Endoplasmic Reticulum Stress Participates in Aortic Valve Calcification in Hypercholesterolemic Animals

Zhejun Cai,* Fei Li,* Wei Gong,* Wanjun Liu, Quanlu Duan, Chen Chen, Li Ni, Yong Xia, Katherine Cianflone, Nianguo Dong, Dao Wen Wang

Objectives—Aortic valve (AV) calcification occurs via a pathophysiological process that includes lipoprotein deposition, inflammation, and osteoblastic differentiation of valvular interstitial cells. Here, we investigated the association between endoplasmic reticulum (ER) stress and AV calcification.

Approach and Results—We identified ER stress activation in AV of patients with calcified AV stenosis. We generated an AV calcification model in hypercholesterolemic rabbits and mice, respectively, and found marked AV ER stress induction. Classical ER stress inhibitor, tauroursodeoxycholic acid, administration markedly prevented AV calcification, and attenuated AV osteoblastic differentiation and inflammation in both rabbit and mouse models of AV calcification via inhibition of ER stress. In cultured valvular interstitial cells (VICs), we found that oxidized low density lipoprotein (oxLDL) caused ER stress in a cytosolic [Ca]21-dependent manner. OxLDL promoted osteoblastic differentiation via ER stress–mediated protein kinase-like ER kinase/activating transcription factor 4/osteocalcin and inositol-requiring transmembrane kinase and endonuclease-1α (IRE1α)/spliced X-box–binding protein 1/Runx2 pathway, and induced inflammatory responses through IRE1α/c-Jun N-terminal kinase and IRE1α/nuclear factor kappa-light-chain-enhancer of activated B cells signaling in VICs. Inhibition of ER stress by either tauroursodeoxycholic acid or 4-phenyl butyric acid could both suppress oxLDL–induced osteoblastic differentiation and inflammatory responses in VICs.

Conclusions—These data provide novel evidence that ER stress participates in AV calcification development, and suggest that ER stress may be a novel target for AV calcification prevention and treatment. (Arterioscler Thromb Vasc Biol. 2013;33:2345–2354.)

Key Words: aortic valve, calcification of endoplasmic reticulum stress inflammation osteogenesis oxidized low density lipoprotein tauroursodeoxycholic acid

Aortic valve (AV) calcification and its subsequent calcified AV stenosis (CAVS) are common clinical problems. Approximately 25% of adults >65 years old have some degree of AV sclerosis (defined as valvular thickening or calcification without significant obstruction),1 and CAVS is present in >4% of very elderly patients,1 which has become the most common indication for valve replacement and the second most common indication for cardiac surgery in the United States and Europe.2,3 Although the disease is associated with increased cardiovascular events and mortality, there currently is no effective therapy other than surgical or interventional AV replacement.4 In the past decade, increasing evidence suggests that AV calcification is not simply a passive degenerative process, but an active disease progress similar with atherosclerosis, including lipoprotein deposition, chronic inflammation, and osteoblastic differentiation of valvular interstitial cells (VICs).5,6 The endoplasmic reticulum (ER) is a crucial luminal network for protein synthesis, folding, and transportation. Conditions disrupting the ER homeostasis cause accumulation of unfolded and misfolded proteins in the ER lumen, create a state defined as ER stress, and activate a complex signaling network termed the unfolded protein response, which consists of 3 branches, protein kinase-like ER kinase (PERK), inositol-requiring transmembrane kinase and endonuclease-1α (IRE1α), and activating transcription factor 6 (ATF6).6 So far, numerous studies have shown that ER stress is linked with a variety of pathophysiologic conditions, such as diabetes mellitus, obesity, cancer, atherosclerosis, myocardial injuries, and inflammatory conditions.7–10 However, whether ER...
stress is also involved in the pathogenesis of AV calcification remains unknown.

Histological data demonstrate the presence of oxidized low-density lipoprotein (oxLDL) in calcified AV, indicating its pathophysiological role in the development of AV calcification. Furthermore, several groups have recently demonstrated that oxLDL could trigger ER stress in endothelial cells, thus playing an important role in the development of atherosclerosis. These studies suggest that ER stress may be induced during AV calcification.

Recent studies reported that ER stress-mediated activation of activating transcription factor 4 (ATF4) and spliced X-box–binding protein 1 (XBP1s) are transcriptional activators that control the important osteoblastic differentiation factors osteocalcin and Runx2. Moreover, an emerging function of ER control the important osteoblastic differentiation factors osteo-

Activation of ER Stress in Calcified AV of Hypercholesterolemic Animals

We examined whether ER stress was induced in AV leaflets of AV calcification animals. Results showed that both HC+vitD2 treatment in rabbits and HC treatment in ApoE−/− mice markedly increased the AV area (AVA) and AVA indexed by body surface area in HC+vitD2-treated rabbits decreased markedly compared with the normal controls (Table 1). We also observed a significant increase in transvalvular peak jet velocity and a marked decrease in AVA and AVA index after 24 weeks of HC diet treatment in ApoE−/− mice (Table 1).

We next assessed the degree of calcification. Alizarin red staining revealed increased calcium deposit in HC+vitD2 rabbits compared with normal controls (Figure 2A and 2B). The transition from VICs to myofibroblasts is also a critical change in valvular pathology. We examined myofibroblasts in AV by immunohistochemical staining using α-smooth muscle actin (α-SMA) antibody. AV in control animals exhibited sparse α-SMA positive myofibroblasts, whereas HC+vitD2 treatment markedly increased the α-SMA positive staining area (Figure III in the online-only Data Supplement). Similarly, we also observed significant calcium deposits in AV of HC diet–treated ApoE−/− mice by alizarin red staining (Figure 2A and 2B). The expression of α-SMA was markedly increased in HC-treated ApoE−/− mice (Figure IV in the online-only Data Supplement).

High-Cholesterol Diet Induced Significant AV Calcification in Animals

We used 2 different classical animal models of AV calcification, rabbits and mice, which are similar to human clinical condition. After 12 weeks of high-cholesterol (HC)+vitamin D3 (vitD3) diet, increased echogenicity occurred in AV of HC+vitD3-treated rabbits (Figure II in the online-only Data Supplement). AV area (AVA) and AVA indexed by body surface area in HC+vitD3-treated rabbits decreased markedly compared with the normal controls (Table 1). We also observed a significant increase in transvalvular peak jet velocity and a marked decrease in AVA and AVA index after 24 weeks of HC diet treatment in ApoE−/− mice (Table 1).

