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Superoxide production: A procalcifying cell signalling event in osteoblastic differentiation of vascular smooth muscle cells exposed to calcification media

THIBAULT SUTRA[1], MARION MORENA[1,2], ANNE-SOPHIE BARGNOUX[1,3], BERTRAND CAPORICCIO[1], BERNARD CANAUD[2,4], & JEAN-PAUL CRISTOL[1,3]

[1]*EA 4188 Nutrition humaine, Biodisponibilité et Athérogénèse, Institut Universitaire de Recherche Clinique, Université Montpellier 1, France,* [2]*Institut de Recherche et Formation en Dialyse, Montpellier, France,* [3]*Laboratoire de Biochimie, and* [4]*Service de Néphrologie-Hémodialyse et Soins Intensifs, Centre Hospitalier Universitaire Lapeyronie, Montpellier, France*

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Abstract

Recent studies showed that hydrogen peroxide (H_2O_2) enhanced bone markers expression in vascular smooth muscle cells (VSMCs) implicated in osteoblastic differentiation. This study aimed at investigating the role of NAD(P)H oxidase in vascular calcification processes. A7r5 rat VSMCs were incubated with β-glycerophosphate (10 mM) or uremic serum to induce a diffuse mineralization. H_2O_2 production by VSMCs was determinated by chemiluminescence. NAD(P)H oxidase sub-unit ($p22^{phox}$), Cbfa-1, ERK phosphorylation and bone alkaline phosphatase (ALP) expressions were measured by Western blotting. VSMCs exhibited higher production of H_2O_2 and early expression of $p22^{phox}$ with β-glycerophosphate or uremic serum within 24 h of treatment. β-glycerophosphate-induced oxidative stress was associated with Cbfa-1 expression followed by ALP expression and activity, meanwhile the VSMCs expressing ALP diffusely calcified their extracellular matrix. Interestingly, diphenyleneiodonium partly prevented the osteoblastic differentiation. Results from this model strongly suggest a major implication of vascular NAD(P)H oxidase in vascular calcification supported by VSMCs osteoblastic differentiation.

Keywords: *Vascular calcification, uremia, osteoblastic differentiation, NAD(P)H oxidase, β-glycerophosphate, oxidative stress*

Introduction

Vascular calcification [1], a major cardiovascular risk factor, has long been thought to result from passive depositions on necrotic or inflamed zone [2]. During the last decade, it became clear that vascular calcification involves an active and regulated process [3,4] related to the osteoblastic differentiation of vascular myocytes. Indeed, in normal vessel wall, vascular smooth muscle cells (VSMCs) express a contractile phenotype highly specialized to maintain vascular tonus by contraction or relaxation. These VSMCs also regulate extracellular matrix (ECM) mainly constituted by proteoglycans, elastin and type I collagen. However, exposed to pathogenic media (uremia, diabetes mellitus), VSMCs gain a dedifferentiated phenotype characterized by a loss in contractile proteins and an increase in ECM expression. VSMC type is also able to acquire osteoblastic characteristics by expressing bone proteins such as osteopontin [5], type I collagen [6] and bone alkaline phosphatase (ALP) [7]. ECM is therefore profoundly

Correspondence: Jean-Paul Cristol, MD PhD, Laboratoire de Biochimie, Centre Hospitalier Universitaire Lapeyronie, 371 Avenue du Doyen Gaston Giraud, 34295 Montpellier cedex 5, Montpellier, France. Fax: +33 467 338 393. Email: jp-cristol@chu-montpellier.fr

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modified and becomes similar to that of skeletal tissue, allowing calcium deposition leading then to atherosclerosis calcification.

β-glycerophosphate [8], oxidizing LDL [9], homocysteine [10] or hyperglycemia [11] are known to be involved *in vitro* in this osteoblastic differentiation mainly via activation of the Core binding factor alpha 1 (Cbfa-1) transcription factor. Pathologic conditions such as diabetes mellitus and chronic renal failure are associated with an enhanced vascular calcification process detected by imaging or biological markers [12–14]. An enhanced Cbfa-1 expression was reported in these pathologies [15]. In addition, the increase in $CaXPO_4$ product observed in end stage renal failure could further enhance the VSMC mineralization process [16].

