Eur Respir J 2009; 34: 1452–1460
DOI: 10.1183/09031936.00195808
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3-Nitrotyrosine inhibits fibroblast-mediated collagen gel contraction and chemotaxis

H. Sugiura*, X. Liu#, T. Ichikawa*, M. Ichinose* and S.I. Rennard#

ABSTRACT: Reactive nitrogen species induce tissue inflammation and nitrate tyrosine residues of various kinds of proteins. Recent studies have established that the free amino acid form of 3-nitrotyrosine induces cytotoxity and growth inhibition and alters the cellular function in cultured cells. The aim of this study was to evaluate whether 3-nitrotyrosine could affect tissue remodelling in fibroblasts. To accomplish this, human fetal lung fibroblasts (HFL-1) were used to assess the fibroblast-mediated contraction of floating gels and chemotaxis towards fibronectin. In addition, the ability of fibroblasts to release fibronectin, transforming growth factor (TGF)-β1, fibronectin and vascular endothelial growth factor (VEGF) was assessed. 3-Nitrotyrosine significantly inhibited gel contraction ($p<0.01$) compared with control and this inhibition was abolished by nitric oxide synthase (NOS) inhibitor. 3-Nitrotyrosine did not affect TGF-β1 and VEGF but significantly decreased fibronectin release ($p<0.01$) into the media. 3-Nitrotyrosine significantly inhibited chemotaxis towards fibronectin through suppression of $\alpha_5\beta_1$ integrin expression ($p<0.01$). NOS inhibitor also reversed 3-nitrotyrosine-inhibited chemotaxis ($p<0.01$). Finally, 3-nitrotyrosine enhanced the expression of the inducible type of NOS ($p<0.01$) and nitric oxide release ($p<0.01$) through nuclear factor-κB activation. These results suggest that the free amino acid form of 3-nitrotyrosine can affect the tissue repair process by modulating nitric oxide production.

KEYWORDS: Inducible nitric oxide synthase, nitric oxide, reactive nitrogen species, remodelling

AFFILIATIONS
*Third Dept of Internal Medicine, Wakayama Medical University School of Medicine, Wakayama, Japan.
#Pulmonary Medicine, Nebraska Medical Center, Omaha, NE, USA.

CORRESPONDENCE
H. Sugiura
Third Dept of Internal Medicine
Wakayama Medical University School of Medicine
811-1 Kimiidera
Wakayama
Wakayama 641-0012
Japan
E-mail: sugiura@wakayama-med.ac.jp

Received:
Dec 25 2008
Accepted after revision:
April 18 2009
First published online:
May 14 2009

Excessively produced nitric oxide (NO) derived from the inducible type of NO synthase (iNOS) leads to the formation of reactive nitrogen species (RNS), including peroxynitrite and nitrogen dioxide, during inflammatory and immune processes in lung diseases [1]. These RNS are formed from NO and superoxide anions [2] or *via* the H_2O_2/peroxidase-dependent nitrite oxidation pathway [3]. Excessive RNS cause tissue injury, lipid peroxidation and nitration of tyrosine residues [1, 4]. A metabolite generally reflecting the *in vivo* production of RNS is the amino acid derivative 3-nitrotyrosine. The production of 3-nitrotyrosine has been observed in various inflammatory lung diseases, including chronic obstructive pulmonary disease [5, 6], bronchial asthma [5, 7], cystic fibrosis [8] and idiopathic pulmonary fibrosis [9].

3-Nitrotyrosine was thought to be a stable marker of RNS production [10]. However, recent studies have established that, in addition to serving as a ''footprint'' of RNS, the free amino acid form of 3-nitrotyrosine itself induces cytotoxity, growth inhibition and morphological changes, and consequently alters the cellular function in cultured cells [11–13]. These studies suggest that 3-nitrotyrosine may play a critical role in the tissue repair process. Indeed, its abundant production (1–120 μM) has been shown under several pathological conditions, including rheumatoid arthritis [14], liver transplantation [15], septic shock [16] and amyotrophic lateral sclerosis [17]. However, whether 3-nitrotyrosine can affect the tissue repair response remains unknown.

Inflammatory processes are frequently accompanied by alterations in the tissue structure. Such alterations may result from tissue damage due to active proteases or toxic moieties released by inflammatory cells. In addition, mediators released at inflammatory sites are capable of directly altering the cell function, leading to tissue repair and remodelling. In human lung fibroblasts, recent studies showed that cytokine stimulation is associated with iNOS gene expression [18]. Gaseous NO can also stimulate iNOS expression [19]. Furthermore, we have shown

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

This article has supplementary material accessible from www.erj.ersjournals.com

that cytokine-induced NO inhibited collagen gel contraction, which is a novel tissue repair model [20]. These studies suggest that iNOS expression can be stimulated in human lung fibroblasts and NO derived from iNOS may affect the tissue repair process. On the basis of these observations, we hypothesised that the free amino acid form of 3-nitrotyrosine can modulate iNOS expression in human lung fibroblasts and affect the tissue repair process.

The present study, therefore, was designed first to determine whether 3-nitrotyrosine could affect tissue remodelling through an effect on the human fetal lung fibroblast (HFL-1)-mediated contraction of collagen gels and chemotaxis towards chemoattractant. Next, we assessed whether 3-nitrotyrosine can modulate fibroblast release of fibronectin, transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF), which are thought to be critical mediators of tissue remodelling. Finally, we determined whether the effects of 3-nitrotyrosine were mediated through excessive production of NO derived from iNOS.

MATERIALS AND METHODS

Materials

Native type I collagen (rat tail tendon collagen (RTTC)) was extracted from rat tail tendons by a previously published method [21]. Briefly, tendons were excised from rat tails, and the tendon sheath and other connective tissues were removed carefully. Repeated washing with Tris-buffered saline (0.9% NaCl and 10 mM Tris, pH 7.5) was followed by dehydration and sterilisation with 50%, 75%, 95% and pure ethanol. Type I collagen was then extracted in 6 mM hydrochloric acid at 4°C. The collagen concentration was determined by weighing a lyophilised aliquot from each lot of collagen solution. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) consistently demonstrated no detectable proteins other than type I collagen.

Commercially available reagents were obtained as follows: 3-nitrotyrosine, indomethacin, N^G-mono-methyl-L-arginine acetate salt (L-NMMA), sodium nitroprusside (SNP), L-N^6-(1-iminoethyl) lysine (L-NIL), 3,3′,5,5′-tetramethylebenzidine, lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St Louis, MO, USA); caffeic acid phenethyl ester (CAPE) and nuclear factor (NF)-κB inhibitors were from Calbiochem (La Jolla, CA, USA); Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were from Invitrogen Life Technologies (Grand Island, NY, USA).