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

ER Stress Is Activated in AV Leaflets of Patients With CAVS

To determine whether ER stress is induced in patients with AV calcification, we used AV tissues from 10 patients with CAVS, as well as 4 traffic accident victims. In parallel with the increased expression of Runx2 and osteocalcin, we found marked activation of ER stress in calcified AV compared with the control subjects as assessed by PERK and IRE1α phosphorylation, and C/EBP homologous protein (CHOP) expression (Figure 1).

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KDEL and CHOP (Figure 3A). Moreover, immunoblotting showed that important ER stress markers, such as p-PERK and p-IRE1α, and CHOP expression were significantly induced in AV of HC+vitD2-treated rabbits compared with controls (Figure VA and VB in the online-only Data Supplement).

**Prevention of ER Stress by TUDCA Attenuated Diet-Induced AV Calcification in Animals**

TUDCA is a chemical chaperone that has been well demonstrated to be a classical inhibitor against ER stress by improving ER folding capacity both in vitro and in vivo.25–27 We examined whether relieving ER stress by TUDCA could rescue AV calcification. We treated HC+vitD2-fed rabbits with TUDCA (50 mg/kg per day) by oral gavage. In parallel with the attenuated ER stress in HC+vitD2+TUDCA group (Figure 3A), we found TUDCA supplementation also reduced AV echogenicity (Figure II in the online-only Data Supplement) and restored AVA index compared with HC+vitD2 group (Table 1). Furthermore, TUDCA significantly suppressed calcium deposit (Figure 2A and 2B) and α-SMA staining (Figure III in the online-only Data Supplement) induced by HC+vitD2 administration.

We also evaluated whether TUDCA administration could prevent HC diet–induced AV calcification in ApoE−/− mice. Twenty-four consecutive weeks of TUDCA (0.5 g/kg per day) oral gavage significantly reduced levels of AV ER stress markers KDEL and CHOP staining (Figure 3B), prevented transvalvular peak jet velocity increases, restored AVA index (Table 1), and reduced calcium deposits (Figure 2A and 2C) and myofibroblast activation (Figure IV in the online-only Data Supplement) induced by HC diet.

**TUDCA Administration Prevented HC Treatment–Induced Osteoblastic Differentiation in AV Leaflets**

We determined whether ER stress links AV osteoblastic differentiation in AV calcification animals. As shown in Figures IV and V in the online-only Data Supplement, HC+vitD2 diet markedly increased Runx2 staining as well as protein and mRNA expression of important osteoblastic differentiation markers, osterix, Runx2, and osteocalcin, in AV leaflets in rabbits. Consistent with the results in AV calcification rabbits, AV staining of osterix, Runx2, and osteocalcin were all markedly increased after 24 weeks of HC diet treatment in ApoE−/− mice (Figure 4A–4D). As expected, ER stress inhibitor TUDCA

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<tr>
<th>Table 1. Hemodynamic Parameters of Animals After Different Interventions</th>
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<tr>
<td>Rabbits</td>
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<tr>
<td>Control</td>
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<tr>
<td>n=6</td>
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<tr>
<td>Transvalvular peak jet velocity, cm/s</td>
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<tr>
<td>AVA, mm²</td>
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<td>AVA index, AU</td>
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Data are presented as mean±SD. AVA indicates aortic valve area; HC, high cholesterol; TUDCA, tauroursodeoxycholic acid; and VitD2, vitamin D2.

*P<0.05 vs control.

**P<0.01 vs control.

#P<0.05 vs HC+vitD2 in rabbits.

##P<0.01 vs HC in ApoE−/− mice.
administration significantly inhibited these effects in both rabbit and mouse models of AV calcification (Figure 4A–4D; Figures V and VI in the online-only Data Supplement).

**TUDCA Protected Against Leaflet Inflammation in Diet-Induced AV Calcification Animals**

Direct involvement of the ER stress–mediated pathway in the inflammatory response has been described in some reports, but not in a model of AV calcification. We evaluated the effect of in vivo TUDCA administration on the level of inflammation in AV leaflets. Results showed that hypercholesterolemic animal models of AV calcification markedly increased macrophage infiltration in AV leaflets as indicated by macrophage markers RAM11 and F4/80, respectively (Figure 4A and 4D; Figure VII in the online-only Data Supplement). However, TUDCA treatment markedly attenuated AV macrophage infiltration in hypercholesterolemic animals (Figure 4A and 4D; Figure VII in the online-only Data Supplement).

**Effects of TUDCA Are Independent of Serum Lipid or Blood Glucose Levels**

We compared the metabolic parameters among groups and found that there were no differences observed in levels of plasma glucose, total cholesterol, LDL, and triglycerides...
among groups at baseline (Table II in the online-only Data Supplement). Significant increases in serum cholesterol and LDL levels were observed in both animal models of AV calcification after the indicated diet treatments, whereas TUDCA treatment did not alter either the lipid profiles or glucose levels compared with the HC+vitD2 or HC diet–fed animals (Table 2).

**OxLDL Induced ER Stress in VICs in a Cytosolic [Ca]2+–Dependent Manner**

OxLDL presents in calcified AV,11 and therefore we investigated whether oxLDL could trigger ER stress in cultured VICs. Results showed that oxLDL induced marked ER stress in VICs in a dose-dependent manner as evaluated by expression of ER stress markers (Figure VIIIA and VIIIB in the online-only Data Supplement). It has been reported that oxLDL could increase cytosolic [Ca]2+ in various cell types.29,30 We found incubation of oxLDL led to a significant increase in cytosolic [Ca]2+ (Figure VIIIC in the online-only Data Supplement). Moreover, as with oxLDL, incubation of VICs with a calcium ionophore A23187 markedly enhanced ER stress, and, by contrast, pretreatment with calcium chelator BAPTA-AM effectively protected VICs from ER stress induced by both oxLDL and A23187 (Figure VIIID and VIIE in the online-only Data Supplement).

**ER Stress Promoted OxLDL-Induced Osteoblastic Differentiation Via PERK/ATF4/Osteocalcin and IRE1α/XBP1s/Runx2 Signaling in VICs**

It has been reported that the important osteoblastic factors osteocalcin and Runx2 are the downstream targets of ATF4 and XBP1s, respectively, which are activated during ER stress.14–16 We therefore examined whether oxLDLs trigger osteoblastic differentiation in VICs via ER stress activation. As Figure 5A and 5B depicted, small interfering RNA silencing of PERK (Figure 5A in the online-only Data Supplement), or its downstream target ATF4 (Figure 5B in the online-only Data Supplement), significantly suppressed the expression of osteocalcin induced by oxLDLs. Moreover, silencing of IRE1α (Figure 5C in the online-only Data Supplement), or its downstream target XBP1 (Figure 5D in the online-only Data Supplement), could markedly inhibit oxLDL-induced expression of Runx2 (Figure 5E and 5F).

