Lipoxin Alleviates Diabetic Vascular Calcification via the YAP Pathway

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Keywords: vascular smooth muscle cells, vascular calcification, advanced glycation end products, lipoxin, yes-associated protein

Posted Date: December 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1179483/v1

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Abstract

Background: Vascular calcification is highly prevalent in patients with diabetes and has detrimental consequences. However, no effective prevention and treatment methods are currently available. Extensive evidence has demonstrated the protective effect of lipoxin (LX) against vascular diseases. However, whether LX prevents diabetic vascular calcification remains unknown. Here, we tested the hypothesis that LX alleviated osteogenic differentiation and subsequent calcification of vascular smooth muscle cells (VSMCs).

Methods: In vitro, human aortic smooth muscle cells (HASMCs) were incubated in osteogenic medium (OM) with advanced glycation end products (AGEs) and LX to further determine the underlying mechanisms. An in vivo diabetic mouse model was established using a combination of a high-fat diet and multiple formulations of low-dose streptozotocin (STZ). Cell culture, alkaline phosphatase (ALP) staining, ALP activity, Alizarin red staining, von kossa staining, determination of calcium content, western blot analysis, immunohistochemistry, and immunofluorescence staining and statistical analysis were used in our study.

Results: AGEs dose-dependently induced calcification and expression of osteogenesis-related markers, including Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), and type I collagen (COL1), coupled with the activation of yes-associated protein (YAP). Mechanistically, YAP activation enhanced the AGE-induced osteogenic phenotype and calcification, but inhibition of YAP signalling alleviated this trend. Consistent with the in vitro results, diabetes promoted YAP expression as well as the subcellular localisation of the protein in the nucleus in the arterial tunica media. Interestingly, treatment with LX reduced vascular osteogenesis and calcification in diabetic mice, which was correlated with the reduced YAP levels. In addition, LX significantly inhibited COL1 accumulation and modulated the extracellular matrix. Our results further demonstrated that a pharmacological agonist of YAP reversed LX-mediated protection against osteogenic phenotypic conversion and calcification in VSMCs.

Conclusions: These results demonstrate that LX attenuates transdifferentiation and calcification of VSMCs in diabetes mellitus via the YAP signalling axis, suggesting that LX is a potent therapeutic strategy to prevent diabetic vascular calcification.

1. Background

Vascular calcification is a common and severe comorbidity and complication of chronic diseases, including diabetes mellitus [1], chronic kidney disease [2], and ageing [3]. Although vascular calcification has been demonstrated to be an active, cell-mediated, and reversible process [4], no treatments have been developed to prevent, attenuate, or reverse vascular calcification [5]. Vascular calcification is common in diabetics and has detrimental consequences [6], which increases the risk of cardiovascular and all-cause mortality [7, 8] and is an independent risk factor for the prediction of cardiac events [9]. Cumulative
evidence suggests that advanced glycation end products (AGEs), which are metabolites caused by high glucose levels [10], are involved in the initiation and propagation of vascular calcification [11, 12].

Vascular smooth muscle cells (VSMCs) are the main cell type driving vascular calcification [13]. In VSMCs, AGEs prompt vascular calcification via various mechanisms, including promotion of osteogenic phenotypic transition into osteoblast-like cells [14], active extracellular matrix remodelling [15], cell apoptosis [16], and loss of calcification inhibitors [17]. The transdifferentiation of VSMCs into an osteogenic phenotype is characterised by the downregulation of smooth muscle cell-specific contractile markers [18, 19] and upregulation of bone-related proteins [20, 21], including Runx-related transcription factor 2 (Runx2) [22, 23] and its downstream genes [24, 25]. Runx2 is required for osteogenic differentiation and subsequent calcification of VSMCs in both the medial [26] and intimal [27] calcification models. Runx2 is regulated by various signalling pathways, including yes-associated protein (YAP) [28-30]. The main function of YAP is to regulate cell proliferation and organ size [31, 32], but emerging evidence suggests that it may modulate cell differentiation, including osteogenic differentiation in osteoblasts [33], chondrocytes [28], and mesenchymal stem cells [34]. Currently, several lines of evidence have suggested that YAP expression increases in response to arterial injury [35], and YAP inhibition may inhibit phenotypic conversion and maintain the contractile phenotype of VSMCs [36]. Furthermore, diabetes may induce the expression of YAP in retinal tissues of a streptozotocin (STZ)-treated mouse model [37]. Therefore, we speculated YAP levels may increase in diabetic mouse vasculature, and we hypothesised that finding efficient drugs to suppress YAP activation may alleviate osteogenic differentiation and subsequent calcification.

