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Leptin promotes osteoblast differentiation and mineralization of primary cultures of vascular smooth muscle cells by inhibiting glycogen synthase kinase (GSK)-3β

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ABSTRACT

In this study, we begin to investigate the underlying mechanism of leptin-induced vascular calcification. We found that treatment of cultured bovine aortic smooth muscle cells (BASMCs) with leptin (0.5–4 μg/ml) induced osteoblast differentiation in a dose-dependent manner. Furthermore, we found that leptin significantly increased the mRNA expression of osteopontin and bone sialoprotein, while down-regulating matrix gla protein (MGP) expression in BASMCs. Key factors implicated in osteoblast differentiation, including members of the Wnt signaling pathway, were examined. Exposure to leptin enhanced phosphorylation of GSK-3β on serine-9 thereby inhibiting activity and promoting the nuclear accumulation of β-catenin. Transfection of BASMCs with an adenovirus that expressed constitutively active GSK-3β (Ad-GSK-3β S9A) resulted in a >2-fold increase in GSK-3β activity and a significant decrease in leptin-induced alkaline phosphatase (ALP) activity. In addition, qRT-PCR analysis showed that GSK-3β activation resulted in a significant decrease in the expression of osteopontin and bone sialoprotein, but a marked increase in MGP mRNA expression. When taken together, our results suggest a mechanism by which leptin promotes osteoblast differentiation and vascular calcification *in vivo*.

1. Introduction

Obesity is recognized as a significant independent risk factor for cardiovascular disease and associated morbidity and mortality [1,2]. We now know that adipose tissue can actively secrete adipocytokines, including leptin, resistin, and adiponectin which may play direct and indirect roles in the development of cardiovascular disease (CVD). Furthermore, specific adipocytokines are known to circulate at elevated levels in obese individuals and to correlate positively with the risk for CVD [3–8].

Leptin is a secreted protein of approximately 16 kDa that acts on the hypothalamus to regulate energy intake and expenditure [9,10]. Leptin also has proatherogenic effects that have been well described in various mouse models [11–14]. Recently, our lab demonstrated that leptin can promote the calcification of atherosclerotic lesions in ApoE – deficient mice and that this process of leptin-mediated vascular calcification is associated with an increase in the expression of osteoblast – specific markers, including alkaline phosphatase, osteopontin and osteocalcin, within the vessel wall [15].

There are several clinical consequences associated with the presence of vascular calcification [16]. For example, the calcification of coronary arteries contributes to an increased risk of plaque rupture and the possibility of a myocardial infarction/stroke [17,18]. There is also an increased risk of vessel wall dissection during balloon angioplasty or stent placement [19]. In addition, the calcification of cardiac valves can lead to valvular stenosis resulting in the loss of valve mobility which is a major mechanism for valve failure [20].

Previous findings support a role for "osteoblast-like" cells within calcified vessel walls. Indeed, our lab and others have demonstrated that cultured vascular smooth muscle cells (VSMCs) can be induced to undergo a phenotypic transition into osteoblast-like cells [21,22]. However, the mechanism by which this may be occurring is still largely unknown. There are several signal transduction pathways which are thought to be important in the process of osteoblast differentiation. One such pathway is the canonical Wnt signaling pathway. This pathway becomes activated when Wnt ligands (i.e. Wnt1, Wnt3a) bind the cell-surface Frizzled (FZD) receptor that in turn complexes with co-receptors low-density-lipoprotein-receptor-related protein 5/6 (LRP 5/6). The activation of the cell surface FZD/LRP5/6 receptor complex

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mediates the inactivation of GSK-3β. Active GSK-3β can phosphorylate β-catenin and thereby target it for ubiquitination and proteasomal degradation. When GSK-3β is inactivated, β-catenin escapes degradation, accumulating instead in the cytoplasm and eventually translocating to the nucleus. In the nucleus, β-catenin can heterodimerize with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to induce the expression of specific genes [23,24]. The ability of leptin to modulate Wnt-signaling pathways is supported by studies in various cell types including breast cancer cells [25,26], but to date no one has investigated the effect of leptin on Wnt signaling in a model for osteogenic differentiation. In this study, we propose a mechanism by which leptin is promoting osteogenic differentiation and thus vascular calcification *in vivo*.

2. Material and methods

2.1. Materials

Recombinant human leptin and *p*-nitrophenol phosphate substrate kit used to assess ALP activity were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Antibodies for phospho-GSK-3β (Ser9), total GSK-3β, β-catenin and Lamin A/C were obtained from Cell Signaling (Beverly, MA, USA). Ad-CMV-Null and Ad-GSK-3β S9A constructs were purchased from Vector Biolabs (Philadelphia, PA, USA).

