

Effect of calcitonin gene-related peptide on nitric oxide production in osteoblasts: an experimental study

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Abstract

The aim of this study was to investigate the *in vitro* effects and regulatory mechanism of CGRP (calcitonin gene-related peptide) on NO (nitric oxide) production in osteoblasts. MOB (primary human mandibular osteoblasts) and osteoblast-like cells (MG-63) were either cultured with CGRP or co-incubated with inhibitors targeting eNOS (endothelial nitric oxide synthase), iNOS (inducible nitric oxide synthase), nNOS (neuronal nitric oxide synthase) and $[Ca^{2+}]_i$ (intracellular Ca^{2+}). The NO concentration in cell culture supernatants was measured during the first 24 h using the Griess test; cellular NO was marked with the fluorescent marker DAF-FM, DA (3-amino, 4-aminomethyl-2',7'-difluorescein; diacetate) and measured by fluorescence microscopy from 1 to 4 h after treatment. eNOS and iNOS mRNA expression levels were measured by quantitative RT-PCR during the first 24 h after treatment. CGRP-induced NO production in the supernatants was high between 1 to 12 h, while cellular NO was highest between 1 to 2 h after treatment and returned to basal levels by 3 h. Both in MG-63 cells and MOB, the most effective CGRP concentration was 10 nM with a peak time of 1 h. CGRP-induced NO production decreased when eNOS activity was inhibited or when voltage-dependent L-type Ca^{2+} channels were blocked at 4 h. CGRP was not able to induce changes in iNOS or eNOS mRNA levels and had no effect on the cytokine-induced increase of iNOS expression. Our results suggest that CGRP transiently induces NO production in osteoblasts by elevating intracellular Ca^{2+} to stimulate the activity of eNOS *in vitro*.

Keywords: calcitonin gene-related peptide; nitric oxide production; osteoblast; signalling pathway

1. Introduction

CGRP (calcitonin gene-related peptide), a 37-amino acid peptide generated by tissue-specific alternative splicing of the calcitonin gene, has been proposed to play a key role in promoting osteoblast recruitment and osteogenic activity (Irie et al., 2002; Kawase et al., 2005). CGRP has been shown to be expressed in nerve fibres during bone development and regeneration (Imai et al., 1997), and CGRP-knockout mice as well as functional studies have demonstrated that CGRP is an important factor during bone formation and repair (McDonald et al., 2004; Ma et al., 2009). CGRP receptors have been identified both in *in vitro* osteoblast-like cell models as well as downstream signal transduction pathways, such as the cAMP (cyclic adenosine monophosphate) (Villa et al., 2006), $[Ca^{2+}]_i$ (intracellular Ca^{2+}) (Burns et al., 2004) and MAPK (mitogen-activated protein kinase) (Kawase et al., 2003, 2005) pathways. In our previous study, we found that the expression and activity of NOS (nitric oxide synthase) was positively correlated with CGRP expression during bone healing, suggesting that NO (nitric oxide) may act downstream of CGRP to promote and regulate fracture healing (Li et al.,

2009). However, the regulatory mechanism of CGRP on NO production has not been systematically studied in osteoblasts.

NO has recently been reported to have important effects on osteoblast function and biphasic effects on osteoblastic bone regeneration (van't Hof and Ralston, 2001; Kreja et al., 2008). Low NO concentrations, constitutively produced by osteoblasts, have been shown to act as an autocrine stimulator of osteoblast growth and cytokine production (Riancho et al., 1995; Lin et al., 2008), while high NO concentrations, which are stimulated by proinflammatory cytokines, such as IL-1 (interleukin-1), TNF- α (tumour necrosis factor-alpha) and IFN- γ (interferon-gamma) (Chen et al., 2005; Park et al., 2009), have been shown to have inhibitory effects on osteoblast growth and differentiation (Chen et al., 2005; Fukada et al., 2008). These biological characteristics of NO are determined by the mechanisms of NO action. NO secreted from constitutive NOS [cNOS (constitutive nitric oxide synthase), including eNOS (endothelial nitric oxide synthase) and nNOS (neuronal nitric oxide synthase)], which is constitutively expressed at low levels in its tissues of origin and is regulated at the level of enzymatic activity by changes in free $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration), results in elevated cGMP levels and causes activation of cGMP-dependent protein kinases (Feelisch and

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Abbreviations: ALP, alkaline phosphatase; $[Ca^{2+}]_i$, intracellular Ca^{2+} ; cAMP, cyclic adenosine monophosphate; CGRP, calcitonin gene-related peptide; cNOS, constitutive nitric oxide synthase; DA, diacetate; DAF-FM, 3-amino, 4-aminomethyl-2',7'-difluorescein; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN- γ , interferon-gamma; IL, interleukin-1; iNOS, inducible nitric oxide synthase; L-NAME, L-arginine, N^G-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; MOB, primary human mandibular osteoblasts; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NO₂, nitrite; NO₃, nitrate; RQ%, relative quantity; TNF- α , tumour necrosis factor-alpha.

Stamler, 1996). In contrast, NO is secreted from iNOS (inducible nitric oxide synthase) in larger quantities and for prolonged time periods, resulting in cytotoxicity due to oxygen-derived free radicals and lipid peroxidation (Chen et al., 2005). In osteoblasts, eNOS is widely and constitutively expressed; iNOS is only induced by stimulation with proinflammatory cytokines, and nNOS is hardly expressed at all (van't Hof and Ralston, 2001). We propose that CGRP causes changes in NO production in osteoblasts and further explore whether stimulation with CGRP leads to changes in the expression and activity of NOS. We also identify the regulatory mechanism of CGRP action.