Notably, G-protein-coupled receptor (GPCR) ligands have been shown to activate or inhibit YAP in a manner dependent on G protein activation [38], and the receptor of lipoxin (LX) belongs to the GPCR family [39]. LX may exert robust immunoregulatory and anti-inflammatory activities [40] via formyl peptide receptor 2 and A-type lipoxin receptor (FPR2/ALX) [41]. LX is a specialised pro-resolving lipid mediator (SPM) [42] that has recently been shown to play a role in the functioning of vascular wall cells, such as VSMCs [43, 44]. In some studies, LX was reported to modulate the VSMC phenotype [45] and attenuate diabetes-associated atherosclerosis [46]. However, the mechanisms underlying this process remain unclear. Thus, we investigated the protective effect of LX and whether LX may function as a therapeutic target for diabetic vascular calcification.

2. Methods

2.1 Materials

Human aortic smooth muscle cells (HASMCs), smooth muscle cell medium, foetal bovine serum (FBS), smooth muscle cell growth supplement, and penicillin/streptomycin solution were purchased from ScienCell (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibico (Grand Island, NY, USA). AGE-BSA was obtained from Abcam (Cambridge, UK). Lysophosphatidic acid (LPA), L-ascorbic acid and STZ were acquired from Sigma-Aldrich (St. Louis, MO, USA). Atorvastatin (ATV)
was purchased from MCE (New Jersey, NJ, USA). LXA4 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Dexamethasone and β-glycerophosphate were obtained from Aladdin (Shanghai, China). Antibodies against YAP and pYAP were obtained from Cell Signalling Technologies (Danvers, MA, USA). Antibodies against RUNX2 and smooth muscle actin (SMA) were acquired from Abcam (Cambridge, UK). Antibodies against osteopontin (OPN) and type I collagen (COL1) were purchased from Santa Cruz (Santa Cruz, CA, USA).

2.2 Cell culture

HASMCs were cultured in smooth muscle cell medium supplemented with 2% FBS, 1% smooth muscle cell growth supplement, and 1% penicillin/streptomycin solution. All experiments were performed using HASMCs at passage 3–6.

2.3 In vitro calcification of VSMCs

Osteogenic medium (OM; DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 10 nM dexamethasone, and 100 μM L-ascorbic acid) was used to induce VSMC calcification [47]. To explore the role of AGEs in vascular calcification, AGEs were applied to treat VSMCs in OM for 7 [48] or 14 [49] days at different concentrations (0, 25, 50, 100, 200, or 400 μg/mL). Calcification was observed by alkaline phosphatase (ALP) staining, Alizarin red staining, and von Kossa staining.

2.4 Cell viability assay

VSMCs were cultured in 96-well plates for 24 h and treated with different concentrations of AGEs (0–400 μg/mL) or LX (0–100 nM) in OM for 1, 3, and 7 days. After treatment, 10 μL CCK8 (Dojindo Laboratories, Kumamoto, Japan) was added to 100 μL fresh medium in each well and incubated for 2 h at 37 °C. A microplate reader (Tecan, Mannedorf, Switzerland) was used to measure the absorbance at 450 nm.

2.5 Animal and experimental models

Six-week-old male C57BL/6J mice susceptible to STZ were acclimatised for one week to the conditions of the animal room. All animal experiments were performed in compliance with the Animal Ethics Procedures and Guidelines of the People's Republic of China and were approved by the Harbin Medical University Animal Ethics Committee. After one week of adaptation, the mice were randomly divided into four groups: (1) control (CON) group, (2) LX group, (3) diabetes mellitus (DM) group, and (4) DM+LX group. Diabetic mice were induced as previously described [50, 51] and fed with a commercial high-fat diet for four weeks, followed by intraperitoneal injection of low-dose STZ (50 mg/kg) for five days during the last week of the high-fat diet. Nondiabetic mice were fed a normal diet and injected with buffer solution. The diabetic model was successfully established in mice with fasting blood glucose (FBG)
levels of $\geq 11.1$ mM two weeks after the induction of diabetes [52]. Mice were treated with either ethanol (0.1%) or LXA$_4$ (5 $\mu$g/kg) twice weekly by intraperitoneal injection two weeks after injection of the first STZ dose or buffer solution. FBG levels and body weight were monitored after STZ injection during the study to validate the diabetic status of the mice. At the experimental end points, the mice were euthanised, and the aortic arch and descending aorta were dissected under a microscope.

