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Extracellular protein kinase CK2 is a novel associating protein of Neuropilin-1

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ABSTRACT

Neuropilin-1 (NRP1) is a multifunctional transmembrane protein which has a short cytoplasmic region with no particular functional domain, and is considered to act as a co-receptor for both VEGFs and semaphorins. However, the molecular mechanisms by which NRP1 carries out such versatile functions are still poorly understood. Here we identified protein kinase CK2 holoenzyme as a novel NRP1 binding protein by our combined purification strategy using epitope-tag immunoprecipitation followed by reverse-phase column chromatography. Further we showed that CK2 binds to the extracellular domain of NRP1 which is also phosphorylated by CK2 both *in vitro* and *in vivo*. Our findings of novel molecular interactions and modification of NRP1 may provide a new clue to understand the diverse functions of NRP1.

Introduction

Neuropilin-1 (NRP1) was originally discovered as a co-receptor for semaphorin-3A (Sema3A), an axon repellent factor [1]. In addition, NRP1 also acts as a co-receptor for vascular endothelial growth factor (VEGF), a molecule with no sequence or structural homology to Sema3A [2]. Partly due to this interesting property of NRP1, a number of reports have been published and revealed that NRP1 is involved in a wide variety of both physiological and pathological processes; axon guidance [3], angiogenesis [4], tumor progression [5], metastasis [6], immunological maturation [7], and virus entry [8].

Recently it has been revealed that NRP1 functions are intriguingly versatile and vary among the cell types in which NRP1 is expressed [9–12]. Considering its short cytoplasmic region without a specific signalling domain, NRP1 is originally thought to function as a co-receptor working with plexins for semaphorins or with VEGFRs for VEGFs [13]. However the molecular mechanisms by which NRP1 produces such diverse effects are not fully explained by this deduction. This led us to hypothesize that additional molecular interactions between NRP1 and unknown molecules may exist. Therefore, the aim of this study was to uncover novel binding partners of NRP1 in order to understand the complex nature of NRP1 functions.

Materials and methods

Materials and primary cells. We utilized the following commercially available antibodies: anti NRP1 antibody (C-19, Santa Cruz), anti FLAG M2 (Sigma), anti CSNK2A1 (sc-6479, Santa Cruz) and anti CSNK2B (Calbiochem). Anti-Myc agarose was obtained from Clontech. Recombinant CK2 holoenzyme was purchased from KinaseDetect. CK2 inhibitor, DMAT (2-Dimethylamino-4, 5, 6, 7-tetrabromo-1H-benzimidazole) was obtained from Calbiochem. Both human umbilical vein endothelial cells (HUVEC) and human coronary artery smooth muscle cells (CASMC) were purchased from Clonetics. They were cultured in endothelial and smooth muscle cell medium (Clonetics) and used up to passage 5.

Expression vector and adenovirus constructs. Human NRP1 cDNA was obtained as described previously [2]. In this experiment, all constructions were performed using the Gateway system (Invitrogen) according to the manufacturer's instructions. With PCR primers, designed to include the stop codon of NRP1, the amplified fragment was inserted to pENTR/D-TOPO (Invitrogen), named pENTR/NRP1. To generate N-terminal-tagged NRP1, either the FLAG epitope (DYKDDDDK) or Myc epitope (EQKLISEEDL) was inserted just after signal sequence of NRP1 (between Lys^{26} and Cys^{27}) by PCR based mutagenesis using pENTR/NRP1 as a template. To identify the binding site on NRP1 for CK2A1, NRP1 lacking aa 54–274 (del A), NRP1 lacking aa 275–631 (del B), NRP1 lacking aa 632–823 (del C), or

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NRP1 lacking aa 876–923 (del cyto) was generated by PCR using either pENTR–FLAG–NRP1 or pENTR–Myc–NRP1 as a template. All NRP1 constructs were sequence-verified and recombined to the mammalian expression vector, pEF-DEST51 (Invitrogen). Adenovirus constructs were generated using the ViraPower Adenoviral Expression System (Invitrogen) essentially as described by the manufacturer. The supplied pAd/CMV/V5-DEST/lacZ was used as a control (Invitrogen).

Generation of stable cell lines. VEGFR2 cDNA was cloned into pENTR/D-TOPO from HUVEC cDNA. We generated Flp293 cells which stably expressed VEGFR2 using Flp-In system (Invitrogen) as previously described [9].

Identification of NRP1 binding proteins using metabolic labelling and high performance liquid chromatography (HPLC). After metabolic labelling with [^{35}S] methionine/cysteine for 8 h, a 100 mm dish of Flp293/VEGFR2 cells, in which 10 μg of Myc-NRP1 was transiently transfected 2 days beforehand, were lysed with lysis buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP40 and the protease inhibitor cocktail (Nakalai)) with or without phosphatase inhibitors (10 mM sodium fluoride, 1 mM orthovanadate), followed by immunoprecipitation with anti-Myc affinity gel for 1 h at 4 °C. A Myc-tagged unrelated gene (human FAM155B, NM_015686.2) was transfected in Flp293/VEGFR2 and used as a control. After extensive washing, bound proteins were boiled and eluted in sample buffer, followed by SDS–PAGE. The radioactivity was detected using a BAS imaging analyzer (Fuji). For further purification, the immunoprecipitates were eluted with elution buffer (0.3% trifluoroacetic acid (TFA), 0.1% octylglucoside and 5% acetonitrile). Then the eluate was applied to a phenyl reverse-phase HPLC column (4.6 × 250 mm, Nakalai). Fractions were eluted with a linear gradient of 0–80% acetonitrile at a flow rate of 0.5 ml/min. Each fraction was resolved by SDS–PAGE, and the radioactivity was detected by BAS. For identification, 6 × 100 mm dishes of Flp293/VEGFR2 cells transfected with Myc-NRP1 or FAM155B-Myc were immunoprecipitated with anti-Myc affinity gel, and followed by HPLC as same as above. The silver stained band was excised and analyzed by MALDI-TOF/MS (Hitachi High-Tech Manufacturing and Service Co., Ltd.).

Co-immunoprecipitation assay. Flp293/VEGFR2 cells were transfected with Myc-NRP1 or the Myc-tagged deletion mutants using Lipofectamine 2000. Two days after transfection the cells were lysed in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP40 and the protease inhibitor cocktail (Nakalai)). To confirm endogenous binding, HUVEC and CASMC in a 100 mm dish without forced expression were used. We then incubated with anti-NRP1 or control rabbit IgG with protein G Sepharose (GE healthcare) for 1 h at 4 °C. After extensive washing, immunoprecipitated samples were subjected to SDS–PAGE and immunoblotting. Metabolic labelling followed by immunoprecipitation for the deletion mutants of NRP1 was carried as described above.

In vitro kinase assay. HEK293T cells transfected with FLAG-tagged NRP1 were lysed with lysis buffer (10 mM Tris–HCl (pH 7.2), 300 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1% NP40 and the protease inhibitor cocktail (Nakalai)), immunoprecipitated with ConA Sepharose (GE healthcare) for 2 h at 4 °C, followed by elution with 0.5 M mannopiranoside (Sigma). Further purification was performed using immunoprecipitation with FLAG agarose followed by FLAG peptide elution (100 μg/ml). The purified NRP1 was equilibrated in kinase buffer (20 mM MOPS (pH 7.0), 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 20 μM cold ATP and 0.1% NP40), and then incubated with recombinant CK2 holoenzyme (100 ng) and 10 μCi of [γ-^{32}P] ATP (GE healthcare) at 30 °C for 30 min. The CK2 inhibitor, DMAT, was added at 1 μM in reaction buffer. Each sample was boiled in SDS sample buffer for 3 min, and eluted proteins were analyzed by SDS–PAGE. The gel was dried and subjected to autoradiography.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed as described previously [14]. Briefly, purified NRP1 was incubated with CK2 holoenzyme in the presence of [γ-^{32}P] ATP as described above. The reaction mixture was then separated by SDS–PAGE, stained with Coomassie blue, and visualized by autoradiography. The radiolabelled band was excised from the gel and digested with trypsin. The digested sample was hydrolyzed by boiling in 6 M HCl for 60 min at 110 °C. The hydrolysate was lyophilized and resuspended in 5% TFA, 50% acetonitrile containing phosphoamino acid standards, and spotted onto thin-layer cellulose plates. Electrophoresis was performed using pH 1.9 buffer for the first dimension and pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension. The standards were then stained with ninhydrin, and the plates were analyzed by autoradiography.

