Original article

Cyclic-AMP-dependent proliferation of a human osteoblast cell line (HOS cells) induced by hydroxyapatite: effect of exogenous nitric oxide

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Abstract. *Background and Aims of the work*: Nitric oxide (NO) has been reported to enhance the production of cAMP by hydroxyapatite (HA)-induced a human osteoblast cell line (HOS cells). The aim of the present study was to test the hypothesis that exogenous NO may up-regulate the proliferation of hydroxyapatite (HA)-induced HOS cells via the cyclic-AMP-protein kinase A (PKA) pathway. *Methods*: HOS cells were pre-incubated with ODQ (guanylyl cyclase inhibitor), SQ22536 (adenylyl cyclase inhibitor), forskolin (adenylyl cyclase activator), IBMX [phosphodiesterase (PDE) inhibitor], siguazodan (PDE3 inhibitor), rolipram (PDE4 inhibitor), or KT5720 (PKA inhibitor), and then, cultured on the surface of HA with or without the presence of SNAP (NO donor). The HOS cell cultures on the HA surface were added with br-cGMP (cGMP analogue), db-cAMP (cAMP analogue) with or without SNAP. The cell proliferation was assessed by a colorimetric assay. *Results*: The up-regulatory effect of SNAP on HA-induced HOS cell proliferation was suppressed by SQ22536 and KT5720, but enhanced by db-cAMP, IBMX, and rolipram. The HA-induced HOS cell proliferation with or without the presence of SNAP was unaltered by ODQ, br-cGMP and siguazodan. *Conclusion*: These results suggest, therefore, that HA-induced HOS cell proliferation may be mediated by the cAMP-PKA pathway regulated by PDE4 and that exogenous NO may amplify this cyclic nucleotide pathway, thereby augmenting HA-induced HOS cell proliferation. (www.actabiomedica.it)

Key words: cAMP, Hydroxyapatite, osteoblasts, nitric oxide

Introduction

During cellular activation and functions, a complex interaction among intracellular chemicals known as the second messengers, such as cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP), results in upstream and downstream signals which lead to activate target genes. Cyclic AMP produced from ATP by adenylyl cyclases and subsequently, it activates the cAMP-dependent protein kinase A (PKA) pathway (1), whereas cGMP synthesis controlled by guanylyl cyclases activates the cGMP-dependent protein kinases (2) (Fig. 1). Osteoblast activation and functions induced by growth factors, such as parathyroid hormone (3), and cytokines, such as prostaglandin E_2 (PGE_2) (4) are known to be regulated by cAMP. Furthermore, previous reports showed that mouse fibroblast attachment on the cellulose substratum was dependent on the cAMP-PKA pathway (5, 6). However, whether or not attachment, spreading, proliferation and differentiation of osteoblasts on hydroxyapatite (HA)-containing implant materials involve the activation of cAMP-PKA pathway remains unclear.

