Calcification of human vascular smooth muscle cells: associations with osteoprotegerin expression and acceleration by high-dose insulin

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Olesen P, Nguyen K, Wogensen L, Ledet T, Rasmussen LM. Calcification of human vascular smooth muscle cells: associations with osteoprotegerin expression and acceleration by high-dose insulin. *Am J Physiol Heart Circ Physiol* 292: H1058–H1064, 2007. First published October 20, 2006; doi:10.1152/ajpheart.00047.2006.—Arterial medial calcifications occur often in diabetic individuals as part of the diabetic macroangiopathy. The pathogenesis is unknown, but the presence of calcifications predicts risk of cardiovascular events. We examined the effects of insulin on calcifying smooth muscle cells in vitro and measured the expression of the bone-related molecule osteoprotegerin (OPG). Human vascular smooth muscle cells (VSMCs) were grown from aorta from kidney donors. Induction of calcification was performed with β-glycerophosphate. The influence of insulin (200 μU/ml or 1,000 μU/ml) on calcification was judged by measuring calcium content in the cell layer and by von Kossa staining. OPG was measured in the medium by ELISA. Histochemistry was used for determination of alkaline phosphatase (ALP). Bone sialoprotein (BSP) and OPG mRNA expressions were done by RT-PCR. β-Glycerophosphate was able to induce calcification in human smooth muscle cells from a series of donors after variable time in culture. Decreased OPG amounts were observed from the cells during the accelerated calcification phase. High dose of insulin (1,000 μU/ml) accelerated the calcification, whereas lower concentrations (200 μU/ml) did not. Calcified cells expressed ALP and BSP activity in high levels. In conclusion, high concentration of insulin enhances in vitro-induced calcification in VSMCs. Altered OPG levels during the calcification raise the possibility that OPG may have a potent function in regulating the calcification process or it may represent a consequence of mineralization. Effects of insulin and modulations by OPG on the calcification process in arterial cells may play a role in the development of calcifications as part of the diabetic macroangiopathy.

atherosclerosis; macroangiopathy; arterial

DIABETIC PATIENTS harbor linear calcifications in the arterial tunica media with a high prevalence (12, 33). These calcifications are considered to be an element of the diabetic macroangiopathy, a pathological entity, which includes a series of diffuse nonatherosclerotic arterial changes, particularly related to the extracellular matrix (17, 18). The degree of medial calcifications is associated with the glucose tolerance and the duration of diabetes (12). Interestingly, medial calcifications in the femoral artery are strong, independent predictors of cardiovascular mortality in diabetes (5, 13), putatively because the calcifications mark the presence of generalized diabetic macroangiopathy, which may provide a fertile soil for atherogenesis (2). The mechanisms leading to calcium deposits in the arterial media in diabetes are, however, unknown.

Recent data suggest that arterial calcifications occur as the result of an active, regulated process. This idea is based partly on findings of high expression of bone-related macromolecules, such as alkaline phosphatase (ALP), bone sialoprotein (BSP), and bone Gla protein in calcified areas of the arterial wall (3, 8, 23, 30). Moreover, in vitro studies of vascular smooth muscle cells have demonstrated that these cells adjust expression of both Cbfa1 (an osteoblast differentiation-inducing transcription factor) and several bone-related molecules, like BSP, when calcification is induced (7, 22). Furthermore, direct evidence comes from gene knockout experiments of matrix-Gla protein and osteopontin-lacking mice, which develop extensive vascular calcification. Also, osteoprotegerin (OPG)-deficient mice develop arterial calcifications, suggesting that this osteoclast inhibitor, in addition, functions as an inhibitor of vascular calcification (1). The role of OPG is, nevertheless, unclear, since the OPG knockout animals develop severe osteoporosis, raising the suggestion that vascular alterations may be caused by systemic effects of extreme osteoclast overfunction. At present, no information is, however, available concerning the putative relation between OPG- and in vitro-induced calcification in vascular cells. Recent data suggest that OPG may play a special role in arterial disease in diabetes, since increased levels are found in aortic tunica media from diabetic patients (14). Altered arterial OPG concentrations and medial calcifications may thus hypothetically be associated and could be markers of diffuse arterial alterations in diabetes, i.e., diabetic macroangiopathy, reflecting effects of metabolic and hormonal factors on the vasculature.

