Activation of nuclear factor-kappa B accelerates vascular calcification by inhibiting progressive ankylosis protein homolog expression

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Abstract

Vascular calcification is a major risk factor of cardiovascular mortality, particularly for patients with end-stage renal disease and diabetes. Although chronic inflammation is one of the etiologic factors, the underlying mechanism is not fully understood. To clarify this, we studied how nuclear factor-kappa B (NF-κB) induction, a mediator of inflammation, might promote vascular calcification. Activation of NF-κB by tumor necrosis factor (TNF) promoted inorganic phosphate-induced calcification in human aortic smooth muscle cells. Pyrophosphate (an inhibitor of calcification) efflux to the extracellular matrix was suppressed along with the decreased expression of ankylosis protein homolog (ANKH), a transmembrane protein that controls pyrophosphate efflux of cells. The restoration of ANKH expression in these cells overcame the decreased pyrophosphate efflux and calcification. Tristetraprolin, a downstream product of NF-κB activation, may mediate destablization of ANKH mRNA since its knockdown by shRNA increased ANKH expression and decreased calcification. Furthermore, a rat chronic renal failure model, with increased serum TNF levels, activated NF-κB and decreased ANKH levels. In contrast, the inhibition of NF-κB maintained ANKH expression and attenuated vascular calcification both *in vivo* and *in vitro*. Both human calcified atherosclerotic lesions and arteries from patients with chronic kidney disease had activated NF-κB and decreased ANKH expression.

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Thus, TNF-activated NF-κB promotes inflammation-accelerated vascular calcification by inhibiting ankylosis protein homolog expression and consequent pyrophosphate secretion.

Introduction

Vascular calcification is a major risk factor of cardiovascular mortality, particularly for patients with end-stage renal disease (1–4), advanced atherosclerosis, and diabetes (5). Approaches to prevent and/or reverse macrovascular calcification are far from ideal, in part because the mechanism is heterogeneous and complex. Growing evidence suggests that disturbed phosphate (Pi)/calcium metabolism, osteogenic transition of vascular smooth muscle cells (SMCs) and SMC apoptosis are associated with vascular calcification. However, the underlying mechanisms controlling vascular calcification are still unclear.

Increased levels of serum inorganic Pi and chronic inflammation during chronic renal failure (CRF) induce vascular calcification. We and others found that chronic inflammation and oxidative stress play a critical role in vascular calcification. Al-Aly et al. reported that tumor necrosis factor (TNF) induced Msh homeobox 2 (Msx2) expression and activated Wnt signaling to promote arterial calcification in an animal model of type 2 diabetes (6). Treatment with the TNF-alpha neutralizing antibody infliximab significantly abrogated aortic Msx2/Wnt signaling and attenuated aortic calcium accumulation (6). Byon et al. found that the osteochondrocytic transcription factor Runx2 was activated by hydrogen peroxide and supported matrix mineralization in cultured vascular SMCs(7). Recently, we showed that high Pi level induced the production of mitochondrial reactive oxygen species and activated NF-κB to induce vascular calcification in a rat CRF model(8). However, how NF-κB activation promotes vascular calcification is largely unknown.

The transcription factor NF-κB has an important role in inflammation and immune responses. In most unstimulated conditions, NF-κB is retained in the cytoplasm by inhibitor of kappa B proteins (IκBs). NF-κB consists of homo- and heterodimeric complexes of the Rel family proteins, including p50, p52, p65/RelA, c-Rel, and RelB. In mammalian cells, classical NF-κB is a heterodimer of p50 and p65/RelA proteins, in which the p65/RelA subunit has transactivation activity (9, 10). NF-κB activation is associated with the pathogenesis of chronic kidney disease (CKD), atherosclerosis, and diabetes.

Because TNF is one of the most potent activators of NF-κB, we examined whether TNF promotes vascular calcification by activating NF-κB in human aortic SMCs (HASMCs). TNF-induced NF-κB potently enhanced Pi-induced mineralization in HASMCs. Unexpectedly, in addition to inducing pro-osteogenic genes, TNF activation of NF-κB suppressed the expression of progressive ankylosis protein homolog (ANKH), an endogenous inhibitor of calcification, in HASMCs. Moreover, NF-κB activation downregulated ANKH in a rat CRF model. Inhibition of NF-κB activation maintained

ANKH expression and attenuated vascular calcification in the CRF model. Our results suggest that NF-κB plays a critical role in chronic inflammation accelerating vascular calcification by inhibiting ANKH expression. Targeting NF-κB may help to prevent vascular calcification.

Results

TNF accelerates Pi-induced calcification and regulates osteogenic gene expression

Low-grade systemic chronic inflammation (11) is a common feature of end-stage renal disease, diabetes and atherosclerosis (12). Pro-inflammatory cytokines such as TNF enhance mineralization in mouse and bovine SMCs.(13) To explore how chronic inflammation affects vascular calcification, we examined whether TNF could promote Pi-induced calcium deposition in HASMCs. As shown in Figure 1A, TNF alone did not induce calcification in HASMCs, but it significantly potentiated Pi-induced calcium deposition as compared with Pi alone, and this effect was TNF concentration dependent (Figure 1B). Furthermore, the minimum concentration of Pi needed to induce calcification was lower (from 2.6 mM to 2.0 mM) in the presence of TNF (Figure 1C). Next, we used qRT-PCR to analyze the mRNA levels of key mediators associated with osteogenic programming during calcification at 2, 8 and 72 hr. Consistent with previous findings (6, 14), the expression of the pro-osteogenic genes bone morphogenetic protein 2 (BMP2) and Msx2 was induced with TNF added to Pi (Figure 1D and E). Because vascular calcification can be modulated by both positive and negative mechanisms, we also examined the expression of calcification inhibitors. The addition of TNF did not affect osteopontin (OPN) expression with Pi induction (Figure 1F) but significantly inhibited matrix Gla protein (MGP) expression only at 72 hr (Figure 1G). Of note, the expression of ANKH began to decrease at 2 hr and was further reduced at 8 and 72 hr with TNF added (Figure 1H).

Decreased secretion of pyrophosphate (PPi) by ANKH mediates the pro-calcification effect of TNF

ANKH is a multipass transmembrane protein that inhibits mineralization by controlling PPi levels. The deletion of *ANKH* in mouse results in abnormal mineralization in articular cartilage and soft tissues (15). To determine whether TNF promoted vascular calcification by inhibiting ANKH expression, we examined whether ectopic expression of ANKH could inhibit TNF-enhanced vascular calcification in HASMCs by retroviral transduction (Figure 2A). TNF potently enhanced Pi-induced mineralization in HASMCs expressing empty vector, but mineralization was significantly suppressed in HASMCs expressing ANKH (Figure 2B, C). Consistent with reducing ANKH expression, TNF significantly reduced PPi level secretion from both control and Pi-induced calcified cells (Figure 2D). As well, ANKH inhibitor (probenecid, 2 mM) decreased and ANKH overexpression increased PPi secretion (Figure 2E). The effect of probenecid and TNF on Pi-induced calcification was equivalent, and TNF failed to further increase calcium deposition in the presence of probenecid (Figure 2F).