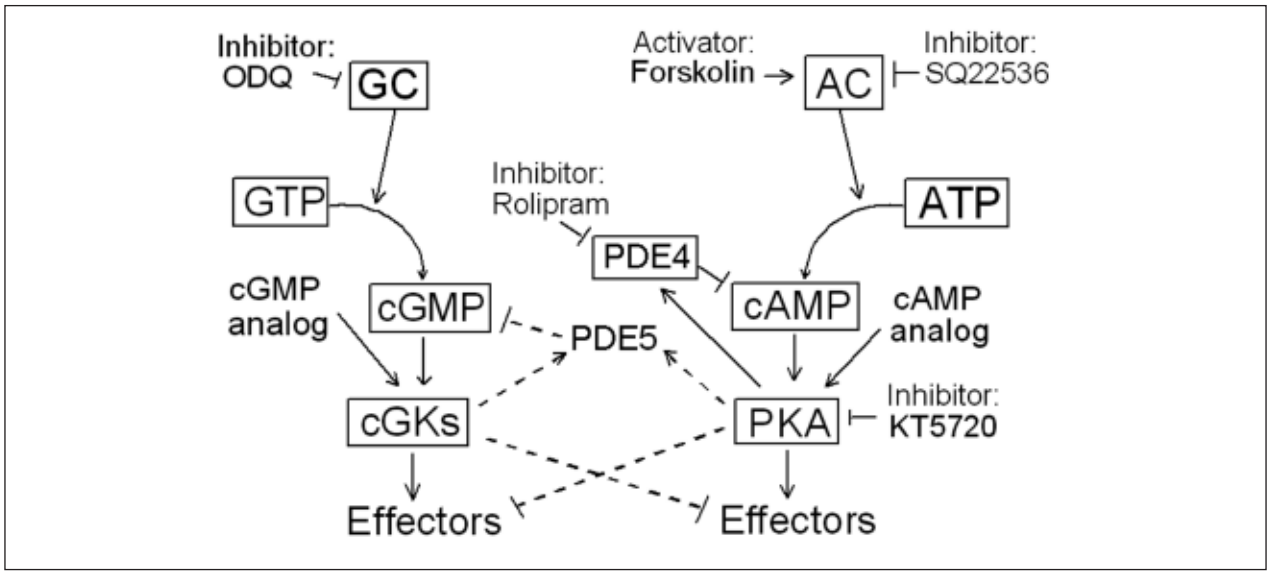

Figure 1. A simplified diagram of cAMP and cGMP pathways and some of its activators, inhibitors and analogs used in the present study. Broken lines represent a possible interplay between these pathways. This diagram does not account for more complex interplay and signaling microdomains. AC = adenylyl cyclase; AMP = adenosine monophosphate; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; cGKs = cGMP-dependent protein kinases; GC = guanylyl cyclase; GTP = guanosine triphosphate; PDE4 and PDE5 = phosphodiesterase isoforms; PKA = protein kinase A

Nitric oxide (NO) is a highly reactive free radical produced as a result of L-arginine metabolism catalyzed by nitric oxide synthases (NOS) and is known to play a crucial role during bone remodeling (7). Indeed, our previous studies showed that the proliferation of a human osteoblast cell line (HOS cells) on the surfaces of HA, a ceramic commonly used for orthopaedics and dental implants (8, 9), is under the regulation of endothelial NOS activities (10). Furthermore, NO enhanced not only the cell proliferation, but also the production of COX-2-mediated prostaglandin E_2 (PGE_2) and cAMP by HA-induced HOS cells (10-12). Therefore, the aim of the present study was to test the hypothesis that NO may enhances HA-induced HOS cell proliferation via enhancement of the cAMP-PKA pathway.

Materials and Methods

Hydroxyapatite

The hydroxyapatite (HA) discs (sintered at 1200°C, 9% final porosity) were cut into pieces with 2 X 2 X 2 mm^3 in size and subsequently autoclaved. In all experiments, each of the HA disc was placed at the well of 96-well plates (Corning, NY, USA).

Cell Cultures

Unless otherwise stated, all materials used in this study were purchased from Sigma, St. Louis, MO, USA. A human osteoblast cell line (HOS cells), obtained from American Type Culture Collection (Rockville, MD, USA), was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin-streptomycin until confluent. After harvesting and washing, a single cell suspension (1 X 10^6 cells/ml) was prepared in the above medium. S-nitroso acetyl penicillamine (SNAP), a NO donor, was dissolved in distilled water to obtain 1 mM as a stock solution and sterilized. Two hundred microliters of cell suspension containing 2 X 10^5 cells were incubated on the surface of the HA-disc with or without the presence of 20 mM SNAP and incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2 as previously described (11).

