Ginkgolide K protects the heart against ER stress injury by activating the IRE1α/XBP1 pathway

Running title: Ginkgolide K protects the heart

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ABSTRACT

BACKGROUND AND PURPOSE

Endoplasmic reticulum (ER) stress is increasingly recognized as an important causal factor of many diseases. Targeting ER stress has now emerged as a new therapeutic strategy for treating cardiovascular diseases. Here we investigated the effects and the underlying mechanism of Ginkgolide K (1,10-dihydroxy-3,14-didehydroginkgolide, GK) on cardiac ER stress.

EXPERIMENTAL APPROACH

Cell death, apoptosis, and ER stress-related signalling pathways were measured in cultured neonatal rat cardiomyocytes (NRCMs), treated with the ER stress inducers tunicamycin, hydrogen peroxide, and thapsigargin. Acute myocardial infarction was established using left coronary artery occlusion in mice, and infarct size was measured by triphenyltetrazolium chloride (TTC) staining. Echocardiography was used to assess heart function and transmission electron microscopy for evaluating ER expansion.

KEY RESULTS

GK significantly decreased ER stress-induced cell death in both in vitro and in vivo models. In ischemic injured mice, GK treatment reduced infarct size, rescued heart dysfunction and ameliorated ER dilation. Mechanistic studies revealed that the beneficial effects of GK occurred through enhancement of inositol-requiring enzyme 1α (IRE1α)/ X box-binding protein-1 (XBP1) activity, which in turn led to increased ER-associated degradation (ERAD)-mediated clearance of misfolded proteins and autophagy. In addition, GK was also able to partially repress the pro-apoptotic action of regulated IRE1-dependent decay (RIDD) and JNK pathway.

CONCLUSIONS AND IMPLICATIONS

In conclusion, GK acts through selective activation of the IRE1α/XBP1 pathway to limit ER stress injury. GK is revealed as a promising therapeutic agent to ameliorate ER stress for treating cardiovascular diseases.
**Keywords** Ginkgolide K · ER stress · IRE1α · XBP1 · ER-associated degradation (ERAD) · Autophagy

**Abbreviations**

GK, Ginkgolide K; ER, Endoplasmic reticulum; NRCMs, neonatal rat cardiomyocytes; ERAD, ER-associated degradation; UPR, unfolded protein response; PERK, pancreatic eIF2-alpha kinase; IRE1α, inositol-requiring enzyme 1α; XBP1, X box-binding protein-1; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; eIF2α, eukaryotic translation initiation factor 2α; ASK1, apoptotic signalling kinase-1; GRP78, glucose regulated protein 78 kD; CHOP, C/EBP homologous protein; RIDD, regulated IRE1-dependent decay; GB, Ginkgolide B; PAF, platelet-activating factor; TRAF2, tumor necrosis factor receptor associated factor 2;

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**Introduction**

The endoplasmic reticulum (ER) is a membranous organelle that plays an important role in the maintenance of cellular processes such as protein processing, calcium homeostasis and lipid biosynthesis. Effective ER function is essential for the folding of secretory and membrane proteins (Wei et al., 1996). A number of cellular stress disturb the balance between the ER folding capacity and the burden of incoming proteins, including oxidative stress, ischemic insult, abnormal ER calcium content, and enhanced expression of normal and/or folding-defective proteins. These events lead to the accumulation of unfolded proteins, a condition referred to as ER stress. ER is capable of sensing a wide variety of perturbations which triggers an adaptive process, known as unfolded protein response (UPR), to maintain ER homeostasis and mitigate or eliminate the stress. The so-called UPR consists of three characteristic components: (1) translational attenuation to reduce the translocation of nascent proteins into the ER, (2) transcriptional activation of genes encoding ER chaperones and enzymes to improve protein folding capacity, and (3) transcriptional activation of genes for components of the ER-associated degradation (ERAD) system and autophagy to degrade the misfolded protein. ER stress engages the ER-located molecular chaperone glucose regulated protein 78 kD (GRP78) and three ER transmembrane proteins-inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 (ATF6), and pancreatic eIF2-alpha kinase (PERK) to mobilise UPR. PERK stimulates phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) as an immediate response. IRE1α is a dual protein kinase/endoribonuclease (RNase), and processes the mRNA encoding unspliced X box-binding protein 1 (XBP1u) to spliced XBP1 (XBP1s) through its RNase activity. XBP1s encodes an active transcription factor which controls the genes encoding proteins involved in protein folding, ERAD, protein quality control and phospholipid synthesis. Meanwhile, ATF6 cooperates with IRE1α for the induction of XBP1 transcription (Sozen et al., 2015). These proteins work in concert to balance the unfolded protein/chaperone system to provide ER homeostasis. If cell fails to recover from ER stress, the UPR represses the adaptive response and triggers apoptosis (Chen et al., 2013).
Malfunction of UPR has been implicated in a wide range of diseases including cancer, diabetes, neurodegenerative diseases, and age-related disorders. Numerous lines of evidence also indicate that UPR and ER stress play an important role in the development of cardiovascular diseases such as ischemia/reperfusion injury (Vekich et al., 2012; Wang et al., 2014), atherosclerosis (Chistiakov et al., 2014; McAlpine et al., 2013) and heart failure (Dickhout et al., 2011; Groenendyk et al., 2013). Although prolonged UPR activation is deleterious, leading to cell death, UPR acts as a protective response at an early stage during cell injury. For UPR action, three ER sensors work synergistically through distinct sets of target genes. Thus, strategies that are able to differentially regulate individual components of UPR to alleviate ER stress may present promising avenues to develop novel therapeutic interventions for cardiovascular diseases.