2. Materials and methods

2.1. Materials

Human CGRP and verapamil were purchased from Sigma-Aldrich. CGRP was dissolved in distilled water to a stock concentration of 100 μ M and stored in 100 μ l aliquots at -70° C. The following reagents were purchased from Beyotime: DAF-FM (3-amino, 4-aminomethyl-2',7'-difluorescein), DA (diacetate); Griess Reagent; L-NAME (L-arginine, N^G-nitro-L-arginine methyl ester); L-canavanine and spermidine. RNAiso Plus, the PrimeScript RT reagent Kit and SYBR[®] Premix Ex Taq II were purchased from TaKaRa.

2.2. Cell culture

MG-63, an osteogenic human osteosarcoma cell line, was obtained from the ATCC (America Type Culture Collection). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% FCS (fetal calf serum), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were sub-cultured every 72 h in a humidified 5% CO₂ incubator. Cells from passages 4–6 were used in the experiments. MOBs (primary cultured human mandibular osteoblasts) were isolated from the mandible of a 24-year-old patient undergoing rebuilding surgery. Following guidelines from the local ethics committee, the patient was informed of the study prior to any surgical procedure and provided consent for use of the materials in this study. Fragments

(2 mm \times 2 mm) of mandibular bone were mechanically isolated and washed several times in PBS to remove blood cells. The fragments were then placed in tissue culture flasks under the same medium and culture conditions as MG-63 cells (Figure 1A). After approximately 2 weeks in culture, bone fragments were discarded, and cells were harvested by trypsinization (Figure 1B). MOBs were identified by ALP (alkaline phosphatase) staining (Figure 1C).

2.3. Determination of NO concentration in cells

Intracellular NO concentrations were measured using a membrane-permeable indicator dye, DAF-FM, which reacts with NO to form a green fluorescent product (Kojima et al., 1999). Cells were seeded on round glass coverslips for 24 h in DMEM containing 10% FCS. After simulating the cells with 10 nM CGRP for 0, 1, 2, 3 or 4 h, individual cultures were incubated for 30 min at 37 $^{\circ}$ C in 10 μ M DAF-FM, washed with DMEM and incubated for an additional 30 min in medium without the dye. Cells were rinsed with D-Hanks (Hyclone), and coverslips were affixed to the flow chamber and mounted on a confocal scanning laser microscopy system (LSCM) coupled to an upright microscope (IX-70, \times 60 objective; Leica, Germany). Fluorescence intensity was recorded at the excitation wavelength of 488 nm, and the intensity at 0 h was set to 1. The single-cell intracellular NO concentration was analysed as the average intensity of DAF-FM fluorescence, and relative increases in the intracellular NO levels were calculated. Only cells not physically touching adjacent cells were selected for analysis with at least 15 cells counted in each view (magnification, \times 200).

2.4. Determination of NO production in cell culture supernatants

MG-63 cells (10^4 /ml) or MOBs (10^4 /ml) were plated in 12-well plates for 24 h and then starved in medium supplemented with 0.1% FCS for 24 h. Cells were incubated with 10 nM of CGRP for 0, 1, 2, 3, 4, 6, 12, 18 or 24 h, and cell culture supernatants were collected and centrifuged at 2000 rev./min for 5 min. NO production was assessed by the Griess reaction (Marzinzig et al., 1997), which measures the stable end product of NO, NO₂ (nitrite)/NO₃ (nitrate). Briefly, 400 μ l samples of conditioned media or NO₂

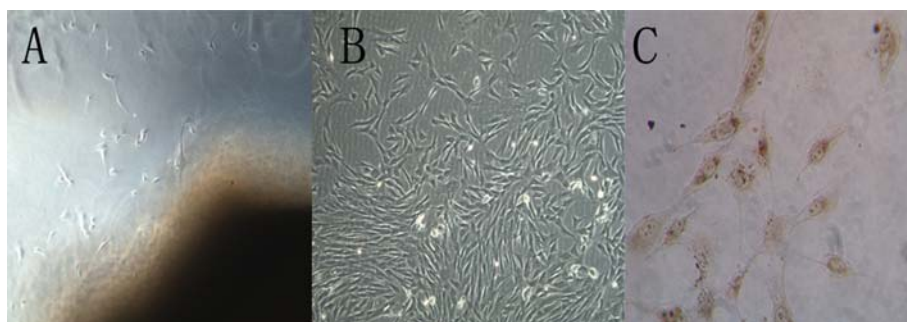


Figure 1 Culture and identification of MOBs
(A) MOBs migrated from bone fragments after incubation in DMEM for 2 weeks (magnification, 100x). (B) MOBs were passaged four times in culture and then produced clustered, spindle-shaped cells (magnification, 100x). (C) MOBs were stained with alkaline phosphatase (magnification, \times 200).

standards (0–100 mM) were mixed with 400 ml of Griess reagent, 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% phosphoric acid. Samples were measured using a microplate reader, and the absorbance at 530 nm was measured against a blank prepared with non-conditioned medium.

To identify which NOS isoforms were affected, we used L-NAME (50 μ M), spermidine (100 μ M) and L-canavanine (500 μ M) to inhibit eNOS, nNOS and iNOS, respectively. Cells were treated with 10 nM of CGRP and the various inhibitors for 4 h. To determine the effect of $[Ca^{2+}]_i$ in CGRP regulation of cNOS, cells were treated with a voltage-dependent L-type Ca^{2+} channel blocker, verapamil (5 μ M), for 4 min. To exclude the influence of any NO synthesis substrates, we co-treated cells with the amino acid L-arginine (20 μ M) as a substrate-rich group.