SQ22536 (an adenylyl cyclase inhibitor) and ODQ (a guanylyl cyclase inhibitor) were dissolved in distilled water and ethanol, respectively. Db-cAMP (a c-AMP analog) and br-cGMP (a cGMP analog) were dissolved in distilled water, whereas forskolin (an adenylyl cyclase activator), IBMX [a non-specific phosphodiesterase (PDE) inhibitor], and KT5720 [a protein kinase A (PKA) inhibitor] were dissolved in DMSO. All inhibitor-containing solutions were adjusted to obtain 1 mM stock solution and filter sterilized. One million cells were incubated in 1 ml of the culture medium containing various concentration of SQ22536 or ODQ for 30 minutes at room temperature. The same number of cells was also incubated in the culture medium containing various concentration of forskolin, KT5720 or IBMX for 1 to 2 hours at room temperature. After washing the HOS cells were cultured with or without the presence of 20 mM SNAP as described above. In other experiment, the single cell solution was added with various concentration of db-cAMP or br-cGMP and the cells were subsequently cultured in the presence of 20 mM SNAP as above. Accordingly, the morphology and growth of cells in the cultures containing 0.001% DMSO were not affected (data not shown). All cultures were in triplicate and each experiment was repeated three times.

Cell proliferation assay

Cell proliferation was determined by a colorimetric assay as described previously (13). Briefly, after harvesting the supernatant, the cells were washed with sterile PBS and dehydrated with 100 μl of 20% methanol for 10 minutes. The cells were then exposed to 100 μl of 0.5% crystal violet for 5 minutes followed by extensive rinsing with PBS. The dye was released from the cells by adding 100 μl of 0.1 M Na citrate in 50% ethanol. The optical density was read at wavelength of 540 nm using mQuant spectrophotometer (Biotek-Instrument, Inc., Winooski, VT, USA). The results were subtracted from the optical density reading of medium only and expressed in absorbance unit.

Statistical analysis

The results were analyzed by a one-way analysis of variance followed by Fischer's least square differences using the SPPS statistical software package (SPSS co., Chicago, USA).

Results

The effect of an adenylyl or guanylyl cyclase inhibitor

HA-induced HOS cell proliferation was suppressed in the presence of SQ22536, but not ODQ, as compared with that without the presence of these inhibitors ($p<0.05$) (Fig. 2). Furthermore, SQ22536 but not ODQ also inhibited the proliferation of HA-induced HOS cells in the presence of SNAP ($p<0.05$) (Fig. 2).

The effect of cyclic nucleotide analogs

In the presence of db-cAMP, HA-induced HOS cell proliferation was enhanced when compared with that without the presence of db-cAMP ($p<0.05$) (Fig. 3). The cAMP analog not only slightly increased the proliferation of HOS cells alone but also amplified the up-regulatory role of SNAP on HA-induced HOS cell proliferation ($p<0.05$). In sharp contrast, br-cGMP failed to alter the HA-induced HOS cell proliferation with or without the presence of SNAP ($p>0.05$) (Fig. 3).

Effect of an adenylyl cyclase activator

As seen in Fig. 4, HA-induced HOS cell proliferation in the presence of forskolin was higher than that without this adenylyl activator ($p<0.05$). Similarly, HA-induced HOS cell proliferation in the presence of SNAP and forskolin was significantly higher than that in the presence of SNAP alone ($p<0.05$).

Effect of phosphodiesterase (PDE) inhibitors

The results showed that IBMX and rolipram, but not siguazodan, enhanced the proliferation of HA-induced HOS cells with or without the presence of SNAP ($p<0.05$) (Fig. 5). Similarly, both IBMX and rolipram, but not siguazodan, up-regulated slightly the spontaneous proliferation of HOS cells alone ($p<0.05$).

