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Nitric oxide inhibits androgen receptor-mediated collagen production in human gingival fibroblasts

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Background and Objective: In our previous study, we found that flutamide [an androgen receptor (AR) antagonist] inhibited the up-regulation of collagen induced by interleukin (IL)-1β and/or nifedipine in gingival fibroblasts. The present study attempted to verify the role of nitric oxide (NO) in the IL-1β/nifedipine-AR pathway in gingival overgrowth.

Material and Methods: Confluent gingival fibroblasts derived from healthy individuals ($n = 4$) and those with dihydropyridine-induced gingival overgrowth (DIGO) ($n = 6$) were stimulated for 48 h with IL-1β (10 ng/mL), nifedipine (0.34 μM) or IL-1β + nifedipine. Gene and protein expression were analyzed with real-time RT-PCR and western blot analyses, respectively. Meanwhile, Sircol dye-binding and the Griess reagent were, respectively, used to detect the concentrations of total soluble collagen and nitrite in the medium.

Results: IL-1β and nifedipine simultaneously up-regulated the expression of the *AR* and type-I collagen α1 [*Col*α1(I)] genes and the total collagen concentration in DIGO cells ($p < 0.05$). IL-1β strongly increased the expression of inducible nitric oxide synthase (*iNOS*) mRNA and the nitrite concentration in both healthy and DIGO cells ($p < 0.05$). However, co-administration of IL-1β and nifedipine largely abrogated the expression of *iNOS* mRNA and the nitrite concentration with the same treatment. Spearman's correlation coefficients revealed a positive correlation between the AR and total collagen ($p < 0.001$), but they both showed a negative correlation with iNOS expression and the NO concentration ($p < 0.001$). The iNOS inhibitor, 1400W, enhanced IL-1β-induced AR expression; furthermore, the NO donor, NONOate, diminished the expression of the AR to a similar extent in gingival fibroblasts derived from both healthy patients and DIGO patients ($p < 0.05$).

Conclusion: IL-1β-induced NO attenuated AR-mediated collagen production in human gingival fibroblasts. The iNOS/NO system down-regulated the axis of *AR*/*Col*α1(I) mRNA expression and the production of AR/total collagen proteins by DIGO cells.

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Our previous study demonstrated that overexpression of the androgen receptor (AR) in gingival tissues is considered to be one of the major causes of the pathogenesis of gingival overgrowth induced by nifedipine, a type-I dihydropyridine calcium-channel blocker (1). We ruled out the possibility of *Porphyromonas gingivalis* as an etiologic factor, and demonstrated that interleukin (IL)-1β is responsible for regulating AR expression in dihydropyridine-induced gingival overgrowth (DIGO) cells (2). Furthermore, the role of the AR in IL-1β-induced collagen expression was verified using the AR antagonist, flutamide. Blockage of the AR in DIGO fibroblasts entirely abolished the stimulatory effect of IL-1β and nifedipine on the expression of connective tissue growth factor and collagen (3). This implies that the AR plays an important role in the development of DIGO.

Inducible nitric oxide synthase (iNOS) and nitric oxide (NO) play certain roles in the inflammatory mechanism of periodontitis. The expression of iNOS protein was significantly higher in tissue samples from periodontitis patients than in those from healthy individuals (4–6). Gingival fibroblasts are one of the sources of NO in the presence of proinflammatory cytokines, including IL-1β, tumor necrosis factor-α and interferon-γ (7).

NO is a free radical and thus participates in many intracellular and extracellular events in tissues. NO was implicated in the pathogenesis of numerous inflammatory and autoimmune diseases (8,9). To date, the precise role of this molecule regarding its biologic interactions as a protective or toxic agent is not entirely clear. It is difficult to reconcile the apparent complexity of interactions of NO with other biomolecules and its dual roles as a cytotoxic agent and a physiologic messenger molecule. NO appears to be a bifunctional modulator of cell death, capable of either stimulating or inhibiting apoptosis (10).

We were interested in the role of NO in DIGO because our previous study implied that elevated AR expression and prominent T-helper cell 1 cytokine-labeled cells are two significant factors in the pathogenesis of nifedipine-induced gingival overgrowth. (1). It was also found that the additive effects of nifedipine and IL-1β on gingival tissues might aggravate gingival overgrowth by augmenting AR activity through the regulation of intracellular [Ca^{2+}] homeostasis (3). On the other hand, NO was found to regulate mitochondrial and cytoplasmic Ca^{2+} signals during T-cell activation and may be linked to lymphocyte dysfunction in rheumatoid arthritis (11). A marked increase in NO production was found in early-stage diffuse cutaneous lesions of systemic sclerosis patients with active fibrosing alveolitis, and constitutive iNOS expression by systemic sclerosis fibroblasts may contribute to the increased production of NO (12,13). NO production and expression of *iNOS* mRNA are related to collagen production of keloid scar formation (14,15), wound healing (16), cultured tendons (17) and pulmonary fibrosis (18).

Since we found that the specific AR genome of gingival fibroblasts derived from DIGO may be a cornerstone in regulating the anabolic arm of tissue overgrowth, we assumed that interactions between the IL-1β, nifedipine, AR/type I collagen α [Colα1(I)]/collagen axis and the iNOS–NO cascade may act as a reciprocal mechanism in regulating collagen overproduction and affect the fibrotic homeostasis of DIGO. In the present study, we determined whether cross-talk occurs between the iNOS–NO and AR/Colα1(I)/collagen cascades in DIGO cells.