The extracellular matrix protein MGP negatively regulates mineralization. Because MGP expression was inhibited by TNF, we examined whether ectopic expression of MGP

attenuated TNF-enhanced mineralization (Supplemental Figure 1A~C). Overexpression of MGP significantly inhibited Pi-induced calcium deposition but was unable to prevent TNF-enhanced calcium deposition. Thus, TNF may promote human SMC calcification by inhibiting ANKH expression, which leads to decreased PPi secretion.

TNF-activation of NF-κB suppresses ANKH level and promotes vascular calcification

Because NF-κB is a major downstream effector, we examined whether TNF activates NF-κB to inhibit ANKH, thereby promoting vascular calcification in HASMCs. TNF strongly induced the expression of NF-κB target genes, including interleukin 8 (IL-8), IκBα, and monocyte chemoattractant protein 1 (MCP-1) (Figure 3A), all associated with vascular inflammation. Furthermore, TNF rapidly induced the phosphorylation and degradation of IκBα in HASMCs; consistently, TNF stimulated the nuclear translocation of p65, which was blocked by IKK inhibition (IKKβVI, 2 μM) (Figure 3B). In accordance, IKK inhibition blocked TNF-induced decrease in ANKH expression (Figure 3C) and PPi secretion (Figure 3D) and pro-calcification effects (Figure 3E).

To determine whether TNF-activated NF-κB was sufficient to promote calcification, we overexpressed Flag-tagged p65 in HASMCs by retroviral transduction (Figure 4A). Overexpression of p65 significantly enhanced Pi-induced mineralization (Figure 4B) and induced IL-8 mRNA level but repressed ANKH level (Figure 4C). To determine whether NF-κB was essential for TNF-enhanced mineralization, we depleted p65 in HASMCs using lentivirus-mediated expression of short hairpin RNA (shRNA) and confirmed the knockdown by western blot analysis (Figure 4D). In contrast to p65 overexpression, p65 knockdown significantly reduced TNF-augmented mineralization in Pi-treated HASMCs (Figure 4E). In accordance, p65 knockdown inhibited TNF-induced IL-8 expression but rescued TNF-decreased ANKH level (Figure 4F). ANKH overexpression in HASMCs prevented p65-augmented calcium deposition (Figure 4G). Therefore, TNF may activate NF-κB to promote calcification by downregulating ANKH expression and PPi secretion.

RNA-destabilizing factor tristetraprolin (TTP) mediates the effects of TNF on ANKH expression and calcification

Genome-wide analysis of the ANKH promoter predicted the existence of a transcription-factor NF-κB binding site (−194~−203 bp), which suggests that TNF might inhibit ANKH transcription through NF-κB. We subcloned the 0.3-kb upstream sequence of the ANKH promoter from the transcription start site (pGL3-ANKH-prom) from human genomic DNA into the pGL3 luciferase reporter. The basal luciferase activity was higher for pGL3-ANKH-prom than the pGL3 control vector in both HASMCs and HEK293T cells (Figure 5A, B). However, TNF was unable to modulate the basal luciferase activity of pGL3-ANKH-prom. As a control, TNF potently stimulated NF-κB luciferase reporter activities in both HASMCs and HEK293T cells. Therefore, NF-κB may not regulate ANKH expression at the transcriptional level.

Next, we analyzed the expression of genes regulating mRNA stability and found that the level of the RNA-destabilizing factor TTP was increased by TNF, which depended on NF-κB activation (Figure 5C). Moreover, shRNA knockdown of TTP (Figure 5D) reversed the

TNF-induced decrease in ANKH expression (Figure 5E) and increase in calcium deposition (Figure 5F). In accordance, overexpression of TTP further augmented Pi− and Pi+TNF-induced calcification (Figure 5G). Thus, TNF-downregulated ANKH expression may be due to TTP-mediated ANKH mRNA degradation.

Inhibition of NF-κB decreases calcium deposition in rat CRF model and human renal arteries

To further test whether NF-κB activation promoted vascular calcification *in vivo*, we used a rat CRF model of vascular calcification induced by an adenine diet. We blocked NF-κB in abdominal aortas using the super-repressor form of IκBα, with serine 32 and 36 replaced by alanines. Abdominal aortas after 3 weeks of the adenine diet were smeared with Ad-IκBα or Ad-GFP mixed with matrix gel. At 6 weeks after the diet, calcium deposition was significantly induced in rat aortas, as assessed by von Kossa staining (Figure 6A). Ad-GFP did not affect calcium deposition, but Ad-IκBα significantly inhibited calcium deposition. ANKH expression was significantly lower in CRF than control aortas with saline treatment (Figure 6A). In contrast, Ad-IκBα but not Ad-GFP treatment rescued the decreased ANKH levels (Figure 6A). To further confirm our results, we examined ANKH expression in aortas by western blot analysis and found that Ad-IκBα maintained the expression of ANKH (Figure 6B). Therefore, NF-κB promoted vascular calcification by inhibiting ANKH expression *in vivo*.

Similar to observations in aortas from patients with CKD (16), serum TNF level was two-fold higher in CRF than normal rats (Figure 6C). In accordance, Pi slightly increased calcium deposition in human renal artery rings at day 7, and TNF significantly accelerated the Pi-induced calcification, which was almost completely reversed by IKK inhibition (IKKβVI, 2 μM) (Figure 6D). ANKH expression in aortic rings was also inhibited by TNF, but Ad-IκBα maintained the expression of ANKH and decreased TNF-accelerated calcification (Supplemental Figure 2).

ANKH expression is inversely associated with NF-κB activities in the human artery wall

To further demonstrate that NF-κB negatively regulated ANKH expression *in vivo*, we investigated a possible inverse relation between NF-κB and ANKH expression in human artery walls of patients with CKD or atherosclerosis. Because the phosphorylation of p65 on Ser536 is associated with NF-κB activities, we used anti-phospho- NF-κB p65 (Ser536) antibodies to determine NF-κB activation. In human atherosclerotic lesions, ANKH protein was weakly detected in areas strongly stained with phosphorylated p65, where von kossa staining showed clear calcium deposition (Figure 7). Decreased ANKH protein level was also observed in calcified aortic walls from CKD patients with increased phosphorylation of p65 as compared with aortic walls from healthy donors. Therefore, NF-κB activation in local inflammation in the human aortic wall may augment calcification by downregulating ANKH.

Discussion

Once considered only passive deposition of calcium phosphate mineral, vascular calcification is now being recognized as an actively regulated process that is finely turned by both inducers and inhibitors of mineralization. Our study provides a novel mechanism of pro-inflammatory cytokines such as TNF exacerbating human SMC calcification by activating NF-κB. TNF-activated NF-κB suppressed the expression of the endogenous inhibitor of mineralization ANKH *in vitro* and *in vivo*. We provide a direct link between chronic inflammation and the osteogenic program attributed to vascular calcification.

