In Dahl salt–sensitive rats, a high-salt diet leads to hypertension, cardiac hypertrophy, and subsequent heart failure, as well as significant increases in both GRP78 and CHOP expression.[33] Consistent with these findings, in mice that received transaortic constriction, GRP78 and CHOP are upregulated.[38] Interestingly, UPR activation has been found in both hypertrophic and failing hearts, whereas the activation of ER-initiated apoptosis CHOP, but not c-Jun N-terminal kinase (JNK) or caspase 12, is found only in failing hearts.[38] These findings suggest that UPR activation is consistently found in hearts subjected to pressure overload. However, when the ER stress is prolonged, the ER-initiated apoptosis signal CHOP is activated in failing mouse hearts induced by pressure overload. To clarify the role of CHOP in hypertrophic and subsequently failing hearts by pressure overload, we performed transaortic constriction using CHOP knockout (KO) mice.[27] We observed that both cardiac hypertrophy and dysfunction were attenuated in CHOP KO mice compared with wild type. In CHOP KO mice, the enhanced phosphorylation of eIF2α, which was attributable to the lack of negative regulation by GADD34, may lead to the global repression of translation. This may explain the mechanism by which cardiac hypertrophy is reduced in pressure-overloaded hearts of CHOP KO mice. Furthermore, microarray analysis revealed that CHOP positively and negatively regulates several proapoptotic and antiapoptotic molecules of the Bcl-2 family. These findings suggest that CHOP might be a promising molecular target for the treatment of cardiac hypertrophy and failure.

Protein accumulation resulting from the impairment of the secretory pathway or mutant protein synthesis also causes heart failure. In transgenic mice systemically expressing a mutant KDEL (Lys-Asp-Glu-Leu) receptor,[48] which is a retrieval receptor for ER chaperones in the early secretory pathway, disturbed recycling of misfolded proteins between the ER and Golgi complex and enhanced expression of CHOP and apoptosis were observed in the mutant hearts. The transgenic mice exhibited dilated cardiomyopathy without obvious findings in other tissues, suggesting that the heart is sensitive to ER stress. The transgenic expression of mutant proteins in neural and cardiac tissues is a good model to test whether intracellular aggregation affects cardiac function.[40,41,49,50]

Viruses exploit the translational machinery of the host cell to synthesize their viral proteins, leading to increased folding pressure on the ER and chaperone upregulation.[51,52] Thus, it is likely that the adaptive and proapoptotic pathways of UPR are involved in the pathophysiology of viral myocarditis. Furthermore, Mao et al demonstrated that ER stress plays an important role in cardiomyocyte apoptosis and the development of dilated cardiomyopathy in rabbits immunized with a peptide corresponding to the β1-aderenoceptor.[53,54] These findings suggest that the UPR plays an important role in the pathophysiology of virus and autoimmune heart diseases.

Ischemic Heart Diseases

In a myocardium that has experienced ischemia/reperfusion (I/R), myocardial death and severe inflammation are induced because of the depletion of oxygen and nutrients, followed by the sudden burden of oxygen free radicals and production of proinflammatory cytokines.[55] Either of these stimuli can potentially induce the adaptive and proapoptotic pathways of UPR. Indeed, increased expression of UPR-related genes is reported in cardiomyocytes near myocardial infarction in mice and humans.[56–58]

Martindale et al demonstrated the role of ATF6, a component of the UPR, in I/R injury using transgenic mice with cardiac-restricted expression of a novel tamoxifen-activated form of ATF6.[59] The tamoxifen-treated transgenic mouse hearts exhibit better functional recovery from ex vivo I/R, as well as significantly reduced necrosis and apoptosis and increased expression of ER-resident chaperones GRP78 and -94. Toko et al demonstrated that the treatment of mice with 4-(2-aminoethyl) benzenesulfonyl fluoride, an inhibitor of ATF6, further reduces cardiac function and increases the mortality rate after myocardial infarction.[58] These findings suggest that ATF6 exerts cardioprotective effects on I/R injury. Furthermore, Glembotski and colleagues showed that ATF6 activation induces numerous genes in cultured cardiomyocytes, including MANF (mesencephalic astrocyte-derived neurotrophic factor).[60] Knockdown of endogenous MANF with microRNA increases cell death on simulated I/R, whereas the addition of recombinant MANF to media protected the cultured cardiac myocytes from simulated I/R-mediated death.[60] It was also shown that activated ATF6 induces the Derlin-3 gene, which encodes an important component of the ERAD machinery. Overexpression of Derlin-3 enhances ERAD and protects cardiomyocytes from simulated ischemia-induced cell death.[61]

In rat neonatal cultured cardiomyocytes, hypoxia increased XBP1 mRNA splicing and GRP78 protein levels.[56] Because infection with a recombinant adenovirus encoding dominant-negative XBP1 augments hypoxia/reoxygenation-induced apoptosis, the XBP1 arm of the UPR may play cardioprotective roles against hypoxic insult. Vitadello et al demonstrated that the overexpression of GRP94, the expression of which is regulated by XBP1 and ATF6, reduces myocyte necrosis caused by calcium overload or simulated ischemia in cardiac H9C2 muscle cells.[62] In addition, it was reported that ischemic preconditioning or postconditioning reduces cardiac damage associated with UPR activation.[63,64] In human heart samples, Severino et al demonstrated that PDI is upregulated 3-fold in the viable periinfarct myocardial region.[65] Adenoviral-mediated PDI gene transfer to the mouse heart results in a 2.5-fold smaller infarct size and significantly reduces cardiomyocyte apoptosis in the periinfarct region and the smaller left ventricular end-diastolic diameter compared with treatment with a transgene-null adenoviral vector.

On the other hand, Terai et al demonstrated that hypoxia induces CHOP expression and the cleavage of caspase 12; this effect is significantly inhibited by pretreatment with a pharmacological activator of AMP-activated protein kinase (AMPK).[37] This finding indicates that the proapoptotic pathways of the UPR are involved in cell death by hypoxic stimuli. In addition, Nickson et al demonstrated that ER stress induces the expression of PUMA, a proapoptotic member of the BCL-2 family, and that the suppression of PUMA expression leads to inhibition of cardiomyocyte apoptosis

induced by a pharmacological ER stressor.[65] Importantly, the targeted deletion of PUMA attenuates cardiomyocyte death and improves cardiac function during I/R periods.[66] Pharmacological ER stressors can induce tribbles (TRB)3 expression in cultured cardiomyocytes, and myocardial infarction results in cardiac ER stress caused by the induction of TRB3.[67] Cardiac-specific overexpression of TRB3 sensitizes mice to infarct expansion and cardiomyocyte apoptosis in the infarct border zone after myocardial infarction. Agents that inhibit TRB3 expression or activity may lead to reduced pathological cardiac remodeling in patients.[67]

Furthermore, microbes activate a transcriptional program through Toll-like receptors (TLRs). TLR signaling modifies both the adaptive and the proapoptotic pathways of the UPR.[68,69] TLR-mediated signaling pathways have been implicated in myocardial I/R injury.[70,71] Disruption of TLR2-mediated signaling may be helpful for the induction of immediate or delayed myocardial protection from I/R injury.[71] Further investigation should identify the possible involvement of TLR-ER signaling in I/R injury.

Potential Cardiotoxicity of the New Cancer Therapy

In recent years, small-molecule inhibitors of protein kinases, including receptor tyrosine kinase inhibitors, have been developed for cancer treatment. Interestingly, Kerkela et al demonstrated that imatinib, the first tyrosine kinase inhibitor to inhibit the activity of the causal fusion protein Bcr-Abl in chronic myeloid leukemia, induces left ventricular dysfunction in the animal model and in some patients.[72] In cardiomyocytes, Kerkela et al showed a relationship between c-Abl activity and ER homeostasis, although the mechanism by which c-Abl inhibition destabilizes the ER has not been identified.[72] This study was the first to show that ER-initiated apoptosis signaling may be involved in the cardiotoxicity of this anticancer drug.[73]

A proteasome inhibitor is another important advance in molecular anticancer therapy for the treatment of multiple myeloma.[73] Because proteasome inhibition causes the accumulation of unfolded proteins, it will activate the UPR. Importantly, treatment with a proteasome inhibitor is associated with a high prevalence of heart failure.[74] Consistent with that clinical report, we showed that proteasome inhibition induces cardiomyocyte death and activates ER stress–induced transcriptional factor ATF6, but not XBP1, leading to a failure to upregulate ER chaperones.[75] Overexpression of GRP78 suppresses both CHOP expression and cardiomyocyte death by proteasome inhibition. These findings indicate that proteasome inhibition perturbs ER homeostasis in cardiomyocytes without the induction of ER chaperones, thereby inducing a cycle of cardiac damage. Chemical ER chaperones or drugs that enhance endogenous ER chaperone function in the heart will be promising candidates to prevent cardiotoxicity by proteasome inhibitors,[76] although it is important to recognize the possibility that chemical chaperones will promote cancer growth.

The UPR was shown to be highly induced in various tumors and closely associated with cancer cell survival.[77] There are a number of drugs under development that aim to treat cancer by inhibiting one or more of the branches of the UPR and enhancing ER stress.[78] Careful monitoring of cardiac function is necessary when using pharmacological agents targeting the UPR.

Atherosclerosis

Advanced atherosclerotic plaques provide a pathophysiological environment that can cause ER stress and activate the UPR; in this environment, oxidized lipids, inflammation, and metabolic stress are induced.[79] Indeed, ER stress is markedly increased in endothelial cells subjected to atherosclerosis-prone shear stress.[80] Consistently, transcript profiles revealed that the most abundant feature of the endothelium of all atherosusceptible regions is the upregulation of genes associated with ER stress.[81] Dong et al demonstrated that the dysfunction of AMPK significantly increases the level of ER stress and suppresses sarcoplasmic reticulum calcium-transporting ATPase (SERCA) activity in endothelial cells.[82] These results suggest that AMPK functions as a physiological suppressor of ER stress by maintaining SERCA activity and intracellular Ca^{2+} homeostasis.