Interestingly, oxidative stress prevailing in these pathologies could participate in the development of atherosclerosis [17,18]. An enhanced production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$) and H_2O_2 within arterial wall by resident macrophages, endothelial cells and VSMCs could be a cell signalling pathway involved in cell cycle regulation, protein kinase activity, cell growth, modification of ECM and gene expression [19]. In addition, it has been recently shown that VSMCs could produce intracellular $O_2^{\bullet -}$ by NAD(P)H-like system via two different homologues of the $gp91^{phox}$ NAD(P)H oxidase sub-unit, Nox2 and Nox4 [20]. Since an inverse relationship was observed between smooth muscle myosin heavy chain SM2 and expression of $p22^{phox}$, another NAD(P)H oxidase membrane sub-unit, oxidative stress may be involved in the loss of contractile phenotype [21]. Oxidative stress may also participate in the transdifferentiation process as suggested by enhanced calcifications when VSMCs are incubated in the presence of H_2O_2 [22]. Very recently, an oxidant generation (particularly H_2O_2) and an over-expression in NAD(P)H oxidase sub-units $p22^{phox}$ and Nox2 have been observed around calcifying foci [23].

Thus, we hypothetized that VSMC-ROS production, partly due to NAD(P)H oxidase activity, was involved in the VSMC transdifferentiation. Therefore, we investigated ROS production and NAD(P)H oxidase expression induced by uremic serum and β-glycerophosphate, a well known osteoblastic differentiation agent, in VSMCs. Increase of vascular calcification was assessed by Cbfa-1 and bone ALP expression/activity. Calcification was confirmed with the Von Kossa staining. Implication of oxidative stress in osteoblastic differentiation was determinated using diphenyleneiodonium (DPI), an uncompetitive inhibitor of flavoenzymes. In order to determine the potential signalling pathway involved, expression of the phosphorylated extracellular signal-regulated kinase (pERK) was also performed.

Materials and methods

Cells and reagents

The aortic rat VSMC line A7r5 was obtained from the American Tissue Culture Collection (Flow Laboratories, Rockeville, MD). All culture reagents were purchased from Invitrogen (Cergy Pontoise, France). Foetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY). β-glycerophosphate, luminol, horseradish peroxidase (HRP) and diphenyleneiodonium (DPI) were obtained from Sigma Aldrich (St Quentin-Fallavier, France). $p22^{phox}$, Cbfa-1, ALP, pERK1/2 and total ERK primary antibodies as well as secondary anti-goat antibody were purchased from Tebu-Bio (Le Perray en Yvelines, France). Enhanced chemiluminescence (ECL) Western blotting detection reagents (Supersignal® West Pico Chemiluminescent Substrate) were provided by Pierce (Perbio Science, Brebières, France). All solvents were obtained from Sigma Aldrich (St Quentin-Fallavier, France).

Collection of pooled uremic and control sera

Uremic sera from 30 haemodialysis patients (mean age of 65 ± 7.2 years old and stable on haemodialysis for at least 2 years) were obtained and pooled from pre-dialysis samples collected as part of the routine monitoring. Control pool serum was obtained from blood samples collected in the preventive medicine centre.

A7r5 cell culture

A7r5 cells were grown in 60-mm-diameter dishes in Dulbecco's modified Eagle's medium (DMEM) containing 1 g/L D-glucose, 26.2 mM Na_2HCO_3, 4 mM L-glutamine, 10% FBS and 1% antibiotic solution (100 U/mL of penicillin and 100 mg/mL of streptomycin). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Before all experiments, the cells were made quiescent by incubation for 48 h in low serum medium (0.5% FBS).

Cell culture with known vascular calcification factors: β-glycerophosphate and uremic serum

β-glycerophosphate. A7r5 were cultured in growth medium or in β-glycerophosphate-enriched medium (DMEM (glucose 4.5 g/l) containing 15% FBS, 10 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin and β-glycerophosphate (10 mmol/L)) as previously reported [8,24], in the presence or absence of DPI (1 μM) for 24 or 72 h. The medium was replaced with fresh medium three times a week. The phosphate concentration used in this work and in previously reported studies seems largely higher than levels currently observed in

uremic serum. However, it has been shown that hyperphosphatemia is not the only calcifying factor present in uremia [15]. In addition, Chen et al. [25] have shown that low-phosphate concentration did not modify the expression of alkaline phosphatase and osteopontin compared with a high concentration.

Uremic serum. A7r5 were incubated for 24 or 48 h with DMEM (glucose 4.5 g/l) containing 100 U/mL penicillin, 100 mg/mL streptomycin plus 10% uremic serum from dialysis patients or 10% serum from healthy control patients. The main biological features of control and uremic serum were reported in Table I.

In the time course experiments, the beginning day of culture in calcification medium was defined as day 0. Cells grown in growth medium were considered as control cells. Cells grown in β-glycerophosphate- or uremic serum-enriched medium (namely calcification media) were considered as treated cells.