### 2.6 Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin sensitivity test (IPIST)

For both IPGTT and IPIST, blood samples were collected from the tail vein after 16 h fasting. To conduct IPGTT, blood glucose levels were assessed as threshold glucose levels (0 min) before intraperitoneal injection of glucose load (2 g/kg) [53]. Blood glucose levels were estimated at 30, 60, 90, and 120 min following injection with a calibrated glucometer (Roche, Basel, Switzerland). Similar to IPGTT, the IPIST was established with intraperitoneal injection of insulin at a dose of 0.75 U/kg [54], after which blood glucose was measured 30, 60, 90, and 120 min after injection.

### 2.7 ALP staining and ALP activity assay

VSMCs were fixed with 4% paraformaldehyde and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium mixture (Beyotime, Shanghai, China) for 30 min. After washing, photographs were taken under a digital microscope (Olympus, Tokyo, Japan). The ALP activity assay kit (Beyotime) uses p-nitrophenyl phosphate as a phosphatase substrate, which turns yellow ($\lambda_{\text{max}} = 405$ nm) when dephosphorylated by ALP. As previously described [55], total proteins of cells were first extracted by centrifugation in lysis buffer, and then their ALP activity was measured colourimetrically. The results were normalised to total protein levels determined using a bicinchoninic acid (BCA) assay kit (Beyotime).

### 2.8 Alizarin red staining, von Kossa staining, and determination of calcium content

VSMCs were fixed with 4% paraformaldehyde and stained with Alizarin red staining solution (Cyagen, Guangzhou, China) for 10 min. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 $\mu$m. After conventional dehydration, sections were stained with Alizarin red solution for 10 min and washed with distilled water. After xylene treatment, the slides were sealed with gel resin.

VSMCs and tissue slices were incubated with 1% silver nitrate solution for 30 min under ultraviolet light. They were then washed with 5% sodium thiosulfate solution for 2 min and counterstained with Nuclear Fast Red for 3 min. Images were obtained using a microscope (Olympus).
Calcium content in VSMCs and aortas was quantified with a calcium colorimetric assay (Beyotime), and total protein concentration was measured using the BCA method. Calcium content was normalised to the total protein concentration.

2.9 Western blot analysis

Proteins were extracted from cells or tissues with RIPA buffer, fractionated by SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in the blocking agent (5% non-fat dried milk in Tris-buffered saline with Tween 20) for 1 h and incubated overnight at 4 °C with a primary antibody. After three cycles of cleaning with Tris-buffered saline-Tween 20, the membranes were incubated with appropriate secondary antibodies and observed by enhanced chemiluminescence. ImageJ software was used for analysis.

2.10 Immunohistochemistry

Briefly, sections were dewaxed, rehydrated, blocked for endogenous peroxidase activity and nonspecific binding, and incubated overnight with primary antibodies (YAP, 1:200) at 4 °C. After three cycles of washing with phosphate-buffered saline, the sections were incubated with secondary antibody at 37 °C for 30 min. Bound secondary antibodies were detected using diaminobenzidine solution.

2.11 Immunofluorescence staining

For immunofluorescence staining analysis, VSMCs and slides were stained with antibodies specific to RUNX2, SMA, or YAP.

2.12 Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8. Independent experiments were performed using different cell batches. Differences between groups were determined using the Student’s t-test or one-way analysis of variance (ANOVA). Results with two or more variables were analysed using a two-way ANOVA. Where normality could not be confirmed, data were analysed using the Mann-Whitney U test. Statistical significance was defined as \( P < 0.05 \).

3. Results

3.1 AGE-induced VSMC calcification
To determine a suitable concentration of AGEs for osteogenic differentiation and the subsequent VSMC calcification, CCK-8 assay was used to test cell viability. We cultured VSMCs with AGEs (0–400 μg/mL) for 1, 3, and 7 days in OM. As shown in Supplementary Figure 1A, AGEs did not affect VSMC viability at all concentrations tested. Thus, we may use 25–400 μg/mL of AGEs in the follow-up study.

To assess the capacity of calcification induced by AGEs in VSMCs, we first investigated whether AGEs may increase the activity of ALP, an early marker of osteogenic differentiation and calcium nodule formation [56]. Therefore, we examined ALP staining and calcium nodule staining in control media (CM) and in OM with AGEs (0–400 μg/mL). ALP activity of VSMCs in the CM group was markedly lower than that in the OM group. Our results further demonstrated that elevated concentrations of AGEs increased ALP activity (Figure 1A). Interestingly, VSMCs in CM were devoid of nodular staining, whereas nodular staining gradually increased with AGE concentration (Figure 1B). In addition, von Kossa staining showed granular calcification localised in the cell and extracellular matrix regions of the cultures (Supplementary Figure 1B). In contrast with 0 μg/mL AGEs, marked augmentation of ALP activity and calcium content was observed when VSMCs were treated with 100, 200, or 400 μg/mL AGEs, as indicated in Figure 1C and 1D.