A growing body of evidence indicates that a key cellular event in the conversion of benign to vulnerable atherosclerotic plaques is ER stress-induced macrophage apoptosis.[83] Interestingly, macrophage and smooth muscle cells in atherosclerotic plaques produce abundant secretory proteins, which potentially induce ER stress in these cells.[79] A cause of macrophage death is the accumulation of free cholesterol in the ER, leading to activation of the UPR and CHOP-induced apoptosis.[84–86] Macrophage fatty acid-binding protein-4 (aP2), a cytosolic lipid chaperone, plays an important role in cellular lipid metabolism and the reception of lipid signals.[87] Erbay et al demonstrated that aP2 is the predominant regulator of lipid-induced macrophage ER stress and that its absence increases bioactive lipids that render macrophages resistant to lipid-induced ER stress.[88] Another study shows that impaired proteasome function promotes features of a more rupture-prone plaque phenotype in apolipoprotein (Apo)E KO mice.[89] These findings suggest that ER stress is closely involved in the development of atherosclerosis and that ER-related molecules are promising candidates for therapy.

We investigated the association between the ER stress and plaque rupture in 152 human coronary artery autopsy samples. We found a strong association between ER stress markers such as CHOP and GRP78 and ruptured atherosclerotic plaques in human coronary artery lesions,[90] suggesting that ER stress is likely involved in the development of plaque rupture in humans. Importantly, Thorp et al provide direct evidence for a causal link between the ER-initiated apoptosis signaling of CHOP and plaque necrosis.[91] It was shown that plaque necrosis and lesion apoptosis are markedly reduced in the CHOP-deficient mice mated with ApoE KO or low-density lipoprotein receptor KO atherosclerotic mice.[91]

Targeting UPR As Potential Therapy in Cardiovascular Diseases

There are 2 main approaches to targeting the UPR for the treatment of cardiovascular diseases. The first approach

Table. Drugs Affecting UPR and ER-Initiated Apoptosis Signaling

Drug	Mechanism of Action	Potential Indication
Phenylbutyrate	Chemical chaperone	Cardioprotection[28,119]; atherosclerosis prevention[88]
Tauroursodeoxycholic acid	Chemical chaperone	Cardioprotection; atherosclerosis prevention[88]
BiP inducer X[76]	GRP78 induction	Cardioprotection
Curcumin[104]	GRP94 induction	Cardioprotection
Salubrinal[93]	Prevention of eIF2a dephosphorylation	Cardioprotection; prevention of cardiac hypertrophy
Sunitinib[96]	IRE1 activation	
EN460	ERO1 inhibitor	ROS reduction[125]
Fused heterocyclic compound	ASK1 inhibitor	Cardioprotection[4,126]
SP600125	Prevention of CHOP induction by stretch	Prevention of cardiac hypertrophy/failure[28] and atherosclerosis
SB203580	CHOP phosphorylation	Prevention of cardiac hypertrophy/failure[28] and atherosclerosis
TNF-α antibody	Prevention of CHOP induction	Prevention of cardiac hypertrophy/failure[28,39] and atherosclerosis
CS-866	Reduction of ER stress by pressure-overload	Cardioprotection[38]
Pravastatin	Reduction of ER stress by pressure-overload	Cardioprotection[39]
Pioglitazone[120]	Reduction of ER stress	Cardioprotection; atherosclerosis prevention
AICAR; metformin	Reduction of ER stress by AMPK activation	Cardioprotection[37,121]; atherosclerosis prevention[82,122]
Isoproterenol	Proteasome activation and assembly	Cardioprotection[107]; atherosclerosis prevention

AICAR indicates 5-aminoimidazole-4-carboxyamide-1-β-D-ribofuranoside.

involves activating components of the adaptive pathway of the UPR to deal with the stress. The second approach is to deactivate the components of the proapoptotic pathways of UPR. Although targeting the UPR components seems promising as a potential therapy for cardiovascular diseases, there are several limitations in our knowledge. For example, the ER stress sensors IRE1α and PERK elicit signaling to maintain ER homeostasis and to induce cell death. Unfortunately, at the present time, the mechanisms by which the signaling switches from cell survival to death are not fully understood. Thus, we cannot know the precise moment to activate or inhibit ER stress sensor proteins for treatment. Although these limitations suggest that it is still at an early stage for potential therapeutic use, the alteration of UPR activation or the reduction of ER stress are still promising therapeutic goals.

Pharmacological Agents Targeting UPR Component

Pharmacological agents that directly activate or deactivate UPR components will be potentially useful in treating cardiovascular diseases (Table). Potential components of the UPR and ERAD, such as ATF6, IRE1, spliced XBP1, PERK, eIF2α, and the proteasome, could be good initial targets for therapeutic design. For example, activation of ATF6 may exert cardioprotective effects against I/R injury.[36,56,58–61,92] We have demonstrated that enhanced phosphorylation of eIF2α may lead to repressed global protein synthesis and attenuated cardiac hypertrophy in pressure-overloaded hearts of CHOP KO mice,[27] suggesting that enhanced phosphorylation of eIF2α would be a novel therapeutic target for the prevention of cardiac hypertrophy. Salubrinal is a small molecule that prevents dephosphorylation of eIF2α.[93] This compound significantly increases the Grp78/Bip ER chaperone levels and results in attenuated caspase-4–dependent apoptosis in Aβ-treated neurons.[40,94] Although this chemical strategy could be beneficial for treating cardiac hypertrophy, we note that different cell types respond differently to salubrinal.[95] Korennykh et al demonstrated that several kinase inhibitors, including sunitinib, can directly activate IRE1, leading to XBP-1 splicing and a reduction in ER stress.[96] However, we note that patients receiving sunitinib, especially those with a previous history of hypertension and coronary heart disease, are at increased risk for cardiovascular events and should be monitored for exacerbations of their hypertension and for evidence of LVEF dysfunction during treatment.[97,98] Thus, it may be of therapeutic value to separate the intended function of kinase inhibitors from the unintended activation of IRE1. Finally, the UPR components are broadly expressed, and augmentation or inhibition of their activity may lead to unintended toxicity if the agent is not selectively delivered to the cell type or organ of interest.[99] Targeting cell-specific ER component such as CREBH or PARM-1 would be a good strategy.

Although there are no pharmacological agents targeting CHOP, experimental results suggest that the development of CHOP inhibitors would attenuate cardiac hypertrophy and failure and prevent atherosclerosis.[27,91] CHOP activates ERO1α, which catalyzes reoxidation of PDI, resulting in the production of hydrogen peroxide.[100,116] ROS generated as a byproduct of disulfide bond formation in the ER cause oxidative stress and contribute to apoptotic cell death.[9,101] ERO1α may thus be an important mediator of apoptosis downstream of CHOP. Ron and colleagues report that the inhibitor EN460 interacts selectively with the reduced, active form of ERO1α and prevents its reoxidation.[102] This selectivity is explained by the rapid reversibility of the reaction of EN460 with unstructured thiols, in contrast to the formation of a stable bond with ERO1α. Moreover, both the antioxidant *N*-acetylcysteine and the ER stress inhibitor tauroursodeoxycholic acid reverse the reduced cardiomyocyte contractile function elicited by the oxidative stress inducer menadi-

one.[103] Curcumin and masoprocol can serve as lead candidate prophylactics for ROS induced ER stress as they preserve the functional integrity of the PDI.[104] Genetic or chemical intervention to reduce ROS levels improves protein folding and cell survival and may provide an avenue to treat and/or prevent cardiovascular diseases.[9]

Pharmacological agents that indirectly target the UPR and ERAD pathways will also be promising. Cyclic stretching significantly increased CHOP protein levels; this increase is reduced by the addition of JNK inhibitor SP600125 or TNF-α antibody 30 minutes before the stretch stimuli.[28] An inhibitor of p38 mitogen-activated protein kinase, SB203580, abolished the stress-inducible in vivo phosphorylation of CHOP.[105] Thus, these findings suggest that mitogen-activated protein kinase inhibitors may modify components of the UPR pathways. Finally, a fused heterocyclic compound is now under development to inhibit ASK1 activity, a downstream signal of IRE1, because ASK1 is involved in cardiac hypertrophy and failure.[20,106] Interestingly, we have demonstrated that ischemic preconditioning and activation of protein kinase A with isoproterenol or forskolin enhance proteasome assembly and activity.[107] Because proteasome inhibition promotes atherosclerosis and cardiac dysfunction,[46,89] protein kinase A activators may attenuate ER-initiated apoptosis signaling through enhanced proteasome function.

Chemical ER Chaperones

GRP78 is the ER homolog of HSP70 proteins with a conserved ATPase domain and a peptide-binding domain.[7,108] As a chaperone, GRP78 recognizes and binds to proteins with hydrophobic residues in the unfolded regions.[7,108] GRP94 shares common transcriptional regulatory elements with the GRP78 promoter and is coordinately regulated with GRP78. It is reasonable to speculate that overexpression of ER-resident chaperone aids in the folding of proteins and attenuation of ER stress.[109] Indeed, overexpression of GRP78/94 attenuates ER stress and cardiac damage by I/R[62] or proteasome inhibition.[62,75,110] Furthermore, ischemic preconditioning protects hearts against I/R injury associated with the increased expression of GRP78.[63] Consistent with this observation, a small chemical induces GRP78 and protects against ER stress in neurons.[76]

One promising approach is the use of pharmacological agents, such as small chemical chaperones, which can stabilize proteins in their native conformation and rescue mutant protein folding and/or trafficking defects.[111,112] Sodium phenylbutyrate (PBA) is approved for the chronic adjunctive treatment of certain urea cycle disorders.[113] It also functions as a chemical chaperone because of the preferential hydration of the exposed polypeptide backbones and side chains of partially unfolded structures.[114,115] PBA is now used in the clinical setting for the treatment of diseases associated with protein misfolding, such as α1-antitrypsin deficiency and cystic fibrosis.[114,115] Ozcan et al have shown that chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes.[116] Erbay et al have shown that ER modification by chemical chaperones in macrophages and adipocytes has therapeutic efficacy against atherosclerosis in mouse models.[88] Furthermore, PBA rescues ER stress–induced suppression of APP proteolysis and prevents apoptosis in neuronal cells[117] and inhibits adipogenesis by modulating the UPR.[118] Although the authors did not check the UPR pathways, PBA also protects against doxorubicin-induced cardiac injury.[119] Because PBA is clinically approved for some diseases,[113] it will be fascinating to test its efficacy in humans with cardiovascular disease. However, we must emphasize that there are several limitations with these chemicals because of the high doses needed to produce the desired effect. Further studies will be required to elucidate how these chemical chaperones promote protein folding and to develop their analogs with desirable pharmacokinetics for the treatment of cardiovascular diseases.

