Original Article

Ethanol attenuates vasorelaxation via inhibition of inducible nitric oxide synthase in rat artery exposed to interleukin-1β

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Running title: Ethanol attenuates vasorelaxation through iNOS inhibition

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Abstract

Nitric oxide produced by inducible nitric oxide synthase (iNOS) regulates sepsis-induced hypotension. During septic shock, interleukin (IL)-1β is synthesized in endothelial cells and smooth muscle cells by endotoxin. Ethanol suppresses endotoxin-induced hypotension. The present study aimed to elucidate the effect of ethanol on gradual relaxation and iNOS expression induced by IL-1β in isolated rat superior mesenteric arteries (SMAs). Exposure to IL-1β-induced contraction in SMA rings, followed by a gradual relaxation of phenylephrine pre-contracted tone. Contraction was abolished by indomethacin (IM), cycloheximide, and endothelium denudation. In contrast, the gradual relaxation was abolished by NOS inhibitors, cycloheximide, endothelium denudation, and inhibited by ethanol (50 and 100 mM). However, IM had no effect on relaxation. Western blot analysis demonstrated that iNOS expression was induced by IL-1β and was inhibited by ethanol and endothelium denudation. Furthermore, mRNA expression of iNOS, but not endothelial NOS, was inhibited by ethanol. These data suggest that IL-1β-induced contraction is mediated by thromboxane A2, whereas IL-1β-induced relaxation occurs via nitric oxide derived from iNOS. The endothelium plays an important role in vasorelaxation.

Taken together, ethanol inhibits IL-1β-mediated vasorelaxation by suppressing endothelium iNOS expression. This study provides the first evidence of ethanol-induced inhibition of IL-1β-mediated vasorelaxation.

Key words: ethanol, rat, superior mesenteric artery, interleukin-1β, relaxation response

Introduction

Bacterial sepsis is a systemic inflammatory state characterized by vascular smooth muscle dysfunction, leading to hypotension, inadequate tissue perfusion, and multiple organ failure.[1] Inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO) plays a key role in sepsis.[2] Patients with sepsis have elevated levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α).[2] Endotoxemia-associated vascular iNOS expression is induced by a variety of cytokines, including IL-1β and TNF-α, and lipopolysaccharide (LPS), resulting in a high NO production.[3]

LPS and IL-1β activate the expression of iNOS [4, 5] in the rat superior mesenteric artery (SMA) and abdominal aorta. Increased iNOS-mediated NO production suppresses phenylephrine (Ph)-induced vascular contractile responses, resulting in decreased arterial pressure.[3, 6] Thus, in endotoxemia, the impaired contractile function of the smooth muscle and the resulting decreased blood pressure have been attributed to increased NO-mediated dilatation secondary to iNOS activity.

IL-1β is a cytokine produced by monocytes and macrophages that contributes to disease pathogenesis. It is an important mediator of host

responses to infection and injury.[3, 7] Prolonged exposure of blood vessels to IL-1β decreases vascular tone and causes systemic vasodilation. IL-1β can produce a shock-like syndrome, characterized by hypotension and decreased vascular resistance, when administered to animals.[8, 9] Interestingly, ethanol is a recognized immunosuppressant. For example, chronic abuse of ethanol increases susceptibility to bacterial infections and pneumonia.[10, 11] Previous studies have shown that acute *in vitro* administration of ethanol inhibits LPS-mediated induction of iNOS gene expression and release of NO from Kupffer cell, alveolar macrophages, and glial cells in experimental animals.[12-19] Therefore, the increased sensitivity to bacterial infection associated with ethanol seems to be due to ethanol-induced suppression of NO production. On the other hand, Ajisaka et al.[20] reported that acute low-dose ethanol administration also inhibits endotoxin-induced hypotension and inflammatory cytokine responses in intravenously treated rats. Ajisaka et al. suggested that acute ethanol administration may play a protective role in endotoxin-induced shock. Furthermore, Greenberg et al.[21] reported that ethanol suppresses endotoxin-induced hypotension in rats administered LPS intravenously. Greenberg et al. suggested that ethanol may increase morbidity and mortality

during endotoxemia by masking the hypotension and humoral changes characteristic to early endotoxemia. Thus, the explanation for the inhibitory effect of ethanol on endotoxin-induced hypotension is a matter of debate. To obtain a better understanding of the inhibitory effect of ethanol, we aimed to elucidate the mechanism underlying the ethanol-induced suppression of endotoxin-mediated hypotension. We evaluated the effect of ethanol on vascular reactivity induced by IL-1β *in vitro* using isolated rat SMAs.

Methods

Tissue preparation

All procedures were approved by the Committee on Ethics in Animal Experiments of Nara Medical University and were conducted in conformity with institutional guidelines. Male Wistar rats (10–12 weeks old, 330–350 g) were anesthetized by intraperitoneal pentobarbital injection (0.05 g/kg) and sacrificed by exsanguination. SMAs were removed, cut into 1 mm segments, and mounted horizontally on tension hooks with a 50 μm diameter in 4 ml tissue baths containing Krebs-Ringer solution (pH 7.4). Baths were maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

Drug application

IL-1β, Ph, and ethanol were obtained from Wako Pure Chemical (Osaka, Japan). Cycloheximide, 1400W, indomethacin (IM), nitro-L-arginine, and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All reagents were dissolved in distilled water, which was also used as the vehicle in control experiments. Concentrations are reported as the final molar concentration in the bath.