Ginkgolide K (1,10-dihydroxy-3,14-didehydroginkgolide, GK) is a diterpene lactone compound isolated from the leaves of Ginkgo biloba, which is a natural product with a long history of therapeutic application for cardiovascular diseases in humans (Liu et al., 2014). Ginkgo biloba extract EGb761 is a widely used and well-defined extract of Ginkgo biloba leaves (Montes et al., 2015). Many studies have shown that ginkgolides are the most important constituents of Ginkgo biloba which play a vital role in prevention and treatment of many diseases, including cardiovascular diseases (Tosaki et al., 1996, Liu et al., 2012) and neurological disorders (Maclennan et al., 2002). Previous studies have reported that Ginkgolide B (GB) is a natural specific antagonist of the platelet-activating factor (PAF) receptor which exerts protective effects against ischemic injury (Reinstein et al., 2013), arrhythmia (Koltai et al., 1989; Zhao et al., 2013), and inflammation (Liu et al., 2014; Liu et al., 2012). Recently, GB has been reported to attenuate apoptosis as well as ER stress through its antioxidant properties (Li et al., 2013; Zhang et al., 2011). As a derivative compound of GB, GK has been shown to possess protective effects on cerebral ischemia/reperfusion injury (Ma et al., 2012b) and markedly protect PC12 cells against H2O2- or glutamate-induced cytotoxicity by ameliorating mitochondrial dysfunction, oxidative stress and Ca2+ overload (Ma et al., 2012a; Ma et al., 2014). A pharmacokinetic study of GK in rats provided some important information which would promote further study of GK in human diseases (Fan et al., 2015). However, the effects of GK on ER stress remains elusive.
In the present study, we demonstrate that GK enhances IRE1α phosphorylation and Xbp1 mRNA splicing, and partially represses regulated IRE1-dependent decay (RIDD) and the JNK pathway. Relieved ER stress by GK is further confirmed by in vivo studies in mice subjected to acute myocardial infarction (AMI), which is a clinically relevant model to mimic the ER stress-caused cardiac injury. Our findings suggest the IRE1α/XBP1 pathway plays a pivotal role in GK-mediated cardioprotection.

Materials and Methods

Materials

Ginkgolide K (purity > 98%) was obtained from Jiangsu Kanion Pharmaceutical Co., Ltd., China, and freshly prepared before experiments. Tunicamycin (Tm), hydrogen peroxide (H$_2$O$_2$), and thapsigargin (Tg) were purchased from Sigma. Tm and Tg were dissolved in DMSO at a concentration of 5 mg ml$^{-1}$ and 600 μM, respectively.

Other reagents and solvents were of analytical grade.

Primary culture of neonatal rat cardiomyocytes (NRCMs)

NRCMs were isolated from 1-2 day-old Sprague-Dawley rats (obtained from Charles River UK, Ltd.) using the standard enzymatic method described previously (Kimura et al., 2010). Experimental procedures were reviewed and approved by the Ethics Committee, University of Manchester.

Assessment of cell viability using LDH method

To assess cell viability, ER stress was elicited by addition of fresh media containing 2.5 μg ml$^{-1}$ Tm, 300 nM Tg, or 100 μM H$_2$O$_2$. NRCMs were pre-treated with GK at the indicated concentrations for 12 h followed by incubation with Tm, Tg or vehicles (0.05% DMSO in culture media) for 24 h, or H$_2$O$_2$ for 4 h. Cell death was evaluated by measuring released LDH in the media from dead cells using CytoTox 96 Non-Radioactive Cytotoxicity Assay (G1780, Promega) according to the manufacturer’s protocol.
Apoptosis assay

For cultured NRCMs, the fluorescent in situ TUNEL cell death detection kit was used (Roche). Briefly, cells were fixed with 4% paraformaldehyde and then permeabilised. DNA nicks were labelled with terminal deoxynucleotidyltransferase and nucleotide mixture containing fluoresceinisothiocyanate-conjugated dUTP. Nuclei were counter stained with DAPI, and images were collected on a fluorescence microscope (Olympus BX51) and MetaVue Software (Molecular Devices). The percentage of TUNEL-positive was calculated using ImageJ software. The operator and data analysis were blinded.

For paraffin-embedded heart sections, the colorimetric in situ cell death detection kit (Roche Applied Science, USA) was used following the manufacturer’s instructions. Individual nuclei were visualised at a magnification of 400 fold, and the percentage of apoptotic nuclei was calculated in a blinded manner from 6 randomly chosen fields per slide and averaged from four slides per heart for statistical analysis.

PCR and primers

Total RNA were extracted using Trizol reagent. 10 μg of total RNA was subjected to RT-PCR using Superscript III First Strand (Invitrogen). Then 20-50 ng of cDNA was amplified by standard PCR with the HotStarTaq Master Mix Kit (Qiagen) or real-time quantitative PCR (qPCR) using SYBR® Select Master Mix. For standard PCR, the PCR products were analysed in 2% agarose gel, and images were collected with the Bio-Rad ChemiDoc MP imaging system and Image Lab software. qPCR was performed on StepOnePlus Real-Time PCR System by comparative 2-ΔΔCt method. The used primers were synthesised by Sigma as below: for Xbp1, 5’-TTACGAGAGAAACTCATGCGC-3’ and 5’-GGGTCAACTTGTCCAGATGTC-3’; for Edem1, 5’-AAAGCCCTCTGGAGACCTCG-3’ and 5’-AAGGGATTCTTGGTCGCCCTG-3’; for Hrd1, 5’-CCGCTGCTAAAAGATTGCCTG-3’ and 5’-TCTTCTGCAGTGCTCACAGG-3’; for Gapdh, 5’-GTATCCAGGGTGTCTTCTGAG-3’ and 5’-CTCGTGGTACAGATTGCTG-3’; for Lc3b, 5’-TGTCGACTTATTGGAGAGGCGCA-3’ and 5’-TTCACCAACAGGAAGGCTGTC-3’; for Bcl1, 5’-AGGTTGAGGAAAGGCGGAGC-3’ and 5’-TGTCCGACCTTCTTGAGGAGGCGCA-3’; for Beclin1, 5’-AGGTTGAGGAAAGGCGGAGC-3’ and 5’-TGTCCGACCTTCTTGAGGAGGCGCA-3’. The primers of Blos1, Hgnat, Scara3, Pmp22, Pdgfrb, and Col6 were obtained from Qiagen (Cat. No.: QT00102060, QT00175938, QT00194866, QT01298696, QT01063069, QT01599430).
Electrophoretic analysis of Xbp1 mRNA splicing