In vivo kinase assay. HUVEC were transfected with either LacZ or FLAG-NRP1 adenovirus at MOI 25 in 60 mm dishes 2 days before assay. The medium was changed to phosphate-free DMEM (GIBCO) containing 2% dialysed FBS (GIBCO), labelled with ortho [^{32}P] orthophosphate (0.25 mCi/ml, GE healthcare) for 4–12 h. The CK2 inhibitor, DMAT, was added at 1 μM during the labelling period. The labelled cells were washed with sucrose buffer once, lysed with lysis buffer (10 mM Tris–HCl (pH 7.2), 150 mM NaCl, 10 mM sodium fluoride, 1% NP40 and the protease inhibitor cocktail (Nakalai)), and then immunoprecipitated with anti-FLAG agarose for 1 h at 4 °C. The eluted proteins were analyzed by SDS–PAGE. The gel was dried and subjected to autoradiography.

Results

Identification of protein kinase CK2 as a novel NRP1 binding protein

We have previously reported that NRP1 affects VEGFR2 expression before ligand binding and VEGFR2 degradation after forming the receptor complex via unknown mechanisms [9]. Therefore, we presume that NRP1 may have other associating molecules in the presence of VEGFR2.

Screening NRP1 immunoprecipitates from metabolically labelled Flp293/VEGFR2 cells transiently transfected with Myc-NRP1 yielded 3 candidate bands (Fig. 1A, lane 2). We chose Myc-tagged NRP1 because background signal was the lowest among tested epitope tags (data not shown). Interestingly the intensities of these bands decreased if phosphatase inhibitors were added in the lysis buffer (Fig. 1A, lane 4). These target proteins were further purified by reverse-phase HPLC, and the fraction analysis is shown in Fig. 1B. Among the 3 targets, we could clearly separate a 43 kDa NRP1-associated band in fraction 57 and a 25 kDa band in fraction 58. To identify these two bands, a large scale preparation without metabolic labelling was performed and both fractions visualized by silver stain. Although a large amount of immunoglobulin derived from the anti-Myc affinity gel masked the 25 kDa band in fraction 58, the 43 kDa NRP1-associated band was detected by silver stain (Fig. 1C). Peptide mass fingerprinting by MALDI-TOF/MS analysis of the digested 43 kDa band revealed that four peptides (QLYQTLTDYDIR, EAMEHPYFYTVVK, GGPNIITLADIVKDPVSR and EPFFHGHDNYDQLVR) matched to human protein kinase CK2 α subunit (CSNK2A1).

To confirm CSNK2A1 binding to NRP1, co-immunoprecipitation was carried out using Flp293/VEGFR2 transfected with NRP1 as well as HUVEC and CASMC, both of which are reported to endogenously express NRP1. As shown in Fig. 2A, we could detect co-immunoprecipitated CSNK2A1 with NRP1 not only in NRP1 transfected Flp293/VEGFR2 cells but also in HUVEC and CASMC without forced expression. Intriguingly, co-immunoprecipitated CSNK2A1 was more prominent in HUVEC than in CASMC, despite NRP1 is well expressed in both HUVEC and CASMC [9]. To further

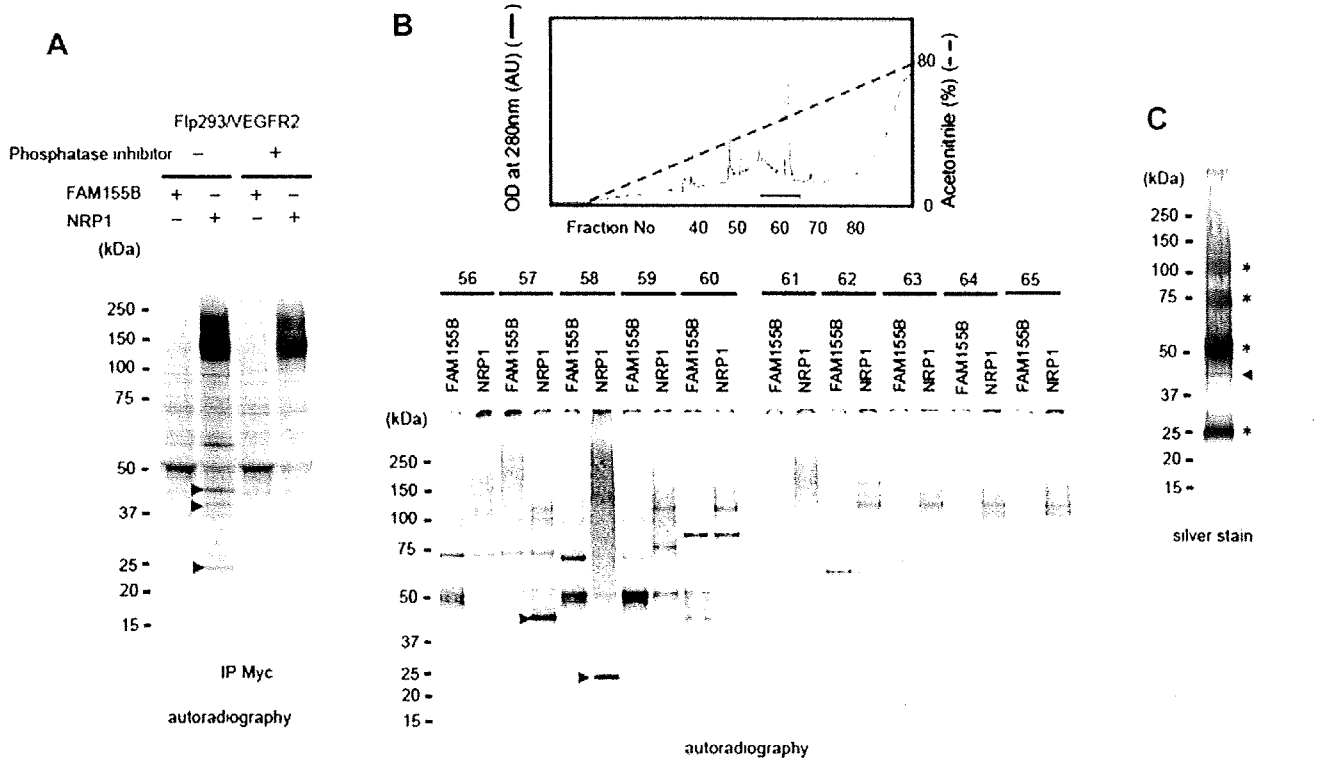

Fig. 1. Identification of protein kinase CK2 as a novel NRP1 binding protein. (A) NRP1 immunoprecipitates using metabolic labelling with ^{35}S in Flp293/VEGFR2 cells showed that 3 candidate bands (arrowheads) were specifically associated with NRP1 (lane 2). Phosphatase inhibitors decreased this interaction (lane 4). Myc-tagged human FAM155B (NM_015686.2) which is unrelated to VEGF signal was used as a control. (B) NRP1-immunoprecipitates were applied to reverse-phase HPLC. The protein was eluted with a linear gradient of 0–80% acetonitrile (Upper panel). Each fraction was separated by SDS–PAGE and visualized by autoradiography (Lower panel). (C) In fraction 57 of large scale preparation without metabolic labelling, the 43 kDa NRP1-associated band (arrowheads) was visualized by silver stain and excised for mass-spectrometry. Asterisks indicated immunoglobulin.

characterize the binding site of NRP1, we constructed several NRP1 deletion mutants (Fig. 2B) which were tested in the co-immunoprecipitation studies. As for intact NRP1, the 43 kDa NRP1-associated band which was revealed to be CSNK2A1 was detected in all constructs except for del B mutant (asterisk in Fig. 2C), suggesting that the interaction between CSNK2A1 and NRP1 occurs at extracellular b1b2 domain of NRP1. It is of note that all three NRP1 associating bands (asterisk and arrowheads in Fig. 2C) disappeared in del B mutant, while they were seen in all other constructs.

Protein kinase CK2 is a holoenzyme which consists of hetero-tetramer of catalytic subunits (α (CSNK2A1) and α′ (CSNK2A2)) and regulatory β (CSNK2B) subunits conforming $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ and $\alpha'_2\beta_2$ combinations. As the molecular weights of CSNK2A2 and CSNK2B are 40 and 25 kDa, respectively, we presume 3 NRP1-associated bands (asterisks in Fig. 1A) may correspond to α, α′ and β subunits. Indeed, co-immunoprecipitation using HEK293T cells transfected with FLAG-NRP1 demonstrated that CSNK2B was associated with NRP1 (Fig. 2D).

From these data, we therefore concluded that protein kinase CK2 holoenzyme can bind to NRP1 via its extracellular domain.