Clinically Approved Pharmaceutical Agents

Some pharmacological agents used in clinical settings may affect UPR pathways. In pressure-overloaded hearts, we found that increased expression of CHOP was partially inhibited by an angiotensin II type 1 receptor antagonist.[38] Additionally, cardiac expression of TNF-α is increased in pressure-overloaded hearts, and TNF-α induces ER stress in cultured neonatal rat cardiomyocytes; both of these effects are significantly inhibited by pravastatin.[39] The antidiabetic agent pioglitazone suppresses ER stress in the liver, which may in part explain the pharmacological effects of pioglitazone in reducing insulin resistance.[120]

Pharmaceutical agents that activate AMPK are other promising agents. Activation of AMPK reduces cardiac ER stress and prevents the progression of heart failure in dogs.[37,121] Furthermore, the reduction of AMPK activity increases ER stress and atherosclerosis in vivo.[82,122] Tempol, which restores SERCA activity and decreases oxidized SERCA levels, markedly reduces the level of ER stress in mouse aortic endothelial cells from AMPKα2-deficient mice. Finally, oral administration of tauroursodeoxycholic acid, a chemical chaperone that inhibits ER stress, significantly reduces both ER stress and aortic lesion development in low-density lipoprotein receptor– and AMPKα2-deficient mice.[82,122] These findings suggest that AMPK activators may reduce ER stress associated with the improvement of cardiovascular disease. Activation of the receptor for glucagon-like peptide-1 reduces ER stress in pancreatic β cells.[123] Because the glucagon-like peptide-1 receptor is expressed throughout the mouse cardiovascular system,[124] it will be interesting to investigate its effects on cardiovascular disease.

Concluding Remarks

Although understanding the pathophysiological role of ER stress in cardiovascular diseases has progressed in recent years, future research needs to address several critical questions. (1) To what extent are the adaptive and proapoptotic pathways of the UPR involved in the pathophysiology of cardiovascular disease? (2) If we activate or deactivate components of UPR, will it improve disease condition? (3) Can we really regulate the cell survival and death signal involved in the UPR? (4) How can we deliver the agent to the targeted tissues? The improved understanding of the underlying molecular mechanisms of the UPR in cardiovascular

diseases will provide new targets for drug discovery and therapeutic intervention.

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Disclosures

None.

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Original article

X-box binding protein 1 regulates brain natriuretic peptide through a novel AP1/CRE-like element in cardiomyocytes

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ABSTRACT

The unfolded protein response (UPR) is triggered to assist protein folding when endoplasmic reticulum (ER) function is impaired. Recent studies demonstrated that ER stress can also induce cell-specific genes. In this study, we examined whether X-box binding protein 1 (XBP1), a major UPR-linked transcriptional factor, regulates the expression of brain natriuretic peptide (BNP) in cardiomyocytes. In samples from failing human hearts, extensive splicing of XBP1 was observed along with increased expression of glucose-regulated protein of 78 kDa (GRP78), a target of spliced XBP1 (sXBP1), suggesting that the UPR was induced in heart failure in humans. Interestingly, quantitative real-time PCR revealed a positive correlation between cardiac expression of GRP78 and BNP, leading us to test the hypothesis that sXBP1 regulates BNP as well as GRP78 in cardiomyocytes. A pharmacological ER stressor caused a dose-dependent increase in the expression of sXBP1 and BNP by cultured cardiomyocytes. Short interfering RNA targeting XBP1 suppressed the induction of BNP expression by a pharmacological ER stressor or norepinephrine, which was rescued by the adenovirus-mediated overexpression of sXBP1. The promoter assay with overexpression of sXBP1 or norepinephrine showed that the proximal AP1/CRE-like element in the promoter region of BNP was critical for transcriptional regulation of BNP by sXBP1. Direct binding of sXBP1 to this element was confirmed by the chromatin immunoprecipitation assay. These findings suggest that ER stress observed in failing hearts regulates cardiac BNP expression through a novel promoter region of the AP1/CRE-like element.

1. Introduction

The endoplasmic reticulum (ER) is an organelle that synthesizes and folds both secretory and membrane proteins [1–3]. Stresses that interfere with ER function lead to the accumulation of unfolded and misfolded proteins, after which the ER transmembrane sensors detect their accumulation and initiate the unfolded protein response (UPR) to maintain ER function [1–3]. Recently, we and others have demonstrated the increased expression of genes targeted by the UPR, such as glucose-regulated protein of 78 kDa (GRP78) and protein disulfide isomerase, in patients with cardiovascular disease, suggesting that activation of the UPR is involved in the pathophysiology of such diseases [1,4,5].

The transcriptional factor X-box binding protein 1 (XBP1) is uniquely regulated by inositol requiring kinase 1α (IRE1α), which is an ER stress sensor conserved in all eukaryotic cells [6]. Interestingly, when IRE1α is activated and senses unfolded proteins in the ER, it promotes an increase of endoribonuclease activity that specifically cleaves the mRNA encoding XBP1 (unspliced XBP1) to form transcriptionally active XBP1 (spliced XBP1) [1–3,6]. Spliced XBP1 (sXBP1) binds to ER stress response elements I and II (ERSE-I; CCAAT (N9)CCACG; ERSE-II; ATTGG(N1)CCACG) and mammalian UPR element (mUPRE; TGACGTGG/A) to regulate a variety of UPR target genes that include ER-resident chaperones and genes involved in ER associated protein degradation and lipid biosynthesis [7,8]. In addition to the induction of UPR-related genes by ER stress, recent studies demonstrated that ER stress also induces unexpected genes in

Abbreviations: BNP, brain natriuretic peptide; cDNA, complementary DNA; ER, endoplasmic reticulum; ERSE-I, -II, ER stress response elements I, II; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP78, glucose-regulated protein of 78 kDa; IRE1α, inositol requiring kinase 1α; mUPRE, mammalian UPR element; NE, norepinephrine; RT-PCR, real-time polymerase chain reaction; siRNA, short interfering RNA; sXBP1, spliced X-box binding protein 1; TU, tunicamycin; uXBP1, unspliced X-box binding protein 1; UPR, unfolded protein response.

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cell type- and condition-specific manner [9,10]. However, the genes altered by the sXBP1 in cardiomyocytes have not been well clarified. In the present study, we found extensive splicing of XBP1 and positive correlation between cardiac expressions of GRP78 and BNP in human normal and failing heart samples. Then we found that sXBP1 regulates BNP expression via a novel region of the BNP promoter in cultured cardiomyocytes. To our knowledge, this is the first information that links the UPR and the natriuretic peptide system which plays an important role in maintenance of the fluid balance and in cardiovascular growth [11].

2. Materials and methods

2.1. Materials

Tunicamycin (TU) and norepinephrine (NE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for XBP1, α- and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody for HP1α was obtained from Sigma Chemical Co. HS-142-1 was kindly provided by Kirin-Kyowa Pharmaceutical Co. (Tokyo, Japan).

2.2. Human heart tissue samples

Human heart tissue samples were studied according to the protocol approved by the Institutional Review Boards of Hayama Heart Center and Osaka University. We used heart samples of left ventricle from 4 patients with heart failure who underwent partial left ventriculoplasty at Hayama Heart Center. Messenger RNA of 2 control left ventricle heart samples were obtained commercially from Clontech Labs (Mountain View, CA, USA) and Stratagene (La Jolla, CA, USA). Tissue samples were frozen at −80 °C until use for extraction of RNA.

2.3. Analysis of XBP1 mRNA processing

Evaluation of XBP1 mRNA splicing was performed as described previously [12]. In brief, splicing of XBP1 mRNA by activated IRE1 creates a frame shift [6].Complementary DNA (cDNA) was prepared from human heart samples and rat cardiomyocytes. Using this cDNA as a template, PCR was performed with specific primers for XBP1 that covered the splicing site. (Human: sense primer: 5′-CCTTGTAGTTGAGAACCAGG-3′ and anti-sense primer: 5′-GGGGCTTGGTATATATGTGG-3′, rat: sense primer: 5′-ACGAGAGAAAACTCATGG-3′ and anti-sense primer: 5′-ACAGGGTCCAACTTGTCC-3′). The human PCR amplification products were 416 (spliced) and 442 (unspliced) base pairs (bp) in size, respectively. The rat PCR amplification products were 290 (spliced) and 264 (unspliced) base pairs (bp) in size, respectively. The products were separated on 5% polyacrylamide gel, visualized under UV light and quantified by densitometry (Scion Image software).

2.4. Preparation of neonatal rat cardiomyocytes

Primary cultures of cardiomyocytes were prepared from neonatal rat hearts as described previously [13]. All procedures were done in accordance with the guiding principles of Osaka University School of Medicine, the "Position of the American Heart Association on Research Animal Use", and the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.5. Immunoblot analysis

Preparation of cardiomyocytes, electrophoresis, immunoblotting, and detection were all done as described previously [13]. Nuclear and cytosolic fractions were separated by Dignam's method [14]. HP1α and beta-actin were used as controls for the nuclear and cytosolic fractions, respectively.

2.6. Quantitative real-time PCR (RT-PCR)

Human or rat samples were prepared according to the Omniscript Reverse Transcription Handbook (QIAGEN Inc.). The primers and probes used for quantification of sXBP1, BNP, GRP78, and GAPDH were all designed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA. https://www.appliedbiosystems.com/). Quantitative RT-PCR was performed as described previously [13].

2.7. RNA interference

We obtained the short interfering RNAs (siRNAs) from B-Bridge International, Inc. to knock-down rat, but not human, XBP1 mRNA (XBP1-1 siRNA: 5′-GAGAAAGCGCUGCGGAGGA-3′, XBP1-2 siRNA: 5′-CUUCAAAGGUAUUCCAAUA-3′) or rat BNP mRNA (BNP siRNA: 5′-CAAACUUGCCACAGUGUAA-3′), as described previously [13]. Bioinformatic analysis reveals that rat siRNA targeting XBP1 did not knock-down human XBP1. Transfection of the siRNAs was performed as described previously [13].

2.8. Adenovirus transduction

Adenoviral vectors containing the genes for LacZ and spliced human XBP1 were prepared as described previously [13]. Adenovirus carrying LacZ or spliced XBP1 was transfected at 24 h after the isolation of cardiomyocytes, and experiments were performed 48 h after transfection.

2.9. Microarray analysis

For microarray analysis, 3 RNA samples of cardiomyocytes transfected with adenovirus carrying LacZ (80 MOI) or spliced XBP1 (80 MOI) for 48 h were used. Cardiac gene expression was determined using GeneChip Rat Genome 230 2.0 Array (Affymetrix). All expression data were normalized by global scaling and analyzed by GeneSpring software (Agilent Technologies).