Cell culture

HFL-1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured on tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ, USA) with DMEM supplemented with 10% FCS, 100 μg·mL^{-1} penicillin, 250 μg·mL^{-1} streptomycin and 2.5 μg·mL^{-1} fungizone. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 4–5 days at a 1:4 ratio. HFL-1 cells were used between the 14th and 18th passages. At 30% confluence, cells were treated with varying concentrations of 3-nitrotyrosine in DMEM containing 10% FCS for 48 h. Cells were then harvested for later assay.

Collagen gel contraction assay

Collagen gels were prepared as described previously [22, 23]. Briefly, RTTC, distilled water and 4 × concentrated DMEM were combined so that the final mixture resulted in 0.75 mg·mL^{-1} collagen, with a physiological ionic strength of 1 × DMEM and a pH of 7.4. Cells were plated in 100-mm dishes in DMEM containing 10% FCS and treated with or without various concentrations of 3-nitrotyrosine for 48 h. To investigate the effect of the NOS inhibitors on fibroblast-mediated gel contraction, L-NMMA or L-NIL was added to the culture media in monolayers with or without varying concentrations of 3-nitrotyrosine. The cells were trypsinised (trypsin-EDTA: 0.05% trypsin, 0.53 mM EDTA-4Na; GIBCO, Invitrogen) and suspended in serum-free DMEM. The cells were then mixed with the neutralised collagen solution so that the final cell density in the collagen solution was 3×10^5 cells·mL^{-1} and the final concentration of collagen was 0.75 mg·mL^{-1}. Aliquots (0.5 mL·well^{-1}) of the mixture of cells in collagen were cast into each well of 24-well tissue culture plates (Falcon) and allowed to gel. After gelation was completed, normally within 20 min at room temperature, the gels were gently released from the 24-well tissue culture plates and transferred into 60-mm tissue culture dishes (three gels in each dish) that contained 5 mL of freshly prepared serum-free DMEM with or without various concentrations of 3-nitrotyrosine. The gels were then incubated at 37°C in a 5% CO_2 atmosphere for 3 days. To investigate the effect of L-NMMA or L-NIL on fibroblast-mediated gel contraction, L-NMMA or L-NIL was added to the culture media after the gels were released. Gel contraction was quantified daily using an Optomax V image analyser (Optomax, Burlington, MA, USA). Data were expressed as percentages of the original gel size.

Measurement of fibronectin, TGF-β1 and VEGF by ELISA

Fibronectin, TGF-β1 and VEGF in the media of the monolayer culture were determined by ELISA according to a previous report [23].

Chemotaxis assay

Cell migration was assessed using the Boyden blindwell chamber (Neuroprobe Inc., Gaithersburg, MD, USA) as previously described [23, 24]. Briefly, 26 μL of serum-free DMEM containing human fibronectin (20 μg·mL^{-1}) was placed into the bottom wells. Polycarbonate membranes with 8-μm pores (Neuroprobe Inc.), which were pre-coated with 5 μg·mL^{-1} gelatin in 0.1% acetic acid, were used. The cells were pretreated with or without various concentrations of 3-nitrotyrosine for 48 h. To investigate the role of NOS, L-NMMA was added to the culture media with or without 3-nitrotyrosine. The cells grown to 75% confluence were rinsed, re-fed with serum-free DMEM and treated with various concentrations of 3-nitrotyrosine at 37°C in a humidified atmosphere of 5% CO_2. The cells were trypsinised and suspended in serum-free DMEM at a density of 1×10^6 cells·mL^{-1}. 50 mL of cell suspension treated with various concentrations of 3-nitrotyrosine in the presence or absence of L-NMMA were then added into each top well. The cells were allowed to migrate at 37°C in a 5% CO_2 atmosphere for 6 h. Cells that had not migrated were scraped off the upper surface of the membrane and the membranes were air dried. The cells were then stained with PROTOCOL (Fisher Scientific, Swedesboro, NJ, USA) and mounted on a glass microscope slide. Chemotaxis was assessed by counting the numbers of cells in five high-power fields.

Wells with serum-free DMEM were used as negative controls and those with chemoattractant alone were used as positive controls.

Western blotting

To investigate the effects of NF-κB activation on iNOS expression, cells were treated with or without various concentrations of CAPE 30 min prior to treatment with 3-nitrotyrosine. After treatment with various concentrations of 3-nitrotyrosine for 48 h in a monolayer culture, cells were washed with 4°C PBS and homogenised in cell lysis buffer (35 mM Tris-HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 1 μM phenylmethylsulfonyl fluoride, 100 $\mu g \cdot mL^{-1}$ aprotinin and 1 $\mu g \cdot mL^{-1}$ leupeptin). Samples were solubilised in SDS-PAGE sample buffer. To investigate NF-κB translocation into the nucleus, cells were seeded in 60-mm dishes at a density of 1×10^5 cells$\cdot mL^{-1}$. At 90% confluence, the cells were treated with 10^{-4} M 3-nitrotyrosine. The cells were harvested at various time points. To obtain the nuclear and cytosolic fractions, a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) was used according to the manufacturer's instructions. Equal amounts of protein were loaded and separated by electrophoresis on 12.5% SDS-PAGE gels. After electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). Primary polyclonal antibodies against human iNOS (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or $\alpha_5\beta_1$ integrin (1:1,000 dilution; Chemicon International, Inc., Temecula, CA, USA), or mouse monoclonal anti-NF-κB p65 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.) were used for detection. Bound antibodies were visualised using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) with a Typhoon Scanner (Amersham Biosciences). The intensity of the detected band was quantified by NIH image.

Quantitative PCR

Fibroblasts were treated with or without 3-nitrotyrosine and were harvested at 12 h. Total RNA was eluted using an RNeasy mini kit (Qiagen Sciences, Valencia, CA, USA). cDNA was generated using reverse transcriptase. The primers for iNOS were designed as follows: forward CCCCACGCTGCATTGG; reverse CACGTGTCTGCAGATGTGTTCA. Gene expression was measured using assays on the above probe and primers and reactions were analysed by using the ABI 7000 Taqman® system (Applied Biosystems, Foster City, CA, USA).

Measurement of nitrite/nitrate

To evaluate nitrite/nitrate production in the monolayer culture, cells were seeded in 6-well tissue plates at a cell density of 1×10^5 cells$\cdot mL^{-1}$. The cells were treated with or without varying concentrations of 3-nitrotyrosine for 48 h and the supernatant was harvested. The concentration of nitrite/nitrate was determined by a Nitric oxide assay kit (Assay Designs, Inc., Ann Arbor, MI, USA).