Measurement of tension

Isometric tension was monitored using a force-displacement transducer (Nihon Kohden Kohgyo Co., Tokyo, Japan). One side of the tension hook was connected and recorded with a pen recorder. The rings were equilibrated for approximately 1 hr, replacing the medium every 15 min, and were adjusted for a resting tension of 0.2 g. Arteries were then precontracted with 10^{-6} M Ph. At peak pre-contraction, 20 ng/ml IL-1β was added to the organ baths. Thereafter, tissue tension was monitored for 3 hr. To investigate the role of iNOS in IL-1β-induced relaxation, the rings were pretreated with cycloheximide, IM, the iNOS inhibitors AMT and 1400W, nitro-L-arginine, or ethanol 30 min before the addition of 10^{-6} M Ph. Ethanol and all drugs were coincubated with IL-1β over the 3 hr period.

To examine the role of the endothelium in IL-1β-induced relaxation, endothelium was denuded by gently rubbing the intimal surface with stainless wire. Removal of the endothelium was confirmed by the loss of the relaxation in response to 10^{-6} M acetylcholine. Relaxation was expressed as the percent contraction in response to 10^{-6} M Ph 3 hr after the addition of IL-1β.

Preparation of isolated SMA for western blotting and RT-PCR analysis

Rat SMAs were isolated and washed with Krebs-Ringer solution. The SMA

specimens were divided into the following three groups: control, IL-1β, or IL-1β and ethanol. In the control group, SMAs were incubated with Krebs-Ringer solution for 3 hr. In the IL-1β group, SMAs were incubated with IL-1β for 3 hr. In the IL-1β and ethanol group, specimens were incubated with ethanol 30 min before incubation with IL-1β and, thereafter, ethanol was co-incubation with IL-1β for 3 hr.

Western blotting

Several specimens from each group were homogenized and centrifuged, and the supernatant was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Samples were transferred onto a polyvinylidene fluoride membrane, which was blocked and incubated with a primary antibody against iNOS (ab15323, Abcam, Cambridge, UK). Membranes were washed with Tris-saline buffer containing 0.05% Tween-20 and incubated with a secondary antibody Rabbit IgG HRP (170-6515, Bio-Rad Laboratories Inc., Hercules, CA, USA). We used an antibody against actin (A2103, Sigma-Aldrich, St. Louis, MO, USA) as a loading control. After an additional wash, the signal was visualized by enhanced chemiluminescence using an LAS-1000 image analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan). The band intensity was calculated using Image J

software (National Institutes of Health, Bethesda, MD, USA).

RNA isolation and quality control

Total RNA was extracted from cell samples using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and treated with DNase I to eliminate contamination from genomic DNA. RNA samples were diluted in Tris-EDTA buffer, and the concentration was measured at a wavelength of 260 nm with a UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan). Real-time RT-PCR analyses were performed using samples with a 260/280 nm absorbance ratio that was between 1.9 and 2.1.

Gene expression analysis

Total RNA (50 ng) was reverse-transcribed using the Superscript First Stand system (Life Technologies) with random hexamers, as described in the manufacturer's protocol. To assess eNOS and iNOS expression, quantitative RT-PCR analysis was performed using the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with a universal PCR master mix according to the manufacturer's specifications. The expression of the following genes was examined using TaqMan probes and primers: eNOS (*NOS3*, assay ID; Rn02132634_s1) and iNOS (*NOS2*, assay ID; Rn00561646_m1). The

expression of *hypoxanthine phosphoribosyl transferase* (*Hprt1*, assay ID; Rn01527840_m1) was used as an endogenous control. For quantification of iNOS gene expression, cDNAs derived from pooled IL-1β-treated lungs were used to generate standard reference curves. For quantification of eNOS expression, we employed the ΔΔCt method, because adequate reference samples for the standard curve could not be found. Data were expressed relative to the value of untreated SMA.

Statistical analysis

Data are presented as the mean ± SEM, and were analyzed using a one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test. A *P*-value less than 0.05 was considered statistically significant.

Results

IL-1β-induced relaxation in SMAs

Ph produced sustained contraction in endothelium-intact or -denuded SMA (data not shown) over a 3 hr period (Figure 1). In contrast, IL-1β induced a peak contraction approximately 30 min after IL-1β. After 1.5 hr, it recovered to levels similar to those at the time of addition (Figure 1). Gradual relaxation followed the observed contraction. IM, an inhibitor of cyclooxygenase, abolished the contractile response seen after the addition of IL-1β, but did not inhibit the gradual relaxation (Figures 1 and 2). Cycloheximide, an inhibitor of protein synthesis, almost completely abolished both the contractile and relaxation response (Figures 1 and 2). Similarly, SMA denudation inhibited both contractile and relaxation responses to IL-1β (Figure 1). Therefore, the following experiments were conducted in the presence of IM to exclude the effect of the contractile response on relaxation.

Nitro-L-arginine, a non-selective NOS inhibitor, and 1400W and AMT, specific iNOS inhibitors, inhibited IL-1β-induced relaxation responses (Figures 3 and 4). The relaxation response was also inhibited by 50 and 100 mM ethanol (Figures 3 and 4).

Expression of iNOS protein in IL-1β-treated SMAs

When SMAs were unstimulated, iNOS was not expressed in the presence or absence of endothelium (Figures 5 and 6). However, IL-1β exposure for 3 hr enhanced iNOS expression in SMAs, both in the presence or absence of endothelium. In SMAs without endothelium, the degree of IL-1β-induced iNOS expression was 30% compared to SMAs with endothelium (Figure 5). IL-1β-induced iNOS expression in SMAs with endothelium was inhibited by co-incubation with 50 mM or 100 mM ethanol for 3 hr, respectively (Figure 6).

