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ORIGINAL ARTICLE

5-Fluorouracil prevents lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophage cells by inhibiting Akt-dependent nuclear factor-κB activation

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Abstract The effect of 5-fluorouracil (5-FU) on the production of nitric oxide (NO) in macrophages was examined by using lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. 5-FU at non-toxic concentrations significantly inhibited NO production in LPS-stimulated RAW 264.7 cells. The inhibition by 5-FU was mediated by attenuated expression of an inducible NO synthase protein and mRNA. 5-FU inhibited the activation of nuclear factor (NF)-κB and the subsequent nuclear translocation. Furthermore, 5-FU inhibited the phosphorylation of Akt, an upstream molecule of NF-κB signaling. 5-FU did not affect a series of mitogen-activated protein kinases. Therefore, 5-FU was suggested to inhibit the LPS-induced NO production in activated macrophages through preventing Akt-dependent NF-κB activation.

Keywords 5-FU · LPS · NO · Akt · NF-κB · iNOS

Introduction

Macrophages can recognize and eliminate tumor cells by using a variety of cytotoxic effectors [1]. Recently, nitric oxide (NO) is paid particular attention in mediating macrophage cytotoxicity. High levels of NO derived from activated macrophages has a potentially cytotoxic/cytostatic effect upon tumor cells [2]. Therefore, the expression of an inducible type of NO synthase (iNOS) in activated macrophages may be cytostatic or cytotoxic for tumor cells [3]. Thus, therapeutical interference with iNOS activity is of considerable interest, especially in tumors, where metastatic activity and host defense seem to be correlated to iNOS expression [2].

The anti-metabolite 5-fluorouracil (5-FU) is one of the most potent anti-tumor and a widely used chemotherapeutic agent, specially for advanced colorectal cancer and some other solid cancers [4]. 5-FU appears to have a complex action including both inhibitory and promoting activity on the NO production in cancer cells. In fact, 5-FU inhibits NO production in human colon adenocarcinoma cells [5] and stomach cancer cells [6] whereas it induces and enhances NO production in gastric cancer cells [7] and liver carcinoma cells [8]. Conflicting evidence still surrounds the effects of 5-FU on NO production in cancer cells. On the other hand, there is no report on the effect of 5-FU on the production of NO and expression of iNOS in activated macrophages. Bacterial lipopolysaccharide (LPS) is known to activate the macrophages and induce the iNOS expression and high NO output [9, 10]. In the present study, we examined the effect of 5-FU in NO production in LPS-stimulated RAW 264.7 macrophage cells. Here, we report that 5-FU inhibits the iNOS expression and NO production in LPS-activated RAW 264.7 macrophages via inhibition of Akt-dependent nuclear factor (NF)-κB activation.

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Materials and methods

Reagents

5-Fluorouracil and LPS from *Escherichia coli* O55:B5 were purchased from Sigma Chemicals, St Louis, MO, USA. A series of antibodies to NF-κB, Akt, p38, extracellular signal regulated kinase (Erk)1/2, stress-activated protein kinase (SAPK/JNK) and their phosphorylated forms were obtained from Cell Signaling Technology, Beverly, MA, USA. An anti-iNOS antibody was purchased from Upstate Inc., Lake Placid, NY, USA.

Cell culture

The murine macrophage cell line, RAW 264.7, was obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, MD, USA) and antibiotics at 37°C under 5% CO_2.

Cell viability

Cell viability was determined by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Chemicon, Temecula, CA, USA) as described elsewhere [11].

Determination of nitrite concentration

Nitric oxide was measured as its end product, nitrite, using Griess reagent as described elsewhere [12, 13].