2.10. Confocal fluorescence microscopy

Cells were plated at a concentration of 1×10^6 cells/plate and viewed with confocal fluorescence microscopy (Radiance 2100 Laser Scanning System, Bio-Rad, Heimei Hempstead, UK), as described previously [13]. Cell surface area was measured using ImageJ (http://rsb.info.nih.gov/ij/) from 30 randomly selected cells per experiment.

2.11. Assessment of cardiomyocyte viability

The viability of cardiomyocytes was evaluated as described previously [13]. The dose of HS-142-1 used in the present study efficiently blocked BNP receptor [15].

2.12. Plasmid construction

Progressive deletion fragments of the 5′ flanking region of the BNP gene were amplified by PCR with sense primers containing an additional Kpn1 site (hBNP-1780F: 5′-GGTACCCCCTGGCAGTGATTATGAGCTTCA-3′, hBNP-238F; 5′-GGTACCGGGACTTGTCTGTGTCTCCA-3′, hBNP-111F; 5′-GGTACCTGATCTCAGAGGCCCGGAATGT-3′, hBNP-101F; 5′-GGTACCGGCCCGGAATGTGGCTGATAAAT-3′) and an antisense primer containing a Hind III site (hBNP+61R; 5′-AAGCTTGCTGCTGCTGCGATGCGTCCGGGTTTGCTT-3′). After digestion with Kpn1 and Hind III, the fragments were inserted between the Kpn1 and Hind

III sites in the firefly luciferase reporter plasmid pGL3-Basic (Promega). A deletion mutant lacking the AP1/CRE-like element was amplified by PCR with a sense primer (hBNP-103F: 5′-GAGGCCCGGAATGTGGCTGATAAAT-3′) and an anti-sense primer (hBNP-112R: 5′-GGGCCCGGGAATGAGCCCCTCCGCGCCT-3′), and was inserted into the pGL3-Basic plasmid. The plasmid for human sXBP1 was a kind gift from Dr. K. Mori (Kyoto University, Japan).

2.13. Luciferase gene reporter assay

Freshly prepared rat cardiomyocytes (4×10^6 cells) were transiently co-transfected with 10 μg of the indicated reporter construct and 10 μg of control vector (Renilla luciferase plasmid pRL-SV40) with or without 10 μg of the effector plasmid carrying sXBP1 cDNA. Electroporation was done at 280 V/300 μF in 0.2-cm cuvettes. Then the cells were plated into fibronectin-coated six-well culture dishes and incubated for 72 h to allow attachment. For the NE experiment, cardiomyocytes were co-transfected with the indicated reporter construct and control vector and maintained for 48 h. Then, they were treated with NE (1 μmol/L) for 24 h. Subsequently, luciferase activity of cell lysates was measured with a luminometer according to the manufacturer's protocol (Dual-Luciferase Reporter Assay; Promega), and reporter activity was calculated as the relative luciferase activity (firefly luciferase/Renilla luciferase) to correct for variations in transfection efficiency.

2.14. Chromatin immunoprecipitation (ChIP) assay

Rat neonatal cardiomyocytes (5×10^6) were transfected with adenovirus carrying human sXBP1 or LacZ and incubated for 48 h before being cross-linked with 1% formaldehyde. The ChIP assay was performed using a Chromatin Immunoprecipitation Assay Kit (Upstate) according to the manufacturer's protocol. The P1/P2 primers used for detection of rat BNP were 5′-GACAACACCAGCTGCAGGATGGGCTTGACGGCAAGT-3′ (rBNP-2863F) and 5′-CCGACCTCTGTGCATCAATGGTA-3′ (rBNP-2706R), yielding a 157 bp product. The P3/P4 primers used for detection of rat BNP were 5-GGAAACAAGGACCTGTTAGTGT-3′ (rBNP-257F) and 5-GGGTGGGGTTATCTCTGATTT-3′ (rBNP-72R), yielding a 185 bp product.

2.15. Statistical analysis

Data are expressed as the mean ± S.E.M. Unpaired Student *t*-test was used to compare the expression of BNP and GRP78 in human hearts. Spearman rank correlation analysis was used to examine the relationship between cardiac mRNA levels of targeted genes. The results of quantitative RT-PCR, cardiomyocyte viability analysis, and the promoter assay were compared by one-way factorial ANOVA followed by Bonferroni's correction. Microarray data was analyzed using unpaired *t*-test. For all analyses, $P<0.05$ was accepted as statistically significant.

3. Results

3.1. Extensive XBP1 splicing and increased expression of GRP78 and BNP in human failing hearts

To investigate activation of the UPR in failing human hearts, we determined the extent of XBP1 mRNA splicing as an indicator of UPR activation in samples of normal and failing myocardium. The clinical characteristics of patients were listed in the Table 1. The major form of XBP1 in normal human hearts was unspliced, while the major form in failing hearts was spliced (Fig. 1A). Consistent with these findings about XBP1 activation, quantitative RT-PCR revealed that expression of GRP78, a target of sXBP1, was significantly higher in failing hearts ($n=4$) than in normal hearts ($n=2$) (Fig. 1B). These findings suggest that the UPR was activated in failing human hearts. In addition, the BNP mRNA level was significantly higher in failing human hearts ($n=4$) compared with that in normal hearts ($n=2$) (Fig. 1C). Importantly, the quantitative real-time PCR revealed a positive correlation between cardiac expressions of GRP and BNP (Fig. 1D). There was no significant correlation between sXBP1 and GRP78 (Fig. 1E) or BNP (Fig. 1F).

Table 1
Clinical characteristics of patients.

Age	Gender	Diagnosis	BNP (pg/mL)	NYHA class	Echocardiographic findings		
					EF (%)	LVDd (mm)	LVDs (mm)
69	M	DCM	465	III	30	86	73
63	M	DCM	621	III	28	68	58
59	M	ICM	104	III	18	71	58
57	M	ICM	166	III	15	77	68

DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; BNP, brain-type natriuretic peptide; NYHA, New York Heart Association function class; LVEF, left ventricular ejection fraction; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension.

3.2. A pharmacological ER stressor increases BNP expression via an XBP1-dependent pathway in rat neonatal cardiomyocytes

Then, we investigated whether ER stress could induce BNP expression via an XBP1 dependent pathway. PCR analysis showed that treatment of cardiomyocytes with tunicamycin (TU), a pharmacological ER stressor, increased the level of mRNA for spliced XBP1 (Fig. 2A). TU increased the protein levels of nuclear, but not cytosolic, XBP1 protein (Fig. 2A) and mRNA levels of BNP (Fig. 2B) in rat cardiomyocytes. When we transfected a different dose of siRNA targeting XBP1 (siRNA-XBP1-1), siRNA targeting XBP1 dose-dependently reduced protein levels of nuclear XBP1 and mRNA levels of BNP by tunicamycin (Fig. 2C). When we transfected 2 different siRNAs targeting XBP1, either of the siRNAs targeting XBP1 efficiently reduced XBP1 protein levels (Fig. 2D). We also found that increased expression of BNP in response to pharmacological ER stress was significantly attenuated by 2 different siRNAs targeting XBP1 (Fig. 2E). These findings suggested that pharmacological ER stress induces BNP expression via an XBP1-dependent pathway in cultured rat cardiomyocytes.

3.3. Adenovirus-mediated overexpression of spliced XBP1 increases BNP expression in cultured rat cardiomyocytes

Next, we investigated the influence of sXBP1 on BNP expression by cultured rat cardiomyocytes. Immunohistological analysis revealed the nuclear localization of XBP1 in rat cardiomyocytes infected with adenovirus carrying sXBP1, but not LacZ (Fig. 3A). The transfection of adenovirus carrying sXBP1 or LacZ did not change the cardiomyocyte size (Fig. 3B). An increase of nuclear XBP1 protein levels by the overexpression of sXBP1 was also confirmed by immunoblot analysis (Fig. 3C). Adenovirus-mediated overexpression of sXBP1 increased mRNA levels of GRP78 (Fig. 3D) and BNP (Fig. 3E) in a dose-dependent manner.

Since the stimuli that enhance BNP expression often activate hypertrophic program, we checked the expression levels of representative hypertrophic genes when cardiomyocytes were transfected with adenovirus carrying LacZ ($n=3$) or sXBP1 ($n=3$): BNP (0.47 ± 0.07 versus 1.47 ± 0.04, $P=0.0002$), GRP78 (0.83 ± 0.12 versus 1.81 ± 0.25, $P=0.02$), β-myosin heavy chain (MHC) (0.93 ± 0.05 versus 1.05 ± 0.04, $P=0.15$), atrial natriuretic peptide (ANP) (0.86 ± 0.08 versus 1.10 ± 0.04, $P=0.06$), α-actin (0.35 ± 0.13 versus 1.46 ± 0.03, $P=0.001$), α-MHC (1.00 ± 0.00 versus 0.73 ± 0.14, $P=0.12$) and sarcoendoplasmic reticulum Ca ATPase (SERCA) (0.96 ± 0.04 versus 0.97 ± 0.19, $P=0.95$). These findings suggest that hypertrophic genes

