

# Inducible nitric oxide synthase (iNOS) and $\alpha$ -melanocyte-stimulating hormones of iNOS origin play important roles in the allergic reactions of atopic dermatitis in mice

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**Abstract:** To elucidate the possible involvement of nitric oxide (NO) derived from inducible NO synthase (iNOS) in the pathogenesis of patients with allergic rhinitis, we used an animal model of atopic dermatitis (AD) induced by epicutaneous sensitization and analysed the differences in ear thickness, the frequency of scratching and plasma levels of ovalbumin-specific immunoglobulin E (OVA-IgE), transforming growth factor (TGF)- $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , adrenocorticotrophic hormone (ACTH) and  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) between control and iNOS<sup>-/-</sup> mice. Eight-week-old control and iNOS<sup>-/-</sup> male C57BL/6j mice were sensitized three times with OVA antigen. Before and after the last skin sensitization, the number of scratching incidents and the thickness of the ear were examined, and the plasma levels of OVA-IgE,  $\alpha$ -MSH, ACTH, TGF- $\beta$  and TNF- $\alpha$  were analysed by ELISA.

Sensitization of mice with OVA resulted in increased plasma levels of OVA-IgE,  $\alpha$ -MSH, ACTH, TGF- $\beta$  and TNF- $\alpha$  in control, but not in iNOS<sup>-/-</sup> mice. The administration of L-nitro-arginine-methyl ester (L-NAME) abolished all the above changes that occurred in the control mice. In addition, iNOS<sup>-/-</sup> mice given  $\alpha$ -MSH exhibited a change similar to that seen in the control, whereas iNOS<sup>-/-</sup> mice given ACTH, TGF- $\beta$  or TNF- $\alpha$  did not demonstrate any changes. These results indicate that symptoms of AD such as scratching can be exacerbated by  $\alpha$ -MSH, which is induced by iNOS-derived NO.

**Key words:**  $\alpha$ -melanocyte-stimulating hormone – atopic dermatitis – immunoglobulin E – inducible nitric oxide synthase – nitric oxide

Accepted for publication 27 July 2011

## Introduction

Nitric oxide (NO) is synthesized by three forms of NO synthases (NOSs) using L-arginine as a substrate (1). Among three isoforms of NOS isozymes, inducible NOS (iNOS) has been known to play important roles in the regulation of inflammatory reactions and bactericidal actions of granulocytes (2). NO derived from iNOS also plays important roles in the modulation of symptoms in patients with inflammatory diseases, including atopic dermatitis (AD) (3), pollinosis and rhinitis (4).

Inducible NOS is induced by various factors, of which the pro-inflammatory cytokines are the most thoroughly investigated (5–7). Specific cytokine combinations, such as interleukin (IL)-1 $\beta$ , IL-2, and tumor necrosis factor (TNF)- $\alpha$  can induce iNOS *in vitro* (7). Although these cytokines are either absent or present only at low levels in AD lesions (8), other proinflammatory cytokines may be involved in the induction of iNOS in AD. iNOS is expressed by dermal endothelial cells or infiltrating cells closely associated with the endothelium; subsequently, iNOS produces a relatively large amount of NO (9).

However, the role of NO in AD, as well as the role of NO in rhinitis, remains to be elucidated. Recent studies demonstrate the development of a mouse model whose symptoms are similar to those in patients with AD (10–12). These mice show increased scratching and marked elevation of immunoglobulin E (IgE) in plasma, particularly when challenged with allergens.

To elucidate the role of NO in the aetiology of AD, we analysed changes in ear swelling, the number of scratching incidents and plasma IgE levels after ovalbumin (OVA) sensitization in mice showing symptoms of AD using L-nitro-arginine-methyl ester (L-NAME), an inhibitor of NOS. In addition, we developed an AD model using iNOS knockout (iNOS<sup>-/-</sup>) mice and observed the influence on symptoms of AD.

## Materials and methods

### Animals

Specific-pathogen-free, 6-week-old male C57BL/6j mice (SLC, Hamamatsu, Japan) and iNOS<sup>-/-</sup> mice (Jackson Laboratories, Bar Harbor, ME, USA) were subjected to experiments according to the Animal Care Regulations of Osaka City University Medical School.

