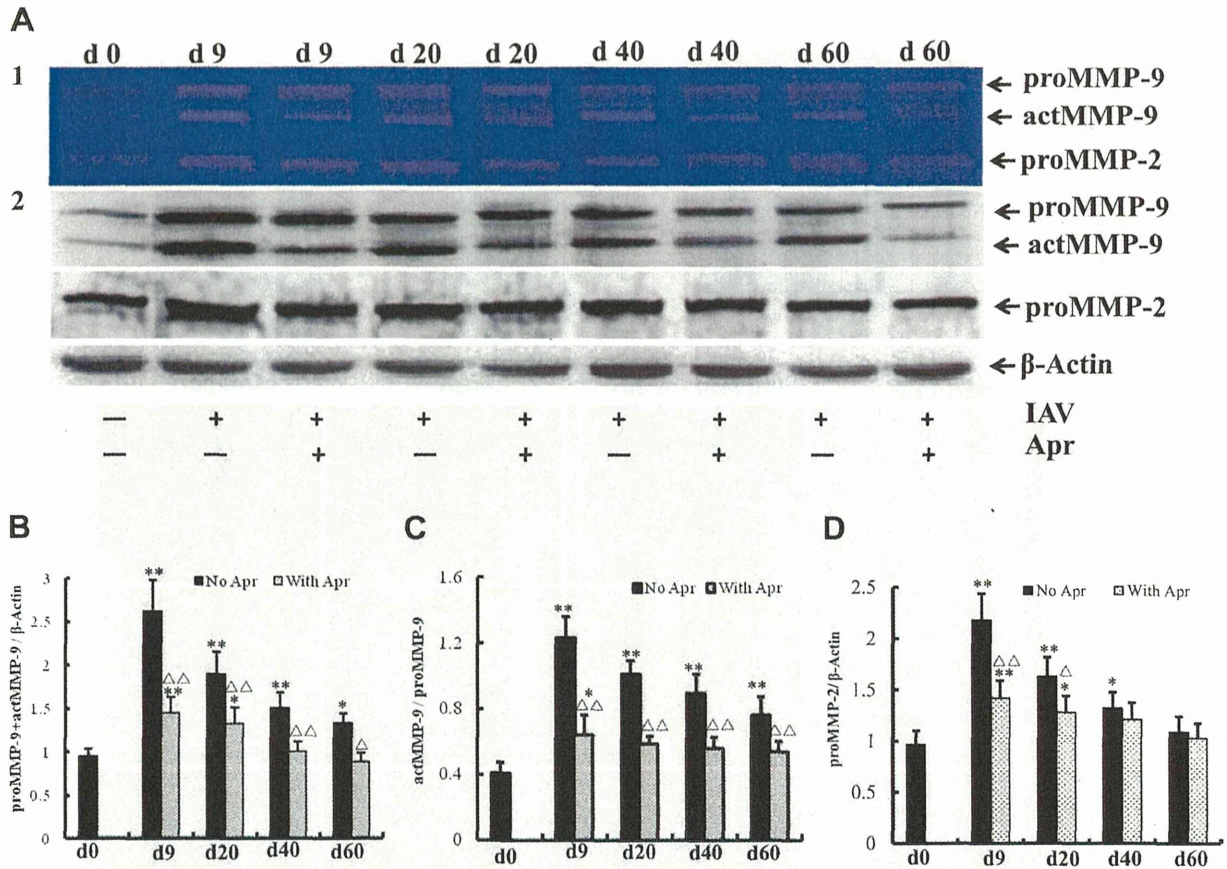

Fig. 3. Upregulation and activation of pro-matrix metalloproteinases (pro-MMPs) in the myocardium and effects of trypsin inhibitor. *A*: kinetics of activity and upregulation of MMPs in hearts as determined by zymography (*1*) and Western blot analysis (*2*) from *days 0* to *60* postinfection. β-Actin was the internal control. *B–D*: densitometry showed significant inhibition of upregulation of pro-MMP9 + active (act)MMP9 (*B*), pro-MMP-2 (*C*), and activation of pro-MMP-9 (*D*) by Apr after IAV infection. Data are averages ± SD of 3 independent experiments from 7–10 mice/group. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. *day 0*; $\triangle P < 0.05$ and $\triangle\triangle P < 0.01$ vs. no Apr treatment at the same time after IAV infection.

degradation, collagen deposition, and Col I and Col III mRNA in the myocardium increased in IAV-induced acute and chronic myocarditis. The differential increase in Col I and Col III mRNA led to a decreased Col I-to-Col III ratio. Fourth, cardiac function was transiently impaired in the acute stage and deteriorated with progressive ventricular dilation during the chronic stage. Finally, trypsin inhibitor aprotinin suppressed pro-MMP-9 activation and cytokine release, alleviated myocardial inflammation, reduced collagen proliferation, and restored collagen metabolism, thus effectively preventing ventricular dilation and DCM for improved cardiac function.

Consistent with our previous study (39), we found that IAV infection significantly upregulated ectopic trypsin, pro-MMPs, and proinflammatory cytokines in acute myocarditis. However, by *day 20* postinfection, although replication of IAV could not be detected in the myocardium, upregulation of trypsin, MMPs, and proinflammatory cytokines persisted during the chronic stage. One possible reason for the persistent upregulation of these factors might be a trypsin-MMP-9-cytokine cycle in the myocardium. Initially, IAV infection induces upregulation of trypsinogen, pro-MMPs, and proinflammatory cytokines through the Toll-like receptor 7/8-myeloid differentiation primary response protein 88-NF-κB/activator protein (AP)-1 signaling pathway in the acute stage (40, 50). Induced trypsinogen is converted to trypsin through autoactivation or by widely distributed endogenous activators (13). Trypsin converts upregulated pro-MMP-9 to active MMP-9 and promotes proinflammatory cytokine secretion through PAR-2 (35, 39, 44). MMP-9 activates proinflammatory cytokines such as IL-6, IL-1β, and TNF-α to active forms (9, 42), which, in turn, promote transcription of trypsinogen and pro-MMPs (50, 51). In this model, the interrelationship of trypsin, MMP-9, and cytokines is a cycle that contributes to persistent upregulation of these factors even after IAV is no longer present in the myocardium. Another reason for the persistent upregulation of these factors might be the effects of inflammatory cells infiltrating the myocardial interstitium. These cells release IL-1β, IL-6, and TNF-α, which enhance the transcription of trypsin and MMPs genes via NF-κB and AP-1 (39, 51). Trypsin and MMP-9 synergistically promote inflammatory cell migration across the ECM to inflammatory loci (39, 53). These migrating cells further induce upregulation of trypsin and MMPs by releasing cytokines in inflammatory loci, leading to persistent upregulation. The colocalization of upregulated trypsin and inflammatory infiltrates we observed during acute and chronic stages suggests a close relationship among these pathogenic factors that results in chronic myocardial inflammation.

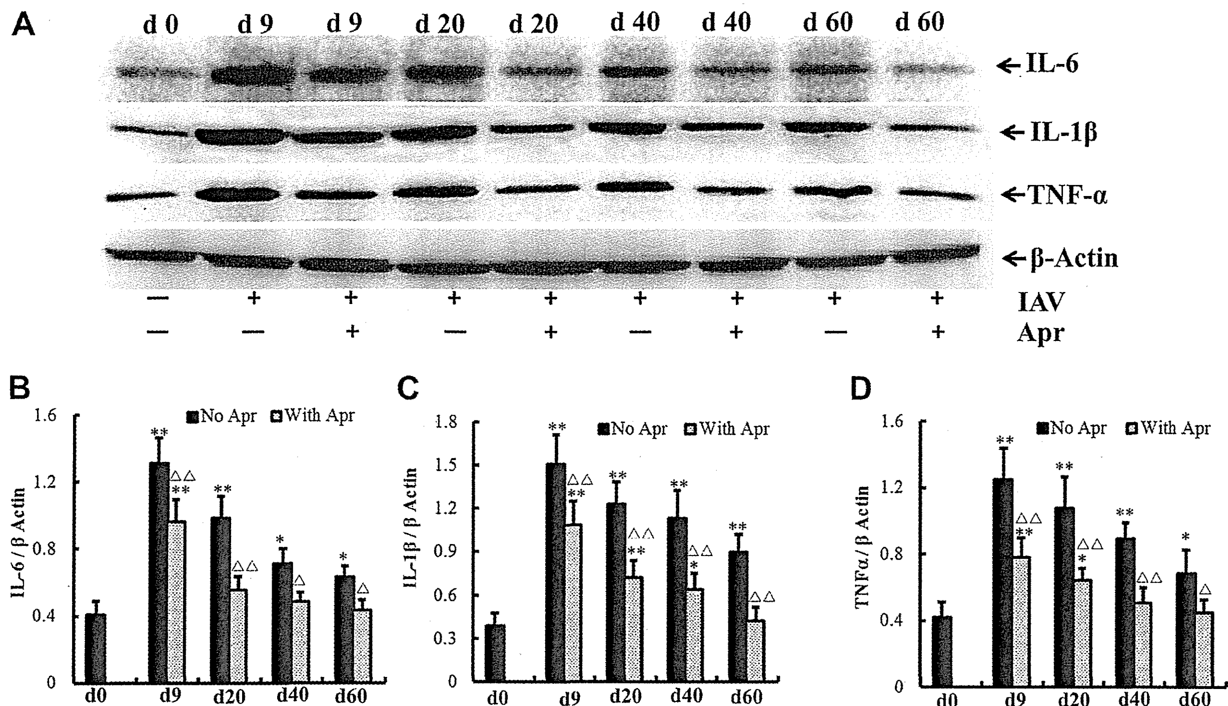

Fig. 4. Cytokine induction in the myocardium and suppression by trypsin inhibitor. *A*: time courses of induction of IL-6, IL-1β, and TNF-α from *days 0* to *60* postinfection by Western blot analysis. β-Actin was the internal control. *B–D*: densitometry showed that Apr significantly inhibited the induction of IL-6 (*B*), IL-1β (*C*), and TNF-α (*D*) during acute and chronic phases of myocarditis. Data are means ± SD of 3 independent experiments from 7–10 mice/group. $*P < 0.05$ and $**P < 0.01$ vs. *day 0*; $\triangle P < 0.05$ and $\triangle\triangle P < 0.01$ vs. no Apr at the same time after IAV infection.

Although IAV replication, myocardial inflammation, and expression of MMPs, trypsin, and proinflammatory cytokines were significantly inhibited by *day 9* postinfection in mice treated with aprotinin, the pathological changes in the myocardium persisted without recovery until after 40–60 days of continuous treatment with aprotinin. These findings suggested that trypsin might be involved in the pathological process throughout acute and chronic phases of myocarditis after IAV infection.

In the trypsin-MMP-9-cytokine cycle, the regulation of trypsin expression is crucial for cycle maintenance. In the acute stage, trypsin was significantly upregulated by IAV infection and peaked on *day 9* postinfection. Induced trypsin activates PAR-2 to evoke cytokine release, which, in turn, upregulates trypsin expression through activation of NF-κB and AP-1 (40, 50). However, PAR-2 is the "sensory" arm of a negative feedback mechanism to downregulate trypsin expression (25). Activated PAR-2 forms a complex with β-arrestin and ERK1/2, which effectively prevents ERK1/2 translocation into the nucleus and after transcription of trypsinogen (6, 47). Trypsin expression gradually declined from *day 20* postinfection accompanied by the clearance of IAV. The final level of trypsin depended on the balance between its up- and downregulation mechanisms.