Material and methods

Participant selection

Six DIGO responders (D1–D6; age range: 51–69 years; mean age, 55 years) and four periodontally healthy individuals (H1–H4; age range: 46–57 years; mean age, 52 years), all nonsmokers, were included in this study (Table 1). Informed consent was obtained from each patient under the surveillance of the Ethics Committee of Taipei Medical University Hospital.

Diagnostic criteria for the DIGO group included individuals who had taken 20–30 mg/d of amlodipine or felodipine (type II dihydropyridines) for 6–36 mo and whose gingival overgrowth was classified as grade II or grade III (DIGO responders) (19). Their plaque-control scores ranged from 70 to 100%, and their gingival bleeding scores ranged from 55 to 92%. Patients were selected for tissue sampling 6 wks after scaling and root planing. The probing depth of the sampled area was still ≥ 5 mm after scaling and root planing.

Healthy gingival tissue was harvested from donors who were undergoing implant surgery or selective oral surgery. Inclusion criteria for healthy

Table 1. Age, gender and gingival overgrowth index of our selected patients

	Age	Mean age	Gender	Gingival overgrowth index
Healthy group				
H1	57	52	M	–
H2	52		F	–
H3	46		M	–
H4	55		M	–
DIGO group				
D1	55	58	M	2
D2	55		M	2
D3	51		M	2
D4	59		F	2
D5	63		M	2
D6	69		M	3

DIGO, dihydropydine-induced gingival overgrowth; F, female; H, healthy; M, male.

individuals were: no antibiotic therapy for any reason in the 3 mo preceding the start of the study; no systemic condition that might contribute to periodontal conditions; and no regular treatment with any nonsteroidal anti-inflammatory drugs. Clinically, the healthy tissue appeared firm and pink, and exhibited no erythematous changes.

Fibroblast cell culture

Gingival fibroblasts obtained from DIGO and healthy tissues were cultured in phenol-red-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Cells between the fourth and eighth passages were used in this investigation. Cells were cultured to 70–80% confluence, and then maintained for 24 h in medium containing 10% charcoal-stripped serum. To prepare the charcoal-stripped serum, FBS was incubated with activated charcoal (10 mg/mL; Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37°C. The serum was then centrifuged and passed through a 0.22-μm filter (Millipore, Molsheim, France). After 24 h, we changed the medium to 0.2% charcoal-stripped serum and continued incubation for another 24 h. After 24 h, gingival fibroblasts were treated with nifedipine and IL-1β.

In order to imitate the serum condition of patients who take dihydropyridine, a concentration of 0.34 μM nifedipine was added to the medium as a serum background agent in this study (20–22). In addition, 10 ng/mL of IL-1β was applied to stimulate both healthy and DIGO human gingival fibroblasts (23). Four different stimuli were used – the negative control, IL-1β, nifedipine and IL-1β + nifedipine – which were added 48 h before a confluent state was reached in each of the healthy and DIGO groups. In our experiment, nifedipine was added 1 h before IL-1β or was added to the medium as a background agent. In addition, cells were collected after 24 h of stimulation, and 10 μM ascorbic acid was added 1 d before the cell pellets were collected for the following experiments.

In order to verify the upstream or downstream position of the iNOS/NO system in regulating the AR/collagen cascade, a single-cell strain was selected from H3 and D6 (gingival overgrowth index grade III) patients for further investigation. The iNOS inhibitor, *N*-(3-aminomethyl)benzylacetamidine (1400W; Sigma-Aldrich, Shanghai, China), at 500 nM, and the NO donor, NONOate (DETA NONOate; Cayman, Ann Arbor, MI, USA), at 500 μM, were added 1 h before nifedipine was applied. We collected *AR* mRNA and AR protein from these cells and then carried out western blotting and real-time PCR.

Quantification of *iNOS*, *AR* and *Col*α1(I) mRNAs

For RT-PCR, total RNA was isolated with the Trizol reagent (Invitrogen, Istanbul, Turkey), and complementary DNA (cDNA) was obtained with a first-strand cDNA synthesis kit (GE Healthcare, Taipei, Taiwan). The real-time PCR was performed using a SYBR mix reagent (ABI, New York, NY, USA) and run for 40 cycles at 95°C for 4 min, 60°C for 1 min and 70°C for 1 min. Each 25 μL of PCR mixture contained a cDNA template, the SYBR mix reagent and 250 nM of each gene-specific primer. The sequence of each gene-specific primer was as follows: 5′-CCTGGCTTCCGCAACTTACAC-3′ (AR forward) and 5′-GGACTTGTGCATGCGGTACTCA-3′ (AR reverse); 5′-TGCAGACACGTGCGTTACTC-3′ (iNOS forward) and 5′-GGTAGCCAGCATAGCGGATG-3′ (iNOS reverse); and 5′-TTCTTGATCCTTTCGTGCCTCCAC-3′ [Colα1(I) forward] and 5′-CTGGTCCCCAAGGCTTCCAAGG-3′ [Colα1(I) reverse]; sequences of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were as follows: 5′-TGCACCACCAACTGCTTAGC-3′ (GAPDH forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (GAPDH reverse). Data of cycle time (CT) values of the primers for AR, iNOS, Colα1(I) and GAPDH for the PCR procedure were collected, and GAPDH was used as the internal control to calculate the CT value.

Measurement of NO concentration

In a confluent state, culture media were collected and NO production was assayed by measuring the stable NO metabolites, nitrite and nitrate. The Griess reagent was used to measure total nitrate/nitrite in a simple two-step process. The first step was the conversion of nitrate to nitrite using nitrate reductase. The second step was the addition of the Griess reagent that converted nitrite into a deep-purple azo compound. In brief, 25-μL samples were harvested from the conditioned medium and treated with 75 μL of the Griess reagent (0.1% sulfanilamide, 0.1% naphthalene-ethylene-diamine dihydrochloride and 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was monitored with a microplate reader. The nitrite concentration was determined using sodium nitrite as the standard.

