Nitric oxide production and cell proliferation by human osteoblasts stimulated with hydroxyapatite

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Abstract

The aim of the present study was to determine nitric oxide (NO) production and cell proliferation by hydroxyapatite (HA)-stimulated human osteoblasts. Human osteoblast-like cells (HOS cell line) were cultured on the surface of HA. Medium or cells alone was used the controls. L-arginine, D-arginine, 7-NI (an nNOS inhibitor), L-NIL (an iNOS inhibitor), L-NIO (an eNOS inhibitor) or carboxy PTIO, a NO scavenger, was added in the HA-exposed cell cultures. Some of the cells were pre-coated with anti-human integrin αV antibody. The levels of NO were determined by the Griess reagent. Cell proliferation was assessed by colorimetric assay. The results showed that increased NO production and cell proliferation by HA-exposed osteoblasts up to day 3 in the cultures was observed. L-arginine, but not D-arginine, upregulated both NO production and proliferation of HA-stimulated cells. Anti-integrin αV antibody or L-NIO suppressed NO production and proliferation of HA-stimulated cells. HA-stimulated osteoblast proliferation was inhibited by carboxy PTIO. The results of the present study suggest, therefore, that HA-stimulated osteoblast proliferation and NO production may be an integrin αV molecule and eNOS dependent mechanism and that HA-stimulated osteoblast proliferation may be regulated by endogenous NO in an autocrine fashion.

Keywords: eNOS; Hydroxyapatite; Integrin αV; Nitric oxide; Osteoblasts

Introduction

Hydroxyapatite (HA) is known as a ceramic material widely used for orthopaedic and dental implants, since this biomaterial has ability to stimulate osteoblast functions *in vitro* and *in vivo* [1]. However, the exact mechanisms by which HA induces osteoblast functions remain unclear. Previous studies indicated that as an initial step, osteoblasts via integrin molecules attach and spread on the surface of protein-coated HA [2,3]. It appears to suggest that signal transduction generated by osteoblast integrin-HA interaction may also induce the production of proteins, such as osteopontin, prostaglandin E_2 (PGE_2) and transforming growth factor-β (TGF-β) which may, in turn, regulate cell proliferation and differentiation [4-6].

Nitric oxide (NO) is a gaseous molecule generated from L-arginine under catalization of nitric oxide synthase (NOS) and it plays a crucial role on the nervous, cardiovascular and immune system [7]. Three isoforms of NOS, i.e., neural NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3), are recognized [8]. Human osteoblasts have been shown to express all NOS isoforms [9]. However, others found that osteoblasts expressed eNOS and iNOS molecules only [10,11]. Proinflammatory cytokines and bacterial lipopolysaccharide increased the iNOS expression [9,10,12], whereas the eNOS activities could be induced by stimulators such as estradiol [13], estrogen [14] and fluid shear stress [15]. These studies indicate that iNOS and eNOS may play a role in bone inflammation and physiology, respectively.

The exact mechanism(s) by which NO regulates the bone formation has been a focus of attentions. For example, Hikiji and colleagues showed that NO directly induces osteoblast proliferation, independent on the presence of cytokines [16]. Other demonstrated, however, that NO stimulates osteoblast proliferation via the induction of PGE_2 production [17]. Indeed, the effect of NO on osteoblast functions

may be dependent on the rate and concentration of this gaseous molecule. Slow and moderate release as well as low concentration of NO may stimulate osteoblast proliferation [9,18], whereas rapid release and high concentration of NO may lead to osteoblast apoptosis, perhaps, via cGMP pathway and the activation of both proapoptotic Bax and Bcl2 protein [18-20]. Whether or not osteoblasts stimulated by HA produces NO which may, in turn, regulate osteoblast proliferation has not been reported. Therefore, the aim of the present study was to determine NO production and cell proliferation by HA-stimulated human osteoblasts and assess whether endogenous NO may regulate HA-stimulated human osteoblast proliferation.