### Sensitization

Experiments were carried out according to the method of Jin et al. (10). The mice were sensitized with OVA (100  $\mu$ g) or saline (100  $\mu$ l) applied to a sterile patch. The patch was placed on each mouse for 1 week and then removed. Two weeks later, an identical patch was reapplied to the same skin site. Each mouse had a total of three 1-week patch exposures separated from each other by 2-week intervals. The mice were challenged 1 day after the third sensitization (day 50) by applying the OVA solution (5  $\mu$ l) to both the dorsal and ventral surfaces of the right ear. Ear thickness was measured with a micrometer, and atopic response was

regarded as the difference between the ear thickness measured before challenge and 24 h after challenge. The frequency of scratching incidents of sensitized mice, such as scratching of the nose, ears and dorsal skin with the hind paws, was measured during a 10-min period. Licking of the belly and dorsal skin during grooming was disregarded. Each occurrence of scratching of the head, neck, dorsal skin, ears and nose was counted to obtain the maximum score. All experiments were performed at the end of the third sensitization.

#### Treatment of animals with L-NAME

The mice were intraperitoneally injected with 20 mg/kg L-NAME (antagonist of NOS; Sigma, St Louis, MO, USA) dissolved in 0.1 M phosphate buffer (pH 7.2) after sensitization on days 7, 28 and 49 (13). The same volume of phosphate buffer was administered to the control group.

#### $\alpha$ -MSH, ACTH, TGF- $\beta$ or TNF- $\alpha$ treatment

Approximately 1  $\mu$ g/mouse of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) (14), 57 ng/mouse of adrenocorticotrophic hormone (ACTH) (15), 100 ng/mouse of transforming growth factor (TGF)- $\beta$  (16) or 100 pg/mouse of TNF- $\alpha$  (17) in 50  $\mu$ l saline was intraperitoneally injected after sensitization on days 7, 28 and 49 in iNOS $^{-/-}$  mice, whereas saline was injected into control iNOS $^{-/-}$  mice.

#### Preparation and staining of dorsal skin

The mice were killed 1 day after the third sensitization. Dorsal skin specimens were fixed in phosphate-buffered (PBS) paraformaldehyde (4%), embedded in frozen Tissue Tek, OCT compound and cut into 5- $\mu$ m-thick sections. The sections of dorsal skin were washed in PBS and subsequently incubated overnight at 4°C with a goat anti-mast cell tryptase (1:50) polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The sections were then washed in PBS and incubated at room temperature for 2 h with FITC-conjugated anti-goat immunoglobulin (1:30) (Dako Cytomation, Glostrup, Denmark). The expression of mast cell tryptase was then evaluated immunohistochemically using a fluorescent microscope.

#### Quantification of neuronal hormones, OVA-specific IgE (OVA-IgE), cytokines, NOx ( $\text{NO}_3^- + \text{NO}_2^-$ ) and histamine, by enzyme-linked immunosorbent assay (ELISA)

Blood samples were taken from the heart 1 day after the third sensitization, and the plasma samples were fractionated. Skin samples were removed 1 day after the third sensitization and immediately frozen in liquid nitrogen and stored at -80°C. For extraction form, the skin was homogenized. After centrifugation, the supernatant was used for examination. The plasma levels of  $\alpha$ -MSH, ACTH, OVA-IgE, TGF- $\beta$ , TNF- $\alpha$  and  $\text{NO}_3^- + \text{NO}_2^-$  and the cutaneous levels of  $\alpha$ -MSH, ACTH and histamine were determined using a commercial ELISA kit ( $\alpha$ -MSH and ACTH – Phoenix Pharmaceuticals Inc., Burlingame, CA, USA; OVA-IgE – Shibayagi Co., Gunma, Japan; TGF- $\beta$  – Promega, Madison, WI, USA; TNF- $\alpha$  – Pierce Biochemistry, Inc., Rackford, IL, USA;  $\text{NO}_3^- + \text{NO}_2^-$  – Dojindo, Kumamoto, Japan; histamine – Bertin Pharma, Montigny le Bretonneux, France) according to the manufacturer's instructions.

#### Statistical analysis

All data are expressed as the mean  $\pm$  SD derived from 10 animals. The results obtained from the animal groups were analysed using either Student's *t*-test or ANOVA using a computer software

program. Differences were considered to be significant when  $P < 0.05$ .

## Results

### Effect of OVA antigen on ear swelling, scratching and plasma OVA-IgE and $\text{NO}_3^- + \text{NO}_2^-$ levels

Ear swelling and the frequency of scratching significantly increased after sensitization of mice with OVA antigen (Fig. 1). In addition, the plasma levels of OVA-IgE and  $\text{NO}_3^- + \text{NO}_2^-$  increased markedly after sensitization.