As expected, AGEs reduced the expression of the smooth muscle cell-specific contractile marker SMA and elevated the expression of osteogenic markers RUNX2, OPN, and COL1 (Figure 1E and 1F). These results indicated that 100, 200, and 400 μg/mL AGEs dramatically induced VSMC calcification. YAP expression was profoundly induced in parallel with the expression of osteogenesis-related markers. Furthermore, YAP serine 127 phosphorylation significantly decreased as the concentrations of AGEs increased (Figure 1G), suggesting that YAP was activated by AGEs.

3.2 AGEs activated YAP under calcifying conditions

To identify the role of AGEs during VSMC calcification, VSMCs induced with 100 μg/mL AGEs were monitored by specific staining and western blotting at the following stages of calcification: the early stage (day 7), the medium stage (day 9), and the late stage (day 14). The staining of ALP was observed at the early stage of calcification in VSMCs induced by 100 μg/mL AGEs (Figure 2A). Calcification induced by 100 μg/mL AGEs was time-dependent. Notably, calcification nodules were not detectable at the early stage of calcification. However, sparse and spotted precipitates were observed in VSMCs and the surrounding extracellular matrix at the medium stage of calcification (Figure 2B and 2C), suggesting the involvement of the extracellular matrix in vascular calcification.

During calcification formation, RUNX2 expression gradually increased, and conversely, the levels of SMA reduced (Figure 2D and 2E). During the early stage of calcification, YAP levels were low. YAP expression was moderately elevated during the middle stage of calcification but significantly elevated during the late stage. As expected, immunoblot analysis validated the decreased expression of phosphorylated YAP (Figure 2F). These findings indicate that YAP activation may play a role in the process of VSMC calcification.
3.3 AGE-induced VSMC calcification through a YAP-dependent pathway

To determine the potential involvement of YAP in AGE-induced VSMC calcification, we applied the YAP inhibitor ATV [57] and the YAP agonist LPA [58] in OM. We observed ATV attenuated calcium deposition, and LPA increased calcified deposits in OM. These findings suggest YAP can modulate VSMC calcification. As expected, AGEs induced severe calcium deposition in OM, which was significantly attenuated by ATV. In addition, LPA further increased calcium deposits in the presence of AGEs, as shown in Figure 3A–C.

To investigate the underlying mechanisms of YAP-mediated VSMC calcification, we examined the protein expression levels. RUNX2 levels were upregulated by LPA, whereas SMA levels were downregulated. However, ATV induced the opposite trend. Furthermore, ATV reversed the AGE-induced increase in RUNX2 levels, and LPA further aggravated the increased levels of RUNX2 in response to AGEs (Figure 3D–F).

3.4 LX attenuated vascular calcification in diabetic mice

To evaluate whether LX may attenuate vascular calcification in DM, a diabetic mouse model was established using a combination of a high-fat diet and multiple low-dose STZ. LX was intraperitoneally injected twice weekly from weeks 6–25 of the experiment, as described in the methods section (Figure 4A). Notably, all diabetic mice had lower body weight and increased FBG levels compared to nondiabetic mice (Figure 4B and Supplementary Figure 2A). Glucose homeostasis was evaluated using IPGTT and IPIST at the end of the study. For IPGTT, compared to nondiabetic mice, the diabetic mice showed delayed glucose clearance and high blood glucose levels for 120 min (Supplementary Figure 2B). For IPIST, all mice exhibited reduced blood glucose levels, but the diabetic mice had higher blood glucose levels than the nondiabetic mice (Supplementary Figure 2C). In both IPGTT and IPIST, the area under the curve (AUC) value in diabetic mice was significantly increased compared with that in nondiabetic mice. The diabetic mice given multiple low-dose STZ following a high-fat diet had impaired glucose tolerance and insulin resistance ($P < 0.0001$) (Supplementary Figure 2D and 2E). Interestingly, treatment with LX had no significant effect on blood glucose levels in IPGTT and IPIST, indicating that the role of LX in diabetic mice was not induced by glycaemic control ($P > 0.05$).