To examine Xbp1 mRNA splicing, the restriction endonuclease Pst I was used to detect Xbp1s by only digesting the Xbp1u isoform as described before (Margariti et al., 2013). Briefly, half of the RT-PCR product was digested with Pst I, and resolved on 2% agarose gels, and visualised with ethidium bromide.

RNA interference

Specific short interfering RNA for IRE1α (siIRE1, Cat. No. SI02937914) and XBP1 (siXBP1, Cat. No. SI01973482) were purchased from Qiagen. Negative control siRNA (scramble, Cat. No. SR-CL000-005) was obtained from Eurogenetec. NRCMs were transfected with siRNA (15 nM) using Lipofectamine LTX and Plus reagents according to the manufacturer’s instructions (Invitrogen). To assess the specific silencing effect of siIRE1 and siXBP1, total IRE1α and XBP1 protein were detected by immunoblot analysis 72 h post-transfection.

Assessment of autophagic flux

Autophagic flux was detected by microtubule-associated protein 1 light chain 3 type 2 (LC3-II) and p62 turnover in the presence and absence of a cysteine protease inhibitor E64d (10 μg·ml⁻¹, Calbiochem) and an aspartic protease inhibitor pepstatin A (10 μg·ml⁻¹, Sigma) (Mizushima et al., 2007). NRCMs were pre-treated with E64d and pepstatin A for 2 h to inhibit lysosomal proteases followed by incubation with or without 10 μM GK for 12 h and then with or without Tm for additional 24 h incubation. At the end point, LC3-II and p62 levels were analysed by Western blot.

Animals and treatment

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee and the Ethics Committee of Chinese Academy of Medical Sciences and Peking Union Medical College. All studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath et al., 2010).
Male adult ICR mice (8–10 weeks old) were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The experiments were performed in the Experimental Animal Center of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. The animals were housed under controlled conditions at a room temperature of 25 °C in a 12 h light/dark cycle with ad libitum access to water and food.

Mice were anaesthetised with 2% isoflurane inhalation using an isoflurane delivery system. AMI was performed by permanent left descending coronary artery (LCA) occlusion as previously described (Gao et al., 2010). All of the animals were randomly assigned to three experimental groups (n=12 per group): (i) Sham group (undergoing the same surgical procedure except for occlusion); (iii) AMI+Vehicle group; (iii) AMI+GK group. All animals were monitored and received one dose of buprenorphine (0.3 mg·kg⁻¹) within 6 h post-surgery. GK was dissolved in Tween 80 (<20%, Sigma) before administration. Mice in AMI+GK 10 group were injected intraperitoneally with GK (10 mg·kg⁻¹ body weight) immediately after LCA occlusion. The mice in Sham and AMI+Vehicle groups were administered with a corresponding dose of the vehicle. Another dose was administered at 12 h after surgery. Mice were subjected to 24 h of myocardial ischemia followed by echocardiographic and infarct size analysis. At the end of the study, animals were killed by cervical dislocation. The tissue samples for transmission electron microscopy (TEM), TUNEL, and Western blot analysis were prepared from the non-infarcted region adjacent to the border zone of myocardial infarcts at the anterior wall of left ventricle. The operator and data analysis were blinded.

**Assessment of infarct size**

Myocardial infarct size was determined by triphenyltetrazolium chloride (TTC) staining. Briefly, the hearts were frozen shortly and sliced into five 2-mm transverse sections. The sections were incubated at 37 °C with 1% TTC in phosphate buffer (pH 7.4) for 15 min; fixed in 10% formaldehyde solution, photographed with a digital camera (Canon E450) and calculated in a blinded manner using ImageJ software. The infarct size was expressed as a percentage of left ventricle circumference.
All mice underwent transthoracic echocardiography at 24 h after surgery (prior to killing) using the Vevo 770 High-Resolution In Vivo Micro-Imaging System (FujiFilm VisualSonics Inc.). Left ventricular internal diameter end diastole (LVIDd) and end systole (LVIDs), were measured perpendicularly to the long axis of the ventricle. Ejection fraction (EF) was calculated automatically by the echocardiography software according to LVIDd and LVIDs. Pulse wave velocity was used to measure aorta blood flow velocity (AV). The operator and data analysis were blinded.

**Transmission electron microscopy**

Small heart samples about 1 mm³ were fixed immediately by immersion in 4% ice-cold glutaraldehyde, postfixed for 1 h in 1% OsO₄ in 0.1 M cacodylate buffer, dehydrated, and embedded in Epon 812 at 60 °C for 48 h. Routine 60-nm ultrathin sections were cut and mounted on coated grids, and stained with 2% uranyl acetate and 0.2% lead acetate. The examination was performed in a blinded manner with a transmission electron microscope (H-7650, Hitachi).