MTT assay

For monitoring cell viability, the 3-nitrotyrosine-treated cells were incubated with MTT solution at a final concentration of 1 $mg \cdot mL^{-1}$ for 4 h at 37°C. After incubation, dimethyl sulfoxide was added into each well. The absorbance of each sample at 570 nm was determined by a spectrophotometer using a reference wavelength of 630 nm.

Statistical analysis

Data were expressed as mean±SEM. Multiple comparisons of experimental values among the groups were evaluated by one-way ANOVA followed by Bonferroni's *post hoc* test to test for multiple comparisons and the Mann–Whitney U-test for single comparisons. Probability values of <0.05 were considered significant.

RESULTS

At first, we examined the effect of 3-nitrotyrosine on the cell viability. 3-Nitrotyrosine did not affect the cell viability in either the collagen gel culture condition or the chemotaxis condition (see supplementary fig. 1).

To investigate the 3-nitrotyrosine modulation of collagen gels, cells cast in collagen gels were floated in media with various concentrations of 3-nitrotyrosine. 3-Nitrotyrosine significantly inhibited the gel contraction compared with control in a concentration-dependent manner (at 10^{-4} M, gel size was 81.0±0.4% *versus* 65.9±0.1% of initial size on day 3; $p<0.01$; fig. 1). The NOS inhibitor L-NMMA (10^{-4} M) completely abolished the inhibitory effect of 3-nitrotyrosine on gel contraction compared with the 3-nitrotyrosine-treated group (on day 3, gel size was 71.5±0.7% *versus* 88.6±1.2% of initial size; $p<0.01$; fig. 2a), while the cyclooxygenase inhibitor indomethacin (2×10^{-6} M) had no effect on the 3-nitrotyrosine-mediated inhibition of gel contraction (on day 3, gel size was 86.4±1.0% *versus* 88.6±1.2% of initial size; fig. 2b). To investigate whether iNOS was related to the L-NMMA-mediated effect, the effects of a specific iNOS inhibitor L-NIL on the 3-nitrotyrosine-mediated inhibition of gel contraction was assessed. As shown in figure 3, L-NIL significantly reversed the 3-nitrotyrosine-mediated inhibition of gel contraction in a concentration-dependent manner (at 10^{-5}–10^{-4} M; $p<0.01$). To investigate the role of iNOS in the tissue repair, we treated the cells with LPS to stimulate the iNOS expression. LPS significantly augmented iNOS expression ($p<0.01$; supplementary fig. 2a). LPS significantly attenuated fibronectin release in the media compared with control (4,217±464 *versus* 8,131±585 ng$\cdot$culture^{-1}; $p<0.01$; supplementary fig. 2b). L-NIL significantly restored the LPS-attenuated fibronectin release (6,750±391 *versus* 4,217±464 ng$\cdot$culture^{-1}; $p<0.01$; supplementary fig. 2b).

It is known that fibronectin and TGF-β1 are involved in tissue repair and are able to enhance collagen gel contraction by human lung fibroblasts. VEGF is believed to contribute to vascular cell proliferation and may be a mediator of tissue repair and remodelling. To determine whether 3-nitrotyrosine could contribute to the release of these mediators, the release of these three mediators in the monolayer culture was evaluated. 3-Nitrotyrosine (10^{-5}–10^{-4} M) significantly decreased fibronectin release in the monolayer culture ($p<0.01$; fig. 4a), while it did not affect the release of TGF-β1 (fig. 4b) and VEGF (fig. 4c) in the monolayer culture.

Because fibroblast migration from neighbouring connective tissue into sites of inflammation plays an important role in tissue repair in response to injury, we assessed the effect of

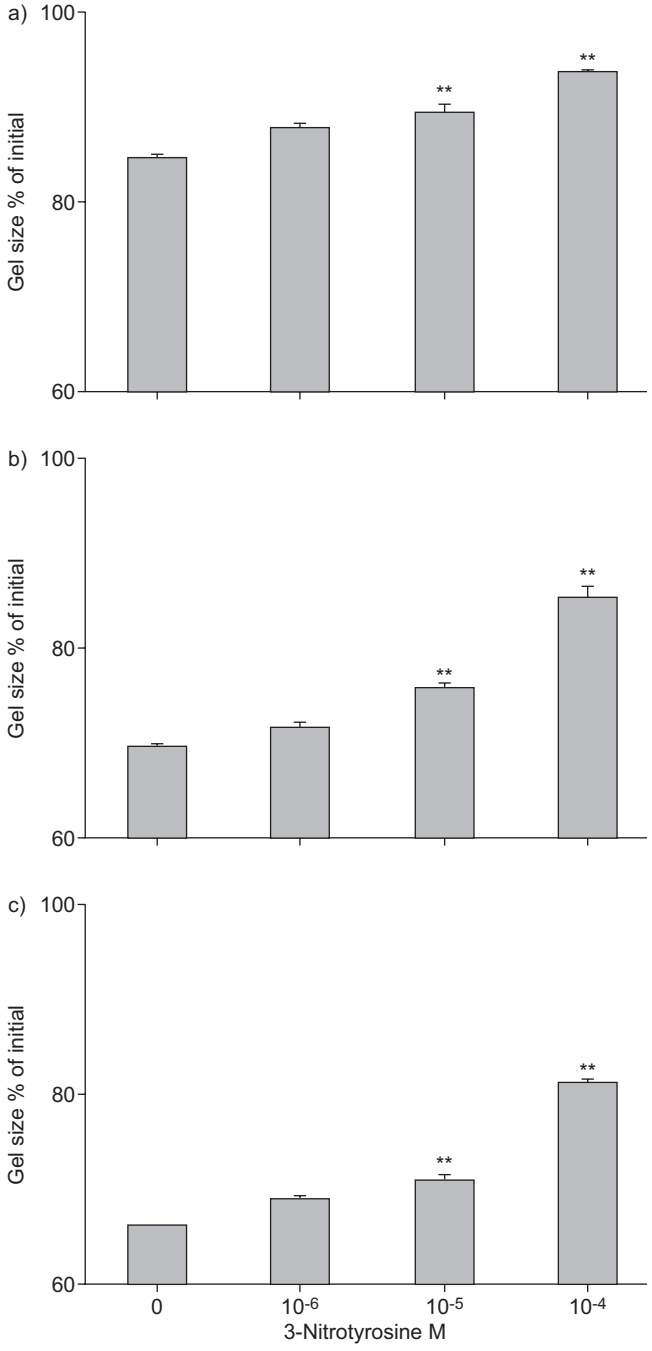

FIGURE 1. Effect of 3-nitrotyrsoine on collagen gel contraction by human fetal lung fibroblasts. Gel size was measured on a) day 1, b) day 2 and c) day 3. All values are mean±SEM for four separate experiments, each performed in triplicate. **: $p<0.01$ compared with control.