**Inhibition of ER Stress Reduced OxLDL-Induced Osteoblastic Differentiation and Inflammation in VICs**

We further investigated whether inhibition of ER stress would protect VICs from oxLDL-induced osteoblastic differentiation and inflammation. We found that TUDCA and 4-phenyl butyric acid (4-PBA), 2 classical ER stress inhibitors with distinct chemical structures, markedly attenuated ER stress triggered by oxLDL in VICs (Figure 6A and 6B; Figure XII in the online-only Data Supplement). We assayed the effects of TUDCA and 4-PBA on JNK and NF-κB–mediated proinflammatory roles of JNK and NF-κB in VICs. As expected, IRE1α inhibition also reduced oxLDL-induced nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα) degradation and NF-κB p65 nuclear translocation (Figure 5F and 5G).

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**ER Stress Is Involved in OxLDL-Activated Inflammation Through IRE1α-Mediated JNK and NF-κB Signaling in VICs**

We further explored whether oxLDL could lead to inflammation via the ER stress–mediated IRE1α pathway.31 Results showed that small interfering RNA silencing of IRE1α markedly reduced JNK phosphorylation triggered by oxLDL (Figure 5E). Meanwhile, as expected, IRE1α silencing also reduced oxLDL-induced nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα) degradation and NF-κB p65 nuclear translocation (Figure 5F and 5G).

Furthermore, IRE1α inhibition was associated with reduced expression of inflammatory cytokines induced by oxLDL in VICs, including interleukin (IL)-6, IL-8, and monocyte chemotactic protein-1 (MCP-1) (Figure X in the online-only Data Supplement). The proinflammatory roles of JNK and NF-κB in VICs were supported by the effect of the JNK inhibitor SP600125 and NF-κB inhibitor BAY 11-7082, which, in part, inhibited the oxLDL-induced expression of IL-6, IL-8, and MCP-1 (Figure XIA–XIC in the online-only Data Supplement). Moreover, BAY 11-7082 incubation also significantly suppressed osteocalcin and Runx2 expression induced by oxLDL (Figure XID in the online-only Data Supplement), suggesting a role of NF-κB signaling in osteoblastic differentiation in VICs.

### Table 2. Serum Lipid Profiles and Glucose Levels in Animals After Different Intervention

<table>
<thead>
<tr>
<th></th>
<th>Rabbits</th>
<th>ApoE−/− Mice</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HC</td>
</tr>
<tr>
<td></td>
<td>n=6</td>
<td>n=7</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.61±0.89</td>
<td>5.73±0.84</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>1.30±0.21</td>
<td>25.17±3.88**</td>
</tr>
<tr>
<td>LDL-c, mmol/L</td>
<td>0.72±0.18</td>
<td>19.43±3.42**</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>0.85±0.23</td>
<td>1.25±0.33</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. HC indicates high cholesterol; LDL-c, LDL cholesterol; TC, total cholesterol; TG, triglyceride; TUDCA, tauroursodeoxycholic acid; and VitD2, vitamin D2.

**P<0.01 vs control.
†Not significant vs HC+vitD2 in rabbits or HC in ApoE−/− mice.
exposure to oxLDL (Figure 6D; Figure XIV A in the online-only Data Supplement) and simultaneously prevented the NF-κB p65 activation induced by oxLDL (Figure 6E and 6F; Figures XIVB and XV in the online-only Data Supplement). Moreover, as depicted in Figure XVI in the online-only Data Supplement, oxLDL significantly increased the expression of IL-6, IL-8, and MCP-1, and the effects could be markedly suppressed by TUDCA and 4-PBA.

Finally, we evaluated whether ER stress inhibition could prevent mineralization of VICs induced by oxLDL. TUDCA and 4-PBA treatments significantly attenuated oxLDL-induced mineralization and alkaline phosphatase.
(ALP) activity in VICS (Figure XVIIA and XVIIIB in the online-only Data Supplement). Consistently, Alizarin staining showed that TUDCA and 4-PBA markedly attenuated VICS mineralization induced by oxLDL (Figure XVIIIC in the online-only Data Supplement).

Discussion
Despite the clinical importance of heart disease and intense study in this field, the mechanisms underlying the development of AV calcification remain unclear. The present study suggests the important role of ER stress in the pathogenesis of AV calcification. We identified significant ER stress induction in calcified AV of patients with CAVS. In our hypercholesterolemia-induced AV calcification animal models, we also found ER stress activation in AV leaflets. Inhibition of ER stress by TUDCA administration was associated with significant benefits on AV calcification and could markedly attenuate AV osteoblastic differentiation and inflammation. Our in vitro experiments subsequently indicated that oxLDL causes ER stress in VICS by increasing cytosolic [Ca]2+. Furthermore, we provide evidence that oxLDL induces osteoblastic differentiation via ER stress–mediated PERK/ATF4/oestocalcin and IRE1α/XBP1s/Ranx2 pathways, and oxLDL promotes inflammatory responses via ER stress–mediated IRE1α/JNK and NF-kB signaling. Inhibition of ER stress by either TUDCA or 4-PBA could suppress osteoblastic differentiation, inflammatory response, and mineralization induced by oxLDL in VICS.

AV Calcification and ER Stress Activation
AV calcification development shares some similarities with atherosclerosis and arterial calcification. It has been widely accepted that ER stress plays an important role in atherosclerotic plaque progression and in vitro data indicate the potential role of ER stress in arterial calcification development. However, whether there is a link between ER stress and AV calcification has not been reported to date. In this present work, we used calcified AV tissue samples from patients with CAVS, as well as 2 classical hypercholesterolemic AV calcification animal models to explore their links.

Lipoprotein deposition, osteoblastic differentiation, and inflammation all markedly contribute to AV calcification and its subsequent CAVS in humans. In this study, we identified upregulation of ER stress markers in patients with significant CAVS. Moreover, we observed that ER stress was markedly induced in AV leaflets in 2 distinct AV calcification models of 2 species, further suggesting that ER stress activation in AV leaflets potentially correlates with AV calcification development.