Next, we evaluated the effect of LX on medial arterial calcification in diabetic mice. Calcium precipitation is a major event in VSMC calcification. Von Kossa staining and alizarin red staining in the aortas indicated multifocal calcification lesions in diabetic mice (Figure 4C and 4D). However, LX treatment significantly lowered aortic calcification in the DM+LX group compared to that in the DM group. Elastin Van Gieson staining also demonstrated that vessels of diabetic mice exhibited more flattening and degrading elastin fibres and collagen accumulation compared to those of the nondiabetic mice (Figure 4D). Aortic calcium content analysis further confirmed that calcium deposition was higher in diabetic mice than in the nondiabetic mice. However, treatment with LX mitigated these effects (Figure 4E).
Transdifferentiation of the osteogenic phenotype of VSMCs is another key event in vascular calcification. LX alleviated the loss of the contractile phenotype markers SMA and the gain of the osteogenic marker RUNX2, as demonstrated by IF and WB (Figure 4F–H). Interestingly, in healthy control mouse vessels, YAP was expressed slightly. However, the YAP levels in diabetic mice increased significantly and were primarily localised in the nuclei but were reduced moderately with LX treatment (Figure 4F and 4I). Consistent with the in vitro data during osteogenic phenotypic drift of AGE-induced VSMCs, YAP expression was profoundly induced in parallel with the expression of osteogenesis-related markers.

3.5 LX inhibited AGE-induced calcification in VSMCs

We first confirmed the effect of LX on cell viability using CCK-8 assay. The results suggested that LX at the concentrations used had little effect on VSMC viability, as compared to OM alone (Supplementary Figure 3A).

We observed that staining intensity decreased with increasing LX concentration, as depicted by ALP staining, Alizarin red staining, and von Kossa staining (Figure 5A, 5B, and Supplementary Figure 3B). These quantitative results of ALP activity and calcium content further demonstrated the dose-dependent inhibitory effect of LX on AGE-induced VSMC calcification. For example, compared to VSMCs treated with 0 nM LX, VSMCs treated with 100 nM LX showed significantly decreased ALP activity (1.609 ± 0.4541 U/mg protein) and calcium content (45.870 ± 5.655 μg/mg protein) (Figure 5C and 5D).

We then explored whether low doses of LX distinctly affect differentiation to an osteogenic phenotype in AGE-induced VSMCs. The expression of the contractile and osteogenic phenotype genes was changed significantly after LX treatment in AGE-induced VSMCs (Figure 5E and 5F). Inhibition of osteogenic signalling by LX was accompanied by YAP inhibition (Figure 5G).

3.6 LX exerted an anti-calcification role by inhibiting AGEs-induced YAP activation

To confirm that LX may suppress calcification induced by AGEs via YAP, VSMCs were cultured with AGEs plus LX or LPA. AGEs enhanced ALP staining, Alizarin red staining, and von Kossa staining. However, LX alleviated the enhanced staining induced by AGEs, and this was rescued by LPA (Figure 6A–C).

LX significantly ameliorated AGEs-induced osteogenic transdifferentiation in VSMCs, and the decreasing trend was counteracted by LPA (Figure 6D–G). As depicted in Figure 6H, YAP was predominantly localised to the nucleus in the cultured VSMCs, in line with previous results [35]. AGEs enhanced YAP and RUNX2 staining; however, LX alleviated this effect. As expected, LPA may rescue the LX-mediated decreased trend of YAP and RUNX2 expression.

4. Discussion
There are three major striking observations in our results that support the novel protective effect of LX against diabetic vascular calcification via YAP. First, the expression and subcellular localisation of YAP in the nucleus is enhanced in VSMCs induced by AGEs and in vascular tissues of diabetic mice, accompanied by increased expression of osteogenic markers. Second, this study is the first to demonstrate that LX attenuates diabetic vascular osteogenesis and calcification via YAP signalling and subsequent coordinated expressional regulation. Third, the underlying mechanism involves extracellular matrix remodelling. We propose a novel mechanism for the protective effect of LX against vascular calcification by inhibiting extracellular matrix generation.

To our knowledge, this is the first study to evaluate YAP expression in AGE-induced VSMCs. We observed AGEs had a dose-dependent effect on YAP expression, which increased with increasing AGE concentrations (Figure 1E and 1G). In addition, the phosphorylated YAP (pYAP, S127) signal also decreased with increasing AGE concentrations, suggesting that YAP signalling is activated during diabetic conditions. Our results showed AGEs enhanced calcium deposition (Figure 1B, 1D, and Supplementary Figure 1B) and osteogenic phenotypic markers (Figure 1E), and these correlated with the upregulation of YAP accumulation. We further excluded the possibility that AGEs induced calcium deposition by influencing VSMC viability (Supplementary Figure 1A).

