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3 Interleukin-18 enhances vascular calcification and osteogenic differentiation of vascular
4 smooth muscle cells through TRPM7 channel activation
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Abstract

Objective—Vascular calcification (VC) is an important predictor of cardiovascular morbidity and mortality. Osteogenic differentiation of vascular smooth muscle cells (VSMCs) is a key mechanism of VC. Recent studies show that Interleukin-18 (IL-18) favors VC while transient receptor potential melastatin 7 (TRPM7) channel upregulation inhibits VC. However, the relationship between Il-18 and TRPM7 is unclear. We questioned whether IL-18 enhances VC and osteogenic differentiation of VSMCs through TRPM7 channel activation.

Approach and Results—Coronary-artery calcification (CAC) and serum IL-18 were measured in patients by CT scanning and ELISA respectively. Primary rat VSMCs calcification were induced by high inorganic phosphate and exposed to IL-18. VSMCs were also treated with TRPM7 antagonist 2-aminoethoxy-diphenylborate (2-APB) or TRPM7 siRNA to block TRPM7 channel activity and expression. TRPM7 currents were recorded by patch-clamp. Human studies showed that serum IL-18 levels were positively associated with coronary artery calcium scores (r=0.91 P<0.001). In VSMCs, IL-18 significantly decreased expression of contractile markers alpha smooth muscle actin (α-SMA), smooth muscle 22 alpha (SM22α) and increased calcium deposition, alkaline phosphatase activity and expression of osteogenic differentiation markers bone morphogenetic protein-2 (BMP2), Runx2, and osteocalcin (P<0.05). IL-18 increased TRPM7 expression through ERK1/2 signaling activation and TRPM7 currents were augmented by IL-18 treatment. Inhibition of TRPM7 channel by 2-APB or TRPM7 siRNA prevented osteogenic the IL-18 increase of differentiation and calcification of VSMCs.

Conclusions—These findings suggest that CAC is associated with increased IL-18 levels. In addition, IL-18 enhances VSMCs osteogenic differentiation and subsequent VC induced by β-GP via TRPM7 channel activation. Therefore, IL18 may contribute to VC in pro-inflammatory conditions.

Abbreviations

VC, Vascular calcification

CKD, chronic kidney disease

VSMCs, Vascular smooth muscle cells

IL-18, Interleukin-18

TRPM7, transient receptor potential melastatin 7

β-GP, β-glycerophosphate

ALP, alkaline phosphatase

siRNA, small interfering RNA

α-SMA, alpha smooth muscle actin

SM22α, smooth muscle 22 alpha

BMP2, bone morphogenetic protein-2

2-APB, 2-aminoethoxy-diphenylborate

Vascular calcification (VC) is a pathological process that occurs in many diseases such as hypertension, diabetes and chronic kidney disease (CKD) [1]. It directly correlates with an elevated risk of cardiovascular morbidity and mortality [2, 3]. Previously, VC was considered as an inevitable process due to passive precipitation of calcium and phosphate. VC is now believed to be a complex and actively regulated process sharing similarities with bone formation [1, 4]. Numerous risk factors, including hyperphosphatemia, have been implicated in VC[5]. Our recent study demonstrated that β-glycerophosphate stimulation of vascular smooth muscle cells (VSMCs) induced osteogenic differentiation and calcification[6]. Although the primary mechanism of VC is VSMCs transformation to osteo-/chondrocytic-like cells [7, 8], underlying processes of VC remain elusive in pro-inflammatory conditions.

Inflammation plays a vital role in chronic renal failure (CRF) and is associated with a high incidence of VC[9]. Interleukin-18 (IL-18) is a proinflammatory cytokine that belongs to the IL-1 superfamily and is produced by macrophages and other cells including VSMCs [10, 11]. IL-18 binds to its IL-18 receptor, and together with IL-12, induces cell-mediated immune inflammatory response[12]. Usually, IL-18 is activated by the inflammasome. Previous studies demonstrated that in CRF, plasma IL-18 levels were elevated and considered as a strong predictor for poor outcomes of CRF patients[13, 14]. Interestingly, recent studies showed that elevated plasma levels of IL-18 were significantly associated with VC in CKD stage 3 and 4 patients [15]. Accordingly, IL-18 might be involved in the process of VC, and the underlying mechanisms need to be further studied.