Although the above data implicate ER stress in induction of calcified AV, how ER stress is initiated is still unknown. To date, oxLDL is associated with proinflammatory and mineralization promoting properties to induce AV calcification. Our current results add another dimension to oxLDL and provide evidence that oxLDL contributes to ER stress in AV calcification. Here, we showed that oxLDL stimulation causes strong ER stress in VICS. As previously documented, increased cytosolic [Ca]2+ hampers ER abilities of protein folding, causes unfolded or misfolded protein accumulation, and could finally lead to ER stress.

We hypothesized that oxLDL stimulated ER stress in VICS via increasing cytosolic [Ca]2+. Indeed, incubation of oxLDL increased cytosolic [Ca]2+ in VICS, which is consistent with recent studies in which oxLDL increases cytosolic [Ca]2+ in smooth muscle cells and endothelium cells. Providing additional support to this hypothesis, we demonstrated that administration of A23187, a calcium ionophore, could also strongly induce ER stress in VICS. Moreover, we found that oxLDL or A23187-induced ER stress could be attenuated by reducing cytosolic [Ca]2+. These findings suggest that oxLDL-induced ER stress in VICS is mediated in a cytosolic [Ca]2+-dependent manner.

ER Stress Links Osteoblastic Differentiation and Inflammation
Activation of osteoblastic differentiation in VICS has been proven to play a central role during AV calcification development. ATF4 and XBP1s are critical transcription factors downstream of PERK and IRE1α signaling branches of ER stress, and they play important roles in the recovery of damaged cells exposed to ER stress. Recent studies indicate that ATF4 and XBP1s mediate not only ER stress responses but also osteoblastic differentiation because they are transcriptional activators of osteocalcin and Runx2, the central regulators of osteoblastic differentiation. Using small interfering RNA silencing strategy, we provided evidence that ER stress–mediated PERK/ATF4 and IRE1α/XBP1s signaling is responsible for osteocalcin and Runx2 expression, and thus directly contributes to oxLDL-induced osteoblastic differentiation in VICS.

Recent studies revealed that chronic inflammation contributes to the development of AV calcification. Accumulating infiltrated inflammatory cells are major sources of matrix metalloproteinases and cysteine endopeptidases, which degrade collagen and elastin in the valvular extracellular matrix, thereby resulting in valvular dysfunction and further activation of osteoblastic differentiation. As a growing body of evidence suggests that ER stress can lead to inflammation, ER stress may also contribute to AV calcification via promotion of inflammatory responses. We explored the role of the IRE1α pathway, shown to be linked with JNK and NF-kB signaling. We found that JNK was activated by oxLDL, in agreement with previous reports, and that silencing IRE1α blocked the phosphorylation of JNK. Furthermore, NF-kB nuclear translocation induced by oxLDL could also be prevented by IRE1α silencing. Both NF-kB and JNK activation induced the expression of the inflammatory genes. Our data further suggest that IRE1α silencing could, in part, inhibit the oxLDL-induced inflammatory genes expression, in agreement with the role of IRE1α/JNK and IRE1α/NF-kB, in inflammation.

The NF-kB pathway regulates expression of a wide variety of genes that are involved in inflammatory responses. Moreover, it has been reported that NF-kB signaling mediates Runx2 and osteocalcin expression, and promotes osteoblastic differentiation. In our experiment, we showed that NF-kB inhibition significantly suppressed oxLDL-stimulated inflammatory responses, and the expression of Runx2 and osteocalcin as well. These results indicate that NF-kB activation mediates oxLDL-induced proinflammatory effects and osteoblastic differentiation in VICS.
Inhibition of ER Stress as a Therapeutic Approach to AV Calcification

Chemical chaperones, such as TUDCA and 4-PBA, enhance the ER folding ability to prevent protein aggregation, and thus have the ability to alleviate ER stress. In this study, we found TUDCA and 4-PBA protected against oxLDL-induced ER stress in VICs. Concomitant with reduced ER stress, these 2 structurally distinct ER stress inhibitors significantly suppressed osteoblastic differentiation and inflammatory responses induced by oxLDL in VICs, and therefore add strength to the proposal that the oxLDL-triggered osteoblastic differentiation and inflammation in VICs is, at least in part, induced via ER stress.

Altogether, the results provide support to the hypothesis that ER stress in AV leaflets is associated with AV calcification development. Furthermore, these implications raise a crucial question: whether reducing AV ER stress could be used as a strategy to alter the progression of AV calcification. The chemical chaperone TUDCA has been proven to be protective in various diseases, such as diabetes mellitus, obesity, and atherosclerosis, via prevention of ER stress. We also found that TUDCA protected against hypercholesterolemia-induced AV calcification in animal models. TUDCA administration significantly reduced leukotriene ER stress activation, prevented osteoblastic differentiation and inflammation, and attenuated AV calcification development. These protective effects were independent of serum lipid or glucose metabolism and were demonstrated to act via inhibition of AV ER stress, further implicating the involvement of ER stress in AV calcification development.

The most striking result in the present study was that TUDCA administration could significantly reduce the development of AV stenosis, which might lead to a novel therapeutic approach. Several clinical trials indicate that ER stress–inhibitor chemical chaperones could improve liver and muscle insulin sensitivity in obese subjects, while ER stress plays an essential role in insulin resistance and diabetes mellitus development. Our study provides evidence that ER stress inhibitors might be protective against AV calcification in humans.

Many other clinical conditions other than hypercholesterolemia may contribute to the development of AV calcification. Studies indicate that chronic kidney disease is closely associated with AV calcification; increased parathyroid hormone and inflammation might contribute to this. Recently, Zhou et al. reported that chronic kidney disease could lead to vascular ER stress activation and cause insulin resistance. Although further studies are required, this may provide additional evidence supporting our findings and suggests that ER stress may also play an important role during chronic kidney disease–induced AV calcification. Patients with bicuspid AV malformation develop AV calcification and stenosis spontaneously at an early age. Local blood turbulence and inflammation may be the major contributor to the development of AV calcification in bicuspid AV malformation, and the role of ER stress in such a condition needs further investigation.