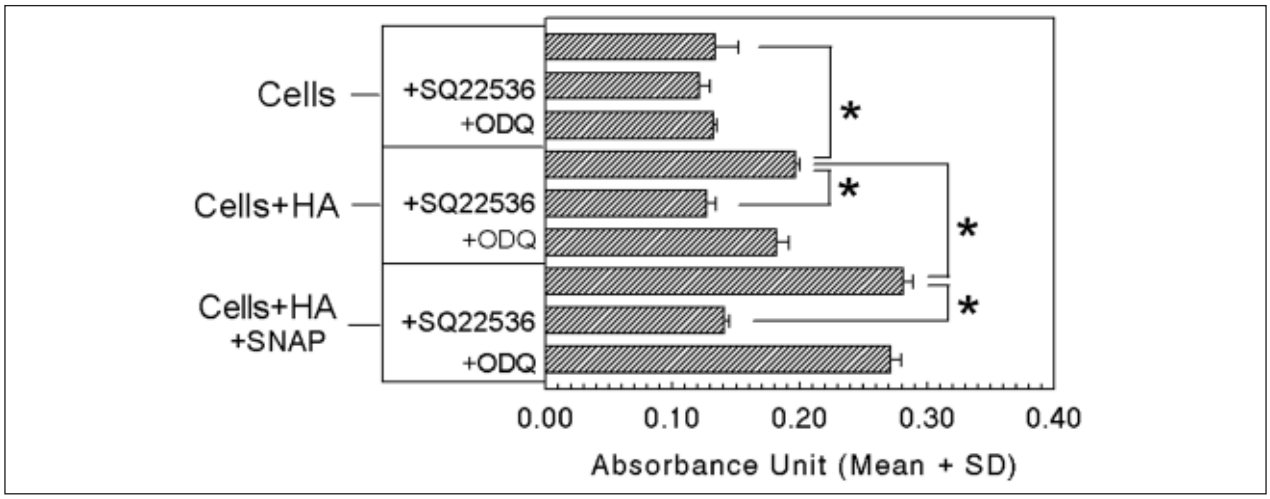

Figure 2. The effect of SQ22536 (an adenylyl cyclase inhibitor) or ODQ (a guanylyl cyclase inhibitor) on HA-stimulated HOS cell proliferation with or without the presence of SNAP (NO donor). The concentration of SQ22536, ODQ and SNAP were 100 μM, 4 μM, and 20 μM, respectively. (*) = significant difference at $P<0.05$

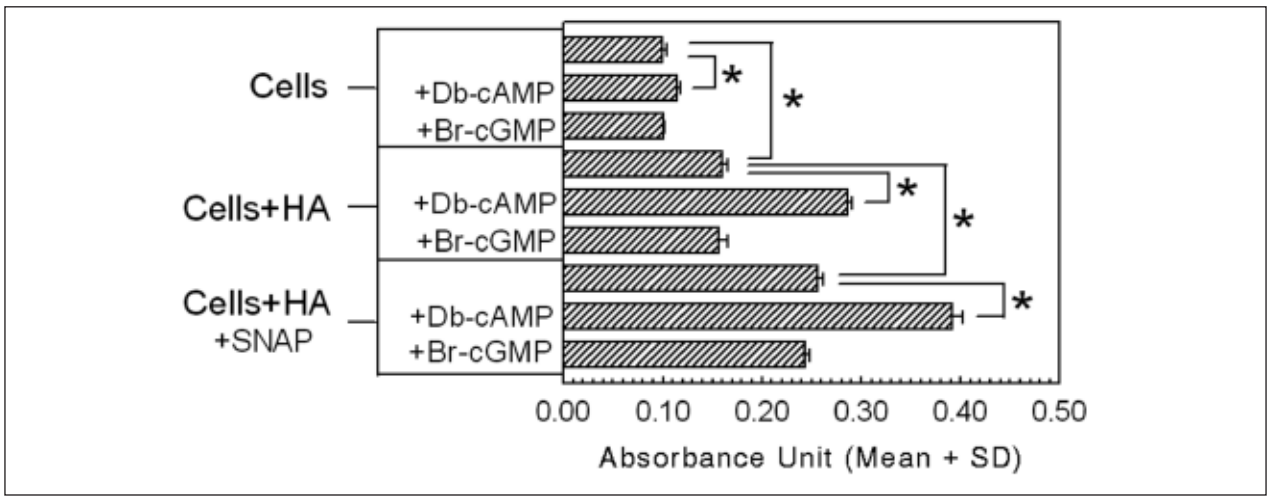

Figure 3. The effect of bromo-8-cGMP (br-cGMP) and dibutyryl cAMP (db-cAMP), a cGMP and cAMP analog respectively, on HA-stimulated HOS cell proliferation with or without the presence of SNAP (NO donor). The concentration of br-cGMP and db-cAMP were 10 μM, whilst that of SNAP was 20 μM. (*) = significant difference at $P<0.05$