Determination of chemiluminescence intensity as an indicator of H_2O_2 production

Luminol-enhanced chemiluminescence was used to determine H_2O_2 production in cells cultured both in control and calcification media. After 24, 48 or 72 h culture (in uremic serum- or β-glycerophosphate-enriched medium, respectively), the cells were placed in RPMI medium containing 0.2 mM luminol and 0.1 g/L HRP and the H_2O_2 production was measured by means of a luminometer Victor^2 Wallac 1420 (Perkin Elmer SAS, Villebon sur Yvette, France). After chemiluminescence assay, the medium was discarded and cells were dissolved in 1 N NaOH. Then, lysed cells were centrifuged at 3000 g for 10 min at 4°C and protein concentration was measured by the Lowry et al. [26] method. Each chemiluminescence measure was carried out in triplicate and the experiments were repeated 5-times. Results were expressed as counts/10 min/μg of protein.

In order to define maximal chemiluminescence intensity, preliminary kinetic analyses were performed with calcification medium including 1, 2, 3 and 6 days of incubation. According to these preliminary results, 24, 48 and 72 h were chosen.

Table I. Analysis of pooled control sera (blood samples collected in the preventive medicine centre) and uremic sera (blood samples collected from 30 haemodialysis patients).

	Control serum	Uremic serum
Urea (mM)	5.40 ± 0.25	21.80 ± 1.10
Creatinine (μM)	74 ± 1	739 ± 26
Calcium (mM)	1.92 ± 0.35	2.03 ± 0.05
Phosphorus (mM)	1.21 ± 0.01	1.49 ± 0.09
Homocysteine (μM)	11.90 ± 0.20	27.10 ± 2.42
ALP (U/l)	62 ± 21	90 ± 52

In absence of cells, no luminol-induced chemiluminescence could be evidenced, ruling out an auto-oxidation in the medium or serum itself. Indeed, in the absence of cells, only a very low chemiluminescence signal could be recorded with RPMI (68 ± 3.3 counts/s), control (73 ± 1.7 counts/s) and β-glycerophosphate medium (74 ± 1.6 counts/s) or control (59 ± 3.3 counts/s) and uremic serum (63 ± 1.8 counts/s), corresponding to the blank (67.2 ± 9.9 counts/s) of the luminomètre Victor^2 Wallac 1420 (Perkin Elmer SAS, Villebon sur Yvette, France). By contrast in the presence of cells, chemiluminescence signal is regularly comprised of between 3000–12 000 counts/s.

Determination of alkaline phosphatase activity

After incubation in control or calcification media, cells were washed twice with phosphate-buffered saline solution (PBS). Cells were suspended in ice cold buffer consisting of 1% Triton X-100, 0.9% NaCl, then lysed cells were centrifuged at 5000 g for 10 min at 4°C. The alkaline phosphatase activity was measured by a kinetic colorimetric method on an Olympus AU 2700 (Rungis, France). The results were expressed as unit/g of protein.

Western blotting of NAD(P)H oxidase sub-unit, calcification markers and pERK1/2

After incubation in control or calcification media, in the presence or absence of NAD(P)H oxidase inhibitor when expression of calcification markers was assessed, cells were washed twice with phosphate-buffered saline solution (PBS), scraped from the culture vessels and collected. Harvested cells were suspended in ice cold buffer consisting of 0.1 mM Tris-HCl, 0.5% Triton X-100, 120 mM NaCl, 25 mM KCl, 2 mM $CaCl_2$, 1 mM phenylmethylsulphonyl fluoride, 10 μM leupeptin and 1 μM pepstatin. Then, lysed cells were centrifuged at 5000 g for 15 min at 4°C and the protein concentration was determined by Lowry method protein assay. Equal amounts of sample proteins (50 μg) were electrophoretically separated on a 12% sodium dodecyl sulphate–polyacrylamide gel. Proteins were electrically transferred to nitrocellulose membrane and the membrane was incubated overnight in blocking solution (5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBS-T)). Subsequently, monoclonal goat primary antibodies against NAD(P)H oxidase sub-unit p22^{phox}, ALP, Cbfa-1 or pERK1/2 were added at 4°C for 1 h. Since Nox1 and Nox4, the two natural catalytic isoforms present in VSMC, are functionally and structurally related to p22^{phox}, which stabilizes this complex through a proline rich region, only the p22^{phox} sub-unit has been explored. The antibody-antigen complexes were detected by incubating the membranes with horseradish conjugated secondary antibody (Tebu-bio) for 1 h at room

temperature. Reactive bands were visualized by the ECL-enhanced chemiluminescence method. The signal intensities were determined by imaging the films with a Molecular Dynamic densitometer and analysed by the Bio1D software. Values are the means ± SD calculated from three experiments.