Evidence suggests that AV calcification possesses characteristics of arterial calcification, and both share similar epidemiological risk factors. Inflammation and osteoblastic differentiation play a key role in the development of both these conditions. In the absence of in vivo data, Masuda et al. and Saito et al. reported that stearate and bone morphogenetic protein 2 stimulated osteoblastic differentiation in vascular smooth muscle cells via activating ER stress. This is similar to our study that shows that oxLDL triggers osteoblastic differentiation via ER stress–mediated PERK/ATF4/ostecalcin and IRE1α/XBP1s/Runx2 signaling in VICs. However, arterial calcification and AV calcification development are mediated through differential processes. In AV calcification, VICs should first be activated by various stimuli to become α-SMA–containing activated VICs, which are then further differentiated into osteoblast-like VICs, whereas vascular smooth muscle cells are already α-SMA positive and can be directly stimulated into osteoblast-like cells. With respect to these important differences between arterial calcification and AV calcification, future studies are required to verify the links between ER stress and arterial calcification in vivo.

Although LDL and oxLDL are closely associated with AV calcification and CAVS development, recent randomized clinical trials of statins on CAVS led to conflicting results, which did not show protective effects on CAVS development. In addition, recent studies indicate statin therapy for vascular calcification seemed to paradoxically accelerate vascular calcification. In addition, the well-established lipid lowering and anti-inflammatory effects of statins, several reports suggested that statins could also trigger ER stress. This implies that induction of ER stress by statins may be one of the mechanisms limiting their therapeutic efficacy in CAVS. Moreover, our study showed that inhibition of ER stress did not alter lipid profiles, suggesting this as a potential approach to managing osteogenic differentiation, independent of classical risk factors.

Limitations

One limitation in our study is the limited number of human normal AV samples available. Our previous report demonstrated that valve tissues of patients with cardiomyopathy receiving heart transplantation strongly exhibit ER stress activation because of severe heart failure, and therefore these samples are unfortunately not suitable to serve as a negative control in our study. Furthermore, we only used small interfering RNAs against ER stress signaling to support the potential roles of ER stress–mediated osteoblastic differentiation and inflammation in AV calcification in vitro. Because IRE1α or XBP1 deficiency both cause embryonic lethality in mice, and PERK or ATF4 deficiency in mice spontaneously develop diabetes mellitus or skeletal dysplasia, respectively, these potential targets are thus unavailable or not suitable for in vivo study.

In summary, we demonstrate that ER stress is involved in the pathogenesis of AV calcification. Specifically, oxLDL promotes osteoblastic differentiation and inflammation, at least in part via activating ER stress, and ultimately promotes AV calcification. Inhibition of ER stress markedly relieved ER stress, inhibited osteoblastic differentiation and inflammation in VICs, and attenuated AV calcification development in hypercholesterolemia animals. The proposed mechanism is outlined in Figure XVIII in the online-only Data Supplement.
These results collectively indicate that ER stress may be a novel target against AV calcification.

Acknowledgments
We acknowledge the extensive assistance with echocardiography from Drs Xiaojun Bi, Han Chen, and Jing Zhao. We also thank Dr Jia Wei for graphic assistance.

Sources of Funding
This work was supported by the National Natural Science Foundation of China (81000097 and 31103031), the “973” Program (2012CB518004), and the Zhejiang Provincial Natural Science Foundation of China (LQ13H020003).

Disclosures
None.

References
Significance

Aortic valve (AV) calcification and its subsequent calcified AV stenosis are associated with increased cardiovascular events and mortality without effective therapy other than surgical or interventional AV replacement. The work presented in this report suggests that endoplasmic reticulum (ER) stress signaling is involved in the development of AV calcification. The authors demonstrate that ER stress is activated in calcified AV leaflets from human samples as well as animal models of AV calcification. This work indicates that ER stress directly contributes to osteoblastic differentiation and inflammatory responses in valvular interstitial cells. Most importantly, ER stress inhibition effectively prevented AV calcification in animal models. ER stress inhibitors have already been approved by Food and Drug Administration, suggesting they are conceivable treatment for patients with AV calcification. Further work would be needed to assess whether ER stress inhibition is a safe and effective treatment strategy for patients with AV calcification.
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Arterioscler Thromb Vasc Biol. 2013;33:2345-2354; originally published online August 8, 2013; doi: 10.1161/ATVBAHA.112.300226

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Material

Endoplasmic Reticulum Stress Participates in Aortic Valve Calcification in Hypercholesterolemic Animals

Zhejun Cai, Fei Li, Wei Gong, Wanjun Liu, Quanlu Duan, Chen Chen, Li Ni, Yong Xia, Katherine Cianflone, Nianguo Dong, Dao Wen Wang
Supplemental Figure I

Identification of porcine valvular interstitial cells (VICs). Cultured VICs were identified by α-SMA and vimentin.
Supplemental Figure II

Echocardiographic images and echogenicity data of rabbits with different interventions. (A) Echocardiographic images of control (left panels), HC+vitD₂ (middle panel), and HC+vitD₂+TUDCA aortic valves (right panels) after 12 weeks of intervention. (B) Echogenicity increased in aortic valves from HC+vitD₂ rabbits compared with the control group. TUDCA greatly attenuated this effect (Control: n=6; HC+vitD₂: n=7; HC+vitD₂+TUDCA: n=7; **P<0.01 vs. control; ##P<0.01 vs. HC+vitD₂).
Supplemental Figure III

AV myofibroblast transition of rabbits with different interventions. Representative immunohistochemical staining of α-SMA in AV leaflets of control group (A), HC+vitD2 group (B), and HC+vitD2+TUDCA group (C). Scale bars: 200 μm. (D) TUDCA treatment markedly reduced number of myofibroblasts in AV induced by HC+vitD2 diet in rabbits (Control: n=6; HC+vitD2: n=7; HC+vitD2+TUDCA: n=7; **P<0.01 vs. control; ###P<0.01 vs. HC+vitD2).
Supplemental Figure IV

AV myofibroblast transition of ApoE−/− mice with different interventions. Representative immunohistochemical staining of α-SMA in AV leaflets of control group (A), HC group (B), and HC+TUDCA group (C). Scale bars: 100 μm. (D) TUDCA treatment markedly reduced the number of myofibroblasts in AV induced by HC diet in ApoE−/− mice (Control: n=15; HC: n=15; HC+vitD2+TUDCA: n=15; **P<0.01 vs. control; ###P<0.01 vs. HC).
Supplemental Figure V

TUDCA inhibited AV ER stress activation and osteoblastic differentiation in HC+vitD₂ fed rabbits. (A) Representative immunoblotting image of ER stress and osteoblastic differentiation markers of AV leaflets in rabbits with different interventions. TUDCA significantly prevented ER stress activation (B), and osteoblastic differentiation markers Runx2 and osteocalcin protein (C) and mRNA (D) expression induced by 12 weeks of HC+vitD₂ administration in rabbits (**P<0.01 vs. control; ##P<0.01 vs. HC+vitD₂).
Supplemental Figure VI