**Western blot**

After treatment, proteins were extracted from NRCMs or heart tissue in Triton lysis buffer (20 mM Tris HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylsulphonylfluoride, 10 µg·ml⁻¹ leupeptin, 10 µg·ml⁻¹ aprotinin). Cytochrome c release was measured by a mitochondria Isolation Kit (Pierce, USA). Equal protein extracts (20 µg) were subjected to Western blot analysis with antibodies against C/EBP homologous protein (CHOP), cleaved Caspase 3, cleaved PARP, Beclin 1, LC3A/B, p62, phospho-PERK, PERK, Bcl-2, ATF6, activating transcription factor 4 (ATF4), eIF2α, phospho-eIF2α, apoptotic signalling kinase-1 (ASK1), phospho-ASK1, JNK, phospho-JNK, Ubiquitin (Cell Signalling); cytochrome c, COX4, GRP78, EDEM1, IRE1α, phospho-IRE1α (Ser724), GAPDH (Abcam); Bax (Santa Cruz) and XBP1s (BioLegend). Immunoblots were detected by enhanced chemiluminescence with anti-mouse or anti-rabbit immunoglobulin G coupled to horseradish peroxidise as the secondary antibody (Amersham-Pharmacia).
Data analysis

Data are expressed as the means ± SEM and assessed with a two-tailed Student’s t-test or a one-way ANOVA followed by Tukey’s post hoc test using GraphPad Prism software version 6.0 (GraphPad Software). The post hoc tests for multiple comparisons were performed only if F achieved P<0.05 and there was no significant variance in homogeneity. A value of P<0.05 was considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

Results

**GK alleviated ER stress-induced injury in NRCMs**

To evaluate the effects of GK on ER stress, we used Tm to mimic ER stress injury in NRCMs, and degree of cell death was measured using the LDH method. The results showed that Tm treatment at 2.5 μg·ml⁻¹ for 24h induced prominent cell death, however, GK pre-treatment at the doses of 10, 20 μM significantly decreased the percentage of cell death (Fig. 1A). Next, we examined whether GK could prevent cytotoxicity exerted by the other ER stressors, such as Tg and H₂O₂. We observed that Tg or H₂O₂ markedly increased the percentage of cell death in NRCMs, whereas 10 μM GK significantly reduced the percentage of cell death (Fig. 1B, C). Pre-treatment with 20 μM GK further decreased cell death in NRCMs. TUNEL staining was performed to assess the occurrence of apoptosis in NRCMs. The assay demonstrated that TUNEL-positive cells in GK pre-treatment with 10 and 20 μM were significantly fewer than the Tm-treated group (Fig. 1D, E). Western blot analysis showed that pre-treatment with GK reduced the level of the ER stress-induced apoptosis mediator CHOP in a dose-dependent manner (Fig. 1F, G), and the level of cleaved Caspase-3 and cleaved PARP was also reduced after pre-treatment with 10 and 20 μM GK, compared to Tm control (Fig. 1F, H, I). Moreover, 10 μM GK increased the expression of anti-apoptotic protein Bcl-2, thus decreasing the ratio of Bax/Bcl-2 following Tm induced ER stress. GK also blocked Tm-induced cytochrome c release from mitochondria, as indicated by a decrease in the ratio of cytosolic cytochrome c to mitochondrial cytochrome c (Fig. 1J-L). In addition, we found that treatment with GK alone at the indicated concentrations for up to 36h did not affect the viability of NRCMs as well as the levels of Bcl-2, Bax, cytochrome c and cleaved Casepase 3 (supplementary Fig. S1), suggesting that GK per se has no cytotoxicity.
GK promoted IRE1α activation and Xbp1 splicing in Tm treated NRCMs

To investigate the mechanism whereby GK prevents cell death in response to ER stress, we next examined the effects of GK on the UPR signal in stressed cardiomyocytes. Western blot analysis showed that Tm treatment for 24h increased GRP78 level and activated three arms of the UPR compared to non-treated vehicle control (Fig. 2A-H). In comparison with Tm control, 12h pre-treatment with 10 μM GK further enhanced GRP78 expression, and increased phosphorylation at Ser 724 of IRE1α, whilst there was no significant effects on the active form of ATF6, the phosphorylation of PERK and eIF2α as well as ATF4, suggesting that GK could selectively activate the IRE1α branch in the UPR. To verify the activation of the IRE1α branch, we assessed the mRNA level of Xbp1s, which is readout of the RNase activity of activated IRE1α. The Xbp1 mRNA splicing assay was performed as shown in Fig. 2I, and the mRNA level of Xbp1s in NRCMs treated with 10 μM GK was nearly 2-fold higher than the Tm control, indicating increased IRE1α RNase activity (Fig. 2I). Consistent with these findings, western blot analysis confirmed that 10 μM GK treatment increased the protein expression of XBP1s (Fig. 2J). These results prompted us to explore whether GK alone can modulate the ER stress signalling pathways. The results in supplementary Figure S2 showed that GK alone had no effects on ER stress marker proteins (supplementary Fig.S2A). Taken together, these data suggest that GK promotes activation of the IRE1α/XBP1 pathway to protect NRCMs from sustained ER stress.

