, cells were collected for use in experiments. Changes in expression levels of MAPK signaling pathway-associated proteins such as p-JNK, JNK, p-p38 and p38 were assessed using western blotting. Band density was semi-quantified using Image J software and were presented as fold change versus control (untreated). Semi-quantified data was presented as mean ± SD (n=3). *P<0.05 control vs. HT and IF vs. HT (pJNK/JNK), **P<0.01 control vs. HT + IF and IF vs. HT + IF (pJNK/JNK). *P<0.05 control vs. HT and IF vs. HT (p-p38/p38), **P<0.01 control vs. HT + IF and IF vs. HT + IF (p-p38/p38). (B) Cells were pre-treated with 10 μM SP600125 for 1 h, then exposed to HT (44˚C; 20 min) with IF (500 μM). DNA fragmentation was performed following 6 h incubation. The results were presented as mean ± SD (n=3); significance was assessed using one-way ANOVA with Tukey's multiple comparison test. *P<0.05 HT vs. HT + IF, **P<0.01 HT + SP600125 vs. HT + IF, ***P<0.001 HT vs IF and SP600125 (either treatment alone or in combination). IF, isofraxidin; HT, hyperthermia; p, phosphorylated.

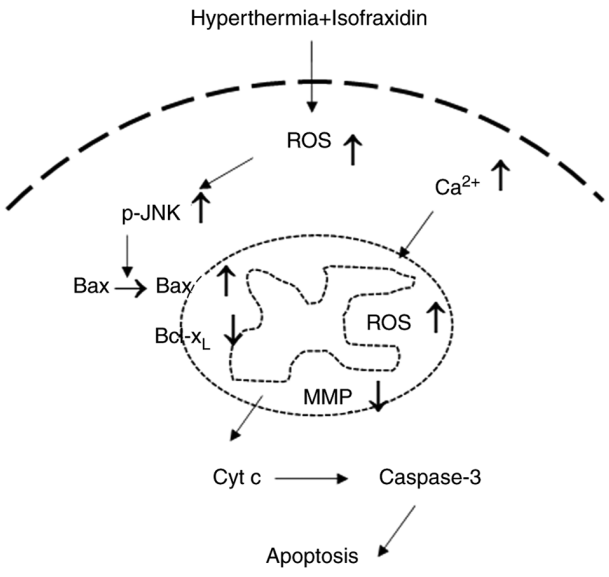

Figure 7. Isofraxidin enhances hyperthermia-induced apoptosis via a ROS mediated mitochondria-caspase-dependent signaling pathway in U937 cells. ROS, reactive oxygen species; MMP, mitochondrial membrane potential; Cyt c, cytochrome c; p, phosphorylated.

The apoptotic process occurs via two distinct yet tightly interconnected pathways: Intrinsic pathway (mitochondrial) and extrinsic pathway (death receptors). There are specific proteins in each of these pathways. The key step of the intrinsic apoptosis pathway is permeabilization of the mitochondrial outer membrane (MOMP), which results in MMP loss, then release of certain apoptogenic factors from mitochondria. Cytochrome c, one such protein, binds to the adapter Apaf-1, which recruits cytosolic pro-caspase-9 into a heptameric complex, called the apoptosome. Active caspase-9 cleaves and activates effector caspase-3 and caspase-7 (34,35). In the present study, HT-induced MMP loss was further promoted in IF + HT combination treatment cells. Moreover, the release of cytochrome c from mitochondria to cytosol was clearly observed in HT-treated cells and expression of cytosolic cytochrome c was further enhanced in IF + HT combination treatment cells. In the extrinsic apoptosis pathway, stimulation of death receptors such as CD95 (APO-1/Fas) and TNF-associated apoptosis-inducing ligand receptors results in formation of the death-inducing signaling complex, following activation of the initiator caspase-8. Activated caspase-8 propagates the apoptotic signal either by direct activation of downstream

effector caspases such as caspase-3 or by truncation of BH3 interacting-domain death agonist (Bid) (36). Bid assists in activation and translocation of Bax to the mitochondria, thus triggering the intrinsic pathway (37). In the present study, protein expression levels of cleaved caspase-3 and caspase-8 were induced in HT-treated cells and markedly enhanced by IF pre-treatment following HT exposure. Taken together, these data demonstrated that IF may elevate HT-induced apoptosis in a caspase-dependent mitochondrial signaling pathway.