Effect of IL-1β on iNOS and eNOS mRNA expression in SMAs

Real time PCR analysis revealed that eNOS mRNA expression was slightly, although no significantly, decreased in IL-1β treated SMAs, as compared to untreated SMAs. However, iNOS mRNA expression was enhanced in a time dependent manner when SMAs were expressed to IL-1β for 1 or 3 hr. Moreover, pretreatment with 50 or 100 mM ethanol for 3 hr suppressed IL-1β-induced iNOS mRNA expression (Figure 7).

Discussion

In the present study, we showed that IL-1β induces early contraction of SMAs, followed by a gradual relaxation. SMA contraction was abolished by IM, an inhibitor of cyclooxygenase, and cycloheximide, an inhibitor of protein synthesis. These data suggest that the contractile response was caused by cyclooxygenase-induced thromboxane A2, which produces a contractile response. Thromboxane A2 is reported to regulate vascular tone via its effect on the expression of iNOS.[22] Therefore, our experiments were conducted in the presence of IM to exclude the effect of thromboxane A2 on relaxation.

IL-1β-induced relaxation was not inhibited by IM; therefore, relaxation was likely not caused by cyclooxygenase products, including prostacyclin, which is known to induce relaxation. In contrast, relaxation was abolished by cycloheximide, the iNOS inhibitors, AMT and 1400W, and the non-selective NOS inhibitor, nitro-L-arginine. These results suggest that IL-1β-induced relaxation is induced by iNOS, which is produced by *de novo* protein synthesis. This enhancement leads to the generation of large amounts of NO. This conclusion is supported by the IL-1β-induced enhancement in iNOS mRNA and protein expression. In rat SMAs and thoracic aorta, LPS causes relaxation via

vascular iNOS expression,[4, 23] in line with our results. However, IL-1β was shown to relax isolated rabbit mesenteric arteries by a prostaglandin-dependent, NO-independent mechanism.[24] This may be due to species differences.

IL-1β-induced relaxation responses were inhibited by endothelium denudation. These data suggest that the relaxation response is endothelium-dependent. Lu et al.[25] reported that IL-1β can decrease Ph-induced contraction in rat aortic rings with endothelium, and that removal of the endothelium eliminated this effect. Additionally, Vo et al.[4] found eNOS inhibition in the endothelium by specific inhibitors suppresses LPS-induced iNOS expression, thereby delaying the associated vascular effects during endotoxemia. These data suggest that eNOS activation is an essential step in the induction of iNOS expression. Therefore, in the present study, the loss of IL-1β-induced relaxation in the absence of endothelium is likely due to the loss of NO derived from eNOS activation by endothelium denudation. Our data indicated that iNOS was expressed in denuded SMA, suggesting that iNOS expression in smooth muscle cells was not involved in relaxation. Nevertheless, this possibility is unlikely because eNOS mRNA expression was not increased by IL-1β exposure. Alternatively, immunofluorescence analyses in rat SMAs

revealed that iNOS was expressed in both endothelial and smooth muscle cells after IL-1β treatment.[26] Thus, a more plausible explanation is that iNOS expressed in endothelial cells may be largely involved in relaxation.

In the present study, ethanol inhibited IL-1β-induced relaxation. Several mechanisms may regulate ethanol-induced suppression of relaxation. First, IL-1β-induced relaxation is regulated by iNOS-mediated NO release. NO induces relaxation via cyclic guanosine monophosphate (cGMP), through the activation of soluble guanylate cyclase (sGC) in smooth muscle cells. Ethanol may impair the ability of the vasculature to vasodilate, by preventing NO-mediated sGC activation in smooth muscle cells. However, this is unlikely, because ethanol does not inhibit sodium nitroprusside-induced relaxation, which induces sGC activation.[27] Alternatively, ethanol could inhibit eNOS activity, thereby decreasing eNOS-derived NO and downregulating IL-1β-induced iNOS induction. Indeed, ethanol inhibits endothelium-dependent relaxation in response to acetylcholine, which is mediated by eNOS-derived NO.[27] However, this is also unlikely, because eNOS mRNA was not inhibited by ethanol in the present study. Ethanol may inhibit the upregulation of iNOS expression induced by IL-1β. The present study indicated that ethanol suppresses IL-1β-induced

iNOS mRNA expression 1 and 3 hr after treatment and iNOS protein expression 3 hr after treatment. It is reasonable that IL-1β induced iNOS mRNA expression 1 hr after addition, because relaxation was observed at 1.5 hr, at which point iNOS protein was expressed. Furthermore, in porcine smooth muscle cells and lung alveolar macrophages, ethanol negatively regulates iNOS transcription and protein production.[28, 29] These studies also support the possibility that ethanol inhibits iNOS protein expression. Although iNOS is expressed in both endothelial and smooth muscle cells after IL-1β treatment,[26] ethanol may have inhibitory effects on endothelial cell iNOS expression, and not smooth muscle cells, because the relaxation response was not observed in denuded SMAs. Thus, the present study shows that the endothelium plays a critical role in the *in vitro* inhibitory effect of ethanol on IL-1β-induced vasorelaxation. Husain et al.[30, 31] reported that chronic ethanol ingestion induces hypertension in rats. Chronic ethanol ingestion is related to aortic inflammation, oxidative endothelial injury, and downregulation of the aortic endothelial NO-generating system leading to a loss of the endothelium-dependent vascular relaxation response and hypertension in rats.[30] Thus, the endothelium also plays a major role in chronic ethanol-induced hypertension. In experiments similar to those conducted in the

present study, when the endothelium was intact or was not injured, ethanol may have played a protective role in endotoxin-induced hypotension. However, in experiments involving chronic ethanol ingestion, when the endothelium is injured, ethanol seems to be related to an increase in blood pressure. Further investigation is required to determine the validity of this explanation.