Quantification of total collagen in the supernatant

A Sircol Soluble Collagen Assay kit (Biocolor, Carrickfergus, County Antrim, UK) was used to detect the concentration of the supernatant of both the healthy and DIGO groups. Three-hundred microlitres of the Sircol dye reagent and 300 μL of the supernatant were mixed in new 1.5-mL Eppendorf tubes (Eppendorf, Singapore) for sample preparation. After regular processing (3), 200 μL of each sample was transferred to a 96-well plate for measurement of the absorbance at 595 nm and to produce a standard curve.

Western blot analysis

Cell pellets collected during cell culture were used for protein extraction. Twenty microliters of 2 × sample buffer [0.125 mM Tris-base, 4% (weight by volume) sodium dodecyl sulfate (SDS), 2% mercaptoethanol and 10% glycerol] was added to dissolve the cell pellet, the sample was placed into boiling water for 5 min, cooled on ice for another 5 min and spun down at 13,400 *g* and 4°C for 15 min. The supernatant was transferred to a new 1.5-mL Eppendorf tube. A

Bio-Rad protein assay kit (Bio-Rad, Shanghai, China) was used for protein quantification. Each protein sample (50 μg/line) was electrophoresed on an 8% SDS-polyacrylamide gel at 120 V. The separated proteins were then electroblotted onto a polyvinylidene difluoride (PVDF) transfer membrane. The PVDF membrane was soaked in Tris-Buffered Saline Tween-20 (TBS-T) containing 5% skimmed milk and incubated with the corresponding first antibody (AR, rabbit, SC-815; Santa Cruz Biotechnology, Santa Cruz, CA, USA; iNOS, rabbit, ab3523; Abcam, Interlab Co., Ltd, Taipei, Taiwan; and α-tubulin, cp06; Calbiochem, Taipei, Taiwan) which was diluted in TBS-T containing 5% skimmed milk (AR, 1 : 200 dilution; iNOS, 1 : 200 dilution; and α-tubulin, 1 : 3000 dilution). A horseradish peroxidase-conjugated secondary antibody (1 : 10,000 dilution; Sigma) was used to detect the bound primary antibody. Immune complexes were visualized by enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. The intensity of the bands was scanned and analyzed by a Gel-Pro analyzer (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical procedures

The statistical software SPSS version 12.0 (SPSS, Chicago, IL, USA) was used. Intergroup and intragroup differences in the results of the quantitative real-time PCR and western blot analysis were analyzed using the Mann–Whitney *U*-test because of limited samples. The Student's *t*-test was used to analyze the nitrite and total collagen concentrations in the supernatant of a multiple-patient analysis and in a single-cell strain study of the effects of inhibitory iNOS and NO promotion on *AR* mRNA expression and AR protein production. In addition, Spearman's correlation coefficient was used to analyze the possible reciprocal relation of *iNOS* mRNA/nitrite concentration and *AR*/*Col*α1(I) mRNA and the downstream relationship of the AR protein/total collagen concentration in the supernatant.

Results

In the present study, the addition of IL-1β + nifedipine showed an activation effect on the expression of *AR* mRNA and on the production of AR protein by DIGO cells. The addition of IL-1β resulted in a slight decrease in the expression of *AR* mRNA in both groups (Fig. 1A; $p < 0.05$). However, when nifedipine + IL-1β were added, the expression of *AR* mRNA was strongly enhanced in both groups, especially in DIGO cells ($p < 0.05$). Regarding the expression of AR protein in the two groups, the different stimulants regulated its production similarly as for the expression of *AR* mRNA. The median value of DIGO cells was threefold higher than that of the healthy group (Fig. 1B).

Figure 2 shows the effects of the four different stimulatory conditions on the expression of *Col*α1(I) mRNA and on the concentration of soluble collagen in the two groups. The addition of IL-1β slightly decreased the expression of *Col*α1(I) mRNA and total collagen production in both groups, compared