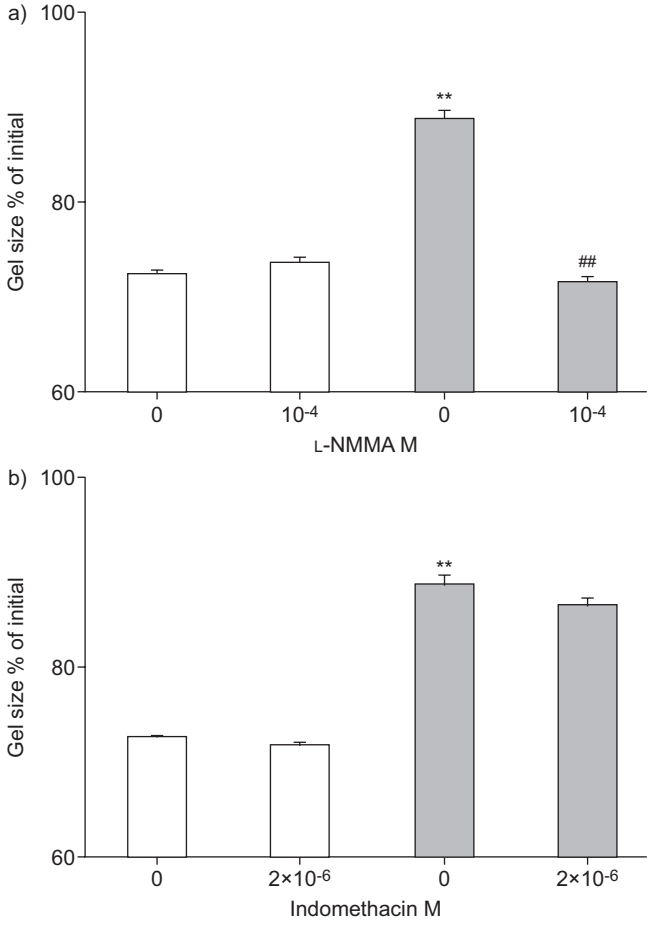

FIGURE 2. Effect of N^G-mono-methyl-L-arginine acetate salt (L-NMMA) and indomethacin on 3-nitrotyrosine-inhibited collagen gel contraction by human fetal lung fibroblasts. Fibroblasts were cast into collagen gels and maintained in floating culture in medium with (■) or without (□) 3-nitrotyrosine at 10^{-4} M in the presence or absence of a) the nitric oxide synthase inhibitor L-NMMA or b) the cyclooxygenase inhibitor indomethacin. All values are mean±SEM for four separate experiments, each performed in triplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with 3-nitrotyrosine-treated group.

3-nitrotyrosine on HFL-1 chemotaxis. Using fibronectin (20 $\mu g \cdot mL^{-1}$) as the chemoattractant, 3-nitrotyrosine significantly inhibited fibroblast chemotaxis compared with control in a concentration-dependent manner (at 10^{-4} M, 248±24 cells in five high power fields (HPF) *versus* 95.0±14 cells in five HPF; $p<0.01$; fig. 5a). The NOS inhibitor L-NMMA (10^{-4} M) completely abolished the 3-nitrotyrosine-mediated inhibition of chemotaxis towards fibronectin ($p<0.01$; fig. 5a). We also investigated the effect of NO on fibroblast-mediated chemotaxis. The NO donor SNP inhibited the chemotaxis in a concentration-dependent manner (at 10^{-3} M, 223±14 cells in five HPF *versus* 108±9.8 cells in five HPF; $p<0.01$; fig. 5b). To explore the possible mechanism of inhibition of chemotaxis by 3-nitrotyrosine, we investigated the effect of nitrotyrosine on the expression of $\alpha_5\beta_1$ integrin, which is a receptor for fibronectin. As shown in figure 5c, 10^{-4} M 3-nitrotyrosine decreased the expression of $\alpha_5\beta_1$ integrin (both $p<0.05$).

To clarify which mechanisms are related to the 3-nitrotyrosine-augmented iNOS expression, we investigated the effect of 3-nitrotyrosine on NF-κB activation, which is thought to be the most important pathway in the regulation of iNOS expression. Fibroblasts were incubated with 3-nitrotyrosine and examined for the translocation of NF-κB p65 into the nucleus. There was no significant change in the translocation of NF-κB into the nucleus from 0 to 120 min without 3-nitrotyrosine treatment (*e.g.* at 0 min *versus* 60 min, 1.00±0.0 *versus* 1.04±0.15 relative intensity of

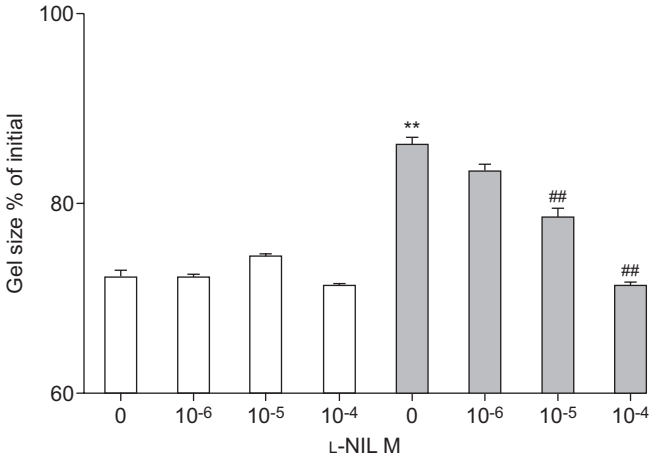

FIGURE 3. Effect of various concentrations of L-N^6-(1-iminoethyl)lysine (L-NIL) on 3-nitrotyrosine-mediated inhibition of gel contraction. Fibroblasts were maintained in floating culture in medium with (■) or without (□) 3-nitrotyrosine at 10^{-4} M in the presence or absence of varying concentrations of L-NIL. All values are mean±SEM for four separate experiments, each performed in triplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed group.