Compensatory hyperinsulinemia prevails in Type 2 diabetes as a result of insulin resistance, especially in skeletal muscles. Since epidemiological studies have shown an independent relation between cardiovascular diseases and hyperinsulinemia (6, 28), insulin has been suggested as a mediator of vascular alterations in diabetes. Moreover, animal models of hyperinsulinism, as, for example, the Zucker rat, show diffuse arterial changes of the same diffuse type as seen in patients with diabetes (17, 18), as recently shown by Sista et al. (26). These changes may at least partly reflect direct insulin effects, since the vasculature is an insulin target organ and both endothelial and smooth muscle cells express insulin receptors. In vitro, insulin influences mitogenesis and chemotaxis in vascular smooth muscle cells (15, 19, 29), although new evidence questions these effects in human vascular cells (9, 27). Interestingly, insulin decreases the production of hyaluronic acid (4) and OPG (14) in short-term experiments with human
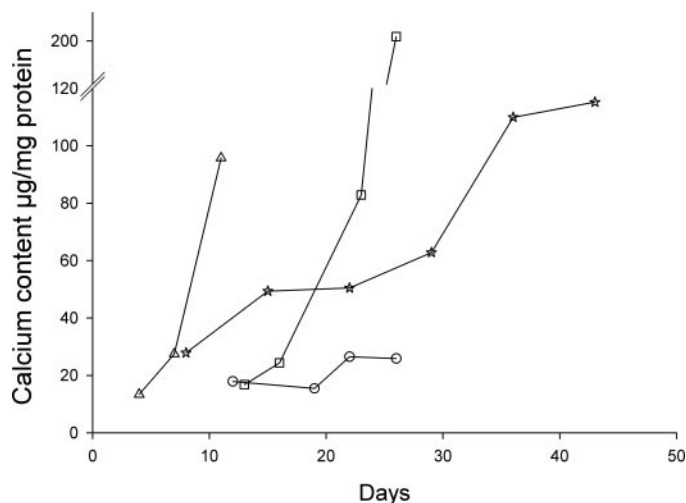
Fig. 1. Calcium accumulates variably in the cell layer of β-glycerophosphate (8 mM)-treated vascular smooth muscle cells (VSMCs) from different donors. Cells were treated with β-glycerophosphate, after which the calcium content in the cell layer was measured at the indicated time points. Results from four donors with variable responses are shown: △, early rapid calcification; □, abrupt calcification after longer time; ☆, slow but continuous increase in calcium content; ○, almost no calcification, despite prolonged incubation. The calcium content from non-β-glycerophosphate-treated control cells from each donor never exceeded 10 μg/mg protein (not shown). Results are mean values from 4–6 cultures for each time point.

vascular smooth muscle cells. The influence of insulin on the calcification-related molecule OPG suggests that an interplay between these molecules may influence the occurrence of medial calcifications in diabetes; however, no data concerning this idea are available.

The objective of the present study was to study the possible relation between in vitro-induced calcification of smooth muscle cells and OPG. Moreover, we aimed to investigate putative effects of insulin on the calcification process.

MATERIALS AND METHODS

Human vascular smooth muscle cell culture. Human vascular smooth muscle cells were grown from explants of normal aortic tissues, obtained from excess donor vasculature at kidney transplantations. The cells were cultivated as previously reported (4). For experiments, cells were grown in DMEM (Sigma D5523) containing 10% FBS and were used between *passages 3* and *6*. The quantitative part of this study included experiments with cells derived from eight different aortic donors. The local ethical committee approved the growing of cells.

Induction of calcification of human vascular smooth muscle cells. Single-cell suspensions (4×10^4 cells/well) were placed in culture plates in DMEM containing 10% FBS (growth medium). After adherence, cells were switched to calcification medium consisting of the growth medium supplemented with 8 mM β-glycerophosphate. Medium was replaced twice a week. For time course experiments, the first day of culture in the calcification medium was defined as *day 0*. For calcium and protein measurements, 6×24-well plates were used for six time points. Each plate contained between four and six wells for each treatment group (calcification medium, calcification medium in presence of insulin and control growth medium). For RNA isolation cells were seeded in six-well plates.

Quantitation of calcium and protein content. The cultures were decalcified with 0.6 N HCl for 24 h. The calcium content of the HCl supernatant was determined by the *o*-cresolphthalein Complexone method (calcium kit 587-A, Sigma). After decalcification, the cells were washed with PBS and solubilized with 0.1 N NaOH-0.1% SDS. Total protein content was measured using MicroBCA Protein Assay Reagent Kit (Pierce) with bovine serum albumin as standard. The calcium content of the cell layer was normalized to total protein content.

Cytochemical staining for mineral deposit and ALP. Von Kossa staining was used to confirm the presence of mineral deposits. Cell cultures were stained with 5% silver nitrate for 30 min. The calcium phosphate salts were demonstrated as black stain. Activity of ALP was demonstrated in cultured cells after being washed with PBS and incubated with substrate consisting of 2 mg/ml naphthol AS-BI phosphate sodium salt (Sigma N-2250) and 1 mg/ml fast red violet LB salt (Sigma F-1625) in 0.1 M Tris buffer (pH 8.2) at 37°C for 30 min and washed with PBS, followed by fixation in 4% formaldehyde buffer for 10 min. Cells were washed in distilled water, counterstained with Mayer hematoxylin for 2 min, and finally washed in water.