2.5. Assays of eNOS and iNOS mRNA expression

Cells were seeded, starved and treated with 10 nM of CGRP for 0, 6, 12, 18 or 24 h. eNOS and iNOS mRNA expression levels were detected by quantitative RT-PCR. To assay for the regulation of CGRP on iNOS mRNA expression (Park et al., 2009), cytokine-induced groups were used as positive controls, which included stimulation with TNF- α (25 ng/ml), IFN- γ (100 U/ml) and both TNF- α (25 ng/ml)+IFN- γ (100 U/ml) at 24 h; negative control groups were treated with DMEM medium. For each time point and loading treatment, six mRNA samples from each group were extracted using RNAiso Plus and reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa).

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β -actin were used as internal controls. Primers were designed with the Primer Express software using published human sequences in GenBank and synthesized by TaKaRa: eNOS forward, 5'-CCTCTAAGGTGATCCCTGTCA-3', reverse, 5'-GCATCCTGCCA-GTTTGAGGT-3' (223 bp); iNOS forward, 5'-TGTTGGGTTCTCT-CTGCCATA-3', reverse, 5'-ACGATTCCAGCCTGATACGC-3' (157 bp); β -actin forward, 5'-CATGTACGTTGCTATCCAGGC-3', reverse, 5'-CTCCTTAATGTACGCACGAT-3' (250 bp); GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3', reverse, 5'-ATGGTGGTGAAGACGCCAGT-3' (142 bp).

Quantitative PCR was performed using SYBR Premix Ex Taq II. Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 s denaturation at 95°C, 30 s annealing at 61°C and 45 s elongation at 72°C and a terminal elongation for 5 min at 72°C. All samples and standards were run in triplicate. Gene expression was quantified using the C_T (comparative threshold cycle) method and reported as the fold difference relative to GAPDH and β -actin; the obtained RQ% (relative quantity) of target mRNA was calculated relative to a control sample set to 1.

2.6. Statistical analysis

All results are expressed as the mean \pm S.E.M. The data were analysed using a one-way analysis of variance (ANOVA) and Newman-Keuls-Student's *t* test. $P < 0.05$ or $P < 0.01$ was considered significant.

3. Results

3.1. Effect of CGRP on the NO concentration in osteoblasts

To accurately measure temporal NO concentrations in cells simulated by CGRP, the membrane-permeable indicator DAF-FM, DA was used. This probe is converted by intracellular esterase into DAF-FM, which reacts with NO to form a green fluorescent product. The absorbance of this fluorescent product was then imaged and measured by laser scanning confocal microscopy. As shown, CGRP exhibited a dose-dependent effect (0.1–100 nM) with an effective concentration of 10 nM in both MG-63 cells (Figure 2A) and MOBs (Figure 2B); we subsequently only used 10 nM in all our experiments. The most effective CGRP treatment time point was 1 h in both cell types. Stimulation of MG-63 and MOBs with 10 nM CGRP at 1 h caused peak increases of 323% and 224% in NO concentration, respectively. The NO concentration returned to basal levels after cells were treated for 3 h, but this stimulatory effect was 1 h longer in MG-63 cells than in MOBs.

3.2. Time-dependent effect of CGRP on NO production in cell supernatants

To evaluate long-term NO production in cells, we measured NO_2^-/NO_3^- concentrations (stable NO end products) in the cell supernatants. As shown, NO concentrations increased linearly in the control groups during the first 24 h with little difference in NO production between MG-63 cells (Figure 3A) and MOBs (Figure 3B) at 24 h, NO production was, respectively, 5.95 and 5.35 μ M. CGRP, however, induced a significant increase in NO production between 1 and 6 h, causing increases of 67.5% in MG-63 cells ($P < 0.01$) and 87.6% in MOBs ($P < 0.01$) at the 4-h time point. This maximum difference point of 4 h was designed as the critical observation point in all subsequent experiments. After incubation for 12 h, CGRP-stimulated NO production in experimental groups coincided with the linear increase in control groups.

3.3. Effect of CGRP on the activity of NOS isoforms

To further explore which NOS isoforms are simulated by CGRP in osteoblasts, we treated cells with inhibitors of eNOS (L-NAME), iNOS (L-canavanine) and nNOS (spermidine) to block CGRP induction. At the same time, we added a NO substrate, L-arginine, to find the CGRP induction change in substrate sufficient conditions. In both MG-63 cells (Figure 4A) and MOBs (Figure 4B), NO production decreased when eNOS was blocked, but not when either iNOS or nNOS was blocked (numbers 1 to 4), suggesting that eNOS is responsible for NO production in osteoblasts. CGRP-induced NO production is effectively blocked 48.3% ($P < 0.01$) and 52.3% ($P < 0.01$) by the eNOS inhibitor L-NAME in both MG-63 and MOBs, respectively (numbers 5 to 8). In substrate-sufficient conditions (numbers 9–13), CGRP-induced NO production was not significant when compared with the