2.2. Isolation of vascular smooth muscle cells

VSMCs were isolated from fresh bovine aortas that were obtained from a local slaughter house (Highland Packers LTD, Hamilton, ON) as previously described [21,27].

2.3. Measurements of alkaline phosphatase activity

To assess the extent of osteoblast differentiation, BASMCs were seeded onto 24-well plates at a concentration of 2.5×10^4 cells/well and then cultured for 8 days in differentiating media (DMEM containing 10% FBS, 10 mM β-glycerophosphate and 0.5 mM ascorbic acid) in the absence or presence of 2 μg/ml leptin. In some experiments, cells were infected with either 10 MOI of a control adenovirus (Ad-CMV-Null) or an adenovirus that constitutively expressed active GSK-3β by substitution of the serine residue with an alanine residue at position 9 (Ad-GSK-3β S9A). Cells were infected for 24 h before being cultured in the absence or presence of 2 μg/ml leptin. Cells were harvested by being lysed in 1% Triton X-100, 0.9% NaCl for one hour on ice. ALP activity was assessed at 405 nm using a *p*-nitrophenol phosphate substrate kit. Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) was used to quantify protein concentrations and ALP values were normalized to protein (U/mg of protein).

2.4. qRT-PCR

BASMCs were plated onto 60 mm dishes at a concentration of 2.5×10^5 cells/plate. When cells reached 70% confluency, they were allowed to continue differentiating in 10% FBS, 10 mM β-glycerophosphate and 0.5 mM ascorbic acid in either the absence or presence of leptin (2 μg/ml) for 8 days in order to measure mRNA expression levels of osteopontin, bone sialoprotein and MGP. Media was changed every 3–4 days for up to 8 days. Briefly, cellular RNA was isolated using an RNeasy mini kit (Qiagen, Mississauga, ON, Canada). RNA was quantified by measuring the absorbance at 260 nm and RNA purity was verified by calculating the ratio of the absorbance at 260 and 280 nm (A_{260}/A_{280}). cDNA was then reverse transcribed from 4 μg of RNA using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR analysis was performed on the ABI PRISM 7300 (Applied Biosystems, USA) using iQ SYBR Green Supermix (Bio-Rad), 4 μg cDNA and 500 nM foward and reverse primers. See Table 1 for specific primers and protocols.

2.5. von Kossa staining

BASMCs were plated onto coverslips at a concentration of 5×10^4 cells in a 24-well plate. Cells were cultured in DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate and 0.5 mM ascorbic acid in the absence or presence of leptin (0.5–2 μg/ml). Media was changed every 3–4 days up to 12 days. Mineralization was assessed by von Kossa staining as previously described [27].

2.6. Preparation of cell-free lysates and nuclear extracts

BASMCs were plated onto 60 mm dishes at a density of 2.5×10^5 cells/plate and then cultured in DMEM containing 10% FBS. Once cell cultures reached 70% confluency, cells were washed twice in 1× phosphate buffered saline (PBS) and cultured in DMEM containing 0.5% FBS for an additional 24 h before being treated for various periods of time with leptin (2 μg/ml). Cells were then rinsed twice with ice-cold 1× PBS before being lysed in lysis buffer (1% sodium deoxycholate, 1% Triton X-100, 50 mM Tris HCl pH7.2, 0.25 EDTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF, 0.1 mM Na_3VO_4) and isolated using a cell scraper. To obtain nuclear extracts, cells were first resuspended in a hypotonic buffer (10 mM Hepes–KOH (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1 mM Na_3VO_4, 0.5 mM PMSF, 10 μg/ml aprotonin, 2 μg/ml pepstatin A and 2 μg/ml leupeptin) to release the cytoplasmic proteins. To then isolate nuclear proteins, the pellet was resuspended in 20 mM Hepes-KOH (pH 7.9) containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF 10 μg/ml aprotonin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin and 25% glycerol. Bio-Rad DC protein assay was used to determine protein concentrations.

2.7. Western blot analysis

Cell-free lysates or nuclear extracts were prepared as described above and western blot analysis was performed as previously published [21]. Protein bands were visualized by an enhanced chemiluminescent system. Results were quantified using ImageJ version 1.32j software (Wayne Rasband, National Institute of Health, USA).