Histochemical analysis

After 10 days of culture in control or β-glycerophosphate-enriched medium, calcium deposition on A7r5 cells was assessed by Von Kossa staining (30 min, 5% silver nitrate), as previously described [27]. Black colour determined diffusely *in vitro* calcification throughout the cell layer.

Statistical analysis

All data were expressed as mean ± SD and were analysed for statistical significance by ANOVA least significant difference. These analyses were performed with the StatView version 5.0 software.

Results

β-glycerophosphate and uremic serum induced an over-expression of p22^{phox}, a NAD(P)H oxidase sub-unit, in VSMC

We evaluated the effect of β-glycerophosphate and pooled uremic serum on expression of NAD(P)H oxidase p22^{phox} sub-unit by A7r5 cells.

β-glycerophosphate, an osteoblastic differentiation agent, significantly induced an early increase in p22^{phox} expression after 24 h of incubation (percentage of p22^{phox} increase compared to control conditions: 94%, $p < 0.001$), this expression being further enhanced after 72 h. In addition, our results showed that basal rate of p22^{phox} expression by the cells in control conditions was constant whatever the time of incubation (see Figure 1A).

Incubation of the cells in the presence of pooled uremic serum (see Table I for composition), known to induce osteoblastic differentiation in bovine VSMC, resulted in a significant increase in p22^{phox} expression after 24 h of incubation (106%, $p = 0.0226$) compared with pooled control serum (see Figure 1B), this over-expression being sustained at 48 h.

β-glycerophosphate and uremic serum enhanced luminol-induced chemiluminescence, indicating an over-production of H_2O_2 by VSMC

The chemiluminescence intensity measured in A7r5 cells cultured in β-glycerophosphate-enriched medium was significantly enhanced after 24 h (67%, $p = 0.0134$), this over-production being sustained at 72 h compared with control cells (see Figure 2A).

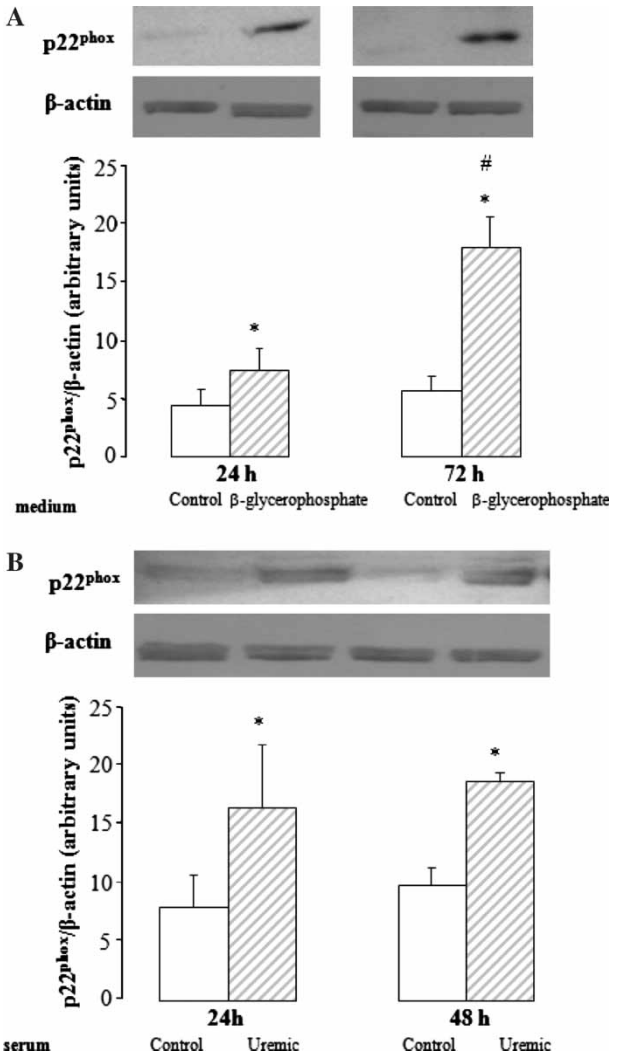

Figure 1. Effect of β-glycerophosphate and uremic serum on expression of p22^{phox}, a NAD(P)H oxidase sub-unit, in vascular smooth muscle cells. A7r5 cells were cultured (A) in control or β-glycerophosphate-enriched medium for 24 and 72 h and (B) in control or uremic serum for 24 and 48 h. Expression of NAD(P)H oxidase sub-unit p22^{phox} was then determined using 50 μg of protein extracts which were analysed by polyacrylamide gel electrophoresis. All intensities were expressed as arbitrary units from whole-cell lysates of A7r5 cells. Values are the means ± SD calculated from three experiments. * $p < 0.05$ vs (A) control medium or (B) control serum and # $p < 0.0001$ vs (A) β-glycerophosphate-enriched medium at 24 h.