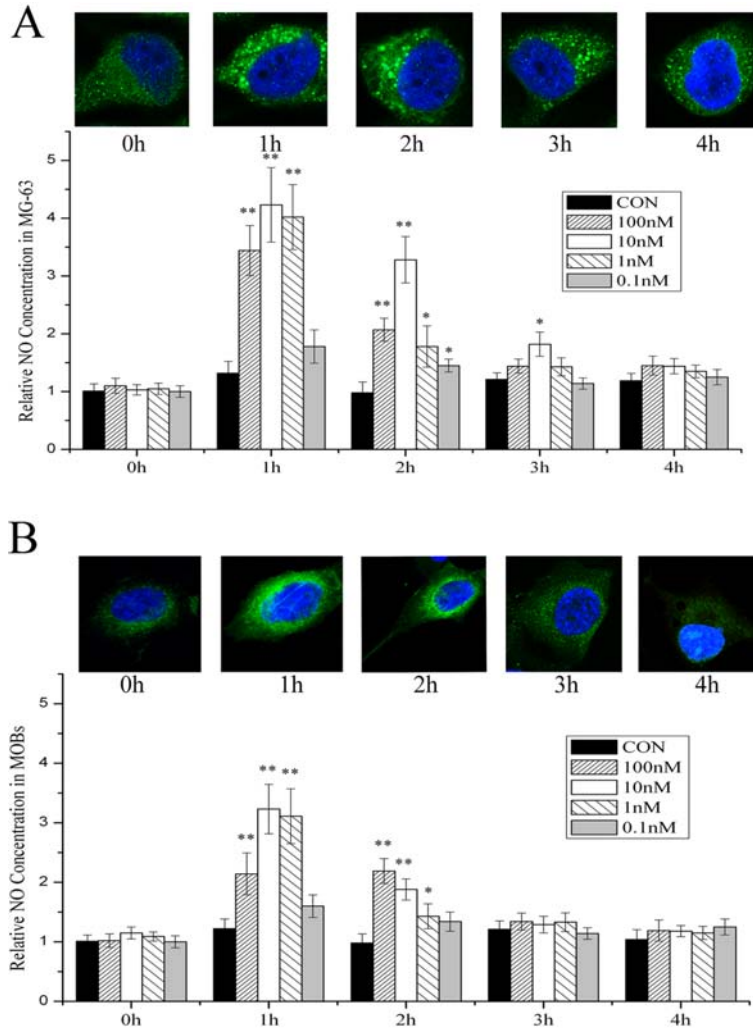


Figure 2 Time-dependent effect of CGRP on NO concentration in MG-63 cells (A) and MOBs (B)

Cells were incubated with CGRP (0.1–100 nM) from 0 to 4 h. NO concentrations in cells were detected using DAF-FM and measured by confocal scanning laser microscopy. Cell fluorescence images in which cells were stimulated by 10 nM CGRP are shown above the columns. Data are presented as the relative absorbance value compared with control groups at 0 h and expressed as the mean \pm S.E.M. for each time point, $n=8$; * $P<0.05$, ** $P<0.01$.

conditional control groups (number 9). Interestingly, the eNOS inhibitor L-NAME was still able to block CGRP induction under this condition.

3.4. Effect of $[Ca^{2+}]_i$ in CGRP-induced NO production

Given that eNOS is the NOS isoform responsible for CGRP-induced NO production in osteoblasts, we wanted to explore the underlying regulatory mechanism. As Ca^{2+} is a key factor in eNOS activity and can be induced by CGRP in osteoblasts, we blocked Ca^{2+} using verapamil, a voltage-dependent L-type Ca^{2+} channel blocker. Verapamil decreased basal levels of NO both in MG-63 cells by 56.1% ($P<0.01$, Figure 5A) and in MOBs by 45.0% ($P<0.01$, Figure 5B), respectively, after stimulation for 4 h. NO levels returned to normal by blocking L-type Ca^{2+} channels, but CGRP-induced NO production decreased 58.9% ($P<0.01$) in MG-63 cells and 36.4% ($P<0.05$) in MOBs. This result suggests that

Ca^{2+} is a necessary signal for CGRP-dependent regulation of eNOS activity.

3.5. Time-dependent effect of CGRP on eNOS and iNOS mRNA expression

Except for its regulatory role on the activity of NOS, CGRP may up-regulate NOS expression to induce further increases in NO production. We thus performed quantitative RT-PCR to determine the effect of CGRP on eNOS and iNOS mRNA expression levels, although it has not been verified that nNOS is expressed in osteoblasts. As shown, we did not observe any statistically significant difference in either eNOS or iNOS mRNA levels between 0 and 24 h after treatment (Figure 6). In addition, we only observed a mild increase of eNOS expression in MG-63 cells at 12 h compared with control groups ($P<0.05$, Figure 6A); eNOS mRNA expression did not increase in MOBs (Figure 6B).

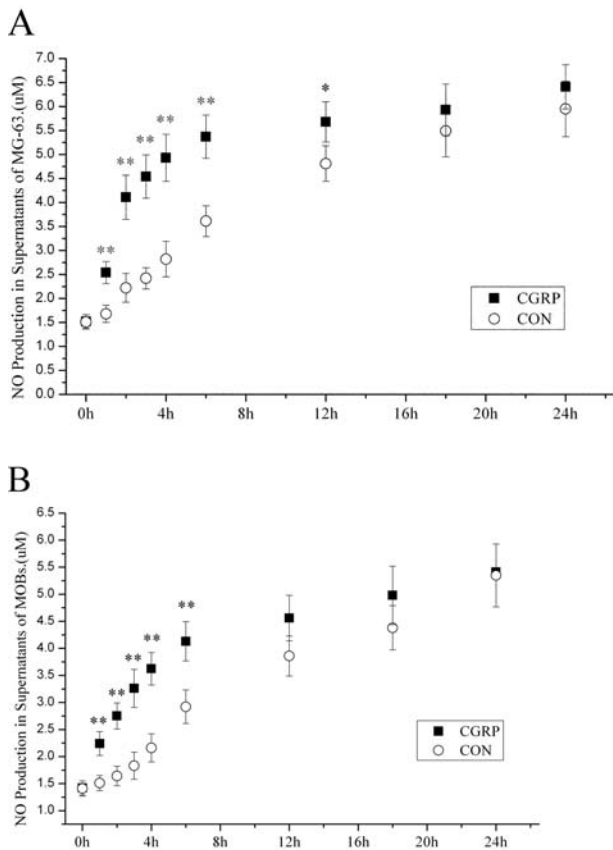


Figure 3 Time-dependent effect of CGRP on NO production in MG-63 (A) and MOB cell supernatants (B)