NRP1 is phosphorylated by CK2 in vitro and in vivo

As CK2 is reported to be an extracellular kinase [15] and phosphatase inhibitors affected the interaction between CK2 and NRP1 (Fig. 1A), we hypothesized that NRP1 is a substrate for CK2. As shown in Fig. 3A, the purified recombinant NRP1 was strongly phosphorylated by holoenzyme CK2 *in vitro* and this phosphorylation was significantly inhibited by adding the CK2 inhibitor, DMAT. Using purified deletion mutants of NRP1 as a substrate for the *in vitro* kinase assay, phosphorylation of NRP1 by CK2 was completely absent in del B mutant (Fig. 3B), suggesting that CK2 phosphorylates NRP1 at its extracellular b1b2 domain in addition to being the binding site. Phosphoamino acid analysis of digested phosphorylated NRP1 revealed that the phosphorylation of NRP1 by CK2 holoenzyme occurred in both threonine and serine residues, but not tyrosine residue (Fig. 3C).

As phosphorylation of NRP1 has not been reported before, we further checked this phosphorylation of NRP1 *in vivo*. HUVEC transfected with either FLAG-tagged NRP1 adenovirus or LacZ expressing adenovirus, were metabolically labelled with ortho ^{32}P, then subjected to immunoprecipitation by anti-FLAG Ab or anti-NRP1 Ab. As shown in Fig. 3D, the distinct single band which corresponds to NRP1 in molecular weight was clearly labelled by ^{32}P, and that this phosphorylation was not affected by VEGF. In addition, the extent of phosphorylation of NRP1 was decreased by the pre-incubation of cells with DMAT, the CK2 inhibitor (Fig. 3E). These data suggest that NRP1 is phosphorylated by CK2 *in vivo*.

Discussion

In this study, we identified the novel NRP1 binding protein, CK2 holoenzyme in our combined purification strategy using epitope-tag immunoprecipitation followed by reverse-phase HPLC. We also demonstrated that NRP1 is phosphorylated by CK2 both *in vitro* and *in vivo*.

Although NRP1 is considered to work as a co-receptor that binds extracellular ligands and forms receptor complexes with

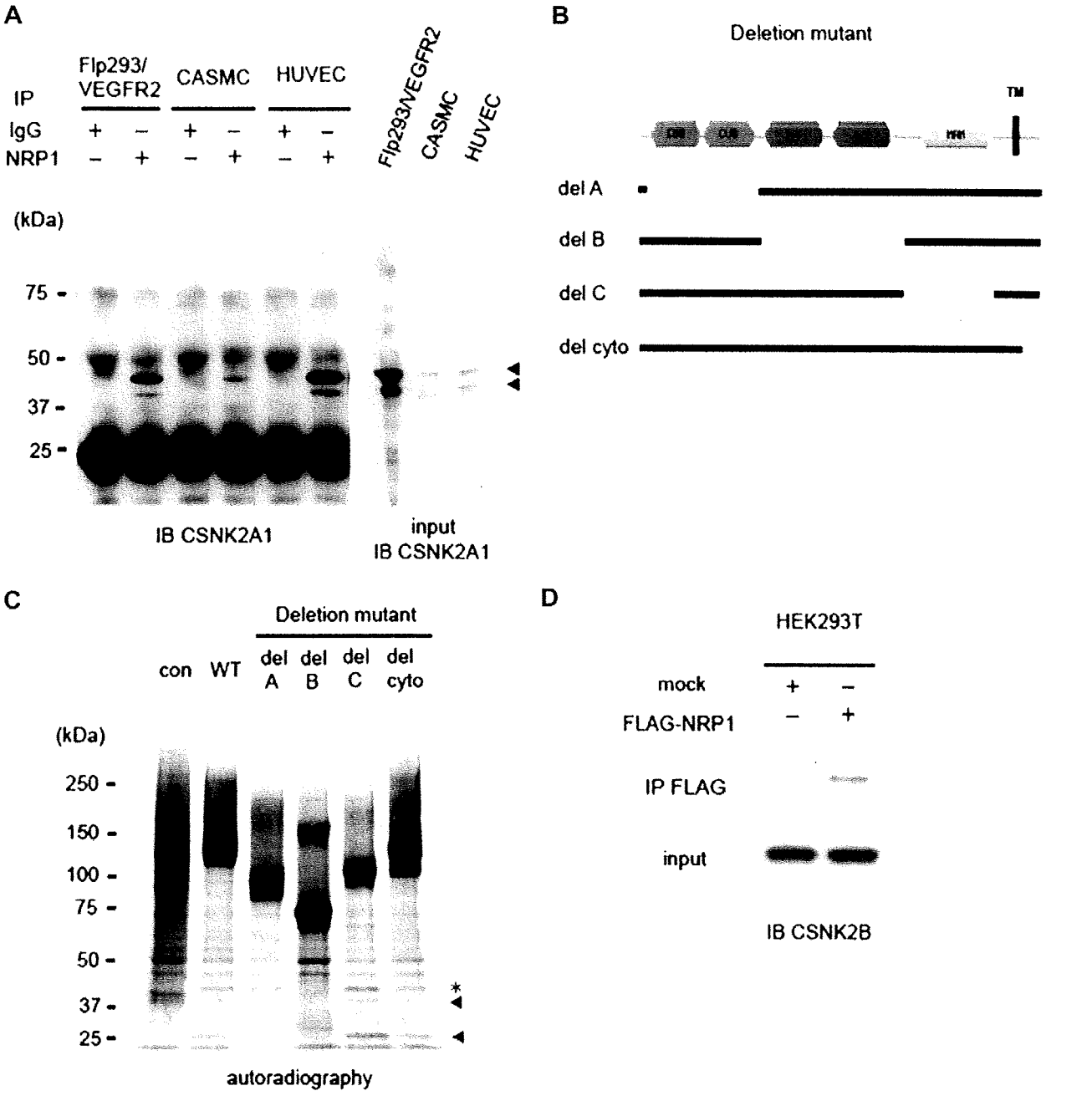

Fig. 2. Protein kinase CK2 binds NRP1 via its extracellular domain. (A) CSNK2A1 (arrowheads) was co-immunoprecipitated with NRP1 in either Flp293/VEGFR2 transfected with NRP1, human umbilical vein endothelial cells (HUVEC) or human coronary artery smooth muscle cells (CASMC). As input, 5% of whole cell lysates were used (right panel). Non-specific IgG was used as an immunoprecipitation control. (B) Schematic model of the NRP1 deletion mutants. CUB; complement protein subcomponents Clr/Cls, Urchin embryonic growth factor and Bone morphogenic protein domain, MAM; Meprin/A5-protein/PTPmu domain, TM; transmembrane. (C) NRP1 immunoprecipitates using metabolic labelling with ^{35}S showed 3 NRP1-associating bands (arrowheads) could be detected in all constructs except for del B mutant. (D) Co-immunoprecipitation using HEK293T cells transfected with FLAG-NRP1 demonstrated that CSNK2B was associated with NRP1.

the signalling receptors involved (VEGFRs or plexins), this model does not fully explain all the lines of evidence relating to the functions of NRP1 in endothelial cells and other cell types [7,16,17]. NRP1 was reported to be able to homodimerize or oligomerize through its binding to heparan sulfate [18]. Further, we and others recently reported that NRP1 is modified by negatively charged-glycosaminoglycan chains [9]. Recently, Sarris et al. demonstrated that NRP1 localises in immunological synapse where multi-signalling molecules can form a complex and initiate an immune response [11]. All this evidence suggests that NRP1 acts like scaffold protein which can form a multi-signalling molecular complex [12]. Therefore, the identification of novel molecular interactions with NRP1 is likely to provide a new aspect to help understand NRP1 functions.

Here we identified CK2 as a novel associating partner of NRP1 and this interaction occurs at the extracellular domain of NRP1. Protein kinase CK2 is present in the cytoplasm, nuclei, and several other organelles. In addition, this enzyme has been recently found bound to the external side of the cell membrane where it acts as an ectokinase phosphorylating several extracellular proteins [19–21].