NF-κB/lamin A/C), whereas 10^{-4} M 3-nitrotyrosine significantly enhanced the translocation of NF-κB into the nucleus at 60 min as shown in figure 6a ($p<0.01$). Furthermore, a novel NF-κB inhibitor, CAPE, significantly suppressed the 3-nitrotyrosine-augmented iNOS expression (at 0.3–1.0 μg·mL^{-1} CAPE; $p<0.01$; fig. 6b). These results suggest that 3-nitrotyrosine stimulated NF-κB activation and augmented iNOS expression through NF-κB activation.

To clarify whether 3-nitrotyrosine augments the expression of iNOS, fibroblasts were incubated with 3-nitrotyrosine and examined for the expression of iNOS mRNA and protein. As shown in figure 7a and b, 10^{-4} M 3-nitrotyrosine significantly enhanced the expression of iNOS mRNA and protein ($p<0.01$). Furthermore, NO release in the media of the HFL-1 cell culture was investigated. 3-Nitrotyrosine significantly enhanced the release of NO in the media of the HFL-1 culture in a concentration-dependent manner (at 10^{-4} M; $p<0.01$; fig. 7c).

Because 3-nitrotyrosine is incorporated into α-tubulin in various types of cells and changes the cellular function, we investigated the incorporation of 3-nitrotyrosine into α-tubulin by western blotting. 3-Nitrotyrosine was not incorporated into α-tubulin in HFL-1 cells assessed by western blotting (supplementary fig. 3).

DISCUSSION

The present study demonstrated that the free amino acid form of 3-nitrotyrosine inhibits the fibroblast-mediated contraction of three-dimensional collagen gels and fibroblast chemotaxis towards fibronectin. 3-Nitrotyrosine also significantly decreased fibronectin release into the supernatant of the monolayer cultures. The NOS inhibitors L-NMMA and L-NIL recovered the 3-nitrotyrosine-inhibited gel contraction, mediator production and chemotaxis towards fibronectin. Furthermore, 3-nitrotyrosine enhanced the expression of iNOS protein through NF-κB activation and NO production. These results suggest that

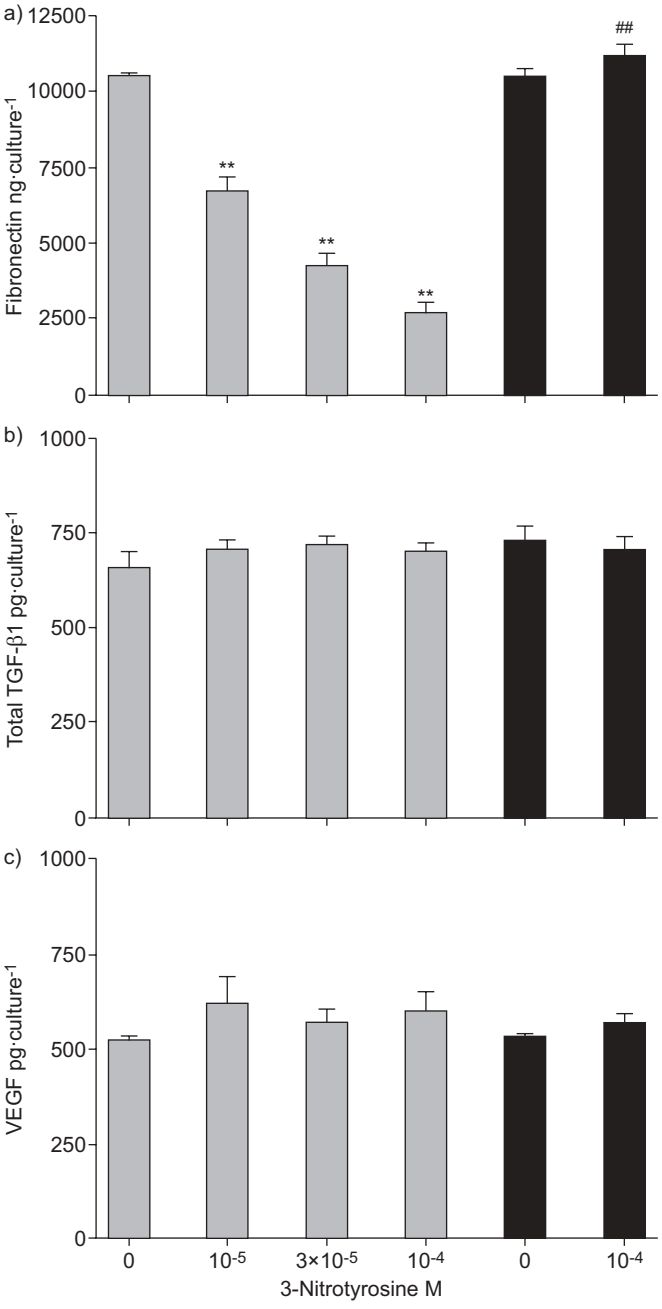

FIGURE 4. Effect of 3-nitrotyrosine on a) fibronectin, b) transforming growth factor (TGF)-β1 and c) vascular endothelial growth factor (VEGF) release. The effect of N^G-mono-methyl-L-arginine acetate salt at 10^{-4} M was also tested (■). Media were assayed by ELISA. All values are mean±SEM for four separate experiments, each performed in duplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed (10^{-4} M) group.

3-nitrotyrosine can affect fibroblast-mediated repair processes and that the mechanism of this effect depends on the generation of NO.

3-Nitrotyrosine is a marker of nitration of the free amino acid form of tyrosine or tyrosine residues of proteins [4]. Recently, it has been reported that the free amino acid form of 3-nitrotyrosine is not only a marker of RNS, but also induces

