Induction of intracellular heat-shock protein 72 prevents the development of vascular smooth muscle cell calcification

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Aims
Vascular calcification (VC) is a significant contributor to cardiovascular mortality in patients with chronic kidney disease (CKD) and coronary artery disease (CAD). Osteo/chondrocytic transformation and simultaneous dedifferentiation of smooth muscle cells (SMCs) are important in the pathogenesis of VC. Heat-shock protein 72 (HSP72) is a cardioprotective inducible heat-shock protein that functions as a molecular chaperone. However, its role in the development of accelerated vascular dysfunction and calcification is largely unexplored.

Methods and results
We describe for the first time marked reduction in HSP72 expression in arteries from patients with CKD and CAD, compared with healthy controls, in vivo. Induction of HSP72 by heat-shock treatment (HST) significantly prevented the development of calcification of human aortic smooth muscle cells (HA-SMCs), in vitro. These anti-calcific effects were abolished following treatment with both quercetin, an HST inhibitor, and HSP72 siRNA knockdown. Induction of HSP72 suppressed Cbfa-1-dependent osteo/chondrocytic transformation and stabilized SMC contractile phenotype through the myocardin–serum response factor (SRF) pathway. Co-immunoprecipitation studies demonstrated physical association between SRF and HSP72. Furthermore, organ culture of arteries from CKD and CAD patients showed that these arteries retained their ability to induce HSP72 following HST, despite initially reduced expression.

Conclusion
Our study shows for the first time that intracellular HSP72 may function as a central regulator of molecular pathways involved in the development of VC. We suggest treatment strategies that up-regulate HSP72 as a new approach to inhibit VC.

Keywords
Vascular calcification • Heat shock protein • Core binding factor alpha 1 • Serum response factor • Smooth muscle cells

1. Introduction
Vascular calcification (VC) is highly correlated with cardiovascular mortality. It is particularly common in patients with coronary artery disease (CAD) and occurs at an accelerated rate in chronic kidney disease (CKD) as well as in diabetes and hypertension.1,2 These conditions can lead to chronic vascular stress that can cause local and diffuse calcification. The deposition of calcium minerals in the media of arteries ultimately results in vascular stiffening, increased pulse wave velocity, and pulse pressure.3

There is increasing evidence that osteo/chondrocytic transformation of vascular smooth muscle cells (VSMCs) and their dedifferentiation from a contractile to proliferative phenotype is important in the initiation and progression of VC. In vitro and in vivo studies have shown that this process can be driven under hyperphosphatemic conditions in the presence or absence of hypercalcaemia seen in CKD.4,5

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Unfortunately, there are currently no ideal therapies directed at the prevention or treatment of VC to combat this growing problem in clinical practice today.

Emerging evidence has demonstrated a cardioprotective role for the inducible protein, heat-shock protein 72 (HSP72). HSP72 belongs to the HSP70 family of proteins that function as molecular chaperones and protect newly synthesized proteins from aggregation and improper folding. Animal studies have shown that induction of HSP72 by heat-shock treatment (HST) reduces myocardial tissue damage and improves myocardial function following ischaemic injury. Clinical studies have reported an inverse relationship between circulating HSP72 vs. evidence of CAD and degree of atherosclerosis. Furthermore, a recent study has reported significantly lower HSP72 levels in blood monocytes from predialysis patients, and even lower levels in haemodialysis patients. This study is particularly significant since dialysis patients are well known to exhibit accelerated VC. However, the role of intracellular HSP72 in VC is a largely untouched area and little is currently known about the molecular mechanism underlying its protective effects.

We previously demonstrated that HSP72 can prevent vascular permeability changes in certain acute inflammatory processes, including anaphylactic shock and blood–brain barrier disruption, by stabilizing intracellular and membrane-bound proteins. In this study, we show for the first time that CKD and CAD are a state of endogenous HSP72 deficiency. Induction of HSP72 by HST in human aortic smooth muscle cells (HA-SMCs) prevented the development of VC through the inhibition of osteo/chondrocytic transformation and stabilization of SMC contractile phenotype, in vitro. These protective effects were abolished by quercetin, a chemical inhibitor of inducible HSPs, and following HSP72 siRNA studies. Furthermore, we show that arteries taken from CKD and CAD patients retain their ability to induce HSP72.

2. Methods

2.1 Human samples

Human samples were collected from healthy people donating a kidney, CKD patients undergoing a renal transplant, and CAD patients undergoing a coronary artery bypass operation. Human artery collection was performed at the University Hospitals Coventry and Warwickshire, UK. Ethical approval was obtained from the Coventry Research Ethics committee, UK and institutional review board approval for use of the human tissues at the Brigham and Women’s hospital was obtained from Partners Human Research Committee, USA. We declare that all work with human samples conform with the principles outlined in the Declaration of Helsinki.

