Original Research

Intermedin$_{1–53}$ attenuates vascular smooth muscle cell calcification by inhibiting endoplasmic reticulum stress via cyclic adenosine monophosphate/protein kinase A pathway

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Abstract

We previously reported that endoplasmic reticulum (ER) stress-mediated apoptosis participated in vascular calcification. Importantly, a novel paracrine/autocrine peptide intermedin$_{1–53}$ (IMD$_{1–53}$) in the vasculature inhibited vascular calcification in rats. But the mechanisms needed to be fully elucidated. Vascular smooth muscle cells (VSMCs) calcification was induced by $CaCl_2$ and β-glycerophosphate. Tunicamycin (Tm) or dithiothreitol (DTT) was used to induce ER stress. We found that IMD$_{1–53}$ (10^{-7} mol/L) treatment significantly alleviated the protein expression of ER stress hallmarks activating transcription factor 4 (ATF4), ATF6, glucose-regulated protein 78 (GRP78) and GRP94 induced by Tm or DTT. ER stress occurred in early and late calcification of VSMCs but was inhibited by IMD$_{1–53}$. These inhibitory effects of IMD$_{1–53}$ were abolished by treatment with the protein kinase A (PKA) inhibitor H89. Pretreatment with IMD$_{1–53}$ decreased the number of apoptotic VSMCs and downregulated protein expression of cleaved caspase 12 and C/EBP homologous protein (CHOP) in calcified VSMCs. Concurrently, IMD$_{1–53}$ restored the loss of VSMC lineage markers and ameliorated calcium deposition and alkaline phosphatase activity in calcified VSMCs as well. The observation was further verified by Alizarin Red S staining, which showed that IMD$_{1–53}$ reduced positive red nodules among calcified VSMCs. In conclusion, IMD$_{1–53}$ attenuated VSMC calcification by inhibiting ER stress through cAMP/PKA signalling.

Keywords: intermedin$_{1–53}$, vascular calcification, endoplasmic reticulum stress, apoptosis

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Introduction

Endoplasmic reticulum (ER) stress is a subcellular pathological process, whereby the protein processing capacity of the ER is exceeded by the expression of nascent proteins, which results in accumulation of unfolded or misfolded proteins.[1] The mutant ER client proteins trigger the unfolded protein response (UPR) to maintain ER homeostasis. The UPR is initially an adaptive response by increasing expression of ER chaperones, accelerating degradation of unfolded proteins and inhibiting protein translation.[1,2] Prolonged and/or excessive ER stress induces proapoptotic molecules expression including C/EBP homologous protein (CHOP), caspase-12 (ER stress-specific) and c-JUN NH_2-terminal kinase (JNK) and leads to apoptosis.[3]

Growing evidence from experimental and clinical research indicates that ER stress-induced apoptosis is involved in several processes of cardiovascular diseases such as atherosclerosis, vascular calcification (VC), myocardium ischemic-reperfusion injury and ischemic heart disease.[4–6] Inhibiting ER stress may play a key role in treatment of these diseases.[7,8]

Intermedin (IMD) or adrenomedullin 2 (ADM2), an important paracrine/autocrine factor discovered in 2004,[9,10] is a new member of the calcitonin/calcitonin gene-related peptide (CGRP) family. Human prepropeptide (prepro-IMD) could be cleaved between arginine residues at positions 93 and 94, resulting in the production of a longer bioactive fragment of prepro-IMD (95–147),

namely IMD_{1-53}.[11] The biological effects of IMD are mediated by the calcitonin receptor-like receptor (CRLR; seven trans-membrane G protein-coupled receptor), whose ligand-binding specificity is regulated by receptor activity-modifying proteins (RAMPs).[12] The main pharmacological effects of IMD are attributed to the activation of cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signalling.[13]

IMD exists extensively in the cardiovascular system, kidney, lung, pancreas and other organs.[13] Peripheral administration of IMD decreases aortic resistance, lowers arterial pressure and reduces the myocardial infarction size.[13] Therefore, IMD might be a cardiovascular protective factor. Interestingly, we found that the level of IMD mRNA and protein is downregulated in the calcified aorta and plasma, and IMD_{1-53} ameliorated VC in an *in vivo* model of rats induced by vitamin D_3 plus nicotine.[14] However, the detailed mechanism of IMD_{1-53} inhibiting VC has not been investigated.

Our previous study found that IMD_{1-53} attenuated ischemia-reperfusion-induced myocardial apoptosis by inhibiting ER stress.[6] In this study, we investigated whether IMD_{1-53} inhibited VC by inhibiting ER stress. We studied the effect of IMD_{1-53} on ER stress in vascular smooth muscle cells (VSMCs) and the underlying signalling pathway in calcified VSMCs *in vitro*.

Materials and methods

Materials

Synthetic human IMD_{1-53} was from Phoenix Pharmaceuticals (Belmont, CA, USA). β-Glycerophosphate, tunicamycin (Tm), dithiothreitol (DTT), H89 and Alizarin Red S were from Sigma (St. Louis, MO, USA). The calcium assay kit was from Biosino Biotechnology and Science (Beijing, China). The alkaline phosphatase (ALP) assay kit was from Beijing BHKT Clinical Reagent Co., Ltd (Beijing, China). Primary antibodies for glucose-regulated protein 78 (GRP78), GRP94, activating transcription factor 4 (ATF4), ATF6, osteocalcin and smooth muscle 22 (SM22) α were from Abcam (Cambridge, UK); α-actin, osteopontin (OPN), B-cell lymphoma (Bcl)-2, Bax, GAPDH and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies anti-mouse and anti-rabbit IgG were from Jackson Immuno Research Laboratories, Inc. (CA, USA). Nitrocellulose membrane was from Hybond-C (Amersham Life Science, UK). The enhanced chemiluminescence and Hoechst staining kits were from Applygen Technologies (Beijing, China). Other chemicals and reagents were of analytical grade.