### Effect of L-NAME on ear swelling, scratching and plasma OVA-IgE levels

Ear swelling and the frequency of scratching for the OVA-sensitized mice decreased significantly after the administration of L-NAME, an inhibitor of NOS (Fig. 2). L-NAME administration also attenuated the increase in plasma levels of OVA-IgE.

### Effect of iNOS on the change induced by sensitization with OVA antigen

It has been well documented that NO derived from iNOS plays important roles in inflammatory reactions (18,19). In this study, we analysed changes in symptoms and inflammatory factors in control versus iNOS $^{-/-}$  mice before and after sensitization with OVA (Figure S1). Ear swelling and the frequency of scratching in the sensitized animals were significantly suppressed in iNOS $^{-/-}$  mice. The plasma levels of OVA-IgE,  $\alpha$ -MSH, ACTH, TGF- $\beta$  and TNF- $\alpha$  and the cutaneous level of  $\alpha$ -MSH and ACTH increased in the wild mice, but demonstrated low levels in iNOS $^{-/-}$  mice.

### Effects of mast cell tryptase and cutaneous histamine levels on the change induced by sensitization with OVA antigen

The OVA-sensitized wild-type mice showed a significantly increased expression of mast cell tryptase and higher cutaneous histamine levels, whereas OVA-sensitized iNOS $^{-/-}$  mice did not show such an increase (Fig. 3).

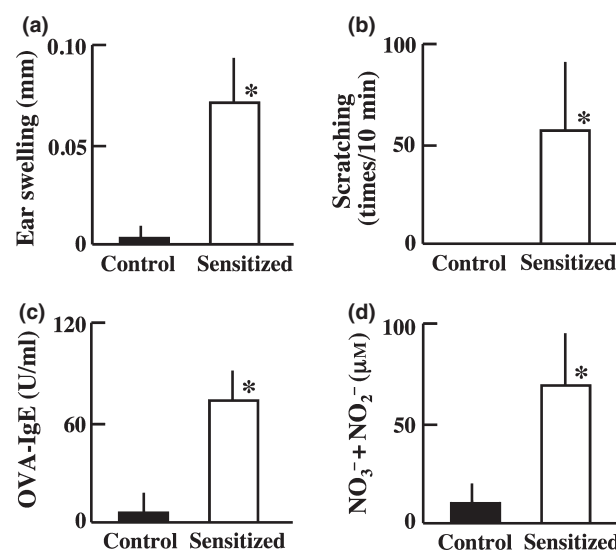


**Figure 1.** Ear swelling (a), the number of scratching incidents (b) and plasma levels of ovalbumin-IgE (c) and  $\text{NO}_3^- + \text{NO}_2^-$  (d) in control and the sensitized mice. Ear swelling, scratching time and the plasma levels of various factors were determined as described in the text. Values are presented as the mean  $\pm$  SD of the values from 10 animals. \* $P < 0.05$  in comparison with the control mice.

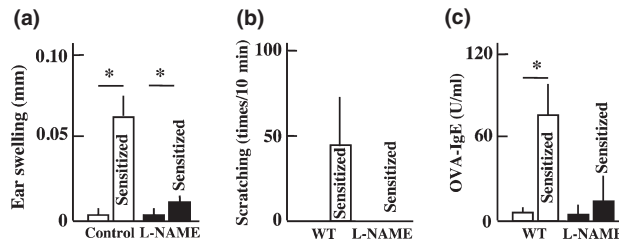


**Figure 2.** Ear swelling (a), the number of scratching incidents (b) and plasma levels of ovalbumin (OVA)-IgE (c) of control and the sensitized mice. L-NAME was injected intraperitoneally in sensitized mice. Values are increased in the sensitized mice in comparison with the control mice. However, injection of L-NAME attenuated the increase in ear swelling, scratching time and plasma OVA-IgE levels. Values are presented as the mean  $\pm$  SD from 10 animals. \* $P$  < 0.05 in comparison with the control mice.