2.2 Human aortic smooth muscle cells

Commercially available HA-SMCs were obtained from three different age-matched sources (Lot: 0295, 0573, 3523; ScienCell Research Lab, Carlsbad, CA, USA). HA-SMCs were cultured in 5% CO2/37°C incubator and grown with the SMC medium (SMCM) containing 0.5 mM phosphate and 1.6 mM calcium. HA-SMCs were seeded until 50% confluency and placed in opti-MEM I Reduced Serum (Invitrogen, CA, USA). After a washout period of 4 h, control samples were collected (0 h) and remaining arterial explants were incubated at 41°C for 30 min. Following HST, arterial rings were cultured for 24 and 48 h. PCR was used to detect HSP72 mRNA expression following human arterial organ cultures and this method is discussed in further detail in the supplemental Methods section.

2.3 Long-term calcification model, in vitro

HA-SMCs were grown to 80% confluence and then treated with control or calcification medium containing 5 mM calcium chloride (CaCl2; Cat No. 223506; Sigma, MO, USA) and β-glycerolphosphate disodium (Cat No. G9422; Sigma) for 21 days.

2.4 Long-term HST model, in vitro

HA-SMCs were grown to 80% confluence before commencing HST. HST was performed as previously described. Briefly, cells were placed into a pre-heated incubator at a temperature of 43°C for 30 min daily for 21 days.

2.5 Antibodies and assay kits

HSP72 antibody (Cat No. SPA-810F; AssayDesigns, MI, USA) was used at a concentration of 1:1000 for WB and at 1:200 for HIC. Osteocalcin (OC; Cat No. sc-74495; Santa Cruz, CA, USA) was used at a concentration of 1:500 for WB. Serum response factor (SRF) (Cat No. sc-56779; Santa Cruz Technology) was used at a concentration of 1:200 for WB. Cbfα1 antibody (Cat No. 05-1478; Millipore, MA, USA), Myocardin antibody (R&D Systems, MN, USA), Actin antibody (Cat No. MAB1501; Millipore), and α-smooth muscle actin (α-SMA) antibody (Cat No. A-5228; Sigma) were used at a concentration of 1:1000 for WB.

Alkaline phosphatase (ALP) was measured by Sensolyte® pNPP Alkaline Phosphatase Assay Kit (Ana Spec, CA, USA). Calcification was assessed using Alizarin red staining and the Arsenazo III (Fisher Scientific, USA) method (see Supplementary Methods for further details).

2.6 Arterial explants organ culture

Epigastric arteries (CKD patients) and mammary arteries (CAD patients) were dissected and arterial rings were placed in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, CA, USA). After a washout period of 4 h, control samples were collected (0 h) and remaining arterial explants were incubated at 41°C for 30 min. Following HST, arterial rings were cultured for 24 and 48 h. PCR was used to detect HSP72 mRNA expression following human arterial organ cultures and this method is discussed in further detail in the supplemental Methods section.

2.7 HSP72 siRNA transfection

HSP72 siRNA was purchased from Invitrogen (Part No. 4392422). Cells were seeded until 50% confluence and placed in opti-MEM I Reduced Serum Medium (Cat No. 31985-062, Invitrogen). Transfection was achieved using Lipofectamine (vector) reagent (Cat No. 15338-100, Invitrogen) and PLUS reagent (Cat No. 11514-015) at manufacturer’s recommended concentrations. For all experiments, 400 nM HSP72 siRNA was used with a transfection time of 24 h. Scrambled siRNA (Cat No. sc-37007, Santa Cruz) was used at a concentration of 400 nM in all experiments as a negative control.

2.8 Protein analysis

Detection of HSP72 on paraffin-embedded sections of human arteries was performed by immunohistochemistry. Western blotting and immunoprecipitation (IP) techniques were used for protein analysis of cell lysates. Protocols used for these techniques are provided in the supplementary methods section.

2.9 Statistical analysis

All experiments were performed at least three times and the results were expressed as the mean ± standard error. Analysis was performed using descriptive statistics, two-tailed paired t-test, or one-way ANOVA followed by Tukey’s post-hoc analysis as stated in the representative figure legend. P-values < 0.05 were considered statistically significant.
3. Results

3.1 CKD and CAD are a state of HSP72 deficiency associated with VC, *in vivo*

To evaluate whether HSP72 deficiency is involved in VC seen in CKD and CAD, we investigated the expression profile of HSP72 in human arteries from CKD and CAD patients compared with healthy controls. We show for the first time that HSP72 is expressed in the tunica media containing predominantly SMCs in human arteries (*Figure 1A*). Arteries from CKD and CAD patients showed markedly reduced expression with significant calcification compared with arteries from healthy controls. Calcification was assessed by Alizarin red staining. Western blot analysis confirmed higher expression of HSP72 in arteries from healthy controls compared with arteries from patients with CKD and CAD (*Figure 1B* and *C; *P* < 0.05). Demographic and clinical data of patients included in this analysis are provided in Table 1. These observations suggest that reduction in HSP72 levels may contribute to the development of accelerated VC. Note that CAD patients undergoing a bypass surgery included in this study have normal renal function (*Table 1*). Student’s *t*-test was used for statistical analysis.