IRE1α/XBP1 plays a pivotal role in GK mediated cytoprotection

To further confirm the involvement of the IRE1α/XBP1 pathway in GK-mediated cytoprotection in cardiomyocytes, we used siRNA to silence IRE1α or XBPI in NRCMs. Western blot analysis demonstrated a significant knockdown of IRE1α or XBPI in NRCMs (Fig. 3A). Cell death analysis by the LDH method showed that IRE1α or XBPI knockdown nearly abolished the protective effects of GK in Tm challenged NRCMs (Fig. 3B). Same results were also revealed in the TUNEL assay. As showed in Fig. 3C-D and supplementary Fig.S3, the beneficial effects of 10 μM GK was blunted in the presence of siIRE1 or siXBPI. Taken together, these results substantiate the notion that IRE1α/XBP1 pathway plays a critical role in GK-mediated cytoprotection.
GK partially repressed IRE1α-induced RIDD activity and ASK1/JNK activation

Prolonged ER stress allows IRE1α to exert its RNase activity on many ER-localised mRNAs and some antiapoptotic miRNAs. This phenomenon is referred to as RIDD which is commonly regarded as a mechanism of IRE1α-dependent apoptosis (Byrd et al., 2013; Ghosh et al., 2014). In this study, we examined the RIDD activity by qPCR analysis using mRNA expression of 6 RIDD-specific substrates. We found that ER stressors Tm notably reduced Blos1, Hgnat, Scara3, Pmp22 and Col6 in NRCMs (Fig. 4A); and Tg reduced Blos1, Hgnat, Pdgfrb and Pmp22 (supplementary Fig. S4B), which was in line with previous studies (Hollien et al. 2009; Mendez, R. et al. 2013; Maurel, M. et al. 2013). In contrast, we observed that 10 μM GK elevated Blos1, Hgnat and Scara3 in Tm treated NRCMs, and 4μ8C, a known IRE1α inhibitor, was also found to significantly elevate the levels of Blos1, Hgnat, Scara3, Pmp22 and Col6 in Tm treated NRCMs (Fig. 4A). In addition, GK alone had no effect on mRNA expression of RIDD substrates (supplementary Fig.S4A). Collectively, these data demonstrate that GK partially represses IRE1α-induced RIDD activity in Tm treated NRCMs.

Activated IRE1α can form a hetero-oligomeric complex with tumour necrosis factor receptor associated factor 2 (TRAF2) which stimulates activation of ASK1 and downstream JNK to promote apoptosis (Chen et al., 2013). Thus, we investigated the effects of GK on the ASK1/JNK pathway. As shown in Fig. 4B-D, Tm treatment increased the phosphorylation level of ASK1 and JNK compared to the non-treated vehicle control. However, compared to the Tm control, GK treatment significantly decreased the level of phospho-JNK, but not the level of phosphor-ASK (Fig. 4B-D).

GK stimulated ERAD in Tm treated NRCMs

Proteins that fail to correctly fold or assemble in the ER are retro-translocated into the cytosol for polyubiquitination, and thereafter degraded by the proteasome system to preserve protein homeostasis via a process known as ERAD (Amm et al., 2014; Christianson et al., 2014). It is generally agreed that ubiquitin conjugation to substrates drives the retro-translocation process and transfers the ubiquitinated substrates to the cytosolic 26S-proteasome for digestion. Thus, levels of ubiquitin-conjugated proteins can reflect the activity of degradation (Su et al., 2010; Zhang et al., 2014). As shown in Fig 5A and supplementary Fig.S2B, Western blot analysis using ubiquitin antibody for detecting ubiquitinated proteins...
demonstrated that pre-treatment with 10 μM GK profoundly reduced the level of ubiquitin-conjugated proteins compared to the Tm control, suggesting a positive effect of GK to remove ubiquitinated proteins following Tm induced ER stress. In order to exclude the possibility due to a decline in the onset of ubiquitination, two important components of the ERAD machinery were examined; an ubiquitin ligase HMG-CoA reductase degradation protein 1 homolog (HRD1) and ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1). PCR results showed that 10 μM GK treatment increased Edem1 and Hrd1 mRNA compared to the Tm control (Fig. 5B, C). Western blot analysis showed that Tm (N-linked glycosylation blocker) considerably elevated the level of non-glycosylated EDEM1 protein, and treatment with 10 μM GK further enhanced the level of EDEM1 protein, regardless of glycosylated and non-glycosylated (Fig. 5D, E). These results clearly manifest that GK stimulates ERAD to eliminate misfolded protein in ER stressed NRCMs.

**GK prompted autophagic flux in Tm treated NRCMs**

Although ERAD has been regarded as a primary approach for the degradation of misfolded proteins, recent reports have shown that autophagy is an alternative cellular approach to eliminate unfolded proteins (Amm et al., 2014; Groenendyk et al., 2013; Margariti et al., 2013). We showed by quantitative PCR that 10 μM GK treatment increased the transcriptional level of autophagy markers Beclin1 and LC3B when compared to Tm control (Fig. 6A, B). In addition, Western blot analysis demonstrated induced protein expression of Beclin1 after 10 μM GK treatment, indicating activation of the autophagy process (Fig. 6C, D). Furthermore, detection of the autophagosome-specific isoform of LC3 (known as LC3-II) and p62 was performed to assess the autophagic flux in the presence of lysosome-mediated proteolysis inhibitors, pepstatin A and E64d. In the presence of Tm, 10 μM GK induced a notable elevation in LC3-II (Fig. 6E, F). Meanwhile, 10 μM GK induced additional accumulation of LC3-II protein in the presence of pepstatin A (10 μg ml⁻¹) and E64d (10 μg ml⁻¹) (Fig. 6E, F), suggesting a genuine increase in LC3-II. The observation of decreased p62 after 10 μM GK treatment confirmed that GK improved activation of autophagy in NRCMs (Fig. 6E, G). Of note, 10 μM GK alone had no effect on expression of LC3 and p62 with or without treatment of pepstatin A and E64d. (supplementary Fig.S2 C-F). These results indicate that GK boosts an autophagic response in stressed NRCMs.
**GK exerted cardioprotection and attenuated ER stress-induced apoptosis in AMI mice**