MOMP leads to both caspase-dependent and -independent cell death signaling and is therefore a key cell life-or-death decision checkpoint. This checkpoint is controlled by the Bcl-2 family of proteins (30). This family is subdivided into three key groups on the basis of their function: Pro-apoptotic BH3-only (Noxa, Bid, Bim, p53 upregulated modulator of apoptosis, Bad, Bcl-2-modifying factor, Hrk and Bcl-2-interacting killer), pro-survival (Mcl-1, Bcl-2, Bcl-xL) and pore-forming proteins (Bax and Bcl-2 homologous antagonist/killer). In the apoptotic process, large amounts of Bax/Bak are bound by Bcl-2 survival factors, BH3-only proteins displace large numbers of Bax/Bak molecules from Bcl-2 and therefore increase the number of pore-forming proteins available for activation of MOMP (38,39). The mitochondrial translocation of Bax is key to the release of cytochrome c during the apoptotic process (40,41). In the present study, the protein expression levels of Bcl-xL were significantly decreased in IF + HT combination treatment cells compared with the HT group. The protein expression levels of Bax in the total cell lysate was not significantly affected by IF in response to HT; however, translocation of Bax from the cytosol to the mitochondria was observed in HT-treated cells and IF + HT combination-treated cells. The data demonstrated that IF pre-treatment potentiated HT-triggered translocation of Bax. Taken together, these data demonstrated that IF enhanced HT-induced apoptosis by decreasing protein expression levels of anti-apoptotic Bcl-xL and increasing translocation of Bax and the release of cytochrome c.

MAPKs are a family of protein kinases that are responsible for transduction of intracellular signaling. They also participate in a range of physiological regulatory activities. Activation of MAPK signaling pathways, including JNK, p38 and ERK, serves a key role in modifying cell death induced by cellular stresses. It has been reported that stress-activated JNK and p38 kinase phosphorylate Bax; phosphorylation of Bax leads to mitochondrial translocation of activated Bax and initiation of mitochondria-dependent apoptosis in cells treated with cell death stimulants (42). To assess the molecular mechanism of IF in response to HT-induced apoptosis, protein expression levels of JNK and p38 were assessed. IF + HT combination treatment markedly increased phosphorylation levels of JNK and p38 compared with the HT group. JNK activation has been reported to serve an essential role in HT-induced apoptosis (43). To evaluate the role of JNK in IF-mediated apoptotic enhancement, a JNK inhibitor (SP600125) was used in U937 cells. The JNK inhibitor markedly decreased IF-mediated apoptotic enhancement following HT treatment, indicating that the JNK pathway may be involved in the enhancement of apoptosis by IF.

Ca^{2+} is a second messenger in cells that translates extracellular stimuli into intracellular activities, which are important for the regulation of physiological processes (44). Excessive elevation or prolonged activation of Ca^{2+} signaling causes cell death (45,46). It has been reported that oxidative stress and intracellular Ca^{2+} overload are connected in HT-mediated cell death (45,46). It is well established that intracellular Ca^{2+} overload triggers apoptosis via the mitochondria-dependent pathway (47). The mitochondria-dependent apoptosis pathway involves numerous events, such as generation of ROS, the opening of permeability transition pores, MMP loss, release of cytochrome c, expression of Bcl-2 family members and activation of caspase-9 and caspase-3 (48,49). Moreover, calcium increase is secondary to oxidative stress-induced mitochondrial damage. Therefore, the association between increased calcium release and ER stress should be part of future study of the combination of drugs and HT. In the present study, the data supported the classic mitochondria-dependent apoptotic pathway, which was promoted by HT with or without IF pre-treatment in U937 cells. The enhanced intracellular Ca^{2+} overload in HT + IF-treated cells triggered apoptosis via the mitochondria-dependent pathway.

To the best of our knowledge, the present study is the first to demonstrate the effect of IF on HT-induced apoptosis. These findings suggested that IF significantly enhanced HT-induced apoptosis via caspase-mitochondria dependent pathways such as loss of MMP, excessive ROS generation, mitochondrial translocation of Bax, release of cytochrome c and caspase-3 activation. HT was not only cytotoxic to cancer cells but also enhanced fluidity and permeability of the plasma membrane, which promotes drug delivery (50). Therefore, increased IF delivery into cells by HT may be a possible mechanism underlying the enhancement of apoptosis demonstrated by combined treatment (Fig. 7). In the present study, data were collected mainly using U937 cells; lack of evaluation using a normal cell line is as a limitation of the present study. However, it is important to note that a previous study also reported that IF exhibits low toxicity towards BEAS-2B normal lung epithelial cells compared with the lung cancer A549 cell line (6). Furthermore, our previous study also demonstrated that IF serves as an antioxidant and protects cells against radiation-induced cell death (3). These reports suggested that IF does not induce cytotoxic effects in normal cells. These data suggested that IF might be an effective thermo-sensitizer for cancer therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Hypotheses and experiments were conceived by QLZ. Experiments were performed by PL, MUR, PJ, KA, ZGC and QLZ. Data analysis and manuscript preparation were

performed by TK, JIS, KN, MUR and QLZ. QLZ and PL confirm the authenticity of all the raw data. 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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