The transient receptor potential melastatin 7 (TRPM7) cation channel, a member of the TRP melastatin subfamily, is a Mg^{2+}- and Ca^{2+}- permeable ion channel covalently coupled to an alpha-type Ser/Thr protein kinase domain. TRPM7 has been found in VSMCs and plays an important role in the transdifferentiation of the VSMC phenotype[16, 17]. Montezano AC et al. demonstrated that TRPM7 is critically involved in VSMCs differentiation to an osteogenic phenotype, a process that is regulated by magnesium[17]. In another study, Zhang et al. showed that up-regulation of TRPM7 channel by angiotensin II (Ang II) contributed to the development of a proliferative phenotype of aortic VSMCs [18]. Similarly, Lin J et al. showed that TRPM7 channel activation inhibited ox-LDL-induced proliferation and migration of VSMCs via MEK-ERK pathways[19]. Thus, all these recent findings indicate that TRPM7 is a potential and important molecular regulator of VC[17, 20]. However, whether TRPM7 takes part in osteogenic differentiation and calcification of VSMCs enhanced by IL-18 has not been explored.

In the present study, we investigated whether IL-18 enhanced VC and osteogenic differentiation of VSMCs and explored the potential role of TRPM7 channel in this process.

Materials and Methods

Materials and Methods are detailed in the online-only Data Supplement.

Results

Serum levels of IL-18 are associated with VC in humans

To evaluate the relationship between serum levels of IL-18 and VC, we assessed serum levels of IL-18 in different VC groups. 64 participants entered into this study. The baseline characteristics were shown in Table 1. Based on the coronary-artery calcium scores, five groups were defined. We found that 41 (64%) participants had coronary-artery calcification. Serum levels of IL-18 were progressively elevated with the increasing severity of VC (Figure

176 1A). Moreover, A Spearman correlation analysis showed that the serum IL-18 levels were
177 significantly associated with the coronary-artery calcium scores (r= 0.91, P<0.001) (Figure
178 1B).

IL-18 enhances VC in cultured VSMCs

180 To evaluate whether IL-18 directly promotes VC, VSMCs were incubated in Dulbecco's
181 modified Eagle's medium without β-GP, and treated with or without IL-18 for 14 days
182 respectively. The results showed no significant difference of Alizarin red S staining, calcium
183 deposition and ALP activity, indicating no direct effect of IL-18 on VC (Figure 2A-2C). We
184 then exposed rat VSMCs to increasing concentrations of IL-18 in calcifying medium
185 containing β-GP for 14 days. The calcium deposition and ALP activity were assessed to
186 reflect VC. As shown in Figure 2D, 2E, 5ng/ml IL-18 did not significantly influence calcium
187 deposition and ALP activity of VSMCs (P>0.05). In contrast, calcium deposition and ALP
188 activity of VSMCs were gradually enhanced by the supplementation with increasing
189 concentrations (from 10 to 100ng/ml) of IL-18 (P<0.01). These results indicated that IL-18
190 augmented VC in a dose-dependent manner. Furthermore, VSMCs were treated with
191 100ng/ml IL-18 in the β-GP calcifying medium for different time of treatment. IL-18
192 significantly enhanced calcium deposition and ALP activity at different stimulation time points
193 compared with the group cultured without IL-18 (P<0.05, Figure 2F, 2G). These results
194 suggest that IL-18 also enhanced VC in a time-dependent manner.

IL-18-enhances osteogenic differentiation of VSMCs

196 We further test whether IL-18 enhances osteogenic differentiation of VSMCs. BMP2 is a
197 wildly used marker for osteogenic differentiation of VSMCs[21]. Firstly, we demonstrated that
198 IL-18 alone did not influence the expression of BMP2 (Figure 2H). Then we exposed rat
199 VSMCs to increasing concentrations of IL-18 in calcifying medium containing β-GP for 14
200 days. As shown in Figure 2I-2J, compared with the group cultured without IL-18, the mRNA
201 and protein levels of BMP2 were increased in VSMCs stimulated with increasing IL-18
202 concentrations (from 10 to 100ng/ml) (P<0.01). However, no significant difference was found
203 in VSMCs when treated with 5ng/ml IL-18 (P>0.05). Primary rat VSMCs were also treated
204 with 100ng/ml IL-18 in the calcifying medium for different time periods and results showed
205 that IL-18 significantly enhanced the mRNA and protein levels of BMP2 at different
206 stimulation time points (from 4 to 14 days) as compared with the group cultured without IL-18
207 (P<0.05, Figure 2K-2L). Our findings demonstrated that IL-18 enhanced osteogenic
208 differentiation of VSMCs both in a dose-dependent and time-dependent manner.

