Article

Effects of Activin A on the Activities of the Mouse Peritoneal Macrophages

Xuejun Zhang[1], Yang Li[2], Guixiang Tai[1], Guiyue Xu[1], Pengyu Zhang[1], Yu Yang[1], Fengxue Lao[1] and Zhonghui Liu[1, 3]

Activin A is a kind of pre-inflammatory factor that belongs to the transforming growth factor-β (TGF-β) superfamily. To investigate the effect and mechanism of activin A on the activities of mouse macrophages, the secretion of NO in the supernatant of cultured mouse peritoneal macrophages was examined by NO assay kit, and the expression of iNOS, ActRIIA and ARIP2 mRNA in mouse peritoneal macrophages was analyzed by RT-PCR. The results showed that activin A stimulated the secretion of NO and the expression of iNOS mRNA in non-activated mouse macrophages in a time- and dose-dependent manner. In contrast, activin A in the same concentration inhibited the secretion of NO in LPS-activated mouse macrophages in a dose-dependent manner. ActRIIA was highly expressed on macrophages, and activin A upregulated the ActRIIA mRNA expression in macrophages. Anti-ActRIIA antibody can block the secretion of NO from the macrophages stimulated by activin A. Furthermore, RT-PCR analysis revealed that activin A enhanced the ARIP2 mRNA expression in macrophages. These results indicated that Activin A may be a weak activator compared with LPS to mouse macrophages, and activin A may modulate the secretion of NO through ActRIIA-ARIP2 signal pathway in mouse macrophages. *Cellular & Molecular Immunology*. 2005;2(1):63-67.

Key Words: activin A, ActRIIA, macrophage, RT-PCR, NO

Introduction

Activin A is also called immunologic inhibitor-P (1), as a kind of pre-inflammatory factor, it has attracted more attention in recent years. In particular, activin A is connected with fibrosis of inflammatory process, a lot of experiments have confirmed that activin A is an important factor of inducing fibrosis of liver of animals (2). Related study also showed that activin A could inhibit the IL-1β secretion from macrophages stimulated by LPS (3). However, so far it is not clear whether activin A can directly affect the function of macrophages and its regulation mechanism. In this study, we found that type IIA of activin receptor (ActRIIA) was highly expressed in mouse macrophages, furthermore, the signal transduction of ActRIIA was controlled by activin receptor-interacting protein (ARIP), which was found in recent years (4). Therefore, that activin A regulates the activities of macrophages in the state of physiology and pathology will help us to understand the effects and mechanisms of activin A as a kind of pre-inflammatory factor.

Materials and Methods

Animals

C57BL/6 mice were obtained and maintained in the Laboratory Research Center, Jilin University. Female mice were used at 4-6 weeks of age.

Activin A-stimulating macrophages assay

Mouse peritoneal macrophages were obtained by lavaging the peritoneal cavity with 5 ml of sterile IMDM medium and the cells were washed three times with IMDM medium. All the cells were cultured in a humidified incubator containing 5% CO_2 at 37°C for 1 h. Non-adherent cells were washed

[1]Department of Immunology, School of Basic Medical Sciences, Jilin University, Changchun 130021, China.

[2]The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, P.R.China.

[3]Corresponding to: Dr. ZhongHui Liu, Department of Immunology, School of Basic Medical Sciences, Jilin University, Changchun 130021, China. Tel: +86-431-561-9476, Fax: +86-431-563-9362, E-mail: liuzh@mail.jlu.edu.cn.

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Abbreviations: ActRIIA, type II A of activin receptor; ARIP, activin receptor-interacting protein; NO, nitric oxide; LPS, lipopolysaccharide; iNOS, inducibal nitric oxide synthase.