A diabetic mouse model was established using a combination of a high-fat diet and multiple low-dose STZ, which imitated the natural history of DM (impaired glucose tolerance and insulin resistance) (Supplementary Figure 2B–E). The model also exhibited similar metabolic features, including significantly elevated levels of AGEs [59, 60], which gradually developed vascular calcification in the media [61]. YAP was expressed at low levels and localised mostly in the cytoplasm of vascular tissues of nondiabetic mice (Figure 4I), which is consistent with the results of the only other study related to YAP and vascular calcification [62]. In contrast, YAP was expressed at high levels and localised in the nucleus in the arterial tunica media of diabetic mice, coupled with the in vitro results indicating that YAP upregulation was induced by local VSMCs (Figure 1E). These results implied diabetic conditions caused YAP activation, which further induced enhanced vascular calcification. A previous study confirmed that VSMCs expressed higher levels of YAP in response to injury stimuli to further contribute to neointima formation [35]. Extended periods of increased YAP expression led to vascular diseases, such as vascular calcification and neointima formation. This novel finding has promising implications for preventing diabetic vascular calcification by targeting YAP.

Osteogenic phenotype shift in VSMCs is a key event in the mechanism of vascular calcification [63]. Accumulating evidence has confirmed that YAP plays a crucial role in the phenotypic conversion of VSMCs, which modulates vascular remodelling [3, 35]. Nevertheless, the mechanism by which YAP modulates osteogenic reprogramming remains to be clarified.

Osteogenic phenotypic transition is characterised by the downregulation of contractile markers and upregulation of osteogenic-related markers [3, 63]. Our results illustrated that AGEs significantly induced osteogenic phenotypic transition in vitro, and this was accompanied by YAP upregulation (Figure 1E). We
further identified that pharmacological inhibition of YAP mitigated this trend, suggesting that AGES induced osteogenic phenotypic switching via YAP signalling (Figure 3D). Consistent with these results, we also found that in vivo YAP upregulation was related to enhanced trans-differentiation to the osteogenic phenotype of VSMCs in diabetic vessels (Figure 4E–I). Our results support the notion that YAP signalling is a major driver of the osteogenic phenotypic conversion of VSMCs. This finding agrees with previous reports showing that suppression of YAP maintains smooth muscle cell-specific contractile markers [64-66].

However, the only other study associated with YAP and vascular osteogenic differentiation showed that VSMCs lost the contractile phenotype and underwent osteogenic differentiation in SM-YAP/Taz-KO mice, followed by vascular calcification. These apparent discrepancies could be due to the different states of VSMCs, including pathological or physiological conditions. In VSMCs of normal mature arteries, YAP is retained in the cytoplasm and interacts with DVL3 to avoid nuclear translocation and osteogenic differentiation. However, in many disease states, YAP is recruited to the nucleus [67-69]. These results further confirmed that subcellular localisation of YAP affected its ability to function, which is in line with previous studies [70, 71]. The results of this study show that the subcellular localisation of YAP in the nucleus is increased in the vasculature of diabetic mice (Figure 4I) and in AGE-induced VSMCs (Figure 6H), which offers a novel mechanism for its nuclear accumulation.

Resolvin, protectin, and maresin, with LX, are referred to as SPMs, which carry out robust immunoregulatory and proresolving activities [72-74]. The link between LX and vascular calcification has not been previously investigated; however, SPMs and their receptors prevent vascular calcification [75-77]. We have demonstrated for the first time that LX has a protective effect against vascular calcification in response to diabetes in vivo and in vitro. In a mouse model, arterial medial calcification induced by chronic dysregulated glucose metabolism due to diabetes was alleviated by LX (Figure 4C–E). Similar to the in vitro data, LX inhibited AGE-induced VSMC calcification in a dose-dependent manner (Figure 5B, 5D, and Supplementary Figure 3B). We also observed that adding the YAP agonist masked the protection of LX from calcification in VSMCs (Figure 6B and 6C). Despite in rodent models, studies on the benefits of eicosapentaenoic acid (EPA) [78, 79], the precursor of SPMs, and results describing its clinical effect on vascular calcification have been conflicting [80, 81]. One explanation for this discrepancy is the distinct components and variables of EPA. However, the present results offer another plausible explanation that EPA supplementation enhanced the formation of multiple SPMs, including LX [82], which directly prevented vascular calcification.

We observed that LX attenuated the increased expression of osteogenesis-related markers and the decreased expression of smooth muscle cell-specific contractile marker in vivo in diabetic mouse vessels (Figure 4F and 4G). Similar results were observed in vitro in VSMCs induced by AGEs (Figure 5E and 5F). These trends were reversed by LPA (Figure 6D–F), suggesting that suppression of VSMC trans-differentiation to an osteogenic phenotype via YAP is a pivotal mechanism by which LX inhibits VSMC calcification. Our results are the first to demonstrate how LX regulates calcium deposition in VSMCs in response to diabetic conditions and confirms YAP as a key driver of this process. Several reports have
shown that LX mediates a protective role in vascular disease [46, 83, 84], and this is consistent with the role of LX in vascular calcification observed in diabetic mouse vessels.