Immunoblotting

RAW 264.7 cells were cultured with 5-FU (500 μM) for 1 h in a 35 mm plastic dish (4×10^5 cells/dish) and stimulated with LPS (1 μg/ml) for various time. The immunoblotting method was described previously [13]. Briefly, the cell lysates were extracted by the lysis buffer containing 0.5 M Tris–HCl, 4% sodium dodecylsulfate (SDS) and 2-mercaptoethanol, and boiled at 80°C for 5 min. The protein concentration of the samples was determined by the BCA protein-assay reagent (Pierce, Rockford, IL, USA). An equal amount of protein (20 μg) was analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to a membrane filter. The membranes were treated with an appropriately diluted antibody for overnight. The immune complexes were detected with a 1:5,000 dilution of horseradish peroxidase-conjugated protein G for 1 h and the bands were visualized with a chemiluminescent reagent (Pierce, Rockford, IL, USA).

Luciferase reporter gene assay for NF-κB activation

RAW 264.7 cells (3×10^5/ml) were plated in a 35 mm plastic dish. On the following day, the cells were transfected with 0.5 μg/ml of pNF-κB-TA-luc luciferase reporter genes (Mercury pathway Profiling Luciferase System 2, BD Biosciences Clontech, Palo Alto, CA, USA) and 0.05 μg of pRL-TK plasmid (Promega, Madison, WI, USA) by lipofectamine 2000 transfection reagent (Gibco-BRL, Gaithersburg, MD, USA). The transfected cells were incubated for 24 h, stimulated with LPS (1 μg/ml) for 6 h after pretreatment with 5-FU (500 μM), and lysed with a lysis reagent. The luciferase activity was determined by the dual luciferase-assay kit (Promega, Madison, WI, USA).

Reverse transcription-polymerase chain reaction

RAW 264.7 cells were plated in 35 mm plastic dishes, incubated with 5-FU (500 μM) and stimulated with LPS (1 μg/ml) for 6 h. After the total RNA was extracted by using a RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Semi-quantitative Reverse transcription-polymerase chain reaction (RT-PCR) was carried out by using access quick RT-PCR system (Promega, Madison, WI, USA). RT-PCR reactions were performed using mouse iNOS primers forward 5′-TCT TTG ACG CTC GGA ACT GT and reverse 5′-TCT TGA CCA TCA GCT TGC AA. A total of 30 cycles were run for each experiment. The sample was taken out and run in 1.5% agarose gel. The housekeeping GADPH gene was used as a control.

Immunohistochemistry

RAW 264.7 cells were cultured in eight-chambered slide and incubated with 5-FU (500 μM) for 1 h and then incubated with or without LPS (1 μg/ml) for 1 h. The cells were fixed with methanol at −20°C for 20 min. They were incubated with 10% normal goat serum at room temperature for 30 min to avoid non-specific staining. After that, the cells were incubated with rabbit anti-NF-κB antibody in 1% BSA at 4°C for overnight. Normal rabbit serum was used for a negative control. After treatment with a second antibody, the cells were stained according to the avidin–biotin–peroxide complex (ABC) method using a commercial Vectastain ABC kit (Vector Laboratories, Burlingame,