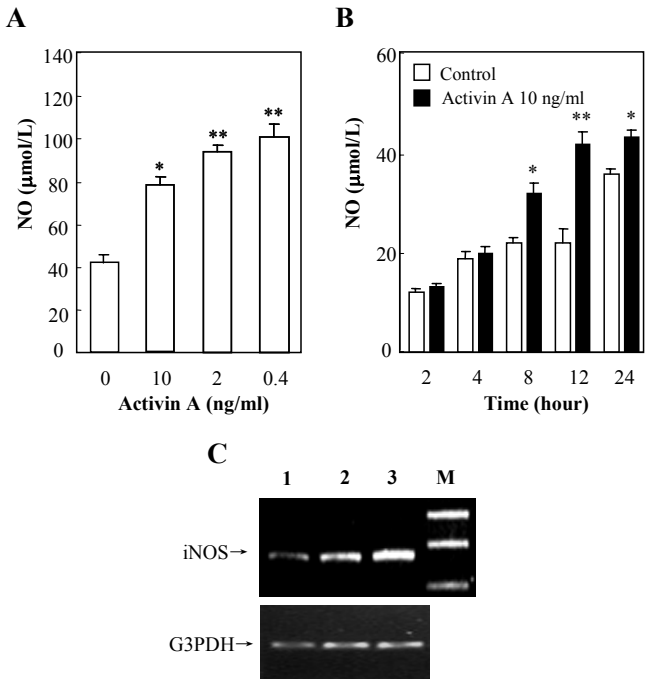

Figure 1. Effects of activin A on the secretion of NO and expression of iNOS mRNA in mouse macrophages. (A) Macrophages were incubated for 24 h with 0.4-10 ng/ml of activin A. (B) Macrophages were incubated with 10 ng/ml of activin A for 2 - 24 h. *$p < 0.05$, **$p < 0.01$ compared with the control of activin A 0ng/ml by Student's *t*-test. (C) Expression of iNOS mRNA in macrophages stimulated with 10 ng/ml and 2 ng/ml of activin A. Lane 1, control; lane 2, activin 2 ng/ml; lane 3, activin 10 ng/ml; M, molecular weight marker.

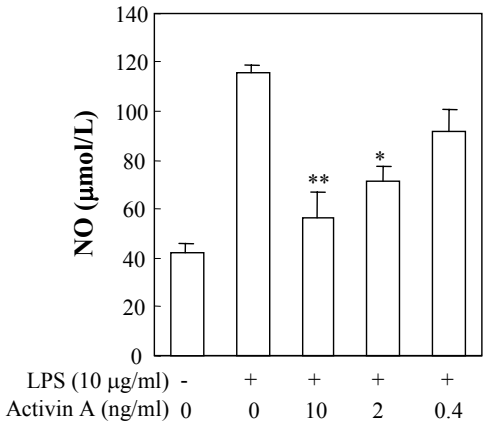

Figure 2. Effects of activin A on the secretion of NO in LPS-activated macrophages. Macrophages were incubated for 24 h with 10 μg/ml of LPS in the presence or the absence of 0.4-10 ng/ml of activin. *$p < 0.05$, **$p < 0.01$ compared with the 10 μg/ml of LPS by Student's *t*-test.

away directly. The adherent cells were seeded in 12-well plates at a density of 0.5×10^5 cell/ml, and activin A was added into the wells at different concentration to stimulate the cells for 24 h. The supernatants were collected by centrifugation at 10,000 rpm for 20 min, then stored at -30°C for analysis of NO by NO assay kit.

Assay of NO level

NO levels of culture supernatants were determined by NO kit according to the manufacturer's protocol. The optical densities of the assay samples were measured at 550 nm. NO concentrations were calculated by comparison with standard samples.

Analysis of iNOS, ActRIIA and ARIP2 mRNA expression by RT-PCR

Total RNA was isolated by Trizol reagent according to manufacturer's protocol (Invitrogen). iNOS, ActRIIA, and ARIP2 cDNA fragments were amplified by one step RT-PCR Kit (Takara) using 1 μg of total RNA. The primers are ActRIIA: 5'-ATT GGC CAG CAT CCA TCT CTT G-3' and 5'-TGC CAC CAT CAT AGA CTA GAT TC-3'; ARIP2: 5'-GGA GAG CAG TCA GAT ATG AAC GG-3' and 5'-CTT GTG GCA ATA CTT CTC TGG TG-3'; iNOS: 5'-CAC AAG CTG GCT CGC TTT GC-3' and 5'-TGG CCC TGC TCC CCG TGG AGC; G3PDH: 5'-GAT TGT TGC CAT CAA CGA CC-3' and 5'-GTG CAG GAT GCA TTG CTG AC-3'. G3PDH was used as positive control. The PCR products were analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide.

Western blot for ActRIIA

The macrophages were incubated in a lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, and 1 μg/ml aprotinin). The lysate was centrifuged at 12,000 rpm for 30 min. The supernatants were harvested, and the proteins were fractionated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Then the membranes were incubated with an anti-ActRIIA antibody (diluted 1:1,000), followed by incubation with a horseradish peroxidase-conjugated second antibody. Labeled proteins were detected by chemiluminescence (ECL-Plus; Amersham Pharmacia Biotech) (5).

Statistical analysis

Data were presented at the mean ± SD. Comparisons between groups were conducted by student's *t*-test.