VSMC culture *in vitro*

Male Sprague–Dawley (SD) rats (150 ± 10 g) were from the Animal Center, Health Science Center, Peking University (Beijing, China). All animal procedures were complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (documentation No. 55, 2001) and the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The explant culture of VSMCs was described previously[14] with minor modification. Briefly, male SD rats thoracic aortas were cut into small pieces after the removal of endothelium and adventitia, placed in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) and incubated at 37°C in an incubator containing 95% air and 5% CO_2. VSMCs migrating from explants were collected and maintained in growing medium (DMEM containing 10% FBS). The α-actin testing of cultured cells confirmed a positive response. VSMCs at passages five to eight were used for experiments.

Treatment of cells

For calcification, confluent VSMCs were treated with 2.5 mmol/L $CaCl_2$ and 5 mmol/L β-glycerophosphate. ER stress was induced in VSMCs by treating them with ER stress inducers Tm (1 μg/mL) or DTT (2 mmol/L). Cells were incubated with buffer alone, Tm/DTT, IMD_{1-53} (10^{-7} mmol/L) + Tm/IMD_{1-53} (10^{-7} mmol/L) + DTT, respectively. Tm and DTT were administered after the addition of IMD_{1-53} for 30 min, and then the cells were incubated for 3 days for Western blot analysis. To investigate the effect of IMD_{1-53} on calcifying media-stimulated ER stress, VSMCs were preincubated with the IMD_{1-53} (10^{-7} mmol/L) for 30 min; after co-incubation for 6 h or 6 days, cells were collected for Western blot analysis. Each experiment was performed at least three times with triplicate cultures used in every experiment, unless stated otherwise.[15]

To investigate the signalling pathway of IMD_{1-53} inhibiting ER stress, PKA inhibitor H89 (10 μmol/L) was administered 30 min before the addition of IMD_{1-53}, and the other procedures were same as described above.

Alizarin Red S staining

Alizarin Red S staining was performed as described previously[14] with minor modification. Cultured VSMCs grown in 12-well plates were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 45 min at 4°C, then washed in distilled water and exposed to Alizarin Red S (2% aqueous, Sigma) for 5 min, then washed again with distilled water and observed by microscopy. Positive staining was red/purple.

ALP activity assay

VSMCs were washed three times with cold PBS and were lysed with buffer containing 0.2% NP-40 and 1 mmol/L $MgCl_2$ as we previously described[16] with minor modification. After centrifugation at 8000g for 10 min at 4°C, the supernatant was collected. The protein content of the supernatant was determined by the Bradford method. ALP activity was measured by use of an ALP Colorimetric assay kit (Beijing BHKT Clinical Reagent Co., Ltd, China): The samples of cell lysis solutions (50 μL) were mixed with 500 μL bicarbonate buffer at 37°C for 5 min and then incorporated with pre-warmed disodium phenyl phosphate in aqueous solution (500 μL). After the reaction system was incubated at 37°C for 15 min, 1.5 mL potassium ferricyanide was

added, and absorbance was determined at 510 nm. ALP activity was calculated using phenol solution as a standard, according to the kit's instruction. Data were normalised to levels of total protein.

Quantification of calcium content

After the end of calcification treatment, the medium was removed, and cells were washed three times with cold PBS and decalcified with 0.6 mol/L HCl. Calcium content in the supernatant was determined by the o-cresolphthalein complexone method (Calcium Kit, Biosino Biotechnology and Science, Beijing). Data were normalised to levels of total protein.

Hoechst staining

Hoechst staining of VSMCs was performed by using Hoechst staining kit (Beijing Applygen Technologies) according to manufacturer's instructions. Fixed cells were washed with PBS and stained with Hoechst (5 mg/mL) for 5 min. The cells were washed twice with PBS and then observed under a fluorescence microscope. And the condensed or fragmented apoptotic nuclei were observed. The apoptotic nuclei were counted in at least 200 cells from five randomly selected fields in each treatment and expressed as a percentage of the total number of nuclei counted.

Western blot analysis

Cell extracts were prepared as described[14] and then homogenised in lysis buffer. Equal amounts of protein samples were loaded on 10% gels and then transferred to a nitrocellulose membrane. Then the membrane was incubated with the primary antibodies for GRP78 (1:20,000), GRP94 (1:5000), ATF4 (1:1000), ATF6 (1:1000), β-actin (1:4000), cleaved caspase 12 (1:1500), CHOP (1:1000), Bcl-2 (1:500), Bax (1:1000), α-actin (1:2000), SM22 α (1:2000), OPN (1:500), osteocalcin (1:1000) or GAPDH (1:2000) overnight at 4°C, then secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG) for 1 h. Protein expression was analysed by use of NIH Image and normalised to β-actin expression. All experiments were repeated at least three times.

Statistical analysis

Each experiment was repeated at least three times, and data are expressed as mean ± SD (unless otherwise indicated). Statistical analyses involved the use of GraphPad Prism v5.00 for Windows (GraphPad Software Inc., San Diego, CA, USA). Comparisons were analysed by one-way ANOVA followed by Student–Newman–Keuls test. A $P < 0.05$ was considered statistically significant.

Results

$IMD_{1–53}$ directly inhibited VSMC ER stress induced by Tm and DTT *in vitro*

To determine whether $IMD_{1–53}$ had a direct inhibitory effect on VSMC ER stress, rat VSMCs were incubated with the ER stress inducers Tm or DTT *in vitro*. Compared with the control treatment, Tm treatment significantly upregulated protein levels of the ER stress markers GRP78, GRP94, ATF4 and ATF6, and $IMD_{1–53}$ (10^{-7} mmol/L) significantly attenuated the overexpression of GRP78, GRP94, ATF4 and ATF6 protein by 36.4% ($P < 0.01$), 43.1% ($P < 0.05$), 38.4% ($P < 0.05$) and 41.9% ($P < 0.05$), respectively, as compared with Tm alone. Accordingly, $IMD_{1–53}$ (10^{-7} mmol/L) significantly reduced the upregulated protein of GRP78, GRP94, ATF4 and ATF6 by 47.6% ($P < 0.01$), 33.7% ($P < 0.01$), 45.1% ($P < 0.01$) and 50.2% ($P < 0.05$), respectively, as compared with DTT alone (Figure 1).