IL-18 upregulates expression of TRPM7 in calcifying VSMCs

210 We then investigate whether IL-18 affects TRPM7 expression in calcifying VSMCs.
211 Immunofluorescence analysis showed that TRPM7 expression was significantly increased in
212 the calcifying medium and IL-18 further amplified this expression (Figure 3A). Similar findings
213 of TRPM7 expression were found in both in mRNA and protein levels. (Figure 3B-3C,
214 P<0.05).

IL-18 activates TRPM7 currents in calcifying VSMCs

216 To evaluate the effect of IL-18 on TRPM7 currents, we performed patch-clamp studies to
217 examine TRPM7 currents. The results indicated that TRPM7 currents were significantly
218 activated in VSMCs cultured in the calcifying medium as compared with the control cells
219 (Figure 4A-4B, P<0.05). Moreover, IL-18 made enhancement onTRPM7 currents (Figure 4A-

4B, P<0.05), which implied that IL-18 activated TRPM7 channels and increased TRPM7 currents in calcifying VSMCs.

Inhibition of TRPM7 ameliorates VC enhanced by IL-18

To evaluate whether Inhibition of TRPM7 ameliorates VC enhanced by IL-18, we inhibited TRPM7 channel by using a TRPM7 antagonist, 2-aminoethoxy-diphenylborate (2-APB), and TRPM7 gene knock down by small interfering (si)RNA. Compared with scrambled siRNA, TRPM7 siRNA significantly reduced expression of TRPM7, indicating efficiency of the system (Figure 4C). Histologically, we demonstrated that TRPM7 silencing did not cause detectable spontaneous VSMCs calcification after 14 days culture in the absence of calcifying medium (see Supplemental Figure I). When exposed to calcifying medium, both TRPM7 siRNA and TRPM7 inhibitor 2-APB ameliorated calcification deposition and decreased ALP activity of VSMCs (Figure 4D-F, P<0.05). Moreover, IL-18 enhanced effect on calcium deposition and ALP activity in VSMCs were inhibited by TRPM7 siRNA and 2-APB (Figure 4D-F, P<0.05).

Inhibition of TRPM7 attenuates IL-18-enhanced osteogenic differentiation of VSMCs

We then determine whether inhibition of TRPM7 suppresses IL-18-stimulated osteogenic differentiation of VSMCs. Firstly, we demonstrated that TRPM7 silencing did effectively inhibited osteogenic differentiation of VSMCs after 14 days culture in the calcifying medium and IL-18 (see Supplemental FigureⅡ). Then the flow cytometry results showed that the expression of contractile markers of VSMCs, α-SMA and SM22α, was significantly decreased by β-GP (α-SMA, from 93.4% to 60.4%; SM22α, from 87.2% to 63.6%) and IL-18 further decreased the expression (α-SMA, from 60.4% to 37.9%; SM22α, from 63.6% to 27%, P<0.05) (Figure 5A-5C). However, when treated with 2-APB or TRPM7 siRNA, the percentage of α-SMA and SM22α was elevated (Figure 5A-5C). Moreover, expression of the osteogenic markers BMP2, Runx2 and osteocalcin were induced by β-GP and further enhanced by IL-18 (P<0.05, Figure 5D-5F). 2-APB and TRPM7 siRNA significantly prevented the IL-18 enhanced osteogenic differentiation processes (P<0.05, Figure 5D-5F). The same findings were also demonstrated by western blot analysis (Figure 5G-5L). Interestingly, we found from the western results that 2-APB and TRPM7 siRNA , especially TRPM7 siRNA, mainly inhibited osteogenic differentiation of VSMCs under the calcified condition treated with IL-18 (Figure 5G-5L).