OPG measurements. During the calcification process, conditional medium was collected and stored at −20°C until analysis. The concentration of OPG in the conditional medium was determined by a commercially available sandwich ELISA (catalog no. DY805, R&D Systems, Minneapolis, MN), as previously described (14). The assay employs mouse anti-human OPG as capture antibody and a biotinylated goat anti-human OPG for detection, which is visualized by horseradish peroxidase-conjugated streptavidin.

RNA isolation and quantitative RT-PCR. Total RNA was extracted from cells grown in six-well culture dishes by using 500 μl of TRIzol (Invitrogen) according to the instructions from the manufacturer. The
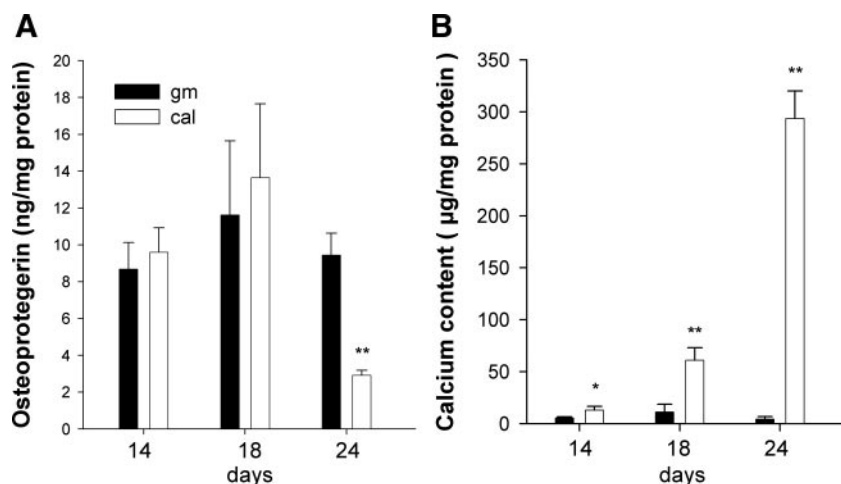

Fig. 2. Osteoprotegerin (OPG) secretion decreases in calcifying human VSMCs. OPG amounts in the medium (*A*) and calcium content in the cell layer (*B*) are shown for different time points in cultures treated with β-glycerophosphate (Cal) and normal growth medium without β-glycerophosphate (GM). *$P < 0.05$ and **$P < 0.01$ compared with control cells. Data are shown as means ± SD of 4 cultures from 1 donor.

Table 1. *Average calcium and protein content in the cell layer together with secreted amounts of osteoprotegerin in cultures of human vascular smooth muscle cells from several donors treated with β-glycerophosphate and insulin*

	Cellular Calcium, μg/mg protein	Cellular Protein, μg/well	Secreted Osteoprotegerin, ng/μg protein
Precalcification			
GM	5.4±3.5 (*n*=8)	183.2±58.2 (*n*=8)	1.37±1.13 (*n*=8)
Cal	14.9±7.2 (*n*=8)*	179.2±60.3 (*n*=8)	1.38±0.94 (*n*=8)
Cal + Ins 200	15.8±2.9 (*n*=4)	194.7±80.1 (*n*=4)†	1.97±1.27 (*n*=4)
Cal + Ins 1,000	28.5±14.4 (*n*=7)†	207.7±67.3 (*n*=7)†	1.06±0.81 (*n*=6)
Calcification			
GM	8.1±5.6 (*n*=8)	232.3±63.8 (*n*=8)	2.71±2.59 (*n*=7)
Cal	90.8±74.7 (*n*=8)*	235.7±71.6 (*n*=8)*	1.32±0.85 (*n*=7)†
Cal + Ins 200	145.0±77.3 (*n*=6)	240.1±85.7 (*n*=6)	1.12±0.84 (*n*=4)
Cal + Ins 1,000	208.0±210.6 (*n*=5)†	238.9±27.1 (*n*=5)†	0.79±0.36 (*n*=4)†

Values are means ± SD; *n*, number of individual artery donors available for paired *t*-test. GM, normal growth medium; Cal, cultures treated with 8 mM β-glycerophosphate; Cal + Ins, Cal with the addition of insulin (200 μU/ml or 1,000 μU/ml). Results are evaluated from a series of experiments: from each donor a large collection of cultures was used and results were averaged (*n* = 4–6 cultures) for each treatment group for each calcification phase (precalcification and calcification) as described in MATERIALS AND METHODS. Precalcification is defined as cellular calcium <20 μg/mg in the Cal group; calcification as cellular calcium >20 μg/mg in Cal group. *$P < 0.01$ vs. GM; †$P < 0.05$ vs. Cal. Paired *t*-test was used to judge differences between treatment groups.