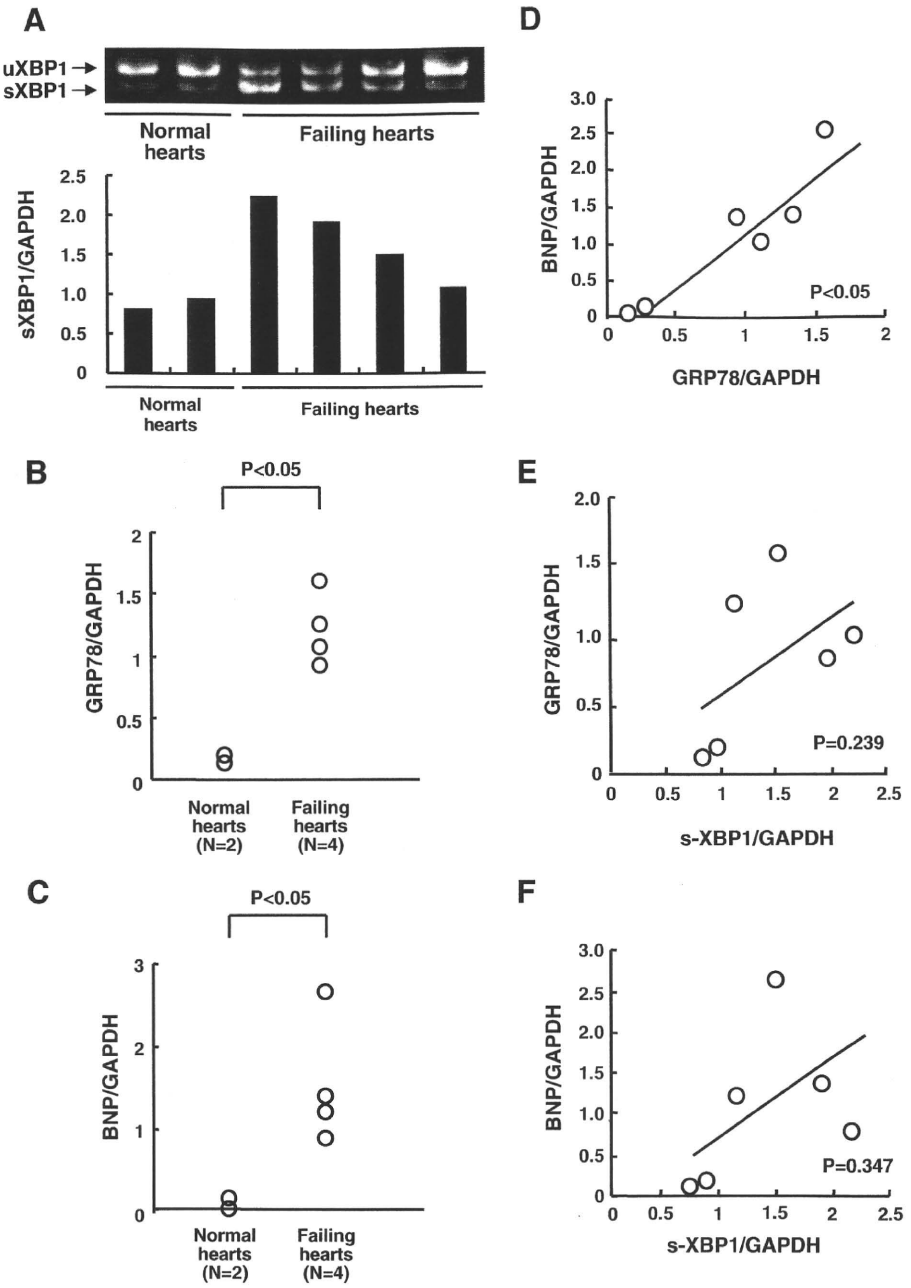


Fig. 1. UPR signaling in human hearts. (A) (Upper panel) Splicing of XBP1 mRNA in myocardial tissue from normal ($n=2$) and failing ($n=4$) human hearts. (Lower panel) Quantitative analysis of sXBP1 in normal and failing human hearts. uXBP1 and sXBP1 indicate unspliced and spliced XBP1, respectively. (B, C) Quantitative analysis of GRP78 (B) and BNP (C) mRNA in normal and failing human hearts. (D) Relationship between the cardiac expression of GRP78 and BNP in normal and failing human hearts. (E, F) Relationship between the cardiac expression of sXBP1 and GRP78 (E) or BNP (F) in normal and failing human hearts. All results of quantitative RT-PCR were normalized for GAPDH expression.

except α-actin did not significantly alter by the overexpression of sXBP1. Furthermore, overexpression of sXBP1 did not alter cardiomyocyte viability, suggesting that sXBP1 did not induce BNP secondary to cellular damage (data not shown). Importantly, we found that overexpression of sXBP1, but not LacZ, could rescue the tunicamycin-mediated enhancement of BNP that was blocked by siRNA for XBP1 (Fig. 3F). We could not detect the binding of XBP1 to the proximal AP1/CRE-like element in the BNP promoter in response to a pharmacological stressor (data not shown).

3.4. Spliced XBP1 binds to an AP1/CRE-like element in the BNP promoter region and increases its promoter activity

To investigate whether or not sXBP1 activated the transcription of BNP, we performed a number of luciferase reporter assays, in which cultured cardiomyocytes were co-transfected with a series of reporter plasmids containing fragments of the BNP 5′-flanking region (from −1780 to −101) and the pGL3 vector with or without sXBP1. Under baseline conditions, transfection of longer reporter plasmids containing the promoter region (−238 to −111) increased luciferase activity compared with transfection of the reporter plasmid lacking the promoter region (−238 to −111) or the empty pGL3-Basic plasmid (Fig. 4A left). These findings suggested that the proximal region (−238 to −111) was essential for BNP promoter activity under baseline conditions. After transfection of the pGL3-Basic vector containing spliced XBP1 (inducible conditions), co-transfection of the reporter gene containing the region (−111 to −101) increased luciferase activity by 3- to 4-fold compared with the control (empty pGL3-Basic vector) or the reporter gene lacking this region (Fig. 4A right). The bioinformatic analysis revealed that the BNP promoter

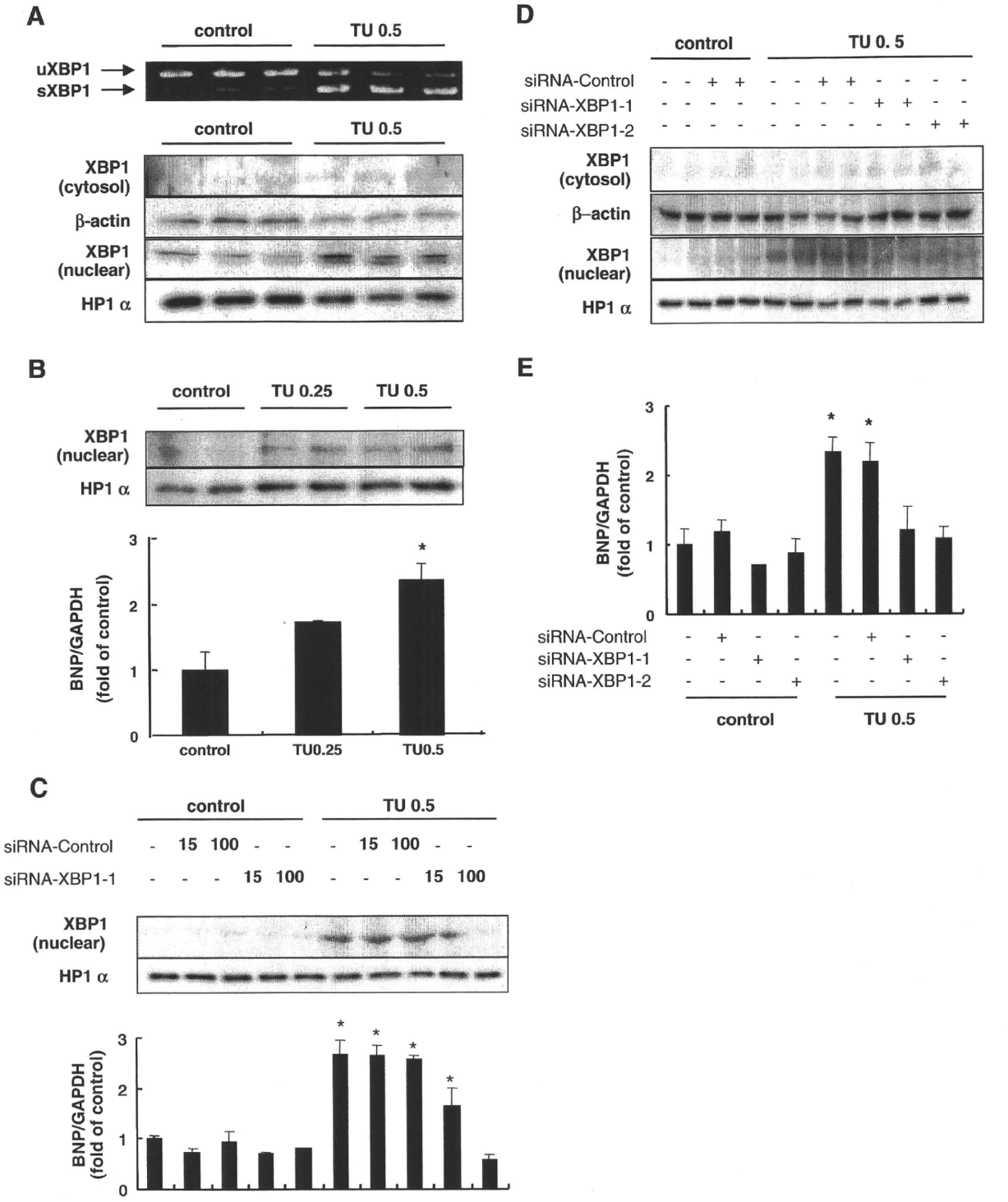


Fig. 2. A pharmacological ER stressor increases BNP expression via an XBP1-dependent pathway in rat neonatal cardiomyocytes. (A) Effects of TU on splicing of XBP1 in cultured cardiomyocytes. Neonatal rat cardiomyocytes were maintained for 24 h and then treated with TU (0.5 μg/mL) for the next 24 h. Evaluation of XBP1 splicing was performed by quantitative RT-PCR (upper panel) and immunoblot analysis (lower panel). (B) Dose-dependent effects of TU (0.25 or 0.5 μg/mL) on protein levels of XBP1 in the nuclear fraction (upper panel) and mRNA levels of BNP (lower panels) in cultured cardiomyocytes. HP1α was used as the internal control for protein levels in nuclear fraction. Results were expressed as the mean ± SEM. *$P<0.05$ versus control. (C) Dose–response effects of siRNA for XBP1 on BNP expression. Neonatal rat cardiomyocytes were maintained for 6 h and then were treated with different dose of control and XBP1 siRNA (15 μmol/L and 100 μmol/L) for 18 h. Subsequently, cardiomyocytes were treated with TU (0.5 μg/mL) for 24 h. (D, E) Effects of 2 different siRNAs targeting XBP1 on the protein level of XBP1 (D) and mRNA level of BNP (E) in cardiomyocytes treated with TU. Experiments were repeated twice independently ($n = 2$–3 per experiment). Results are expressed as the mean ± SEM. *$P<0.05$ versus control.

region (−111 to −101) corresponded to an AP1/CRE-like element, which is well conserved among mammals (Fig. 4B). These findings suggested that the AP1/CRE-like element is essential for BNP promoter activity under inducible conditions.