A7r5 cells cultured in the presence of uremic serum-enriched medium showed the same profile of chemiluminescence as observed with β-glycerophosphate (see Figure 2B). Chemiluminescence intensity was significantly increased as early as 24 h (31%, $p = 0.0214$), this over-production being enhanced at 48 h as compared with pooled control serum.

β-glycerophosphate and uremic serum-induced increase of calcification

To confirm that A7r5 cells cultured with β-glycerophosphate and uremic serum expressed osteoblastic

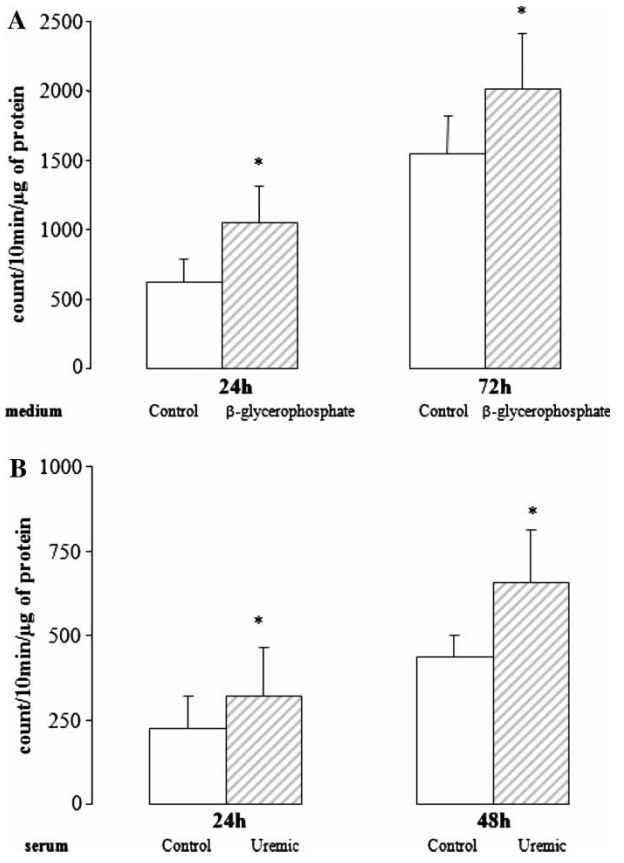

Figure 2. Effect of β-glycerophosphate and uremic serum on H_2O_2 production in vascular smooth muscle cells. A7r5 cells were cultured (A) in control or β-glycerophosphate-enriched medium for 24 and 72 h and (B) in control or uremic serum for 24 and 48 h. Cells were then placed in RPMI medium containing 0.2 mM luminol and 0.1 g/L HRP and the H_2O_2 production was measured by means of a luminometer. All activities were expressed as counts normalized from whole-cell lysates of A7r5 cells and results were expressed as counts/10 min/µg of protein. Values are the means $\pm$ SD calculated from five triplicate experiments. $\star$ $p < 0.05$ vs (A) control medium or (B) control serum.

markers, we examined ALP expression by Western blotting.

Both β-glycerophosphate and uremic serum did not have any significant effect on ALP expression and activity at 24 h of culture, by contrast our results showed a delayed enhanced ALP expression by 77.5% ($p = 0.014$) and 98% ($p < 0.05$), respectively at 72 h with β-glycerophosphate and 48 h with uremic serum (see Table II). Moreover, ALP activity was also analysed in cells cultured in β-glycerophosphate and uremic serum. These two media do not modified ALP activity at 24 h of culture. In agreement with ALP expression, β-glycerophosphate and uremic serum increased ALP activity by 118% ($p < 0.0001$) and 117% ($p < 0.05$), respectively, at 72 h with β-glycerophosphate and 48 h with uremic serum.

Moreover, Von Kossa staining showed a calcium deposit on VSMCs cultured with β-glycerophosphate compared to control cells (see Figure 3) after 10 days of incubation.

H_2O_2 production derived from NAD(P)H oxidase activation is involved in expression of osteoblastic markers

To further investigate ROS production as a contributing factor of VSMC osteoblastic differentiation, we examined the modulation of early markers of osteoblastic differentiation such as Cbfa-1 expression and ERK phosphorylation using β-glycerophosphate-enriched medium.