Cells were incubated with 10 nM of CGRP for 24 h and assayed every hour for the first 4 h and then every 6 h from 6 to 24 h. NO concentrations were detected by the Griess reaction. Data are presented as the absolute value of NO production and expressed as the mean \pm S.E.M., $n=8$; * $P < 0.05$, ** $P < 0.01$.

3.6. Effect of CGRP on cytokine-induced iNOS mRNA expression

Although CGRP does not affect eNOS and iNOS mRNA expression in the first 24 h, we wanted to determine the effect, if any, of CGRP on cytokine-induced increases in iNOS mRNA expression. Regulation of iNOS mRNA levels is an important and effective step in NO production. We used IFN and TNF, previously identified in other osteoblast cell lines, to induce a proinflammatory increase in iNOS mRNA levels. As shown, treating with either cytokine alone had no effect (Figure 7), although TNF treatment did lead to an increase in iNOS mRNA expression in MBOs (Figure 7B). However, co-treating with both IFN and TNF led to a 661% ($P < 0.01$) up-regulation of iNOS mRNA in MG-63 cells (Figure 7A) and a 521% ($P < 0.01$) increase in MBOs (Figure 7B). Finally, when we used CGRP to stimulate cytokine-induced groups, we also did not observe any statistically significant differences compared with the cytokine-induced groups, suggesting that CGRP does not affect cytokine-induced iNOS mRNA expression, at least *in vitro*.

4. Discussion

We demonstrated that incubation of osteoblast-like cells with CGRP leads to a transient time-dependent increase in NO production, primarily through eNOS present in cells. In addition, we elucidated the mechanism of eNOS-mediated NO production via stimulation of CGRP on intracellular Ca^{2+} levels.

CGRP has been considered to be an endogenous mitogen activator in osteogenic cells because of its effects on cell proliferation (Huebner et al., 2008). Villa et al. (2006) reported that CGRP treatment caused an increase in osteoblast proliferation through the cAMP-dependent signalling pathway, and we have found that CGRP also increases MG-63 cell proliferation through regulation of AP-1 (activator protein-1) (L. Yan, T. Yinghui, Y. Bo, Z. Gang, W. Xuefei and L. Min, unpublished work). Although the major effect of NO appears to be inhibition of cell proliferation in many cell types (Chen, et al., 2005; Tamashiro et al., 2010), there are several reports indicating that the NO produced constitutively by constitutive NOS may have a stimulatory action and may act as a stimulator of osteoblast growth and cytokine production. Because of the positive correlation reported between NO and CGRP expression in bone callus (Li et al., 2009), we hypothesized that NO may also mediate CGRP-induced osteoblast proliferation or differentiation *in vitro*. In this study, we chose MG-63 and MOB cells to investigate this hypothesis. MG-63 express low basal levels of alkaline phosphatase, osteocalcin, synthesize types I and III collagen as immature marrow stromal cells (Ryu et al., 2009) and have been reported to be affected by CGRP in studies (Kawase et al., 2003, 2005; Bums et al., 2004). MOB cells are more representative and similar to our experiment *in vivo* (Li et al., 2009).

NO present in cell supernatants rapidly reacts with oxygen to yield the stable metabolites NO_3 and NO_2 , which can easily be measured by the Griess reaction (Feelisch and Stamler, 1996). In our study, we found that treatment with 10 nM of CGRP induced a significant increase in NO production between 1 to 12 h both in MG-63 cells and MOB cells, which recovered after 12 h (comparing between CGRP-stimulated groups and control groups). Therefore, we initially concluded that CGRP promoted NO production in osteoblasts, at least *in vitro*. However, the amount of accumulated NO_3 and NO_2 did not accurately reflect the amount of NO present in the cell. We thus used DAF-FM DA to quantitatively image the concentration of NO in the cell by assaying fluorescence photodensity (Kojima et al., 1999). Owing to fluorescence attenuation, we had to limit the time of observation to 4 h. Using this method, we found significant increases in fluorescence intensity within 2 h of CGRP stimulation. The most effective concentration that induced NO production was 10 nM, the same concentration that effectively promotes cell proliferation (Villa et al., 2006). It appeared that MG-63 cells were more sensitive than MOB cells to secrete NO upon CGRP treatment. We could not detect any statistically significant differences among all of the groups after 2 h of treatment, suggesting that any increases in NO production observed in cell supernatants up to 12 h occurs during the first 2 h. We thus assumed that CGRP-induced increases in NO concentration are directly due to transient and temporal production from cNOS. At the same time, we found that increases in NO