In conclusion, in rat SMA, ethanol was considered to inhibit IL-1β-mediated vasorelaxation probably by suppressing endothelium iNOS expression. This study provides the first evidence of ethanol-induced inhibition of IL-1β-mediated vasorelaxation.

Conflict of Interest

The authors declared no conflicts of interest.

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Figure Legends

Figure 1.

Representative traces showing the effect of inhibitors and endothelium denudation on IL-1β-induced relaxation of Ph pre-contracted rat SMAs.

Representative traces showing the effect of inhibitors and endothelium denudation on IL-1β–induced relaxation of Ph precontracted rat SMAs. (a) Time control trace showing a sustained contractile response to Ph over a 3 hr period. (b) A contractile response followed by a gradual relaxation response induced by IL-1β. (c) Absence of IL-1β–induced contractile response in the presence of IM. (d) Absence of IL-1β–induced relaxation in the presence of Chx and (e) Absence of IL-1β–induced contractile and relaxation responses in endothelium-denuded SMAs. The arrow and dashed line indicate when IM and Chx, and IL-1β were added, respectively.

SMA: superior mesenteric arteries; Ph: phenylephrine; IL: interleukin; IM: indomethacin; Chx: cycloheximide.

Figure 2.

Summary data showing the effects of various inhibitors and endothelium denudation on IL-1β-induced relaxation. Results represent the mean ± SEM, and are expressed as a percent of the contraction elicited by Ph. †$P < 0.01$ vs. control.

SEM: standard error of mean; IL: interleukin.

Figure 3.

Representative traces showing the effect of inhibitors and ethanol on IL-1β-induced relaxation responses to Ph pre-contracted isolated rat SMAs in the presence of IM. Representative traces showing the effect of inhibitors and EtOH on IL-1β–induced relaxation responses to Ph precontracted isolated rat SMAs in the presence of IM. (a) Gradual relaxation response induced by IL-1β. (b) Absence of IL-1β–induced relaxation in the presence of L-NA. (c) Absence of IL-1β–induced relaxation in the presence of 1400W. (d) Absence of IL-1β–induced relaxation in the presence of AMT. (e and f) Inhibition of IL-1β–induced relaxation by 50 mM (e) and 100 mM (f) EtOH. The arrow and dashed line indicate when IM and various inhibitors and IL-1β were added, respectively.

SMAs: superior mesenteric arteries; Ph: phenylephrine; IL: interleukin; IM: indomethacin; Chx: cycloheximide L-NA: nitro-L-arginine; AMT; 2-amino-5,6-dihydro-6-methyl-AQ3 4H-1,3-thiazine hydrochloride; EtOH; ethanol.

Figure 4.

Summary data showing the effects of various NO synthase inhibitors and ethanol (50 mM and 100 mM) on IL-1β-induced relaxation. Results represent the mean ± SEM, and are expressed as a percent of the contraction elicited by Ph. *$P < 0.01$ vs. IM and IL-1β.

Figure 5.

Effect of endothelium denudation on IL-1β-induced iNOS expression

Effect of endothelium denudation on iNOS protein expression induced by IL-1β. iNOS and actin were evaluated by western blot analysis in IL-1β-treated SMAs with or without endothelium (upper). The relative iNOS protein expression is expressed as a percent of the maximal iNOS band (lower). *$P < 0.01$ vs. intact

IL-1β.

iNOS: inducible nitric oxide synthase; IL: interleukin.

Figure 6.

Effect of EtOH on IL-1β-induced upregulation of iNOS expression.

Effect of ethanol (50 and 100 mM) on iNOS expression induced by IL-1β. iNOS and actin were analyzed by western blot in IL-1β-treated SMAs with endothelium (upper). The relative iNOS protein expression was expressed as a percent of the maximal iNOS band (lower).*$P < 0.05$ vs. IL-1β.

EtOH: ethanol; IL: interleukin; iNOS: inducible nitric oxide synthase; SMAs: superior mesenteric arteries.

Figure 7.

Relative mRNA expression profile of iNOS and eNOS in SMAs.

Expression levels were determined by real-time PCR, and then normalized to the expression of *Hprt1*. (A) With regard to iNOS, data are expressed as a comparative ratio to IL-1β-treated rat lung. (B) For quantification of eNOS expression, we employed the ΔΔCt method, because adequate reference

samples for the standard curve could not be found. Data were expressed relative to the value of untreated SMA.*; $P < 0.05$ versus IL-1β 3hr.

mRNA: messenger RNA; eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; SMAs: superior mesenteric arteries; PCR: polymerase chain reaction; HPRT: hypoxanthine phosphoribosyl transferase; IL: interleukin; $\Delta\Delta C_t$: comparative cycle threshold; N.D.: not detected.N.D.; not detected.