2.8. GSK-3β activity assay

BASMCs were infected with either 10 MOI Ad-CMV-Null or Ad-GSK-3β S9A. To measure GSK-3β activity, whole cell lysates were collected in GSK-3 lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 10 mM DTT, 1 mM benzamidine, 0.1× Roche PhosSTOP, 1 mM Na_3VO_4,

Table 1
Primers and Protocols for qRT-PCR.

Genes	Primer sequence	Annealing temperature (°C)
Osteopontin	Fwd: CCGCCGCAGACCAAGGAAAAAT Rev: AACTGGAAGGGCGGAGGCAAT	60
Bone sialoprotein	Fwd: ATGGGACTACCCCACCACCGT Rev: CCCCACGAGGATCTCCGTTCTCA	62
Matrix gla protein	Fwd: GAGCTCAACCGGGAAGCTTGTG Rev: CCTCGGCGCTGCCGGAAATAA	61
GAPDH	Fwd: ATGGCCTTCCGCGTCCCCACTCC Rev: AGCCAAATTCATTGTCGTACC	60–62

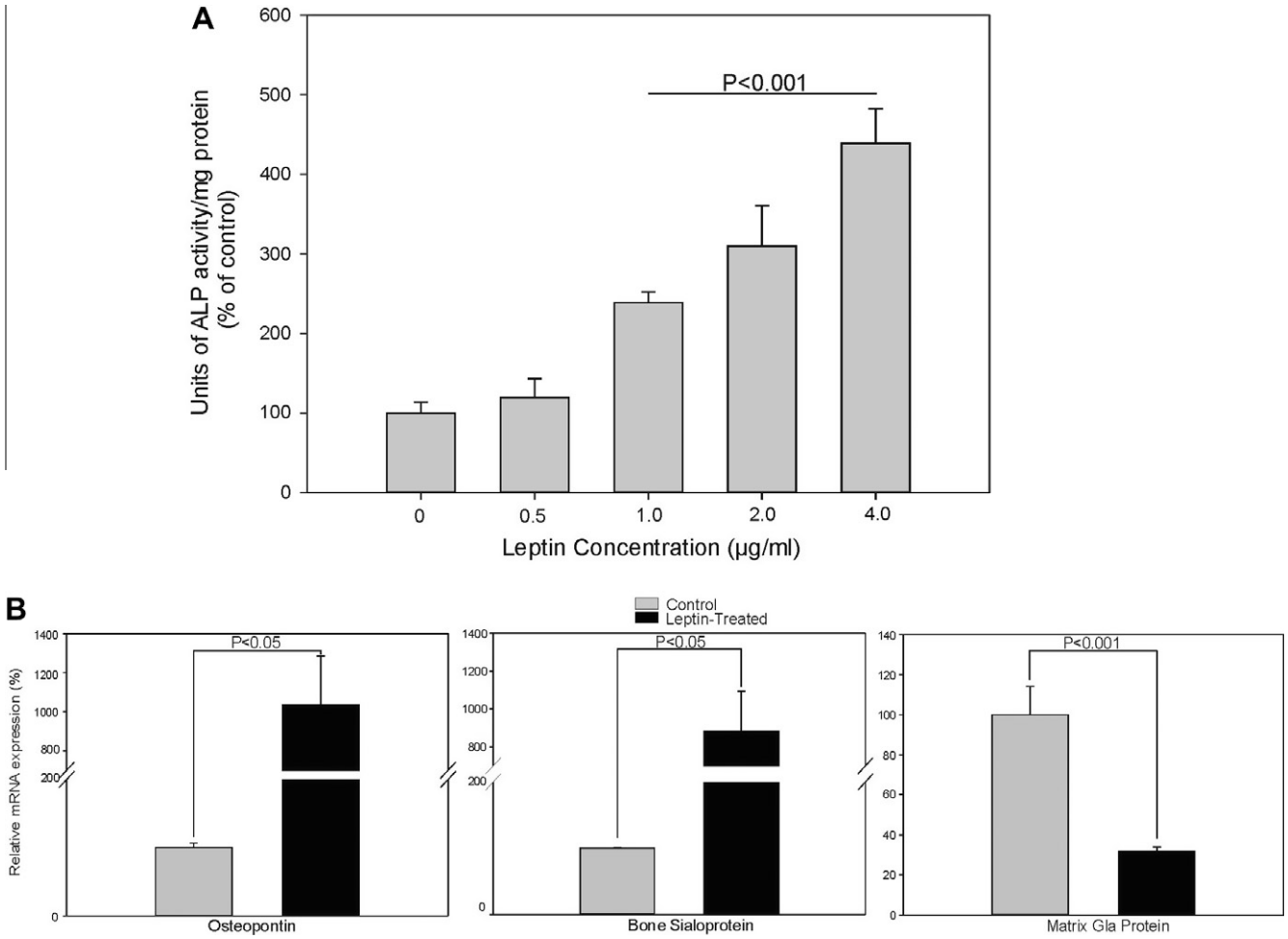

Fig. 1. The effect of leptin on osteoblast differentiation in primary cultures of BASMCs. (Panel A) BASMCs were cultured in the presence of 0–4 μg/ml leptin for 8 days before assessing ALP activity as an index of osteoblast differentiation. (Panel B) Expression of osteoblast-specific markers was analyzed by qRT-PCR in BASMCs cultured in the presence of 2 μg/ml leptin for 8 days. $P < 0.001$ compared to BASMCs cultured in the absence leptin.

and 1 mM PMSF) on day 3, 5 and 7 and then immunoprecipitated overnight with 0.625 μg purified mouse anti-GSK-3β antibody (BD Biosciences, Mississauga, ON). GSK-3 activity was then assayed as previously described [28].

2.9. Statistical analysis

Data are expressed as mean ± standard error (S.E.M) of at least three or four independent experiments. A significance of differences was determined using an unpaired student's *t*-test of equal variance. qRT-PCR data are expressed with adjusted *p*-values using the Benjamin-Hochberg False Discovery Rate.

3. Results and discussion

3.1. Effect of leptin on osteoblast differentiation and mineralization