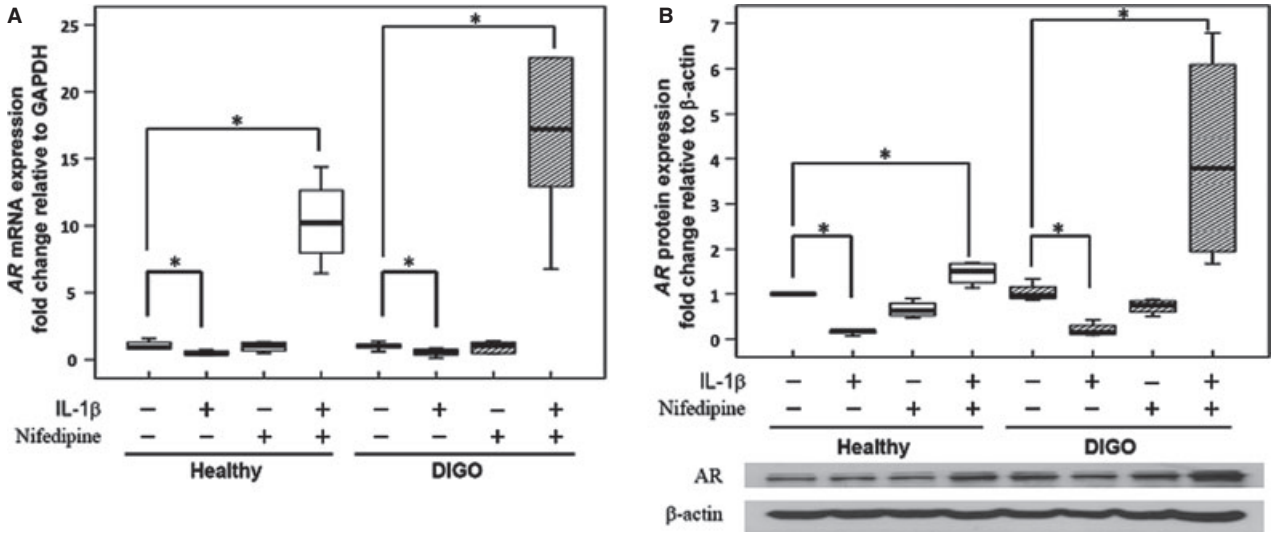

Fig. 1. In each group, the expression of androgen receptor (*AR*) mRNA showed a slight decrease after stimulation with interleukin (IL)-1β (10 ng/mL) and nifedipine (0.34 μM). The expression of *AR* mRNA increased dramatically when stimulated simultaneously with IL-1β + nifedipine (A). Each box represents values extending from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths, plotted as a ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. *$p < 0.05$, according to the Mann–Whitney *U*-test. Compared with their baseline samples, expression of AR protein in healthy cells and in dihydropyridine-induced gingival overgrowth (DIGO) cells were significantly higher than in the control groups when stimulated with IL-1β + nifedipine (B). The median value was almost threefold higher than that of the healthy group. Each box represents the values extending from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths, plotted as a ratio of β-actin expression. *$p < 0.05$, according to the Mann–Whitney *U*-test.

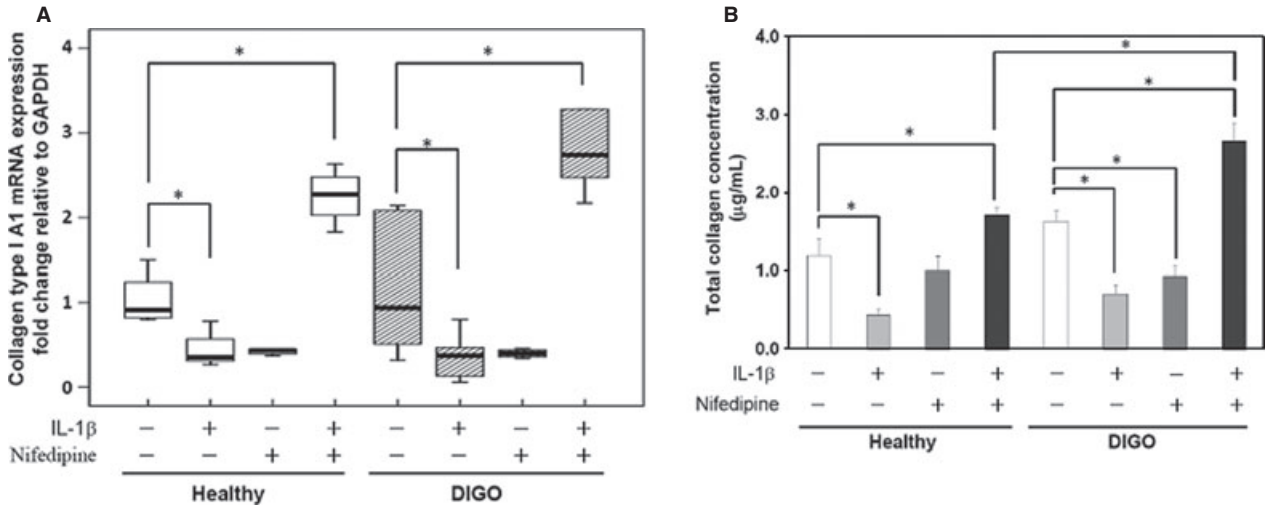

Fig. 2. When cells were stimulated with interleukin (IL)-1β + nifedipine, the expression of type-1 collagen-α [*Col*α1(I)] mRNA was significantly enhanced in both cells (A). Each box represents the values extending from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths, plotted as a ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. *$p < 0.05$, according to the Mann–Whitney *U*-test. The mean concentration of soluble collagen in the supernatant of healthy and dihydropyridine-induced gingival overgrowth (DIGO) cells is shown (B). In contrast to this result, we found that the collagen concentration was significantly higher in both groups when cells were simultaneously stimulated with IL-1β + nifedipine ($p < 0.05$). Compared with the results of *Col*α1(I) mRNA expression, DIGO cells produced more soluble collagen than did healthy cells ($p < 0.05$). *$p < 0.05$, Student's *t*-test.

with their controls (Fig. 2A and 2B; $p < 0.05$). The addition of nifedipine + IL-1β significantly increased the expression of *Col*α1(I) mRNA in both groups (Fig. 2A; $p < 0.05$), but no significant difference was found between the two types of cells. However, under simultaneous stimulation with both drugs, DIGO cells showed greater production of total collagen than did healthy cells (Fig. 2B; $p < 0.05$).

Considering the inflammatory effect of IL-1β on both types of cells, the expression of *iNOS* mRNA and the mean nitrite concentration were significantly enhanced by IL-1β (Fig. 3A and 3B; $p < 0.05$). Nevertheless, the addition of nifedipine drastically reduced the effect of IL-1β to a level even lower than the baseline condition ($p < 0.05$). There were no intergroup differences in the expression of iNOS mRNA expression or in the mean nitrite concentration between healthy cells and DIGO cells under any conditions.