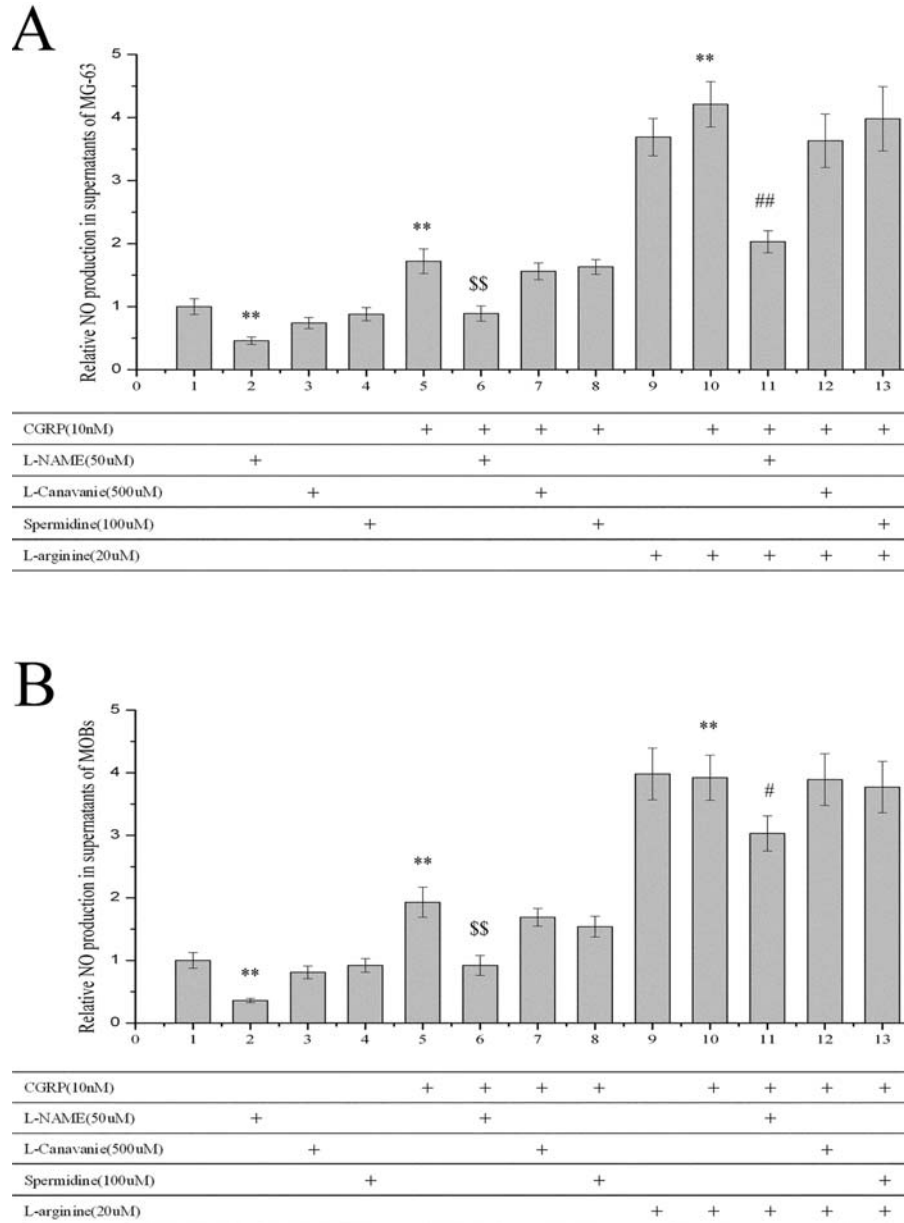


Figure 4 Effect of inhibitors of NOS isoforms on CGRP-induced NO production in MG-63 cells (A) and MOB3 (B)

Cells were treated as described and detected at the 4-h time point. NO concentration in the supernatants were detected and measured as described in Figure 3. Data are presented as the relative NO production compared with control groups (first column) and expressed as the mean \pm S.E.M; $n=8$; * $P<0.05$, ** $P<0.01$ compared with control groups; \$ $P<0.05$, \$\$ $P<0.01$, compared with CGRP+groups; # $P<0.05$, ## $P<0.01$, compared with CGRP+L-arginine+groups.

were temporal and low in concentration, suggesting that NO may be a downstream messenger to mediate CGRP effect. Despite having cytotoxic effects, NO is involved in many signalling transduction pathways, including cGMP-dependent, EGFR (epidermal growth factor receptor) and MAPK signalling pathways (Chen et al., 2005; Lo and Hung, 2006; Ptasinska et al., 2007). Once NO is demonstrated to be the second messenger of CGRP signalling, we can further explore the effects of CGRP on these other signalling pathways.

Three isoforms of NOS have been identified thus far, and both eNOS and iNOS are constitutively expressed in osteoblasts (Helfrich et al., 1997). Although little is known about the expression of nNOS in bone and cultured bone-derived cells, nNOS mRNA has been detected by RT-PCR in bone (Schmidt et al., 1992; Chen et al., 2005). Here, we applied specific inhibitors of these three NOS isoforms to identify which isoform mediates increases in NO production in osteoblasts. We found that inhibiting the activity of eNOS leads to statistically significant decreases in basal NO levels