Effect of protein kinase A (PKA) inhibitor

KT5720 suppressed HA-induced HOS cell proliferation as compared with HA-induced HOS cells with or without the presence of SNAP alone ($p<0.05$), but db-cAMP and forskolin failed to ablate this suppressive effect of KT5720 ($p<0.05$) (Fig. 6). In contrast, KT5720 failed to alter the spontaneous proliferation of HOS cells ($p>0.05$).

Discussion

The present study demonstrated that HOS cell proliferation on the surfaces of HA was regulated by a cAMP but not cGMP-dependent mechanism. These results are in accordance with previous studies showing that mouse fibroblast attachment on a cellulose substratum was dependent on the cAMP pathway (5,6). It should be noted, however, that the cAMP

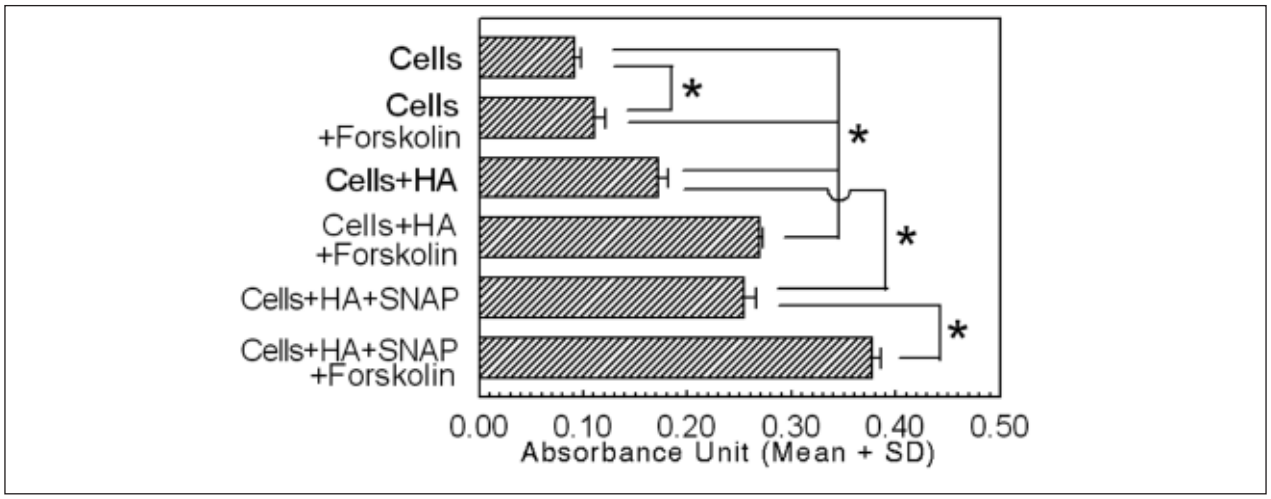

Figure 4. The effect of forskolin (an adenylyl cyclase activator) on HA-stimulated HOS cell proliferation with or without the presence of SNAP (NO donor). The concentration of forskolin and SNAP were 10 μM and 20 μM, respectively. (*) = significant difference at $P<0.05$