Spearman's correlation coefficients of all mRNAs, proteins and total collagen concentrations are shown in Table 2. We found that correlations of the *AR* mRNA/AR protein/*Col*α1(I) mRNA/total collagen concentration axis were all positive ($r = 0.710$–0.917, $p < 0.001$). However, correlations between the variables of *iNOS* mRNA/nitrite concentration and *AR* mRNA/AR protein/*Col*α1(I) mRNA/total collagen concentration were negative ($r = -0.659$ to -0.826, $p < 0.001$). The correlation between *Col*α1(I) mRNA and the total collagen concentration was $r = 0.777$ ($p < 0.001$).

Figure 4A shows that the expression of *AR* mRNA by DIGO cells was stronger than that of healthy cells ($p < 0.05$) under stimulation with both IL-1β and nifedipine. However, the addition of 1400W to the cell cultures overwhelmingly increased the expression of *AR* mRNA by DIGO cells. The addition of IL-1β, nifedipine and 1400W enhanced the expression of *AR* mRNA in DIGO cells by threefold more than found in healthy cells ($p < 0.05$). Figure 4B shows a significant difference in the production of the AR protein between healthy and DIGO cells when they were simultaneously stimulated with IL-1β, nifedipine and 1400W.

Following addition of the NO donor, the significant differences in expression of *AR* mRNA between healthy cells and DIGO cells in both groups of the baseline control and with concurrent stimulation with nifedipine and IL-1β vanished (Fig. 5A). NONOate inhibited the expression of *AR* mRNA in both types of cells. The expression of the AR protein by DIGO cells was significantly reduced to even less than the baseline level (Fig. 5B).

Discussion

In the present study, the expression of *AR* mRNA, AR protein, *Col*α1(I) mRNA and total collagen was more prominent in DIGO cells than in healthy cells; however, the expression of *iNOS* mRNA and nitrite concentration of both types of cells was similar. This study highlights differences between DIGO cells and healthy cells, which, from an anabolic

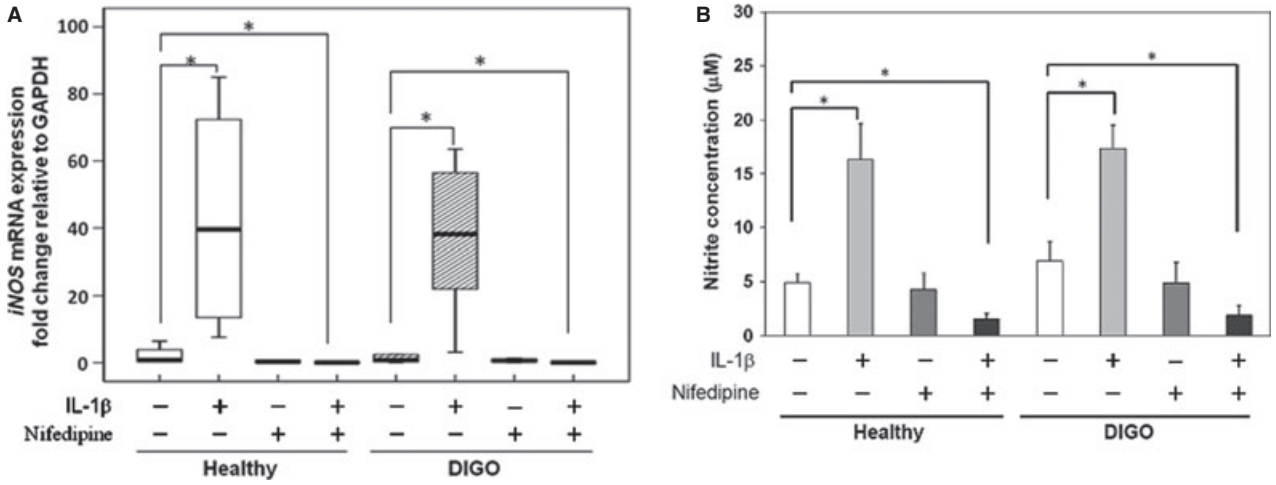

Fig. 3. Compared with the control sample in each group, inducible nitric oxide synthase (*iNOS*) mRNA expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) significantly increased after stimulation with interleukin (IL)-1β (10 ng/mL), but decreased (but not to a significant level) when nifedipine (0.34 μM) was added (A). When both IL-1β and nifedipine were added simultaneously, the expression of *iNOS* mRNA significantly decreased in both groups ($p < 0.05$). Each box represents values that extend from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths, plotted as a ratio of GAPDH expression. *$p < 0.05$, according to the Mann–Whitney *U*-test. The mean nitrite concentration in the culture supernatant of healthy and dihydropyridine-induced gingival overgrowth (DIGO) cells under four different types of stimulation are depicted in (B). In reference to control samples, the mean nitrite concentration significantly increased in both healthy and DIGO cells after stimulation with IL-1β (10 ng/mL) alone. However, the nitrite concentration significantly decreased in both groups when simultaneously stimulated with IL-1β and nifedipine. The nitrite concentration in both groups showed no significant difference under any circumstances. *$p < 0.05$, Student's *t*-test.