amount of isolated RNA was ensured by measuring the optical density at 260 and 280 nm. Total RNA (1 μg) was used with random hexamers and murine Moloney leukemia virus-reverse transcriptase (GIBCO-BRL) for reverse transcription in 30 μl reaction mixture. The quantitative RT-PCR procedure was performed as previously described (14). Real-time PCR was performed in 25 μl amplification mixture containing 12.5 μl SYBR Green Supermix (Bio-Rad), 2 μl cDNA from RT solution, and 10 pmol primer sets. The relative standard curves were generated by using a sample cDNA with high RNA content diluted 5- to 125-fold. PCR was performed with two primer sets, specific for human BSP (5′-TGG AAA TCG TTT TAA ATG AGG ATA and 5′-AAG CAA TCA CCA AAA TGA AGA CT), OPG (5′-GGA ACC CCA GAG CGA AAT AC and 5′-CCT GAA GAA TGC CTC AC), and β-actin (5′-GTC CAG ACG CAG GAT GGC ATG and 5′-CTA CAA TGA GCT GCG GTG GGC). Standards and samples were run in duplicate. The efficiency of the PCR amplifications for BSP, OPG, and β-actin were >98%. Results are expressed as the ratio of OPG mRNA units to β-actin mRNA units.

Statistical analysis and assessment of results from multiple donors. As mentioned in RESULTS, the rate of calcium accumulation differed considerably between experiments (i.e., donors). To be able to combine the results from different experiments for statistical evaluation, we divided each experiment into two periods: a precalcification phase, during which the average calcium concentration in the β-glycerophosphate-treated control wells was below 20 μg/mg protein, and a

calcifying phase, where the average calcium content in the β-glycerophosphate-treated control cultures was above 20 μg/mg protein. An average result from each treatment modality for each donor for each calcification phase (when available) was calculated and used for presentation.

All results are expressed as means ± SD. Differences between groups were tested by ANOVA and Student's *t*-test (paired, whenever relevant). A two-tailed *P* value <0.05 was considered significant.

RESULTS

In vitro calcification of human vascular smooth muscle cells. The addition of β-glycerophosphate to cultures of vascular smooth muscle cells induced calcification, as judged by the accumulation of calcium in the cell layer. However, the rate of calcium deposit varied extensively between different experiments, using different donors (4 examples are shown in Fig. 1). Most experiments were characterized by an initial period of varying length, with only slowly rising calcium levels, followed by a second phase, with cellular calcium concentrations above 20 μg/mg protein. However, some cultures had a continuous slow course, and cells from a few donors did not incorporate significant amounts of calcium, even after prolonged incubation periods with β-glycerophosphate (Fig. 1).