3.2 Induction of HSP72 inhibits the development of vascular SMC calcification, *in vitro*

We developed a long-term *in vitro* calcification model using HA-SMCs cultured in the calcification medium for 21 days. Preliminary time- and dose-dependent studies showed that the calcification medium
Inducible HSP72 is an inhibitor of vascular calcification

3.3 Induction of HSP72 inhibits osteo/chondrocytic transformation of HA-SMCs under pro-calcific stress, in vitro

In order to assess whether HSP72 preventative effects against calcification occurred through the process of osteo/chondrocytic transformation, we evaluated expression levels of Cbpα1, the master regulator of bone-differentiation, and its downstream regulated proteins, OC and ALP. Cbpα1, OC, and ALP (Figure 3A) levels were upregulated in HA-SMCs grown in our long-term calcification medium, suggesting osteo/chondrocytic transformation from a SMC phenotype. However, induction of HSP72 by HST inhibited upregulation of these markers. These effects were abolished following HSP72 siRNA knockdown. Taken together, these results support the notion that HSP72 may prevent the development of calcification through the suppression of HA-SMC phenotypic adaptation to osteo/chondrocytic-like cells.

3.4 Induction of HSP72 stabilizes SMC contractile phenotype under pro-calcific stress, in vitro

Myocardin is the master regulator of the SMC contractile phenotype and functions as a cofactor for SRF to regulate transcription of smooth muscle contractile genes, including α-SMA. Myocardin, SRF, and α-SMA expression were suppressed in our long-term calcification model, suggestive of HA-SMC dedifferentiation from a contractile phenotype (Figure 3B). However, HST fully restored these markers under pro-calcific stress. These SMC stabilizing effects were abolished by HSP72 knockdown by siRNA. Our data point to a SMC contractile stabilizing role of HSP72 involved in its anti-calcific effects (Figure 3B; \( P < 0.05 \)).

To further investigate the molecular mechanism of HSP72 in the stabilization of SMC phenotype, we assessed HSP72, SRF, and myocardin protein–protein interactions. Using co-immunoprecipitation (Co-IP) studies, we demonstrate that induction of HSP72 results in SRF–HSP72 protein–protein interactions. Using co-immunoprecipitation (Co-IP) studies, we demonstrated that induction of HSP72 through Co-IP studies, we demonstrated that induction of HSP72 results in SRF–HSP72 protein–protein interactions. Using co-immunoprecipitation (Co-IP) studies, we demonstrated that induction of HSP72 results in SRF–HSP72 protein–protein interactions.

Table 1 Summary of demographic and clinical data of patients included for arterial HSP72 expression analysis

<table>
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<tr>
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<th>CAD</th>
<th>CKD</th>
<th>P-value</th>
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<td>Age (mean ± SD; range; yrs)</td>
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<td>On dialysis</td>
<td>0.09 –</td>
</tr>
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</table>

The table summarizes the demographic and clinical data of patients included for arterial HSP72 expression analysis. The data includes the number of patients, age, weight, gender, ethnicity, presence of hypertension, diabetes, smoking, creatinine levels, and eGFR, categorized into three groups: Healthy control (C), CAD, and CKD. The P-values for comparisons between groups are also provided.
Figure 2 Induction of HSP72 prevents the development of smooth muscle cell calcification, in vitro. (A) Human-aortic smooth muscle cells (HA-SMCs) grown in calcification medium in the absence of heat shock treatment (HST) developed severe calcification with marble red appearance. HST significantly inhibited the development of calcification with the absence of marble red staining. Both quercetin, an inhibitor of inducible heat shock proteins and HSP72 siRNA abolished the calcification preventative effects of HST. Calcification was qualitatively assessed by Alizarin red staining and quantitatively using the Aresenazo III method \( n = 6 \). ** \( P < 0.01 \), target groups vs. control group; *\( P < 0.05 \), target groups vs. calcification medium treated group. Calcification medium: 5 mM β-Glycerolphosphate + 5 mM CaCl\(_2\); scale bar: 100 μm. (B) HSP72 was significantly increased following HST in both the calcification medium treatment and non-calcification medium treatment groups. No induction was observed when HA-SMCs were exposed to calcification promoting treatment alone. However, HST with the addition of quercetin inhibited induction of HSP72 \( n = 3 \). *\( P < 0.05 \). (C) HSP72 was significantly increased following HST and HSP72 siRNA inhibited induction of HSP72. Scrambled SiRNA did not inhibit the induction of HSP72 under HST. Of note, exposure time of the blots in figure (B) was longer than in figure (C) to illustrate that very slight expression of HSP72 in control can be detected only by significant over-exposure of the blot. Calcification medium: 5 mM β-Glycerolphosphate + 5 mM CaCl\(_2\); \( n = 3 \) **\( P < 0.01 \), target groups vs. control group; *\( P < 0.05 \), target groups vs. heat shock treated group; #\( P < 0.05 \), target groups vs. calcification medium treated and HST group. One-way ANOVA followed by Tukey’s post-hoc analysis was used for statistical analysis.
3.5 Organ culture of arteries from CKD and CAD patients retains their ability to induce HSP72 mRNA following HST