Recent data have implicated the inflammatory cytokines IL-1, IL-6 (17), transforming growth factor β (18), and TNF(13) as key contributors to the pathogenesis of vascular calcification(11). Increased serum level of TNF has been detected in diabetic, atherosclerotic and CRF patients. Neutralizing antibody against TNF greatly abrogated vascular calcification in an animal model *in vivo* (6). The mechanisms described so far involve increased apoptosis and induction of alkaline phosphatase (ALP) and osteogenic genes such as Runx2 and Msx2 (6, 13, 19, 20). However, how the endogenous inhibitors of calcification are regulated by inflammatory cytokines is less defined. Yao et al.(21) showed that the procalcific effect of IL-6 is mediated by increasing the level of heat shock protein 70 to antagonize MGP function, an endogenous inhibitor of mineralization. From a mechanistic viewpoint, our data provide further evidence that an inflammatory factor contributes to vascular calcification by regulating the osteogenic program through both mediators and regulators. TNF increased the level of the procalcific factor BMP-2 and decreased that of inhibitors of calcification, ANKH and MGP, in the presence of the calcification inducer Pi. The upregulation of BMP-2 by TNF agrees with previous study (14).

ANKH was originally identified as a multipass transmembrane protein that exports inorganic PPi to the extracellular space to inhibit hydroxyapatite formation and prevent calcium deposition in skeletal tissue (15). Mutations in ANKH result in 2 distinct calcification disorders: craniometaphyseal dysplasia (22, 23) and familial calcium pyrophosphate dihydrate deposition disease (24, 25). Recent study suggested that extracellular PPi generation mediated by both ANKH- and adenosine triphosphate release-dependent mechanisms is a critical regulator of SMC calcification (26). In our study of HASMCs, TNF–NF-κB signaling markedly repressed ANKH expression and PPi secretion and promoted calcification. In contrast, we and others (19) found that TNF–NF-κB signaling markedly increased ALP activity (data not shown), which can locally degrade PPi as a necessary step to permit vertebrate biomineralization. Thus, TNF-decreased PPi level may result from depressed ANKH expression and enhanced ALP activity. Additionally, the restoration of ANKH expression greatly rescued PPi secretion and TNF-induced calcification, which may be a way to counteract TNF-augmented calcification regardless of ALP activity. Furthermore, TNF could not further increase Pi-induced calcification in the presence of ANKH inhibition, which indicates that reducing PPi secretion is a major mechanism of TNF-augmented calcification. We further verified the inverse association of NF-κB activation and ANKH expression in a CRF animal model with high serum level of TNF. Collectively, these data strongly indicate an important TNF–NF-κB–ANKH-PPi axis attributed to vascular calcification. Because aortas from patients with CKD showed

enhanced TNF level (16) and our CRF rats showed a two-fold increase in serum TNF level. Together with the presented results showed a role for TNF in vascular calcification, and thus prompted further research with TNF blockers on treating or preventing vascular calcification *in vivo*.

We found decreased MGP expression with TNF treatment. The depletion of MGP was previously found to promote vascular calcification *in vivo* (27). However, MGP overexpression failed to prevent the procalcific effect of TNF in our *in vitro* assay of HASMCs, possibly because overexpressed MGP has full function only after it is posttransationally modified by a vitamin K-dependent gammaglutamyl carboxylase into γ-carboxyglutamic acid (Gla) residues(28). However, gammaglutamyl carboxylase is not expressed in SMCs (29) and is absent in culture media. As well, the ectopic expression of MGP may be not strong enough to antagonize TNF-promoted calcification. Nevertheless, our results suggest that chronic inflammation may promote vascular calcification by inhibiting the effect of mineralization inhibitor ANKH.

TNF has been found to downregulate the expression of COL1A2, FGF10 and MyoD through mechanisms including transcriptional repression (30), physical interfering interaction (31), and posttranscriptional downregulation (32). Although we predicted potential NF-κB binding sites located within the promoter region of ANKH, we were unable to identify the direct transcriptional repression of ANKH by NF-κB using luciferase reporter assays. Instead, consistent with Carballo and King (33, 34), we found that TNF stimulated the expression of the RNA-destabilizing factor TTP. Indeed, analysis of ANKH mRNA level revealed at least 15 AU-rich elements (AUUUA) within the 3′-UTR, which allows for TTP binding and promotes ANKH mRNA degradation. With knockdown of TTP by shRNA, the TNF-induced decrease in ANKH expression and increase in calcium deposition were reversed, and overexpression of TTP promoted calcification. Therefore, TTP mediates the effects of TNF on ANKH expression and calcification.

Our study provides direct evidence that TNF-mediated NF-κB activation aggravates human vascular calcification via TTP-dependent repression of ANKH, the endogenous mineralization inhibitor. These data may shed light on understanding the complexity of vascular calcification during CRF, diabetes and atherosclerosis.

Methods

Materials

Sodium phosphate (Na_2HPO_4 and NaH_2PO_4) was from Fisher Scientific (Fair Lawn, USA); minimum essential medium alpha (MEM-α), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were from GIBCO (Grand Island, USA). TRIzol was from Invitrogen (Carlsbad, USA); the luciferase assay system was from Promega (Madison, USA); Alizarin red stain, cetylpyridinium chloride, and adenine were from Amresco (Ohio, USA); and IKK inhibitor (IKKβVI) and primers for PCR were from Sigma-Aldrich (St. Louis, USA). Antibodies for ANKH, phosphor-p65 (s536), IκBα, β-actin, and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, USA); p65 was

from Millipore (Billerica, USA); phosphor-IκBα was from Cell Signaling (Danvers, USA), and α-tubulin was from Sigma-Aldrich.

Patients

We excised a 2- to 3-mm circumferential segments of renal arteries from healthy donors during renal transplantation. Arteries of uraemic patients were from clinical discards of the slim abdominal aorta strip excised for aortic inosculation. Atherosclerotic tissue samples were obtained from patients by directional atherectomy according to protocols approved by the Medical Ethical Committee of the Health Science Center of Peking University, which comply with the principles outlined in the Declaration of Helsinki (35). All patients gave their informed consent for use of the tissue biopsy before surgery. Institutional Review Board approval was granted to our study.

Animal model

We used the animal vascular calcification model with male Wistar rats (n=32, VitalRiver Laboratories, Beijing). Eight-week-old rats were pair-fed with standard chow containing 1.2% calcium and 0.6% phosphorus for the control group or 0.75% adenine (Sigma-Aldrich, St. Louis, USA), 1.2% calcium and 1% phosphorus for the CRF group for 6 weeks as described previously (8, 36) (serum biochemical parameters are in supplemental table 1). After 3 weeks, rats were anesthetized with pentobarbital sodium by intraperitoneal injection (40 mg/kg), then underwent surgery. The left renal artery was the center marker, and ~2.0 cm of abdominal aorta was exposed. Adenovirus (Ad)-IκBα or Ad-GFP (5×10^9 plaque forming units) was mixed with 200 μl matrix gel (Sigma) and smeared around the exposed abdominal aorta. The abdominal aortas were excised for western blot analysis and immunostaining. All surgical procedures and postoperative care were performed in accordance with guidelines of Peking University Animal Care and Use Committee.

Cell and vascular tissue culture

HASMCs were from Invitrogen (Carlsbad, USA) and grown in a humidified 5% CO_2 incubator at 37°C in MEM-α supplemented with 15% FBS and 1% penicillin-streptomycin. The HASMCs were infected by retroviral vector and lentiviral victor as described (37), then cultured with calcifying medium with 3.0 mM inorganic phosphate (Pi). Human embryonic kidney 293T (HEK293T) cells were obtained from the American Type Culture Collection (ATCC).