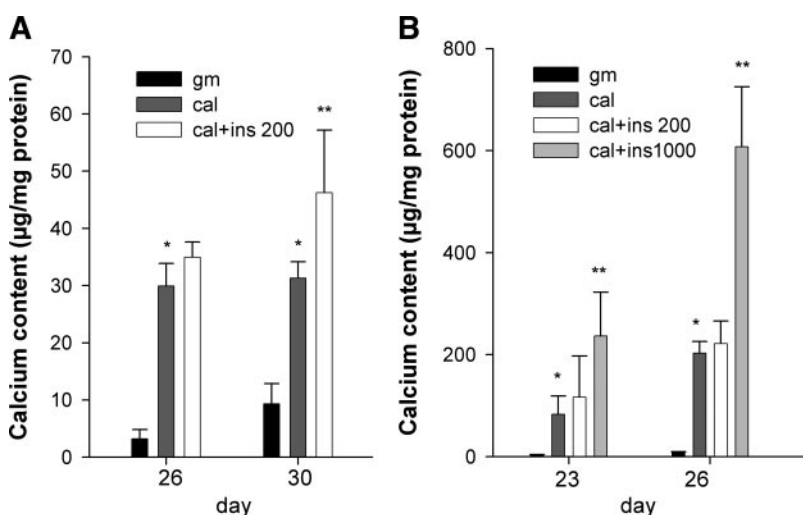

Fig. 3. Insulin increases calcification in the cell layer of β-glycerophosphate-treated VSMCs. Cellular calcium content from experiments with two donors is shown: an example of a late calcifying culture with rather low calcium values (*A*) and an early calcifying culture with higher calcium incorporation (*B*). Cells were treated for the indicated time periods. GM, results from control cells (normal growth medium); Cal, the addition of β-glycerophosphate; Cal + Ins 200 and Cal + Ins 1,000, results from β-glycerophosphate-treated cultures, which, in addition, received 200 μU/ml and 1,000 μU/ml insulin, respectively. *$P < 0.01$ when compared with control cells. **$P < 0.01$ vs. β-glycerophosphate-treated cells. Data represent means ± SD (*n* = 4–6 cultures).
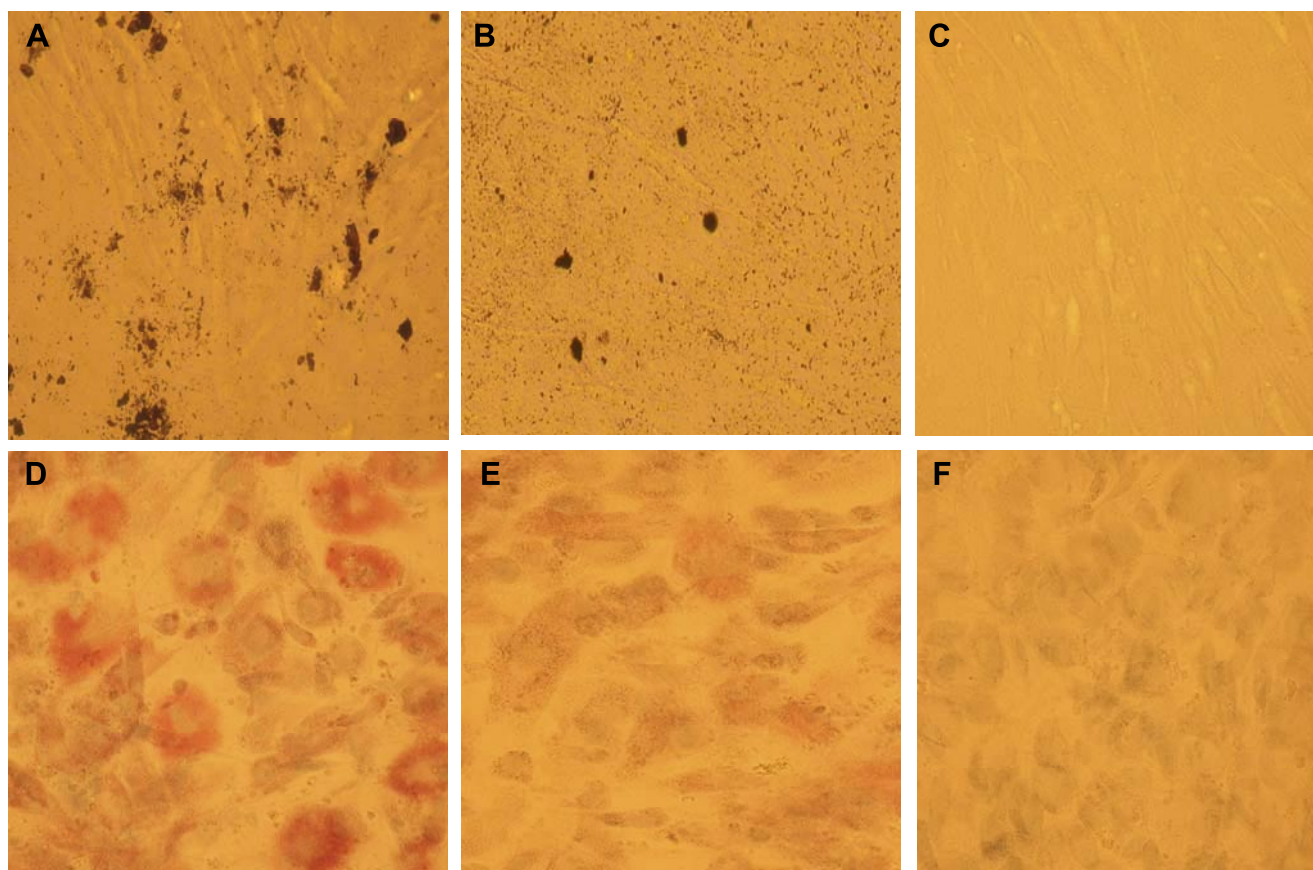
Fig. 4. Calcium deposits and alkaline phosphatase expression in insulin- and β-glycerophosphate-treated human VSMCs. *A–C*: von Kossa staining. *D–F*: cytochemical detection of alkaline phosphatase activity. *A* and *D*: cells treated with β-glycerophosphate and 1,000 μU/ml insulin. *B* and *E*: cells only treated with β-glycerophosphate. *C* and *F*: control cells. Cells were incubated for 36 days.