Numerous studies provide important evidence for the critical role of ER stress in cardiomyocytes apoptosis after myocardial infarction (Luo et al., 2015; Mitra et al., 2013). Thus, the AMI model is an ideal tool to study ER stress in the context of heart disease. In the present study, the AMI model was induced by coronary ligation in 8-10 week old male ICR mice to evaluate the effects of GK on ER stress-mediated injury in vivo. After 24h of coronary ligation, a myocardial infarct size of up to 33.1% of LV circumference was observed in AMI+Vehicle mice. Intraperitoneal injection of GK with two doses of 10 mg·kg⁻¹ immediately and 12h after coronary ligation was shown to significantly diminish the infarction size (Fig.7A). As shown in Fig.7B-F, echocardiography analysis exhibited that heart function in AMI+Vehicle mice was compromised, as evidenced by significantly increased LVIDd and LVIDs, lower EF and AV. However, 10 mg·kg⁻¹ GK treatment considerably improved heart function with decreased LVIDd and LVIDs as well as increased EF and AV. TUNEL assay on heart sections displayed that the percentage of TUNEL-positive cells in AMI+GK10 group was significantly lower than that in AMI+Vehicle group, indicating decreased apoptosis by GK treatment (Fig.7G, H).

**GK activated IRE1α in ER stressed myocardium of AMI mice**

The *in vitro* results described above inspired us to examine the effects of GK on ER stress in the AMI mice. ER expansion was investigated using TEM. TEM analysis demonstrated normal ER and mitochondria in sham hearts, while more dilated ER was observed in hearts from AMI+Vehicle mice (Fig.8A). In contrast, the ER in myocardium from mice treated with 10 mg·kg⁻¹ GK appeared fairly normal with no significant expansion (Fig.8A). Western blot analysis demonstrated a significantly raised level of the ER stress maker proteins GRP78 and CHOP, total and phosphorylation of IRE1α, XBPIs, and autophagy marker proteins LC3II and p62 in AMI mice (Fig.8B-I). As compared to AMI vehicle group, 10 mg·kg⁻¹ GK treatment considerably decreased CHOP and p62 but augmented phosphorylation of IRE1α, XBPIs and LC3II. These results demonstrate the beneficial effects of GK on relieving ER stress through the IRE1α branch in an AMI animal model.
Discussion and conclusion

*Ginkgo biloba* extractions, especially ginkgolides including ginkgolide A, B, C, and J have been reported to possess potent cardioprotective properties via inhibiting platelet-activating factor (PAF), scavenging free radical to protect isolated heart against ischemia (Varga *et al.*, 1999; Pietri *et al.*, 1997; Tosaki *et al.*, 1996; Koltai *et al.*, 1989). In the present study, we demonstrate that GK, a newly isolated constituent in ginkgolide family, protects NRCMs and mice myocardium against ER stress-induced apoptosis. The selective activation of the IRE1α/XBP1 pathway in the UPR plays a pivotal role in the beneficial effects of GK on ER stress. These results suggest that GK confers profound cytoprotection in response to ER stress.

As the most ancient of three ER transmembrane sensors, the IRE1α protein plays a central role in the UPR. IRE1α senses unfolded proteins either directly or indirectly through its ER luminal domain that becomes oligomerised during stress (Chen *et al.*, 2013; Han *et al.*, 2009). The oligomerisation allows trans-autophosphorylation and the resultant conformational change leads to IRE1α activation. In our experiments, GK markedly activated the IRE1α arm of the UPR in both *in vivo* and *in vitro* models, as evidenced by higher level of phosphorylated IRE1α and Xbp1 mRNA splicing, thus increasing the protein expression of XBP1s, which is a transcriptional factor with target genes, including Grp78 (Lee *et al.*, 2003; Yoshida *et al.*, 2001; Hosoi *et al.*, 2012). In this study, we observed an increase in GRP78 level that may be due to de novo synthesis as a result of activating IRE1α/XBP1s pathway.

In yeast, IRE1 is activated through a two-step mechanism, namely dissociation of GRP78 and direct interaction with unfolded proteins at its luminal domain (Bravo *et al.*, 2013). In the past, the dissociation of GRP78 had been thought to equally regulate the activation of these three sensors. However, differences in the responses of these sensors to the GRP78 dissociation have been recognised and distinct activation kinetics for each sensor were also reported (DuRose *et al.*, 2006). It remains unclear whether a similar mechanism is applicable to IRE1α in mammalian cells. Some studies suggested that mammalian IRE1α activation strongly depends on the dissociation of GRP78 rather than a direct interaction with unfolded proteins (Oikawa *et al.*, 2009; Oikawa *et al.*, 2012). Preferential activation of UPR signalling branches by alternative types of ER stress have also been studied (DuRose *et al.*, 2006). The structural differences between IRE1α and PERK play a role in their differential activation kinetics (DuRose *et al.*, 2006). In contrast to IRE1α and PERK, less structure-function information is available for the luminal portion of the type II transmembrane protein ATF6. Apart from the
dissociation of GRP78, ATF6 activation is negatively regulated by intra- and intermolecular disulphide bridge formation (Nadanaka et al., 2007; Tsukumo et al., 2007). Therefore, individual branches may be specialised to respond to particular stressors.