Inhibition of TRPM7 decreases IL-18-stimulated TRPM7 channel currents of VSMCs

Both 2-APB and TRPM7 siRNA attenuated TRPM7 channel currents in VSMCs cultured with calcifying medium (P<0.05, Figure 6A-6B). Furthermore, in calcifying VSMCs treated with IL-18, the TRPM7 channel currents were also decreased by 2-APB and TRPM7 siRNA (P<0.05, Figure 6C-6D).

ERK signaling is involved in the process of IL-18 enhanced TPRM7 expression

We have demonstrated that IL-18 enhanced TRPM7 expression (Figure 3). To explore the potential role of ERK signaling in IL-18 enhanced TRPM7, we evaluated the expression of p-ERK and total ERK. The results showed that the ratio of p-ERK/total ERK was increased by β-GP, and further elevated by IL-18 (P<0.05, Figure 6 E). However, this effect was blocked by ERK1/2 inhibitor U0126 (Figure 6E). Moreover, we found that U0126 decreased IL-18 enhanced TRPMP7 expression, while 2-APB and TRPM7 siRNA had no effect on the ratio of p-ERK/total ERK (Figure 6 F-G). These findings indicate that IL-18 enhanced TRPM7 expression via ERK1/2 signaling activation.

Discussion

The main findings of our study demonstrate that IL-18 is associated with VC in humans and that it enhanced VC and osteogenic differentiation of VSMCs through processes that involve TRPM7 channel activation.

VC is a dynamic and actively regulated process influenced by various factors, including inflammatory mediators [22]. Recent findings demonstrated that IL-18 is associated with VC in CKD patients [15, 23] where a decrease in glomerular filtration rate (GFR) is accompanied with a rise in IL-18 levels. Furthermore, CKD patients with higher concentrations of IL-18 had higher hospitalization rates, and higher cardiovascular morbidity and mortality [24-26]. Valente AJ et al. found that in vitro IL-18 induced cardiac fibrosis migration and differentiation through cytoplasmic adapter protein TRAF3IP2[27]. In another study, Harrison et al found that epithelial-derived IL-18 regulated Th17 cell differentiation[28]. Their conclusions support the notion that IL-18 plays a key role in cell differentiation. In this study, we evaluated osteogenic differentiation of VSMCs by detecting the main osteogenic marker BMP2. Although up-regulation of BMP2 is demonstrated to promote calcification, the crucial mechanism of calcification is still unclear. Interestingly, our findings showed that IL-18 alone has no direct effect on VC and BMP2 expression. However, under calcifying condition, the pro-calcific effect of IL-18 and accelerating osteogenic differentiation of VSMCs were greatly activated. And such processes mainly involved in TRPM7 channel activation. It indicates that IL-18 plays pro-calcific effect and accelerates osteogenic differentiation of VSMCs should under calcified condition.

Increased pulse wave velocity (PWV) is an independent risk of cardiovascular morbidity and mortality among patients with CKD [29] and is an effective indicator of arterial stiffness and VC [30]. Porazko T et al. firstly reported that IL-18 was positively correlated with PWV [23]. However, whether IL-18 directly enhances VC has not been explored before. In this study, we demonstrated for the first time that IL-18 directly enhanced VC and osteogenic differentiation of VSMCs. In the present study, we unravel some novel mechanisms through TRPM7 in IL-18-mediated VC. Previous studies suggested that IL-18 accelerated arterial injury in CKD through the induction of lymphocyte differentiation to Th-1 cells which express IL-18 receptor[31]. However, this hypothesis has not been confirmed as other findings demonstrated that the effect of IL-18 was mainly on atherosclerosis [32, 33]. Recent studies have shown that the pathologic process of VC is different from atherosclerosis. The bone-vascular axis is an important mechanism of VC [34]. Lowering serum levels of LDL-c by statins, the most effective method for suppressing atherosclerosis among lipid lowering treatments, did not attenuate the progression of VC [35]. More importantly, recent studies found that statins promoted VC independent of their plaque-regressive effects [36]. In the present study we have further investigated putative mechanisms of VC, focusing on IL-18 and TRPM7.