cAMP/PKA signalling was involved in the inhibitory effects of $IMD_{1–53}$ on VSMC ER stress induced by Tm or DTT *in vitro*

To investigate the signalling pathway by which $IMD_{1–53}$ inhibited Tm- or DTT-stimulated ER stress, VSMCs were preincubated with the PKA inhibitor H89 (10 μmol/L) for 30 min before $IMD_{1–53}$ treatment. H89 preincubation blocked the inhibitory effects of $IMD_{1–53}$ on Tm or DTT-induced protein expression of GRP78, GRP94, ATF4 and ATF6 by 40.2 ($P < 0.01$), 129 ($P < 0.01$), 72.7 ($P < 0.05$) and 107% ($P < 0.05$) and by 109 ($P < 0.05$), 46.2 ($P < 0.01$), 69.4 ($P < 0.05$) and 75.8% ($P < 0.05$) (Figure 2). Tm- or DTT-induced ER stress in cultured VSMCs was not affected by H89 alone. These results suggested that cAMP/PKA pathway involved in the inhibitory effect of $IMD_{1–53}$ on VSMC ER stress induced by Tm or DTT.

cAMP/PKA signalling was involved in the inhibitory effect of $IMD_{1–53}$ on ER stress induced by calcifying media

To investigate whether $IMD_{1–53}$ could alleviate ER stress induced by calcification of VSMCs, cells were treated with $CaCl_2$ and β-glycerophosphate for 6 h and 6 days. With $IMD_{1–53}$ treatment, protein levels of the ER stress markers with calcification were decreased by 39.3 ($P < 0.05$), 45.5 ($P < 0.01$), 44.1 ($P < 0.05$) and 28.6% ($P < 0.05$) at 6 h and by 34.3 ($P < 0.05$), 70.4 ($P < 0.01$), 67.0 ($P < 0.01$) and 35.5% ($P < 0.05$) at day 6 (Figure 3).

Next, we confirmed whether cAMP/PKA signalling was involved in the inhibitory effects of $IMD_{1–53}$ on ER stress induced by calcifying media. Calcified VSMCs were preincubated with the PKA inhibitor H89 (10 μmol/L) for 30 min before $IMD_{1–53}$. Treatment with H89 inhibited the $IMD_{1–53}$ reduction of protein levels of ER stress markers (Figure 4), which demonstrated that cAMP/PKA pathway involved in the inhibitory effect of $IMD_{1–53}$ on VSMC ER stress induced by calcifying media.

$IMD_{1–53}$ inhibited apoptosis of calcified VSMCs

Previously, we reported that ER stress-mediated apoptosis contributed to VC.[5] In this study, we tested whether $IMD_{1–53}$ could alleviate ER stress-induced apoptosis in calcified VSMCs. Calcification increased apoptotic nuclei (Figures 5a and b) and elevated Bax protein level but decreased Bcl-2 protein level, for decreased ratio of Bcl2 to Bax in