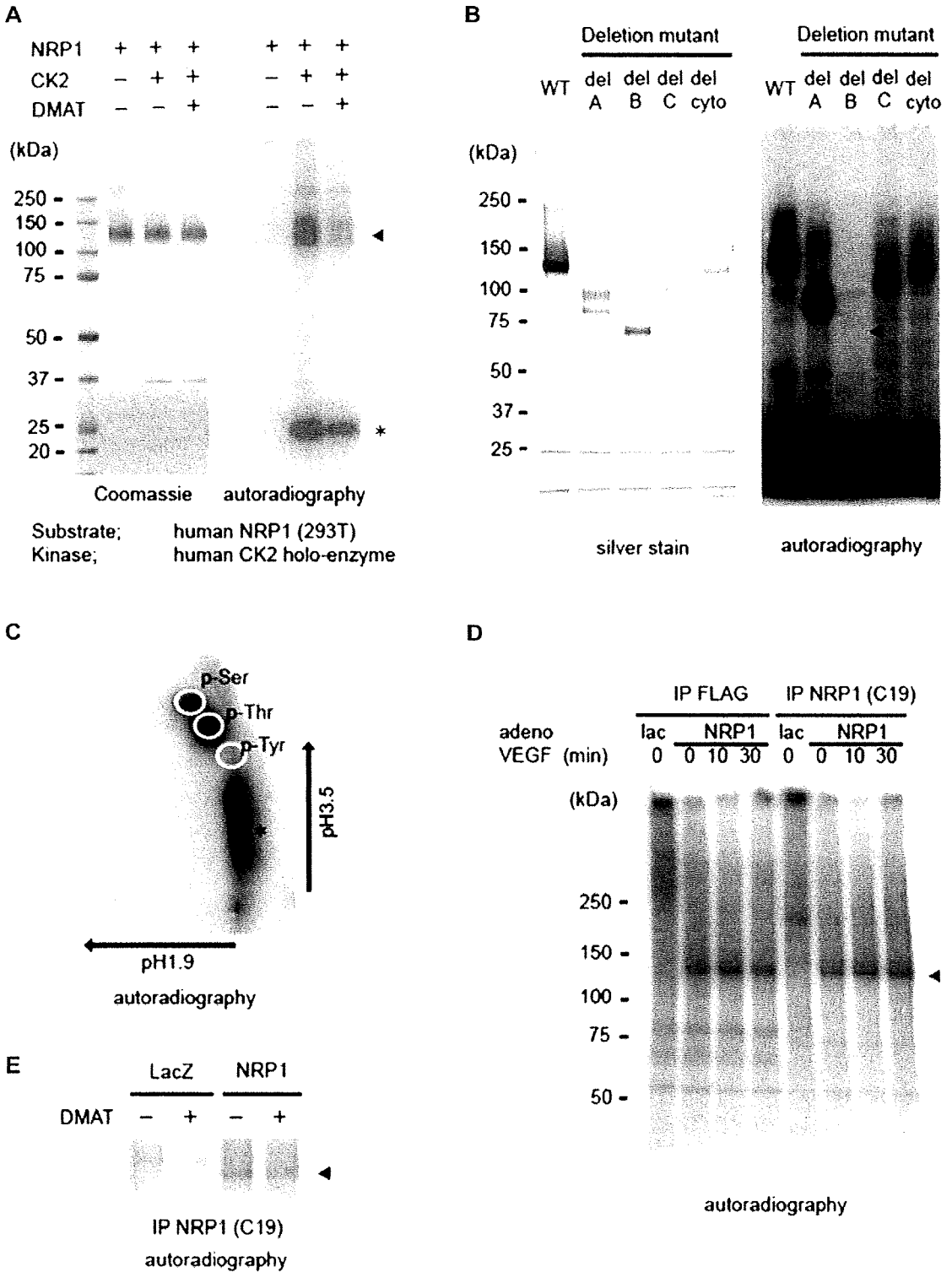

Fig. 3. Protein kinase CK2 phosphorylates NRP1 at its extracellular domain *in vitro* and *in vivo*. (A) FLAG-NRP1 purified from HEK293T was incubated with recombinant CK2 holoenzyme in the presence of [γ-^{32}P] ATP at 30 °C for 60 min, and then the reaction mixture was analyzed by SDS–PAGE, stained with Coomassie blue, and visualized by autoradiography. NRP1 was clearly phosphorylated by CK2, this phosphorylation was significantly blocked by the CK2 inhibitor, DMAT (1 μM). Asterisk shows autophosphorylated CK2β subunit (CSNK2B). (B) Deletion mutants of FLAG-NRP1 purified from HEK293T were used for the *in vitro* kinase assay as described in (A). Phosphorylation of NRP1 by CK2 was completely absent in the del B mutant. Arrowhead shows absent phosphorylation in del B mutant. (C) Phosphoamino acid analysis of phosphorylated NRP1. Phosphorylated NRP1 *in vitro* was excised from the gel, digested with trypsin, and hydrolyzed in hydrochloric acid. The resulting phosphoamino acids were separated by thin-layer electrophoresis using pH 1.9 for the first dimension and pH 3.5 for the second dimension. Autoradiography shows radiolabelled material co-migrating with the phosphoserine (pSer) and phosphothreonine (pThr) standards, but not with phosphotyrosine (pTyr) standard. Asterisk shows incomplete hydrolysis. (D) *In vivo* ^{32}P labelling in HUVEC transfected by either LacZ or FLAG-NRP1 adenovirus demonstrated that NRP1 was phosphorylated *in vivo* (arrowhead). VEGF (50 ng/ml) was added as indicated time course. (E) CK2 inhibitor, DMAT pretreatment (1 μM) decreased NRP1 phosphorylation *in vivo*. The experimental protocol was same as in (D).

Protein kinase CK2 is a holoenzyme which consists of heterotetramer of catalytic and regulatory subunit, and previous reports suggested that expression of both subunits is necessary for the appearance of the ectopic enzyme as an ectokinase [15]. Therefore our findings that NRP1 immunoprecipitates contains 3 bands (which corresponds to catalytic subunits α, α′ and regulatory β subunit) is consistent with the biology of ectokinase CK2.

The identification of an ectokinase with catalytic properties on the cell surface shed new light on the potential role of extracellular phosphorylation on a number of physiological and pathological processes [22]. Protein kinase CK2 is one of the major ectokinases and CK2-mediated extracellular phosphorylation has been reported in regulating cellular functions; cell adhesion [19,20], neurite regeneration [21] and lytic activity [23]. As NRP1 was originally considered as an adhesion molecule [24], and known to interact with L1CAM adhesion molecule [25], the interaction between CK2 and NRP1 or CK2 mediated NRP1 phosphorylation might be involved in regulating cell–cell interactions.

In summary, we identified ectokinase CK2 as a novel binding partner for NRP1, and that this protein kinase phosphorylates NRP1 *in vitro* and *in vivo*. Although future investigation will be needed to clarify the physiological importance of this interaction/phosphorylation of NRP1 by CK2, these data add new insight into the understanding of the biology of NRP1.

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Review article

ER stress in cardiovascular disease

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ABSTRACT

The endoplasmic reticulum (ER) is an organelle involved in protein folding, calcium homeostasis, and lipid biosynthesis. Various factors that interfere with ER function lead to accumulation of unfolded proteins, including oxidative stress, ischemia, disturbance of calcium homeostasis, and overexpression of normal and/or incorrectly folded proteins. The resulting ER stress triggers the unfolded protein response (UPR) that induces signal transduction events to reduce the accumulation of unfolded proteins by increasing ER resident chaperones, inhibiting protein translation, and accelerating the degradation of unfolded proteins. However, if stress is severe and/or prolonged, the ER also initiates apoptotic signaling that includes induction of the pro-apoptotic transcriptional factor C/EBP homologous protein, activation of c-Jun amino-terminal kinase, and cleavage of caspase-12. These ER-initiated events lead to cell death via mitochondria-dependent and -independent apoptotic pathways. Furthermore, the B cell lymphoma 2 family of proteins expressed on the ER and mitochondria are also involved in regulating cell death due to ER stress. Thus, the ER is now recognized as a vitally important organelle that can decide cell survival or death. Recent animal and human studies have revealed that the UPR and ER-initiated apoptosis are implicated in the pathophysiology of various cardiovascular diseases, including heart failure, ischemic heart disease, the development of atherosclerosis, and plaque rupture. Improved understanding of the molecular mechanisms underlying UPR activation and ER-initiated apoptosis in cardiovascular disease will provide us with new targets for drug discovery and therapeutic intervention.

Contents

1. Introduction

The endoplasmic reticulum (ER) is an organelle that has an essential role in multiple cellular processes, such as the folding of secretory and membrane proteins, calcium homeostasis, and lipid biosynthesis [1,2]. A variety of insults can interfere with ER function, leading to the accumulation of unfolded and misfolded proteins in the ER. When ER transmembrane sensors detect the accumulation of unfolded proteins, the unfolded protein response (UPR) is initiated to cope with the resulting ER stress. If ER stress is prolonged or overwhelming, however, it can induce cell death. Recent studies have suggested that the UPR and ER-initiated apoptosis are implicated in the pathophysiology of various human

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diseases, including cardiovascular disease, neurodegenerative disease, diabetes mellitus, and liver disease [1-3]. This review summarizes (1) the molecular mechanisms of the UPR and ER-initiated apoptosis and (2) their involvement in the pathophysiology of cardiovascular disease.