Incubation of VSMC for 24 h with β-glycerophosphate resulted in a 3-fold increase in ERK1/2 phosphorylation ($p < 0.0001$) (see Figure 4) and in a 2.5-fold increase in Cbfa-1 expression ($p < 0.001$) (see Figure 5).

Interestingly, DPI prevented the β-glycerophosphate-induced increase in ERK1/2 phosphorylation and Cbfa-1 expression (see Figures 4 and 5).

Discussion

This study aimed at exploring the implication of NAD(P)H oxidase in the osteoblastic differentiation of VSMC during the process of *in vitro* vascular calcification. Our results clearly showed an increase in NAD(P)H oxidase expression and in H_2O_2 generation, indirectly measured with a chemiluminescence method, by VSMC after 24 h of culture in calcification conditions such as β-glycerophosphate or uremic serum. This increase of chemiluminescence intensity was associated with an early and late

Table II. Effect of β-glycerophosphate and uremic serum on expression and activity of ALP in vascular smooth muscle cells. A7r5 cells were cultured in control or β-glycerophosphate-enriched medium for 24 and 72 h and in control or uremic serum for 24 and 48 h. Expression of ALP was then determined by polyacrylamide gel electrophoresis. All intensities were expressed as arbitrary units (AU). ALP activity was performed on cell lysate by a colorimetric method. The results were expressed as Unit/g of protein (U/g).

	Serum				Medium			
	Control		Uremic		Control		β-glycerophosphate	
	24 h	48 h	24 h	48 h	24 h	72 h	24 h	72 h
ALP (AU)	19.9 $\pm$ 5.7	20.0 $\pm$ 6.6	28.2 $\pm$ 4.6	39.8 $\pm$ 4.3$\star$	27.9 $\pm$ 9.3	31.7 $\pm$ 17.1	32.4 $\pm$ 6.4	77.1 $\pm$ 8.0$\star$
ALP (U/g)	5.03 $\pm$ 1.2	4.73 $\pm$ 1.57	5.67 $\pm$ 1.53	10.3 $\pm$ 2.2$\star$	5.9 $\pm$ 2.3	6.7 $\pm$ 2.1	8.6 $\pm$ 1.9	14.6 $\pm$ 3.2$\star$

$\star$ $p < 0.05$ vs control medium at 72 h or control serum at 48 h.

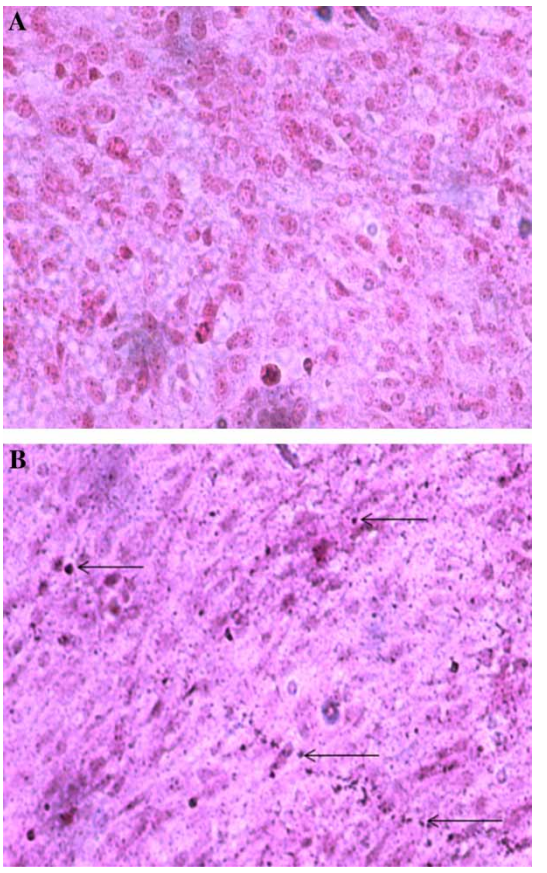

Figure 3. Von Kossa staining of A7r5 cultured in control or β-glycerophosphate-enriched medium. A7r5 cells were cultured for 10 days in the presence or absence of β-glycerophosphate-enriched medium. Von Kossa staining of A7r5 was then assessed and showed diffuse calcification (black colour for a positive detection) on ECM of the cells. Panels depict cells that are grown in the absence (A) or presence (B) of β-glycerophosphate. Cell cultures were observed by light microscopy ($\times$40 magnification).