In the present study, both pro-MMP-2 and pro-MMP-9 in the myocardium were upregulated during acute and chronic phases. However, only pro-MMP-9 was activated, probably because pro-MMP-2 is resistant to activation by trypsin (19). MMP-9 also cleaves Col I and Col III, whereas MMP-2 does not (36). Trypsin has three major isoforms. In cardiac tissue, $trypsin_2$ is the major isoform and its abundance is nearly double that of $trypsin_1$, whereas $trypsin_3$ is barely detectable (39). $Trypsin_2$ degrades Col I and is an efficient activator of a prourokinase cascade and procollagenases (33). Consistent with the kinetic expression of trypsin and MMP-9 that we observed in the myocardium, circulating ICTP, which reflects degradation of Col I, increased prominently in early stages and then slightly in late stages. In response to increased collagen degradation, the lost collagen was replaced by newly synthesized collagen, which was distributed around inflammatory loci in the acute phase and extensively proliferated across the ventricular interstitium in the chronic phase. Col I and Col III are the main components of the myocardial ECM. During collagen proliferation, Col I and Col III mRNA (especially Col III) was significantly increased. The differential increase of Col I and Col III mRNA led to a decreased Col I-to-Col III mRNA ratio in the myocardium. The increased ICTP and decreased relative proportion of Col I and Col III mRNA indicated that in ECM remodeling, properly cross-linked Col I was increasingly degraded and replaced with poorly cross-linked Col III. Col I provides substantial tensile and stiffness to prevent slippage and overstretching of myocytes, whereas Col III has greater elasticity (54). These changes might promote ventricular myocyte slippage and chamber dilation that contribute to wall thinning and systolic dysfunction in the development of DCM.

The reasons for the differential increase in Col I and Col III expression after IAV infection might be different molecular mechanisms. IL-6, IL-1β, and TNF-α were persistently upregulated during acute and chronic phases after IAV infection. Previous studies (10, 32) have indicated that IL-1β and IL-6

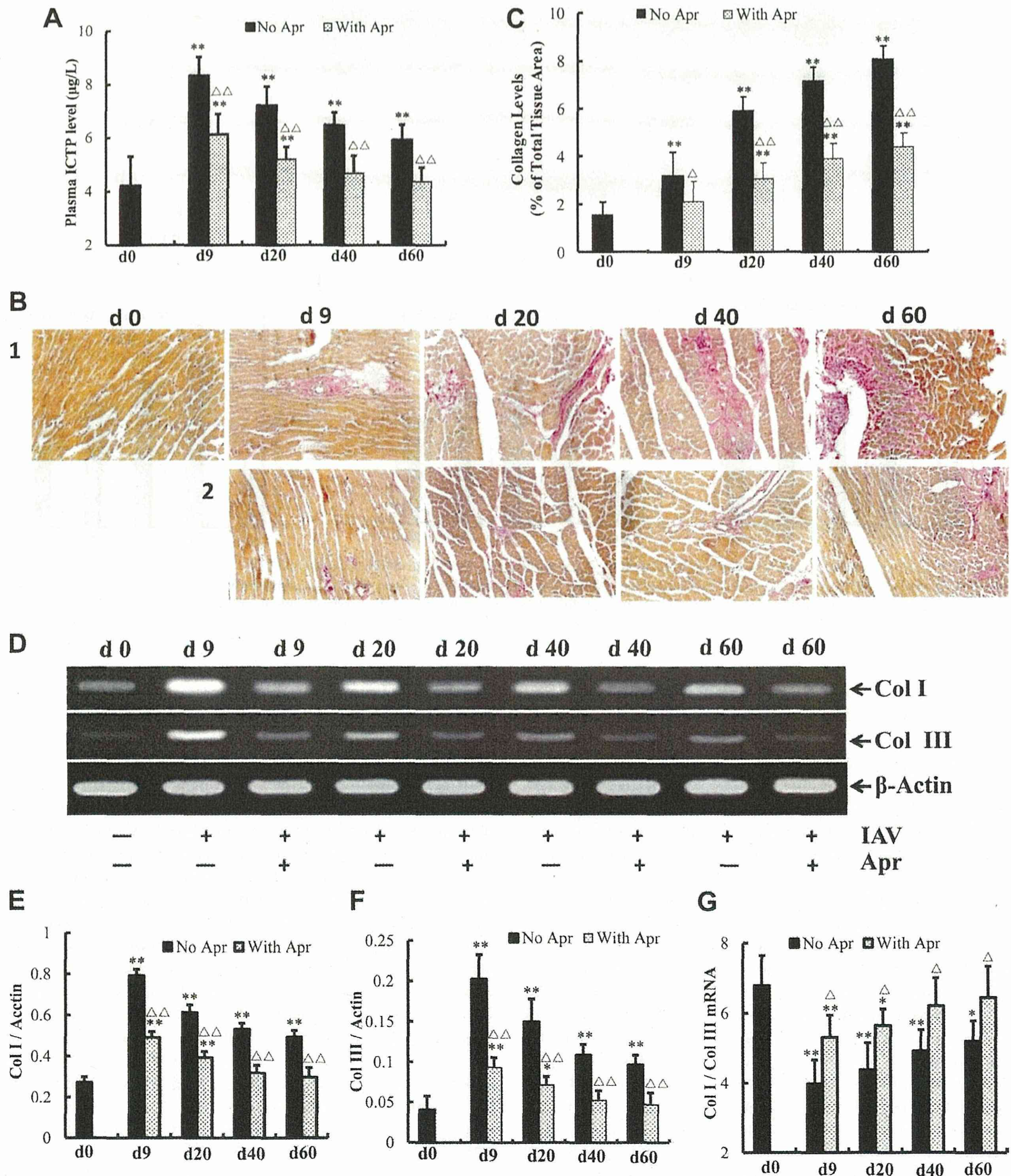

Fig. 5. Increased collagen type I (Col I) degradation, upregulation of collagen synthesis, and effect of trypsin inhibitor. *A*: Col I degradation as measured by plasma Col I cross-linked carboxy-terminal telopeptide (ICTP) during acute and chronic phases (n = 7–10 mice/group). Apr effectively prevented degradation. *B*: proliferation of collagen in the myocardium as identified by van Gieson staining from *days 0* to *60* without (*1*) and with (*2*) Apr treatment. Magnification: ×100. *C*: quantification of collagen as percent area (n = 5 mice/group). *D*: synthesis of Col I and collagen type III (Col III) as assessed by Col I and Col III mRNA using RT-PCR. β-Actin was the internal control. *E–G*: densitometry showed significantly upregulated Col I (*E*) and Col III (*F*) mRNA and a decreased Col I-to-Col III mRNA ratio (*G*) in IAV-induced acute and chronic myocarditis. Data are means ± SD of 3 independent experiments from 7–10 mice/group. *P < 0.05 and **P < 0.01 vs. *day 0*; △P < 0.05 and △△P < 0.01 vs. no Apr treatment at the same time point.

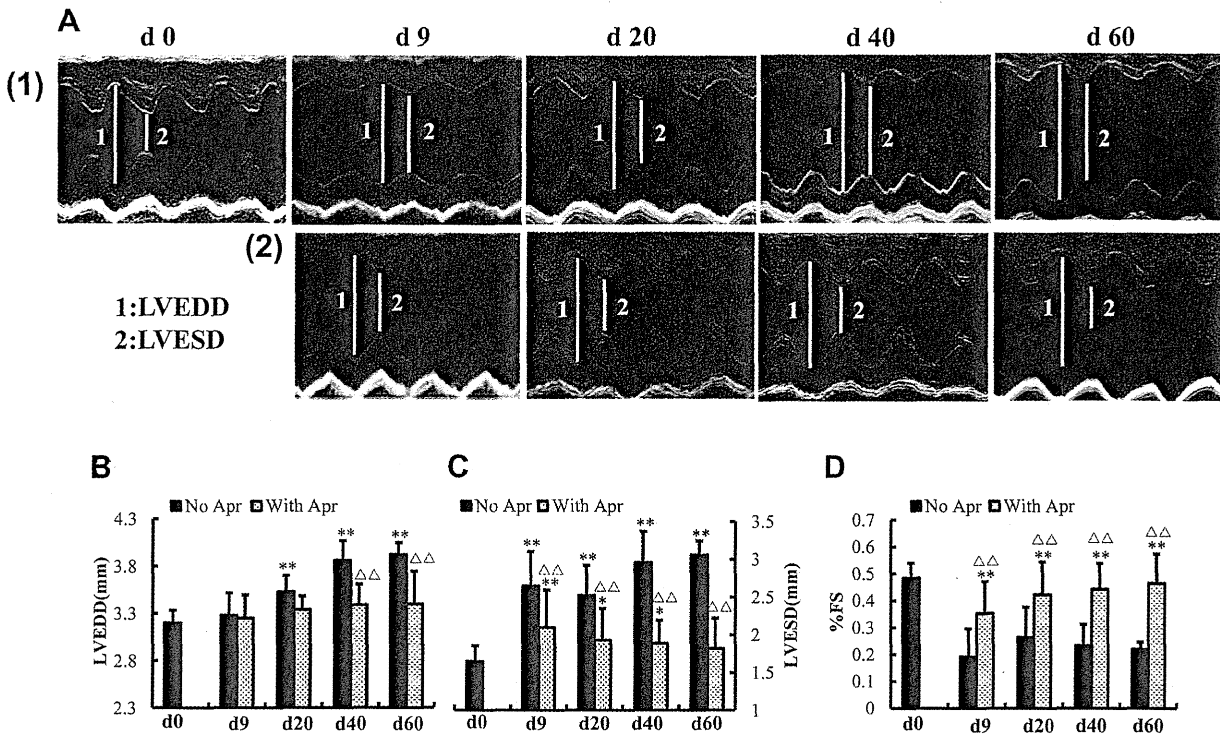

Fig. 6. Progressive left ventricular (LV) dilation and restoration by trypsin inhibitor. *A*: representative M-mode echocardiogram images of mice from *days 0* to *60* postinfection without (*1*) or with (*2*) Apr treatment. *B–D*: measurements of LV end-diastolic dimension (LVEDD; *B*), LV end-systolic dimension (LVESD; *C*), and fractional shortening (FS; in %; *D*) at the indicated time points showed inhibition of LV dilation and improvement of LV function with Apr after IAV infection. n = 7–10 mice/group. $^*P < 0.05$ and $^{**}P < 0.01$ vs. *day 0*; $^{\triangle\triangle}P < 0.01$ vs. no Apr at the same time of observation.

induce collagen synthesis through upregulation of Col I and Col III genes. In contrast, TNF-α reduces Col I mRNA levels and the steady state of Col I mRNA but has no effect on Col III mRNA (30, 43). In our study, differential regulatory effects of cytokines on Col I and Col III mRNA might have resulted in the shift of the Col I-to-Col III mRNA ratio in the development of DCM after IAV infection.

Trypsin is an efficient activator of a prourokinase cascade (33). Plasminogen is the primary physiological substrate of urokinase. Plasminogen is activated in patients with acute viral myocarditis at all stages of the clinical course (7). Plasmin can directly degrade basement collagen type IV (27) and activate MMP-9 (12). The combined upregulation of trypsin and plasmin activity causes synergistic proteolytic degradation of the vascular basement and the myocardial ECM. In addition to trypsin, aprotinin also inhibits plasmin activity, thus effectively protecting the heart from inflammatory injury and myocardial remodeling.