We have previously shown that the adipocytokine, leptin, can promote the vascular calcification of atherosclerotic lesions in ApoE – deficient mice. We demonstrated that the increase in vascular calcification was associated with an increase in the presence of osteoblast-specific markers (e.g. alkaline phosphatase, osteocalcin and osteopontin) within the vessel wall [15]. Vascular calcification is a tightly-regulated, cell-mediated process that recapitulates the progression of bone mineralization and several theories exist to describe the process by which vascular calcification may be occurring [16]. Vascular calcification may result from the loss of calcification inhibitors such as MGP [29,30], the nonspecific entrapment of nucleation complexes that arise due to bone resorption [31,32] or the phenotypic transition of a subpopulation of VSMCs into bone-forming (osteoblast-like) cells [21–33]. Our *in vivo* findings suggest that leptin enhances the expression of osteoblast-specific markers within the vessel wall by inducing the differentiation of VSMCs into osteoblast-like cells.

Our lab and others have previously demonstrated that primary cultures of BASMCs can be induced to undergo a phenotypic transition into "osteoblast-like cells" [21,27,34,35]. To determine if leptin promotes osteoblast differentiation in primary cultures of BASMCs, we assessed ALP activity as an index for osteoblast differentiation. BASMCs were cultured in the absence or presence of increasing concentrations of leptin (0.5–4 μg/ml) for 8 days. As seen in Fig. 1A, ALP activity was significantly, and dose-dependently increased when BASMCs were cultured in the presence of leptin with a 4.4 ± 0.2-fold ($P < 0.001$) increase in ALP activity at 4 μg/ml. Similar results were found when we used qRT-PCR to examine the effect of leptin on several other osteoblast-specific markers (Fig. 1B). We observed a significant increase in the expression of both osteopontin and bone sialoprotein expression but a significant decrease in the mRNA levels of the calcification inhibitor, MGP. To determine if leptin has the same effect on mineralization, BASMCs were cultured in the absence or presence of increasing doses of leptin (0.5, 1, and 2 μg/ml) for 12 days. von