2. The endoplasmic reticulum

The ER is recognized as the organelle involved in the synthesis and folding of secreted and membrane-bound proteins and thus is the first part of the secretory pathway [1-3]. The ER supports the biosynthesis of approximately one third of all cellular proteins in a typical eukaryotic cell [4]. To achieve the proper folding of proteins, the lumen of the ER is a special environment [1,2], e.g., the ER has the highest calcium concentration within the cell. Both protein folding reactions and the functioning of various calcium-dependent ER resident chaperones require a high level of calcium. Furthermore, an oxidizing environment inside the ER is crucial for the formation of disulfide bonds that is required for the proper folding of proteins. As a consequence of these special requirements, ER function is highly sensitive to stresses that disturb calcium homeostasis or alter the intraluminal redox status.

3. UPR signaling

When ER stress occurs, three ER transmembrane sensors are activated to initiate adaptive responses [1-3]. These sensors include protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1), and the transcriptional factor activating transcription factor 6 (ATF6). These UPR sensors are located with their N-terminus inside the lumen of the ER and their C-terminus in the cytosol, thereby connecting the ER with the cytosol. All three sensors are maintained in an inactive state through the interaction of their N-terminus with glucose-regulated protein 78 kDa (GRP78) [5]. When unfolded proteins accumulate in the ER, GRP78 releases these sensors to allow their oligomerization and thereby initiates the UPR [5] (Fig. 1).

PERK is a serine threonine kinase that phosphorylates eukaryotic translation initiation factor 2α (eIF2α) after the onset of ER stress to shut off mRNA translation and reduce the protein load on the ER. Paradoxically, however, several mRNAs require the phosphorylation of eIF2α for their translation, including the transcriptional factor ATF4 that induces UPR-related genes to reduce the level of unfolded proteins in the ER.

IRE1α is the most fundamental ER stress sensor and is conserved in all eukaryotic cells. Interestingly, activation of IRE1 elicits endoribonelease activity that specifically cleaves the mRNA encoding the transcriptional factor X-box binding protein 1 (XBP1). This unconventional splicing reaction is required for the translation of transcriptionally active XBP1. Active (spliced) XBP1 binds to ER stress response elements I and II (ERSE-I: CCAAT(N9)CCACG; ERSE-II: ATTGG(N1)CCACG) and to the mammalian UPR element (mUPRE: TGACGTGG/A) to regulate a variety of UPR-related genes [6]. Recent studies showed that XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks, although it has not been well identified how XBP1 regulates genes in cardiomyocytes [7].

ATF6 is a basic ZIP family transcriptional factor that binds to ERSEs in the promoter region of UPR-related genes. ER stress induces the release of GRP78 from ATF6 and thus permits the translocation of ATF6 from the ER to the Golgi apparatus, where S1P- and S2P-mediated proteolytic cleavage produces a transcriptionally active cytosolic fragment. ATF6 activates a subset of UPR-related genes, including XBP1. The three arms of the UPR (including ATF4, XBP1, and ATF6) coordinately regulate the transcription of various genes encoding ER chaperones and protein folding enzymes in order to reduce the accumulation of unfolded proteins.

4. ER-associated degradation

Another mechanism that reduces the level of misfolded and unfolded proteins in the ER is degradation via the ER-associated protein degradation (ERAD) pathway [1,2]. Most ERAD substrates are ubiquitinated before undergoing degradation by proteasomes. The ERAD mediates retro-translocation of unfolded proteins into the cytosol where these proteins are degraded by the ubiquitin-proteasome machinery.

5. ER-initiated apoptotic signaling

When the UPR fails to control the level of unfolded and misfolded proteins in the ER, ER-initiated apoptotic signaling is induced. Interestingly, all of the ER sensor proteins are responsible for apoptotic signaling as well as for the UPR, but it remains unclear how the cell makes a decision between survival and death (Fig. 2).

IRE1α-dependent apoptotic signaling occurs via diverse pathways. IRE1α interacts with the adaptor protein TNF receptor-associated factor (TRAF) 2. IRE1α and TRAF2 then interact with a mitogen-activated protein kinase kinase kinase, ASK1, which subsequently phosphorylates JNK [8,9].

C/EBP homologous protein (CHOP) is the one of most thoroughly investigated molecules among those involved in ER-initiated apoptotic signaling. CHOP is a pro-apoptotic bZIP transcriptional factor that is mainly regulated by ATF4- and ATF6-dependent pathways [1,2].

Table 1
ER stress and cardiovascular disease.

Diseases	Role of ER stress	Target protein	Refs.
Hypeterophic heart	• Pressure-overload to heart induces UPR	• GRP78	[22]
Failing heart	• ER stress is induced in human failing hearts	• GRP78	[22,23]
	• Pressure-overload to heart finally leads to cardiac apoptosis associated with CHOP induction	• CHOP	[22]
	• Impairment of a retrieval receptor for ER chaperones causes heart failure and CHOP induction	• KDEL/CHOP	[24]
Autoimmune cardiomyopathy	• Autoimmune cardiomyopathy induced by beta-adrenergic receptor peptide is associated with ER stress	• p38/CaMKII • ATF6/GRP78/CHOP	[25]
Alcoholic cardiomyopathy	• Alcohol induces myocardial ER stress	• GRP78/CHOP	[26]
Ischemic heart	• PDI is induced in cardiomyocytes near myocardial infarction in humans	• PDI	[45]
	• GRP94 plays cardioprotective role against hypoxic insult	• GRP94	[46]
	• ATF6 protects the myocardium from ischemic/reperfused myocardium	• ATF6/GRP78/GRP94	[47]
	• Hypoxia induces CHOP and caspase 12 activation, which is inhibited by AMP-activated kinase	• ATF6/GRP78/GRP94	[51]
	• PUMA inhibits cardiomyocyte cell death by ER stress	• PUMA	[52,53]
Cardiotoxicity of anti-cancer drug	• Imatinib induces cardiomyocyte cell death associated with ER stress and JNK activation	• JNK	[31]
	• Proteasome inhibition induces cardiomyocyte cell death via CHOP	• CHOP	[35]
Atherosclerosis	• Oxidative stress causes macrophage apoptosis via CHOP	• CHOP	[39]
	• UPR and ER-initiated apoptosis in macrophage in atherosclerotic lesions		[39,40]
	• Increased CHOP induction in ruptured atherosclerotic plaques	• CHOP	[39,43]

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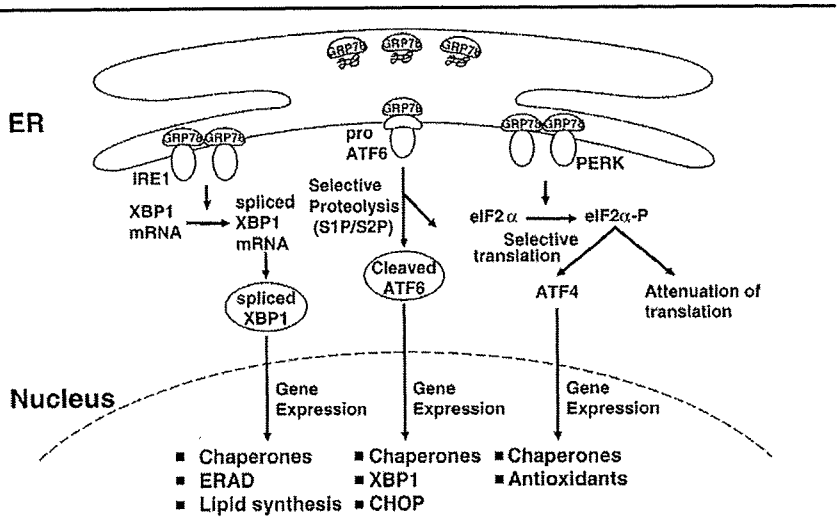

Fig. 1. The unfolded protein response. When ER stress occurs, GRP78 dissociates from the three ER transmembrane sensors (including protein kinase-like ER kinase (PERK), inositol requiring kinase 1 (IRE1), and the transcriptional factor activating transcription factor 6 (ATF6)), allowing their activation. PERK phosphorylates eukaryotic translation initiation factor 2 (eIF2α) after the onset of ER stress and consequently shuts off mRNA translation. However, eIF2α phosphorylation also induces the translation of a transcriptional factor (ATF4) that induces the UPR-related genes. Activation of IRE1 elicits endoribonuclease activity that specifically cleaves the mRNA encoding the transcriptional factor X-box binding protein 1 (XBP1). This unconventional splicing reaction is required for translation of transcriptionally active XBP1 to induce UPR-related genes. When ER stress occurs, ATF6 translocates from the ER to the Golgi apparatus, where S1P- and S2P-mediated proteolytic cleavage produces a transcriptionally active cytosolic fragment. ATF6 activates a subset of the UPR-related genes, including XBP1. The three arms of the UPR, including ATF4, XBP1, and ATF6, coordinately regulate the transcription of UPR-related genes encoding ER chaperones and protein folding enzymes to reduce the accumulation of unfolded proteins.