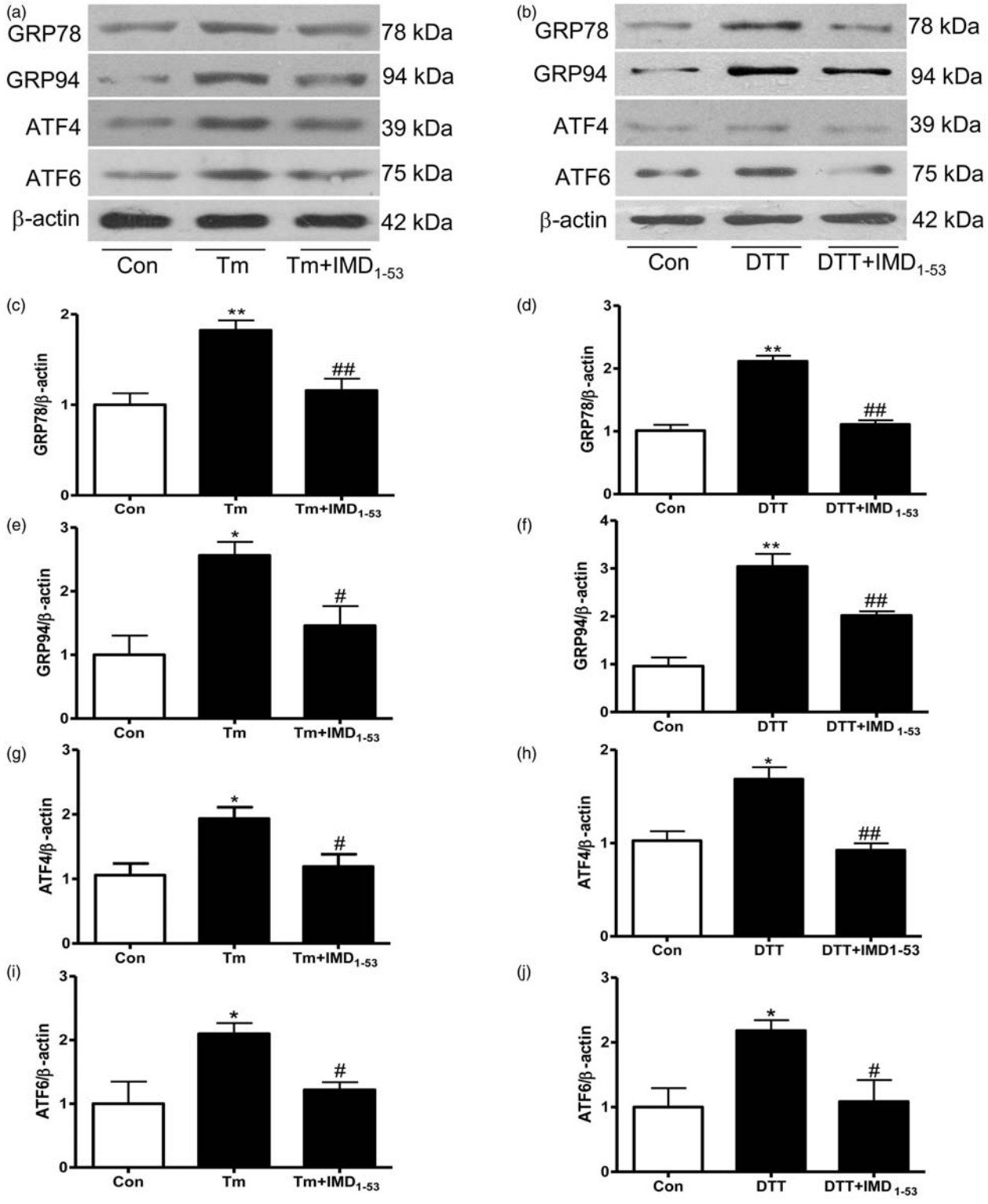

Figure 1 IMD_{1-53} inhibited tunicamycin (Tm)- or dithiothreitol (DTT)-induced endoplasmic reticulum (ER) stress in vascular smooth muscle cells (VSMCs) *in vitro*. (a, b) Western blot analysis of protein levels of GRP78, GRP94, ATF4, ATF6 (β-actin was a loading control) and (c–j) quantification. Data are mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (Con); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Tm or DTT. IMD_{1-53}: $intermedin_{1-53}$

calcified VSMCs by 68.2% ($P < 0.01$) (Figures 5c to e) after incubation with calcifying media 6 days. These findings were reversed with IMD_{1-53} treatment by 2.39-fold ($P < 0.01$). Importantly, IMD_{1-53} treatment greatly attenuated the calcification-induced protein levels of CHOP and cleaved caspase 12, specific indexes of apoptosis induced by ER stress, by 32.3 ($P < 0.01$) and 25.5% ($P < 0.05$), respectively (Figures 5c, f and g).

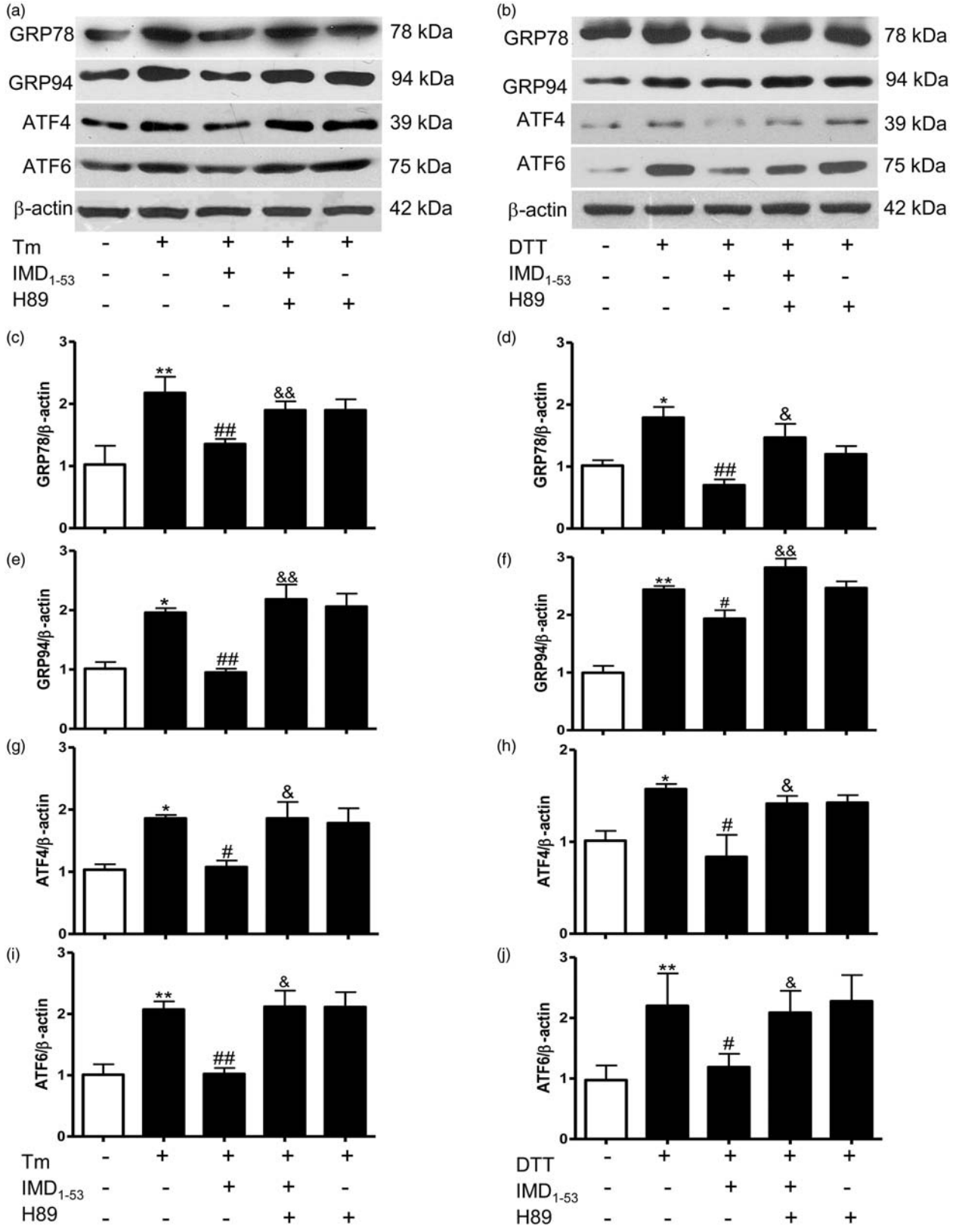

Figure 2 H89, a protein kinase A (PKA) inhibitor (10 μmol/L), attenuated the effect of $IMD_{1–53}$ (10^{-7} mol/L) inhibiting Tm- or DTT-induced ER stress *in vitro*. (a, b) Western blot analysis of protein levels of GRP78, GRP94, ATF4, ATF6 (β-actin was a loading control) and (c–j) quantification. Data are mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus Con; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Tm or DTT; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus Tm + $IMD_{1–53}$ or DTT + $IMD_{1–53}$