No specific treatment is currently available for the prevention of IAV-induced myocarditis and subsequent DCM. Heymans et al. (12) reported that suppression of MMP-9 activity reduces the cardiac inflammatory response, protecting mice against cardiac injury, dilatation, and failure during viral myocarditis. However, Cheng et al. (3) indicated that MMP-9 is

Table 1. *Effects of the trypsin inhibitor Apr on kinetics of cardiac function and LV posterior wall thickness after influenza A virus infection*

	Day 0	*Day 9*		*Day 20*		*Day 40*		*Day 60*	
Parameters	Control	No Apr	With Apr	No Apr	With Apr	No Apr	With Apr	No Apr	With Apr
n	10	8	9	7	9	7	9	7	9
Ejection fraction, %	70.51 ± 9.06	49.94 ± 8.26†	60.36 ± 6.46*‡	52.35 ± 11.01†	61.89 ± 8.87*‡	49.28 ± 5.08†	63.27 ± 12.71§	47.67 ± 8.53†	65.05 ± 9.26§
Stroke volume, µl	44.56 ± 3.74	31.42 ± 3.43†	37.42 ± 4.34†§	31.82 ± 5.55†	38.25 ± 3.31†§	32.48 ± 3.97†	40.67 ± 6.09§	31.61 ± 4.36†	42.49 ± 4.90§
Cardiac output, ml/min	24.06 ± 3.63	18.22 ± 2.45†	21.55 ± 3.08*§	18.27 ± 2.60†	21.89 ± 2.01§	18.20 ± 2.50†	22.92 ± 2.75§	18.06 ± 3.32†	23.87 ± 2.47§
LV posterior wall thickness at end diastole, mm	0.74 ± 0.07	0.76 ± 0.08	0.75 ± 0.06	0.69 ± 0.08	0.73 ± 0.07	0.64 ± 0.07*	0.73 ± 0.06‡	0.61 ± 0.08†	0.71 ± 0.09‡
LV posterior wall thickness at end systole, mm	1.15 ± 0.07	1.17 ± 0.07	1.16 ± 0.06	1.13 ± 0.06	1.15 ± 0.07	1.03 ± 0.08†	1.14 ± 0.05§	0.98 ± 0.06†	1.14 ± 0.06§

Values are means ± SD; *n*, number of surviving mice. $^*P < 0.05$ and $^†P < 0.01$ compared with *day 0*; $^‡P < 0.05$ and $^§P < 0.01$ compared with no aprotinin (Apr) treatment at the same time of observation.

essential for viral clearance by promoting immune cell recruitment in the early phase of infection. Therefore, MMP-9 inhibition might impair host defense and enhance infection dissemination. In our study, administration of the trypsin inhibitor aprotinin effectively suppressed IAV replication and the inflammatory response throughout acute and chronic phases by inhibiting trypsin-mediated activation of IAV hemagglutinin and interrupting the trypsin-MMP-9-cytokine cycle. Thus, inhibition of MMP-9 activity by aprotinin will not impair the host defense against viral infection. In addition, recent studies (2, 34, 52) have reported that PAR-2 is involved in innate immune responses during RNA virus infection and enhanced cardiac remodeling in the injured heart. Activation of PAR-2 negatively regulates the Toll-like receptor-3-dependent antiviral pathway with reduced expression of interferon-β (34). PAR-2 knockout mice are protected from H1N1/PR8 IAV virus-induced lethality and coxsackievirus B3-induced myocarditis (34, 52). Overexpression of PAR2 in mice induced cardiac fibrosis, inflammation, and heart failure (2). As trypsin is one of the most potent activators of PAR-2, aprotinin can partially abrogate the pathological roles of PAR-2 induced by trypsin.

Study limitations. Our findings suggested that trypsin is a key factor in acute and chronic stages of myocarditis after IAV infection. An experiment in which aprotinin treatment started from *day 9* postinfection could further confirm the protective effects of trypsin inhibitor during chronic stage but was not performed. Thus, we cannot exclude the contribution of reduced acute myocarditis by aprotinin treatment for the prevention of DCM.

Conclusions. The results of the present study suggested that ectopic trypsin in the myocardium was involved in acute and chronic myocardial inflammation by promoting IAV infection and initiating a trypsin-MMP-9-cytokine cycle and promotes progressive cardiac dilation through mediation of collagen remodeling. Thus, trypsin might play important roles in the development of DCM after IAV infection. Aprotinin prevented the progression of myocarditis to DCM by suppressing IAV infection, interrupting the trypsin-MMP-9-cytokine cycle and restoring collagen metabolism. Our findings suggest that inhibition of trypsin activity might be a promising therapeutic approach for the prevention of DCM after IAV infection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.-Y.P., H.K., and J.-H.Z. conception and design of research; H.-Y.P., H.-M.S., and L.-J.X. performed experiments; H.-Y.P. and J.-H.Z. drafted manuscript; H.-Y.P., H.-M.S., L.-J.X., M.P., Y.-P.W., H.K., and J.-H.Z. approved final version of manuscript; M.P. and Y.-P.W. analyzed data.

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Oral Administration of *Lactobacillus pentosus* Strain S-PT84 Enhances Anti-Influenza Virus-Specific IgG Production in Plasma after Limited Dose of Influenza Virus Vaccination in Mice

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Abstract

Background: It has been reported that various *Lactobacillus* species enhance antigen-specific antibody production after viral infection and/or vaccination in animals and humans. In this study, the effect of oral administration of *Lactobacillus pentosus* strain S-PT84 on subcutaneous administration of limited dose of 2.5 ng of influenza A virus (IAV) (H1N1) split hemagglutinin (HA) vaccine was studied in mice.

Methods: Seven-week-old BALB/c female mice were fed the AIN-93M diet with or without 0.186% S-PT84 for 6 weeks ad libitum and then given subcutaneous injection of IAV/California/7/2009 (H1N1) HA vaccine at doses of 2.5–15.0 ng/mice and boosted on day 28. Two weeks after the last vaccination, the mice were sacrificed under anesthesia, and the amount of anti-HA-specific IgG in plasma was measured by ELISA.

Results: The levels of anti-HA-specific IgG in plasma were significantly higher in the S-PT84 group than in the control group without S-PT84 treatment. Anti-HA-specific IgA levels in nasal washes were under the detection level in both groups.

Conclusion: Oral administration of S-PT84 enhanced the production of HA-specific IgG antibody in plasma after subcutaneous vaccination, even at the limited dose of 2.5 ng of IAV (H1N1) HA in mice, suggesting that S-PT84 has a potent adjuvant activity against IAV HA.

Introduction

Influenza is an acute viral infection that results in high morbidity and significant mortality, particularly in infants and the elderly populations [1]. They sometimes have a high risk of fatal influenza pneumonia and thus vaccination is recommended for prevention. The World Health Organization has recommended use of oseltamivir (Tamiflu®, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) and zanamivir (Relenza®, Glaxo Smith Kline K.K., Tokyo, Japan) for the treatment of influenza. These antiviral neuraminidase inhibitors are useful options for seasonal influenza infections in the world [2]. However, Shinahara et al. recently reported that administration of these inhibitors decreases anti-IAV HA-specific IgG and IgA production in plasma and nasopharyngeal secretions, probably because of inhibition of viral replication and limited production of viral antigens [3]. The limited viral antigens do not induce sufficient acquired immunity in patients treated with anti-viral neuraminidase inhibitors, resulting in a high frequency of influenza virus re-infection in the subsequent year [3].

Lactic acid bacteria (LAB) are widely used as a health food ingredient and could modulate mucosal and systemic immune responses [4,5]. Oral administration of live *Lactobacillus casei* Shirota significantly increases the survival rate of neonatal and infant mice infected with IAV by stimulating IL-12 production and NK activity in the lung [6]. Oral administration of heat-killed *Lactobacillus plantrum* L-137 [7] and *Lactobacillus pentosus* strain b240 [8] enhances type 1 interferon production and increases the production of anti-IAV IgG in plasma and IgA in bronchoalveolar lavage fluid, respectively, and prolongs the survival period of mice infected with IAV. These studies showed that oral administration of live and nonviable *Lactobacillus* species enhances the protective effects against airway infection of IAV.

It has recently been reported that oral administration of probiotic LAB enhances vaccination-induced antibody production. *Lactobacillus acidophilus* enhances rotavirus-specific antibody after vaccination of rotavirus in the neonatal gnotobiote pig [9]. Davidson et al. reported that volunteers receiving *Lactobacillus* GG show higher protective titers after administration of live attenuated IAV/H3N2 vaccine compared to a placebo group after vaccination [10]. Moreover, Boge et al. reported that daily consumption of a probiotic drink increases relevant specific antibody responses to influenza

vaccination in healthy volunteers over 70 years of age [11]. Therefore oral administration of *Lactobacillus* species might be effective in preventing influenza virus infection by stimulating antibody production.

We previously reported that *Lactobacillus pentosus* strain S-PT84 enhances splenic natural killer (NK) activity and exhibits anti-allergic effects by modulating T-helper1/T-helper2 (Th1/Th2) balance [12]. Moreover, it has been reported that oral administration of S-PT84 enhances interferon-α production from plasmacytoid dendritic cells against an insufficient dose of hemagglutinating virus of Japan (HVJ) infection [13], and that intranasal administration of S-PT84 protects against influenza infection [14]. However, the effects of S-PT84 ingestion on limited dose of IAV HA vaccination and HA-specific antibody induction have not yet been examined. In this study, the effect of oral administration of S-PT84 on the induction of IAV HA-specific antibody in mice immunized by IAV HA vaccine in the nanogram range was investigated.

Materials and Methods

Animals

BALB/c female mice, 7 weeks old, were obtained from Japan SLC, Inc. (Shizuoka, Japan). Mice were housed and fed a commercial diet (CE-2) and tap water ad libitum for 1 week at 25±1 °C and 60% ± 5% humidity under a 12 h light-dark cycle before experimentation. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996).

Bacterial strains and bacteria preparation

S-PT84 was isolated from Kyoto pickles "SHIBAZUKE" [12] and was cultivated in a medium containing glucose and yeast extract (Aromild™, SK yeast extract Hi-K) at 37 °C for 24 hours. Cultured bacteria were collected by centrifugation at 9,190 x g for 5 min, washed twice with sterile saline and once with distilled water, and heat-killed at 95 °C for 5 min. Heat-killed S-PT84 was lyophilized for use in the experiments.

Vaccine

The influenza antigen used in the present studies, IAV/California/7/2009(H1N1) virus processed for HA vaccine (split-product, 0.636 μg protein/0.341 μg HA/mL), was purchased from DENKA SEIKEN Co., Ltd. (Tokyo, Japan).

Procedure and sample collection

BALB/c mice were fed on a commercial diet (AIN-93M, Oriental Yeast Co. Ltd., Tokyo, Japan) with or without 0.186% S-PT84 and tap water ad libitum for 6 weeks. Mice were given subcutaneous vaccinations (2.5–15.0 ng HA/100 μL) and boosted on day 28. Two weeks after the last vaccination, the mice were sacrificed under anesthesia. Blood samples were collected from the vein. Blood was centrifuged (2,500 g, 10 min) at 4 °C, and the supernatant was collected.