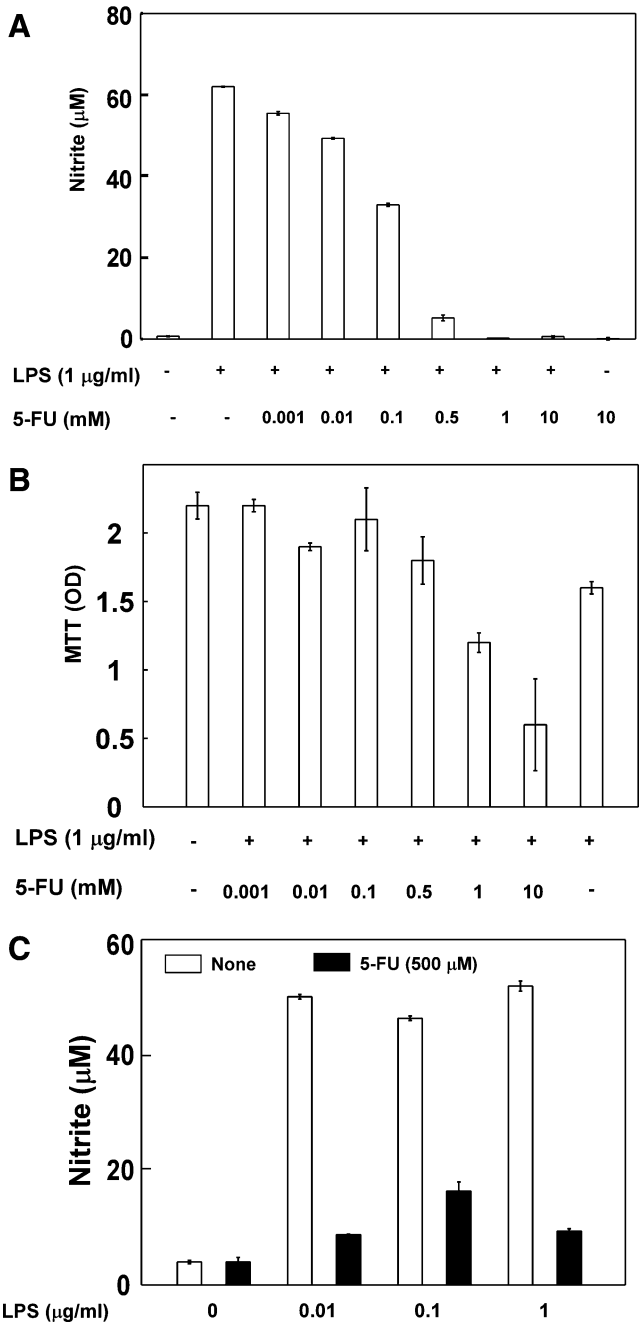

Fig. 1 **a** Effect of 5-fluorouracil (5-FU) on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cells. RAW 264.7 cells were incubated with 5-FU at 1, 10, 100, 500 μM, 1 and 10 mM for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. The nitrite concentration in the culture supernatant was determined for NO production. Experimental data represent the mean of triplicate determinations ± standard deviation (SD). **b** Effect of 5-FU on the MTT activity of LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were incubated with 5-FU at 1, 10, 100 or 500 μM, 1 and 10 mM for 1 h and then stimulated with LPS (1 μg/ml). The MTT activity was determined by the optical density at 570 nm 24 h after LPS stimulation. The experimental results are expressed as the mean of triplicate determinations ± SD. Experiments were carried out three times. **c** Effect of 5-FU on NO production in RAW 264.7 cells stimulated with various concentrations of LPS. RAW 264.7 cells were incubated with 5-FU at 500 μM for 1 h and then stimulated with LPS at 0.01, 0.1 and 1 μg/ml for 24 h. The nitrite concentration in the culture supernatant was determined for NO production. Experimental data represent the mean of triplicate determinations ± SD

CA, USA) and visualized by 3,3′-diaminobenzidine (Vectastain DAB kit, Vector Laboratories, Burlingame, CA, USA). The photograph was taken by the Fujix digital camera HC-2500 under an Olympus BX50 microscope. The digital images were processed in brightness by the computer software.

Statistical analysis

Statistical significance was determined by Student's *t*-test.

Results

Inhibition of LPS-induced NO production by 5-FU

The effect of 5-FU on LPS-induced NO production was studied by using RAW 264.7 cells (Fig. 1a). RAW 264.7 cells were stimulated with LPS (1 μg/ml) for 24 h in the presence of various concentrations of 5-FU and the nitrite concentration in the supernatant was measured for NO production. 5-FU at 500 μM significantly inhibited LPS-induced NO production and the inhibition was roughly concentration-dependent.