OPG secretion decreases in calcifying cultures. OPG was measured in the medium of calcifying cultures, and a typical example of results can be seen in Fig. 2. No difference in the OPG levels was seen in the β-glycerophosphate-containing medium vs. control medium at stages where the cellular calcium was low or only moderately increased. However, at a later stage, when calcium levels (Fig. 2*B*) were high, there was a large, significant decrease in the secretion of OPG (Fig. 2*A*). These results could also be observed when results from several donors were statistically evaluated, as seen in Table 1. In other experiments, we determined the relation between OPG production and different cell densities. It was found that the rate of OPG synthesis is negatively associated with cell numbers. Smooth muscle cells were seeded to a final density at 10^3, 10^4, and 10^5 cells/ml, and OPG production expressed per cell (in pg·ml^{-1}·day^{-1}·cell^{-1}) was 9.78, 8.44 ($P < 0.01$ compared with 10^3 cells/ml), and 1.62 ($P < 0.01$ compared with 10^3 cells/ml), respectively. Furthermore, the effects of different concentrations of PO_4^{-3} and β-glycerophosphate on OPG in smooth muscle cells were also compared, and no significant differences were found after 1, 2, and 3 mM of PO_4^{-3} and 4, 8, and 12 mM of β-glycerophosphate were added to the control medium (PO_4^{-3}: 0.9 mM) for 24 h (data not shown).

Insulin accelerates vascular calcification. Results from experiments with the addition of insulin to calcifying cells from two different donors are shown in Fig. 3. Both in cells with moderately elevated calcium levels and in cells that have achieved higher calcium levels, it can be seen that cultures treated with insulin incorporate more calcium than control cells. The combined results from a large series of experiments with cultures from different donors can be seen in Table 1. In line with results in Fig. 3, Table 1 shows that the calcium content in cultures treated with high doses of insulin (1,000 μU/ml) was statistically significantly increased, whereas a lower insulin dose showed only a trend toward augmented levels in late calcification. The combined results also show that, in the precalcification period, there was higher protein content in insulin-treated cultures when compared with the cultures without insulin (Table 1). No significant differences in protein content were, however, seen when cultures at later stages of calcification were considered (Table 1). In addition to biochemical analysis, calcium deposits were also identified by von Kossa staining, which supported the finding of increased calcifications of cells treated with insulin (Fig. 4). A large number of granular formations developed in cultures treated with β-glycerophosphate, and both size and amounts seemed to be increased when insulin was present in the calcification medium. No deposits were seen in cells cultured in the normal growth medium (Fig. 4). We also found high ALP expression in calcifying cultures (Fig. 4), whereas cultures without the addition of β-glycerophosphate had very low levels, as judged by light microscopy. Similar to calcium deposits, increased staining was present in the insulin-treated cultures. BSP mRNA was measured by real-time PCR, and in calcifying
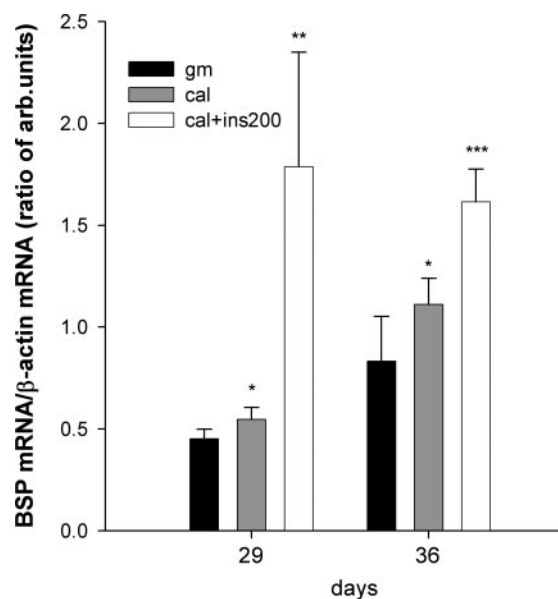
Fig. 5. Expression of bone sialoprotein (BSP) mRNA in calcifying VSMCs is enhanced by insulin. Cells were treated for the indicated time periods, RNA was isolated, and BSP mRNA was analyzed. Data represent means ± SD (n = 4 cultures). No difference in the amount of β-actin was seen between the different groups, when expressed per μg total RNA. arb, Arbitrary. *$P < 0.05$ when compared with control cells. **$P < 0.05$ and ***$P < 0.01$ when compared with β-glycerophosphate-treated cells (Cal).

cells, there was a significant increased BSP mRNA level versus control cells. In the presence of insulin (1,000 μU/ml), the expression of BSP was strongly upregulated (Fig. 5).