Deletion of the CHOP gene protects cells against death induced by pharmacological ER stressors and accumulation of defectively folded proteins and ischemia [10,11]. One important pathway by which CHOP induces apoptosis is regulation of the balance between pro-apoptotic and anti-apoptotic proteins from the Bcl-2 family. CHOP mediates the transcriptional repression and activation of proteins from this family to repress the expression of anti-apoptotic Bcl-2 [12]. CHOP also mediates direct transcriptional induction and translocation to the ER membrane of BIM, a pro-apoptotic BH3-only protein from the Bcl-2 family, in response to ER stress [13].

Interestingly, recent evidence suggests that proteins from the Bcl-2 family can affect ER-initiated apoptosis through calcium signaling [14,15]. In response to ER stress, Bak and Bax (which are pro-apoptotic proteins from the Bcl-2 family) on ER membrane

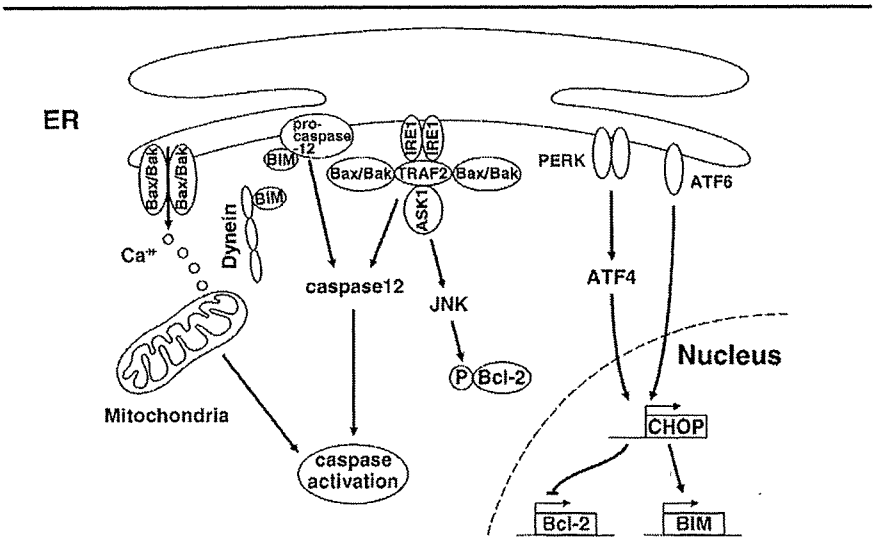

Fig. 2. ER-initiated apoptosis. IRE1a interacts with the adaptor protein TNF receptor-associated factor (TRAF) 2. IRE1α and TRAF2 the interact with ASK1, which subsequently phosphorylates JNK. Activation of JNK induces apoptotic cell death though the phosphorylation of several proteins from the Bcl-2 family. C/EBP homologous protein (CHOP) is a pro-apoptotic bZIP transcriptional factor that is mainly regulated by ATF4- and ATF6-dependent pathways. CHOP represses the expression of anti-apoptotic protein Bcl-2. In addition, CHOP mediates direct transcriptional induction and translocation to the ER membrane of BIM, a pro-apoptotic BH3-only protein from the Bcl-2 family, in response to ER stress. During ER stress, Bak and Bax on the ER membrane undergo oligomerization to release calcium into the cytosol, which also activates both mitochondria-dependent and -independent caspases. Also, the BH3-only protein BIM translocates from the dynein-rich compartment to the ER membrane and activates caspase-12. Bak/Bax also influence UPR by stabilizing the active form of ER stress senor IRE1. Caspase-12 is possibly regulated by BIM or by IRE1-TRAF2-dependent pathways, but the precise mechanism remains under investigation.

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oligomerize to release calcium into the cytosol, which promotes both mitochondria-dependent and -independent caspase activation [16]. NIX (another BH3-only protein) is localized on the ER and mitochondrial membranes and it induces apoptotic cell death by promoting calcium overload in the ER and by increasing mitochondrial outer membrane permeability, probably in coordination with Bak and Bax [17]. In Bak−/− and Bax−/− cells, the IRE1-dependent UPR activation is impaired, suggesting that Bak/Bax influence the UPR to stabilize the active form of ER stress senor IRE1 [17]. The role of Bcl-2 family members from the ER membrane in cardiovascular disease is currently under intense investigation.

Processing of caspase-12 has been reported during ER stress [1,2]. ER stress causes pro-caspase 12 to be cleaved and activated, which in turn activates caspase-9 and caspase-3 thereby leading to mitochondria-independent cell death. Although caspase-12 is possibly regulated by calpain or by IRE1-TRAF2-dependent pathways, the precise mechanism involved remains under investigation [18,19]. Since most humans lack caspase-12, it has been proposed that caspase-4 mediates ER stress-induced apoptosis in human cells [20].

6. ER stress and cardiovascular disease

Recently, the UPR and/or ER-initiated apoptosis have been implicated in the pathophysiology of various human diseases, including cardiovascular diseases such as cardiac hypertrophy, heart failure, atherosclerosis, and ischemic heart disease (Table 1).

7. Cardiac hypertrophy and heart failure

One histological finding characteristic of failing hearts is morphological development of the ER, suggesting that ER overload occurs in this condition [21]. Oxidative stress, hypoxia, and enhanced protein synthesis in failing hearts could all potentially enhance ER stress. In patients with heart failure, we and others have shown a marked increase of GRP78 expression, suggesting that UPR activation is associated with the pathophysiology of heart failure in humans [22,23]. In mice, we have examined the changes of UPR activation and ER-initiated apoptosis signaling after transverse aortic constriction (TAC) [22]. In this model, the mice developed cardiac hypertrophy and failure at 1 and 4 weeks after TAC, respectively. Interestingly, activation of the UPR was found in both hypertrophic and failing hearts, while activation of CHOP related to ER-initiated apoptosis, but not JNK or caspase-12, was only found in failing hearts. These data suggest that UPR activation is persistent in hearts subjected to pressure overload. When the ER stress is excessively prolonged, however, ER-initiated apoptotic signaling (CHOP activation) occurred in mice failing hearts due to pressure overload. These findings suggest that the UPR and ER-initiated apoptosis co-exist in failing hearts and that the CHOP-dependent cell death pathway may be involved in the transition from cardiac hypertrophy to heart failure. We are now investigating the causal relationship between CHOP and the development of heart failure and are attempting to identify the downstream signaling pathway of CHOP, including Bcl-2 family proteins, in this model.

The Lys-Asp-Glu-Leu (KDEL) receptor, a retrieval receptor for ER chaperones in the early secretory pathway, is involved in protein quality control by the ER. Hamada et al. established transgenic mice with systemic expression of mutant KDEL [24]. They observed ubiquitinated protein aggregates, enhanced expression of CHOP, and apoptosis in the hearts of these mice. The transgenic mice developed dilated cardiomyopathy without obvious changes in other tissues, suggesting that the heart is very sensitive to ER stress. Recent studies have also demonstrated the UPR and activation of ER-initiated apoptotic signaling in models of autoimmune cardiomyopathy [25] and alcoholic cardiomyopathy [26].

Heart failure is associated with abnormal calcium handling [27]. Recently, the expression of sarco/endoplasmic reticulum calcium-ATPase isoform 3f (SERCA3f) was up-regulated in human failing hearts [23]. Interestingly, the overexpression of SERCA3f induces the increase in the XBP1 splicing and GRP78 expression in cardiomyocytes, suggesting that SERCA3f would be involved in the ER stress in failing human hearts. Further investigation will be required to clarify whether compartmented regulation of calcium would affect the ER stress-related signaling.

Recently, ASK1 is reported to be essential for the ER stress-induced cell death [9,28]. Since ASK1 knockout mice showed less cardiac dysfunction and cardiac apoptosis cell death after TAC in which ER stress is markedly induced [22,29], it is interesting to investigate whether activation of ASK1 would be involved in the development of heart failure.