Enzyme-linked immunosorbent assay (ELISA)

Anti-influenza HA-specific antibodies in plasma were measured by ELISA [15]. Briefly, 96-well plates (Nunc, Naperville, IL) were coated with HA vaccine and bovine serum albumin (BSA, 0.1 μg/well each) in PBS overnight at 4 °C. After the pre-coating, it was blocked with 1% BSA in 50 mM Tris–HCl (pH 8.0) containing 0.14 M NaCl and 0.05% Tween 20 (TTS) for 1 hour at room temperature. The plasma diluted with TTS containing 1% BSA was added to each well and incubated for 3 hours at room temperature. The plate was washed six times with TTS containing 1% BSA and incubated with goat anti-mouse IgG antibodies conjugated with horseradish peroxidase (Bethyl Laboratories Inc., Montgomery, TX, USA) for 2 hours at room temperature. Color was developed by the addition of TMB substrate (Bethyl Laboratories), according to the instructions provided by the manufacturer. The chromogen produced was measured at 450 nm using a SPECTRA max PLUS384 AutoReader (Molecular Devices, Tokyo, Japan). Antibody titers were defined as the reciprocal of the highest dilution of sample for which the optical density (OD) was at least twice the OD of the negative control samples before vaccination.

Statistical analysis

The significance of differences in values between the control group and S-PT84 group was determined using Student's t-test. P-values less than 0.05 were considered significant.

Results

Doses of vaccination of IAV/California/7/2009(H1N1)

The induced levels of HA-specific IgG antibody in plasma were analyzed to detect the optimal dose of IAV/California/7/2009(H1N1) to evaluate the effect of *Lactobacillus pentosus* S-PT84 as an adjuvant. HA-specific IgG antibody in plasma was increased by IAV HA vaccination in a dose-dependent manner in the range between 2.5 and 15.0 ng (Figure 1). HA at 2.5 ng, even at the lowest dosage, could induce production of HA-specific IgG in plasma, and that dose may be appropriate for examining the effect of LAB on enhancing HA-specific IgG production in plasma. Therefore, the minimal dose of 2.5 ng HA was used to investigate the adjuvant effect of LAB.

Effect of *Lactobacillus pentosus* S-PT84 on the production of HA-specific IgG in plasma

Mice were fed the AIN-93M diet with or without 0.186% S-PT84 for 6 weeks. In the control group treated with two subcutaneous injections of IAV HA at 2.5 ng alone, a small amount of antigen-specific IgG antibody production was detected in plasma. The S-PT84 group of mice treated with continuous feeding of the AIN-93M diet with 0.186% S-PT84 during the experimental period also received two subcutaneous injections of IAV HA at 2.5 ng. HA-specific IgG antibody levels in plasma were higher in the S-PT84 group than in the control group (Figure 2A). On the other hand, HA-specific IgA in nasal washes was under detection levels in both groups (data not shown). The induced HA-specific IgG titers in plasma were significantly higher in the S-PT84 group than in the control group (p=0.007) (Figure 2B).

Discussion

In this study, oral administration of S-PT84 enhanced HA-specific IgG induction in plasma, but not HA-specific IgA induction in nasal wash, after subcutaneous administration of limited dose of IAV (H1N1) HA in mice. The findings suggest that S-PT84 has an adjuvant effect against IAV HA on plasma IgG induction, even at minimal dose. Nonaka et al. reported that S-PT84 induces cytokine

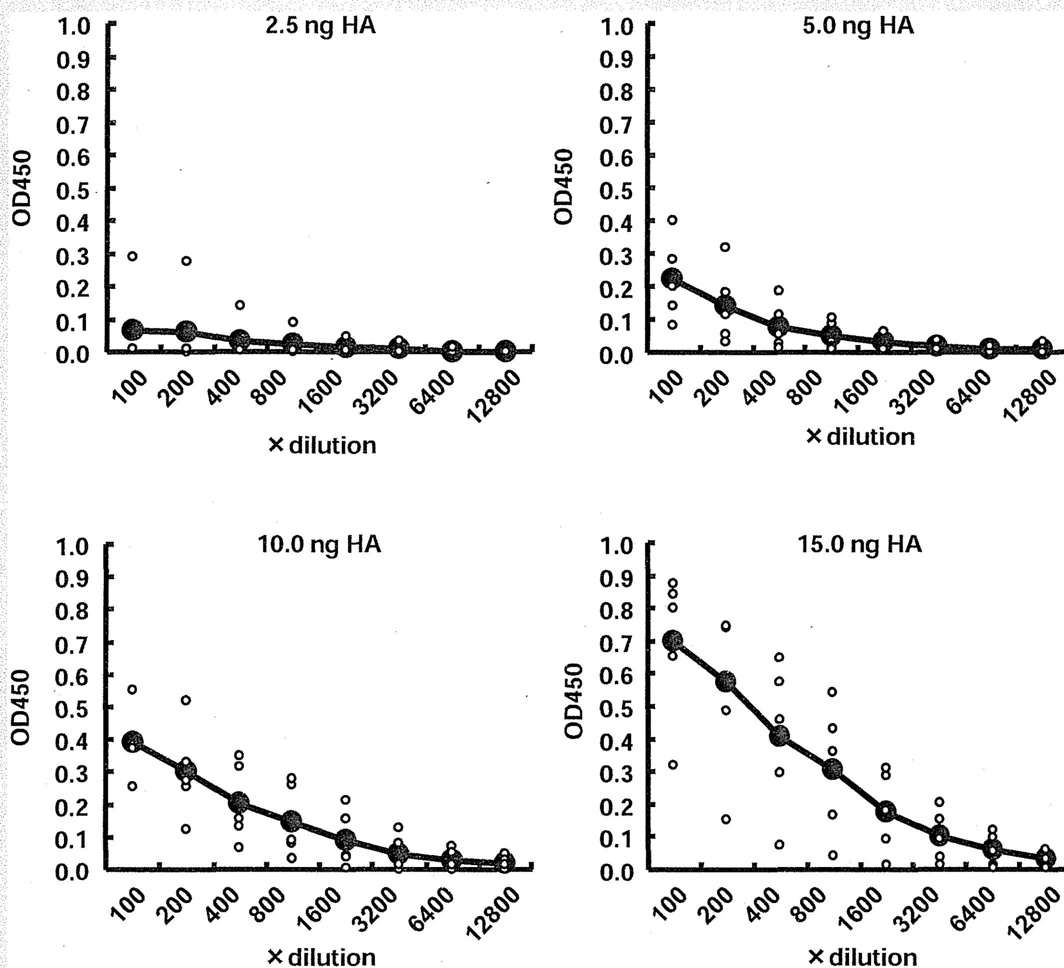

Figure 1: The amount of HA-specific IgG production by vaccination. Mice were fed an AIN-93M diet and given subcutaneous vaccination at doses between 2.5 and 15.0 ng HA and boosted on day 28 from the initial vaccination. Two weeks after the last vaccination, plasma was collected from each mouse, and the amount of HA-specific IgG antibody was measured by ELISA, as described in Materials and Methods. Values are means of 5 mice in each group. Open circles are individual data, closed circles are mean values.

production from peritoneal macrophages in vitro and enhances the activity of NK cells *in vivo* [12]. Koizumi et al. also reported that S-PT84 stimulates IFN-γ and IL-12 production through Toll-like receptor (TLR)-2 and TLR-4 on dendritic cells [16]. Moreover, Izumo et al. reported that *Salmonella*-specific antibody is enhanced by S-PT84 ingestion in *Salmonella* typhimurium-infected mice [17]. Accordingly, we thought that antigen-presenting cells were activated by ingestion of S-PT84 in this experiment, and production of HA-specific IgG in plasma may be enhanced, as in a previous report [17]. It is well known that influenza vaccines administered intramuscularly or subcutaneously induce a predominantly IgG-mediated protection in the systemic immune compartment, but this systemic immunization offers inadequate induction of IgA in airway mucosa [18]. Therefore, oral administration of S-PT84 might affect HA antigen-induced dendritic cell-mediated systemic immunity and induce HA-specific IgG in plasma, but not affect mucosal immunity and HA-specific IgA production.

The present data suggest that S-PT84 enhances antibody production as an adjuvant with limited dose of influenza viral antigen. Hui-Tsu et al. reported that the induction of HA-specific antibody in plasma was enhanced by IAV HA (H1N1) vaccination at 10 ng dose in mice, and the survival rate was also significantly improved by this vaccination [19]. The optimal density (OD) level of HA-specific IgG was similar compared with our present study. Moreover, the strain of IAV/California/7/2009 (H1N1) was same in both experiments. Therefore, we speculate that HA-specific antibody production level by the killed bacterial adjuvant S-PT84 might be sufficient for the viral protection and then an ample titer was obtained for influenza virus exclusion. It suggests that S-PT84 may decrease the risk of influenza virus re-infection treated with anti-viral neuraminidase inhibitors.

As a next step, it should be to investigate whether oral administration of S-PT84 together with the vaccination on limited dose of HA antigen can protect IAV infection, and/or whether oral administration of S-PT84 can induce sufficiently HA-specific IgG antibody treated with antiviral neuraminidase inhibitors after IAV infection.

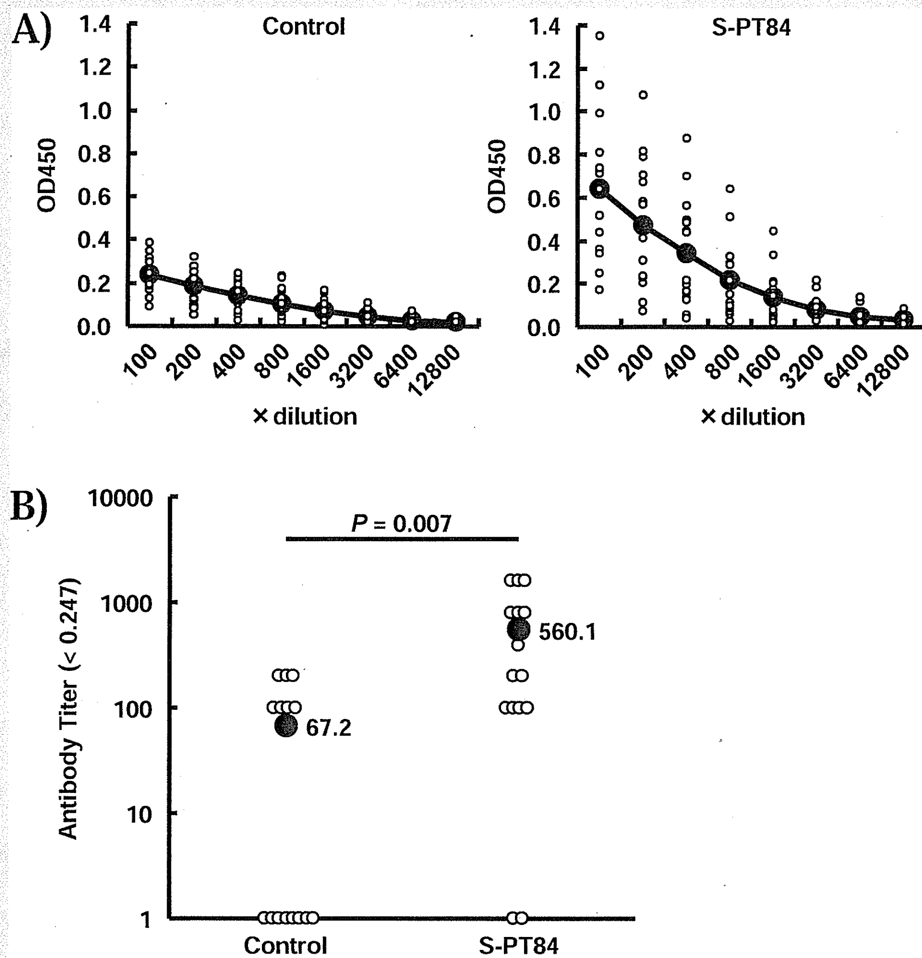

Figure 2: Effect of *Lactobacillus pentosus* strain S-PT84 on HA-specific IgG production by vaccination. Mice were fed an AIN-93M diet with or without 0.186% S-PT84 for 6 weeks followed by subcutaneous vaccination of 2.5 ng HA and boosted on day 28 from the initial vaccination. Two weeks after the last vaccination, plasma was collected from each mouse, and the amount of HA-specific IgG Antibody (Ab) **(A)** was measured by ELISA and expressed as the titer **(B)**, as described in Materials and Methods. Values are means of 15 mice in each group. Significant differences are seen between the control group and the S-PT84 group. Open circles are individual data, closed circles are mean values.