Next, we transfected reporter plasmids containing the BNP promoter region (−1780 to +63) with and without the AP1/CRE-like element (−111 to −101). Deletion of the AP1/CRE-like element resulted in a significant decrease of luciferase activity under inducible

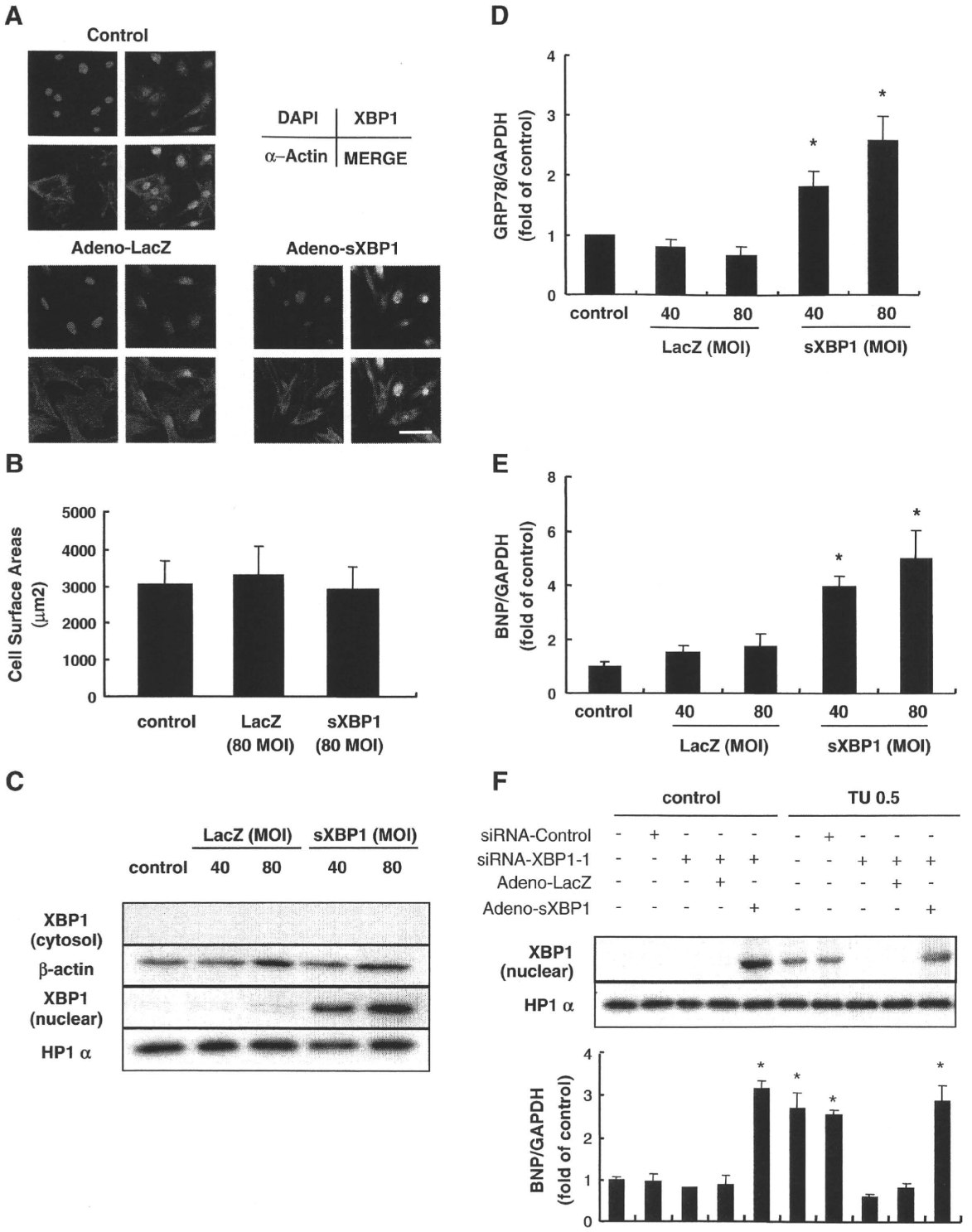


Fig. 3. Adenovirus-mediated overexpression of spliced XBP1 increases BNP expression in neonatal rat cardiomyocytes. (A) Representative immunohistochemistry of XBP1 expression in cultured rat cardiomyocytes. Confocal fluorescence microscopy shows XBP1, α-actin, and DAPI by green, red and blue staining, respectively. Bar indicates 20 μm. (B) Effects of adenovirus-mediated overexpression of LacZ or spliced XBP1 on cardiomyocyte size. (C) Immunoblot analysis of XBP1 in the nuclear and cytosolic fractions. HP1α and β-actin were used as the internal controls for the nuclear and cytosolic fractions, respectively. XBP1 in cytosolic fractions was not observed due to the rapid degradation by proteasome. Effects of overexpression of spliced XBP1 on GRP78 (D) and BNP (E) mRNA levels in cultured cardiomyocytes. MOI indicates multiplicity of infection. (F) Effects of overexpression of XBP1 on mRNA level of BNP that is blocked by siRNA for XBP1. Cardiomyocytes were treated with siRNA targeting XBP1 for 24 h and were treated with tunicamycin (0.5 μg/mL) for the next 24 h. Thereafter, they were incubated with an adenoviral vector carrying LacZ (80 MOI) or spliced XBP1 (80 MOI) for 48 h. Experiments were repeated twice independently ($n=2$–3 per experiment). Results were expressed as the mean ± SEM. *$P<0.05$ versus control.

conditions by overexpression of sXBP1 (Fig. 4C), but not baseline ones.

We also examined the role of XBP1 in BNP expression in response to NE. We found that the NE (1 μmol/L) for 24 h increased protein levels of XBP1 in the nuclear fractions and BNP expression in cultured cardiomyocytes, which was significantly attenuated by siRNA targeting XBP1. The quantitative real-time analysis revealed that the siRNA targeting sXBP1 did not block the enhancement of ANP by NE

(Control: 1.00 ± 0.16; NE: 1.61 ± 0.19; NE + siRNA XBP1: 1.67 ± 0.31; $n = 3$ in each group), suggesting that XBP1 is not involved in hypertrophic program in response to NE. These findings suggest that XBP1 is involved in BNP expression in response to NE as well as a pharmacological ER stressor. Deletion of the proximal AP1/CRE-like element resulted in a significant decrease of luciferase activity in response to NE (Fig. 4D).

Finally, we performed the ChIP assay to determine whether sXBP1 binds to the AP1/CRE-like element of the endogenous BNP promoter in vivo. Chromatin from cardiomyocytes transfected with adenovirus carrying LacZ or sXBP1 was immunoprecipitated with an antibody directed against XBP1. PCR products were obtained by using primers that covered the AP1/CRE-like element (P3/P4), but not with primers covering the −2863 to −2706 region (P1/P2) (Fig. 4E).

3.5. Induction of BNP by pharmacological ER stress does not affect the viability of cultured cardiomyocytes

We investigated the effect of the induction of BNP by pharmacological ER stress on cell viability. The siRNA targeting BNP reduced the BNP mRNA level by 93% ($n = 4$). There was no difference of cell viability when cardiomyocytes were treated with the siRNA targeting BNP (Fig. 5A) or a BNP receptor antagonist (HS-142-1) followed by a pharmacological ER stressor (Fig. 5B). These findings suggested that the induction of BNP by ER stress did not directly prevent cardiac cell death by ER stress.

4. Discussion

4.1. The UPR in heart failure

The ER is an organelle with a role in protein folding, calcium homeostasis, and lipid biosynthesis. Failing hearts show oxidative stress, hypoxia, and enhanced protein synthesis, any of which could potentially lead to ER dysfunction [1–3]. Indeed, we and others have found extensive splicing of XBP1 and increased expression of GRP78 in failing human hearts, suggesting that the UPR is activated in diseased hearts [6,7,16]. Consistent with previous reports, we also found an increase of BNP mRNA levels in myocardial samples from failing human hearts, which is widely used as a marker of heart failure [11,17]. Although the UPR triggers signaling that induces genes to maintain ER function, recent studies have shown that the UPR is also linked to other physiological systems [9,10]. Interestingly, we found a positive correlation between cardiac expression of GRP78 and BNP in human samples. These findings led us to hypothesize that BNP as well as GRP78 are commonly regulated by an UPR-dependent mechanism, although BNP has not previously been recognized as one of the targets of the UPR. Disappointingly, cardiac expressions of sXBP1 did not correlate with those of GRP78 or BNP probably due to the multiple factors that would modify their cardiac expressions in the clinical settings.

4.2. BNP expression is regulated by XBP1 in cardiomyocytes

To investigate whether BNP expression was regulated by ER stress, we treated cultured cardiomyocytes with a pharmacological ER stressor, tunicamycin, which is an inhibitor of glycosylation. This agent dose-dependently increased the expression of BNP in cultured cardiomyocytes, suggesting that BNP expression is upregulated in response to ER stress. We also confirmed that sXBP1 had a crucial role in the cardiac expression of BNP as well as GRP78 in response to a pharmacological ER stressor by experiments using 2 different siRNAs against XBP1 or an adenovirus vector carrying sXBP1. Since we showed that overexpression of sXBP1 increased BNP expression without affecting cardiomyocyte viability, the elevation of BNP expression did not seem to be due to cellular damage caused by adenoviral transfection.

Many hypertrophic stimuli can enhance BNP expression along with both the activation of hypertrophic program and the increase in cardiomyocytes size [18]. Thus, we performed microarray analysis to check whether overexpression of sXBP1 could alter the representative hypertrophic gene expressions. We found that these genes except α-actin did not significantly alter by the overexpression of sXBP1. Interestingly, bioinformatic analysis reveals that there exists AP1/CRE-like element in the promoter region of α-actin (−520 to −513). These results would strength the findings that sXBP1 could regulate the expression of some genes through the AP1/CRE-like element. Furthermore, the overexpression of sXBP1 did not change the size of cultured cardiomyocytes and the siRNA for sXBP1 did not block the enhancement of ANP by NE. These findings also suggest that sXBP1 enhances BNP expression without altering hypertrophic program.

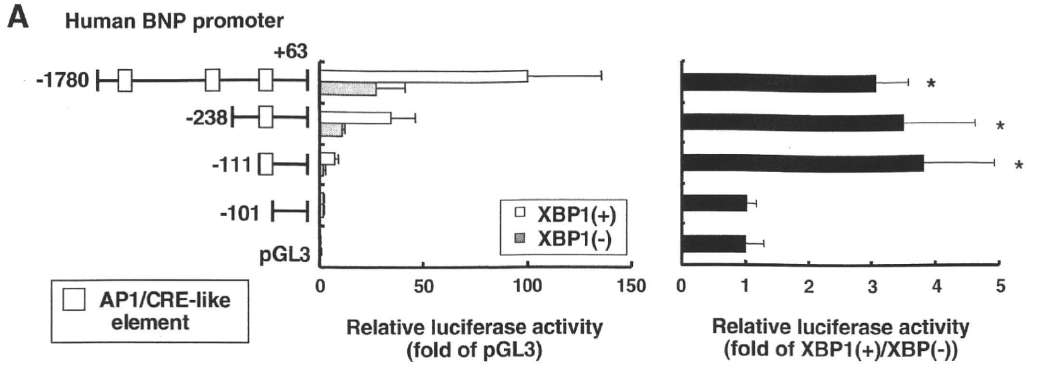
In addition to sXBP1, several factors are involved in the transcription of genes targeted by the UPR [1–3]. These UPR-linked transcriptional factors independently, and sometimes cooperatively, regulate the target genes in response to ER stress [1–3]. Since 2 different siRNAs targeting XBP1 almost completely suppressed BNP expression by tunicamycin and overexpression of sXBP1 could rescue the tunicamycin-mediated enhancement of BNP that was blocked by siRNA targeting XBP1, sXBP1 seems to be an essential regulator of BNP transcription in response to ER stress.