Materials and methods

Hydroxyapatite

The HA discs (9% porosity and sintered at 1200°C), a kindly gift from Dr. Radzali Othman (School of Materials and Mineral Resources Engineering, USM, Malaysia), 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 in the well of 96-well plates (Corning, NY, USA).

Cell Cultures

A human osteoblast-like cell line, HOS cells, were purchased from American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 1% penicillin-streptomycin (Sigma) until confluent. After harvesting and washing, a single cell suspension (1 X 10^6 cells/ml) was prepared in the above medium. Two hundred microliters of cell suspension containing 2 X 10^5 cells/well were plated on the surface of the HA-disc and incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2. Medium only and cell suspension plated in

the wells were used as controls. The NO production and cell proliferation were assessed daily as described below.

In other experiments, the cultures were added with various concentrations of L- or D-arginine (Sigma). Carboxy PTIO, an NO scavenger, in various concentrations was also added in the cultures of HA-stimulated cells. In order to determine the role of NOS isoforms, various concentrations of 7-NI, an nNOS inhibitor, L-NIL, an iNOS inhibitor, or L-NIO, an eNOS inhibitor, were added in the cultures. Carboxy PTIO and NOS inhibitors were purchased from Sigma and diluted in sterile PBS. Furthermore, prior to HA exposure, the cells were incubated with various concentration of anti-human αV-integrin antibody (Santa Cruz Biotech, California, USA) for 1 hour at the room temperature. The cells were then washed 3 times and then cultured on the HA disc as above. All cultures were in triplicates and incubated for 3 days after which NO production and cell proliferation were determined as described below.

No assay

The levels of nitrite, as representing that of NO, were determined from the culture supernatants by the Griess reaction as previously described [21]. Briefly, 100 μl of the supernatants were mixed with equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid) and the color was read by μQuant spectrophotometer (Biotek-Instrument Inc., Vermont, USA) at 540 nm. Sodium nitrite was used to prepare a standard curve for nitrite levels. All reagents for NO assays were purchased from Sigma.

Cell Proliferation assay

Cell proliferation was determined by a colorimetric assay using crystal violet as a dye [22]. Briefly, after harvesting the supernatant and gently washing with sterile PBS, the cells were dehydrated with 100 µl of 20% methanol for 10 minutes and then the solution was carefully aspirated. 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 µQuant spectrophotometer (Biotek-Instrument, Inc.). The results were subtracted by the optical density reading of medium only.

Statistical analysis

The results were analyzed using repeat measurement test for the data derived from the HA-stimulated osteoblast proliferation and NO production. A one-way analysis of variance followed by Fischer's least square differences was used to analyze the data from the remaining experiments. The data analysis was carried out using a statistical software package (SPSS co., Chicago, USA).

Results

NO levels and cell proliferation

The cells cultures were incubated for 3 days and both NO levels from the culture supernatant and cell proliferation were assessed daily. As seen in Fig. 1, NO could be detected in the culture supernatant of cells alone and HA-stimulated cells HA at day 1 and significantly increased at day 2 and 3 ($p<0.05$). There was no significant different between NO levels produced by cells alone and HA-stimulated cells at day 1 ($p>0.05$). However, the production NO by HA-stimulated cells at day 2 and 3 in the cultures was higher than that by cells alone ($p<0.05$). Furthermore, gradually increased cell proliferation up to day 3 was observed in the cultures of cells alone or

HA-stimulated cells ($p<0.05$) (Fig. 1). Proliferation of HA-stimulated cells at day 2 and 3, but not at day 1, was higher than that of cells alone ($p<0.05$).