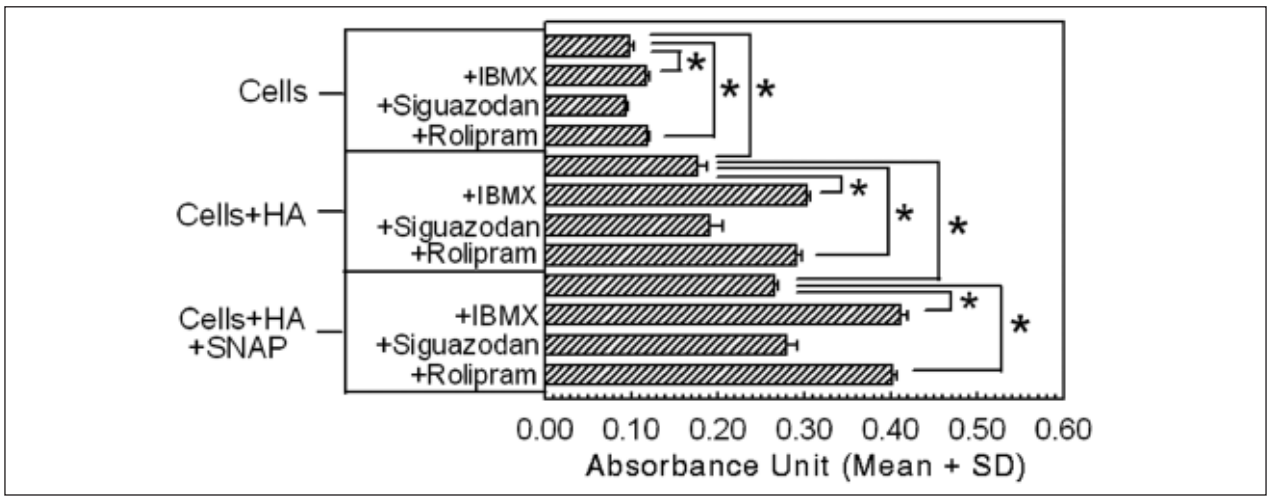

Figure 5. The effect of IBMX (a non-specific PDE inhibitor), siguazodan (a PDE3 inhibitor) and rolipram (a PDE4 inhibitor) on HA-stimulated HOS cell proliferation with or without the presence of SNAP (NO donor). The concentration of IBMX, siguazodan and rolipram were 100 μM, whilst that of SNAP was 20 μM. (*) = significant difference at $P<0.05$

analog alone was able to augment, but SQ22536 failed to alter the proliferation of HOS cells, suggesting that endogenous cAMP may not play a crucial role in HOS cell growth. If so, the cAMP pathway might be activated as a result of HA-HOS cell interaction as previous suggested (12). Of interest, the present study also demonstrated that the up-regulatory role of exogenous NO on HA-induced HOS cell proliferation was inhibited by an adenylyl cyclase inhibitor but enhanced by a cAMP analog, suggesting a possible direct effect of NO on adenylyl cyclase as previous reported (14). Indeed, it may be the case when one has to explain the results of the present study showing that exogenous NO did strengthen the ability of forskolin in augmenting HA-induced HOS cell proliferation.

Phosphodiesterase (PDE), a family of proteins consisting of, at least, 11 isozymes, catalizes the hydrolysis of cAMP to inactive 5'-adenosine monophos-