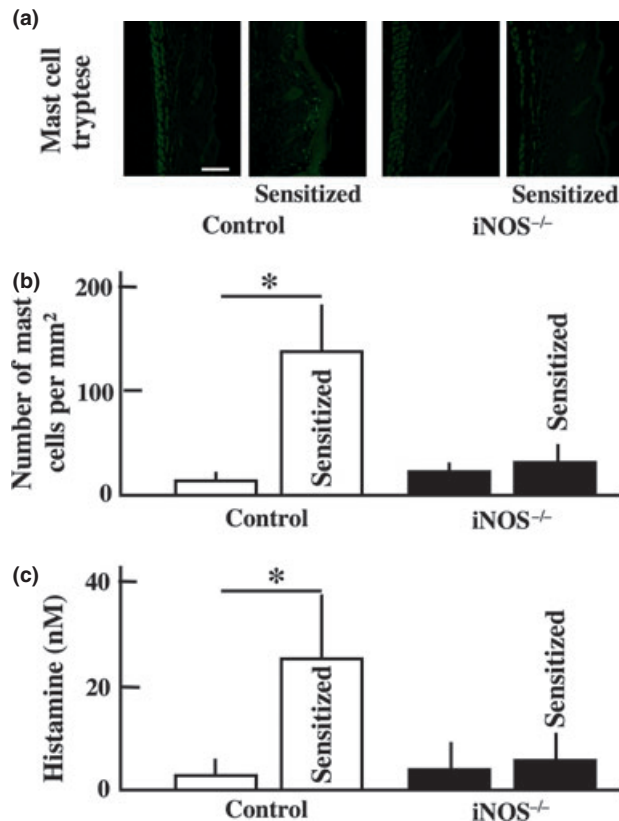


**Figure 3.** The expression of mast cell tryptase (a), the number of mast cells (b) and the levels of histamine (c) in dorsal skin of the control and sensitized mice. C57BL/6j and iNOS<sup>-/-</sup> mice were killed 1 day after the third sensitization, and dorsal skin specimens were frozen, cut into thin sections and stained by routine histological methods. The data show one typical experiment from 10 animals. Scale bar = 10  $\mu$ m. Values are presented as the mean  $\pm$  SD from 10 animals. \* $P$  < 0.05 in comparison with the control mice.

#### Effects of $\alpha$ -MSH, ACTH, TGF- $\beta$ or TNF- $\alpha$ treatment on ear swelling and plasma levels of OVA-IgE in iNOS<sup>-/-</sup> mice

The OVA-sensitized wild-type mouse showed significantly increased ear swelling and plasma OVA-IgE levels, whereas OVA-sensitized iNOS<sup>-/-</sup> mice did not show an increase. However, OVA-sensitized iNOS<sup>-/-</sup> mice treated with  $\alpha$ -MSH showed significantly increased ear swelling and plasma OVA-IgE levels in comparison with the non-treated OVA-sensitized iNOS<sup>-/-</sup> mice

(Figure S2). In contrast, ear swelling and plasma OVA-IgE levels in mice treated with ACTH, TGF- $\beta$  or TNF- $\alpha$  did not change in comparison with the non-treated OVA-sensitized iNOS<sup>-/-</sup> mice.

#### Discussion

Nitric oxide has been found to be important in a number of physiological processes, including vasodilation, neurotransmission, blood coagulation and immune regulation. Furthermore, iNOS is a major producer of NO when it is activated by various cytokines or bacterial lipopolysaccharides. Therefore, one of the methods used to examine the anti-inflammatory activity of a compound is the measurement of its suppressive effects on the synthesis of iNOS (20).

Recently, innate immunity has been suggested to play an important role in the pathogenesis of AD (21,22). In infants with AD, the serum levels of nitrate are increased and correlated with clinical severity (3). Furthermore, the extent and intensity of iNOS immunoreactivity are higher in AD lesions compared with lesions of contact dermatitis (3). Moreover, some reports have shown that when given topically or intrasessionally, NOS inhibitor/NO inhibitor can induce remission of active AD lesions (24). The increase of NOS in the epidermis stimulates NO production that may subsequently lead to vasodilation, erythema and oedema. Other important inflammatory and immune responses may result from an increase in NO, such as disturbing the balance between T-helper (Th) 1 and Th 2 cell types (25). Therefore, it is possible that elevated NO in atopic skin may participate in the pathogenesis of AD.

Sugita et al. (26) reported that iNOS downregulates the ear-swelling response by suppressing dendritic cell migration, and they observed enhanced ear swelling when they blocked the iNOS activity using an iNOS-specific inhibitor by contact hypersensitivity. However, we observed the ear swelling to be suppressed by OVA sensitization in iNOS<sup>-/-</sup> mice or iNOS inhibitor treatment mice. This study used a type I allergic (immediate allergic) induction model by OVA sensitization. Therefore, the crisis mechanism was thought to be different from that associated with type IV allergy (delayed allergy), but the details remain unclear.