TUDCA administration reduced Runx2 expression in AV leaflets of HC+vitD\(_2\) fed rabbits. Representative immunohistochemical staining of Runx2 in AV leaflets of control group (A), HC+vitD\(_2\) group (B), and HC+vitD\(_2\)+TUDCA group (C). Scale bars: 200 μm. (D) HC+vitD\(_2\) diet significantly induced AV Runx2 expression in rabbits, while TUDCA treatment markedly reduced the effect (Control: n=6; HC+vitD\(_2\): n=7; HC+vitD\(_2\)+TUDCA: n=7; **P<0.01 vs. control; ##P<0.01 vs. HC+vitD\(_2\)).
Supplemental Figure VII

TUDCA administration reduces AV macrophage infiltration induced by HC+vitD2 diet in rabbits. Macrophage (RAM11) staining in AV of control group (A), HC+vitD2 group (B), and HC+vitD2+TUDCA group (C). Scale bars: 200 μm. (D) HC+vitD2 diet significantly increased AV macrophage infiltration, while TUDCA effectively suppressed this effect (Control: n=6; HC+vitD2: n=7; HC+vitD2+TUDCA: n=7; **P<0.01 vs. control; ##P<0.01 vs. HC+vitD2).
**Supplemental Figure VIII**

OxLDL-induced ER stress in VICs is cytosolic [Ca\(^{2+}\)]\(_i\) dependent. (A) Immunoblotting for ER stress markers of VICs exposed to oxLDL at different concentrations. (B) Blots were scanned and relative expression levels were normalized against β-actin. OxLDL-induced ER stress in VICs in a dose dependent manner. (C) Incubation with oxLDL increased cytosolic [Ca\(^{2+}\)]\(_i\) to 1.73 fold vs. control. (D) Immunoblotting for ER stress markers of VICs exposed to oxLDL and calcium ionophore A23187, with or without calcium chelator BAPTA-AM pretreatment. (E) As with oxLDL, A23187 triggered ER in VICs, while BAPTA-AM abolished the ER stress-stimulating effects of A23187 and oxLDL in VICs (n=3 for each experiment; *P<0.05 vs. control; **P<0.01 vs. control; ###P<0.01 vs. oxLDL; ††P<0.01 vs. A23187).
Supplemental Figure IX

Silencing efficacy of indicated siRNAs. SiRNAs against PERK (A), ATF4 (B), IRE1α (C), and XBP1 (D) reduced expression of the indicated proteins.
IRE1α signaling mediates oxLDL-induced inflammatory gene expression in VICs. IRE1α silencing suppressed oxLDL-induced IL-6, IL-8 and MCP-1 mRNA expression (n=3 for each experiment; Scr siRNA indicates scrambled siRNA which served as control; **P<0.01 vs. Scr siRNA; ###P<0.01 vs. oxLDL+Scr siRNA).

Supplemental Figure X
Supplemental Figure XI

JNK and NF-κB signaling mediate oxLDL-induced inflammation and osteoblastic differentiation in VICs. Pretreatment of VICs with JNK inhibitor SP600125 or NF-κB inhibitor BAY 11-7082 markedly suppressed oxLDL-induced IL-6 (A), IL-8 (B), and MCP-1 (C) mRNA expression. (D) NF-κB inhibitor BAY 11-7082 significantly inhibited oxLDL-induced Runx2 and osteocalcin expression in VICs (n=3 for each experiment; **P<0.01 vs. control; #P<0.05 vs. oxLDL; ##P<0.01 vs. oxLDL).
Supplemental Figure XII

4-PBA attenuates ER stress induced by oxLDL in VICs. Immunoblotting showed that 4-PBA pretreatment markedly inhibited oxLDL-induced ER stress in VICs (n=3 for each experiment; **P<0.01 vs. control; ## P<0.01 vs. oxLDL).
Supplemental Figure XIII

4-PBA attenuates osteoblastic differentiation induced by oxLDL in VICs. Immunoblotting showed that 4-PBA pretreatment markedly inhibited oxLDL-induced Runx2 and osteocalcin expression in VICs (n=3 for each experiment; **P<0.01 vs. control; ##P<0.01 vs. oxLDL).
Supplemental Figure XIV

4-PBA attenuates oxLDL-induced JNK and NF-κB activation in VICs. TUDCA prevented JNK phosphorylation (A) and NF-κB p65 nuclear translocation (B) in VICs (n=3 for each experiment; **P<0.01 vs. control; ##P<0.01 vs. oxLDL).
Supplemental Figure XV

TUDCA prevents oxLDL-induced NF-κB p65 nuclear translocation in VICs. Representative immunocytofluorescence staining for NF-κB p65 in VICs. TUDCA pretreatment markedly attenuated NF-κB p65 nuclear translocation stimulated by oxLDL.
Supplemental Figure XVI

ER stress inhibition attenuates oxLDL-induced inflammation in VICs. TUDCA (A) and 4-PBA (B) preincubation could both markedly down-regulate mRNA expression of IL-6, IL-8, and MCP-1 stimulated by oxLDL (n=3 for each experiment; **P<0.01 vs. control; ###P<0.01 vs. oxLDL).
Supplemental Figure XVII

ER stress inhibition prevents oxLDL-induced mineralization in VICs. (A) TUDCA and 4-PBA could both reduced mineralization (A) and ALP (B) activity induced by 7-day incubation of oxLDL in the presence of 2mmol/l β-glycerophosphate, 100 nmol/l dexamethasone, and 50 μg/ml ascorbic acid (C) Alizarin staining for calcification nodule formation in VIC cells with different interventions (n=3 for each experiment; **P<0.01 vs. control; ###P<0.01 vs. oxLDL).
Supplemental Figure XVIII

The proposed mechanisms of ER stress promotion of AV calcification. OxLDL increase cytosolic [Ca\textsuperscript{2+}] to trigger ER stress in VICs. This leads to the activation of PERK/ATF4 and IRE1\(\alpha\)/XBP1\(s\) signaling branches of ER stress to induce osteocalcin and Runx2 expression, and promotes osteoblastic differentiation. Meanwhile, activation of the IRE1\(\alpha\) pathway promotes expression of inflammatory response markers via JNK and NF-\(\kappa\)B signaling. The enhanced inflammatory responses and osteoblastic differentiation could then accelerate AV calcification development.
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### Supplemental Table II