Accumulating evidence suggests that VC is a process similar to bone formation[1, 4]. TRPM7, a Mg^{2+}- and Ca^{2+}- permeable ion channel, has been found to be involved in the proliferation and migration of human osteoblasts [37]. Interestingly, recent studies also demonstrated that TRPM7 is a regulator of VSMCs differentiation[17, 20] and that it plays a role in Ang II induced hypertension[38]. Our data showed that the mRNA/protein levels and the currents of TRPM7 were significantly increased in calcifying VSMCs, which were further enhanced by IL-18. These effects were inhibited by TRPM7 inhibitor 2-APB and TRPM7

siRNA. Furthermore, we found that inhibition of TRPM7 attenuated osteogenic differentiation of VSMCs induced by β-GP and enhanced by IL-18. Our findings indicated that TRPM7 channel activation was involved in the process of VC and osteogenic differentiation of VSMCs induced by β-GP and enhanced by IL-18.

TRPM7 is a member of the TRP melastatin subfamily that mediates capacitative Ca^{2+} and Mg^{2+} entry into the cells. TRPM7 combines structural elements of both an ion channel and a protein kinase[39]. Through Ca^{2+} and Mg^{2+} signals, TRPM7 channels participate in many physiological and pathological processes such as hypertension, atrial fibrillation, cancer, and ischemic stroke[40, 41]. A series of factors have been found to regulate TRPM7 channels including Mg^{2+} or Mg-ATP, extracellular pH, angiotensin II, and bradykinin[16, 40, 42]. Among these factors, Mg^{2+} is the most important regulator. August et al. identified that the transporter activity of TRPM7 was decreased by calcifying medium containing a high-normal (3.0mmol/L) level of magnesium[17]. In that study, TRPM7 was considered to play a protective role in VC which is in contrast to our study. Loïc Louvet et al. also showed in their study that inhibition of TRPM7 led to the inefficiency of Mg^{2+} (1.5 and 2 mmol/L) to prevent VC[20]. However, the concentration of magnesium exogenously added in those and other studies was higher than the values observed in patients taking Mg-based phosphate binders[43, 44]. . It is known that physiological concentration of Mg^{2+} is 0.8 to 1.0mM and in apparently healthy Western people, the concentration of Mg^{2+} even ranges between 0.6 and 0.7 mM[45]. Under many pathological conditions such as hypertension, heart failure, and atherosclerosis, patients are prone to magnesium deficiency[46]. And in these important pathologies, TRPM7 channels are often activated[19]. It is shown that magnesium deficiency is associated with IL-18 enhancement in insulin resistance[47]. So, we presume that in the process of IL-18 enhanced VC, extracellular Mg^{2+} may decrease, and TRPM7 expression compensatory increase. However, we did not measure the concentration of extracellular Mg^{2+} and this hypothesis needs further demonstration[43, 44]. Moreover, we found that TRPM7 siRNA is more effectively inhibited osteogenic differentiation of VSMCs than 2-APB especially under the calcified condition treated with IL-18. This implies that inhibiting TRPM7 expression maybe more important than inhibiting its activity. Or it indicates that 2-APB, the non-specific inhibitor of TRPM7, not effectively for inhibiting osteogenic differentiation. Other specific TRPM7 inhibitors are needed to verify our findings.

ERK1/2 signaling is involved in regulating VSMC function including proliferation, cell survival, inflammation and apoptosis in response to diverse stimuli[48]. Zhang et al. demonstrated that Ang II induced increased expression of TRPM7 in VSMCs and that this effect was required for ERK1/2 signaling[18]. In another study, Lin et al. demonstrated that TRPM7 channel regulates ox-LDL-induced proliferation and migration of VSMCs via MEK-ERK pathways. However, whether ERK1/2 signaling is involved in the process of IL-18 enhanced TRPM7 activation in VC has not been explored. It has been shown that IL-18 plays a role in ERK1/2 pathway[49]. In our study, we found that IL-18 activated ERK1/2 signaling during the process of VC and osteogenic differentiation accompanied with TRPM7 activation. Using the ERK1/2 signaling inhibitor U0126 significantly decreased IL-18 enhanced TRPM7 expression. Hence, our findings showed that IL-18 influenced TRPM7 channel activation and subsequently accelerated VC by activating ERK1/2 signaling. However, further clarification is needed.