The results of this study showed that OVA-sensitized iNOS<sup>-/-</sup> mice failed to upregulate the plasma  $\alpha$ -MSH, TGF- $\beta$  and TNF- $\alpha$  levels. Preliminary experiments using NOC7, a potent NO donor, revealed that instillation of the agent also upregulated the plasma  $\alpha$ -MSH, TGF- $\beta$  and TNF- $\alpha$  levels in non-sensitized mice, although the response was fairly small in comparison with that induced by OVA sensitization. These observations are consistent with the hypothesis that the OVA-sensitized inflammatory reactions underlie the mechanism for NO-dependent signalling in the cellular responses to activate the hypothalamo-pituitary pro-opiomelanocortin (POMC) system, thereby enhancing the secretion of  $\alpha$ -MSH. Our experiments revealed that OVA sensitization increased the plasma concentration of  $\alpha$ -MSH in normal mice, but not in iNOS<sup>-/-</sup> mice. Furthermore, atopic symptoms appear when OVA-sensitized iNOS<sup>-/-</sup> mice were given  $\alpha$ -MSH. However, the symptoms did not appear in the administration of TGF- $\beta$  and TNF- $\alpha$ . Therefore, NO (iNOS origin) is thought to activate the Pro-opiomelanocortin (POMC) system and increase the secretion of  $\alpha$ -MSH sequentially, thus resulting in a worsening of atopic symptoms. The molecular mechanism for iNOS-dependent signalling after OVA sensitization to the hypothalamo-pituitary pro-opiomelanocortin system should be studied further.

The ACTH showed an increase by sensitization, and this increase was suppressed in iNOS<sup>-/-</sup> mice. However, the increase in the ACTH levels was lower than that of the  $\alpha$ -MSH level in both the plasma and skin. This indicates that there is selectively more  $\alpha$ -MSH in the cleavage of POMC. In addition, the OVA-sensitized iNOS<sup>-/-</sup> mice treated with ACTH did not show any exacerbation of the skin symptoms. This suggests that the aggravation of inflammation in the sensitized mouse depends significantly on the  $\alpha$ -MSH level.

$\alpha$ -MSH may thus play an important role in the modulation of allergic inflammation (27–29). In addition, mast cells play an important role in the onset of AD. Mast cells express MC1R and MC5R, both of which are  $\alpha$ -MSH receptors on the cell surface, and  $\alpha$ -MSH regulates the release of histamine from mast cells through MC1R and MC5R (30). In these reports, iNOS expression was observed to increase in pollinosis, contact sensitization and AD. In addition, NO in the iNOS pathway induced  $\alpha$ -MSH expression.  $\alpha$ -MSH stimulated the release of histamine via MC1R and MC5R, which are expressed on the surface of mast cells, and it may thus be a possible mechanism leading to the scratching observed in the mice. In fact, iNOS<sup>-/-</sup> mice, which were sensitized in Cry j I, have been reported to exhibit an increase in sneezing after the administration of  $\alpha$ -MSH (27). However, the frequency of sneezing after  $\alpha$ -MSH administration in sensitized iNOS<sup>-/-</sup> mice is not predictable when comparable with the sensitized control mice (data not shown).  $\alpha$ -MSH is the exclusive cause

of the phenotype, but further examination is necessary to explore the possible contribution of other factors.

In addition, mast cells synthesize and release a variety of cytokines and chemokines after stimulation (31).  $\alpha$ -MSH controls cytokines and chemokines through MC5R (32). We previously reported the expression of local MC5R to be related to the increased frequency of sneezing and control of the immune system. In this regard, NO produced from the iNOS pathway may thus be a regulatory factor regarding both sneezing and the immune system.

An examination of the peptides derived from POMC reveals some problems. The cleavage of POMC in humans differs from that in mice. The production of  $\beta$ - and  $\gamma$ -MSH occurs in humans, but not in mice or rats because of the absence of previous enzymatic site in the rodent POMC (33,34). Therefore, further detailed examinations will be necessary in humans.

In summary, these findings suggest that  $\alpha$ -MSH affects cytokine production and mediator release from mast cells through MC5R. Furthermore, the NO produced by the iNOS pathway may therefore regulate the downstream effectors of  $\alpha$ -MSH.

### Author contributions

Kumi Orita and Keiichi Hiramoto performed the research, wrote the article and analysed the data; Keiichi Hiramoto, Hiromi Kobayashi and Masamitsu Ishii designed the research study; and Atsuo Sekiyama and Masayasu Inoue contributed essential reagents or tools.

### Conflict of interests

The authors have no conflict of interest.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Ear swelling (A), the number of scratching incidents (B) and plasma levels of OVA-IgE (C),  $\alpha$ -MSH (D), ACTH (E), TGF- $\beta$  (F) and TNF- $\alpha$  (G) and cutaneous levels of  $\alpha$ -MSH (H) and ACTH (I) in control and sensitized mice.