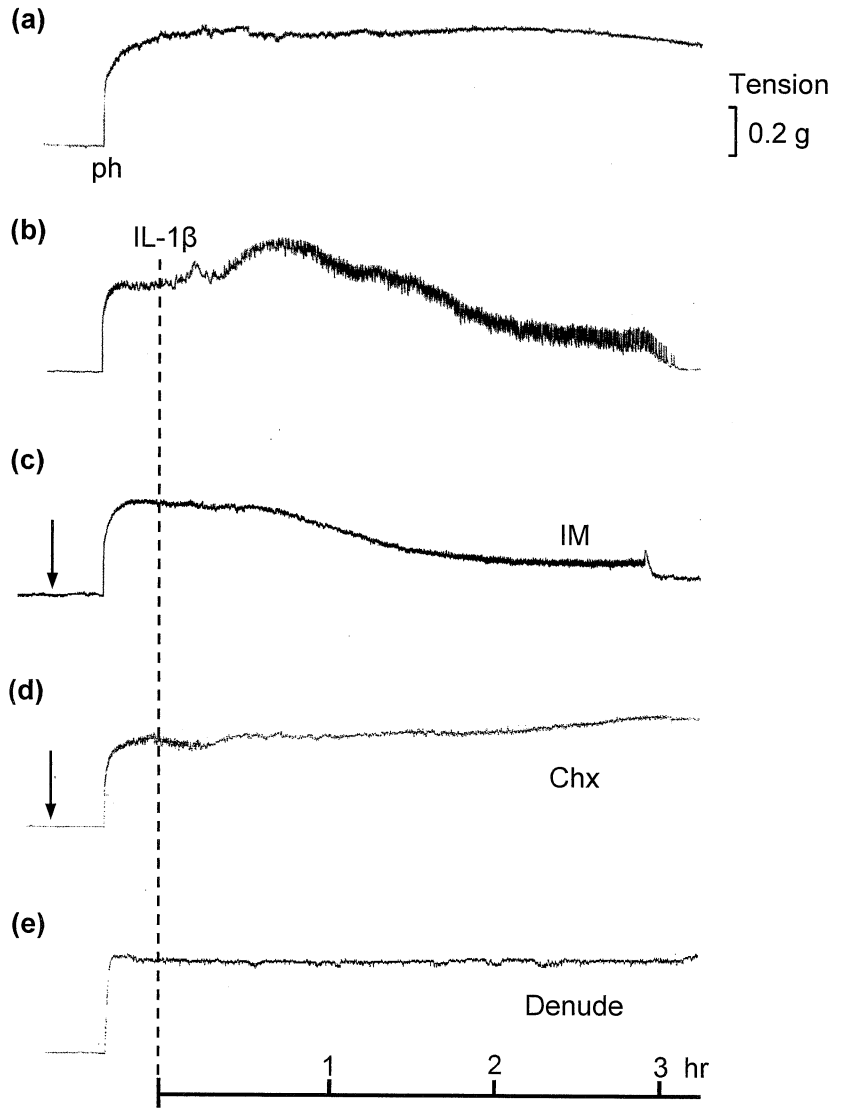


Figure 1

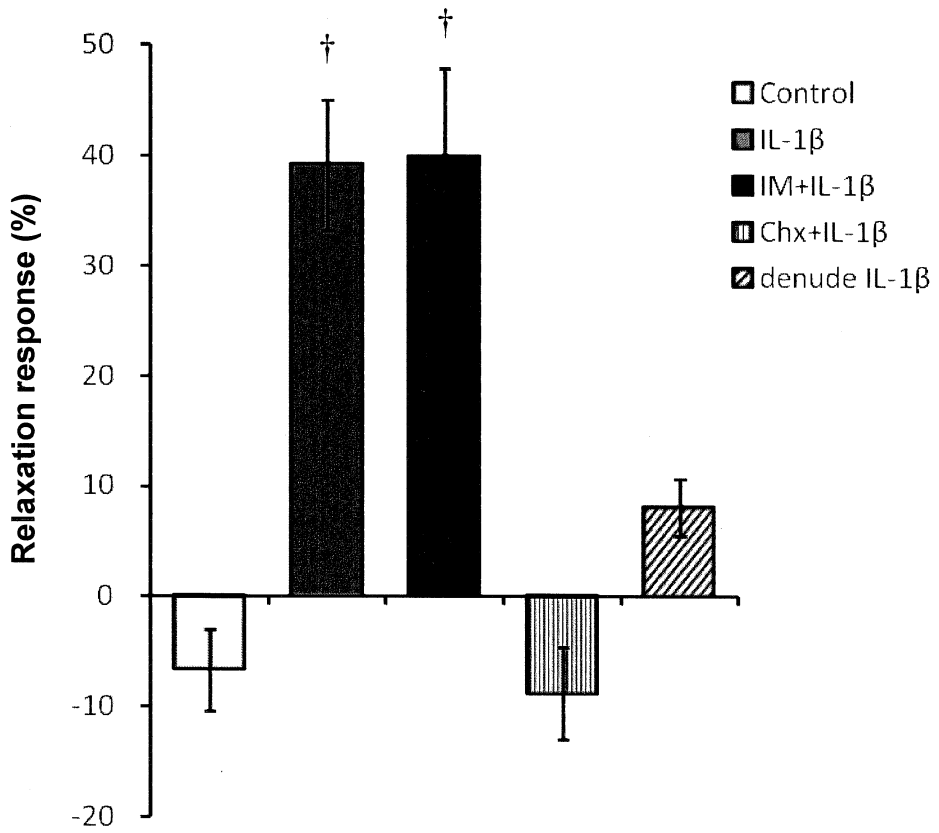

Relaxation response (%)
50
40
30
20
10
0
-10
-20
†
†
Control
IL-1β
IM+IL-1β
Chx+IL-1β
denude IL-1β