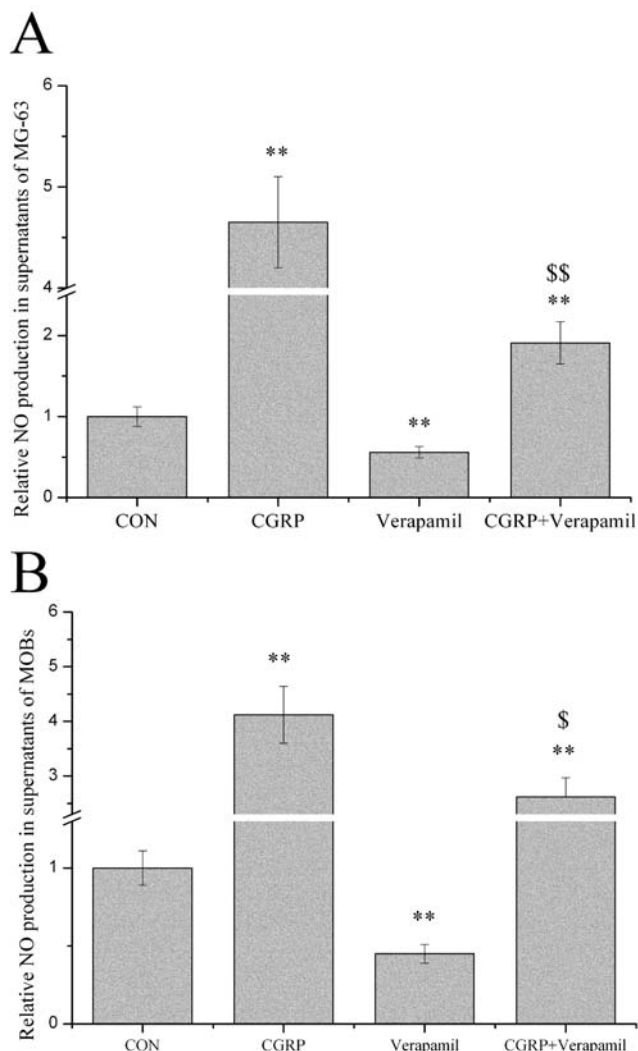


Figure 5 Blocking effect of verapamil on the CGRP-induced NO concentrations in MG-63 cells (A) and MOB cells (B)

Control groups were treated with DMEM, CGRP-stimulated groups were incubated with 10 nM CGRP, verapamil groups were incubated with 5 μ M verapamil and experimental groups were co-incubated with CGRP and verapamil for 4 h. The NO concentration in the supernatants was detected and measured as described in Figure 3. Data are presented as the relative NO production compared with control groups and are expressed as the mean \pm S.E.M., $n=8$; * $P<0.05$, ** $P<0.01$ compared with control groups; \$ $P<0.05$, \$\$ $P<0.01$, compared with CGRP-stimulated groups.

compared with control groups and was able to recover CGRP-induced NO production. It was demonstrated that eNOS is the isoform responsible for maintaining basal NO levels, and CGRP induces increases in NO levels. To exclude the influence of deficiency of NO donors, we co-incubated cells with 0.1 nM CGRP and 20 μ M L-arginine. With sufficient NO donor, CGRP does not induce a higher NO production, but the NO production level can be blocked by inhibiting eNOS. This attenuation of NO may be related with the autoregulatory feedback loop, in which increased NO levels limit additional NO production (Rogers and Ignarro, 1992). CGRP-induced NO production is a normal biological activity of the cell, but increases in NO production lead to cell apoptosis. After blocking intracellular Ca^{2+} using verapamil,

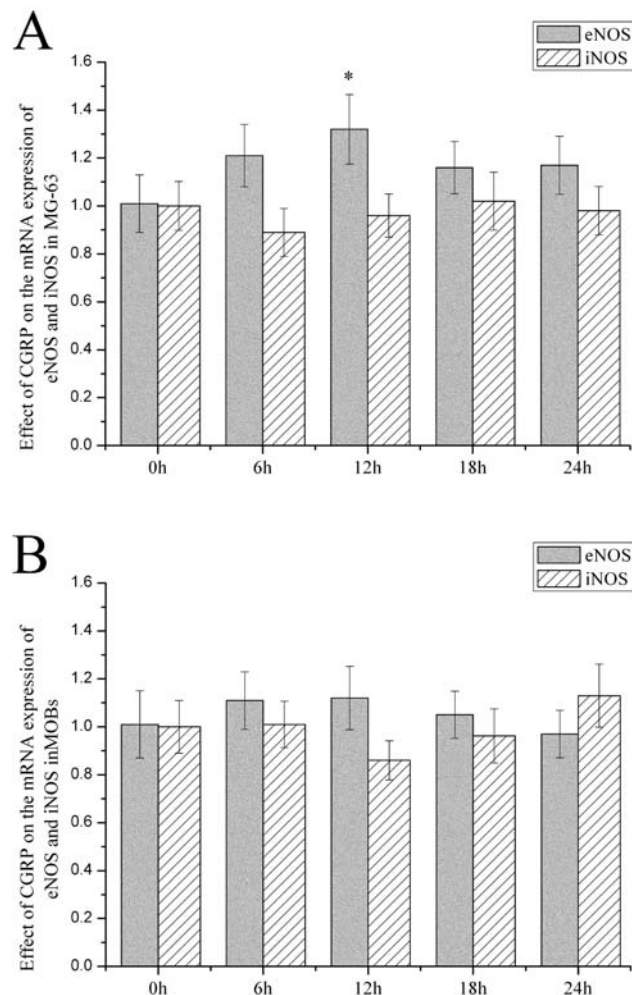


Figure 6 Time-dependent expression of eNOS and iNOS mRNA in MG-63 cells (A) and MOB cells (B) after CGRP treatment