Serum lipid profiles and glucose levels in animals at baseline

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<td>TG (mmol/l)</td>
<td>0.75±0.22</td>
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TC: total cholesterol; TG: triglyceride; LDL-c: LDL cholesterol

Data are presented as mean ± SD.
### Supplemental Table III

Serum calcium, phosphate, and PTH levels in rabbits after different interventions

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<td>Calcium (mg/dl)</td>
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Data are presented as mean ± SD. ** P<0.01 vs. control; n.s. not significant vs. HC+vitD2. PTH: parathyroid hormone.
Supplemental Methods

Human AV samples

This study complied with the Declaration of Helsinki, and was approved by the Review Board of Tongji Hospital and Tongji Medical College, and the subjects recruited to the study provided written informed consent. We obtained calcified AV from ten patients undergoing valve replacement surgery due to symptomatic, severe CAVS without moderate or severe aortic regurgitation or mitral valve diseases. In addition, patients with a history of myocardial infarction, renal insufficiency were excluded (Detailed characteristics were listed in Supplemental Table I). Four normal AV were from age-matched traffic accident victims. Tissue samples were obtained and kept frozen in liquid nitrogen and then stored at -80°C until use.

Experimental animal model

All animal experimental protocols complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal studies were approved by the Institutional Animal Research Committee of Tongji Medical College.

For experiments in rabbits, male New Zealand White rabbits weighing 2.5-3.0 kg were obtained from the Experimental Animal Center of Tongji Medical College (Wuhan, China) and were housed at the animal care facility of Tongji Medical College at 25°C with 12/12 h light/dark cycles. The method to induce AV calcification was applied as described previously. After initial quarantine, rabbits were randomly assigned into three groups and received different diet feeding for a 12-week protocol: control group (n=6) receiving normal diet, HC+vitD2 group (n=7) receiving 0.5% cholesterol plus vitamin D2 50,000 IU/day, and HC+vitD2+TUDCA group (n=7) receiving HC+vitD2 diet supplemented with 50mg/kg/day TUDCA dissolved in water. Vitamin D2 and TUDCA were supplied by oral gavage. Animals without TUDCA treatment received equal amount of vehicle by oral gavage.

For experiments in mice, 8-week old ApoE−/− mice weighing 18-22g were
purchased from Vital River Laboratory Animal Technology Co. Ltd., China, and were housed at the animal care facility of Tongji Medical College at 25°C with 12/12 h light/dark cycles. AV calcification was generated by a 24-week protocol as described previously. After initial quarantine, mice were randomly assigned into three groups: control group (n=15) receiving normal chow, HC group (n=15) receiving 0.25% cholesterol diet, and HC+TUDCA group (n=15) receiving HC diet supplemented with 0.5g/kg/day TUDCA by oral gavage. Animals without TUDCA treatment received equal amount of vehicle (drinking water) by oral gavage.

**Isolation of LDL and preparation of oxLDL**

LDL (density=1.019-1.063 g/ml) were isolated from pooled human plasma by sequential ultracentrifugation as previously described. For oxidation, LDL was incubated with 10 mmol/l CuSO₄ for 18 h at 37°C and terminated by adding 1 mmol/l EDTA. OxLDL was then dialyzed, sterilized by filtering, and diluted to 1 mg/ml in PBS. The prepared oxLDL was stored at 4°C and used within 2 weeks.

**Cell culture and treatment**

VICs were isolated from AV leaflets of adult pigs from a local slaughterhouse by collagenase digestion as previously described. The VICs phenotype was identified by immunocytofluorescence with antibodies against α-SMA (1:50 dilution, Boster, China) and vimentin (1:50 dilution, Boster) (Supplemental Figure I). All experiments were performed with cells in passages 2-4. After confluence, cells were incubated with oxLDL at the concentrations for 12 h. If needed, pharmacological reagents, including 10 μmol/l BAPTA-AM (Sigma-Aldrich, St. Louis, MO), 10 μmol/l SP600125 (Merck, Germany), 10 μmol/l BAY 11-7082 (Merck), 1 μmol/l TUDCA (Calbiochem, Germany), and 2.5 mmol/l 4-PBA (Calbiochem) were added 1 h prior to the addition of 100 μg/ml oxLDL or 5 μmol/l A23187 (Sigma-Aldrich). Cells were starved in serum-free DMEM for 6 h before experiments. For mineralization experiments, VICs were seeded in six-well plates. After 70% confluence, cells
were incubated with indicated interventions in DMEM supplemented with 5% FBS, 2 mmol/l β-glycerophosphate (Sigma-Aldrich), 100 nmol/l dexamethasone (Sigma-Aldrich), and 50 μg/ml ascorbic acid (Sigma-Aldrich) for 7 days.

Cell transfection of siRNAs
SiRNAs against indicated proteins were a pool of three sequences provided by Ribobio, China, which were designed against *Sus scrofa* PERK (XM_003124925.1), *Sus scrofa* IRE1α (XM_003131282.1), *Sus scrofa* ATF4 (NM_001123078.1), and *Sus scrofa* XBP1 (NM_001142836.1). Scrambled siRNAs (Ribobio) were used as normal control. After 30-40% confluence, VICs were transfected with scrambled or indicated siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and Opti-MEM (Gibco, Carlsbad, CA) according to manufacturer’s recommendations, and changed the medium after 6 h.

Immunoblotting
Tissue samples and cells were homogenized and subsequently performed for western blotting as previously described. The following antibodies were used: p-IRE1α (1:2000 dilution, PA1-16927) from Pierce, Rockford, IL; p-PERK (1:500 dilution, sc-32577), PERK (1:1000 dilution, sc-13073), IRE1α (1:1000 dilution, sc-20790), C/EBP homologous protein (1:500 dilution, sc-575), X-box binding protein 1 (XBP1, 1:500 dilution, sc-7160), Runx2 (1:1000 dilution, sc-10758), osteocalcin (1:1000 dilution, sc-30045), p-JNK (1:500 dilution, sc-6254), lamin B1 (1:1000 dilution, sc-56145) and β-actin (1:1000 dilution, sc-47778) from Santa Cruz Biotechnology, Santa Cruz, CA; JNK (1:500 dilution, BS1544), Inhibitor of NF-κB α (IκBα, 1:500 dilution, BS1190) and NF-κB p65 (1:500 dilution, BS1257) from Bioworld, Atlanta, GA. β-actin was used as normalization for total protein or cytosolic protein determination, and lamin B1 for nuclear protein determination. In phosphorylation induction assay, indicated total protein expression was used as normalization. Bands were quantified by densitometry.
using Quantity One Software (Bio-Rad, Hercules, CA).