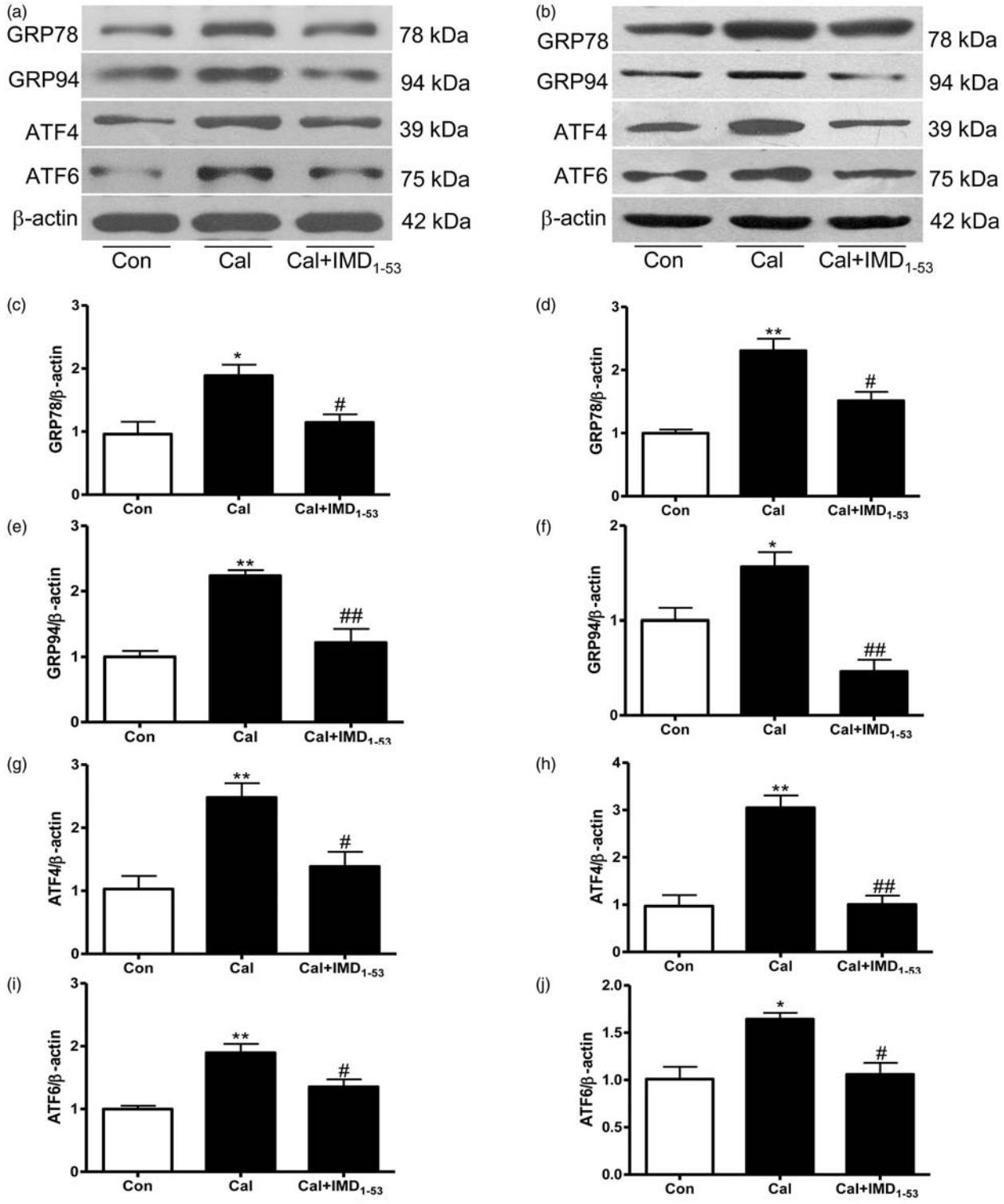

Figure 3 IMD_{1-53} (10^{-7} mol/L) attenuated ER stress induced by calcification in cultured VSMCs. (a, b) Western blot analysis of protein levels of GRP78, GRP94, ATF4, ATF6 (β-actin was a loading control) at 6 h (calcifying stage) and on day 6 (calcified stage), respectively, and (c–j) quantification. Data are mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus Con; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus calcification (Cal)

IMD_{1-53} inhibited the VSMC contractile phenotype transforming into an osteoblast-like phenotype and attenuated VSMC calcification *in vitro*

The VSMC phenotype switch is a key step in VC.[17] We examined VSMC phenotype markers in calcifying VSMCs. Protein levels of the VSMC phenotype markers α-actin and SM22 α were decreased in calcified VSMCs and were rescued with IMD_{1-53} treatment by 1.68-fold and 1.79-fold (both $P < 0.01$), respectively (Figures 6a to c). However, the protein levels of the osteoblast-like cell markers OPN