Figure 2

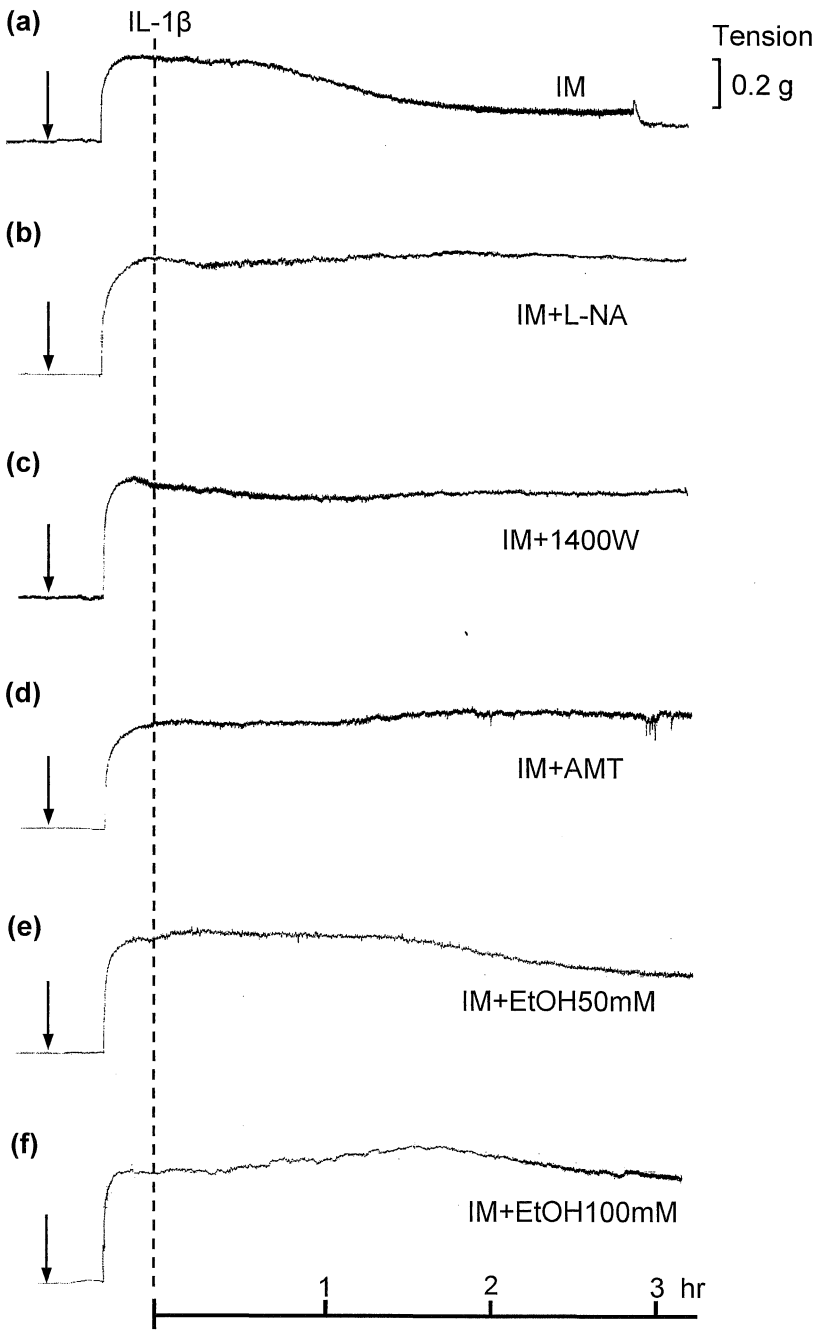

(a)
IL-1β
Tension
0.2 g
IM
(b)
IM+L-NA
(c)
IM+1400W
(d)
IM+AMT
(e)
IM+EtOH50mM
(f)
IM+EtOH100mM
1
2
3 hr