As can be seen in Fig. 6 (and Table 1), insulin addition at high concentrations led to a further decrease of the previously mentioned downregulation of OPG synthesis induced by calcification. However, insulin had no effects in cultures grown in calcification medium in the precalcification phase (Table 1).
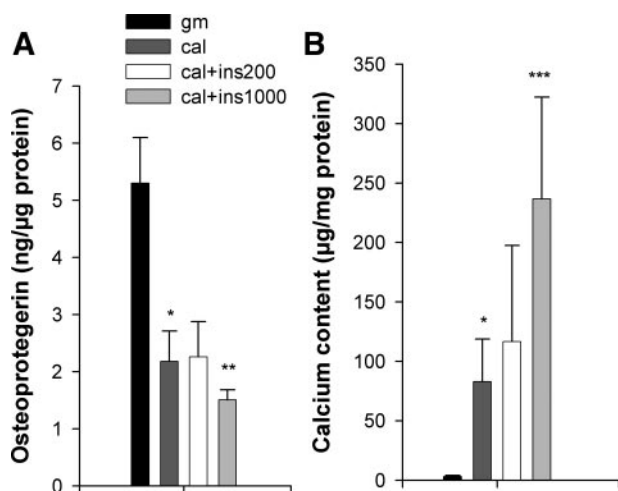

Fig. 6. Insulin decreases OPG secretion parallel to calcium incorporation. Cells were treated for 25 days, and OPG content in the medium (*A*) as well as calcium content in the cell layer (*B*) was measured. *$P < 0.01$ when compared with control cells; **$P < 0.05$ when compared with β-glycerophosphate-treated cells (Cal); ***$P < 0.01$, result from β-glycerophosphate-treated cells with addition of 1000 μU/ml insulin vs. β-glycerophosphate-treated cells without insulin. Data are represented as means ± SD (n = 4–6).
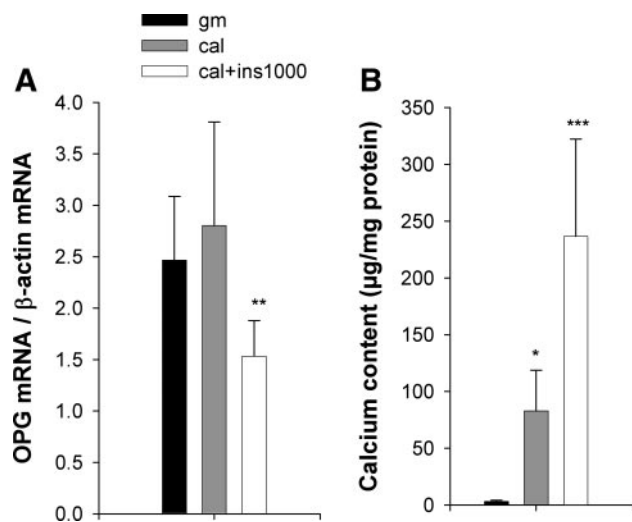

Fig. 7. Insulin decreases OPG mRNA in calcifying VSMCs. Cells were incubated for 18 days, RNA was isolated, and OPG mRNA/β-actin mRNA was determined (*A*) together with cellular calcium content in parallel cultures (*B*). Data are presented as means ± SD (n = 4 cultures). No differences in the amount of β-actin between the different groups were seen when expressed per μg total RNA. *$P < 0.01$ when compared with control cells (GM); **$P < 0.05$ vs. Cal; ***$P < 0.01$ result from Cal + Ins 1,000 vs. Cal.

The effect of high-dose insulin was paralleled when OPG mRNA amounts were considered (Fig. 7).

DISCUSSION

In the present investigation, we experienced that it is possible to induce calcification by β-glycerophosphate treatment in primary cultures of human vascular smooth muscle cells, although we found that the rate and the extent of calcium accumulation in the cell layer vary greatly between experiments. Several different versions of in vitro models of vascular calcifications have been reported (including β-glycerophosphate treatment) (7, 16, 24), and it seems that different calcification profiles may be achieved, although data on variation are sparse, especially concerning human cells. Nevertheless, one study agrees with the present finding, describing calcification in cells from two human donors where calcification was obtained after 1 and 3 wk, respectively (34). In contrast, β-glycerophosphate-induced calcification in bovine vascular cells is seen with less variability, and it seems that bovine cells calcify at a much quicker rate (24). In addition to differences between species, it is clear that there is also a large difference in calcification profile between different experimentally induced calcification pathways. Cells harvested from these nodules by collagenase treatment can calcify spontaneously (31) or after induction with phosphate, and the process develops rather quickly (7) (calcifying vascular cells), whereas induction with β-glycerophosphate develops with a more variable rate, putatively relating to the expression level of ALP. The variability in both systems using human cells probably reflects differences of age, sex, and genetic constitution between donors. Moreover, it is likely that factors related to the selection of cells in the initial harvest of tissue may play a role (e.g., origin is intima/media, etc.). In both models, it seems that calcium accumulation is paralleled with an increase in ALP activity and BSP expression, as seen in the present study and as previously reported

(22, 24). The large variation provides an experimental challenge when trying to extract information about molecular mechanisms from the calcification process. Nevertheless, our results, combining data from several donors, show that vascular calcification is associated with altered OPG levels and that insulin at high doses can accelerate the process.