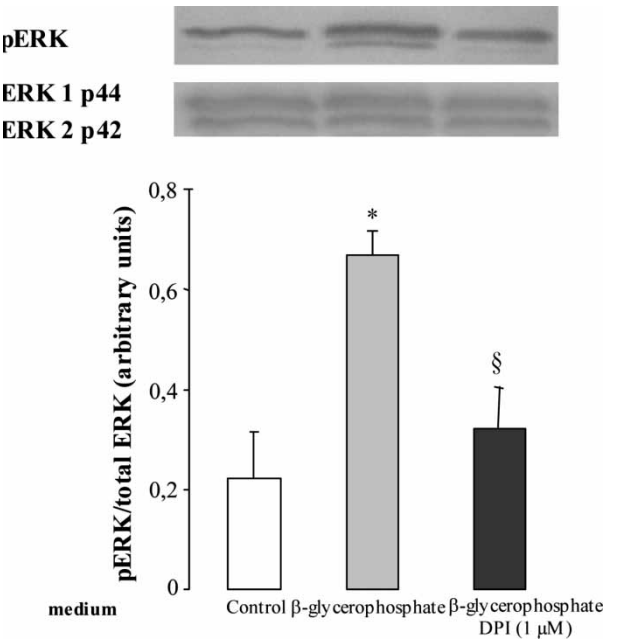

Figure 4. Effect of β-glycerophosphate with or without diphenyleneiodonium, an inhibitor of NAD(P)H oxidase, on phosphorylation of ERK in vascular smooth muscle cells. A7r5 cells were cultured for 24 h in the presence or absence of β-glycerophosphate-enriched medium with or without diphenyleneiodonium (DPI) (1 μM). Phosphorylation of ERK was then determined using 50 μg of protein extract which were analysed by polyacrylamide gel electrophoresis. All intensities were expressed as arbitrary units from total ERK of A7r5 cells. Values are the means $\pm$SD calculated from three experiments. $\star$ $p < 0.0001$ vs control medium and § $p = 0.0003$ vs β-glycerophosphate-enriched medium.

modulation of osteoblastic differentiation markers such as ERK1/2 phosphorylation, Cbfa-1 expression and ALP expression and activity. The osteoblastic transformation was further confirmed by Von Kossa staining, showing a diffuse deposit of calcium after 10 days of culture in β-glycerophosphate-enriched medium. The link between oxidative stress and osteoblastic differentiation was further demonstrated by the reduction in Cbfa-1 expression using DPI as a NAD(P)H oxidase inhibitor.

Vascular calcification in uremic patients has long been regarded as a passive process in which disturbances in calcium phosphorus metabolism played a central role. However, expression of bone matrix proteins by VSMC in arteries recently suggested that vascular calcification was not simply passive but rather an active, cell-mediated process secondary to VSMC transdifferentiation into osteoblast-like cells [28]. Interestingly, this osteoblastic differentiation leading to media calcification is also observed in diabetic patients in the absence of calcium abnormalities. *In vitro* transdifferentiation processes could be achieved by inorganic phosphate donors such as β-glycerophosphate or uremic serum. Indeed, several groups showed that incubation of VSMCs with β-glycerophosphate was associated with high levels in nuclear Cbfa-1 and ALP expression [8,29]. This latter protein is largely involved in bone mineralization and was identified in vascular-calcified lesions such as atherosclerosis, but not in control arterial wall [30]. Here, we reported for the first time that β-glycerophosphate and uremic serum induced not only *in vitro* diffuse calcification but also an early induction of NAD(P)H oxidase system, demonstrated by $p22^{phox}$ expression, associated with an increase of chemiluminescence intensity. This endogenous increase of chemiluminescence intensity induced by calcifying conditions and generated from an over-expression and activity of NAD(P)H oxidase complex is in agreement with a previous study showing that exogenous H_2O_2 could initiate osteoblastic differentiation [22]. These results strongly suggest that NAD(P)H oxidase may be an early target for β-glycerophosphate or uremic serum. Indeed, previous studies have clearly demonstrated an NAD(P)H oxidase-dependent ROS over-production in uremia [31–33] or related disorders [34]. The hypothesis of an oxidative stress-induced calcification