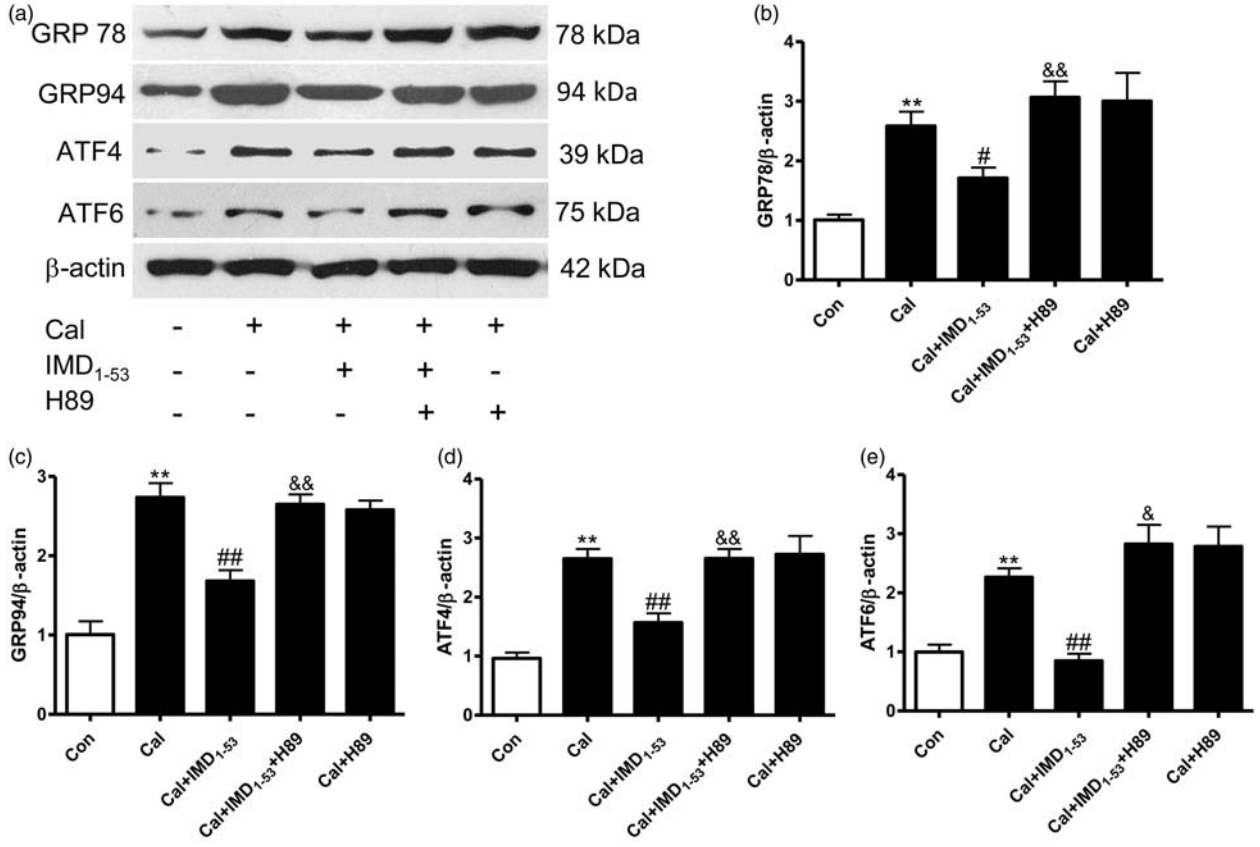

Figure 4 IMD_{1-53} (10^{-7} mol/L) inhibited ER stress activation with calcification through the cAMP/PKA signaling pathway. (a) Western blot analysis of protein levels of GRP78, GRP94, ATF4, ATF6 (β-actin was a loading control), and (b–e) quantification. Data are mean ± SD, $n = 3$, $^{**}P < 0.01$ versus Con; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Cal; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus Cal + IMD_{1-53}

and osteocalcin were increased in calcified VSMCs and were decreased with IMD_{1-53} treatment by 61.7 ($P < 0.05$) and 52.6% ($P < 0.01$) (Figures 6a, d and e).

Next, we studied the effects of IMD_{1-53} on VSMC calcification *in vitro*. Alizarin Red S staining showed that IMD_{1-53} treatment significantly decreased calcium phosphate salt deposition in calcified VSMCs (Figure 6f). Concurrently, IMD_{1-53} reduced calcium content by 52.8% (Figure 6g) and ALP activity by 39.7% (both $P < 0.05$) (Figure 6h) after incubation with calcifying media 14 and 3 days, respectively.

Discussion

In the present study, we found that IMD_{1-53} protected against VSMC calcification by inhibiting VSMC ER stress in the *in vitro* model. IMD_{1-53} directly inhibited Tm- or DTT-induced ER stress in VSMCs *in vitro*. Use of the PKA inhibitor H89 antagonised the effect of IMD_{1-53} inhibiting ER stress. Interestingly, IMD_{1-53} reversed the upregulated protein levels of ER stress markers in calcified VSMCs *in vitro*, reduced calcified VSMC apoptosis, retarded contractile VSMC transforming into osteoblast-like cells and ameliorated VSMC calcification. This is the first report of IMD_{1-53} activating cAMP/PKA signalling to inhibit VSMC ER stress and attenuate VC.

The ER is one of the largest organelles and sometimes described as the factory of the eukaryotic cell. Most lipids, glycans and about one-third of proteins are synthesised, modified and processed in this organelle. ER stress that disrupts ER function can occur in response to various cellular stressors such as hypoxia, ischemia and pharmacological agents (ER stress inducers such as thapsigargin, Tm and DTT), which lead to the accumulation of misfolded and unfolded proteins in the ER.[1]

The ER contains three transmembrane sensors that detect unfolded or misfolded proteins accumulated in ER lumen and initiate three signalling pathways: double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), ATF6 and inositol-requiring enzyme 1.[18] All three pathways can be activated to deal with accumulated unfolded or misfolded proteins and resume the cellular homeostasis in the early stage of ER stress. However, if the ER stress is severe and/or prolonged, the ER initiates apoptotic cell death signalling by CHOP, caspase-12 and JNK-signalling pathways. Recently, proapoptotic pathways of ER stress have been implicated in the pathophysiologic aspects of cardiovascular diseases, including coronary heart disease, atherosclerosis, vascular calcification, hypertension and cardiomyopathy,[19] and inhibiting ER stress could protect against cardiovascular disease. Our previous study indicated that IMD protected the myocardium against ischemia–reperfusion injury by inhibiting ER stress.[6] However, the regulation effects of autocrine/paracrine factors on ER stress in vascular physiologic and pathophysiologic processes need further study.