The main branch of the human renal aorta was isolated during nephrectomy in patients with renal cancer without diabetic or renal complications, and adventitia was removed, and then maintained in a humidified 5% CO_2 incubator at 37°C in DMEM containing 1% penicillin-streptomycin. Ad-IκBα (10^8 pfu/ml) was used to infect the vascular tissue on the first day of culture, then vascular tissue was moved to calcification medium with or without TNF (10 ng/ml) on the second day. After 2-week culture, von Kossa staining, calcium deposition analysis and IHC were performed to analyze calcification and ANKH and phospho-p65 levels.

Histological observation

Specimens of calcifying arteries and atherosclerotic plaques were fixed in 4% formalin, embedded in paraffin and examined with IHC or immunofluorescence. Corresponding normal IgG was a negative control. Semiquantitative analysis of the staining involved *Image-Pro Plus* (Media Cybernetics, Bethesda, USA)..

Quantification of calcium deposition

To assess the mineralization in *in vitro* experiments, cells were induced for 7 days and stained with 2% Alizarin red (Sigma-Aldrich), then destained with 10% cetylpyridinium chloride for 30 min at room temperature, and the concentration was determined by measuring absorbance at 562 nm. For *ex vivo* (human) and *in vivo* (rat) measurement, vascular tissues from nephrectomy and the animal model were dissolved in HNO_3 and the calcium content was measured as we described previously (38), or fixed and sectioned, and calcium deposition was detected by von Kossa staining.

Vector construction

The 0.3-kb promoter for the coding sequence of human *ANKH* gene was synthesed by Sunbiotech Co. (Beijing) and then sub-cloned into pGL3 luciferase reporter vector (Promega, Madison, USA). Full-length *ANKH* mRNA with an additional kozak sequence from the human genome was amplified by RT-PCR (primers: 5′ACCGGTGCCACCATGGTGAAATTCCCGGCGCT, and 5′GAATTCTTATTCATTCTCCTCTCTCATT), then sub-cloned into a pQNCX2 retroviral vector. For viral infection, HASMCs were plated overnight and then infected with retroviruses in the presence of polybrene (6 μg/ml, Sigma-Aldrich) for 6 h. The p65 small hairpin RNA (shRNA) with the target sequence 5′-GGATTGAGGAGAAACGTAA-3′ was subcloned into the pLKO Lentiviral vector from Addgene (Cambridge, USA) and retrovirus packaging was as described (37). The plasmid pSingle-tTS-TTP-shRNA was kindly provided by Dr. Wen-Gong Wang (Peking University). The Ad-IκBα virus was kindly provided by Dr. Nan-Ping Wang (Peking University).

Transfection and luciferase activity assay

At 24 h after plating, cells were co-transfected with use of Lipofectamine 2000 (Invitrogen) with pGL3-ANKH-prom and the transfection control β-gal expression vector. Then, 6 h later, transfection media was replaced with DMEM containing 10% FBS. On the following day, cells were treated with vehicle or TNF. Cell lysates were harvested 48 h after transfection. Luciferase activity of cell lysates was assayed by use of the Dual Luciferase Reporter kit (Promega, Madison, USA).

PPi assay

Medium PPi levels were measured by use of PPiLight™ Pyrophosphate Detection Kit (Lonza, Rockland, USA) according to the instruction. Briefly, add 20 μl of PPiLight detection reagent to 40 μl of medium sample, after 10 min incubation at room temperature, measure the initial steady-state bioluminescence with the luminometer, as basal level. In another tube add 20 μl of PPiLight converting reagent to 40 μl of medium sample and

incubate at room temperature for 30 min, then add 20 μl of PPiLight detection reagent and incubate for 30 min, finally read luminescence and the value was subtracted from the original ATP-dependent bioluminescence value to yield PPi-dependent bioluminescence.

Real-time RT-PCR

RNA was isolated by use of TRIzol regent according to the manufacturer's directions and quantified by UV spectrophotometry, then extracted mRNA was reverse transcribed with 4 μg denatured RNA and 50 ng random hexamers (Invitrogen) in a 20-μl total volume containing 200 U Super Script III reverse transcriptase. Real-time PCR involved the Single Colour Real-Time PCR detection system (BioRad, Hercules, USA) with 1 μl cDNA and 1x SYBR Green dye. The housekeeping gene 18S rRNA was used for internal normalization. Oligonucleotides synthesized by Sigma-Aldrich are in supplemental table 2.

Statistics

Data are reported as mean ± SEM. Unpaired Student t test was used for analysis of 2 groups and one-way or two-way ANOVA for analysis of 3 or more groups, then Bonferroni's multiple comparison tests as applicable. $P<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard Abbreviations and Acronyms

ANKH	progressive ankylosis protein homolog
TNF	tumor necrosis factor
NF-κB	nuclear factor-kappa B
IκBα	inhibitor of kappa B alpha
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
HASMC	human aortic smooth muscle cell
CRF	chronic renal failure
CKD	chronic kidney disease
TTP	tristetraprolin

BMP2	bone morphogenetic protein 2
MSX2	Msh homeobox 2
OPN	osteopontin
MGP	matrix Gla protein
EV	empty vector

References

1. London GM, Guerin AP, Marchais SJ, Metivier F, et al. Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality. Nephrol Dial Transplant. 2003; 18:1731–1740. [PubMed: 12937218]

2. Blacher J, Guerin AP, Pannier B, Marchais SJ, et al. Impact of aortic stiffness on survival in end-stage renal disease. Circulation. 1999; 99:2434–2439. [PubMed: 10318666]

3. Goodman WG, Goldin J, Kuizon BD, Yoon C, et al. Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. N Engl J Med. 2000; 342:1478–1483. [PubMed: 10816185]

4. Guerin AP, London GM, Marchais SJ, Metivier F. Arterial stiffening and vascular calcifications in end-stage renal disease. Nephrol Dial Transplant. 2000; 15:1014–1021. [PubMed: 10862640]

5. Reaven PD, Sacks J. Coronary artery and abdominal aortic calcification are associated with cardiovascular disease in type 2 diabetes. Diabetologia. 2005; 48:379–385. [PubMed: 15688207]

6. Al-Aly Z, Shao JS, Lai CF, Huang E, et al. Aortic Msx2-Wnt calcification cascade is regulated by TNF-alpha-dependent signals in diabetic Ldlr–/– mice. Arterioscler Thromb Vasc Biol. 2007; 27:2589–2596. [PubMed: 17932314]

7. Byon CH, Javed A, Dai Q, Kappes JC, et al. Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. J Biol Chem. 2008; 283:15319–15327. [PubMed: 18378684]

8. Zhao MM, Xu MJ, Cai Y, Zhao G, et al. Mitochondrial reactive oxygen species promote p65 nuclear translocation mediating high-phosphate-induced vascular calcification in vitro and in vivo. Kidney Int. 79:1071–1079. [PubMed: 21368742]

9. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol. 1998; 16:225–260. [PubMed: 9597130]

10. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell. 2002; 109 (Suppl):S81–96. [PubMed: 11983155]

11. Shao JS, Cheng SL, Sadhu J, Towler DA. Inflammation and the osteogenic regulation of vascular calcification: a review and perspective. Hypertension. 55:579–592. [PubMed: 20101002]