The effects of L- and D-arginine

Since NO is synthesized from L-arginine, the next experiments were to assess the effects of exogenous L-arginine and its derivative, D-arginine, on NO production and cell proliferation of HA-stimulated osteoblasts. After the addition of 10 μM of L-arginine, NO levels in the cultures of HA-stimulated cells were higher than those in the cultures of HA-stimulated cells without L-arginine ($p<0.05$) (Fig. 2). Further enhancement of NO production in the cultures of HA-stimulated cells was observed after the addition of 100 μM of L-arginine ($p<0.05$). In sharp contrast, no significant difference in NO production between the cultures of HA-stimulated cells with and without D-arginine could be found ($p>0.05$) (Fig. 2). Furthermore, HA-stimulated cell proliferation in presence of 10 μM, but not 1 μM, of L-arginine was higher than that of HA-stimulated cells alone ($p<0.05$) (Fig. 2). Adding 100 μM of L-arginine in the cultures of HA-stimulated cells induced higher NO production than and the cultures with 10 μM of L-arginine or without L-arginine ($p<0.05$). However, increased concentration of exogenous D-arginine failed to augment HA-stimulated cell proliferation ($p>0.05$).

The effect of anti-human integrin αV antibody

Prior to HA exposure, osteoblasts were incubated with anti-human integrin αV antibody and both NO levels and cell proliferation were then assessed. The results showed that increased concentration of the antibody resulted in increased suppression of NO production by HA-stimulated cells ($p<0.05$) (Fig. 3). Interestingly, NO levels produced cells precoated with 20 μg of antibody and then stimulated with HA were similar with those produced by non-precoated cells alone ($p>0.05$).

Likewise, precoating the cells with the antibody resulted in profound suppression of HA-stimulated cells proliferation ($p<0.05$) (Fig. 3). When the cells pre-coated with 20 μg of anti-human integrin αV, proliferation of HA-stimulated cell proliferation was not significantly different than that of non precoated cells alone ($p>0.05$).

The effects of NOS inhibitors

In order to determine the role of NOS isoforms, specific NOS isoform inhibitors were added in the culture of HA-stimulated cells. The results showed that increased concentration of 7-NI, an nNOS inhibitor or L-NIL, an iNOS inhibitor, did not alter the NO production by the cultures of HA-stimulated ($p>0.05$) (Fig. 4). However, suppression of NO production by HA-stimulated cells after adding L-NIO, an eNOS inhibitor, could be seen in a dose dependent fashion ($p<0.05$). One hundred micro molars of L-NIO did induce lower NO production by HA-stimulated cells than by cells alone ($p<0.05$). Furthermore, there was no significant difference between HA-stimulated cell proliferation with and without increased concentration of 7-NI or L-NIL ($p>0.05$) (Fig. 5). However, HA-stimulated cell proliferation was suppressed by increased concentration of L-NIO ($p<0.05$). The proliferation of HA-stimulated cells in the presence of 100 μM of L-NIO was significantly lower than that of cells alone ($p<0.05$).

The effects of carboxy PTIO

As seen in Fig. 6, carboxy PTIO suppressed HA-stimulated osteoblast proliferation, in a dose dependent mechanism ($p<0.05$). Proliferation of HA-stimulated cells in the presence of 100 μM of carboxy PTIO was significantly lower than that of cells alone ($p<0.05$).

Discussion

The ability of HA to induce osteoblasts function is well known. For example, the proliferation of osteoblasts exposed to nanophase HA ceramics [23], HA-coated titanium [24] or HA cements [25] were higher than osteoblasts alone. The present study also showed that proliferation of human osteoblast-like cells exposed to HA at day 2 in the cultures was higher than that of cells alone. The HA-induced osteoblast proliferation at day 3 was increasingly higher than that at day 2. These results indicate that HA augmented osteoblast proliferation. Of interest, NO production by HA-stimulated human osteoblasts at day 2 in the cultures as seen in the present study was higher than that by cells alone and increasingly higher than that at day 3, demonstrating that Stimulation with HA may promote osteoblasts to produce NO. Taken together, the results of the present study demonstrate that HA may stimulate human osteoblasts to proliferate and produce NO, concomitantly.