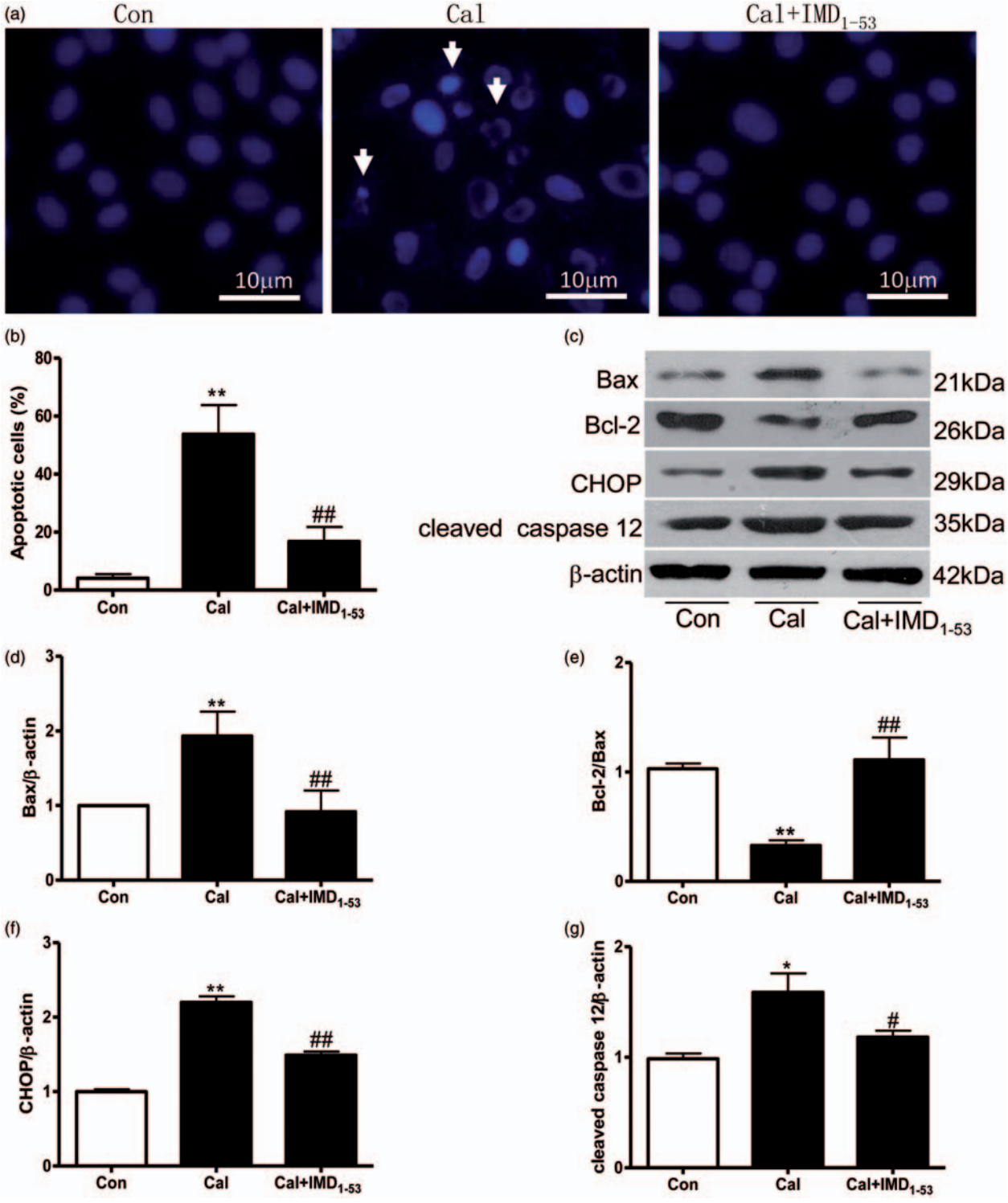

Figure 5 $IMD_{1–53}$ (10^{-7} mol/L) attenuated VSMC apoptosis during calcification. (a) Hoechst staining of VSMCs with or without $IMD_{1–53}$ (10^{-7} mol/L) during calcification (arrows, apoptotic cells). Magnification × 400. (b) The apoptotic nuclei were counted in at least 200 cells from five randomly selected fields in each treatment and expressed as a percentage of the total number of nuclei counted. (c) Western blot analysis of protein levels of Bax, Bcl-2, C/EBP homologous protein (CHOP), cleaved caspase 12 (β-actin was a loading control) and (d–g) quantification. Data are mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus Con; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Cal. (A color version of this figure is available in the online journal)

To investigate the effect of $IMD_{1–53}$ on ER stress, we used an inhibitor of N-glycosylation (Tm) and a reducing agent (DTT) to induce ER stress in cultured VSMCs. $IMD_{1–53}$ directly inhibited the Tm- and DTT-increased levels of ER stress factors in VSMCs. ER stress markers such as GRP78, CHOP and cleaved caspase-12 were upregulated in calcified VSMCs, and $IMD_{1–53}$ inhibited their upregulation. Simultaneously, $IMD_{1–53}$ ameliorated transformation