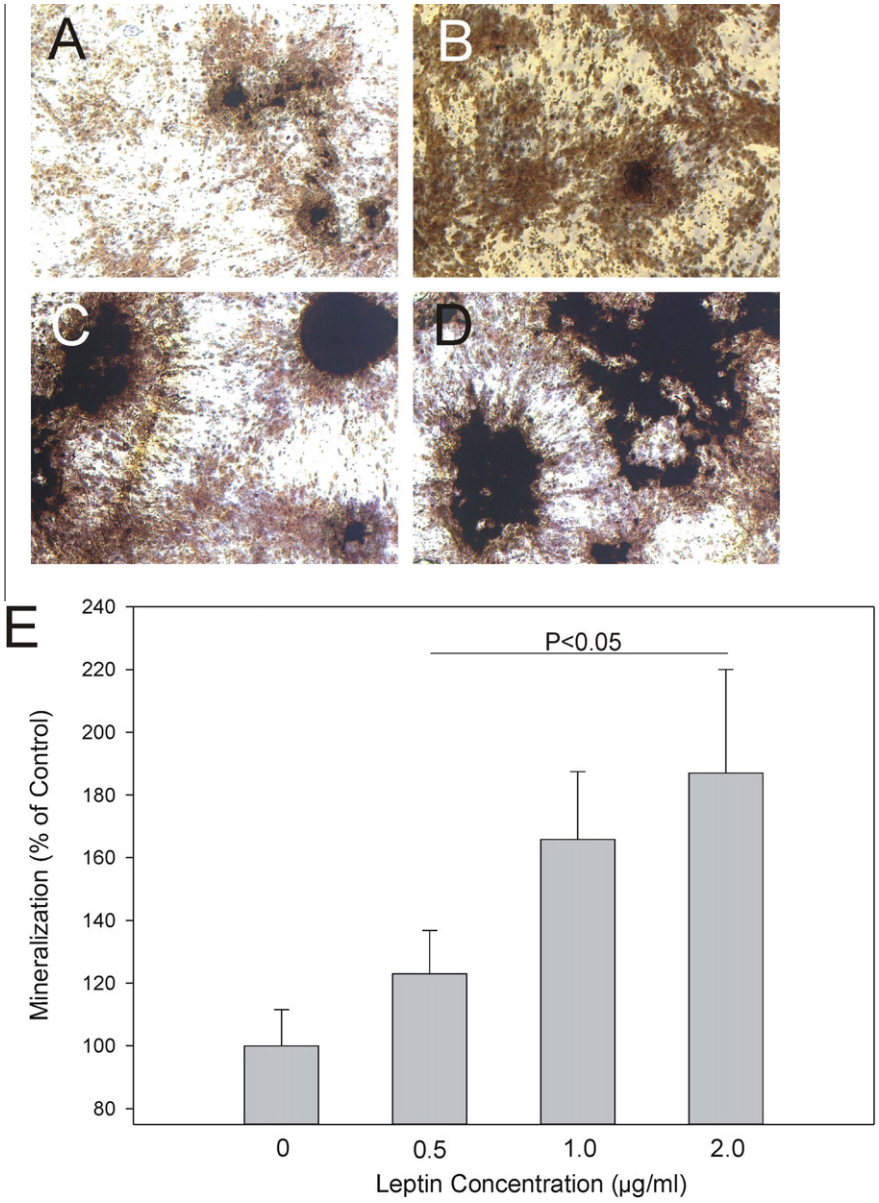

Fig. 2. The effect of leptin on mineralization of primary cultures of BASMCs. (Panel A–D) BASMCs were cultured in the absence or presence of 0.5, 1.0 or 2.0 μg/ml leptin, respectively. Twelve days later, the cells were stained by von Kossa to assess the degree of mineralization (Panel E). $P < 0.05$ when compared to cells cultured in the absence of leptin.

Kossa staining was used to assess the extent of mineralization (Fig. 2A). As seen in Fig. 2B, there was a dose-dependent increase in the degree of mineralization (2-fold at the highest concentration tested). When taken together, these findings confirm leptin's ability to promote the osteogenic differentiation and mineralization of cultured VSMCs.

3.2. Effect of leptin on GSK-3β phosphorylation and β-catenin nuclear accumulation

Since vascular calcification shares many similarities with the process of bone formation, we examined the role of Wnt signaling in our *in vitro* model of osteogenic differentiation in an effort to delineate the mechanism of leptin-induced osteoblast differentiation. BASMCs were treated with leptin for increasing periods of time and cell lysates were analyzed by western blot to determine the effect on GSK-3β. As shown in Fig. 3A, leptin induced the phosphorylation of GSK-3β at serine 9 by 3.2 ± 0.63-fold ($P < 0.05$) at 80 min when compared to untreated controls. Leptin-induced phosphorylation of GSK-3β on serine 9 was transient, peaking at 80 min and returning to control levels after 100 min (data not shown). In a dose response experiment, maximal GSK-3β phosphorylation was observed when BASMCs were treated with 2 μg/ml leptin (Fig. 3B). This dose was used in all subsequent experiments to evaluate the role of leptin in Wnt signaling. The inactivation of GSK-3β by the canonical Wnt ligands is associated with the cytosolic accumulation and subsequent nuclear translocation of β-catenin. To determine whether, in our model of osteoblast differentiation, leptin's ability to promote GSK-3β phosphorylation also results in the nuclear accumulation of β-catenin, we performed immunoblot analysis on nuclear extracts of BASMCs treated with leptin for up to 12 h. As shown in Fig. 3C, treatment with leptin resulted in the nuclear accumulation of β-catenin. The *in vitro* activation of Wnt signaling has previously been shown to upregulate the osteogenic differentiation of mesenchymal stem cells [36,37] and the Wnt signaling pathway has been shown to be active in the calcified vasculature of diabetic low density lipoprotein receptor – deficient ($LDLR^{-/-}$) mice [38]. Our findings suggest