To exclude the possibility that the inhibition of NO production was due to a cytotoxic action of 5-FU, the cell viability of 5-FU-treated RAW 264.7 cells was measured by the MTT assay (Fig. 1b). RAW 264.7 cells were incubated with 5-FU at concentrations of 1, 10, 100, 500 μM, 1 and 10 mM for 24 h. The MTT activity did not change up to 500 μM of 5-FU, at which it remarkably inhibited the LPS-induced NO production. 5-FU at 500 μM was used for further characterization unless otherwise stated.

Next, the inhibitory action of 5-FU on NO production in RAW 264.7 cells stimulated with various concentrations of LPS was examined (Fig. 1c). RAW 264.7 cells were pretreated with 5-FU for 1 h and then stimulated with LPS. 5-FU inhibited the NO production in RAW 264.7 cells treated with 10, 100 or 1,000 ng/ml of LPS. LPS at 1 μg/ml was used in the following experiments for maximal NO production in RAW 264.7 cells.

Time course of 5-FU-mediated inhibition of the NO production

The time-course of 5-FU-mediated inhibition of the NO production was studied (Fig. 2). RAW 264.7 cells were pretreated with 5-FU for 1 h and then stimulated with LPS. LPS did not trigger significant NO production in 5-FU-pretreated RAW 264.7 cells. On the other hand, LPS clearly induced NO production 6 h after the stimulation and the NO production increased up to 24 h in LPS-stimulated RAW 264.7 cells. 5-FU completely inhibited the LPS-induced NO production even 24 h after LPS stimulation.

Inhibition of iNOS protein and mRNA by 5-FU

The effect of 5-FU on the expression of iNOS protein and mRNA was examined. RAW 264.7 cells were pretreated with 5-FU for 1 h and then stimulated with LPS. The iNOS protein was analyzed by immunoblotting with anti-iNOS antibody (Fig. 3a). 5-FU-pretreated RAW 264.7 cells did not express iNOS proteins in response to LPS, although LPS clearly induced the iNOS expression in untreated cells. Subsequently, the effect of 5-FU on the level of iNOS mRNA was examined by a semi-quantitative RT-PCR (Fig. 3b). Whereas LPS definitely augmented the iNOS mRNA level in RAW 264.7 cells, 5-FU pretreatment inhibited the augmentation of iNOS mRNA. In addition, there was no significant difference in the expression of housekeeping GADPH gene as the control.

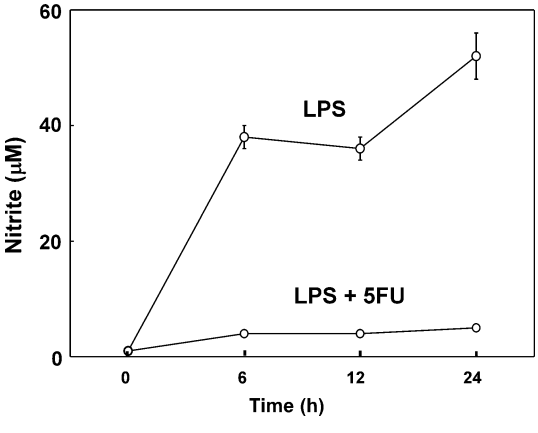

Fig. 2 Time-course of lipopolysaccharide (LPS)-induced nitric oxide production and its inhibition by 5-fluorouracil (5-FU). RAW 264.7 cells were pretreated with 5-FU 500 μM for 1 h and then stimulated with LPS (1 μg/ml). The nitrite concentration was determined 0, 6, 12 and 24 h after LPS stimulation. The experiment data are expressed as the mean of triplicate determinations ± standard deviation. One of three independent experiments is shown