Figure 3

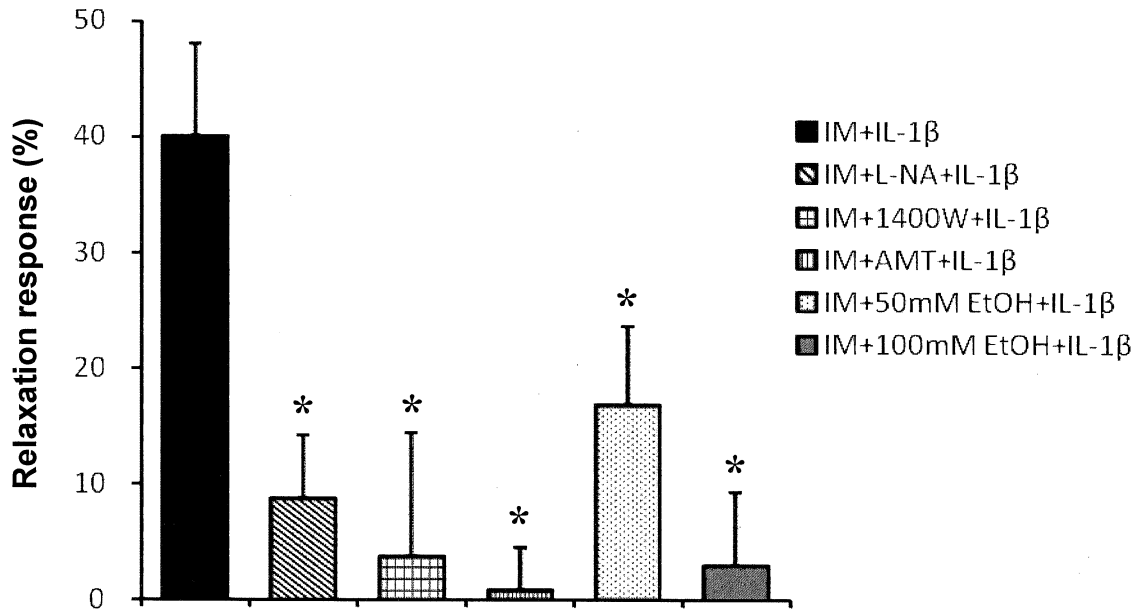

Relaxation response (%)
50
40
30
20
10
0
*
*
*
*
*
IM+IL-1β
IM+L-NA+IL-1β
IM+1400W+IL-1β
IM+AMT+IL-1β
IM+50mM EtOH+IL-1β
IM+100mM EtOH+IL-1β

Figure 4

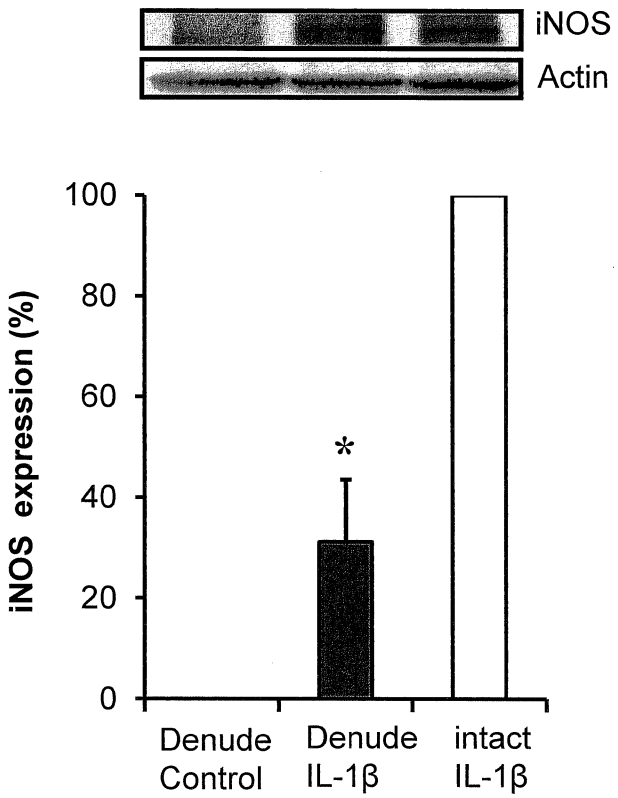

iNOS
Actin
100
80
60
40
20
0
iNOS expression (%)
*
Denude Control
Denude IL-1β
intact IL-1β

Figure 5

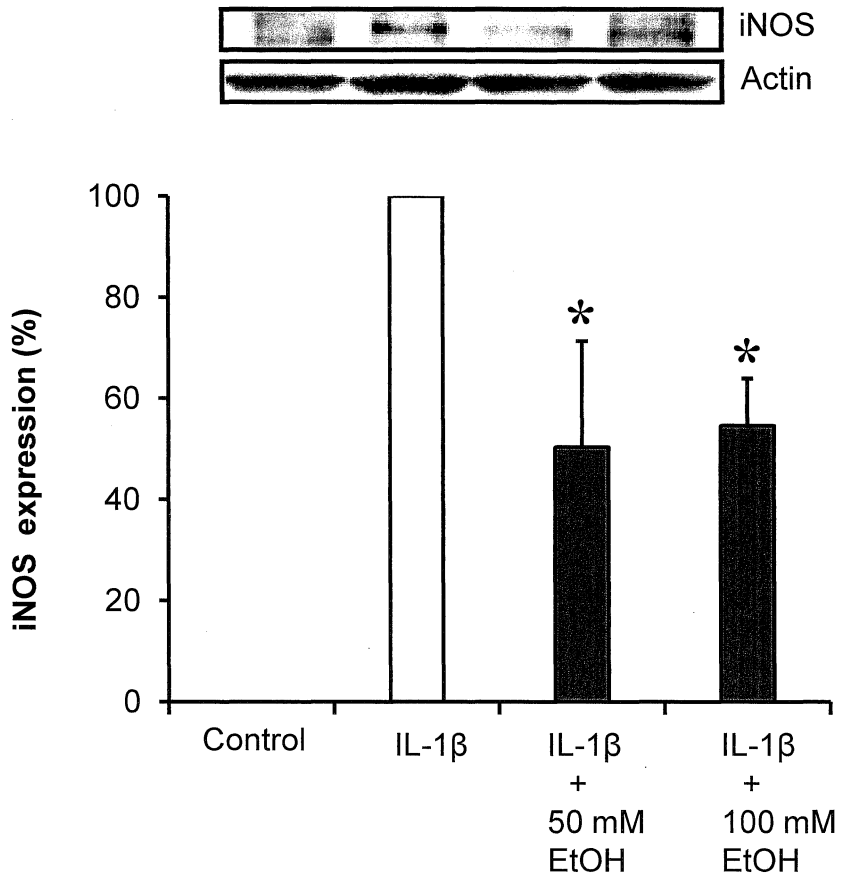
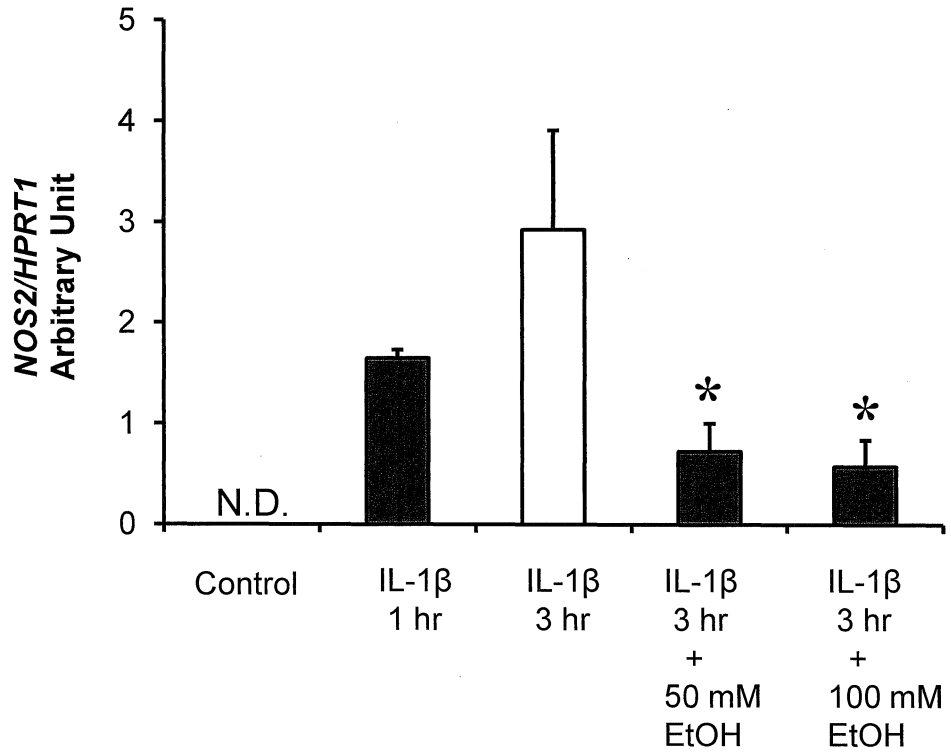