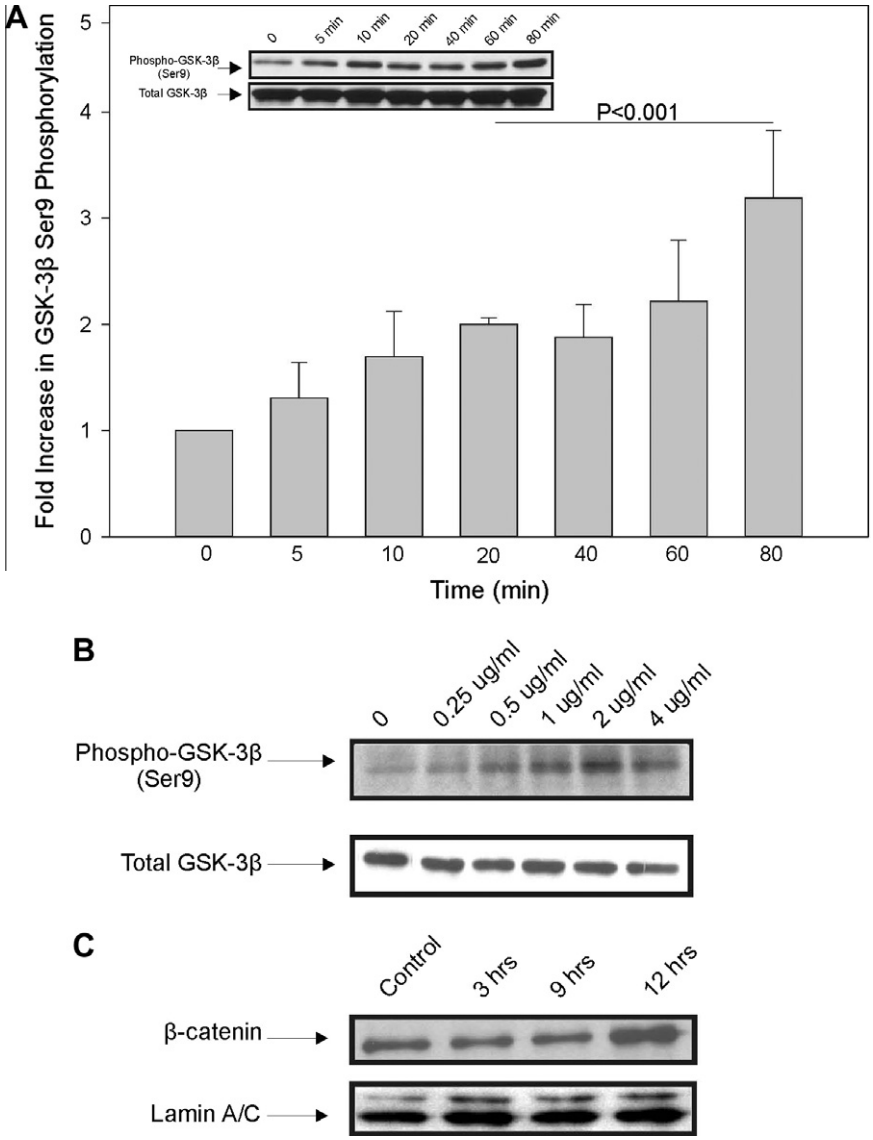

Fig. 3. The effect of leptin on GSK-3β phosphorylation and β-catenin accumulation in primary cultures of BASMCs. (Panel A) BASMCs were cultured in the presence of 2 μg/ml leptin for increasing periods of time up to 80 min. (Panel B) BASMCs were cultured in either the absence or presence of 0.25, 0.5, 1, 2 or 4 μg/ml leptin for 80 min. Cell lysates were prepared and immunoblotted for either phospho-GSK-3β (Ser9) or total GSK-3β. (Panel C) Nuclear extracts cultured in the presence of leptin for 3, 9 or 12 h were isolated and immunoblotted for β-catenin or Lamin A/C. $P < 0.05$ when compared to control conditions in which cells were not treated with leptin.

that leptin may be inducing its osteogenic effects by acting on key players of the canonical Wnt signaling pathway.