The presence of L-arginine for NO synthesis is prerequisite [7]. Therefore, the next experiments were to assess the effect of exogenous L-arginine on HA-induced human osteoblast proliferation and NO production. The present study revealed that increased concentration of exogenous L-arginine up-regulated both NO levels and proliferation of HA-stimulated osteoblasts. The results of the present study are in accordance with previous findings showing that L-arginine modulates osteoblast proliferation and alkaline phosphatase activities as well as NO production [26,27]. The exact mechanism by which exogenous L-arginine elevated HA-stimulated osteoblast proliferation and NO production seen in the present study needs to be investigated further. One possibility is that exogenous L-arginine may stimulate high levels of insulin-like growth factor-I (IGF-I) which may in turn modulate osteoblast proliferation [28]. Concurrently, exogenous L-arginine may act as a signal to elevate the production of intracellular cationic amino acid transporter 2 (CAT2), an

essential protein for L-arginine uptake, thereby increasing L-arginine metabolism and hence, NO production [29].

The exact mechanism by which concurrently increased NO production and osteoblast proliferation after stimulation with HA occurs is not well understood. Osteoblast-derived integrin molecule family have been shown to attach on the serum-derived extracellular matrix (ECM) proteins absorbed onto HA. This integrin-ECM interaction generates signal transduction leading to osteoblast proliferation and differentiation [2,3]. Indeed, Matsuura and colleagues have shown that osteoblast attachment and spreading on HA surface was mediated by integrin αV molecule subunits [30]. To test a possibility that both increased cell proliferation and NO production by HA-stimulated human osteoblasts were mediated by this molecule, osteoblasts cells were incubated with anti-integrin αV antibody prior to stimulation with HA. The results in the present study showed that both NO production and cell proliferation of HA-exposed osteoblasts was suppressed in the presence of anti-integrin αV antibody, indicating that the both proliferation and NO production by osteoblasts on HA may be mediated by integrin αV molecules as previously described [30]. Activation of integrin molecules on osteoblasts by ECM leads to the activation of MAP kinase cascade and AP-1 family of transcription factors necessary for cell growth response [31]. Therefore, that osteoblast-derived integrin αV-HA interaction may initiate signal transduction leading to induce osteoblast proliferation and NO production seen in the present study is imminent. Furthermore, RGD motif-containing fibronectin and vitronectin, which are members of ECM proteins, have been found to be absorbed onto biomaterials and to bind with osteoblast-derived integrin αV subunits [2,3]. However, whether or not these ECM proteins were absorbed onto HA surface where they subsequently interacted with osteoblast-derived integrin αV subunits seen in the present study remains to be clarified further.

The next experiments were to assess a type of NOS isoforms responsible for the production of NO in HA-exposed human osteoblasts. The results of the present study showed that increased concentration of L-NIO, but not 7-NI and L-NIL, suppressed both proliferation and NO production of HA-stimulated osteoblasts, suggesting that eNOS, but not nNOS and iNOS, was activated by HA-osteoblast interaction. These results are not unexpected, since eNOS along with iNOS are expressed by human osteoblasts [9-11]. Activation of eNOS plays a crucial role during bone formation and remodeling as seen by the fact that bone formation in eNOS deficient mice was lacking [32]. Osteoblast proliferation and differentiation induced by estradiol [13] and fluid shear stress [15] were also dependent on eNOS activities. Thus, one may assume that following osteoblast-HA interaction, signal transduction generated from activated integrin αV molecules may up-regulate eNOS activities to produce NO and stimulate cell proliferation. This notion remains to be further investigated further, however.