Cells were incubated with 10 nM CGRP and assayed at 0, 6, 12, 18, and 24 h after treatment. Expression of eNOS and iNOS mRNA was detected by quantitative fluorescent PCR. RQ% of target mRNA was calculated and expressed as the mean \pm S.E.M., $n=6$; * $P<0.05$, ** $P<0.01$, compared with control groups.

a voltage-dependent L-type Ca^{2+} channel blocker, CGRP-induced NO production was also recovered by inhibiting eNOS. Burns et al. (2004) reported that in MG-63 cells, CGRP stimulates Ca^{2+} discharge from intracellular stores through a cAMP-independent mechanism and subsequently stimulates Ca^{2+} influx through L-type voltage-dependent channels, explaining the effect of CGRP on NO production in osteoblasts. Additionally, we observed a gradual rise of eNOS mRNA expression at 12 h in MG-63 cells treated with CGRP, but otherwise did not observe any statistically significant difference between MG-63 cells and MOB cells. Because it only took place at one observation point and in just one kind of cell, we cannot say that CGRP regulates eNOS mRNA expression in osteoblasts. We thus conclude that CGRP causes increases in NO production by regulating eNOS activity, which is dependent on increases in intracellular Ca^{2+} levels in osteoblasts.

Unlike cNOS, iNOS is primarily regulated at the level of transcription and is activated by proinflammatory cytokines, such

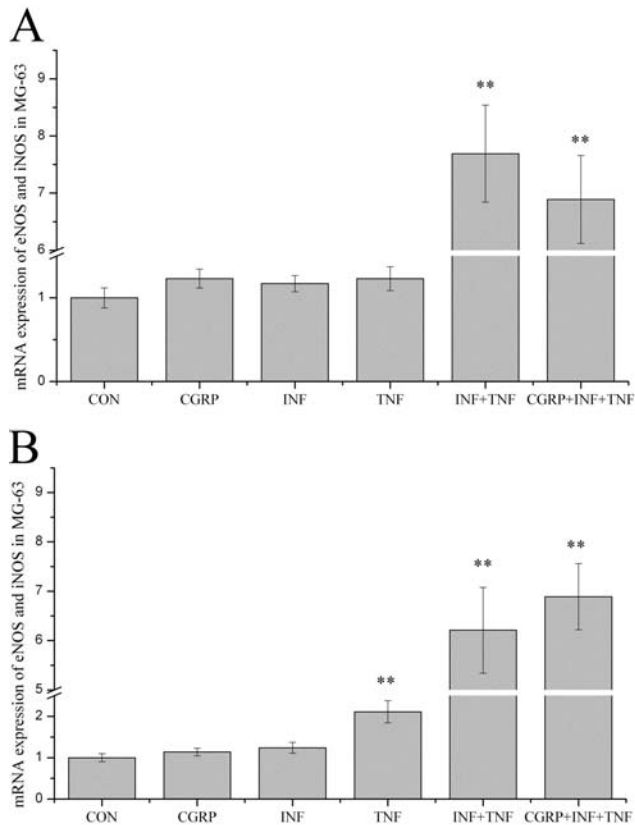


Figure 7 Cytokine-induced iNOS mRNA expression in MG-63 cells (A) and MOB (B)

Control groups were treated with DMEM, CGRP-stimulated groups were incubated with 10 nM CGRP, IFN-stimulated groups were incubated with 10 nM IFN, TNF-stimulated groups were incubated with 10 nM TNF, the co-induced group was incubated with both IFN and TNF and the experimental groups were incubated with CGRP+IFN+TNF for 24 h. iNOS mRNA expression levels were detected by quantitative fluorescent PCR. RQ% of target mRNA was calculated and presented as the mean±S.E.M., $n=6$; * $P<0.05$, ** $P<0.01$, compared with control groups.

as IL-1, TNF- α and IFN- γ (Chen et al., 2005; Park et al., 2009). To identify whether CGRP can regulate the expression of different iNOS isoforms and affect cytokine-induced iNOS activity, we compared iNOS mRNA levels in osteoblasts stimulated with CGRP and those stimulated with TNF, IFN and TNF/IFN as a positive control. We observed iNOS mRNA up-regulation in both MG-63 cells and MBOs with TNF/IFN stimulation and in MBOs with TNF stimulation. However, we did not observe any up-regulation of iNOS mRNA expression or any effect of CGRP on cytokine-induced iNOS mRNA expression. It may be possible that *in vivo*, CGRP indirectly regulates iNOS expression by recruiting inflammatory cells and stimulating growth or inflammatory factors, although CGRP is not known to regulate the iNOS signalling pathway in osteoblasts *in vitro*.

In summary, CGRP transiently induces NO production in osteoblasts by elevating intracellular Ca^{2+} to stimulate the activity of eNOS *in vitro* within the first 2 h of treatment. Increased NO levels and extended incubation time appear to not be cytotoxic to osteoblasts; on the contrary, our results were consistent with a role for CGRP in promoting cell proliferation in either a time- or concentration-dependent manner. This temporal,

CGRP-dependent induction of NO could play a role in the mitogenic effects of CGRP on osteoblast growth. Future work will aim to identify the components up-regulated by NO during osteoblast proliferation and differentiation.

Author contribution

Li Yan designed the experiments and was in charge of the cytological experiments, data statistics and paper writing. Tan Yinghui was responsible for the financial support, experimental design guidelines and article review. Yang Bo was in charge of the molecular biology experiments. Zhang Gang was in charge of literature review and article review. Xiao Xian was responsible for the molecular biological experiments. Zheng Lu was in charge of the cytological experiments.

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