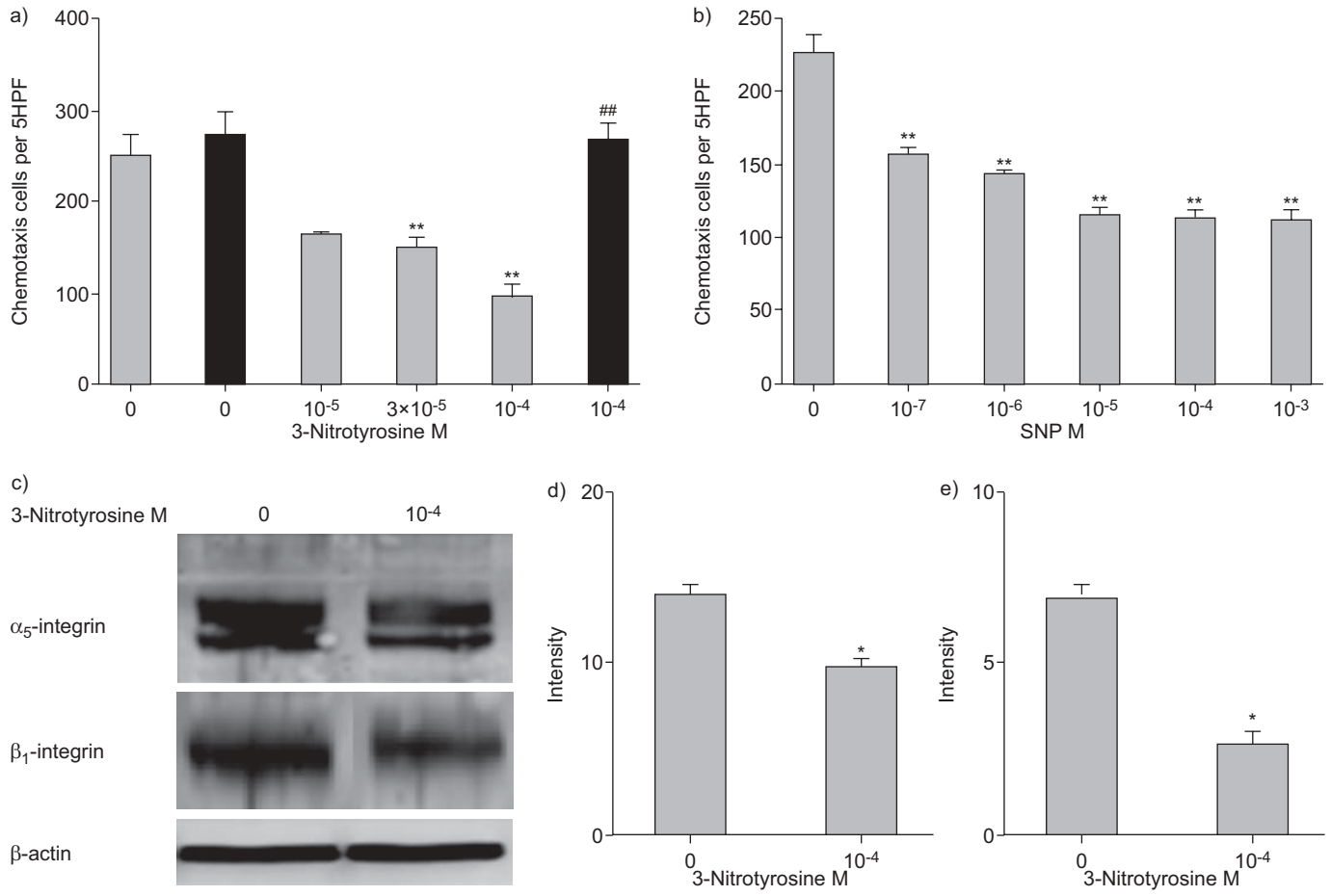

FIGURE 5. Effect of a) 3-nitrotyrosine or b) the nitric oxide donor sodium nitroprusside (SNP) on chemotaxis, and c) effect of 3-nitrotyrosine on expression of $\alpha_5\beta_1$ integrin in human fetal lung fibroblasts. Fibroblasts were exposed to various concentrations of a) 3-nitrotyrosine or b) SNP and assayed for chemotaxis towards fibronectin in the Boyden blindwell chemotaxis assay. The effect of N^G-mono-methyl-L-arginine acetate salt at 10^{-4} M was also tested (■). c) The treated cells were analysed by western blotting for the expression of $\alpha_5\beta_1$ integrin. Band intensity was quantified by NIH image for d) α_5-integrin and e) β_1-integrin. All values are mean±SEM for four separate experiments. *: $p<0.05$ compared with control; **: $p<0.01$ compared with control; ##: $p<0.01$ compared with 3-nitrotyrosine-treated (10^{-4} M) group.

cytotoxity, growth inhibition and morphological changes, and consequently alters the cellular function in cultured cells [11–13]. Our findings demonstrate that 3-nitrotyrosine leads to the decreased release of fibronectin that drives fibroblast-mediated repair responses. Fibronectin, a multifunctional glycoprotein involved in tissue remodelling, is a chemoattractant for lung fibroblasts [25] and can be released in increased amounts from fibroblasts [25] and epithelial cells [26, 27] in response to a variety of cytokines. According to a previous study, both the cellular type and plasma type of fibronectin enhanced collagen gel contraction [28]. In the present study, 3-nitrotyrosine reduced fibronectin production and NOS inhibitor reversed the 3-nitrotyrosine-reduced fibronectin production, suggesting that NO can reduce fibronectin production in human lung fibroblast.

RNS have a variety of biological effects, including tissue injury, lipid peroxidation and nitration of protein tyrosine residues. We have shown that RNS are excessively produced in the airways of asthmatics [5] and that endogenously produced RNS cause airway inflammation in late allergic response model [29]. Recently, we have shown that peroxynitrite, one of the RNS, stimulates fibroblasts. Peroxynitrite stimulates the differentiation of fibroblasts into myofibroblasts and extracellular matrix protein production *in vitro* [23, 30], suggesting that RNS induce fibroblast-mediated profibrotic responses. In the current study, free 3-nitrotyrosine caused the inhibition of tissue remodelling *in vitro*. 3-Nitrotyrosine produced by RNS may counteract the RNS-mediated profibrotic responses.

NO is synthesised in a variety of cell types by the enzyme NOS, which exists in constitutive and inducible isoforms [29]. It has been reported that inflammatory cytokines can induce iNOS in human pulmonary fibroblasts [18]. In the current study, 3-nitrotyrosine induced iNOS protein through NF-κB activation in human lung fibroblasts. 3-Nitrotyrosine can also stimulate NO production in fibroblasts. Furthermore, the NOS inhibitor L-NMMA and the iNOS inhibitor L-NIL diminished the 3-nitrotyrosine-induced inhibition of collagen gel contraction, chemotaxis and fibronectin production, suggesting that 3-nitrotyrosine might affect the tissue repair process through NO production derived from iNOS.

The regulation of fibroblast recruitment *in vivo* is likely to depend on both the chemotactic factors and inhibitors.