The results of the present study indicate that blocking eNOS activities resulted in suppressed NO production and cell proliferation by HA-exposed human osteoblasts. Therefore, it was necessary to assess whether increased HA-stimulated osteoblast proliferation was due to the action of increased levels of NO by adding the HA-stimulated osteoblasts with carboxy PTIO. This NO scavenger is a water soluble derivative of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) which oxidizes NO to generate nitrogen dioxide, NO_2, and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTIO) [33,34]. The resulting NO_2 may either react further with NO to form N_2O_3 which reacts rapidly with water to yield NO_2^-, or NO2 may dimerize to form N_2O_4, which spontaneously dismutates to yield NO_2^- and NO_3^-. Therefore, measuring nitrite levels in the presence of carboxy-PTIO is unnecessary. When increasing concentration of was added into the HA-stimulated osteoblasts, cell proliferation was suppressed in a dose dependent fashion, suggesting that up-

regulated HA-stimulated osteoblast proliferation was due to NO action. The results of the present study support previous studies showing that NO stimulates osteoblast proliferation and differentiation [16,17]. There have, however, been conflicting reports in assessing the exact mechanisms of NO-regulated osteoblast functions. One study suggested that NO directly facilitates osteoblast proliferation and differentiation [16], whereas other has shown that NO may stimulate the production of PGE_2 which in turn modulates osteoblast functions [17]. Hence, further studies are needed to delineate the exact mechanism by which NO regulated HA-stimulated osteoblast proliferation in the present study.

In conclusion, the present study showed that following exposure with HA, elevated NO production and osteoblast proliferation were observed. Exogenous L-arginine further promoted HA-stimulated osteoblast NO production and proliferation. Furthermore, HA-stimulated osteoblast proliferation and NO production were dependent on integrin αV molecules and eNOS activities. Deletion of NO levels by a NO scavenger resulted in suppressed HA-stimulated osteoblast proliferation. Therefore, these results suggest that upon exposure to HA, human osteoblasts may proliferate and produce NO in an integrin αV molecule and eNOS dependent mechanism and that HA-stimulated osteoblast proliferation may be regulated by endogenous NO in an autocrine fashion.

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Legend of figures

Fig. 1. Mean and standard deviation of NO production and cell proliferation by HA-stimulated human osteoblasts.

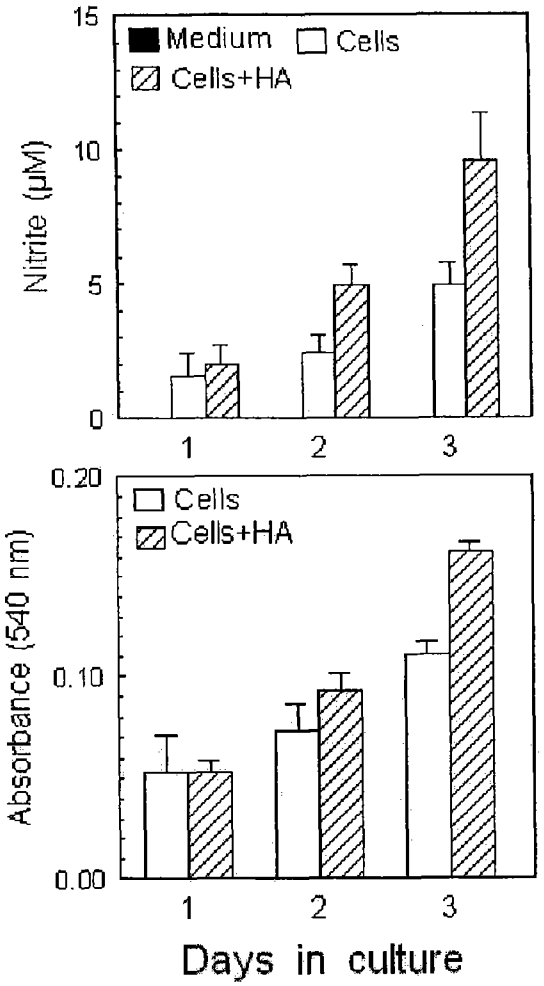
Fig. 2. Mean and standard deviation of NO production and cell proliferation by HA-exposed human osteoblasts in the presence of L-arginine or D-arginine. Cells alone or HA-stimulated cells with or without L-arginine or D-arginine were cultured for 3 days.