**Measurement of cytosolic [Ca\(^{2+}\)]\(_i\)**

Cytosolic [Ca\(^{2+}\)]\(_i\) was determined using Fluo-3/AM (Invitrogen) under a fluorometer as previously described \(^7^,\text{ }^8\). Briefly, VICs were incubated with a loading solution containing HEPES-buffered saline (HBS; 135 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l MgCl\(_2\), 1.5 mmol/l CaCl\(_2\), 11.6 mmol/l HEPES and 11.5 mmol/l glucose, pH=7.3) supplemented with 5 mmol/l Fluo-3/AM, 0.02% pluronic F-127 (Sigma-Aldrich) and 1 mg/ml BSA for 30 min and then incubated in the loading solution without Fluo-3/AM for 30 min. The fluorescence was recorded at 495 nm and 525 nm for excitation and emission wavelengths, respectively. Results were normalized on protein levels, and expressed in ratio of control as previously reported \(^7^,\text{ }^8\).

**Immunocytofluorescence**

Cells grown on cover glass slides were washed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min and then in 0.25% Triton-X 100 for 10 min. After blocking with 3% BSA for 30 min, cells were incubated in indicated antibodies at 4°C overnight. The slides were then revealed with appropriate FITC or Cy3-conjugated secondary antibodies for 1 h at 37°C and DAPI (Sigma-Aldrich) for nuclear counter staining, and were subsequently visualized under a Nikon DXM1200 fluorescence microscope. Image-Pro Plus (Media Cybernetics, Bethesda, MD) was applied for image merging.

**Calcium content determination in VICs**

Calcium deposition in the plates was quantified as previously described \(^9\). Cells were decalcified by 0.6 mol/l HCl solution, and the supernatant was subsequently subjected to calorimetrical quantification using the ocresolphthalein method. The result was calibrated by protein amount and then normalized to the controls.

**ALP activity**

ALP activity was measured using p-nitrophenyl phosphate as the substrate using the commercial kit from Wako, Japan.
Quantitative PCR for mRNA analysis

Total RNA was isolated and reverse transcribed to cDNA as previously described\textsuperscript{10}. The cDNA was subsequently subjected to quantitative PCR using SYBR Premix Ex Taq kit (TAKARA, Japan) and specific primers for rabbit Runx2 (F: 5’- GCA GTT CCC AAG CAT TTC ATC-3’ and R: 5’- GTG TAA GTA AAG GTG GCT GGA TA-3’); osteocalcin (F: 5’ GCT CAC CCT TCG TGT CCA AG-3’ and R: 5’- CCG TCG ATC AGT TGG CGC-3’); β-actin (S: 5’- CAC CCT GAT GCT CAA GTA CC-3’ and R: 5’- CGC AGC TCG TTG TAG AAG G-3’); porcine IL-6 (F: 5’-ATC AGG AGA CCT GCT TGA TG-3’ and R: 5’-TGG TGG CTT TGT CTG GAT TC-3’); IL-8 (F: 5’-TCC TGC TTT CTG CAG CTC TC-3’ and R: 5’-GGG TGG AAA GGT GTG GAA TG-3’); MCP-1 (F: 5’-GTC ACC AGC AGA CCT GCT GGT GAC TAC CTC-3’ and R: 5’-GAC CTG ACC GAC TAC CTC-3’); and β-actin (F: 5’-GAC CTG ACC GAC TAC CTC-3’ and R: 5’-GCT TCT CCT TGA TGT CCC-3’). Results were normalized to β-actin expression and calculated by the ΔΔCt method.

Echocardiography

Transthoracic echocardiography was performed at baseline and at 12 weeks of respective treatment. Animals were sedated with intramuscular injection of 30 mg/kg ketamine and 3.5 mg/kg xylazine. A comprehensive echocardiographic study, including two-dimensional imaging, Doppler imaging and M-mode imaging, and subsequent data calculations were performed by operator blinded to the treatment assignment as described previously\textsuperscript{11} using the Vevo 2100 system\textsuperscript{11}. A semi-quantitative index (range 0-3) for estimating rabbit AV echogenicity, an index of valve thickening and calcification, as follows: 0-absent, 1-mild; 2-moderate, with preserved motility/opening; 3-severe, with decreased motility/opening, was applied as previously reported\textsuperscript{11}. Data were analyzed on a blinded fashion.

Histopathology and immunohistochemistry

After final transthoracic echocardiographic assessment, animals were euthanized by intravenous injection of a lethal dose of pentobarbital sodium.
(100 mg/kg), and AV were then rapidly harvested, rinsed in PBS, fixed in 4% paraformaldehyde in PBS, and embedded in paraffin for histopathological analyses. 3 μm sections were subsequently stained with alizarin red. Immunohistochemical staining were performed using the following antibodies: KDEL (1:100 dilution, Santa Cruz, sc-33806 and sc-58774 identifying multiple proteins which indicate ER stress activation), CHOP (1:100 dilution, Santa Cruz, sc-575 and sc-7351), α-SMA (1:100 dilution, Sigma-Aldrich, sc-58774), osterix (1:100 dilution, Santa Cruz, sc-22536-R), Runx2 (1:100 dilution, Santa Cruz, sc-10758 and sc-8566), osteocalcin (1:100 dilution, Santa Cruz, sc-30045), F4/80 (1:100 dilution, Santa Cruz, sc-25830), RAM11 (1:100 dilution, Dako, Denmark, M0633). Image-Pro Plus (Media Cybernetics) was applied to determine quantitative results (positive staining area/total AV area) for 2 sections of each AV leaflet, and at least 2 of the 3 AV leaflets for each animal were analyzed. Investigators performing the analyses were blinded to the study groups.

**Determination of serum cholesterol, LDLs, triglyceride, and glucose levels**

Animals underwent a 14-15 h fast before blood samples were collected. Serum cholesterol, LDLs, triglyceride, and glucose levels were determined using indicated kits from Biosino, China, according to the manufacturer’s instructions.

**Statistical analysis**

All values are presented as mean±SD. After confirming that all variables were normally distributed using the Kolmogorov-Smirnov test, statistical differences were evaluated by Student t-test or ANOVA followed by Bonferroni’s multiple comparison test. \( P<0.05 \) was accepted as statistically significant.
References