**Figure S2.** Ear swelling (A, C, E, G) and plasma levels of OVA-IgE (B, D, F, H) in control and sensitized mice.

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**Figure S1.** Ear swelling (A), the number of scratching incidents (B) and plasma levels of OVA-IgE (C),  $\alpha$ -MSH (D), ACTH (E), TGF- $\beta$  (F) and TNF- $\alpha$  (G) and cutaneous levels of  $\alpha$ -MSH (H) and ACTH (I) in control and sensitized mice. iNOS<sup>-/-</sup> mice were used for the sensitized mice. Values are increased in the sensitized mice in comparison with the control mice. Values in the sensitized iNOS<sup>-/-</sup> mice shown are significantly decreased in comparison with the sensitized control mice. Values are presented as the mean  $\pm$  SD from ten animals. \* P<0.05 in comparison with the control mice.

**Figure S2.** Ear swelling (A, C, E, G) and plasma levels of OVA-IgE (B, D, F, H) in control and sensitized mice.  $\alpha$ -MSH (A, B), ACTH (C, D), TGF- $\beta$  (E, F), or TNF- $\alpha$  (G, H) was injected intraperitoneally in the sensitized mice. Values were increased in the sensitized mice in comparison with the control mice. Values in the sensitized iNOS<sup>-/-</sup> mice shown are significantly decreased in comparison with the sensitized control mice. However, injection of  $\alpha$ -MSH increased ear swelling and OVA-IgE levels. Injection of ACTH, TGF- $\beta$  or TNF- $\alpha$  did not cause a change. Values are presented as the mean  $\pm$  SD from ten animals. \* P<0.05 in comparison with the control mice.