Cumulative evidence has demonstrated that the extracellular matrix plays a role in vascular calcification [85-87]. We observed more collagen deposition in the calcified arteries of diabetic mice than in control mice. Moreover, elastic Van Gieson staining results also showed that elastin fibres were flattened and degraded in the diabetic vasculature (Figure 4D). Consistent with previous studies, our results further illustrated the link between arterial medial calcification and altering the extracellular matrix, such as elastin degradation [88] and collagen deposition [89] in the diabetic arterial wall.

Notably, LX prevented collagen deposition in diabetic mice, as depicted by Van Gieson staining. In addition, western blot results showed that the diabetic aorta treated with LX exhibited less COL1, which accounted for 65% of the total collagen in the normal vessel[90] (Figure 4F). In vitro, LX dose-dependently decreased COL1 expression induced by AGEs in VSMCs. Consistently, a previous study demonstrated that the receptor of LX, Fpr2, favoured COL1 production [91]. In diabetic atherosclerotic models, LX has been shown to alleviate collagen content [46]. Our results extended the findings that LX exerted a protective effect in vascular diseases by inhibiting collagen accumulation. The mechanism by which LX regulates collagen content in VSMCs requires further exploration. Our study suggests that LX regulates COL1 expression via YAP signalling in VSMCs. In cancer-related studies, YAP signals have been shown to correlate with the modulation of the fibrous collagen networks in fibroblasts and remodelling of the cancer-associated matrix [92]. This is in line with literature showing that YAP signalling plays a role in extracellular matrix remodelling, especially in regulating collagen production [92].

5. Conclusion

This study provides several new insights into the role of YAP in vascular medial calcification; it also suggests LX as a therapeutic strategy for diabetic vascular calcification. Our study used ample evidence to confirm that the diabetic mice and AGE-induced VSMCs exhibited extensive osteogenesis and calcification, coupled with more increased YAP expression. In addition, we provide evidence that LX prevents diabetic vascular calcification via YAP, which potentially explains the mechanism by which LX regulates collagen content. Our study therefore shows that preventing diabetic vascular calcification via the LX and YAP pathways represents a novel therapeutic option for the prevention and treatment of vascular disease.

Abbreviations

| Abbreviation | Description                |
|--------------|----------------------------|
| AGE          | Advanced glycation end     |
| ALP          | Alkaline phosphatase       |
| ANOVA        | Analysis of variance       |
| Abbreviation | Description |
|--------------|-------------|
| ATV          | Atorvastatin |
| AUC          | Area under the curve |
| BCA          | Bicinchoninic acid |
| CM           | Control media |
| COL1         | Type I collagen |
| DM           | Diabetes mellitus |
| DMEM         | Dulbecco's modified Eagle's medium |
| EPA          | Eicosapentaenoic acid |
| FBG          | Fasting blood glucose |
| FBS          | Foetal bovine serum |
| GPCR         | G-protein-coupled receptor |
| HASMC        | Human aortic smooth muscle cell |
| IPGTT        | Intraperitoneal glucose tolerance test |
| IPIST        | Intraperitoneal insulin sensitivity test |
| LPA          | Lysophosphatidic acid |
| LX           | Lipoxin |
| OM           | Osteogenic medium |
| OPN          | Osteopontin |
| RUNX2        | Runt-related transcription factor 2 |
| SD           | Standard deviation |
| SMA          | Smooth muscle actin |
| SPM          | Specialised proresolving lipid mediator |
| STZ          | Streptozotocin |
| VSMC         | Vascular smooth muscle cells |
Declarations

Ethics approval and consent to participate

All animal experiments were performed in compliance with the Animal Ethics Procedures and Guidelines of the People's Republic of China and were approved by the Harbin Medical University Animal Ethics Committee.

Consent for publication

Not applicable for this article

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81671794 and 81970297).

Authors' contributions

BH and MY designed the study; BH, GW, XM, and DL performed experiments. BH, MY, QL, XH, and XW analysed data; BH, MY, QL, RZ, JH and BY edited the article. All authors read and approved the final manuscript.

Acknowledgements

We thank professor Shaohong Fang for helping us to revise the manuscript and give valuable suggestions in experimental design.

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Figures
Figure 1

Advanced glycation end products (AGEs) induced vascular smooth muscle cell (VSMC) calcification.