In contrast to its pro-survival role through splicing XBP1, activated IRE1α can interact with TRAF2 which leads to activation of ASK1 and downstream JNK to induce pro-death signalling. The IRE1α-JNK connection provides mechanistic insight that the UPR is able to regulate anti- and pro-apoptotic molecules to determine divergent fates of stressed cells. c-Jun N-terminal inhibitory kinase (JIK) and Jun activation domain-binding protein 1 (JAB1) have been identified as IRE1α-interacting molecules, and they might influence the shift of the UPR to either pro-survival or pro-apoptosis signalling by association with or dissociation from IRE1α (Oono et al., 2004). It is also pointed out that small chemicals might interrupt the IRE1α/TRAF2 interaction to selectively utilise the beneficial effects following IRE1α activation. These small chemicals might not interfere with Xbp1 mRNA splicing, but could prevent the activation of the pro-apoptotic processes (Szegezdi et al., 2006). The present study shows that GK treatment does not considerably affect the phosphorylation of ASK1; however, it significantly decreases the level of phospho-JNK. The mechanism underlying reduced phospho-JNK by GK treatment requires further investigation. A reasonable hypothesis would be that GK might have effects on other pathways, such as JNK phosphatases, which can negatively regulate JNK phosphorylation, thus blunting phospho-JNK level whereas phospho-ASK level remaining unchanged.

Furthermore, IRE1 is able to exert protective or pro-apoptotic effects via splicing of Xbp1 mRNA and the induction of RIDD, respectively (Chen et al., 2013). It is believed that modulating different sites of IRE1 can differentially regulate Xbp1 splicing and RIDD activity. IRE1 activation requires its dimerisation, oligomerisation and subsequent trans-autophosphorylation, which leads to a conformational change on its RNase domain and an increased RNase activity. Our study demonstrated that GK augmented IRE1α autophosphorylation in response to ER stress stimuli; the increase of autophosphorylation correlated with enhanced Xbp1 mRNA splicing. Conversely, RIDD activation and JNK phosphorylation were decreased by GK treatment. Akin to the beneficial effects of GK, recent studies also reported that IRE1 modulators, such as quercetin (Wiseman et al., 2010), 1NM-PP1 (Han et al., 2009) and F6 peptide (Bouchecareilh et al., 2011), were able to enhance Xbp1 mRNA splicing but attenuate ER stress-mediated RIDD and JNK activation.

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The study by Wiseman et al showed that binding of quercetin at the dimer interface of IRE1’s kinase extension nuclease (KEN) domain enhances IRE1 dimerization, which activates its RNase activity, leading to increasing Xbp1 mRNA splicing (Wiseman et al., 2010). Moreover, Han et al pointed out that an ATP-competitive inhibitor 1NM-PP1, was able to facilitate dimerization/oligomerization of a mutant form of IRE1 with an altered kinase domain, thus increasing IRE1 RNase activity and Xbp1 mRNA splicing, but not affecting the degradation of RIDD substrates (Han et al., 2009). In addition, it has been suggested that IRE1 dimerization/oligomerization could influence its RNA substrate specificity (Tam et al., 2014). However, the extent to which oligomerization dictates the substrate specificity remains elusive. Taking together, we hypothesise that GK likely interacts with IRE1α, which induces IRE1α phosphorylation and subsequent conformational change of its RNase domain. This allosteric change could enhance RNase activity of IRE1α for Xbp1 mRNA splicing. Nevertheless, the interaction between GK and IRE1α remains to be investigated.

In summary, this study presents convincing evidence that GK can selectively activate the IRE1α/XBP1 pathway to ameliorate ER stress for treating cardiovascular diseases (Fig. 9).

**Authorship contribution statement**

Shoubao Wang, Zhenzhong Wang, Xin Wang designed the research study

Xin Wang, Guanhua Du and Wei Xiao supervised the project

Shoubao Wang, Zhenzhong Wang and Jing Guo performed the experiments

Shoubao Wang, Jing Guo, Qiru Fan analysed data.

Xin Wang, Guanhua Du, Wei Xiao and Qiru Fan contributed essential technic, reagents or tools

Xin Wang, Shoubao Wang, Jing Guo, Gina Galli drafted the manuscript.

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Conflict of interest

The authors declare no conflict of interest related to this study.

References


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Figure 1. GK decreased ER stress-induced cell death and apoptosis in NRCMs.

NRCMs were pre-treated with or without GK at the indicated concentrations (5, 10, 20 μM, respectively) for 12 h followed by treatment with ER stressors (Tm, 2.5 μg ml-1, 24 h; Tg, 300 nM, 24 h; H2O2 100 μM, 4 h). (A-C) ER stressors-induced cell death was measured by the LDH method. (D, E) Apoptosis was detected by the TUNEL assay (Scale bar, 40 μm) and the TUNEL-positive cells were quantified. (F-L) Western blot analysis showed that Tm-induced CHOP, cleaved Caspase 3, cleaved PARP and cytosolic cytochrome c were suppressed by pretreatment with GK; Tm supressed Bcl-2 and mitochondrial cytochrome c were increased by pretreatment with GK. COX4 and GAPDH, which are exclusively expressed in the mitochondria and cytosol, respectively, were used as controls for loading and fractionation. Data are presented as means ± SEM (n = 5); *P<0.05. Cyt c, cytochrome c; cyto, cytosolic; mito, mitochondrial.
Figure 2. GK promoted IRE1α activation and Xbp1 mRNA splicing in Tm treated NRCMs.

NRCMs were pre-treated with or without 10 μM GK for 12 h followed by treatment with 2.5 μg ml⁻¹ Tm for 24 h. (A-H) Western blot analysis was performed on cell lysates. Tm induced p-IRE1α, but not ATF6, p-PERK, p-eIF2α and ATF4, was increased by GK. (I) mRNA level of Xbp1 was amplified by PCR followed by splicing assay. The digestion products of Xbp1u and Xbp1s by Pst I were analysed by agarose gel. (J) XBP1s protein level was analysed by Western blot. Data are presented as means ± SEM (n = 5); *P<0.05.
Figure 3. Knockdown of IRE1α or XBP1 abolished GK-mediated cytoprotection.