8. Potential cardiotoxicity of new anticancer therapy

Tyrosine kinase inhibitors have had a great impact on the treatment of some cancers. Interestingly, Kerkela et al. demonstrated that imatinib (a tyrosine kinase inhibitor which inhibits the causative fusion protein Bcr-Abl in chronic myeloid leukemia) induced left ventricular dysfunction in animals and in some patients [30]. Using cultured cardiomyocytes, they showed that imatinib could cause cell death via the induction of ER stress and subsequent activation of JNK. Although further clinical observation is required, this study is the first to show that ER-initiated apoptotic signaling could possibly be involved in the cardiotoxicity of anticancer therapy [31]. Recently, sunitinib, another tyrosine kinase inhibitor, has been shown to activate IRE1 to cleave mRNA of XBP1 directly by promoting its oligomerization [32]. Since sunitinib is closely associated with the heart failure, its effect on IRE1 might contribute to the development of heart failure [33].

Another important new molecular-targeting anticancer agent is a proteasome inhibitor used for the treatment of multiple myeloma [34]. Since activation of the proteasome system can reduce ER stress via degradation of unfolded proteins, its inhibition may cause the accumulation of unfolded proteins and lead to ER stress in various organs including the heart. Indeed, proteasome inhibitor therapy has been reported to be associated with a high prevalence of heart failure [34]. Consistent with this clinical report, we previously found that proteasome inhibition induced cardiomyocyte death and activated ER stress-induced transcriptional factor ATF6, but not XBP1, leading to failure to achieve the up-regulation of ER chaperones [35]. ER-initiated apoptotic signals (including CHOP, JNK, and caspase-12) were activated by proteasome inhibition, while a short interfering RNA targeting CHOP, but not pharmacological blockade of caspase-12 or the JNK pathway, attenuated cardiomyocyte death. In addition, the overexpression of GRP78 suppressed both CHOP expression and cardiomyocyte death produced by proteasome inhibition. These findings indicate that proteasome inhibition disturbs ER homeostasis in cardiomyocytes without the concomitant induction of ER chaperones, which induces a vicious cycle of cardiac damage. A chemical ER chaperone or a drug that enhances endogenous ER chaperone activity in the heart could be promising candidates to prevent the cardiotoxicity of proteasome inhibitor therapy [36].

9. Atherosclerosis

A growing body of evidence indicates that ER stress plays a crucial role in both atherosclerosis and plaque rupture. Macrophages and smooth muscle cells are key players in the development of atherosclerosis. Interestingly, the macrophages and smooth muscle cells in atherosclerotic plaques produce high levels of secretory proteins, which causes ER stress in these cells [37]. Furthermore, advanced atherosclerotic plaques have a pathophysiological environment that causes ER stress and activates the UPR due to the presence of oxidized lipids, inflammation, and metabolic stress [37]. Oxidized

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lipids have been shown to induce ER stress and apoptosis in human aortic smooth muscle cells [38]. We have also demonstrated that oxidized lipids cause the death of macrophages via a CHOP-dependent pathway, with this process being inhibited by antioxidants [39]. In addition, Feng et al. demonstrated that the UPR is activated in cholesterol-fed macrophages, resulting in increased CHOP expression [40]. These potential causes of ER stress in atherosclerotic plaques could lead to the death of macrophages and smooth muscle cells, which would contribute to plaque instability.

Indeed, XBP1 was highly expressed at branch points and areas of atherosclerotic lesions in the arteries of ApoE knockout mice [41]. Zhou et al. demonstrated that markers of the UPR and ER-initiated apoptosis are greatly increased in macrophages from both early and advanced atherosclerotic lesions in a murine model of atherosclerosis [42]. They concluded that the development of atherosclerotic lesions is likely to be associated with both the UPR and ER-initiated apoptosis [42]. Acute coronary syndrome is primarily related to rupture of an unstable atherosclerotic plaque that leads to thrombus formation and occlusion. We previously investigated the association between ER stress and plaque rupture in 152 human coronary artery autopsy samples. In the cap region, there was a strong association between the expression of ER stress markers such as CHOP and GRP78 by macrophages and rupture of the atherosclerotic plaque [39], suggesting that ER stress is probably involved in the occurrence of plaque rupture. Recently, Thorp et al. provided direct evidence of a causal link between an ER-initiated apoptotic signal (CHOP) and plaque necrosis, showing that both plaque necrosis and cellular apoptosis were markedly reduced in CHOP-deficient mice mated with ApoE or Ldlr knockout atherosclerotic mice [43]. One possible hypothesis is that the ER has differing effects on atherogenesis in relation to lesion stage. At an early stage, the UPR may protect smooth muscle cells and macrophages against death related to ER stress caused by the enhanced synthesis of collagen and cytokines. However, when ER stress is prolonged or severe, it causes cell death and this makes plaques more vulnerable. In patients with advanced disease, CHOP is a possible therapeutic target for preventing plaque progression and rupture.

10. Ischemic heart disease

In ischemic/reperfused (I/R) myocardium, oxygen and energy substrates are depleted, followed by a sudden increase of oxygen free radicals, and either of these stimuli can potentially induce the UPR and/or ER-initiated apoptotic signaling. Indeed, increased expression of UPR-related genes has been reported in cardiomyocytes from near the site of myocardial infarction in mice and humans [44,45].

In cultured neonatal rat cardiomyocytes, hypoxia induces UPR activation as evidenced by an increase of XBP1 mRNA splicing and GRP78 protein [44]. After these cardiomyocytes were infected with a recombinant adenovirus encoding dominant-negative XBP1, hypoxia/reoxygenation-induced apoptosis showed an increase, suggesting that the XBP1 arm of the UPR may have a cardioprotective role against hypoxic insults. Vitadello et al. demonstrated that the overexpression of GRP94, the expression of which is regulated by XBP1 and ATF6, reduces necrosis due to calcium overload or simulated ischemia in H9C2 cardiac myocytes [46]. To investigate the role of ATF6 (one arm of the UPR) in I/R injury, Martindale et al. generated transgenic (TG) mice featuring cardiac-restricted expression of a novel tamoxifen-activated form of ATF6 (ATF6-MER) and examined whether ATF6 protects the myocardium from I/R injury [47]. The hearts of tamoxifen-treated TG mice showed increased expression of ER-resident chaperons GRP78 and GRP94 and also exhibited better functional recovery after ex vivo I/R, as well as displaying significantly less necrosis and apoptosis. They concluded that the UPR is activated in the heart during I/R and that the ATF6 arm of the UPR may consequently induce the expression of proteins which reduce I/R injury. They recently showed that mesencephalic astrocyte-derived neurotrophic factor produced in ATF6-dependent manners would modulate myocardial damage during ischemia [48]. In addition, it has been reported that ischemic preconditioning or postconditioning reduces cardiac damage associated with UPR activation [49,50]. In human heart samples, Severino et al. demonstrated that protein disulfide isomerase (PDI) is 3-fold up-regulated in the viable peri-infarcted myocardium [45]. Adenoviral-mediated transfer of the PDI gene resulted in a 2.5-fold smaller infarct size, significantly reduced cardiomyocyte apoptosis in the peri-infarct region, and a smaller left ventricular end-diastolic diameter versus mice treated with a transgene-null adenoviral vector. These findings suggest that the UPR is induced in I/R hearts and that activation of the UPR, including the increased expression of ER-resident chaperones and PDI, plays a protective role against I/R injury.

On the other hand, Terai et al. demonstrated that hypoxia induced ER stress and found that hypoxia-induced CHOP expression and cleavage of caspase 12 were significantly inhibited by pretreatment with a pharmacological activator of AMP-activated protein kinase [51]. This indicates that ER-initiated apoptotic signaling is involved in cell death after a hypoxic insult. In addition, Nickson et al. demonstrated that ER stress induces the expression of PUMA, a pro-apoptotic member of the Bcl-2 family, and that the suppressing PUMA expression leads to inhibition of cardiomyocyte apoptosis induced by a pharmacological ER stressor [52]. Importantly, targeted deletion of PUMA reduces cardiomyocyte death and improves cardiac function during I/R [53]. These findings suggest that ER stress-initiated apoptotic signaling mediates cell death in I/R myocardium. Thus, the important question is raised as to whether ER stress ameliorates or exacerbates I/R injury? Since ER stress sensor proteins can induce both the UPR and ER-initiated apoptotic signaling, cell survival or death is likely to depend on how much and how long cardiomycoytes are exposed to various stimuli induced by I/R.