3.3. Role of GSK-3β in leptin-mediated osteoblast differentiation

To determine if GSK-3β plays a direct role in osteoblast differentiation, BASMCs were infected with an adenovirus expressing constitutively active GSK-3β (Ad-GSK-3β-S9A) or an empty control adenovirus (Ad-CMV-Null). In this cell culture system, total GSK-3β over expression was achieved and sustained for up to 7 days post infection when compared to cells infected with a control adenovirus (Fig. 4A). GSK-3β activity was increased more than 2-fold at 3, 5 and 7 days post infection, compared with cells that were infected with the control adenovirus (Fig. 4A). We then examined the effect of constitutive GSK-3β activity on the mRNA expression of osteoblast-specific markers after 8 days. Constitutive GSK-3β expression significantly decreased osteopontin and bone sialoprotein expression while increasing MGP expression (Fig. 4B). These results suggest that GSK-3β plays a role in osteoblast differentiation. Next, we employed the adenovirus system in an ALP assay (Fig. 4C). BASMCs were once again infected with either Ad-CMV-Null or Ad-GSK-3β S9A and 24 h later, cells were cultured in the absence or presence of 2 μg/ml leptin for 8 days. Cells infected with Ad-CMV-Null and treated with leptin showed a 4.8-fold increase in ALP activity compared to cells cultured without leptin (63.9 ± 3.43 U/mg vs. 13.2 ± 1.63 U/mg). However, cells infected with Ad-GSK-3β S9A and also treated with leptin did not show the same increase in ALP activity as Ad-CMV Null-infected and leptin-treated BASMCs. Ad-GSK-3β S9A-infected and leptin-treated BASMCs showed only a 3.0-fold increase in ALP activity compared to the cells cultured without leptin (50.6 ± 2.63 U/mg vs. 17.1 ± 0.84 U/mg). BASMCs infected with Ad-GSK-3β S9A and treated with leptin had a significantly attenuated increase in ALP activity compared to cells infected with a control adenovirus and also treated with leptin ($P = 0.01$). These results suggest that GSK-3β also plays a role in leptin-mediated osteoblast differentiation.

The mechanism(s) by which leptin modulates GSK-3β phosphorylation/activity to promote the osteogenic differentiation of

VSMCs remains to be determined. It is well established that leptin can regulate many signaling pathways including JAK/STAT, mitogen-activated protein kinases (MAPK), suppressors of cytokine signaling (SOCS) and phosphatidylinositol-3-kinase and insulin receptor substrate proteins (PI3K/IRS) [39]. More recently, Yan et al. [26] demonstrated that leptin induces epithelial-mesenchymal transition in breast cancer cells by modulating Akt/GSK-3β and MTA1/Wnt1 axes to mediate β-catenin activation. Alternatively, in hepatocellular carcinomas, it has been shown that Erk (extracellular-regulated kinase) acts to prime GSK-3β for subsequent phosphorylation at serine 9 by p90RSK. This event results in the inactivation of GSK-3β and the subsequent accumulation of β-catenin [40]. Our lab has previously shown that the treatment of cells with the MEK inhibitor, PD98059, dose-dependently attenuates ALP activity by up to 60% suggesting that leptin's ability to promote the osteoblast differentiation of primary BASMCs is, at least in part, Erk 1/2 – dependent [15]. It is possible that leptin induces the phenotypic transition of smooth muscle cells by modulating Erk/GSK-3β to mediate β-catenin activation. Osteoblast differentiation is a complex process that depends on