OPG was first discovered as an osteoclast inhibitor in 1997 (25). Later observations suggested that the molecule also may work as an inhibitor of vascular calcification, since approximately one-third of OPG knockout mice develop calcium deposits in the arterial wall (1). This indication is in line with the fact that OPG is present in the vasculature in mice (1) and humans (14). OPG was, moreover, found to be overexpressed in relation to calcified areas and also in certain places in atherosclerotic tissue, as seen in immunohistochemical studies (3, 30), whereas in biochemical measurements, we did not find OPG amounts in altered amounts in atherosclerotic areas (14). In the present study, we found a downregulation of OPG amounts in the medium from cells during accelerated calcification. Whether the increased calcification could be related to the lack of calcification inhibition by OPG remains to be seen, but the finding does, at least, clearly show that the amount of OPG is strongly related to the calcification process in vitro. The biological effect of OPG is mediated through its binding and neutralization of either receptor activator of NF-κB ligand (RANKL) or TNF-related apoptosis-inducing ligand (TRAIL), two members of the TNF-α superfamily (32). Both molecules have been found in vascular cells, and at least RANKL has been suggested to be involved in vascular calcification (10, 20). In this study, we attempted to measure TRAIL and RANKL in conditioned media, but we were not able to find sufficient amounts for immunological detection (data not shown). Downregulation of OPG during calcification could, nevertheless, lead to altered activity of the two TNF-related molecules, but further studies to elucidate this hypothesis are needed. Interestingly, we did not observe differences in the expression of OPG mRNA between calcifying and control cells at the time where low OPG protein levels were seen in the cells. This may indicate that the level of OPG is regulated differently, at both the transcriptional, translational, and possibly also posttranslational level by different stimuli. Increased degradation of the molecule in calcifying cultures could, for example, be a possibility, whereas regulation by insulin is at the RNA level, as we also previously observed (14).

In the present study, we observed that insulin at a high concentration accelerates the calcification process in vitro. This induction was related to overexpression of bone-related molecules, i.e., ALP and BSP, and OPG downregulation. We found a slight increase in cellular protein content of insulin-treated cultures in the precalcification period (<20 μg calcium/mg protein). It is noteworthy that the total cellular amount in the cultures does not alter much throughout the experiments, indicating that the experiments are carried out during a steady-state period. The result could indicate that insulin may influence the balance between cell proliferation and apoptosis. Recent studies, which did not observe any effects of insulin on the proliferation or apoptosis of vascular smooth muscle cells (27), seem, however, to indicate that other factors, i.e., effects on the general protein metabolism, may be more important. The effect of insulin on calcification may relate to its capacity to alter synthesis of both hyaluronan (4), CD44 (21), and OPG

(14) in vascular smooth muscle cells. In line with these previous observations, we observed OPG downregulation by insulin in the calcification period but did surprisingly not find effects of insulin on OPG secretion in the precalcification period. This is in contrast to our previous investigations of acute effects of insulin (14). However, it is important to realize that the earlier studies were performed under serum-free conditions and that the present studies were performed over several weeks and in the presence of serum. The observed effects of insulin on the accumulation of calcium deposits are interesting, since vascular calcifications are observed with increased prevalence in patients with Type 2 diabetes, a group of patients suffering from hyperinsulinism. It should, however, be noted that we only found the cellular effects of insulin at high concentrations. In this context, it is also noteworthy that insulin can lower OPG production in normal vascular smooth muscle cells in serum-free conditions at much lower concentrations, as we previously reported (14). This could indicate that the different effects of insulin may follow dual signaling pathways, depending on concentration.

Medial calcifications in diabetes are good predictors of cardiovascular diseases in patients with Type 2 diabetes (5, 13), putatively because they mark the presence of other elements of the diabetic macroangiopathy of which some are known to accelerate the atherogenic process (2). The calcifications are associated with the duration of diabetes and with the magnitude of the glucose intolerance (11) and may relate to matrix alterations and possible accumulation of bone-related molecules, like OPG (14). In the present work, we investigated the mechanisms behind medial calcifications in diabetes, focusing on one putative factor, i.e., hyperinsulinism. Our data indicate that insulin at high concentrations may accelerate calcifications, which may be related to insulin-induced downregulation of the presumed calcification inhibitor OPG. However, it is clear that this scenario cannot explain the whole picture in the arterial wall in diabetic patients, where increased and not decreased levels of OPG have been found (14). The present observations are, nevertheless, important for future work with calcification models and may provide information relevant to the mechanisms behind medial calcifications in diabetes.

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