11. Concluding remarks

Although our understanding of the pathophysiological role of ER stress in cardiovascular disease has progressed in recent years, many interesting and important issues are still unresolved. The fundamental question remains as to how the cell decides between life and death after the onset of ER stress. We also need to know how ER stress is involved in calcium release during excitation–contraction coupling as well as apoptosis in cardiomyocytes. Recent studies have shown that a chemical ER chaperone can reduce ER stress in some diseases [54]. Since the ER is an organelle that has an essential role in multiple functions of both normal and diseased cells, a tissue- or cell-specific drug delivery system must be developed [55]. Improved understanding of the molecular mechanisms underlying UPR activation and ER-initiated apoptosis in cardiovascular disease will provide us with new targets for drug discovery and therapeutic intervention.

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Carperitide and Adiponectin
—— How Are They Connected Each Other to Benefit Acute Decompensated Heart Failure? ——

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ANP and Heart Failure

The heart has been thought to be merely a pump to collect and circulate blood to and from the heart. However, this is not the case as several investigators have noticed the existence of granules in atrial muscle cells, suggesting that the atrial muscle may produce certain substances. After many years, Matsuo and Kangawa discovered that one of the substances is human atrial natriuretic peptide (ANP, carperitide). This observation opened a new era for cardiology because we noticed that the heart is the secretary organ that controls hemodynamics.

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The main cardiovascular effects of carperitide are vasodilation, natriuretic action and reduction of activity of the sympathetic nervous system and the rennin–angiotensin–aldosterone system. It has been reported that an infusion of carperitide elevates cGMP levels and improves the long-term prognosis of patients with acute decompensated chronic heart failure (CHF).[1] After such devoted works for carperitide, it has been used clinically for the treatment of acute heart failure, including acute decompensated CHF.

Heart Failure and Metabolic Disorder

CHF is primarily characterized by impaired cardiac performance; however, recent accumulated evidence strongly indicates that neurohormonal imbalance, inflammation and metabolic abnormalities contribute to high mortality. Intriguingly, increases in plasma catecholamine and angiotensin II levels are thought to play important roles in the pathophysiology of CHF, and they both culminate in abnormal glucose tolerance. Either transient high glucose exposure or decreased insulin sensitivity, which are known to be major cardiovascular risk factors, can result in cellular injury via the generation of oxidative stress and provocation of myocardial apoptosis.

In contrast, plasma adiponectin levels are reported to be negatively correlated to insulin resistance. Adiponectin is one of the circulating adipocytokines, and it plays an important role in energy homeostasis, regulating insulin sensitivity, lipid metabolism and exerts anti-inflammatory properties. Adiponectin knock-out mice develop severe cardiac hypertrophy and exhibit increased mortality subjected to pressure overload caused by transverse aortic constriction. Conversely, adenovirus-mediated overexpression of adiponectin attenuates cardiac hypertrophy following pressure overload in adiponectin knock-out mice.[2] Therefore, adiponectin and carperitide are also thought to contribute to the pathophysiology of CHF.

ANP and Adiponectin in Patients With CHF

The authors of previous studies have found that carperitide infusion increases plasma adiponectin levels in patients with acute decompensated heart failure (ADHF).[3] However, the effect of carperitide on plasma adiponectin levels in patients with diabetes mellitus (DM) remains unknown.

In this issue of the journal, Yamaji et al evaluated the effect of carperitide on plasma adiponectin levels in ADHF patients with or without DM.[4] They clearly demonstrated that plasma adiponectin levels significantly increased with an increase in ANP and a decrease in BNP 7 days after carperitide infusion. Furthermore, they demonstrated that the adiponectin levels before the proper treatment were slightly lower in ADHF patients with DM, and that the percentage increase in adiponectin levels was significantly greater in ADHF patients with DM than in those without DM. They also demonstrated that both higher plasma aldosterone levels and prevalence of DM were significant independent predictors of a greater percentage increase in adiponectin levels after treatment with carperitide.

Several studies have suggested a positive correlation between plasma levels of ANP and/or BNP and adiponectin in patients with CHF. Therefore, high levels of plasma adiponectin are associated with increased mortality and severity in patients with CHF.

How are both carperitide and adiponectin connected to each other? It has been proposed that cardiac natriuretic peptides have a novel lipolytic and potential lipid-mobilization effect that is mediated by a GC-A receptor.[5,6] Tsukamoto et al demonstrated that normal (10^{-11} mol/L), pathophysiological (10^{-10} mol/L) and pharmacological (10^{-9} mol/L) concentrations of ANP enhanced adiponectin mRNA expression and increased adiponectin secretion via the GC-A/cGMP/PKG-dependent pathway by primary cultured human adipocytes.[7] This indicates that carperitide can affect adipose tissues and increase adiponectin production; however, it is not the case vice versa (Dr Tsukamoto, personal communication).

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How Does Adiponectin Exert the Cardioprotective Effect Under Stress Conditions?

Shibata et al demonstrated that adiponectin activates AMP-activated protein kinase (AMPK), and inhibits the hypertrophic response to α-adrenergic receptor stimulation.[2] Furthermore, AMPK is activated by ischemia–reperfusion, as well as in hearts with pressure overload hypertrophy and subsequent heart failure. AMPK is expressed in various tissues, including the myocardium, and plays a central role in the regulation of energy metabolism under stress conditions.[8]

Interestingly, Eurich et al reported the results of a meta-analysis showing that metformin was the only antidiabetic agent to reduce all-cause mortality without causing any harm in patients who had heart failure and DM.[9] Metformin is known to activate AMPK similar to adiponectin.[10] Recently, Sasaki et al demonstrated that long-term oral administration of metformin decreases apoptosis, inhibits cardiac remodeling and prevents the progression of heart failure in a rapid pacing-induced heart failure dog model, which is considered to be similar to human dilated cardiomyopathy, along with increases in AMPK activation.[11] Furthermore, AICAR, another AMPK activator, had effects almost equivalent to those of metformin, suggesting that AMPK activation plays a primary role in reducing apoptosis and preventing heart failure.

What Mechanisms Following AMPK Activation Are Involved in Cardioprotection?

The first possibility is enhancement of nitric oxide (NO) production. CHF is characterized by impaired cardiac performance, neurohormonal imbalance, inflammation and metabolic abnormalities including abnormal glucose tolerance, which is accompanied by an excess of oxidative stress. The excess of oxidative stress causes the impairments of endothelial cells attached to cardiomyocytes, and the endothelial dysfunction may be involved in the deterioration of CHF. AMPK is known to phosphorylates eNOS, resulting in an increase in NO production, and thus inhibits inflammatory cytokine-induced expression of cell adhesion molecules, and suppresses oxidative stress.

The second possibility is related to the improvement of insulin resistance and metabolic abnormalities. Under normal conditions, the adult heart utilizes predominantly fatty acids to derive the majority of its energy. However, metabolic remodeling such as a marked shift in substrate preference away from fatty acids toward glucose is observed in hypertrophic and failing hearts, and the decrease in fatty acid oxidation is not fully compensated for by an increase in glucose oxidation. Yamauchi et al demonstrated that adiponectin stimulates both glucose metabolism and utilization and fatty-acid oxidation via the AMPK signaling pathway.[12]

The third possibility is the antifibrotic effect. Several studies have indicated that AMPK activation inhibits protein synthesis through effects on both the eEF-2 and mTOR pathways. Furthermore, metformin attenuated fibrosis and reduced the TGF-β1 mRNA level.[11]

How Important Are Plasma Adiponectin Levels in ADHF Patients?

Heart failure itself is an insulin-resistant state, and it is reported that plasma adiponectin levels are negatively correlated with insulin resistance. Although the precise mechanisms are unknown, a decrease in plasma adiponectin levels will worsen the cardiac function in patients with heart failure and DM, and this finding indicates that treatment with carperitide may be useful for ADHF, especially in patients with DM. Further studies will be needed to examine this point.

Recently, adjunctive, acute-phase treatment with carperitide ($0.025\mu g \cdot kg^{-1} \cdot min^{-1}$ for 3 days) after reperfusion therapy in patients with acute myocardial infarction (AMI) reduced the infarct size by 14.7%, increased the left ventricular ejection fraction during the chronic phase, and decreased the incidence of cardiac death and admission to hospital because of heart failure.[13] Kojima et al reported that plasma adiponectin levels in patients with AMI decreased significantly at 24 h and 72 h compared with the levels on admission. The plasma adiponectin levels almost returned to the levels on admission on Day 7 after the onset of AMI. The reduction of plasma adiponectin levels during the course of AMI were significantly correlated to the plasma C-reactive protein levels.[14] These results suggest that the decrease in the plasma adiponectin levels contribute to myocardial damage, resulting in decreased left ventricular function. Therefore, administration of carperitide may decrease the infarct size via adiponectin in patients with AMI. Drugs that increase plasma adiponectin levels, such as carperitide, or activate AMPK, such as metformin, may provide a novel strategy for the treatment of ADHF, including AMI in clinical settings.

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