Table 2. Spearman correlation coefficient of all variables

Spearman correlation coefficient	*iNOS* mRNA expression	*AR* mRNA expression	Collagen α1(I) mRNA expression	Nitrite concentration	AR protein	Total collagen
iNOS mRNA	+1.000	−0.817**	−0.666**	+0.925**	−0.826**	−0.743**
AR mRNA	−0.818	+1.000	+0.710**	−0.841**	+0.831**	+0.772**
Collagen α1(I) mRNA	−0.666**	+0.710**	+1.000	−0.659**	+0.787**	+0.777**
Nitrite concentration	+0.925**	−0.841**	−0.659**	+1.000	−0.815**	−0.709**
AR protein	−0.826**	+0.831**	+0.787**	−0.815**	+1.000	+0.917**
Total collagen concentration	−0.743**	+0.772**	+0.777**	−0.709**	+0.917**	+1.000

AR, androgen receptor; iNOS, inducible nitric oxide sythase.
**p value < 0.001 (two-tailed).
+, Positive correlation; −, Negative correlation.

point of view, were that DIGO cells presented greater AR expression and collagen production in the pathogenesis of fibrotic tissue production. This coincides with the results of our previous study that both nifedipine and IL-1β play important roles in DIGO via AR up-regulation (2). Gingival overgrowth induced by calcium-channel blockers is mainly caused by AR activation and collagen accumulation (3). From the activity of the iNOS/NO axis, DIGO cells possessed a similar response to that of healthy cells in the catabolic pathway.

IL-1β and nifedipine attenuate iNOS/NO synthesis in gingival fibroblasts

There are three isoforms of NOS found in mammals: neuronal (n)NOS, endothelial (e)NOS and iNOS. The constitutive isoforms were originally located in endothelial cells (eNOS) and neurons (nNOS), the activities of which are dependent on elevated intracellular calcium levels modulated by calmodulin. The inducible isoform (iNOS) is generally treated as a calcium-independent, cytosolic enzyme produced largely by cytokine-activated macrophages and many other cells (24). Although iNOS is commonly referred to as being Ca^{2+} independent, the iNOS enzyme co-expressed with Ca^{2+}-binding calmodulin demonstrated that there is some Ca^{2+} dependence in iNOS/NO synthesis (25–27). The addition of EDTA to chelate Ca^{2+} resulted in a significant decrease in the stimulation of iNOS production, by 70%. The dynamic control of intracellular Ca^{2+} can partly regulate the intracellular activity of iNOS through Ca^{2+} -binding proteins.

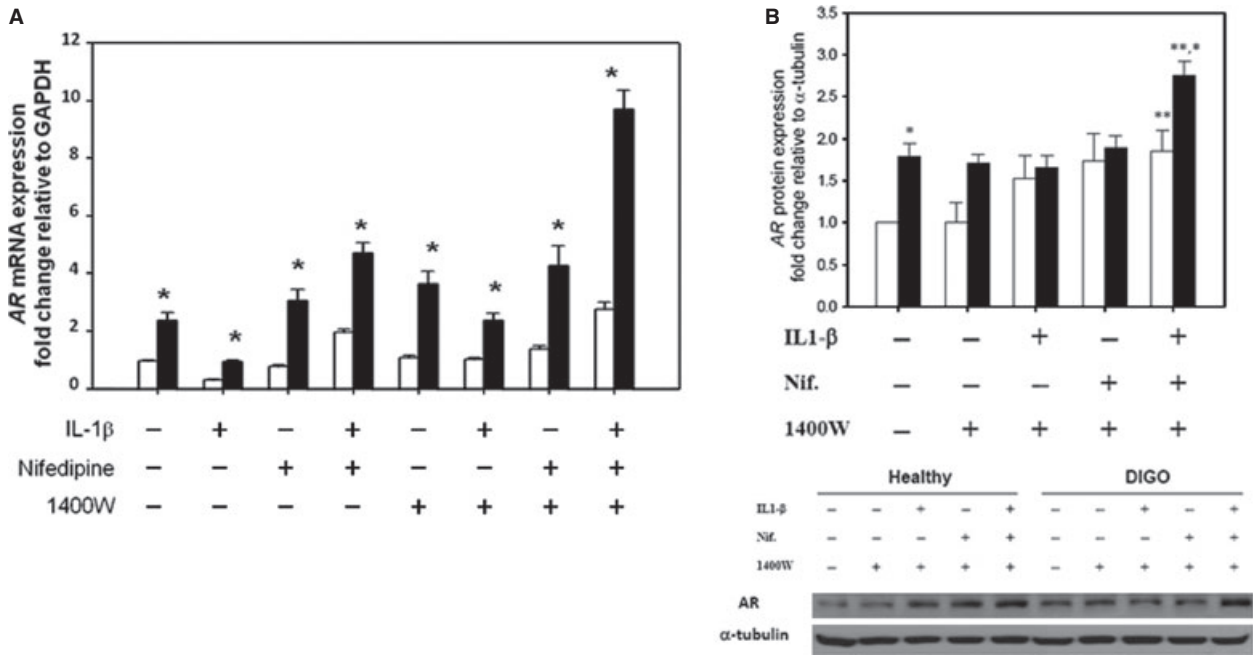

Fig. 4. Stimulation with interleukin (IL)-1β + nifedipine caused a twofold or threefold increase in the expression of androgen receptor (*AR*) mRNA by dihydropyridine-induced gingival overgrowth (DIGO) cells (solid bar) compared with that of healthy cells (empty bar) ($p < 0.05$) (A). However, the addition of an inducible nitric oxide synthase (iNOS) inhibitor (1400W) to cell cultures overwhelmingly increased the expression of *AR* mRNA in DIGO cells. The synergistic addition of IL-1β, nifedipine and 1400W greatly enhanced the expression of AR mRNA in DIGO cells. The application of 1400W significantly increased the production of AR protein in both types of cells. Both groups were normalized to the control of healthy cells as the baseline (B). *Intergroup comparison, $p < 0.05$; intragroup comparison, $p < 0.05$.