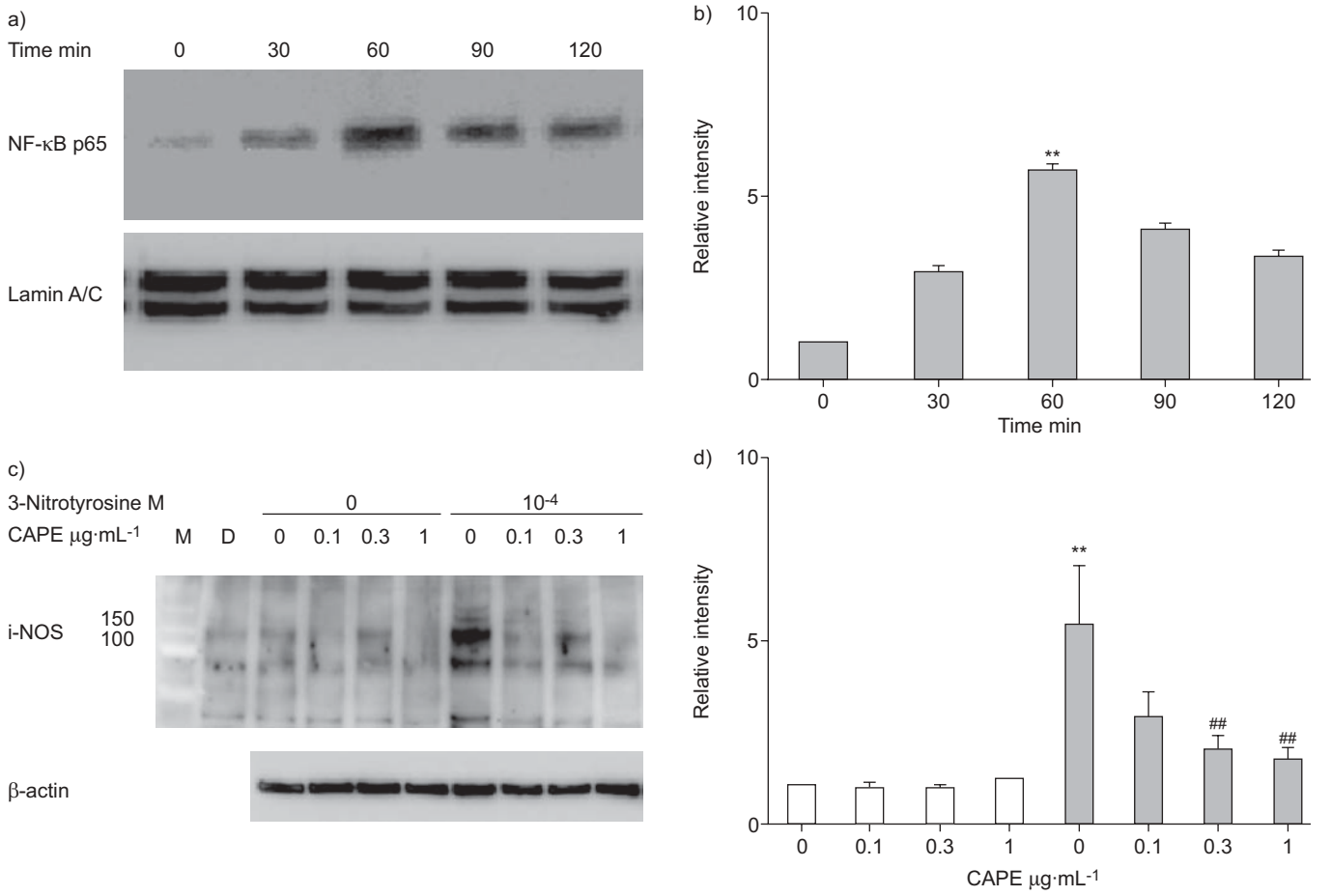

FIGURE 6. Effect of 3-nitrotyrosine on nuclear factor (NF)-κB p65 translocation into the nucleus and effect of an NF-κB inhibitor on the 3-nitrotyrosine-augmented inducible type nitric oxide synthase (iNOS) expression. a and b) Cells were treated with 10^{-4} M 3-nitrotyrosine and harvested at 0–120 min. The amount of NF-κB p65 translocated into the nucleus was assessed by western blotting. c and d) Cells were pretreated with the NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), followed by treatment with (■) or without (□) 3-nitrotyrosine at 10^{-4} M for 48 h. Cells were harvested and iNOS expression was assessed by western blotting. b and d) Band intensity was quantified by NIH image. M: molecular marker lane; D: the same amount of control protein was loaded to prevent ''smiling''. All values are mean±SEM for four separate experiments. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed group.

Fibronectin can augment chemotactic activity in fibroblasts [25]. The current study demonstrated that 3-nitrotyrosine inhibited chemotaxis and NOS inhibitor abolished the 3-nitrotyrosine-induced inhibition of chemotaxis. Exogenously administrated NO also inhibited the fibroblast-mediated chemotaxis. Moreover, 3-nitrotyrosine can stimulate iNOS expression and NO production. Taken together, 3-nitrotyrosine inhibits chemotaxis through NO production. To explore the possible mechanism by which 3-nitrotyrosine inhibits fibroblast-mediated chemotaxis towards fibronectin, we investigated the effect of 3-nitrotyrosine on the expression of $\alpha_5\beta_1$ integrin, which is a receptor for fibronectin. In the present study, we found that 3-nitrotyrosine inhibited the expression of $\alpha_5\beta_1$ integrin. Therefore, 3-nitrotyrosine may inhibit chemotaxis through the suppression of $\alpha_5\beta_1$ integrin expression.

3-Nitrotyrosine could stimulate iNOS protein expression and NO production in the current study. Interestingly, 3-nitrotyrosine can stimulate NF-κB translocation into the nucleus. In general, NF-κB activation is a key process in the regulation of iNOS expression. To our knowledge, this is the first report showing that 3-nitrotyrosine stimulates NF-κB activation in lung fibroblasts. Although RNS are reported to stimulate NF-κB [30], nitrotyrosine derived from RNS may cause inflammation through NF-κB.

In the current study, we attempted to examine the role of NO derived from all types of NOS in the fibroblast function. We found that NO could attenuate the fibroblast function because a nonspecific NOS inhibitor, L-NMMA, restored the 3-nitrotyrosine-inhibited gel contraction (fig. 2a). Then, we found that 3-nitrotyrosine augmented iNOS expression through NF-κB activation. In the next step, we investigated the effects of NO derived from iNOS on the fibroblast function by means of a specific iNOS inhibitor, L-NIL. As we expected, the iNOS inhibitor significantly restored the 3-nitrotyrosine-inhibited gel contraction to the same degree as L-NMMA, as shown in figures 2a and 3, suggesting that NO derived from iNOS is a key mediator in the attenuation of the fibroblast function. Although we only showed that NO derived from iNOS could inhibit the gel contraction in the present study, these findings encourage us to speculate that L-NIL also may inhibit other fibroblast functions, including mediator production, chemotaxis and NO release.