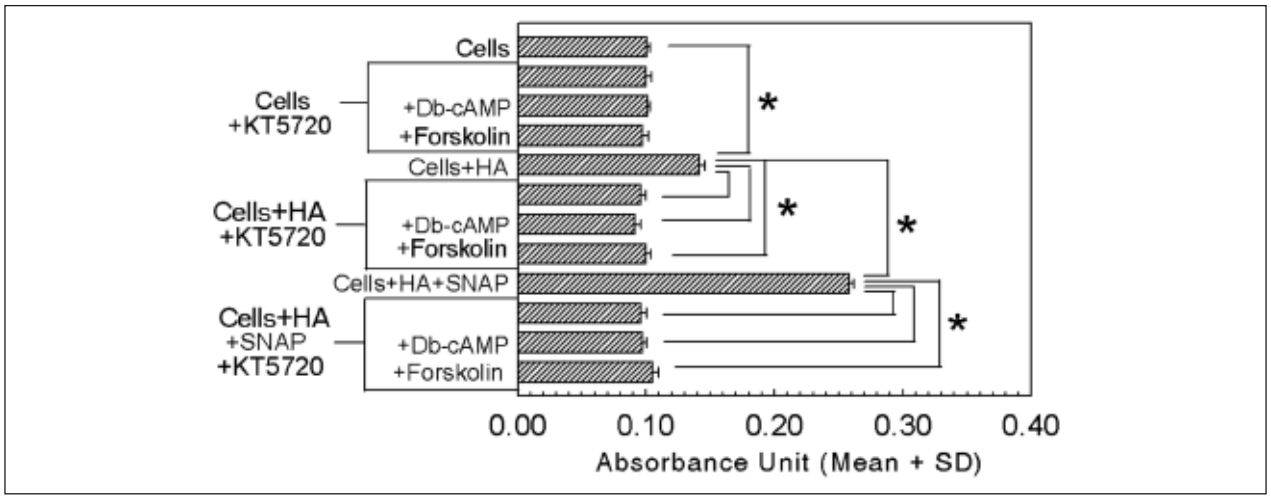

Figure 6. The effect of KT5720 (A PKA inhibitor) on HA-stimulated HOS cell proliferation with or without the presence of SNAP (NO donor). The concentration of KT5720 and SNAP were 1 μM and 20 μM, respectively, whereas that of db-cAMP and forskolin were 10 μM (*) = significant difference at $P<0.05$

phate (5'-AMP) (15). The results of the present study showed that HA-induced HOS cell proliferation might be preferentially regulated by a PDE4 isoform. These results are supported by a previous study indicating that cAMP efflux in human osteosarcoma cells is dependent on PDE4 activities (16). Bone formation in mice regulated by PDE4 has also been reported (17). It seems to suggest, therefore, that HA-HOS cell interaction might generate signals transduction to induce cAMP production (12) and cAMP-specific PDE4 activity concomitantly. In this respect, exogenous NO appeared to augment the levels of cAMP (12) which might in turn up-regulate PDE4 activities in HA-induced HOS cell proliferation as seen in the present study. Alternatively, NO might directly increase the expression of cAMP-specific PDE4 in HA-induced HOS cells as shown by a previous study in rat smooth muscle cells (18). However, this notion remains to be further clarified, since the expression of PDE4 was not assessed in the present study.

The sequence of cAMP activity is the phosphorylation of protein kinase A (PKA) which then activates nuclear transcription factors to induce cell functions, including cell proliferation (19). The present study showed that HA-induced osteoblast proliferation may be dependent on the PKA activity. Failure of the cAMP analog or forskolin to reverse the supressive effect of KT5720 on HA-induced HOS cell proliferation seen in the present study strengthens the speculation that PKA is an indispensable part of a downstream signal that mediates the cAMP-dependent proliferation of HA-induced HOS cells. A previous report showing that cell aggregation on biomaterials such as cellulose substratum was dependent on the activation of the cAMP-PKA pathway (6) highlights the present study. Interestingly, exogenous NO even in the presence of cAMP analog or forskolin failed to overcome the suppressive effect of KT5720 on HA-induced HOS cell proliferation seen in the present, suggesting that exogenous NO might mainly act on the synthesis of cAMP but not on the phophorylation of PKA and/or downstream signals of PKA. This notion needs further studies, however.

In conclusion, HA-induced HOS cell proliferation significantly increased by exogenous NO was inhibited by an adenylyl cyclase inhibitor, a non-specific PDE inhibitor, a PDE4 inhibitor or a PKA inhibitor but enhanced by a cAMP analogue or an adenylyl cyclase activator. The presence of a guanylyl cylase inhibitor, a cGMP analogue or a PDE3 inihibitor failed to alter HA-induced HOS cell proliferation with or without the presence of exogenous NO. The results of the present study suggest, therefore, that HA-induced HOS cell proliferation may be regulated by the cAMP-PKA pathway controlled by PDE4 and that NO may augment HA-induced HOS

cell proliferation via its ability to amplify the magnitude of this cAMP-PKA pathway.