Fig. 3. Mean and standard deviation of NO production and cell proliferation by HA-stimulated human osteoblasts pre-coated with anti-human integrin αV antibody in the presence of L-arginine or D-arginine. Prior to stimulation with HA, cells were pre-coated with anti-human integrin αV antibody. Non pre-coated osteoblasts alone were used as control. All cultures were incubated for 3 days.

Fig. 4. Mean and standard deviation of NO production by HA-stimulated human osteoblasts in the presence of NOS inhibitors. HA-stimulated cell cultures were added with 7-NI (nNOS inhibitor), L-NIL (iNOS inhibitor), and L-NIO (eNOS inhibitor). All cultures were incubated for 3 days.

Fig. 5. Mean and standard deviation of cell proliferation by HA-stimulated human osteoblasts in the presence of NOS inhibitors. Notes of figures are similar to those in Fig. 4.

Fig. 6. Mean and standard of cell proliferation by HA-stimulated osteoblasts in the presence of carboxy PTIO. HA-stimulated cell cultures were added with carboxy PTIO and incubated for 3 days.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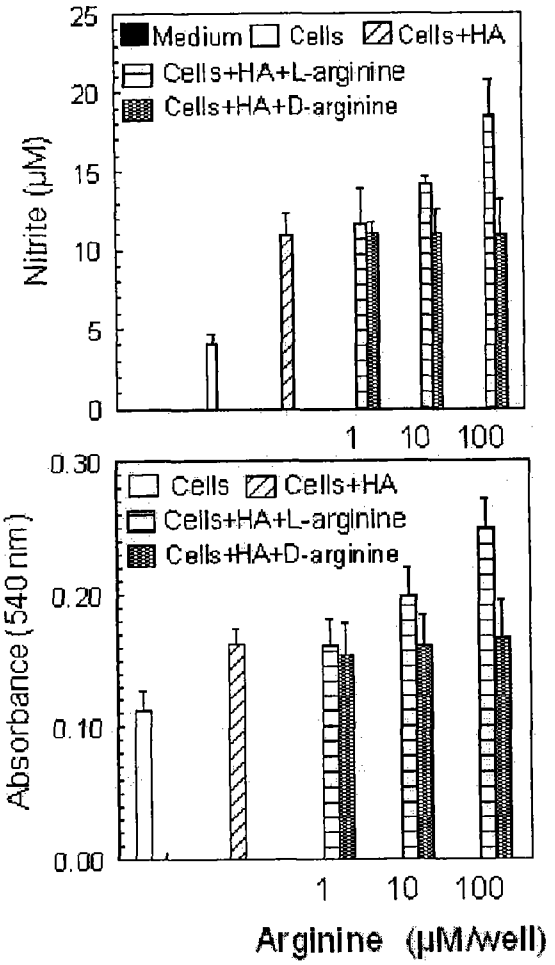