In summary, we demonstrate that IL-18 enhances VC and osteogenic differentiation of VSMCs induced by β-GP through TRPM7 activation. IL-18 alone had no direct effect on VC and osteogenic differentiation of VMSCs. We also found that IL-18 enhanced TRPM7 expression via ERK1/2 signaling activation.

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Disclosures

None.

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Highlights

- Human serum IL-18 levels were positively associated with coronary artery calcium scores.
- IL-18 enhanced the β-GP-induced osteogenic differentiation of VSMCs and subsequent

524   VC. And during this process, TRPM7 expression was significantly increased.

525   ● Treatment with TRPM7 inhibitor 2-APB and TRPM7 siRNA inhibited IL-18-enhanced VC

526   and osteogenic differentiation of VSMCs which were induced by β-GP.

527

Figure legends
Figure 1. Serum levels of IL-18 are associated with vascular calcification (VC) in humans.
(**A**) The serum levels of IL-18 in different VC groups. According to the coronary-artery
calcium scores, the degree of VC was divided: 0 (no calcification), 1 to less than 10 (minimal
calcification), 10 to 100 (mild calcification), 101 to 300 (moderate calcification), and more
than 300 (extensive calcification). We found that the serum levels of IL-18 were gradually
increased accompanied with severity of VC. *P<0.05 vs. no calcification group; #P<0.05 vs.
minimal calcification group; & P<0.05 vs. mild calcification group; @ P<0.05 vs. moderate
calcification group. (**B**) A Spearman correlation analysis showed that the serum levels of IL-
18 were significantly associated with the coronary-artery calcium scores (r= 0.91, P<0.001).
Figure 2. IL-18 enhanced vascular calcification and increases BMP2 mRNA and protein
levels of VSMCs in a dose- and a time-dependent manner. (**A**, **B**, **C**) Quantification of
Alizarin red S staining (×40 magnification), calcium deposition and ALP activity in control
group and IL-18 group (n=5). (**D**, **E**) Quantification of calcium deposition and ALP activity in
VSMCs, respectively. VSMCs were treated with different concentration of IL-18 in the
calcifying medium with β-GP for 14 days (n=6). (**F**, **G**) Quantification of calcium deposition
and ALP activity in VSMCs, respectively. VSMCs were treated with 100ng/ml IL-18 in the
calcifying medium with β-GP for different time of treatment (n=6). (**H**) BMP2 protein
expression was measured by western blot in control group and IL-18 group (n=5).   (**I**, **J**)
Semi-quantification of relative mRNA and protein levels of BMP2. VSMCs were treated with
different concentration of IL-18 in the calcifying medium with β-GP for 14 days (n=5). (**K**, **L**)
Semi-quantification of relative mRNA and protein levels of BMP2. VSMCs were treated with
100ng/ml IL-18 in the calcifying medium with β-GP for different time of treatment (n=5).
Values are means ± SEM, *P<0.05 vs. the group treated in the calcifying medium without IL-
18.
Figure 3. IL-18 upregulated expression of TRPM7 in calcifying VSMCs. (**A**) IL-18
significantly increased expression of TRPM7 in VSMCs. VSMCs were cultured in the control
medium, calcifying medium and IL-18+ calcifying medium for 14 days, then TRPM7
expression was examined by immunofluorescence method. TRPM7 immunoreactivity (red)
was shown in VSMCs. The cells were simultaneously stained to outline the stress fiber of α-
actin (green). Blue indicates nuclei by DAPI staining. The images were taken at 400×
magnification (n=5). (**B**) Semi-quantification of relative mRNA level of TRPM7. VSMCs were
cultured with different mediums for 14 days (n=6). (**C**) Semi-quantification of relative protein
level of TRPM7 (n=6). VSMCs were treated with different mediums for 14 days. Values are
means ± SEM, *P<0.05 vs. control group.
Figure 4. IL-18 activated TRPM7 currents in VSMCs and Inhibition of TRPM7 ameliorated
vascular calcification enhanced by IL-18. (**A**) Electrophysiological recording of TRPM7
currents in VSMCs. VSMCs were incubated in the control medium, β-GP calcifying medium
and β-GP calcifying medium with IL-18 (100ng/ml) for 14 days respectively (n=6). (**B**)
Comparison of average density of TRPM7 currents at +100 mV in VSMCs treated with
different mediums. β-GP significantly increased the currents of TRPM7 and IL-18 further

572 promoted this effect (n=6). (**C**) Knockdown TRPM7 by small interfering (si)RNA. Comparing

573 with the scramble siRNA, TRPM7 siRNA significantly reduced expression of TRPM7 (n=3).