Japan is becoming a super-aging society more rapidly than other countries. Elderly persons have a high risk of influenza-associated pneumonia, which is sometimes fatal. Thus, management of influenza outbreaks is important for them. Indeed, seroconversion and seroprotection are lower in elderly individuals than in adults after influenza vaccination, and their immune functions are weaker than in adults [20]. It is important that preventive action be taken to enhance immune function or provide vaccination for the elderly. In this regard, S-PT84 may have a potential to improve their immune response.

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Serum Starvation Activates NF-κB Through G Protein β2 Subunit-Mediated Signal

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Several cell stresses induce nuclear factor-kappaB (NF-κB) activation, which include irradiation, oxidation, and UV. Interestingly, serum-starving stress-induced NF-κB activation in COS cells, but not in COS-A717 cells. COS-A717 is a mutant cell line of COS cells that is defective of the NF-κB signaling pathway. We isolated genes with compensating activity for the NF-κB pathway and one gene encoded the G protein β2 (Gβ2). Gβ2 is one of the G protein-coupled receptor signaling effectors. In COS-A717 cells, Gβ2 expression is significantly reduced. In Gβ2 cDNA-transfected COS-A717 cells, the NF-κB activity was increased along with the recovery of Gβ2 expression. Furthermore, serum-starving stress induced the NF-κB activity in Gβ2-transfected COS-A717 cells. Consistently, the serum-starved COS cells with siRNA-reduced Gβ2 protein expression showed decreased NF-κB activity. These results indicate that Gβ2 is required for starvation-induced NF-κB activation and constitutive NF-κB activity. We propose that serum contains some molecule(s) that strongly inhibits NF-κB activation mediated through Gβ2 signaling.

Introduction

Nuclear factor-kappaB (NF-κB) is a ubiquitously expressed transcription factor with critical roles in cell survival, proliferation, apoptosis, immune response, and inflammation. NF-κB usually exists as a heterodimer of p50 and p65 (Rel A), and is kept in the cytoplasm through an association with inhibitor of kappaB (IκB) inhibitory proteins. After various stimulations, the serine residues at positions 32 (S32) and 36 (S36) in the IκB proteinare phosphorylated (Brown *et al.*, 1995) by the IκB kinase (IKK) complex (Zandi *et al.*, 1997), and the IκB protein is degraded by the ubiquitin–proteasome pathway (Chen *et al.*, 1995). The IKK complex consists of two catalytic subunits, IKK1 and IKK2 (also referred to as IKKα and IKKβ), and a regulatory subunit, NEMO (Yamaoka *et al.*, 1998). Cytokines and various cell stresses, including irradiation (Criswell *et al.*, 2003), oxidation (Marshall *et al.*, 2000), and UV (Kato *et al.*, 2003), induce NF-κB activation. Serum starvation also activates NF-κB in various cell lines (Ryter and Gomer, 1993; Grimm *et al.*, 1996), indicating that serum contains unknown inhibitor(s) of NF-κB.

On the other hand, constitutively active NF-κB exists in certain normal cells (Pagliari *et al.*, 2000; Lilienbaum and Israel, 2003) and several tumor cells without stimulation (Mori *et al.*, 1999; Lind *et al.*, 2001). However, the mechanism by which NF-κB is constitutively activated in these cells is not known. COS cells have a relatively high level of basal NF-κB activity. We established a mutant cell line, COS-A717, with a defective NF-κB signaling pathway (Kohno *et al.*, 2008). The basal level of NF-κB activity in the COS-A717 cells was reduced by as much as sevenfold, as compared with that in the parental COS cells. Serum starvation induced NF-κB activation in the parental COS cell line, but not in the COS-A717 cell clone. Since the COS-A717 cell clone was constructed by the treatment of COS cells with a frameshift-inducing agent, it is most likely that the NF-κB activating factor(s) expressed in the parental COS cells is not functional in the COS-A717 cells. We previously isolated the B cell activating factor of the TNF family (BAFF) receptor as an NF-κB activator in COS-A717 cells (Kohno *et al.*, 2008). However, the original COS cells do not express BAFF-R, indicating that BAFF-R is not responsible for the defective NF-κB signaling in the COS-A717 cells, and activates NF-κB through a salvage pathway.

In this study, we isolated the guanine nucleotide-binding protein β2 subunit (Gβ2) cDNA as another NF-κB activator by screening a human spleen cDNA expression library. The guanine nucleotide-binding proteins (G proteins) are signal transducers required for various G protein coupled receptor (GPCR)-effector networks (Xie *et al.*, 2000; Wu *et al.*, 2001; Albert and Robillard, 2002). GPCRs 'transduce signals through heteromeric G proteins, and several of them activate NF-κB (Xie *et al.*, 2000; Grabiner *et al.*, 2007; Sun *et al.*, 2009). The heteromeric G proteins consist of α, β, and γ subunits,

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and the α subunit has GTPase activity. When GPCRs interact with their ligands, the active GTP-bound α subunit is released from the heteromeric G protein complex, and Gα and Gβγ induce downstream signaling (Stephens *et al.*, 1994). The Gβ2 expression level in the parental COS cells is much higher than that in the mutant COS-A717 cells. Transfection of a Gβ2 expression plasmid activated NF-κB in COS-A717 cells. The knockdown of Gβ2 expression by siRNA in COS and HT1080 cells reduced the basal NF-κB activity. These results indicate that the activation of the GPCR signal pathway by Gβ2 results in constitutive NF-κB activation in the transformed cells, and the defect of Gβ2 expression is one of the determinants for reduced NF-κB activity in the COS-A717 mutant cells.

Serum starvation activates NF-κB in COS cells, but not in COS-A717 cells. Transfection of COS-A717 cells with Gβ2 restored the starvation-induced NF-κB activation. These results show that NF-κB activation by serum starvation occurs through the Gβ2 signaling pathway, and the inhibitor(s) present in serum sppress the Gβ2 signal. Taken together, our findings suggest that the constitutive NF-κB activation in transformed cells is induced by the GPCR signaling pathway through Gβ2, and that serum contains factor(s) reducing NF-κB activity by suppressing the GPCR signaling.

Materials and Methods

Plasmids and reagents

The human spleen cDNA library was purchased from Life Technologies. The five-tandem κB luciferase reporter vector (5×κB luciferase) was purchased from Stratagene. The IκBα superrepressor (IκBα-SR) expression plasmid was described previously (Sugita *et al.*, 2002). The expression vectors for the dominant negative forms of IKK1 (IKK1.DN), IKK2 (IKK2.DN), and NEMO (NEMO.DN) were kind gifts from Dr. Yamaoka (Tokyo Medical and Dental University, Tokyo, Japan) (Hironaka *et al.*, 2004). The expression vector for the dominant negative mutant of Akt (Akt.DN) was generously provided by Dr. V. Stambolic (Ontario Cancer Institute, Toronto, Canada). Wortmannin was purchased from Sigma.

Cells

The mutant cell lines COS-A717 and COS-A717-GS were described previously (Kohno *et al.*, 2008). The mouse spleen cells were obtained from a C57BL/6 mouse. COS (Kohno *et al.*, 2008), HT1080 (Jones *et al.*, 1975), and HEK293T cells were maintained in the Dulbecco's modified Eagle's medium, and ST1 cells (Yamada, 1996) were maintained in the RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere.

Expression cloning of Gβ2

Isolation of genes with compensating activity for the NF-κB activation pathway was performed according to the previously described method (Kohno *et al.*, 2008). Briefly, COS-A717-GS cells were transfected with a human spleen cDNA library (Life Technologies) using the FuGene 6 reagent. After 48 h of transfection, the top 0.5% fraction of fluorescent cells was collected using a FACStar Plus (Becton, Dickinson and Co.). Plasmids were extracted from sorted cells (Hirt, 1967), amplified in bacteria, and used in three subsequent rounds of flow cytometry-based enrichment. Individual bacterial colonies obtained from the third sorting were grouped into pools of 50 colonies. Positive pools were subdivided further into subpools with half the number of colonies, and were subjected to repeated screening. This process finally yielded independent clones that conferred compensation for the NF-κB activation pathway in COS-A717 cells.

Transfection and luciferase assay

Cells were transfected with a 5×κB-luciferase reporter and a Gβ2 expression plasmid, as indicated in the text and figure legends. Transient transfections were performed using the FuGene 6 reagent (Roche). When necessary, additional DNA (pcDNA3.1) was added to equalize the amount of transfected DNA in each sample. At 48 h post-transfection, the κB-directed expression of firefly luciferase was determined, using luciferase assay reagents (Promega), and the luciferase activities were measured with a BioOrbit 1254 luminometer. The relative transfection efficiency in each sample was determined by measuring the Renilla luciferase activity. The data were normalized per transfection efficiency. Data shown are averages and SD from three independent experiments.

Western blot analysis

Cell extracts were prepared from the cells transfected for the luciferase assay. Cell lysates were resolved by 12.5% SDS PAGE, transferred onto an Immobilon-P membrane (Millipore), and blocked with 5% nonfat dry milk in TBS with 0.5% Tween 20. The blots were incubated with anti-Gβ2, anti-IKK1, anti-IKK2, anti-NEMO, anti-Akt1, anti- IκBα, and anti-phosho S32, and S36-containing peptide of IκBα antibodies (Santa Cruz Biotechnology; refer to Tables 1 and 2), or an anti-β-actin antibody (Chemicon), followed by an incubation with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ig (Amersham Pharmacia Biotech). The blots were visualized with the ECL detection system (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay

Preparation of nuclear extracts for electrophoretic mobility shift assays (EMSAs) was performed as described previously (Sugita *et al.*, 2002). The consensus κB site 5′-AGTTGAGG GGACTTTCCCAGGC-3′ and mutant 5′-AGTTGAGGCGAC TTTCCCAGGC-3′ oligonucleotides were obtained from Santa Cruz Biotechnology. The double stranded oligonucleotides were end-labeled with [γ-^{32}P] ATP, using T4 polynucleotide kinase (Takara). The reaction was conducted in a total volume of 10 μL, using 10 μg of nuclear extract, 1 μg of poly(dI-dC), 20 mM HEPES-NaOH (pH 7.6), 100 mM NaCl, 1 mM DTT, 1 mM PMSF, and 2% glycerol. The binding reaction mixture was incubated with 10,000 cpm of radiolabeled probe for 15 min. For the competition and supershift assays, a 20-fold excess of unlabeled or mutant oligonucleotide, and the antibodies to p65 or p50 (Santa Cruz Biotechnology) were added to the reaction, respectively. The samples were loaded onto a 5% nondenaturing polyacrylamide gel, which was run in a 0.5×TBE buffer. After