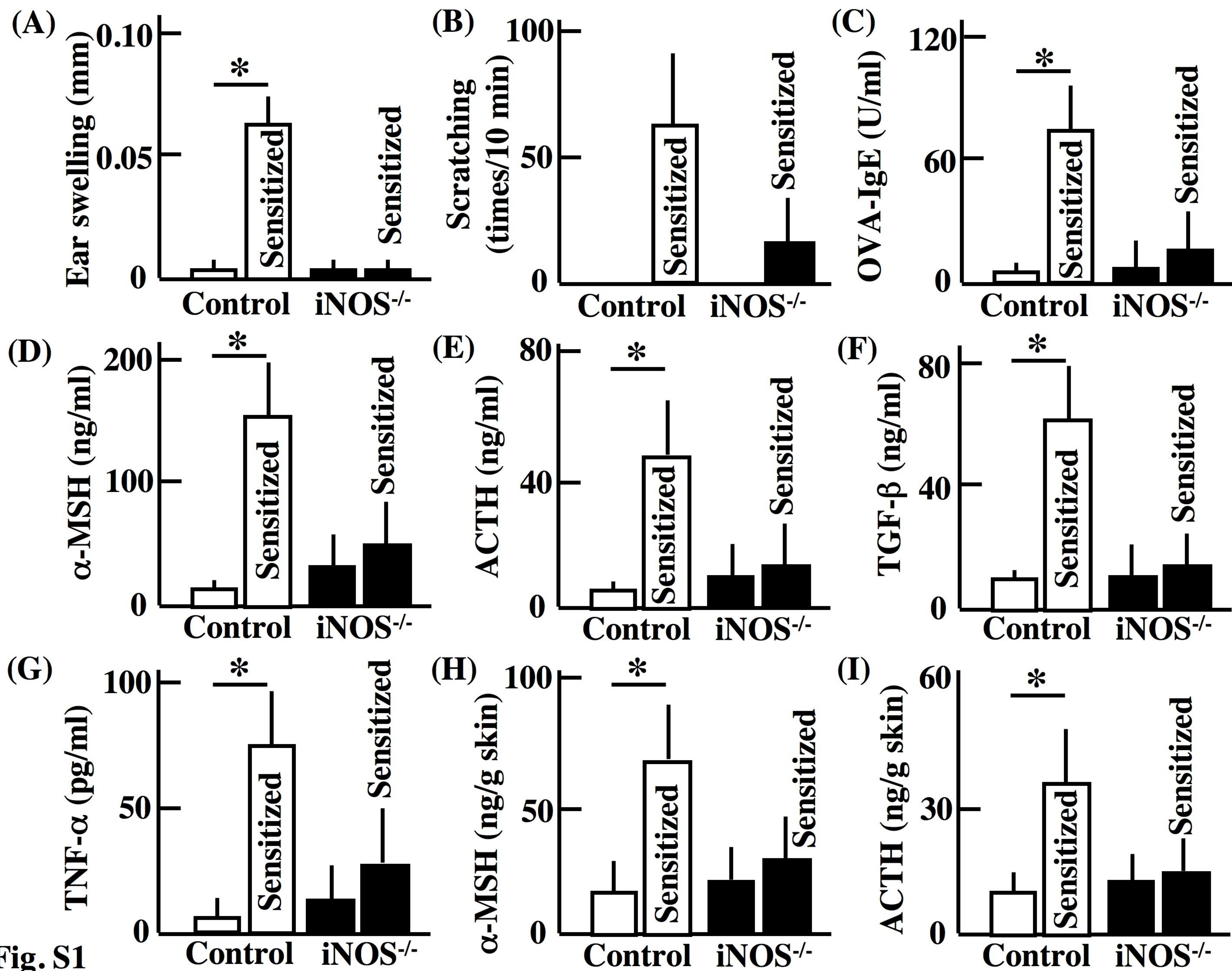


Fig. S1