(A) Staining for alkaline phosphatase (ALP) activity of VSMCs in control medium (CM) and osteogenic medium (OM) supplemented with 0–400 μg/mL AGEs for 7 days. The results represent n = 3 per group. Scale bar = 200 μm. (B) Calcium deposition of VSMCs was evaluated by Alizarin red staining for 14 days.
The results represent $n = 3$ per group. Scale bar = 200 $\mu$m. (C, D) Quantitative analysis of ALP activity for 7 days and calcium content for 14 days in VSMCs was measured using a colorimetric assay ($n = 3$). (E–G) Effects of AGES on the expression of Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), type I collagen (COL1), smooth muscle actin (SMA), and YAP for 14 days. Means ± standard deviation (SD), one-way analysis of variance (ANOVA) followed by Tukey’s test. $^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$ compared with the control cells untreated with 0 $\mu$g/mL AGES.

**Figure 2**

AGES activated YAP under calcifying conditions.

VSMCs induced with 100 $\mu$g/mL AGES were monitored by specific staining and western blot at different stages during calcification formation: the early stage (day 7), the medium stage (day 9), and the late stage (day 14). (A) Staining for ALP activity at different stages. (B) Alizarin red staining at different stages. (C) von Kossa staining at different stages. The results represent $n = 3$ per group. Scale bar = 200 $\mu$m. (D–F) Protein expression of RUNX2, SMA, and YAP was evaluated by western blot under calcifying conditions ($n = 3$), $^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$ vs. early stage of calcification.
Figure 3
AGEs induced VSMC calcification through a YAP-dependent pathway.

(A) Staining for ALP activity of VSMCs treated for 14 days in OM with 100 μg/mL AGEs, 1 μM atorvastatin (ATV), 10 μM lysophosphatidic acid (LPA). (B, C) Alizarin red staining and von Kossa staining of VSMCs treated for 14 days in OM with 100 μg/mL AGEs, 1 μM ATV, and 10 μM LPA. The
results represent \( n = 3 \) per group. Scale bar = 200 \( \mu \text{m} \). (D–G) Protein expression of RUNX2, SMA, and YAP was evaluated by western blot in VSMCs cultured with 100 \( \mu \text{g/mL} \) AGEs, 1 \( \mu \text{M} \) ATV, and 10 \( \mu \text{M} \) LPA. \( N = 3, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 \).

**Figure 4**

Lipoxin (LX) attenuated vascular calcification in diabetic mice.
(A) Schematic representation of experimental design. (B) Fasting blood glucose was measured in nondiabetic and diabetic mice administered vehicle (2% ethanol) or LXA$_4$ from weeks 0–25 (n=7–8 mice per group). (C) Whole-mount aortas stained with von Kossa staining to visualise calcium deposition in the control (Ctrl), LX, diabetes mellitus (DM), and DM+LX groups. Scale bar = 5 mm. (D) Representative Alizarin red staining, von Kossa staining, and elastic Van Gieson staining of aortas isolated from diabetic and nondiabetic mice administered vehicle or LXA$_4$; n=3, scale bar = 100 μm. (E) Aortic calcium content; n=3, means ± SD; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. DM group. (F, G) Aortic protein expression was determined by immunoblotting; n = 3,*$P < 0.05$, **$P < 0.01$ vs. DM group. (H) Aortas were stained by immunofluorescence for DAPI (blue), SMA (red), and RUNX2 (green). Scale bar = 100 μm. (I) Representative cross-sections of aortas immunostained for YAP. Scale bar = 100 μm.
Figure 5

LX inhibited AGEs-induced calcification in VSMCs.

(A) Staining for ALP activity of VSMCs in CM and OM supplemented with 100 μg/mL AGEs or 0–100 nM LX for 7 days. The results represent n = 3 per group. Scale bar = 200 μm. (B) Calcium deposition of VSMCs was evaluated by Alizarin red staining for 14 days. The results represent n=3 per group. Scale bar...
= 200 μm. (C, D) Quantification of ALP activity and deposited calcium normalised to total protein (n = 3). (E–G) Protein expression of RUNX2, OPN, COL1, SMA, and YAP was analysed by western blot. Means ± SD, one-way ANOVA followed by Tukey's test. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control cells treated with 0 nM LX.

Figure 6

LX inhibits AGEs-induced YAP activation.

(A) ALP activity staining in OM treated with 100 μg/mL AGEs, with or without 100 nM LX, or with 10 μM LPA for 7 days. Scale bar = 200 μm. (B, C) Alizarin red staining and von Kossa staining in VSMCs treated with 100 μg/mL AGEs, with or without 100 nM LX, or with 10 μM LPA for 7 days. Scale bar = 200 μm. (D–G) Protein expression was analysed by western blot; n = 3, *P < 0.05, **P < 0.01 vs. AGES+LX. (H) Representative immunofluorescence of RUNX2 and YAP in VSMCs in OM treated with or without LX or LPA. DAPI is shown in blue. Scale bar = 10 μm.

Supplementary Files

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