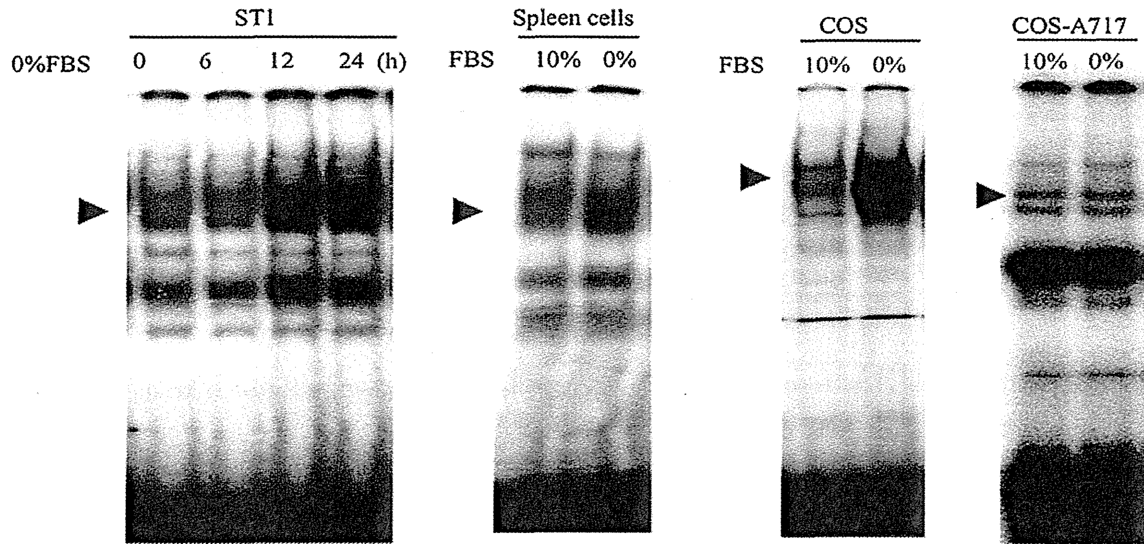


FIG. 1. Serum-starving stress-induced nuclear factor-kappaB (NF-κB) activation. The nuclear extracts were incubated with a ^{32}P-labeled NF-κB consensus oligonucleotide, and analyzed by an electrophoretic mobility shift assay. Nuclear cell extracts from ST1 cells, which were cultured without fetal bovine serum (FBS) for 0, 6, 12, 24 h (*STI panel*). The mouse spleen cells were cultured with 10% FBS or 0% FBS for 24 h (*Spleen cells panel*). Nuclear cell extracts from COS (*COS panel*) and COS-A717 (*COS-A717 panel*) cells, which were cultured with 10% FBS or 0% FBS for 24 h. The *arrowhead* indicates the NF-κB-containing complex.

electrophoresis, the gel was dried and processed for autoradiography.

siRNA

The nucleotide sequences of the two siRNAs for Gβ2 are as follows:

#1 sense 5′-CAUCUGCUCCAUCUACAGCdTdT-3′,
anti-sense 5′-GCUGUAGAUGGAGCAGAUGdTdT-3′;
#2 sense 5′-AGACCUUCAUCGGCCAUGAdTdT-3′,
anti-sense5′-UCAUGGCCGAUGAAGGUCUdGdT;
and sense 5′-GGCUACGUCCAGGAGCGCAdTdT-3′, anti-sense 5′-UGCGCUCCUGGACGUAGCCdTdT-3′ for GFP. The annealed oligonucleotides were transfected by using Lipofectamine 2000 (Invitrogen). Cells were maintained in the Dulbecco's modified Eagle's medium without FBS at the transfection. Cells were harvested after 24 and 48 h after the transfection for COS and HT1080 cells, respectively. For the luciferase assay, cells were transfected with the 5×κB-luciferase reporter using the FuGene 6 reagent, at 6 h after the siRNA transfection.

Results

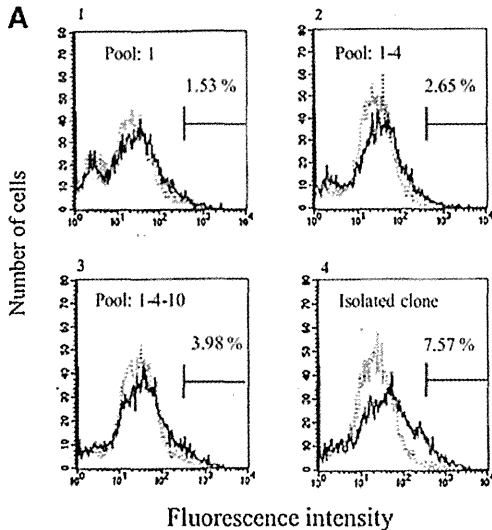
Serum-starving stress induces NF-κB activation

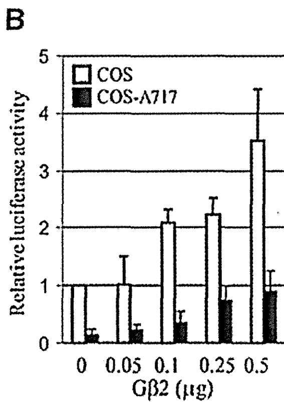
Cell stresses, such as irradiation, UV, and oxidation (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/dna), induce NF-κB activation. Cells are usually cultured with 10% FBS in medium *in vitro*. Serum includes various factors and nutrients for cell survival and proliferation, and thus serum starvation ceases cell proliferation, and then induces cell death. Serum may include factors affecting NF-κB signaling. To address this issue, we analyzed the effects of serum starvation on NF-κB binding to the target sequence by EMSA of nuclear extracts from several cell lines (ST1, COS, and COS-A717) and mouse primary spleen cells. The binding of NF-κB to the target sequence was enhanced by serum starvation in all examined cells, except for the mutant COS-A717 cells, which exhibit defective NF-κB signaling (Fig. 1). These results indicate that serum starvation induces NF-κB activation, suggesting that serum contains unknown factor(s) inhibiting NF-κB activity.

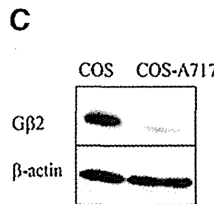
Expression cloning of an NF-κB activating molecule using COS-A717 cells

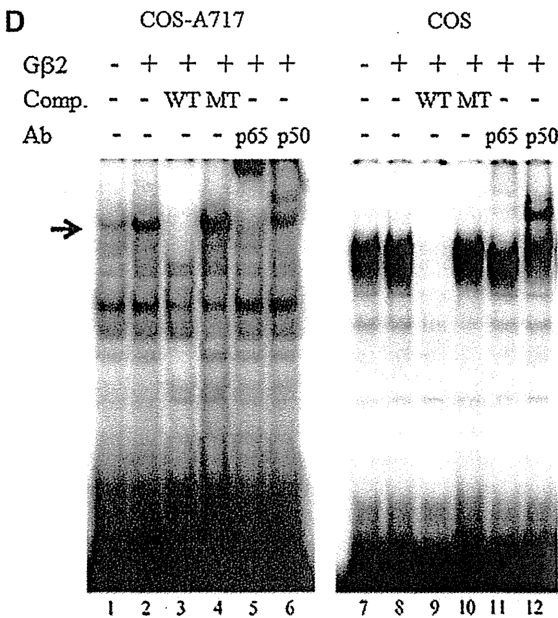
Many transformed cell lines containing the COS cell line have constitutively activated NF-κB signaling. To identify NF-κB activators in the COS cells, a COS-A717 cell derivative containing the GFP gene under the control of the Sp1 site-deleted HIV-1 LTR was constructed, and the cells were designated as COS-A717-GS. The GFP is expressed by NF-κB activation in the COS-A717-GS cells, because the expression from the Sp1 site-deleted HIV-1 LTR is NF-κB dependent. The COS-A717-GS cells were transfected with a human spleen cDNA expression library, and GFP-expressing cells were selected (Fig. 2A). The sequence analysis of the cDNA expressed in the GFP-positive COS-A717-GS cells revealed that it perfectly matched the Gβ2. The expression level of the Gβ2 protein in COS-A717 cells was much lower than that in the parental COS cells (Fig. 2B).

To confirm that Gβ2 activates NF-κB activity, COS and COS-A717 cells were transfected with the Gβ2 expression plasmid, and the NF-κB promoter activity was measured using the 5×κB-luciferase plasmid. Gβ2 activated the NF-κB promoter activity in both COS and COS-A717 cells, in a dose-dependent manner (Fig. 2C). Transfection of the COS-A717 mutant cells with the Gβ2 expression plasmid (0.5 μg) resored the NF-κB activity comparable to the parental COS cells (sevenfold). When 0.5 μg of the Gβ2 expression plasmid was transfected









into the COS cells, the NF-κB activity was also increased by threefold. The DNA-binding activity of NF-κB was elevated by Gβ2 by about 1.7- and 6.2-fold in COS and COS-A717 cells, respectively (Fig. 3D). The complex formation was inhibited by a wild-type κB oligonucleotide competitor, but not by a mutant κB oligonucleotide. The complex was supershifted by both anti-p65 and -p50 antibodies, indicating that the complex consisted of p65 and p50 (Fig. 3D). The Gβ2 transfection activated the NF-κB signal more efficiently in the mutant COS-A717 cells than in the COS cells that originally express Gβ2. These results indicate that Gβ2 activates NF-κB signaling and the defect of Gβ2 expression is one of the determinants for the reduced NF-κB activity in the COS-A717 cells.

Gβ2 is required for NF-κB activation induced by serum starvation

Serum starvation activated NF-κB by 10-fold in COS cells, but had no effect in COS-A717 cells (Fig. 3A). Since the level of the Gβ2 protein is much lower in the COS-A717 cells than in the parental COS cells, we examined whether Gβ2 was involved in the NF-κB activation by serum starvation. Serum starvation elevated the NF-κB activity by fourfold in the Gβ2-transfected COS-A717 cells, indicating that Gβ2 is required for the serum starvation-induced NF-κB activation and that the Gβ2-activated signal is inhibited by the unknown factor(s) present in serum. However, because the level of NF-κB activity in the starved Gβ2-expressing COS-A717 cells was lower than that in the starved COS cells, the COS-A717 cells have additional defect(s) in the NF-κB signal activation.

FIG. 2. G protein β2 (Gβ2) activates NF-κB. **(A)** Identification of Gβ2 by expression cloning. **(A-1)** COS-A717-GS cells were transfected with plasmids obtained from a positive pool of 50 bacterial transformants (pool 1) following four rounds of FACS enrichment. **(A-2)** COS-A717-GS cells were transfected with plasmids from a positive pool (1-4), containing 20 bacteria colonies. **(A-3)** COS-A717-GS cells were transfected with plasmids from a positive pool (1-4-10), containing 10 bacterial colonies. **(A-4)** COS-A717 GS cells were transfected with a Gβ2-encoding clone. **(B)** Western blot analysis of Gβ2 in COS and COS-A717 cells. Proteins were analyzed by immunoblotting with an anti-Gβ2 Ab (*top*) and an anti-β-actin Ab (*bottom*). **(C)** Gβ2-mediated NF-κB activation in COS-A717 cells. COS and COS-A717 cells were transiently transfected with 0.25 μg of the 5×κB-luciferase reporter and the Gβ2 expression construct (0.05, 0.1, 0.25, and 0.5 μg), and then additional DNA (pcDNA3) was added to make the total amount of DNA 1 μg/well. At 48 h post-transfection, the cells were harvested and the luciferase activity was measured. The relative transfection efficiency in each sample was determined by the measurement of the Renilla luciferase activity. The relative luciferase activity in control COS cells (without Gβ2) was set to 1.0. Data shown are averages +SD from three independent experiments. **(D)** Gβ2 induced the NF-κB-binding ability in COS-A717 cells and COS cells. Nuclear proteins from untransfected (*lanes* 1, 7) or Gβ2 transfected (*lanes* 2–6, 8–12) COS-A717 cells or COS cells were isolated. The unlabeled consensus κB oligonucleotide (*lanes* 3, 9) or the mutant κB oligonucleotide (*lanes* 4, 10) was added as a competitor in a 20-fold molar excess to the binding reaction. Abs against p65 (*lanes* 5, 11) and p50 (*lanes* 6, 12) were added to the reaction for a supershift assay. The *arrow* indicates the NF-κB-containing complex.