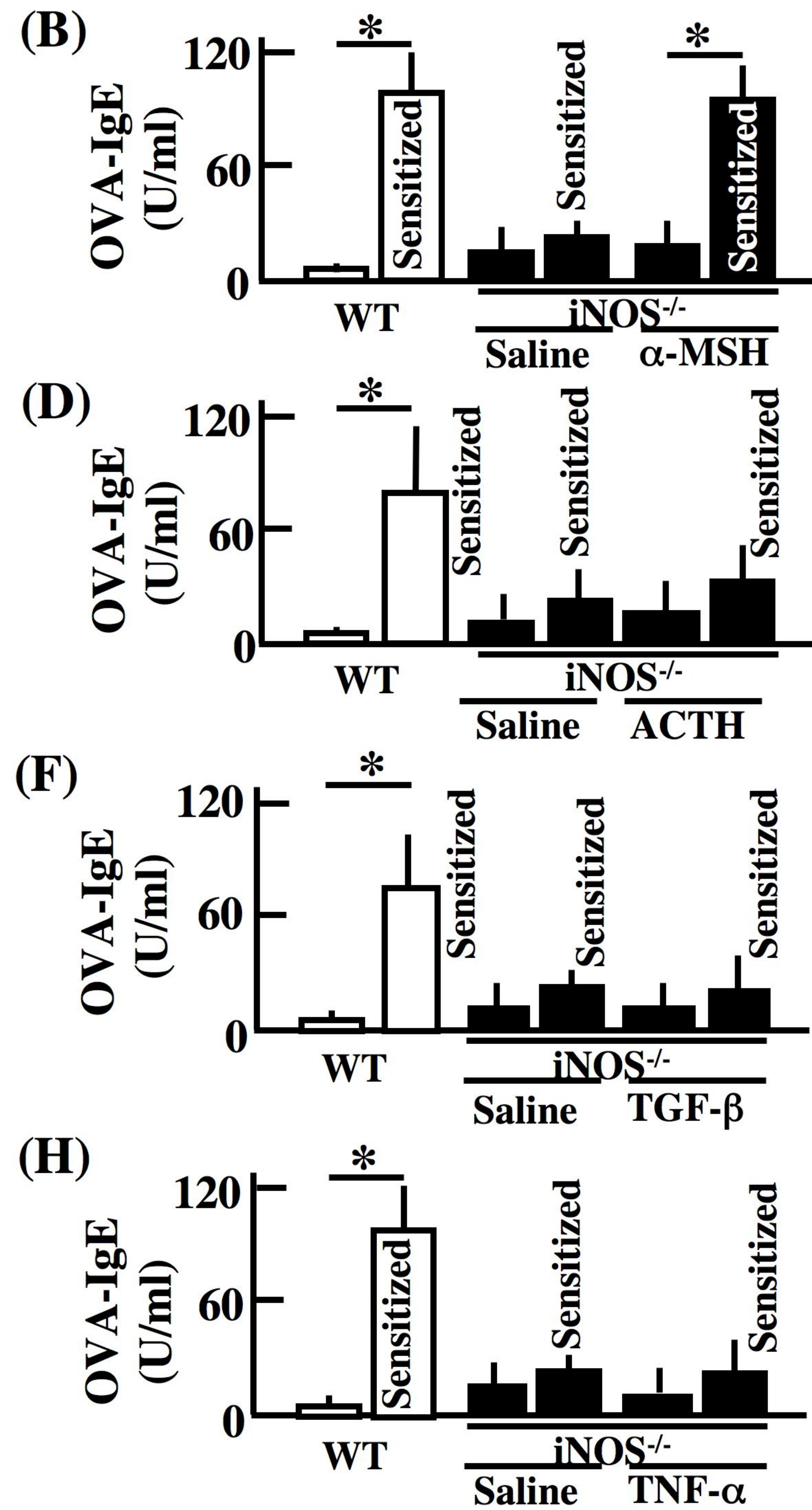
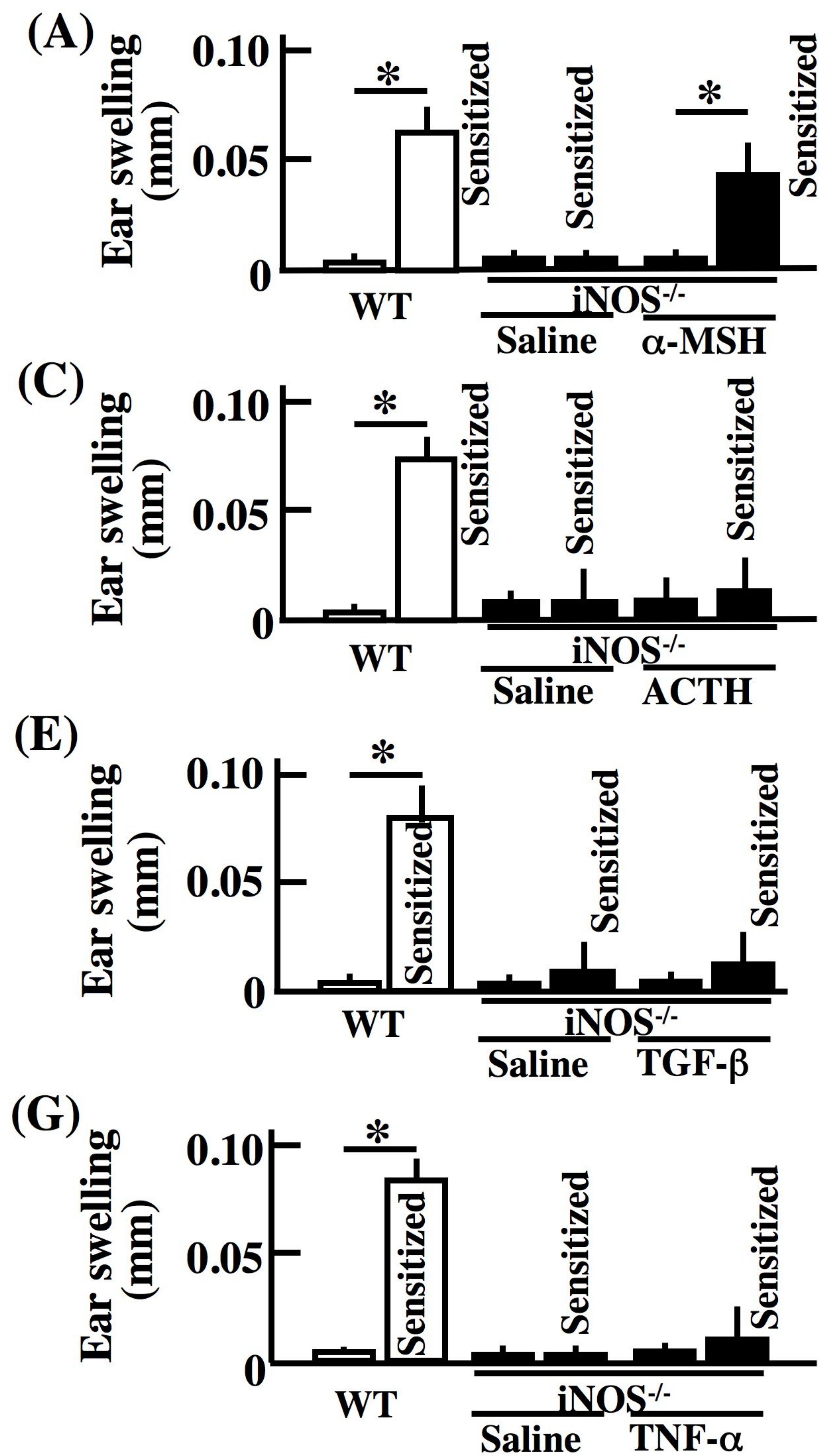


Fig. S2