NRCCMs were transfected with negative control siRNA (scramble), specific siRNA of IRE1α (siIRE1) and XBP1 (siXBP1), respectively, for 72 h, and then stimulated with Tm for 24 h with or without 10 µM GK pretreatment. (A) Western blot analysis demonstrated a significant knockdown of IRE1α and XBP1 in NRCCMs after treatment with siIRE1 or siXBP1. (B) LDH-assay showed that IRE1α or XBP1 knockdown abolished the protective effect of GK in Tm challenged NRCCMs. (C, D) Apoptosis was detected by the TUNEL assay (scale bar, 40 µm) and quantitatively analysed using TUNEL-positive cardiomyocytes percentage per field. The beneficial effect of GK was blunted in the presence of siIRE1 or siXBP1. Data are presented as means ± SEM (n = 5); *P<0.05.
Figure 4. GK partially repressed IRE1α-induced RIDD activity and JNK activation.

(A) RIDD mRNA substrates Blos1, Hgnat, Scara3, Pmp22, Pdgfrb, Col6 were assayed by qPCR. ER stressors Tm notably reduced Blos1, Hgnat, Scara3, Pmp22 and Col6 in NRCMs. 4μ8C, a known IRE1α inhibitor, significantly elevated Blos1, Hgnat, Scara3, Pmp22 and Col6 in Tm treated NRCMs. 10 μM GK elevated Blos1, Hgnat and Scara3 in Tm treated NRCMs. (B-D) Western blot analysis showed that GK treatment repressed the increase in phospho-JNK, but not phosphor-ASK1. Data are presented as means ± SEM (n = 5); *P<0.05.
Figure 5. GK stimulated ERAD in Tm treated NRCMs.

(A) Western blot analysis to detect ubiquitination showed that pre-treatment with 10 μM GK profoundly reduced the level of ubiquitin-conjugated proteins. (B, C) qPCR analysis showed that GK increased the mRNA levels of Edem1 and Hrd1. (D, E) Western blot analysis showed that GK further enhanced the expression of non-glycosylated EDEM1 protein. Data are presented as means ± SEM (n = 5); *P<0.05.
Figure 6. GK prompted autophagic flux in Tm treated NRCMs.

NRCMs were pre-treated with or without 10 μM GK for 12 h, and then treated with or without lysosome inhibitors pepstatin A and E64d for 2 h followed by treatment with 2.5 μg ml⁻¹ Tm for a further 24 h. (A, B) qPCR analysis demonstrated that GK increased the transcriptional level of autophagy markers Beclin1 and Lc3b. (C, D) Western blot analysis demonstrated that GK increased the protein expression of Beclin-1. (E, F) LC3-II was notably elevated by GK, and was further elevated in the presence of pepstatin A and E64d, suggesting a genuine increase in LC3-II. (E, G) Decreased p62 after GK treatment confirmed that GK improved activation of autophagy in NRCMs. Data are presented as means ± SEM (n = 5); *P<0.05.
Figure 7. GK exerted cardioprotection on mice subjected to AMI.

Mice were subjected to AMI by permanent left main descending coronary artery (LCA) occlusion. (A) After 24 h of coronary ligation, myocardial infarct size was determined by TTC staining. The quantitative analysis confirmed a diminished infarction size in GK treatment group. (B-F) Echocardiographic assessment of left ventricular internal diameter end diastole (LVIDd) and end systole (LVIDs), left ventricular ejection fraction (EF) (%) were detected by M-mode images, aorta blood flow velocity (AV) was detected by Pulse wave velocity, GK treatment group exhibited a significantly decreased LVIDd and LVIDs, as well as increased EF and AV, compared to AMI model group. G, H) TUNEL staining was performed on sections from the border zone of heart infarction (scale bar, 100 μm). Bottom images are 1.9x magnifications of the boxed areas in the upper images. Arrows indicated TUNEL positive nuclei. Quantitative analysis indicated decreased apoptosis by GK treatment. Data are presented as means ± SEM (n = 5); *P<0.05.
Figure 8. GK attenuated ER stress and ER expansion in myocardium of mice subjected to AMI.

(A) Ultrastructure of ER and mitochondria of LV papillary muscle was examined by TEM. Middle images (scare bar, 500 nm) are magnifications of boxed areas in the left images (scare bar, 1 μm). Right images are 3x magnifications of boxed areas in the middle images. Representative images showed dilated ER morphology in myocardium from Vehicle mice. The ER structure in myocardium from mice treated with 10 mg kg⁻¹ GK appeared fairly normal with no significant expansion. The arrow heads show the ER morphology. (B) Levels of ER stress maker proteins GRP78, CHOP, total and phosphorylated IRE1α, XBP1s and autophagy marker proteins LC3 and p62, were assessed using Western blot. (C-I) Quantitative analysis showed 10 mg kg⁻¹ GK treatment considerably decreased CHOP and p62 whereas phosphorylation of IRE1α, XBP1s and LC3II was augmented. Data are presented as means ±SEM (n = 5); *P<0.05.
Figure 9. Proposed GK working model.

GK selectively activates the IRE1α/XBP1 pathway, which in turn leads to upregulation of GRP78, ERAD and autophagy influx. In addition, GK is able to partially repress the pro-apoptotic action of RIDD and the JNK. Thus GK can ameliorate ER stress and protect cardiomyocytes.