574 (**D**) Calcium deposition of VSMCs was shown by Alizarin red S staining. VSMCs were treated

575 with different mediums for 14 days (n=4). (**E**) Quantification of calcium deposition in VSMCs.

576 VSMCs were treated with different mediums for 14 days (n=4). (**F**) Quantification of ALP

577 activity in VSMCs (n=4). VSMCs were treated with different mediums for 14 days. Values are

578 means ± SEM, *$P<0.05$ vs. control group; # $P<0.05$ vs. β-GP group; & $P<0.05$ vs. IL-18+β-

579 GP group.

580

581 **Figure 5**. Inhibition of TRPM7 attenuated IL-18 enhanced osteogenic differentiation of

582 VSMCs. (**A**) Analysis of Flow cytometry for α-SMA, SM22α, BMP2, Runx2 and osteocalcin

583 (OCN) expressions, respectively. VSMCs were identified by contractile or osteogenic

584 markers staining (red) versus isotype control (green). X and Y-axis indicates relative

585 fluoresent intensity and percentage of max, respectively (n=5). (**B-F**) The comparison among

586 groups for the expression of α-SMA, SM22α, BMP2, Runx2 and osteocalcin (OCN). (**G**)

587 Western blot analysis of protein levels of α-SMA, SM22α, BMP2, Runx2, OCN and (**H-L**)

588 semiquantitative analysis (n=5). % units indicates the percentage of positive VSMCs. Values

589 are means ± SEM, *$P<0.05$ vs. control group; # $P<0.05$ vs. β-GP group; & $P<0.05$ vs. IL-

590 18+β-GP group.

591

592 **Figure 6**. Inhibition of TRPM7 decreased TRPM7 channel currents of VSMCs enhanced by

593 IL-18. (**A**) Electrophysiological recording of TRPM7 currents and (**B**) the comparison of

594 average density of TRPM7 currents at +100 mV in VSMCs cultured in calcifying medium for

595 14 days (n=6). Values are means ± SEM, *$P<0.05$ vs. β-GP group. (**C**) Electrophysiological

596 recording of TRPM7 currents and (**D**) the comparison of average density of TRPM7 currents

597 at +100 mV in VSMCs cultured in calcifying medium plus IL-18 (100ng/ml) with or without 2-

598 APB or TRMP7 siRNA for 14 days (n=6). Values are means± SEM, *$P<0.05$ vs. β-GP+ IL-18

599 group. (**E**) p-ERK and total ERK protein expression were measured by western blot (n=5).

600 Values are means ± SEM, *$P<0.05$ vs. control group; # $P<0.05$ vs. β-GP group; &$P<0.05$ vs.

601 IL-18+β-GP group. (**F**) TRPM7 protein expression were measured by western blot (n=5).

602 Values are means± SEM, &$P<0.05$ vs. IL-18+β-GP group. (**G**) p-ERK and total ERK protein

603 expression in VSMCs cultured in calcifying medium with 2-APB or TRPM7 siRNA.

604

Table 1 Demographic characteristic of enrolled patients

Variable	Patients (n=64)
Age	62.2±11.3
Male/female	35/29
SBP (mmHg)	148.3±8.4
DBP(mmHg)	85.5±2.6
BMI (kg/m^2)	24.8±3.4
Creatinine (umol/L)	125.7±25.2
UA (µmol/L)	395.7±84.1
HDL-C(mmol/L)	1.1±0.3
LDL-C (mmol/L)	3.3±0.9
TG (mmol/L)	1.7±0.2
TC (mmol/L)	5.0±1.6
FPG (mmol/L)	4.8±2.3

All values are expressed as mean±S.D. BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; UA, uric acid.