Results

Activin A enhances the secretion of NO in the macrophages

To study the effect of activin A on secretion of NO in mouse peritoneal macrophages, we examined the secretion of NO in the culture supernatant of mouse peritoneal macrophages stimulated with activin A alone. The results showed that addition of 10 ng/ml of activin A significantly stimulated secretion of NO in mouse macrophages in the dose- and

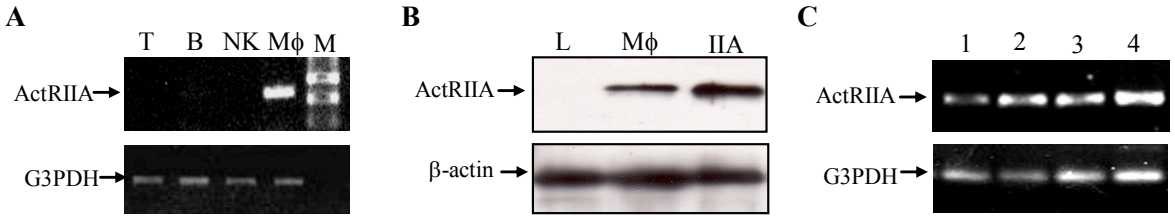

Figure 3. Expression of ActRIIA mRNA in mouse macrophages. (A) ActRIIA mRNA isolated from various immunocytes was identified by RT-PCR. (M, molecular weight marker; Mϕ, macrophages; T, T lymphocytes; B, B lymphocytes; NK, natural killer cells.) (B) Mature ActR II A protein was identified by Western blot. (L, lymphocytes; Mϕ, macrophages; II A, recombinant ActRIIA from COS7 cells.) (C) Effects of activin A on ActRIIA mRNA expression in macrophages. Lane1, control (2 h); lane 2, activin 10 ng/ml (2 h); lane3, control (8 h); lane 4, activin 10 ng/ml (8 h).

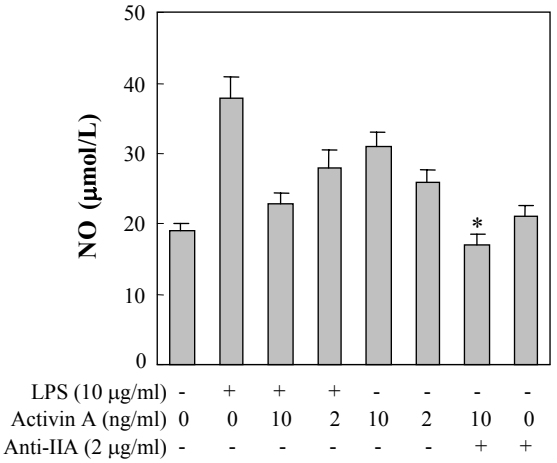

Figure 4. Effects of Anti-ActRIIA antibody on NO secretion in activin A-stimulated macrophages. Macrophages were incubated for 24 h with 0.4-10 ng/ml of activin in presence or absence of 10 μg/ml of LPS or 2 μg/ml of anti-ActRIIA antibody. *$p < 0.05$, compared with the 10 ng/ml of activin A by Student's *t*-test.

time-dependent manner (Figures 1A, 1B). RT-PCR analysis also revealed that macrophages stimulated by activin A highly expressed iNOS mRNA (Figure 1C).

Activin A inhibits the secretion of NO in the LPS-activated macrophages

To explore the effects of activin A on the secretion of NO in activated macrophages, the macrophages were stimulated by LPS (10 μg/ml) with or without activin A (10 ng/ml, 2 ng/ml and 0.4 ng/ml). The results showed that activin A inhibited sectetion of NO from mouse peritoneal macrophages activated by LPS (Figure 2).

Expression of ActRIIA on mouse macrophages

To determine the regulatory mechanism of Activin, the expression of ActRIIA mRNA in mouse macrophages was observed by RT-PCR. The results showed that ActRIIA was highly expressed in mouse macrophages, but not obviously expressed in other immunocytes (Figure 3A). Western bloting further confirmed expression of the mature ActRIIA protein on macrophages (Figure 3B). RT-PCR analysis revealed that 10 ng/ml of activin up-regulated the expression of ActRIIA mRNA in macrophages after cultured for 2 h and 8 h (Figure 3C).

Blocking effects of Anti-ActRIIA antibodies on activin A

To confirm that activin A regulates the NO secretion from macrophages through ActRIIA, the function of ActRIIA was blocked with anti-ActRIIA antibody. After anti-ActRIIA antibody binding to ActRIIA, the secretion of NO from the activin-stimulated macrophages decreased (Figure 4).

Effects of activin A on the expression of ARIP2 mRNA

To further explore the activin signaling pathway, RT-PCR analysis revealed that 10 ng/ml of activin A upregulated the expression of ARIP2 mRNA in macrophages after cultured for 2 h and 8 h (Figure 5). These data suggested that activin A may regulate activities of macrophages through the ActRIIA-ARIP2 pathway.