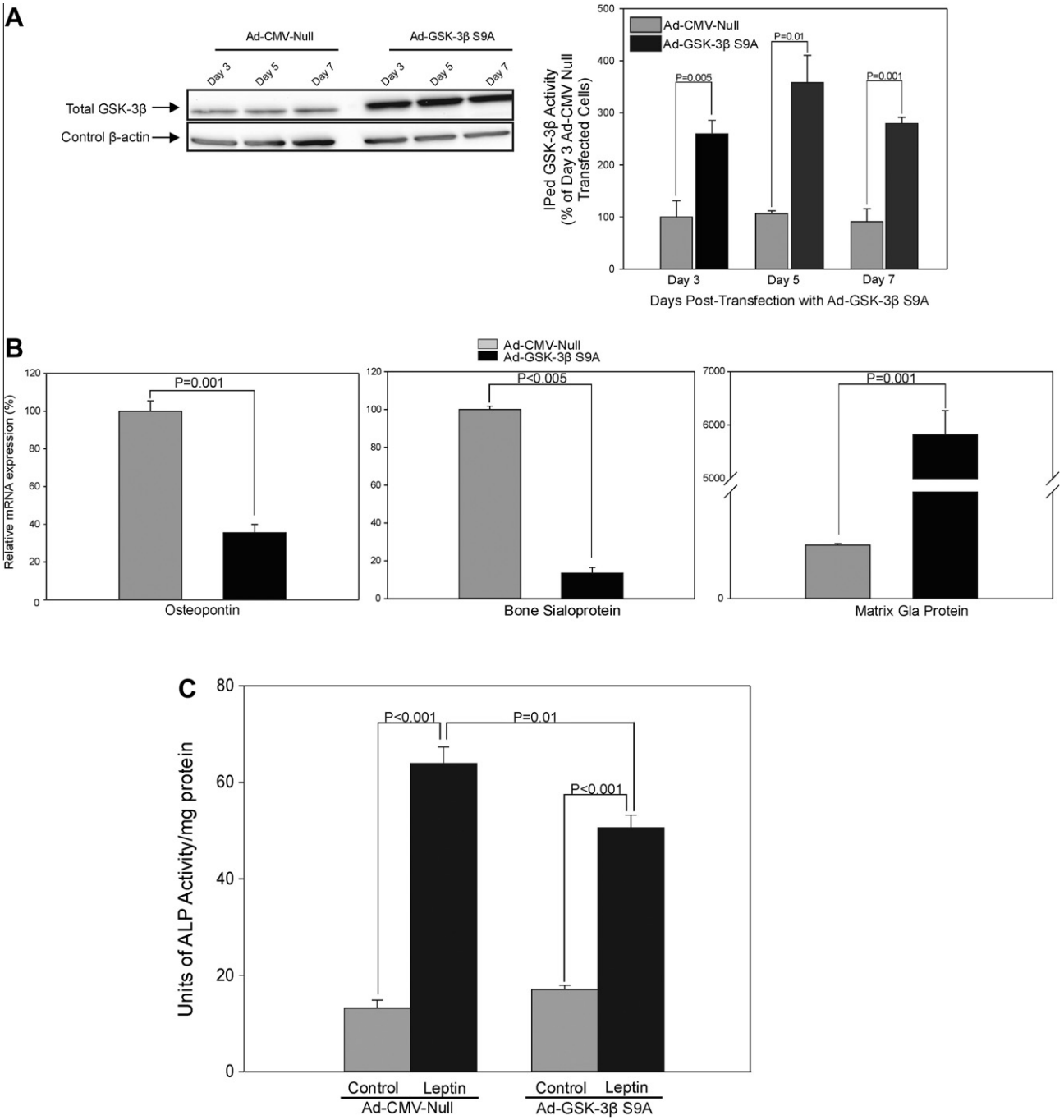

Fig. 4. Transfection of primary cultures of BASMCs with adenovirus that constitutively expresses active GSK-3β. (Panel A) BASMCs were transfected with 10 MOI of either an empty adenovirus (Ad-CMV-Null) or an adenovirus that constitutively expresses active GSK-3β (Ad-GSK-3β S9A) for up to 8 days. Immunoblots were probed with total GSK-3β antibody to verify adenovirus expression and GSK-3β activity was measured by ^{32}P incorporation onto phospho-glycogen synthase peptide-2 substrate. (Panel B) Expression of osteoblast-specific markers was analyzed by qRT-PCR in BASMCs transfected Ad-CMV-Null or Ad-GSK-3β S9A. (Panel C) ALP activity was measured as an index of osteoblast differentiation in BASMCs transfected with Ad-CMV-Null or Ad-GSK-3β S9A and cultured in the absence or presence of 2 μg/ml leptin for 8 days.

temporal expression of various factors and is highly influenced by protein–protein and protein-DNA interactions, and warrants further research.

Clinical trials have demonstrated a positive correlation between circulating plasma leptin levels and CVD. The five year prospective West of Scotland Coronary Prevention Study (WOSCOPS) [8] showed that leptin is a significant risk factor for CVD while a separate finding concluded that plasma leptin levels are a strong predictor for the risk of an acute myocardial infarction [41]. Other studies have shown an association between leptin and the risk for coronary artery calcification in a population of type 2 diabetic and non-diabetic individuals [42,43]. Our findings provide a novel mechanism by which elevated leptin levels, associated with obesity, promote osteoblast differentiation and vascular calcification *in vivo* by regulating GSK-3β activity in VSMCs.

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