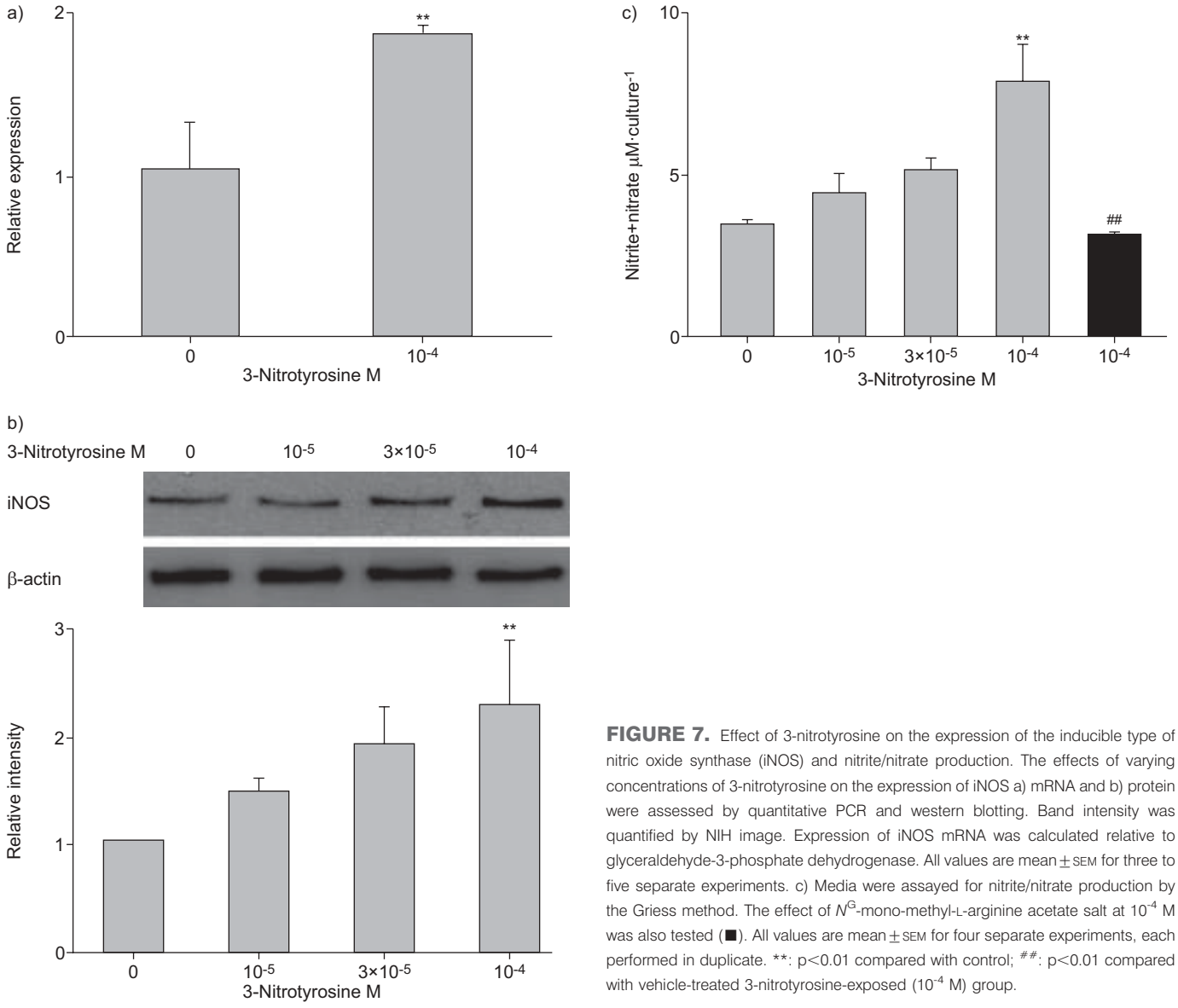

FIGURE 7. Effect of 3-nitrotyrosine on the expression of the inducible type of nitric oxide synthase (iNOS) and nitrite/nitrate production. The effects of varying concentrations of 3-nitrotyrosine on the expression of iNOS a) mRNA and b) protein were assessed by quantitative PCR and western blotting. Band intensity was quantified by NIH image. Expression of iNOS mRNA was calculated relative to glyceraldehyde-3-phosphate dehydrogenase. All values are mean±SEM for three to five separate experiments. c) Media were assayed for nitrite/nitrate production by the Griess method. The effect of N^G-mono-methyl-L-arginine acetate salt at 10^{-4} M was also tested (■). All values are mean±SEM for four separate experiments, each performed in duplicate. **: $p<0.01$ compared with control; ##: $p<0.01$ compared with vehicle-treated 3-nitrotyrosine-exposed (10^{-4} M) group.

It has been reported that 3-nitrotyrosine is incorporated into α-tubulin, induces cell morphology transformation and changes the cell function [13]. Therefore, we investigated whether 3-nirotyrosine attenuates the tissue repair because of the incorporation of 3-nitrotyrosine into α-tubulin. To clarify this, we investigated the incorporation of 3-nitrotyrosine into α-tubulin by western blotting. 3-Nitrotyrosine was not incorporated into the cells (supplementary fig. 3). However, we could not confirm whether the incorporation of 3-nitrotyrosine into α-tubulin is associated with the inhibition of tissue repair observed in the current study because of the lack of a specific tubulin tyrosine ligase inhibitor. The findings that 3-nitrotyrosine is not incorporated into the cells and that NOS inhibitors inhibited the 3-nitrotyrosine-mediated inhibition of tissue repair suggest that the 3-nitrotyrosine incorporation may have had little influence on our current findings.

In summary, our data demonstrate that 3-nitrotyrsine inhibited the fibroblast-mediated contraction of three-dimensional collagen gels, chemotaxis and fibronectin production. 3-Nitrotyrosine also augmented iNOS protein expression through NF-κB activation and the release of NO. These effects of 3-nitrotyrosine were significantly blocked by NOS inhibitor. These results suggest that the free amino acid form of 3-nitrotyrosine can affect the tissue repair process by modulating NO production.

STATEMENT OF INTEREST

A statement of interest for S.I. Rennard can be found at www.erj.ersjournals.com/misc/statements.dtl

ACKNOWLEDGEMENTS

The authors thank B. Bell (Division of Respiratory and Infectious Diseases, Tohoku University Graduate School of Medicine, Sendai, Japan) for reading the manuscript.

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