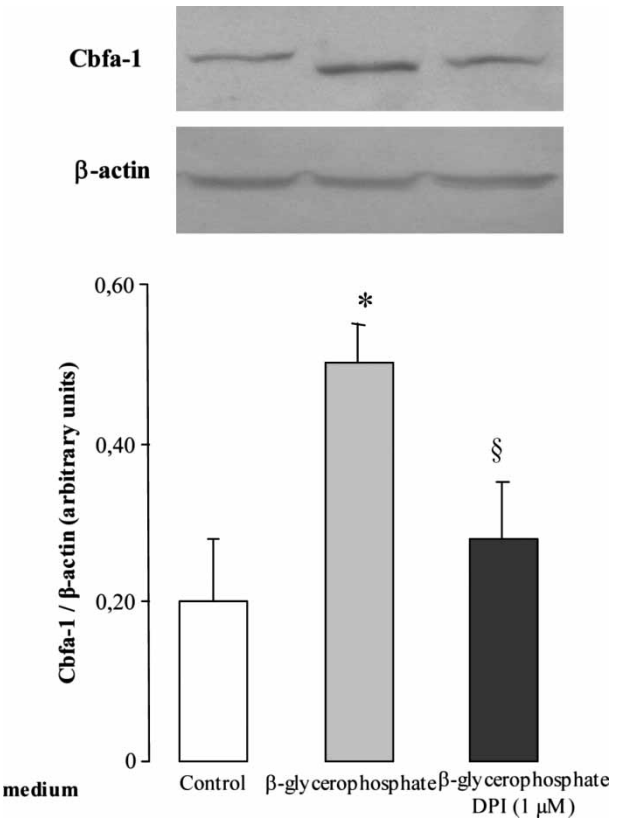

Figure 5. Effect of β-glycerophosphate with or without diphenyleneiodonium, an inhibitor of NAD(P)H oxidase, on expression of Cbfa-1 in vascular smooth muscle cells. A7r5 cells were cultured for 24 h in the presence or absence of β-glycerophosphate-enriched medium with or without diphenyleneiodonium (DPI) (1 μM). Expression of Cbfa-1 was then determined using 50 μg of protein extract which were analysed by polyacrylamide gel electrophoresis. All intensities were expressed as arbitrary units from β-actin of A7r5 cells. Values are the means ± SD calculated from three experiments. $^{\star}p < 0.0001$ vs control medium and § $p = 0.0007$ vs β-glycerophosphate-enriched medium.

is further supported by the association of oxidative stress markers, such as malondialdehyde and hydroperoxides in uremic animal models and recently in haemodialysis patients [35,36].

To test directly whether the NAD(P)H oxidase complex was critical for this H_2O_2 production by VSMCs under calcification conditions, we used DPI as a NAD(P)H oxidase inhibitor [37]. Our results demonstrated a direct role of ROS production mainly originated from NAD(P)H oxidase as an early event in osteoblastic differentiation of VSMCs. Indeed in VSMCs, nuclear Cbfa-1, a key regulatory transcription factor in osteoblastic differentiation [29,38], was activated concomitantly with the NAD(P)H oxidase system (see Figure 1A Figure 5) and partly inhibited with use of DPI (see Figure 5). Similar results have been previously observed in the bone marrow cells since Wang et al. [39] reported that Cbfa-1 expression was induced by $O_2^{\cdot -}$ and accounted for osteoblast marker synthesis and osteogenesis. The ROS-mediated Cbfa-1 activation in such a model is under the dependence of ERK activation, a specific extracellular signal-regulated kinase member of MAPK family sensitive to $O_2^{\cdot -}$ [39]. The relevance of the ERK pathway in VSMC transdifferentiation has been also underlined since fibronectin induces osteoblastic differentiation by activating ERK [40]. In agreement with this observation, our results demonstrated that DPI prevented this β-glycerophosphate-induced ERK activation and Cbfa-1 expression.

Our study acknowledges some limitations concerning the osteoblastic transformation of VSMC. Previous studies have shown that VSMC placed under calcifying conditions lost their lineage markers such as SM22 alpha and smooth muscle alpha-actin [29], suggesting an osteoblastic transformation. Although our study does not provide direct evidence of these osteoblastic transformation by double-staining experiments for VSMC and osteoblast markers, our results strongly support the hypothesis that NADPH oxidase is involved in vascular calcifications.

In conclusion, early NAD(P)H oxidase expression and ROS production induced by β-glycerophosphate appear as a key event in the increase of calcification. Indeed, DPI prevented early ROS production and late ALP expression. It could be suggested that $O_2^{\cdot -}$ produced by NAD(P)H oxidase is involved in ERK phosphorylation and Cbfa-1 activating pathways. The clinical relevance of such an early signalling pathway observed in β-glycerophosphate-induced osteoblastic differentiation needs to be further evaluated in pathological conditions inducing both vascular calcification and oxidative stress such as end stage renal failure and diabetes.

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