Human arteries from healthy controls, CKD and CAD patients were subjected to organ culture and HST (Figure 4). We show that HSP72 mRNA expression was higher in the healthy control group compared with CKD and CAD groups before HST, consistent with our findings from immunohistochemistry and western blot in Figure 1. Down-regulation of HSP72 mRNA in the healthy control group following HST suggests a negative feedback response due to existing high protein expression levels (Figure 4).22

Interestingly, HSP72 mRNA expression peaked at 24 h following HST in the CKD and CAD groups and are consistent with previous studies, in vivo (Figure 4; *P < 0.05). At 48 h following HST, HSP72 mRNA expression declined to near basal levels in the CKD and CAD groups. These results demonstrate that arteries from CKD and CAD patients retain the ability to express HSP72 under HST.

4. Discussion

The data presented in this study suggest that HSP72 is a potential target for the therapeutic treatment of VC. We show for the first time deficient expression of intracellular HSP72 in artery SMCs from patients with CAD or CKD. These arteries retain their ability to induce HSP72 expression. Furthermore, we confirmed induction of HSP72 as a powerful inhibitor of in vitro VSMC calcification by stabilizing the contractile phenotype, regulating the cellular stress response, and inhibiting transformation to the calcifying phenotype.
HSP72 functions at the cellular level to protect cells against many acute and chronic stress conditions and stabilizes the normal physiological function of proteins. Studies have shown an inverse relationship between circulating HSP72 levels and the severity of atherosclerosis. In our study, their vasculo-protective function appears to be unmasked from patients with cardiovascular disease or CKD. Compared with healthy people, we found reduced HSP72 mRNA and protein expression in SMCs of arteries from these patients. Similarly, attenuated cellular HSP72 expression was previously documented in peripheral macrophages of patients with CKD and CAD patients at baseline before HST, consistent with its protein expression patterns in Figure 1. In healthy controls, HSP72 mRNA levels did not increase following HST at 24 h or 48 h, suggesting a negative feedback response due to existing high protein levels. However, HSP72 mRNA levels significantly increased in arteries from CKD and CAD patients after 24 h. HSP72 mRNA levels declined to near basal levels at 48 h following HST. These results indicate that arteries from CKD and CAD patients retain their ability to express HSP72. 

Our results showed that induction of HSP72 using HST inhibited osteogenic transformation via the Cbfa1 pathway and stabilized SMC phenotype with up-regulation of myocardin and α-SMA expression in HA-SMCs, in vitro. We also demonstrated that HSP72 may stabilize the smooth muscle phenotype through its association with SRF in the prevention of calcification. Myocardin is the master regulator of smooth muscle differentiation and interacts with the MAD5 box transcription factor, SRF. SRF binds to a sequence known as CArG-box motif to regulate transcription of smooth muscle contractile genes, including α-SMA. Differentiation of smooth muscle is characterized by high expression levels of smooth muscle contractile genes, while dedifferentiation results in loss of their expression. Loss of myocardin expression has been recently described in calcified arteries from matrix Gla protein deficient (MGP−/−) mice. It is likely that loss of myocardin expression in calcification may alter arterial contractility and response to hyperphosphatemic and haemodynamic stress seen in CKD. This places myocardin/ SRF at a critical nodal point necessary to maintain smooth muscle phenotype and contractile function. Taken together, suppression of osteogenic transformation and simultaneous stabilization of SMC contractile phenotype points to the utilization of multi-modal pathways by HSP72 in the prevention of vascular dysfunction (Figure 5).

Despite suppressed HSP72 expression in medium-sized arteries from CKD or CAD patients, we found that arteries retained their ability to induce HSP72 expression in organ culture following HST. Similar results, with strongly induced vascular HSP72, were described previously in the artery and vein organ culture after hypoxia and HST. Together with similar findings in multiple animal models for cardiac ischaemia and patients with CAD, inducible HSP72 may provide a therapeutic opportunity for progressive artery
Inducible HSP72 is an inhibitor of vascular calcification