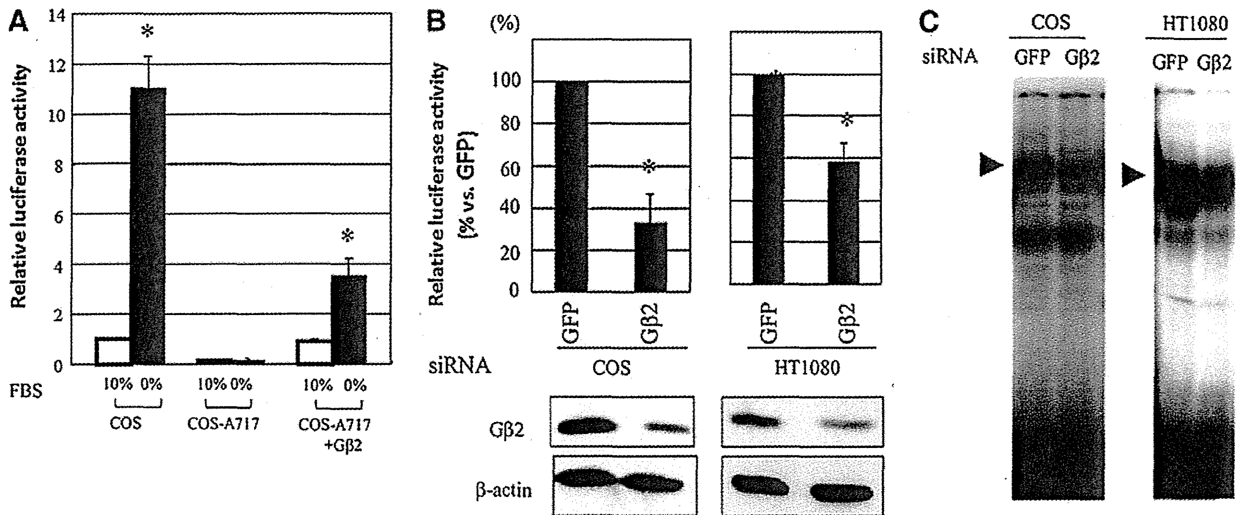


FIG. 3. Gβ2 is required for serum starvation-induced NF-κB activation and for constitutive NF-κB activation in transformed cells. **(A)** The serum-starving stress-induced NF-κB activation was analyzed by a luciferase assay. COS, COS-A717, and Gβ2-transfected COS-A717 cells were transiently transfected with the 5 × κB-luciferase reporter. Six hours after transfection, the cells were washed with phosphate buffered saline (PBS) and incubated with (10%) or without FBS (0%) for 36 h for the luciferase assay. The luciferase activities in COS cells incubated with 10% FBS were set as 1.0. The activations were significant ($^{*}p < 0.05$). **(B)** The Gβ2 siRNA reduced the NF-κB activity in COS and HT1080 cells. The NF-κB activity was determined by transfection with the 5 × κB-luciferase reporter together with the GFP or Gβ2 siRNA, and shown as the % of that in cells transfected with the GFP siRNA. Western blot analyses of Gβ2 (*top*) and β-actin (*bottom*) in cells transfected with the GFP or Gβ2 siRNA#1 were performed. The inhibitions were significant ($^{*}p < 0.05$). **(C)** Nuclear cell extracts were isolated from COS and HT1080 cells transfected with the GFP or Gβ2 siRNA. The *arrowhead* indicates the NF-κB-containing complex.

Gβ2 is involved in NF-κB in HT1080 human fibrosarcoma cell line

HT1080 cells also have a relatively high level of basal NF-κB activity. We examined whether Gβ2 contributes to the constitutive activation of NF-κB in HT1080 cells. Knockdown of Gβ2 expression by siRNA reduced the basal NF-κB activity not only in COS cells, but also in HT1080 cells (Fig. 3B). The siRNA against Gβ2 indeed reduced the Gβ2 protein level. Consistent with the κB promoter activity, the knockdown of Gβ2 inhibited the NF-κB-binding capability to the target sequence (Fig. 3C). These results indicate that Gβ2 is required for the constitutive activation of NF-κB in COS and HT1080 cells, suggesting that Gβ2-mediated signaling contributes to the constitutive NF-κB activation. The serum-deprived Gβ2-mediated NF-kB activation in COS cell was also confirmed using another siRNA (Supplementary Fig. S2).

Impact of IKKs, NEMO, and IκB in Gβ2-induced NF-κB activation

To determine whether the Gβ2 induced NF-κB activation requires IκB phosphorylation, a IκBα-SR with mutations at the inducible phosphorylation sites, S32G and S36A, was coexpressed with Gβ2 in COS-A717 cells. The IκB-SR abolished the Gβ2-induced NF-κB activation in a dose-dependent fashion (Fig. 4A). This result suggests that the phosphorylation of IκBα at S32 and S36 is necessary for the Gβ2-induced NF-κB activation. An important regulator of phosphorylation in the IκB pathway is the IKK complex, which comprises multiple kinases, including IKK1 (IKKα), IKK2 (IKKβ), and NEMO (IKKγ). We examined whether IKK1, IKK2, and/or NEMO were involved in the Gβ2-induced NF-κB activation. Dominant negative forms of IKK1 (IKK1.DN), IKK2 (IKK2.DN), and NEMO (NEMO.DN) were each coexpressed in the Gβ2-transfected COS-A717 cells. As shown in Figure 4B–D, IKK1.DN, IKK2.DN, and NEMO.DN were each able to reduce the Gβ2-induced NF-κB activation in COS-A717 cells in a dose-dependent fashion, indicating that IKK1, IKK2, and NEMO are involved in the Gβ2-induced NF-κB activation. Especially, the IKK2.DN more efficiently suppressed the Gβ2-mediated NF-κB activation than IKK1.DN and NEMO.DN. This result suggests that IKK2 plays an important role in the Gβ2-induced NF-κB activation, like the bradykinin-induced NF-κB activation through Gαq and Gβγ (Xie *et al.*, 2000). To confirm the expressions of the dominant negative and endogenous IKK1, IKK2, NEMO, IκBα, and Akt, COS cells were transfected with the mutant expressing plasmids, and the cell lysates were subjected to Western blotting analysis using their specific antibodies. The descriptions of the dominant negative mutants and their specific antibodies used here are summarized in the Tables 1 and 2, respectively. As shown in Figure 5A, we have confirmed that the anti-IKK1, NEMO, and Akt1 antibodies were able to detect the simian endogenous proteins as well as their human and mouse dominant negative mutants. On the other hand, the antibodies against IKK2 and IκBα reacted to their human dominant negative mutants and the human endogenous proteins in HEK293T cells, but not to the simian endogenous proteins. Considering the different affinities of the antibodies between endogenous simian proteins and their human or mouse counterparts, we could not assess precisely the relative amounts of dominant negative mutants to endogenous proteins in COS cells. However, each

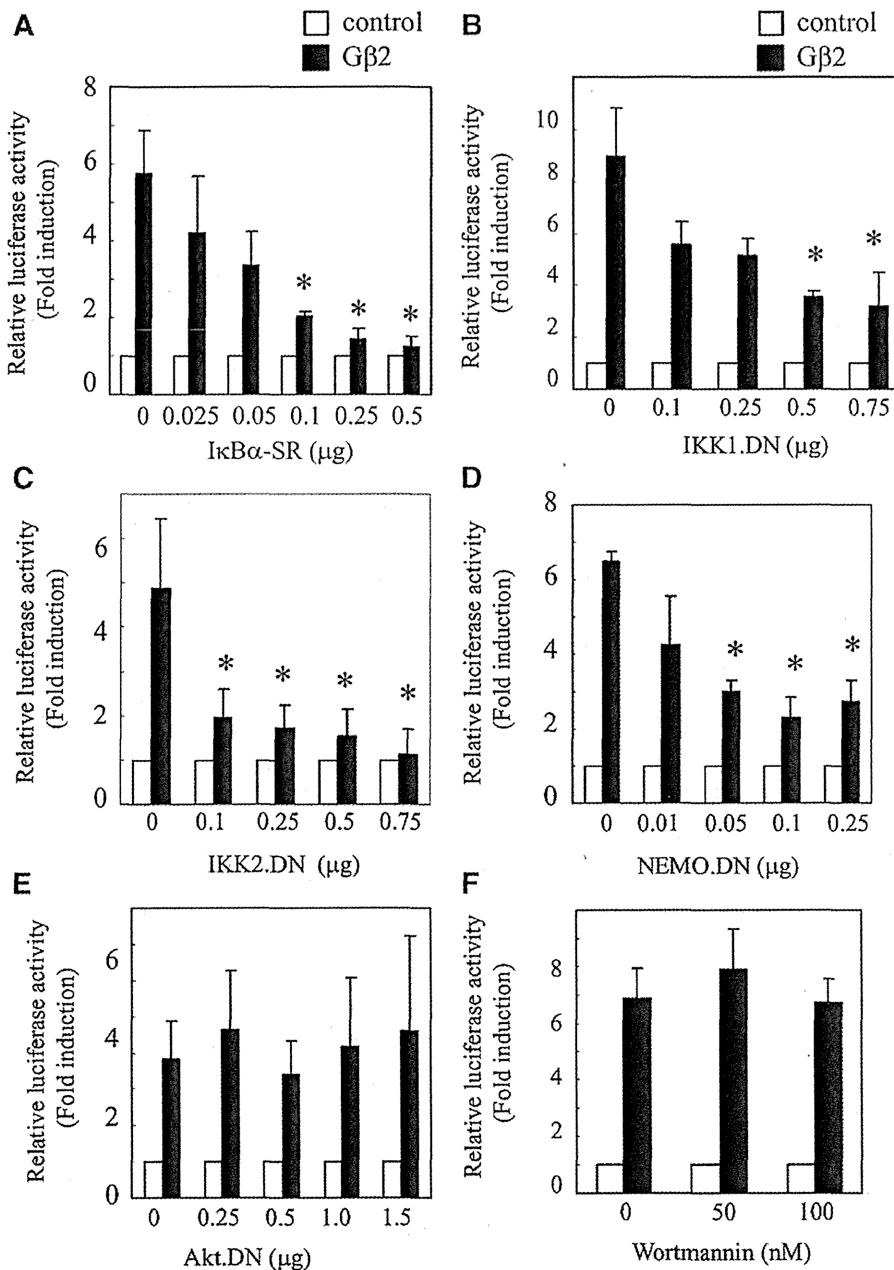


FIG. 4. Involvement of IκB, IκB kinase 1 (IKK1), IKK2, and NEMO in Gβ2-induced NF-κB activation. COS-A717 cells were transfected with 0.25 μg of the 5×κB-luciferase and the mutant expression plasmid of IκBα super-repressor (IκBα-SR) **(A)**, IKK1 (IKK1.DN) **(B)**, IKK2 (IKK2.DN) **(C)**, NEMO (NEMO.DN) **(D)**, or Akt (Akt.DN) **(E)**, together with the Gβ2 construct (0.25 μg) or pcDNA3.1. The pcDNA3.1 plasmid was added to make the total amount of DNA 1 μg/well. COS-A717 cells were cotransfected with 0.5 μg of the 5×κB-luciferase reporter without (control) or with 0.5 μg of the Gβ2 construct. Wortmannin was added 1 h before transfection **(F)**. The relative luciferase activity in the control cells without Gβ2 was set as 1.0. Data shown are averages and SD from three independent experiments. The inhibitions were significant (*$p < 0.05$).