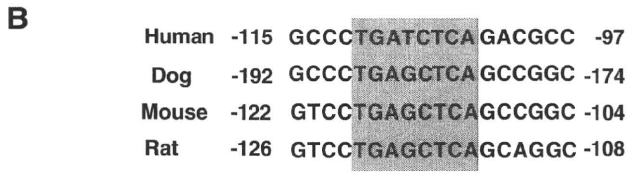
4.3. Spliced XBP1 binds to the BNP promoter response element and increases its promoter activity

Spliced XBP1 is known to bind to certain promoter regions, including ERSE-I, ERSE-II, and mUPRE [7,8], but our bioinformatics analysis revealed that the promoter region of BNP does not include any of these regions. Surprisingly, the promoter assay revealed that the AP1/CRE-like element (−111 to −103) of the BNP promoter was essential for its enhanced activity by spliced XBP1. The ChIP assay also demonstrated that spliced XBP1 bound to the AP1/CRE-like element. AP-1/CRE-like element is the site overlapped by AP-1 and CRE binding sequence [19,20]. Although several factors are reported to bind to this element, to our knowledge, this is the first to show that sXBP1 can bind to this region. Bioinformatic analysis revealed that BNP promoter has 3 different AP-1/CRE-like elements (TGATCTCA at −111; TGAGATCA at −385; and TGACATCA at −1472). Importantly, the promoter assay analysis demonstrated that the only proximal AP-1/CRE-like element has the function to enhance BNP expression in response to spliced XBP-1. Lapointe et al. demonstrated that the proximal AP1/CRE-like element (−111 to −103) is required for BNP expression in response to mitogen activated protein kinase (MAPK) kinase 6 or p38 MAPK [21]. Consistently, another study also demonstrated that only one specific element can play an important role in gene regulation although several of the same elements exist in the promoter region [22].

Although we performed ChIP assay using a pharmacological ER stressor, we could not detect the binding of XBP1 to the proximal AP1/CRE-like element in the BNP promoter (data not shown). One possible explanation for this failure would be due to the difference in sXBP1 levels in experiments using XBP1 overexpression and pharmacological treatment. Furthermore, we tried to use 2 different antibodies against XBP1 for the ChIP assay, but we could detect the binding only when we used the antibody presented in the manuscript. Thus, the technical limitation would be the possible explanation for the failure to detect the binding using a pharmacological ER stressor.

Recent studies demonstrated that NE can induce ER stress in PC12 cells [23,24]. The present study revealed that NE increased protein levels of sXBP1 and that BNP expression in response to NE was blocked by siRNA against XBP1. Since adrenergic systems are activated in patients with heart failure, increased levels of NE might regulate BNP via ER stress related pathways. Since NE can activate both MAPK- and XBP1-dependnt pathways, further investigation will be required for the interaction between sXBP1 and MAPK in the BNP expression through the proximal Ap1/CRE-like element [20,21].


A
Human BNP promoter
+63
-1780
-238
-111
-101
pGL3
AP1/CRE-like element
XBP1(+)
XBP1(-)
0 50 100 150
Relative luciferase activity (fold of pGL3)
0 1 2 3 4 5
Relative luciferase activity (fold of XBP1(+)/XBP(-))


B
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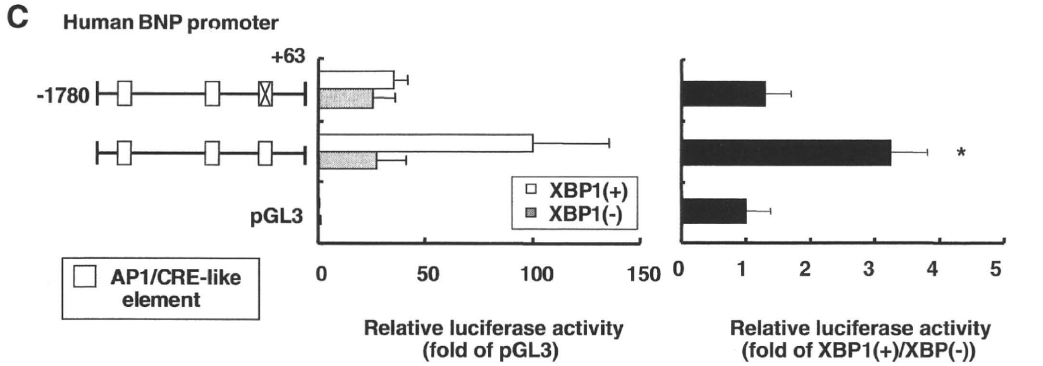
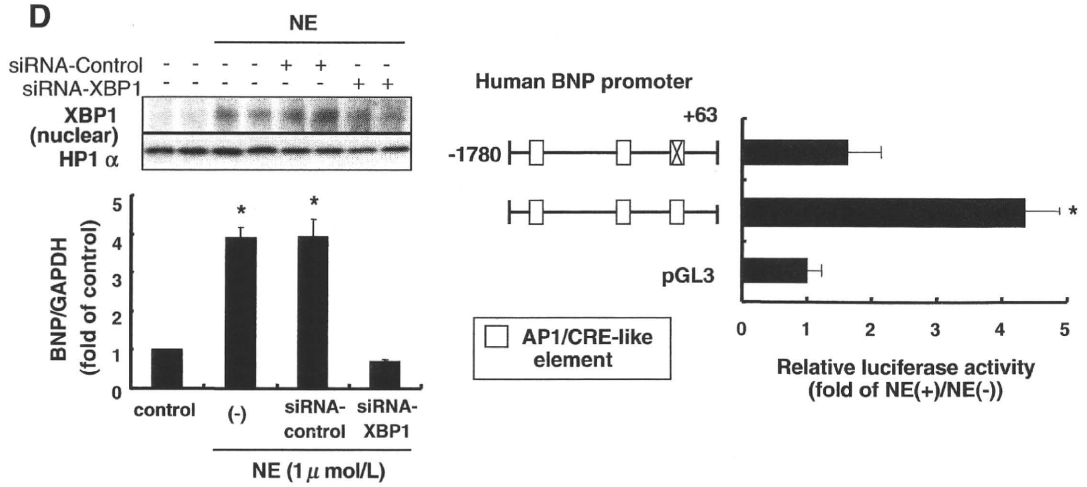
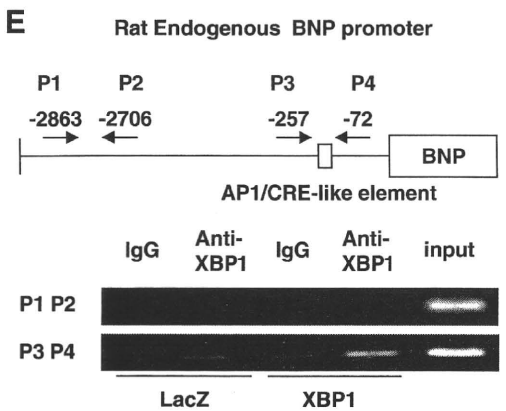
Dog -192 GCCCTGAGCTCAGCCGGC -174
Mouse -122 GTCCTGAGCTCAGCCGGC -104
Rat -126 GTCCTGAGCTCAGCAGGC -108


C
Human BNP promoter
+63
-1780
pGL3
AP1/CRE-like element
XBP1(+)
XBP1(-)
0 50 100 150
Relative luciferase activity (fold of pGL3)
0 1 2 3 4 5
Relative luciferase activity (fold of XBP1(+)/XBP(-))


D
NE
siRNA-Control
siRNA-XBP1
XBP1 (nuclear)
HP1 α
BNP/GAPDH (fold of control)
control
(-)
siRNA-control
siRNA-XBP1
NE (1 μ mol/L)
Human BNP promoter
+63
-1780
pGL3
AP1/CRE-like element
0 1 2 3 4 5
Relative luciferase activity (fold of NE(+)/NE(-))


E
Rat Endogenous BNP promoter
P1 P2 P3 P4
-2863 -2706 -257 -72
BNP
AP1/CRE-like element
IgG Anti-XBP1 IgG Anti-XBP1 input
P1 P2
P3 P4
LacZ XBP1

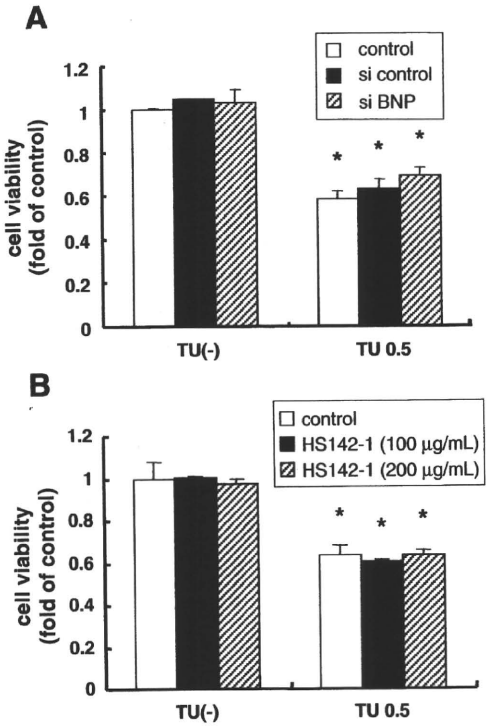


Fig. 5. Induction of BNP by pharmacological ER stress does not affect the viability of cultured cardiomyocytes. Effect of siRNA targeting BNP or HS-142-1, a BNP receptor blocker, on cardiomyocyte death after exposure to pharmacological ER stress with TU. (A) Neonatal rat cardiomyocytes were maintained for 6 h and then treated with BNP siRNA for 18 h. Subsequently, cardiomyocytes were treated with TU (0.5 μg/mL) for 24 h and cell viability was evaluated. (B) Effect of HS-142-1, a BNP receptor blocker, on cardiomyocyte viability after treatment with TU. Neonatal rat cardiomyocytes were maintained for 24 h and then treated with HS-142-1 (100 or 200 μg/mL) for 6 h. Subsequently, the cells were treated with TU (0.5 μg/mL) for 24 h and their viability was evaluated. Three independent experiments were done to assess cell viability ($n=6$ per experiment). *$P<0.05$ versus no treatment.