A study of the role of the intracellular Ca^{2+} ion concentration in the expression profile of NOS isoforms was established with a novel calcium-channel blocker (H-DHMP). The results showed that H-DHPM decreased the increased iNOS in chronic obstructive pulmonary disease (28). In another animal model, a calcium-channel blocker (CV159) was used to evaluate the NO level in the liver with partial hepatic ischemia/reperfusion injury. The results of this study suggested that CV159 exerts protective effects against the toxicity of iNOS-derived NO (29). Obviously, the results shown in Fig. 3A and 3B in our study also imply that the induction of iNOS/NO synthesis by IL-1β was significantly suppressed by the co-administration of IL-1β and nifedipine, or of nifedipine alone, in both cell lines (i.e. derived from healthy and DIGO individuals). In the present multi-patient study, the addition of nifedipine to both cell types dramatically reversed the stimulatory effect of IL-1β on iNOS expression and the nitrite concentration. Although IL-1β is a potent inducer of iNOS expression in both types of gingival cells, the addition of nifedipine abolished the influence for an unknown reason. We inferred that the synergistic reduction of intracellular Ca^{2+} by nifedipine and IL-1β may inversely decrease the expression of iNOS mRNA and NO release and augment AR activity in those cells through regulating intracellular Ca^{2+} homeostasis (3). The simultaneous intake of IL-1β and nifedipine to decrease intracellular calcium ions may attenuate the induction of iNOS/NO by gingival fibroblasts.

iNOS/NO inhibits *AR* mRNA/protein expression in DIGO cells

The intracellular relationship between iNOS and AR in gingival fibroblasts has rarely been discussed. The results of Spearman's correlations (Table 2) showed a negative correlation between iNOS/NO and the AR/Colα1(I)/collagen cascade. Nevertheless, their up-regulatory and down-regulatory relationship was further verified by the inhibitory experiment with iNOS and the use of an NO donor, conducted on two single-cell strains (H3 and D6). The results indicated that application of an iNOS inhibitor (1400W) significantly increased the expression of *AR* mRNA in DIGO cells but had no effect on cells derived from a healthy individual (Fig. 4). When the NO donor was added, the significant difference in expression of *AR* mRNA and AR protein between healthy and DIGO cells was ameliorated (Fig. 5). The production of AR protein in DIGO cells was reduced to a level even lower than that of healthy cells. Both experiments show that the iNOS/NO system plays a role of down-regulating the expression of *AR* mRNA and AR protein in DIGO cells.

The present Spearman correlations also showed that the expressions of *AR*/*Col*α1(I) mRNA, AR protein and

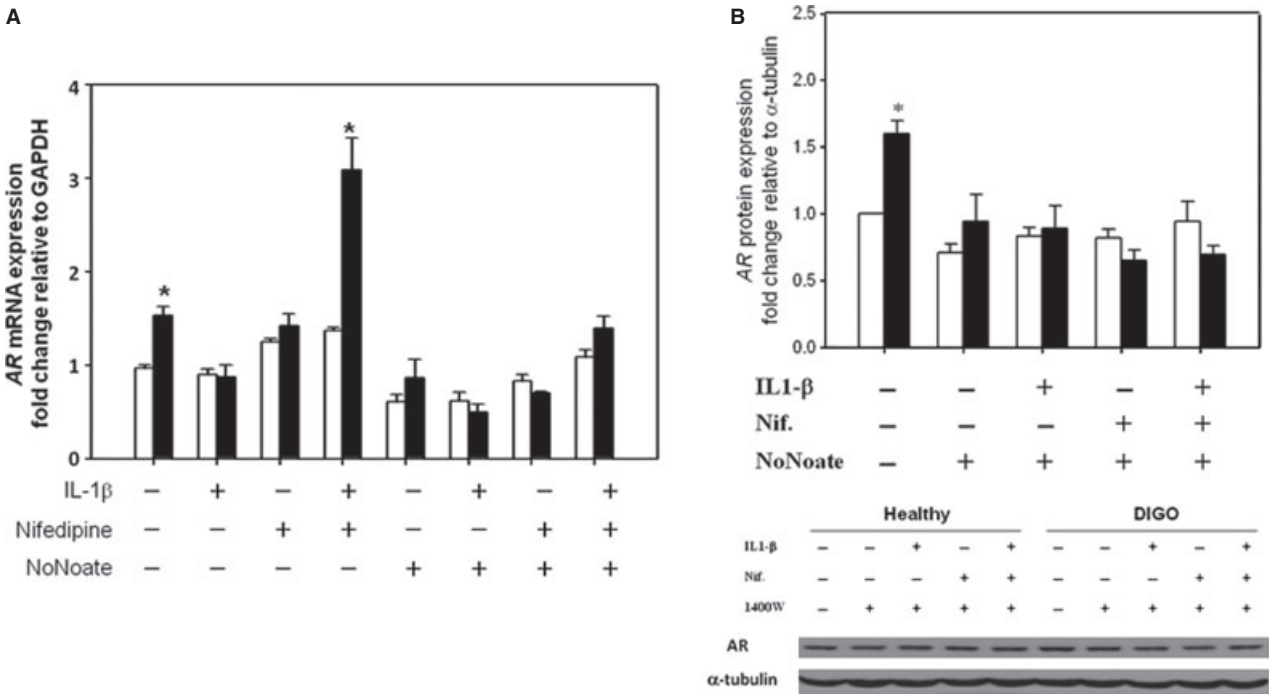

Fig. 5. When a nitric oxide (NO) donor was added, the significant difference of androgen receptor (*AR*) mRNA between healthy cells (empty bar) and dihydropyridine-induced gingival overgrowth (DIGO) cells (solid bar) disappeared (A). The addition of NoNOate inhibited the expression of *AR* mRNA in both types of cells. However, the expression of AR protein by DIGO cells was significantly reduced to a level even less than that of healthy cells (B). Both groups were normalized to the control of healthy cells as the baseline. *Intergroup comparison, $p < 0.05$.