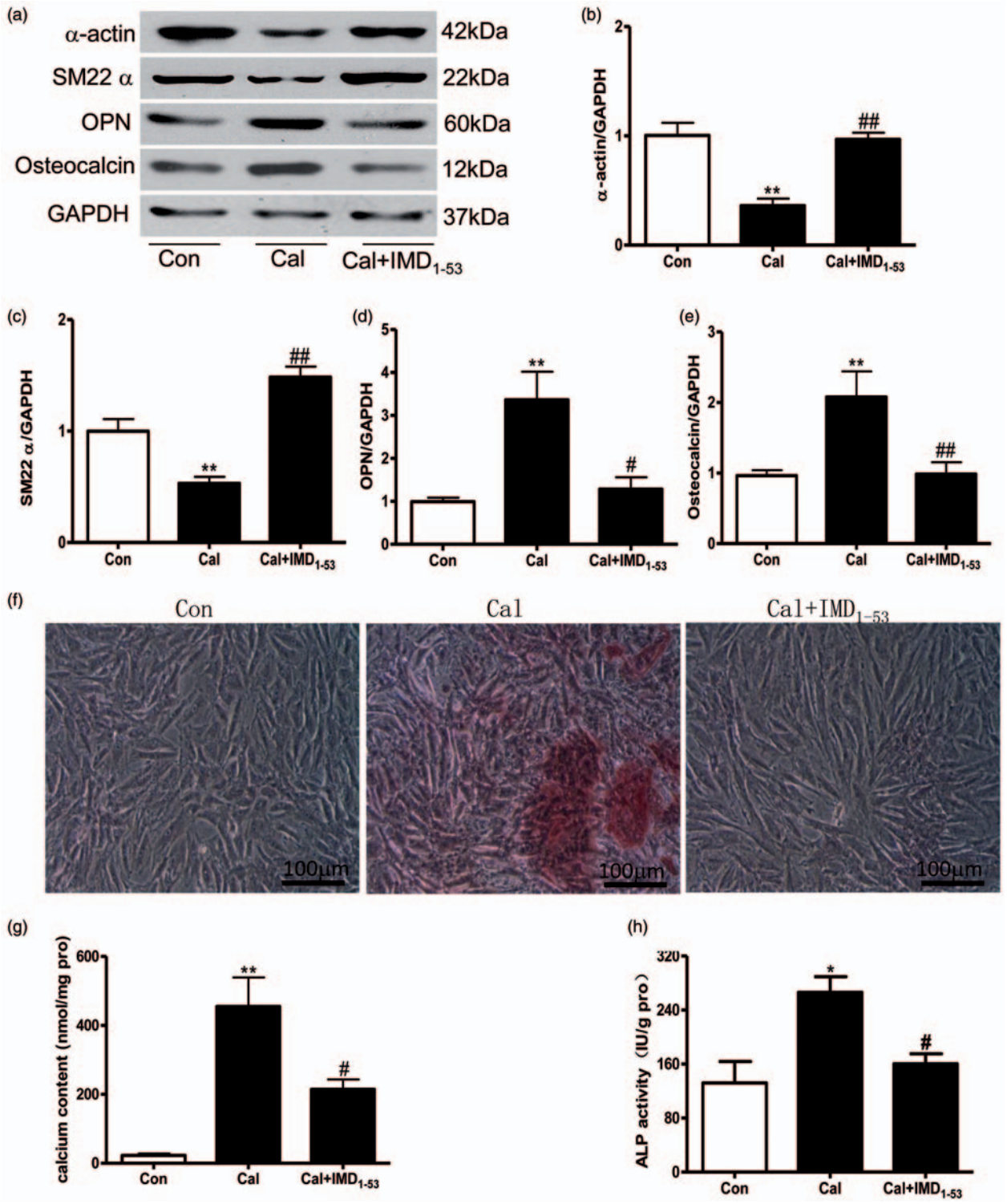

Figure 6 IMD_{1-53} (10^{-7} mol/L) restored VSMC phenotype and attenuated VSMC calcification. (a) Western blot analysis of protein levels of α-actin, smooth muscle 22 (SM22) α, osteopontin (OPN), osteocalcin (GAPDH was a loading control) and (b–e) quantification. Data are mean ± SD, $n = 3$, $^{**}P < 0.01$ versus Con; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Cal. (f) Alizarin Red S staining on day 14 during calcification of calcium deposition in calcified VSMCs with or without IMD_{1-53} treatment. Magnification × 200. IMD_{1-53} decreased calcium content (g) and alkaline phosphatase (ALP) activity (h) in calcified VSMCs. Data are mean ± SD, $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus Con; $^{\#}P < 0.05$ versus Cal. (A color version of this figure is available in the online journal)

of VSMCs into osteoblast-like cells as well as VSMC apoptosis and calcification. Therefore, the vasculo-protective effects of IMD_{1-53} on vascular calcification could be achieved at least in part by inhibiting VSMC ER stress.

In response to ER stress, ATF6 dissociates with GRP78 and translocates to the Golgi lumen where it is cleaved by proteases S1P and S2P, and then the cleaved ATF6 enters the nucleus and activates transcription of ER stress-response

genes such as GRP78 and CHOP. Besides, prolonged translation of ATF4 by PERK signalling was also responsible for CHOP transcription and a caspase-12 pro-apoptotic inducer protein expression.[3] We found that ATF6 and ATF4 upregulated with ER stress induced by ER stress inducers or calcifying media and IMD_{1-53} attenuated their overexpression. These results suggested that IMD_{1-53} inhibited the activation of ER stress in calcified VSMCs and restored the homeostasis of the ER. However, the detailed mechanism needs further study.

IMD acts nonselectively on CRLR/RAMP complex,[12] which is highly expressed in vasculature.[10,20,21] CRLR is one member of seven transmembrane G protein-coupled receptors.[22] It undergoes conformational alterations leading to coupling to Gs, activation of adenylate cyclase and intracellular cAMP.[23] The pharmacological roles of IMD are believed to be attributed to the activation of cAMP/PKA-signalling pathway, which plays an important role in cardiovascular diseases.[24–26] Our previous report indicated that ADM, which shares receptor complexes and structure homology with IMD, attenuates vascular calcification via the cAMP/PKA-signalling pathway *in vitro*.[27,28] In this work, we found that the PKA inhibitor H89 reversed the inhibitory effects of IMD_{1-53} on Tm- or DTT-induced ER stress and on VSMC calcification. The reduced expression of cleaved caspase 12 and CHOP might be due to the activation of cAMP/PKA signalling by IMD_{1-53}. These results suggest that IMD_{1-53} inhibited ER stress-induced apoptosis through the cAMP/PKA pathway. However, the detailed mechanism of the cAMP/PKA-signalling pathway inhibiting VSMC ER stress needs to be investigated further.

In conclusion, we found that IMD_{1-53} protected against VSMC calcification by inhibiting VSMC ER stress, and the signalling pathway of cAMP/PKA was involved at least in part in the inhibitory effect. IMD_{1-53} could attenuate VSMC ER stress-mediated apoptosis. IMD might be a new target to inhibit ER stress and alleviate VSMC calcification.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; J-RC performed the research and participated in data analysis and manuscript writing. X-HD, XT, Y-BZ and YL participated in the research; B-HZ and Y-RY analysed the data. YZ and C-ST participated in designing experiments and revising manuscript. Y-FQ designed and supervised the study, analysed the data and wrote the paper.

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