12. Johnson RC, Leopold JA, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. Circ Res. 2006; 99:1044–1059. [PubMed: 17095733]

13. Tintut Y, Patel J, Parhami F, Demer LL. Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway. Circulation. 2000; 102:2636–2642. [PubMed: 11085968]

14. Csiszar A, Smith KE, Koller A, Kaley G, et al. Regulation of bone morphogenetic protein-2 expression in endothelial cells: role of nuclear factor-kappaB activation by tumor necrosis factor-alpha, H2O2, and high intravascular pressure. Circulation. 2005; 111:2364–2372. [PubMed: 15851600]

15. Ho AM, Johnson MD, Kingsley DM. Role of the mouse ank gene in control of tissue calcification and arthritis. Science. 2000; 289:265–270. [PubMed: 10894769]

16. Koleganova N, Piecha G, Ritz E, Schirmacher P, et al. Arterial calcification in patients with chronic kidney disease. Nephrol Dial Transplant. 2009; 24:2488–2496. [PubMed: 19329792]

17. Parhami F, Basseri B, Hwang J, Tintut Y, et al. High-density lipoprotein regulates calcification of vascular cells. Circ Res. 2002; 91:570–576. [PubMed: 12364384]

18. Watson KE, Bostrom K, Ravindranath R, Lam T, et al. TGF-beta 1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify. J Clin Invest. 1994; 93:2106–2113. [PubMed: 8182141]

19. Lee HL, Woo KM, Ryoo HM, Baek JH. Tumor necrosis factor-alpha increases alkaline phosphatase expression in vascular smooth muscle cells via MSX2 induction. Biochem Biophys Res Commun. 391:1087–1092. [PubMed: 20004646]

20. Son BK, Akishita M, Iijima K, Kozaki K, et al. Adiponectin antagonizes stimulatory effect of tumor necrosis factor-alpha on vascular smooth muscle cell calcification: regulation of growth arrest-specific gene 6-mediated survival pathway by adenosine 5′-monophosphate-activated protein kinase. Endocrinology. 2008; 149:1646–1653. [PubMed: 18174285]

21. Yao Y, Watson AD, Ji S, Bostrom KI. Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix Gla protein. Circ Res. 2009; 105:575–584. [PubMed: 19661459]

22. Nurnberg P, Thiele H, Chandler D, Hohne W, et al. Heterozygous mutations in ANKH, the human ortholog of the mouse progressive ankylosis gene, result in craniometaphyseal dysplasia. Nat Genet. 2001; 28:37–41. [PubMed: 11326272]

23. Reichenberger E, Tiziani V, Watanabe S, Park L, et al. Autosomal dominant craniometaphyseal dysplasia is caused by mutations in the transmembrane protein ANK. Am J Hum Genet. 2001; 68:1321–1326. [PubMed: 11326338]

24. Pendleton A, Johnson MD, Hughes A, Gurley KA, et al. Mutations in ANKH cause chondrocalcinosis. Am J Hum Genet. 2002; 71:933–940. [PubMed: 12297987]

25. Williams CJ, Zhang Y, Timms A, Bonavita G, et al. Autosomal dominant familial calcium pyrophosphate dihydrate deposition disease is caused by mutation in the transmembrane protein ANKH. Am J Hum Genet. 2002; 71:985–991. [PubMed: 12297989]

26. Prosdocimo DA, Wyler SC, Romani AM, O'Neill WC, et al. Regulation of vascular smooth muscle cell calcification by extracellular pyrophosphate homeostasis: synergistic modulation by cyclic AMP and hyperphosphatemia. Am J Physiol Cell Physiol. 298:C702–713. [PubMed: 20018951]

27. Luo G, Ducy P, McKee MD, Pinero GJ, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature. 1997; 386:78–81. [PubMed: 9052783]

28. Wallin R, Cain D, Hutson SM, Sane DC, et al. Modulation of the binding of matrix Gla protein (MGP) to bone morphogenetic protein-2 (BMP-2). Thromb Haemost. 2000; 84:1039–1044. [PubMed: 11154111]

29. Wang Y, Zhen Y, Shi Y, Chen J, et al. Vitamin k epoxide reductase: a protein involved in angiogenesis. Mol Cancer Res. 2005; 3:317–323. [PubMed: 15972850]

30. Kouba DJ, Chung KY, Nishiyama T, Vindevoghel L, et al. Nuclear factor-kappa B mediates TNF-alpha inhibitory effect on alpha 2(I) collagen (COL1A2) gene transcription in human dermal fibroblasts. J Immunol. 1999; 162:4226–4234. [PubMed: 10201951]

31. Benjamin JT, Carver BJ, Plosa EJ, Yamamoto Y, et al. NF-kappaB activation limits airway branching through inhibition of Sp1-mediated fibroblast growth factor-10 expression. J Immunol. 185:4896–4903. [PubMed: 20861353]

32. Guttridge DC, Mayo MW, Madrid LV, Wang CY, et al. NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science. 2000; 289:2363–2366. [PubMed: 11009425]

33. Carballo E, Lai WS, Blackshear PJ. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science. 1998; 281:1001–1005. [PubMed: 9703499]

34. King EM, Kaur M, Gong W, Rider CF, et al. Regulation of tristetraprolin expression by interleukin-1 beta and dexamethasone in human pulmonary epithelial cells: roles for nuclear factor-kappa B and p38 mitogen-activated protein kinase. J Pharmacol Exp Ther. 2009; 330:575–585. [PubMed: 19435930]

35. World Medical Association Declaration of Helsinki. Recommendations guiding physicians in biomedical research involving human subjects. Cardiovasc Res. 1997; 35:2–3. [PubMed: 9302340]

36. Neven E, Persy V, Dauwe S, De Schutter T, et al. Chondrocyte rather than osteoblast conversion of vascular cells underlies medial calcification in uremic rats. Arterioscler Thromb Vasc Biol. 30:1741–1750. [PubMed: 20522801]

37. Park BK, Zhang H, Zeng Q, Dai J, et al. NF-kappaB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. Nat Med. 2007; 13:62–69. [PubMed: 17159986]