iNOS
Actin
iNOS expression (%)
100
80
60
40
20
0
*
*
Control
IL-1β
IL-1β + 50 mM EtOH
IL-1β + 100 mM EtOH

Figure 6

(a)


NOS2/HPRT1
Arbitrary Unit
5
4
3
2
1
0
N.D.
*
*
Control
IL-1β
1 hr
IL-1β
3 hr
IL-1β
3 hr
+
50 mM
EtOH
IL-1β
3 hr
+
100 mM
EtOH

(b)

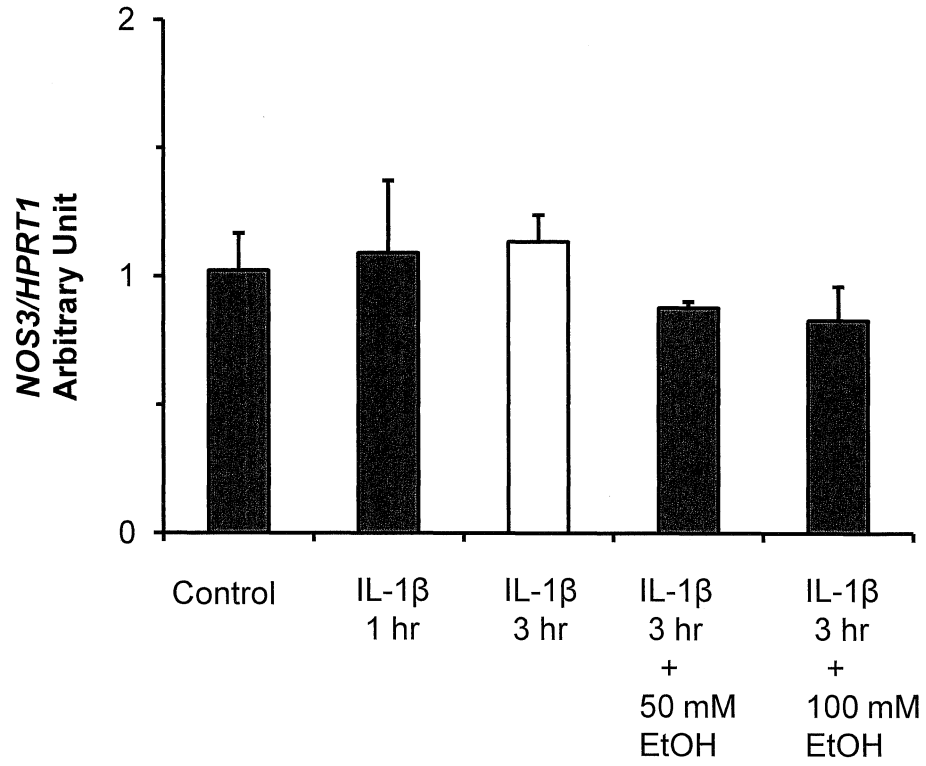

NOS3/HPRT1
Arbitrary Unit
2
1
0
Control
IL-1β
1 hr
IL-1β
3 hr
IL-1β
3 hr
+
50 mM
EtOH
IL-1β
3 hr
+
100 mM
EtOH

Figure 7