Acknowledgements

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References

1. Chin, K-V, Yang W-L, Ravatn R, et al. Reinventing the wheel of cyclic AMP: novel mechanisms of cAMP signaling. *Ann NY Acad Sci* 2002; 968: 49-64.
2. Hofmann F. The biology of cyclic GMP-dependent protein kinases. *J Biol Chem* 2005; 280: 1-4.
3. Swarhout JT, D'Alonzo RC, Selvamurugan N, Partidge NC. Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. *Gene* 2002; 282; 1-17.
4. Kim C-H, Park Y-G, Noh S-H, Kim Y-K. PGE_2 induces the gene expression of bone matrix metalloproteinase-1 in mouse osteoblast by cAMP-PKA signaling pathway. *Int J Biochem Cell Biol* 2005; 37: 375-85.
5. Faucheux N, Correze C, Haye B, Nagel MD. Accumulation of cyclic AMP in Swiss 3T3 cells adhering to a cellulose biomaterial substratum through interaction with adenylyl cyclase. *Biomaterials* 2001; 22: 2993-8.
6. Faucheux N, Nagel MD. Cyclic AMP-dependent aggregation of Swiss 3T3 cells on a cellulose substratum (Cuprophan) and decreased cell membrane Rho A. *Biomaterials* 2002; 23: 2295-301.
7. Van´t Hof RJ, Ralston SJ. Nitric oxide and bone. *Immunology* 2001; 103: 255-61.
8. Dumbleton J, Manley MT. Hydroxyapatite-coated prostheses in total hip and knee arthroplasty. *J Bone Joint Surg Am* 2004; 86-A: 2526-40.
9. Cranin AN, Tobin GP, Gelbman J. Application of hydroxyapatite in oral and maxillofacial surgery. *Compend Contin Educ Dent* 1987; 8: 334-45.
10. Sosroseno W, Sugiatno E, Samsudin AR, Ibrahim MF. The role of nitric oxide on the proliferation of a human osteoblast cell line (HOS cells) stimulated with hydroxyapatite. *J Oral Implantol* 2008; (In press).
11. Sugiatno E, Samsudin AR, Ibrahim MF, Sosroseno W. The effect of nitric oxide on the production of prostaglandin E_2 by hydroxyapatite-stimulated a human osteoblast (HOS) cell line. *Biomed Pharmacother* 2006; 60: 147-51.
12. Sosroseno W, Sugiatno E, Samsudin AR, Ibrahim MF. The effect of nitric oxide on the production of cyclic-AMP by a human osteoblast (HOS) cell line stimulated with hydroxyapatite. *Biomed Pharmacother* 2008; 62: 147-51.
13. Zambonin G, Camerino C, Greco G, Patella V, Moretti B, Grano M. Hydroxyapatite coated with hepatocyte growth factor (HGF) stimulates human osteoblasts in vitro. *J Bone Joint Surg (Br)* 2000; 82: 457-60.
14. Villa-Petroff MG, Younes A, Egan J, Lakata EG, Sollot SJ. Activation of distinct cAMP-dependent and cGMP-dependent pathway by nitric oxide in cardiac myocytes. *Circ Res* 1999; 19: 1093-9.
15. Essayan DM. Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol* 2001; 108: 671-80.
16. Ahlström M, Pekkinan M, Huttunen M, Lamberg-Allardt C. Dexamethasone down-regulates cAMP-phosphodiesterase in human osteosarcoma cells. *Biochem Pharmacol* 2005; 69: 267-75.
17. Kinoshita T, Kobayashi S, Ebara S, Yoshimura Y, Horiuchi H. Phosphodiesterase inhibitors, pentoxifylline and rolipram, increase bone mass mainly by promoting bone formation in normal mice. *Bone* 2000; 27: 811-7.
18. Busch CJ, Liu H, Graveline AR, Bloch KD. Nitric oxide induces phosphodiesterase 4B expression in rat pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: L747-53.
19. Feliciello A, Gottesman ME, Avvedimento EV. cAMP-PKA signaling to the mitochondria: protein scaffold, mRNA, and phosphatases. *Cell Signal* 2005; 17: 279-87.

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