Dicussion

Activins, a homodimer or heterodimer of the βA and βB subunits of inhibin, such as Activin A (βAβA), Activin B (βBβB), Activin AB (βAβB), are multifunctional growth and differentiation factors that belong to the transforming growth factor-β (TGF-β) superfamily (5-7). Activin receptors were identified in gonadal and extragonadal numerous tissueses, exerting various effects as essential regulatory molecules on cell differentiation and growth etc. Two types of activin receptor have been identified, type I and type II, both of which belong to the family of serine/threonine kinase receptors (8). Recent investigations have shown that in the activin-induced signal transduction, activins firstly bind to type II receptor, and then type II receptor phosphorylates type I receptor, resulting in the activation of type I receptor and the signal transduction (4, 9). Once type I receptors form a complex with type II receptors, they become phospho-rylated by constitutively active type II receptors, mainly in

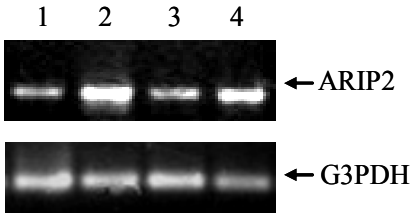

Figure 5. Effects of activin A on ARIP2 mRNA expression in macrophages. Macrophages were cultured in the absence or presence of activin A (10 ng/ml). Lane 1, activin (2 h); lane 2, control (2 h); lane 3, activin (8 h); lane 4, control (8 h).

juxtamembrane GS domain which is rich in glycine, serine and threonine. These findings suggest that type II receptors are the main signal regulators for down-stream signaling. Thus the downstream propagation of the activin-induced signal may be controlled by regulating the action of the type II receptor.

In the recent years, it has been known that NO can mediate macrophage cytotoxicity, regulate blood pressure, and participate in neurotransmission (10, 11), endogenous NO can contribute to destruction of the host's joints and kidneys or to its survival from viral infection (12, 13). NO is a short-lived radical that is produced by enzymatic oxidation of L-arginine (11). We have known so far that the production of NO in the body is regulated through controlling the activity of nitric oxide synthase (NOS), at least two different NO-synthase enzymes exist (14). One is constitutive and requires Ca^{2+} for activity; the other is inducible and acts independent of Ca^{2+}. Inducible NOS was first found in macrophages activated by LPS and IFN-γ; and since then it has been found in many cells (15).

In this study we continue to investigate the expression of inflammatory factor-NO, and detect the effect of activin A on secretion of NO. Activin A in a certain concentration enhanced the secretion of NO and iNOS mRNA expression in the non-activated primary cultured mouse peritoneal macrophages. In contrast, activin A in the same concentration inhibited secretion of NO in the primary cultured mouse peritoneal macrophages activated by LPS. As a pluripotent growth factor, activin A may play important roles for pro-inflammatory or anti-inflammatory response in inflammatory regulation. The different effects of activin A may be correlated with the activated state of macrophages and its secretion of inflammatory factors. These findings have an extremely important significance for further exploring the function of activin A in the state of physiology and pathology.

ActRIIA plays a key role in activin A signal transdution, and is also a receptor that is most clearly studied in mediating activin A signal transduction so far. In our research, we found that ActRIIA was highly expressed on mouse macrophages, but not obviously expressed on other immunocytes. RT-PCR analysis also revealed that activin A upregulated the expression of ActRIIA mRNA in macrophages. After anti-ActRIIA antibody binding to ActRIIA, it blocked the secretion of NO from the activin-stimulated macrophages. These results suggested that ActRIIA might be a major receptor in macrophages to mediate activin signal transduction. On the other hand, in recent studies, a novel finding on the signaling transduction of activin A is that there are a group of activin receptor interacting proteins, which regulate activities of activin receptors in the cells. In previous research, we found that both of ARIP1 and ARIP2 can specifically bind ActRIIA, which control activin's signaling in some cells. Different from ARIP1, ARIP2 is widely expressed in tissues, whereas ARIP1 is abundant only in the tissue of nerve (4, 9). In this study, RT-PCR analysis revealed that activin A upregulated the ARIP2 mRNA expression and ActRIIA mRNA expression simultaneously. These data suggested that the effect of activin A on mouse macro- phages might be through the signal transduction pathway of ActRIIA-ARIP2 to regulate the transcription and secretion of NO.

Above results suggest that activin may be a weak activator, which stimulates the secretion of NO at the physiological concentration. In contrast, it is an inhibitor on pathologically activated macrophages. The action of activin may be the results of competition of different proteins in cross-talking pathways of signal transduction. The activin-signaling transduction mediated by ActRIIA-ARIP2 may be mainly signaling pathway that activin A regulates the activities of macrophages.

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