38. Cai Y, Xu MJ, Teng X, Zhou YB, et al. Intermedin inhibits vascular calcification by increasing the level of matrix gamma-carboxyglutamic acid protein. Cardiovasc Res. 85:864–873. [PubMed: 19910445]
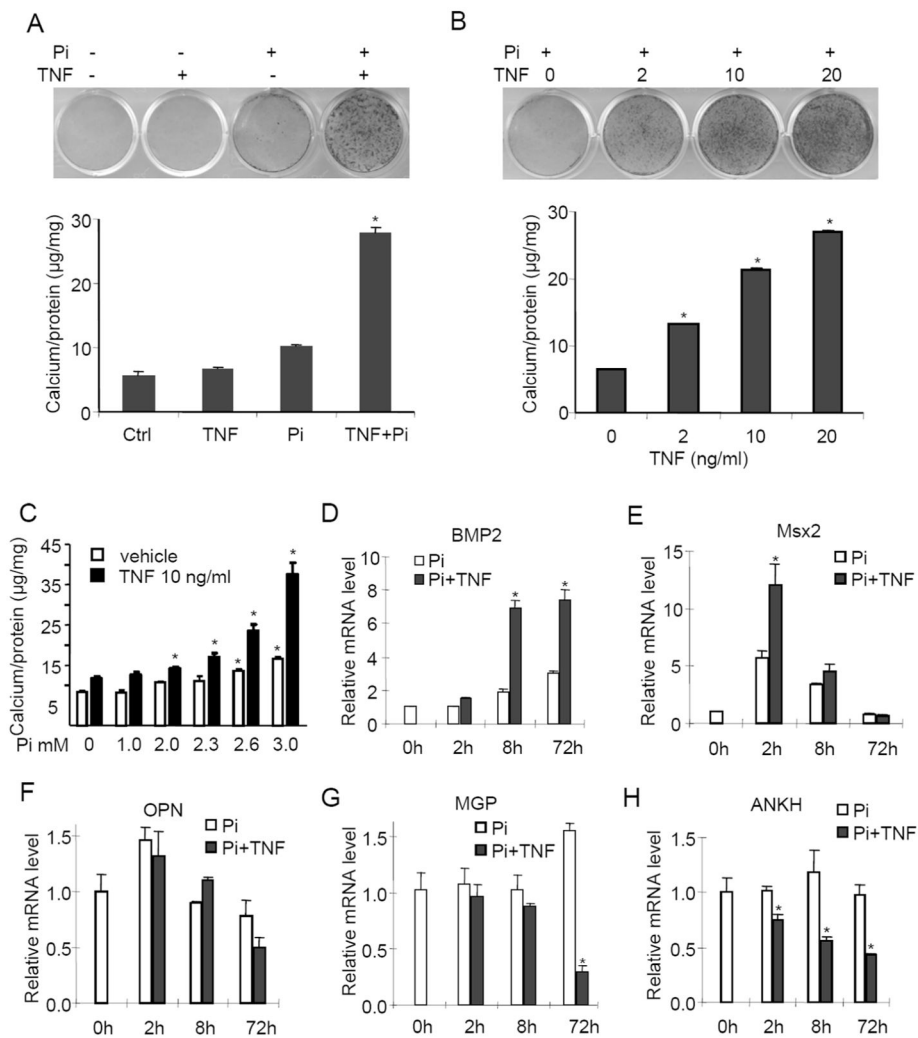
Figure 1.