4.4. Pathophysiological role of BNP induced by XBP1

Since XBP1 mainly regulates UPR-related genes that potentially reduce cell damage due to ER stress, we checked whether the knock-down of BNP mRNA or blockade of the BNP receptor directly influenced cardiac cell viability in response to a pharmacological ER stressor. Our data showed that BNP in response to ER stress did not directly influence cell viability due to ER stress in cardiomyocytes. However, since BNP has an important role in the maintenance of fluid balance and in cardiovascular growth [11], induced BNP by sXBP1 would indirectly contribute to the improvement in cardiac dysfunction in which UPR is activated.

4.5. Conclusion

In the present study, we showed that sXBP1 regulates BNP expression in cultured cardiomyocytes. Regulation of BNP by XBP1 is another intriguing example of integration of the UPR with a range of physiological systems. Furthermore, since BNP is used for the treatment of heart failure [25], drugs targeting spliced XBP1 or the other factors that bind to the AP1/CRE-like element of BNP promoter region could be promising new therapies for heart failure.

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Fig. 4. Spliced XBP1 binds to an AP1/CRE-like element in the BNP promoter region and increases its promoter activity. (A) Mapping of the XBP1-response element in the BNP promoter. Luciferase activity from 3 independent experiments was normalized to Renilla luciferase activity before being compared with the control (pGL3) with and without co-transfection of spliced XBP1. Relative luciferase activity was determined as the average of duplicate measurements in 3 independent experiments. *$P<0.05$ versus control (pGL3). (B) Comparative analysis of the sequence of the AP1/CRE-like element in the BNP promoter. (C, D) Effects of deletion of the AP-1/CRE-like region on BNP promoter activity stimulated by spliced XBP1 or NE (1 μmol/L). The pGL3-BNP-luciferase reporter (−1780/+63) with or without the AP-1/CRE-like element was transfected as described in (A). Experiments were repeated twice independently ($n=2$ per experiment). Relative luciferase activity was determined by averaging duplicate measurements in the 3 independent experiments. *$P<0.05$ versus control (pGL3). (E) Binding of spliced XBP1 to the AP1/CRE-like element in the BNP promoter was demonstrated by the ChIP assay. Chromatin was immunoprecipitated with IgG or an antibody for XBP1. Purified precipitates were analyzed by PCR using primers specific for the AP1/CRE-like element (P3/P4) or the region 3 kb upstream of the BNP promoter (P1/P2).

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Spironolactone use at discharge was associated with improved survival in hospitalized patients with systolic heart failure

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Background The RALES trial demonstrated that spironolactone improved the prognosis of patients with heart failure (HF). However, it is unknown whether the discharge use of spironolactone is associated with better long-term outcomes among hospitalized systolic HF patients in routine clinical practice. We examined the effects of spironolactone use at discharge on mortality and rehospitalization by comparing with outcomes in patients who did not receive spironolactone.

Methods The JCARE-CARD studied prospectively the characteristics and treatments in a broad sample of patients hospitalized with worsening HF and the outcomes were followed with an average of 2.2 years of follow-up.

Results A total of 946 patients had HF with reduced left ventricular ejection fraction (LVEF) (<40%), among whom spironolactone was prescribed at discharge in 435 patients (46%), but not in 511 patients (54%). The mean age was 66.3 years and 72.2% were male. Etiology was ischemic in 39.7% and mean LVEF was 27.1%. After adjustment for covariates, discharge use of spironolactone was associated with a significant reduction in all-cause death (adjusted hazard ratio 0.612, $P = .020$) and cardiac death (adjusted hazard ratio 0.524, $P = .013$).

Conclusions Among patients with HF hospitalized for systolic dysfunction, spironolactone use at the time of discharge was associated with long-term survival benefit. These findings provide further support for the idea that spironolactone may be useful in patients hospitalized with HF and reduced LVEF. (Am Heart J 2010;0:1-7.)

Aldosterone plays an important role in the development and progression of chronic heart failure (HF). It induces vascular damage,[1,2] cardiac hypertrophy,[3-5] and fibrosis.[6-9] Higher level of serum aldosterone has been shown to be an independent predictor of increased mortality risk in patients with HF.[10] The RALES demonstrated that spironolactone reduces the risk of mortality and morbidity in patients with HF and systolic dysfunction.[11] Current guidelines from American College of Cardiology/American Heart Association/AHA and European Society of Cardiology recommend the use of spironolactone in HF patients with reduced left ventricular ejection fraction (LVEF) who were symptomatic under the use of angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) and diuretics.[12,13] However, RALES was performed among carefully selected severe HF patients with current or recent HF of New York Heart Association (NYHA) functional class IV. In addition, it excluded the patients with a serum creatinine concentration of >2.5 mg/dL. Moreover, the use of β-blockers was as low as 10% among the patients enrolled in RALES. Therefore, the patients in the RALES were clearly different from those in the "real world" under current standard practice for HF who are more elderly and have more comorbidities including hypertension, diabetes, and renal dysfunction. However, many patients who received new prescriptions for spironolactone after the publication of RALES have been reported not to have severe HF and about one third had renal dysfunction.[14] These findings indicated that the effect of spironolactone on outcomes needed to be assessed in an unselected population of patients with HF.

The JCARE-CARD studied prospectively the characteristics and treatments in a broad sample of patients hospitalized with HF in Japan from January 2004 to June

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2005, and the outcomes including death and rehospitalization were followed until 2008.[15-20] The JCARE-CARD enrolled 2,675 patients admitted with HF in a Web-based registry at 164 participating hospitals with an average of follow-up of 2.2 years.

The aim of the present study was to analyze the prognostic value of spironolactone on the mortality and rehospitalization by evaluating the relationship between discharge use of spironolactone and clinical outcomes among patients hospitalized with systolic HF registered in the JCARE-CARD database.

Methods

Patients

The details of the JCARE-CARD have been described previously.[15-20] Briefly, eligible patients were those hospitalized because of worsening HF as the primary cause of admission. The study hospitals were encouraged to register the patients as consecutively as possible. For each patient, baseline data included (1) age, sex, and body mass index (BMI); (2) causes of HF; (3) medical history; (4) prior procedures; (5) vital signs at discharge; (6) laboratory data at discharge; (7) echocardiographic data; and (8) medication use at discharge. The data were entered using a Web-based electronic data capture system licensed by the JCARE-CARD (www.jcare-card.jp).

From the database of a total cohort of 2,675 patients registered in JCARE-CARD, the present analysis used the data of 946 patients who had systolic dysfunction defined as LVEF <40% and did not have valvular heart disease as a cause of HF. They were divided into 2 groups according to the spironolactone use (n = 435; 46%) or no spironolactone use (n = 511; 54%) at the time of discharge from the index hospitalization.

Outcomes

The status of all patients was surveyed by June 2008 and the following information of the outcomes was obtained from the participating cardiologists by using a Web-based electronic data capture system: (1) all-cause death; (2) cardiac death, defined as death due to HF, myocardial infarction, and other causes such as pulmonary embolism; (3) rehospitalization due to an exacerbation of HF that required more than continuation of their usual therapy on prior admission; and (4) the composite end point of all-cause death and rehospitalization due to HF. The end points were adjudicated by the cardiologists in each participating hospital. Of 946 patients, 99 patients (10.5%) missed during the follow-up were excluded from the follow-up analysis. Follow-up data were obtained in 847 (89.5%) of 946 patients. Of 847 patients, 396 patients were in the group of spironolactone use and 451 patients were in that of no spironolactone use. Mean postdischarge follow-up was 801 ± 300 days (2.2 ± 0.8 years).

The hypothesis being tested was whether spironolactone use at hospital discharge would be associated with lower mortality and rehospitalization rates during the follow-up compared with no spironolactone use.

Statistical analysis

Patient characteristics and treatments were compared using Pearson χ^2 test for categorical variables, Student t test for normally distributed continuous variables, and Mann-Whitney U test for continuous variables not normally distributed. Only patients who survived the index hospitalization were included in the follow-up analysis. Cumulative event-free rates during the follow-up were derived using the method of Kaplan and Meier. The relationship between the spironolactone use at discharge and outcomes was evaluated among patients with multivariable adjustment. The covariates, age, BMI, serum creatinine at discharge, systolic blood pressure at discharge, LVEF, and medication use (calcium channel blocker, antiarrhythmic, warfarin), were used in developing the postdischarge Cox proportional hazard models.

The results were reported as hazard ratio (HR), 95% CI, and P value. Hazard ratio for outcomes when spironolactone was used was compared with not used. A P value of <.05 was used ~~for criteria~~ for variables to stay in the model. SPSS version 16.0 J for Windows (SPSS, Chicago, IL) was used for all statistical analyses. Q1

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Results

Patient characteristics

The present study included 946 patients with the mean age of 66.3 ± 13.7 years and 72.2% men (Table I). The causes of HF were ischemic heart disease in 39.6%, dilated cardiomyopathy in 36.3%, and hypertensive heart disease in 21.6%. The mean LVEF was 27.1% ± 7.3%.

Characteristics of patients prescribed spironolactone at discharge and those not receiving it prescription were compared in Table I. Patients discharged with spironolactone had significantly higher BMI. Cause of HF, medical history such as hypertension and diabetes, and treatment procedures such as coronary revascularization did not differ between groups. Systolic blood pressure at discharge was significantly lower in patients with spironolactone use. However, diastolic blood pressure was not different. Estimated glomerular filtration rate was significantly lower and the prevalence of renal dysfunction defined as serum creatinine ≥2.5 mg/dL was greater in patients without spironolactone use. Left ventricular end-diastolic and end-systolic diameters were significantly greater in patients with spironolactone use and LVEF tended to be lower, which, however, did not reach statistical significance.

Use of other medications at hospital discharge was compared between groups in Table II. The use of ACE inhibitor or ARB was as high as 90% in both groups, and that of β-blocker was 65%. Importantly, the use of these guideline-based standard medications was similar between spironolactone use and no spironolactone use groups. However, diuretics, antiarrhythmics, and warfa-