TABLE 1. CHARACTERISTICS OF THE DOMINANT NEGATIVE MUTANTS

Molecule	*Species*	*Structure*	*MW (kDa)*	*Tag*
IκBα	Human	S32G, S36A mutant	40	—
IKK1	Human	N145D mutant	85	VSV
IKK2	Human	N145D mutant	87	VSV
NEMO	Mouse	97–412 C-terminal fragment	37	VSV
Akt	Mouse	K179A, T308A, S473A mutant	69	-

MW, molecular weight.

TABLE 2. CHARACTERISTICS OF THE ANTIBODIES

Antigen	*Cross-reactivity*
Human IκBα C-terminal peptide	Human/mouse/rat
Human IKK1 full-length	Human/mouse/rat
Human IKK2 C-terminal peptide	Human/canine
Human NEMO full-length	Human/mouse/rat
Human Akt1 345–480 peptide	Human/mouse/rat
Human phospho-S32 and S36-containing peptide	Human/mouse

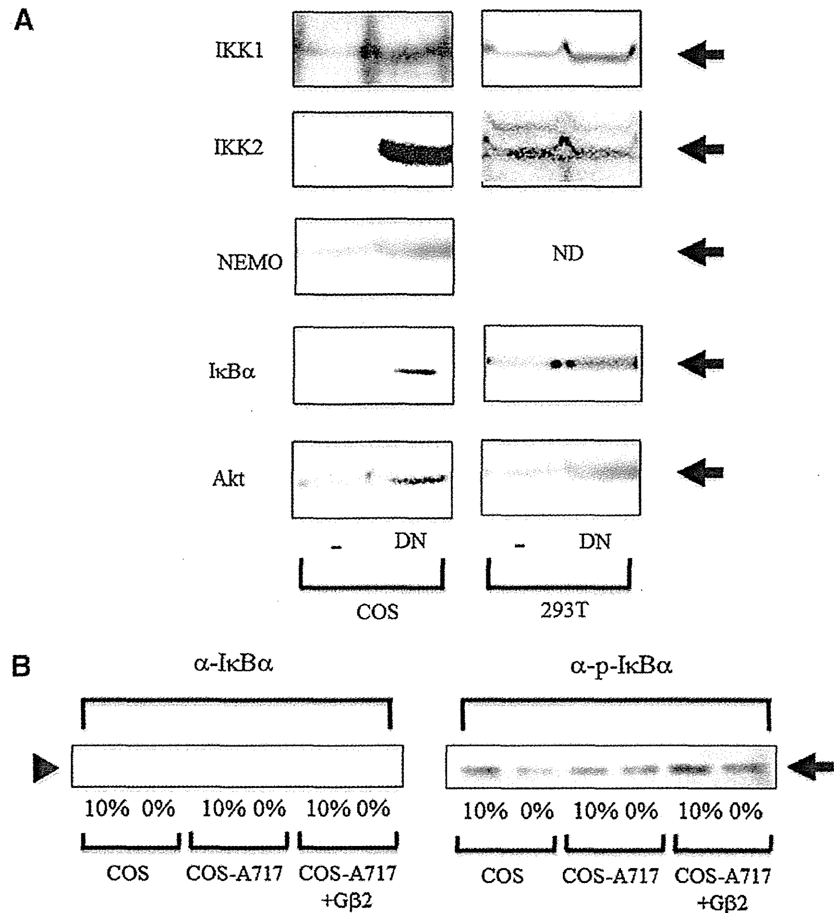


FIG. 5. **(A)** Expressions of the dominant negative and endogenous IKK1, IKK2, NEMO, IκBα, and Akt. COS cells were transfected with 1 μg of the mutant expressing plasmids of IKK1.DN, IKK2.DN, NEMO.DN, superrepressor of IκB (IκB-SR), and Akt.DN (refer to Table 1) in a six-well plate, and the cell lysates were prepared using 100 μL Glo lysis buffer (Promega) after 48h incubation. HEK293T cells were also transfected with the same plasmids, and prepared for Western blot. The description of antibodies is shown in Table 1. The *arrows* indicate the described proteins. N.D: not done. **(B)** COS, COS-A717, and COS-A717-Gβ2 cells were washed with PBS and incubated with (10%) without FBS (0%) for 24 h, and lysed with Glo lysis buffer. The positions of total IκBα, and phospho-IκBα were indicated with *arrowhead* and *arrow*, respectively.

dominant negative mutant is likely to be expressed enough to suppress its endogenous protein.To evaluate the phosphorylation and degradation of IkBα in COS, COSA717, and COSA717-Gβ2 cells by serum deprivation, we carried out Western blotting analysis using their specific antibodies. There were no significant changes in the phosphorylation of IκBα of COS, COSA717, and COSA717-Gβ2 cells by serum deprivation, using a phosphorylation-specific antibody (right panel of Fig. 5B). We could not assess the degradation of IκBα, because the anti-IκBα antibody was actually able to detect the human IκBα protein, but not simian COS IκBα, or because the IκBα expression in COS cell is too low to be detected by this antibody (Fig. 5A and left panel of Fig. 5B). The NF-κB activation by serum deprivation was dependent on IKK1, IKK2, NEMO, and IκBα, and this unique characteristic was not related to the IκBα phosphorylations at S32 and S36.

Because PI3K and Akt are upstream factors of IKKs in the NF-κB activation pathway (Ozes *et al.*, 1999; Romashkova and Makarov, 1999; Xie *et al.*, 2000), we examined whether the Gβ2-induced NF-κB activation occurs through PI3K and Akt activation. However, the PI3K inhibitor,Wortmannin, and a dominant negative mutant of Akt did not affect the Gβ2-induced NF-κB activation in COS-A717 cells (Fig. 4E, F). This result suggests that PI3K and Akt is not involved in the Gβ2-induced NF-κB activation.

Discussion

Many cell stresses activate NF-κB. We have shown here that serum starvation activates NF-κB signal, indicating that serum contains unknown inhibitor(s) of NF-κB signal. Cell stresses, such as radiation (Criswell *et al.*, 2003), oxidation (Marshall *et al.*, 2000), and UV (Kato *et al.*, 2003) positively control the NF-κB signaling. Interestingly, serum negatively regulates the NF-κB signaling, and starvation stress induces NF-κB activation by exclusion of the negative factor of serum.

Serum starvation activated NF-κB signaling in COS cells, but not in COS-A717 cells. The transfection of COS-A717 cells with Gβ2 partially restored the serum starvation-induced NF-κB activation. This result indicates that Gβ2 is required for the starvation-induced NF-κB activation, and the serum inhibitor suppresses the Gβ2-induced signaling pathway (Fig. 6).

Serum starvation of cells is frequently used in many biological experiments, including cell cycle synchronization and induction of apoptosis and autophagy. These biological events induced by starvation unexpectedly include the activation of Gβ2 and NF-κB signals. Therefore, these signaling might affect the synchronization of the cell cycle and the induction of apoptosis and authophagy by starvation, and scientists should consider the effects of the Gβ2 and NF-κB signals in the biological experiments using serum starvation.

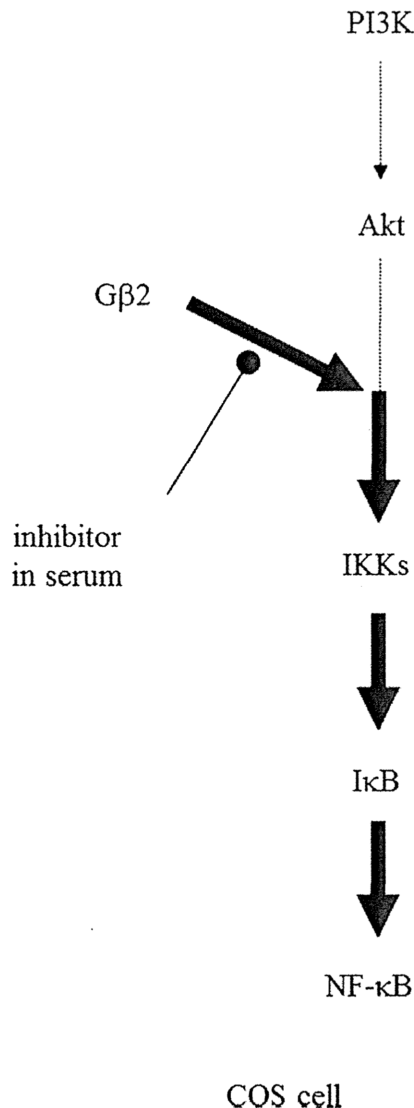


FIG. 6. Signaling pathway of NF-κB activation by Gβ2 or serum starvation.

We are trying to identify the serum inhibitor, and it will provide great impacts into many biological research fields.

NF-κB is constitutively activated in several transformed cell lines, suggesting that NF-κB signaling is involved in cellular transformation. However, the mechanism has not been elucidated yet. COS-A717 cells are mutant cells in which the basal NF-κB activity is much lower compared with the parental COS cells. Here, we showed that COS-A717 cells expressed a lower level of Gβ2 than COS cells, and the transfection of COS-A717 cells with Gβ2 restored the basal NF-κB activity, suggesting that the reduced expression level of Gβ2 is responsible for the defective NF-κB signaling in COS-A717 cells. Furthermore, the knockdown of Gβ2 expression by siRNA reduced the basal NF-κB activity not only in the COS cells, but also in the HT1080 cells, another transformed cell line with constitutively activated NF-κB signaling. These results indicate that Gβ2 is required for the constitutive activation of NF-κB in these transformed cells. This conclusion is strongly supported by previous reports showing that certain GPCR signals or the Gβ1γ2 complex activate NF-κB signaling (Xie *et al.*, 2000; Grabiner *et al.*, 2007; Sun *et al.*, 2009). Furthermore, the Tax oncoprotein of HTLV-1 activates NF-κB (Mori *et al.*, 1999; Gohda *et al.*, 2007) as well as the signals of CXCR4, a GPCR, by binding to the Gβ subunit (Twizere *et al.*, 2007), consistent with our conclusion. Although the Gβγ complex activates NF-κB through PI3K (Stephens *et al.*, 1994; Xie *et al.*, 2000), a PI3K inhibitor did not affect the Gβ2-induced NF-κB activation, suggesting that Gβ2 activates independently of PI3K in the NF-κB activation pathway (Fig. 6).

In summary, this study found that Gβ2-induced signaling activates NF-κB independently of PI3K and Akt in COS cells (Fig. 6). Unknown factor(s) present in serum inhibit the Gβ2-induced signaling. Therefore, serum starvation activates NF-κB by removing the serum inhibitor(s). The Gβ2-induced signaling is the target of the serum inhibitor, because exclusion of the serum inhibitor by starvation elevates NF-κB activity in Gβ2-expressing COS cells, but does not affect in Gβ2-defective COS-A717 cells.

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Disclosure Statement

No competing financial interests exist.

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