Tumor necrosis factor (TNF) promoted human aortic smooth muscle cell (HASMC) calcification and regulated osteogenic gene expression. (A) Primary HASMCs were treated with Pi (3.0 mM) and/or TNF (10 ng/ml) for 7 days, then calcium deposition was analyzed by alizarin red staining. (B) HASMCs were treated with inorganic phosphate (Pi) (3.0 mM) and TNF (0, 2, 10, 20 ng/ml) for 7 days. (C–D) RT-PCR analysis of the expression of anti-osteogenic genes bone morphogenetic protein 2 (BMP2) and Msh homeobox 2 (Msx2). (E–G) RT-PCR analysis of the expression of osteogenic genes osteopontin (OPN), matrix Gla protein (MGP) and ankylosis protein homolog (ANKH). TNF 10 ng/ml, Pi 3.0 mM. (*$P<0.05$ compared to Pi, n=3)
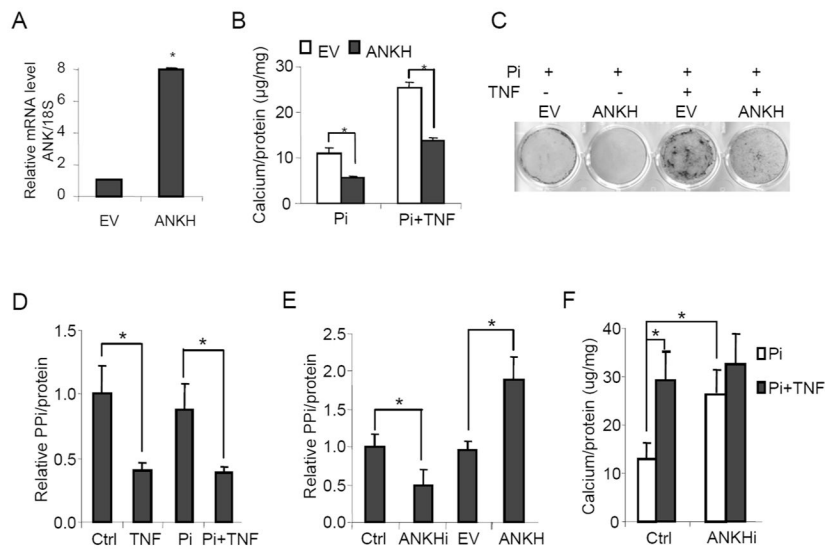
Figure 2.
Decreased secretion of pyrophosphate (PPi) by ANKH mediated TNF-augmented calcification. Real-time RT-PCR identified the overexpression of ANKH (A) by retroviral vector in HASMCs; EV, empty vector. (B) Calcium content assay and (C) Alizarin-red staining of protective effect of ANKH overexpression on calcification induced by Pi (3.0 mM) and/or TNF (10 ng/ml) for 7 days. (D) TNF (10 ng/ml) reduced extracellular PPi levels in both control cells and Pi-induced calcified cells. (E) HASMCs were treated with ANKH inhibitor (ANKHi: probenecid, 2 mM) or overexpressed ANKH for 3 days, and PPi levels in the medium were measured. (F) HASMCs were treated with ANKH inhibitor or vehicle, then with Pi and/or TNF (10 ng/ml) for 7 days. Calcium content was assayed. (*$P<0.05$ n=3)
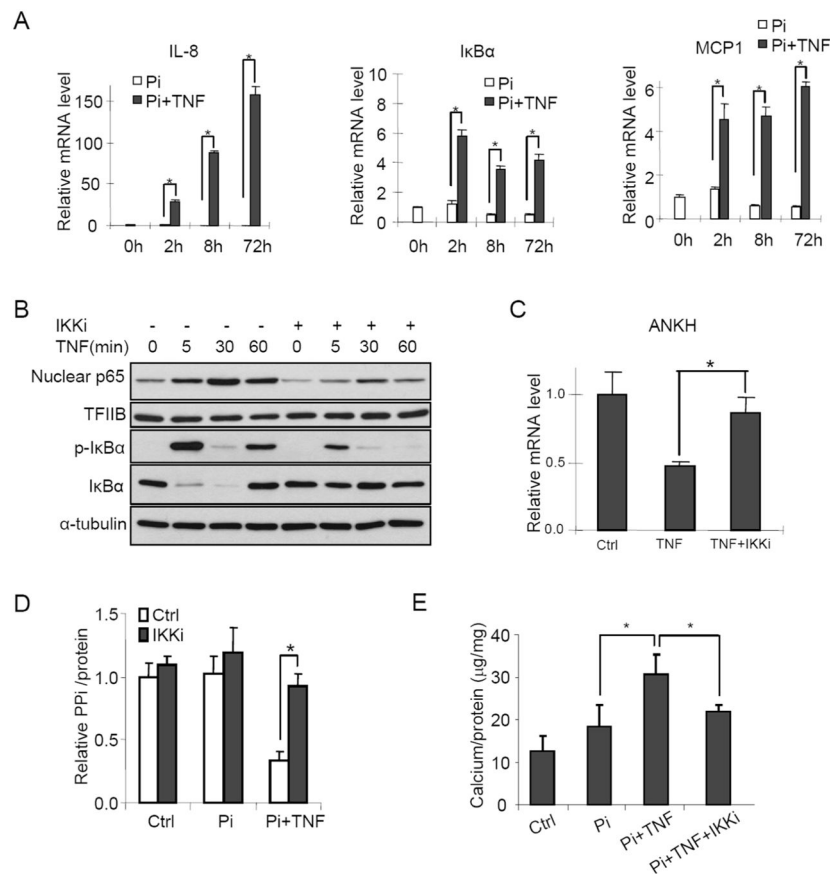
Figure 3.
IKK inhibitor blocked the effects of TNF on ANKH expression and calcification. (A) Real-time PCR analysis of the mRNA levels of interleukin 8 (IL-8), IκBα and monocyte chemoattractant protein 1 (MCP-1). (B) Western blot analysis of TNF (10 ng/ml)-induced translocation of p65, phosphorylation (p) and degradation of IκBα with or without IKK inhibitor (IKKi: IKKβVI, 2 μM) in HASMCs. HASMCs were preincubated with the IKK inhibitor for 30 min and then treated with TNF (10 ng/ml). The internal controls are TFIIB and α-tubulin. (C) Real-time RT-PCR analysis of ANKH mRNA level. HASMCs were preincubated with the IKK inhibitor for 30 min and then treated with Pi and/or TNF (10 ng/ml) for 8 hr, then (D) PPi level and (E) calcium content were measured. (*P<0.05 n=3)
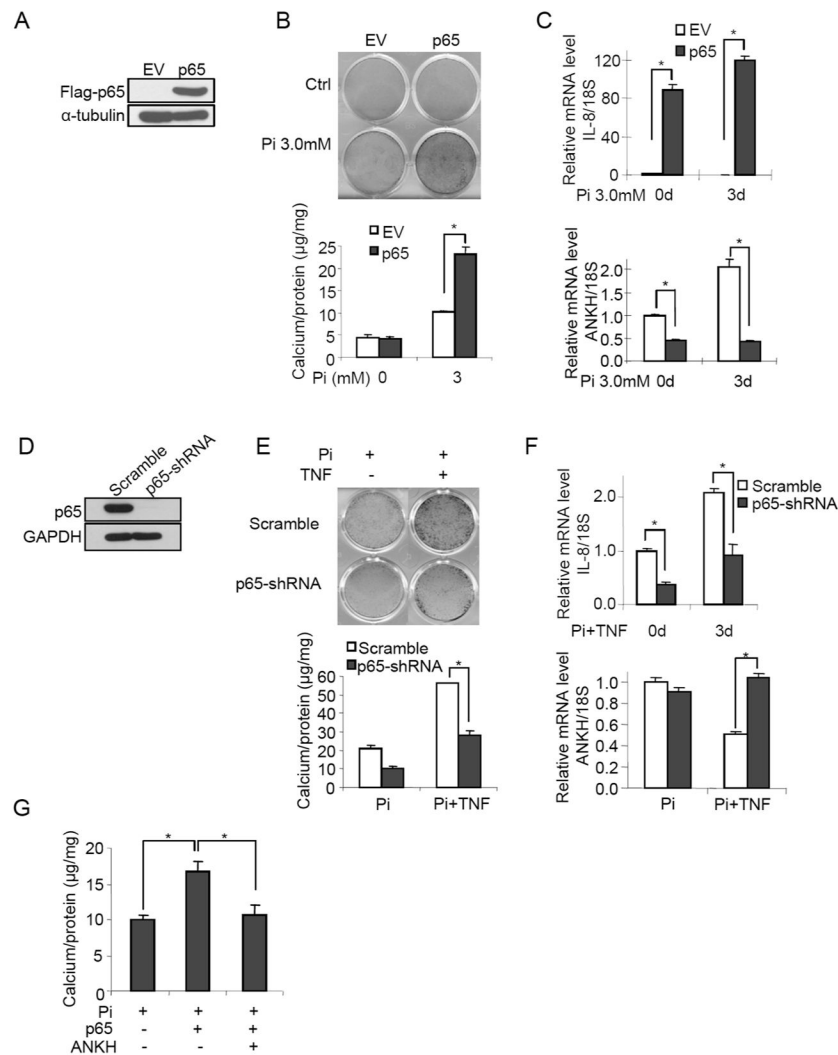
Figure 4.
NF-κB signalling mediated the effects of TNF on ANKH expression and calcification in HASMCs. (A) Western blot analysis confirmed the overexpression of flag-tagged p65 via retrovirus vector in HASMCs; EV, empty vector. (B) HASMCs were stably transfected with EV or p65, then treated with Pi (3.0 mM) for 7 days; Alizarin-red staining and calcium content assay of calcium deposition. (C) HASMCs transfected with EV or p65 were treated with vehicle or Pi for 3 days; RT-PCR analysis