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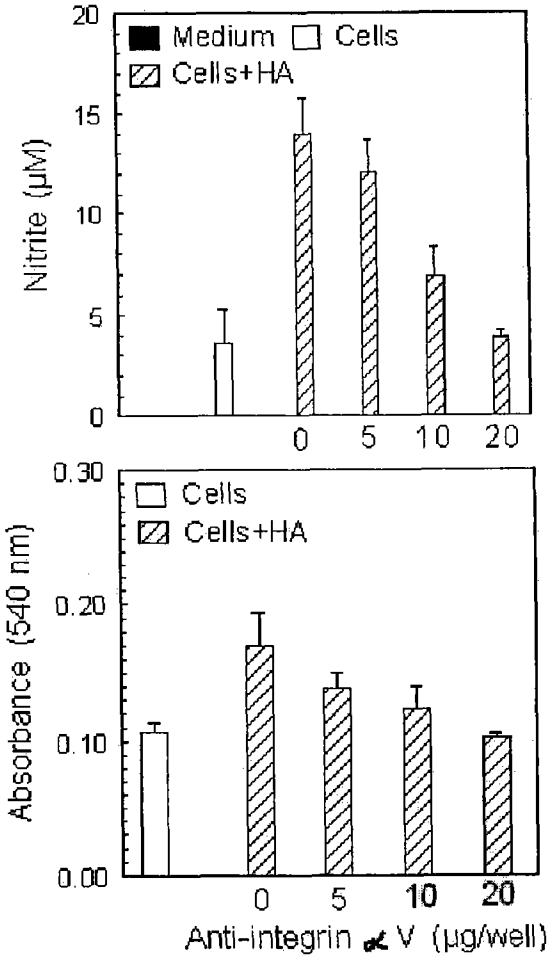
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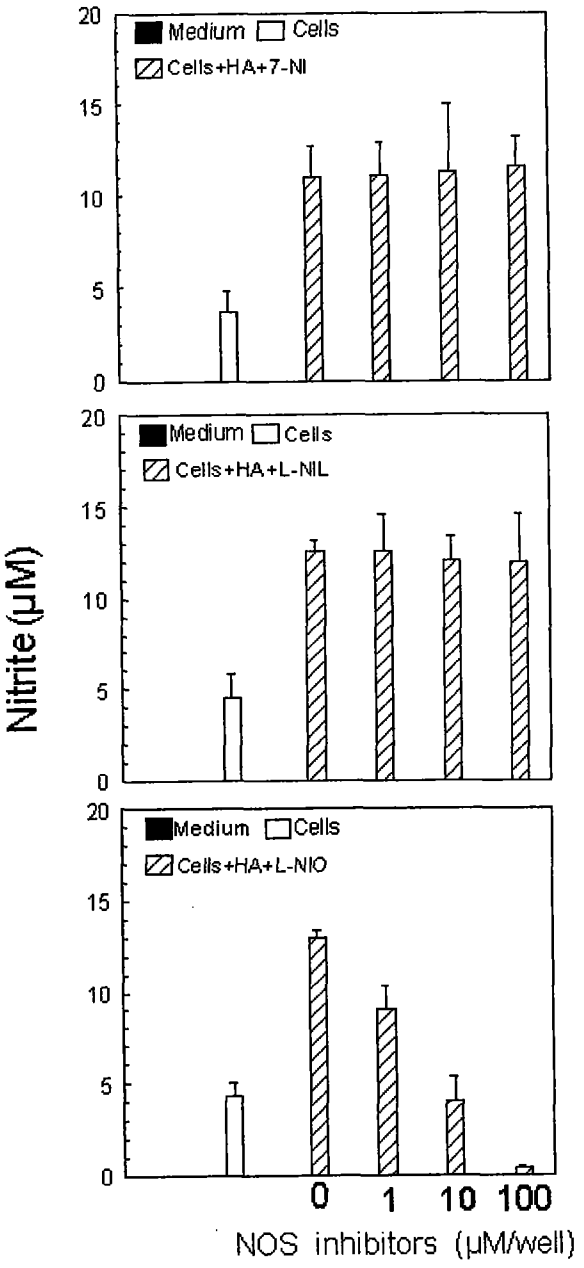
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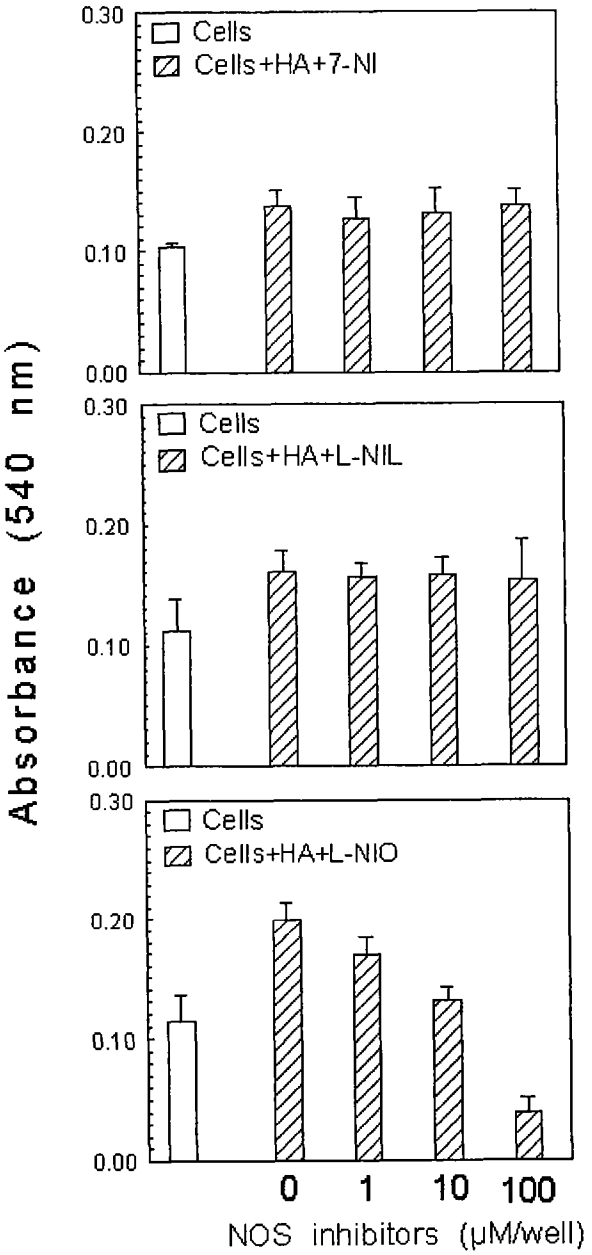
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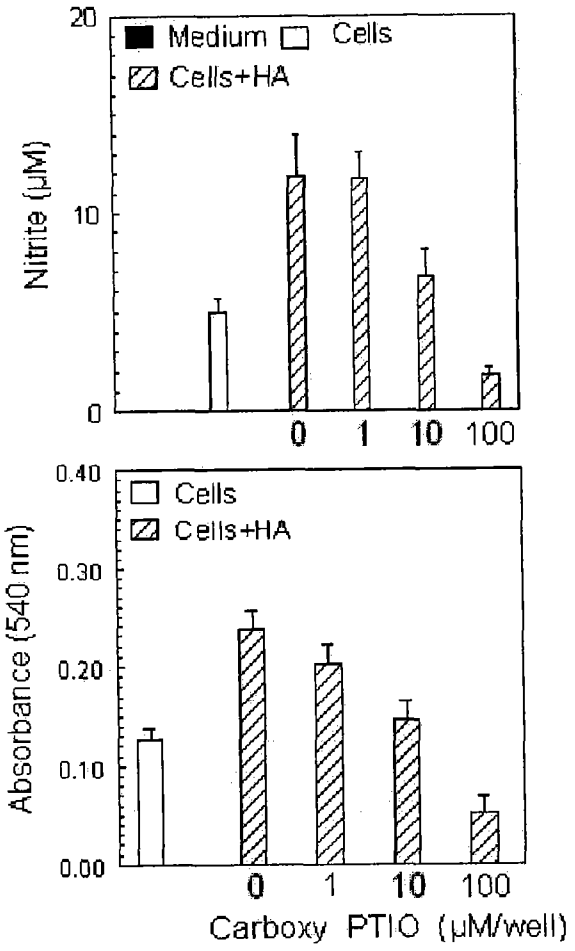
100
NOS inhibitors (μM/well)


20
10
0
Medium
Cells
Cells+HA
Nitrite (μM)
0
1
10
100
0.40
0.30
0.20
0.10
0.00
Cells
Cells+HA
Absorbance (540 nm)
0
1
10
100
Carboxy PTIO (μM/well)