total collagen were positively correlated with each other. The anabolic axis of AR/Colα1(I)/collagen was further confirmed by the pathogenic overgrowth of DIGO tissues in this study. However, the results of a negative correlation between *iNOS* mRNA/nitrite concentrations and *AR* mRNA/AR protein/*Col*α1(I) mRNA/total collagen concentrations also demonstrated reciprocation of the catabolic iNOS/NO and anabolic AR/collagen arms of the pathogenesis of DIGO cells.

iNOS/NO are mandatory in regulating early wound healing (30). In the hypertrophic scarring of wound repair, the ability of NO to significantly inhibit collagen production was observed in dermal fibroblasts (31). NO is a pleiotropic, short-lived secondary messenger that participates in biphasic biological processes such as the regulation of blood vessels, inflammation, bone homeostasis and apoptosis (32–34). NO is able to regulate mitochondrial and cytoplasmic Ca^{2+} signals in T cells under normal conditions (11). NO activates positively charged metal ions of guanylate cyclase, leading to the synthesis of cyclic guanosine monophosphate from guanosine triphosphate. This, in turn, depletes intracellular Ca^{2+} levels by reducing Ca^{2+} release from intracellular reservoirs (35). These actions thereby stimulate increased vascular permeability and exert antiproliferative effects through cellular targeting of downstream effectors (36). Again, in the present study we conjectured that the down-regulation of *AR*/*Col*α1(I) mRNA and collagen synthesis by the iNOS/NO system might also be generated through the event of intracellular Ca^{2+} homeostasis in DIGO gingival cells.

Association of gingival overgrowth and iNOS/NO reactions

Periodontopathic bacteria induce inflammation of periodontal tissues. The cytokines and NO released in periodontal lesions are involved in bacterial infection and are related to the process of inflammation. In a quantitative study of iNOS-positive cells, samples of clinically healthy gingival tissues, plaque-induced gingivitis and localized chronic periodontitis were analyzed using immunohistochemistry. The results indicated that a significant increase in the number of iNOS-positive cells was found in samples with gingivitis and periodontitis. Polymorphonuclear cells expressed significant levels of iNOS and probably represent an important source of NO in human periodontal disease (4). However, in an *in vitro* study, it was found that human gingival fibroblasts can also express increased levels of iNOS and modulate NO synthesis in response to proinflammatory cytokines (7). Our study confirmed that both healthy gingival and DIGO fibroblasts are able to express iNOS and secrete NO to the same degree (Fig. 3). However, the addition of an iNOS inhibitor may significantly stimulate the expression of *AR* mRNA and AR protein in DIGO cells and enhance the differences from those of healthy cells (Fig. 4).

The results are similar to those reported by Fujimori *et al* in which the induction of iNOS in a model of human gingival fibroblasts incubated in medium of co-cultured RAW cells was completely abrogated by incubation with nifedipine (37). On the other hand, the addition of an NO donor may abrogate differences in the expression of *AR* mRNA and AR protein between healthy cells and DIGO cells. This suggests that controlling the iNOS/NO system may be of benefit in controlling DIGO in response to dihydropyridine administration.

Key role of AR in gingival overgrowth

In the last decade, we established a model of IL-1β regulating AR/Colα1(I)/collagen in gingival cells of DIGO patients (1–3). In the present experiment, DIGO fibroblasts were cultured in charcoal-stripped serum for androgen deprivation. The expression of *AR* mRNA and AR protein still dramatically increased when DIGO cells were simultaneously stimulated with IL-1β + nifedipine. This implies that in the absence of androgen, the AR can be activated by an unknown ligand or underlying ligand-independent activation of the AR by phosphorylation of either the AR itself or receptor-associated proteins, such as co-activators (38,39). The AR may act through androgen-independent activation and plays a role as a member of the nuclear receptor superfamily in the etiopathogenesis of DIGO (40). The epigenetic regulation and nuclear factor-κB binding of the AR promoter are currently being investigated in our laboratory.

In this study, the single addition of nifedipine or IL-1β decreased or had no effect on the levels of collagen mRNA and collagen protein (Fig. 2). This result differs from data of our previous study (3), in which the single addition of IL-1β or nifedipine increased procollagen mRNA and soluble collagen protein. The variation may have been a result of the use of different concentrations of nifedipine in these two studies. The present study used a concentration of 0.34 μM nifedipine instead of 10 μM, as in the previous study. Different preparations of culture medium, such as the use of charcoal-stripped serum in the present study, may also have caused differences in the expression of *Col*α1(I) mRNA and total collagen levels compared with those of the previous study. In addition, we speculated that the intracellular scenario of AR and Colα1(I) expression by DIGO cells might not directly reflect the categorization of a gingival overgrowth index in patients with DIGO. Therefore, the choice of different clinical subjects in different studies may have influenced certain aspects of the results.

Conclusions

The activation of iNOS/NO by IL-1β suppressed the expression of *AR*/*Col*α1(I) mRNA and AR/total collagen proteins in DIGO cells. The simultaneous stimulation of IL-1β and nifedipine and inhibition of iNOS inversely increased the overexpression of *AR*/*Col*α1(I) mRNA and AR/total collagen protein production by DIGO cells. Overall, the iNOS/NO cascade down-regulates the axis of *AR*/*Col*α1(I) mRNA and AR/total collagen proteins in collagen homeostasis of DIGO cells.

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