of IL-8 and ANKH mRNA levels. (D) Western blot analysis confirmed p65 knockdown via lentivirus vector in HASMCs. (E) HASMCs stably expressing scramble or p65-shRNA were treated with Pi (3.0 mM) and/or TNF (10 ng/ml) for 7 days; Alizarin-red staining and calcium content assay of calcium deposition. (F) HASMCs expressing scramble or p65-shRNA were treated with Pi and/or TNF (10 ng/ml) for 3 days; RT-PCR analysis of IL-8 and ANKH mRNA levels. (G) HASMCs stably expressing p65 and/or ANKH via retrovirus vector were treated with Pi for 7 days; calcium content assay of calcium deposition. (*$P<0.05$ vs EV or scramble, n=3)
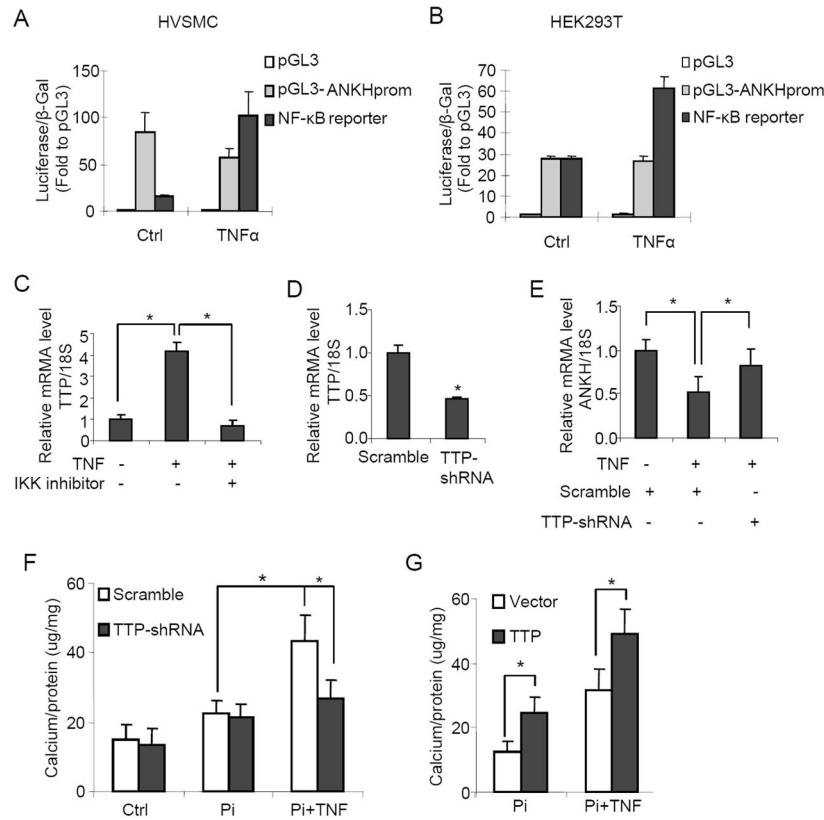
Figure 5.
NF-κB dependent expression of tristetraprolin (TTP) mediated the effects of TNF on ANKH expression and calcification. ANKH-promoter activity assay with pGL3 luciferase reporter in (A) HASMCs and (B) HEK293T cells. (C) IKK inhibitor was added to block NF-κB signal; RT-PCR analysis of TTP mRNA level. After transfection of scramble or TTP shRNA (pSingle-tTS-TTP-shRNA) for 24 hr, RT-PCR analysis of mRNA level of (D) TTP and (E) ANKH with or without TNF (10 ng/ml). (F) Calcium content assay: after transfection of scramble or TTP shRNA for 24 hr, cells were treated with Pi or Pi+TNF (10 ng/ml) for 7 days. (G) Calcium content assay: after transfection of empty or TTP vectors for 24 h, cells were treated with Pi or Pi+TNF (10 ng/ml) for 7 days. (*$P<0.05$, n=3)
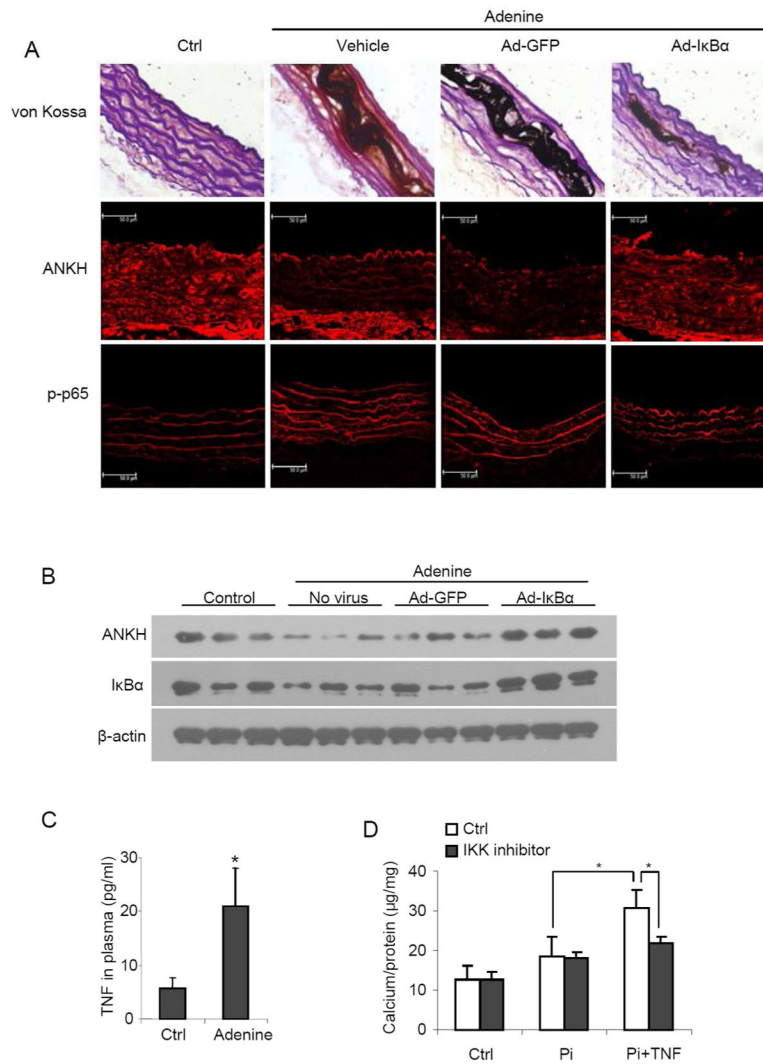
Figure 6.
IκBα increased ANKH expression and decreased calcium deposition in *in vivo* and *ex vivo* models. (A) In the rat chronic renal failure (CRF) model, Ad-IκBα or Ad-GFP were mixed with matrix gel and smeared around the abdominal aorta. Calcium deposition was analyzed by von Kossa staining. Ade, adenine diet at 6 weeks. ANKH and p-p65 levels were detected by immunohistochemical staining; shows one representative image from 6 rats per group (x 400). (B) Western blot analysis of protein levels of ANKH and IκBα in abdominal aortas. (C) Serum TNF level in CRF rats (n=6 rats per group, *P<0.05). (D) Calcium content in human renal artery tissue cultured in DMEM with Pi (3.0 mM) and/or TNF (10 ng/ml) for 2 weeks (n=4 per group, *P<0.05).
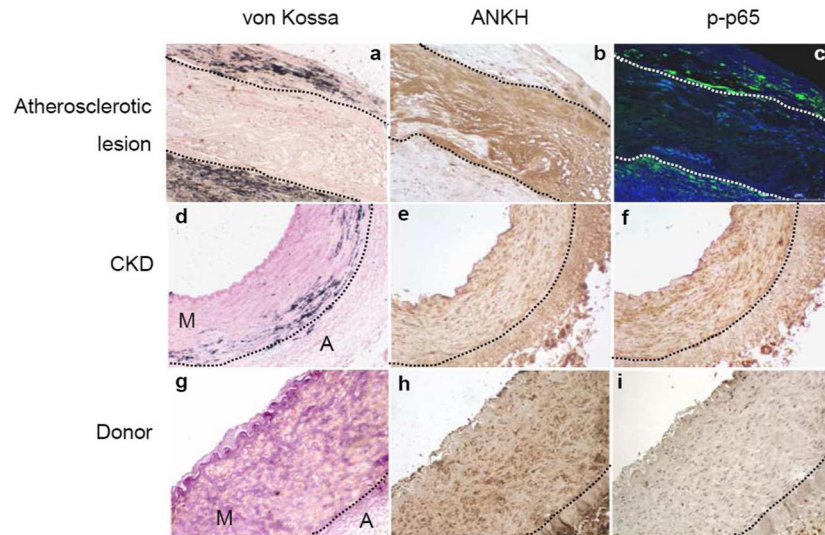
Figure 7.
Calcium deposition, ANKH expression and phosphor-p65 level in the human aortic wall.
(a~c) Atherosclerotic lesion samples were obtained from patients by directional
atherectomy; (d~f) Aortic wall samples were obtained from patients with chronic kidney
disease (CKD); (g~i) Aortic wall samples were obtained from healthy donors during renal
transplantation. (a,d,g) von Kossa staining of calcium deposition. (b,e,h)
Immunohistochemical staining of ANKH protein level. (c,f,i) Immunofluorescence staining
(green) or immunohistochemical staining of p-p65 (s536) level. M= tunica media, A=
adventitia.