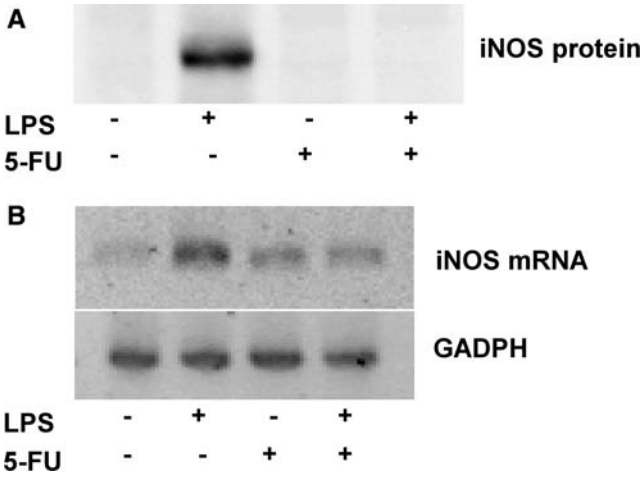

Fig. 3 Effect of 5-fluorouracil (5-FU) on expression of inducible nitric oxide synthase (iNOS) protein and mRNA. RAW 264.7 cells were pretreated with 5-FU 500 μM for 1 h and then stimulated with lipopolysaccharide (1 μg/ml) for 6 h. The expression of iNOS protein and mRNA was detected by immunoblotting and semi-quantitative reverse transcription-polymerase chain reaction

Inhibition of LPS-induced NF-κB activation by 5-FU

5-Fluorouracil is reported to inhibit NO production via inactivation of NF-κB activation in cytokines-stimulated cancer cells [6]. We tried to clarify the involvement of NF-κB in the inhibition of LPS-induced NO production by 5-FU. The effect of 5-FU on LPS-induced NF-κB activation was examined by a NF-κB-dependent luciferase reporter gene assay (Fig. 4). RAW 264.7 cells were pretreated with 5-FU and stimulated with LPS for 6 h and a relative luciferase activity was measured. As shown in Fig. 4, LPS markedly augmented the luciferase activity in RAW 264.7 cells. However, the 5-FU pretreatment definitely inhibited the increase of the luciferase activity in LPS-stimulated RAW 264.7 cells.

Inhibition of LPS-induced NF-κB nuclear translocation by 5-FU

In the preceding section, 5-FU prevented NF-κB activation with the luciferase-activity assay. The effect of 5-FU on nuclear translocation of NF-κB was also examined immunohistochemically (Fig. 5). RAW 264.7 cells were pretreated with 5-FU and stimulated with LPS for 1 h. The nuclei of LPS-stimulated RAW 264.7 cells were positively stained by anti-NF-κB antibody, suggesting nuclear translocation of NF-κB p65. On the other hand, such positive staining was not detected in the 5-FU-pretreated cells, suggesting that 5-FU prevented LPS-induced NF-κB nuclear translocation. In

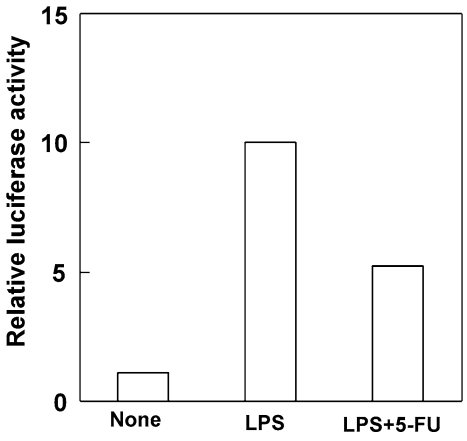

Fig. 4 Effect of 5-fluorouracil (5-FU) on lipopolysaccharide (LPS)-induced nuclear factor (NF)-κB activation. RAW 264.7 cells transfected were incubated with 5-FU (500 μM) for 1 h and then stimulated with LPS (1 μg/ml) for 6 h. The NF-κB activity was expressed as a relative luciferase activity. A typical experiment result of three experiments is shown with the mean of triplicate determinations

addition, 5-FU alone did not affect NF-κB nuclear translocation. Normal rabbit serum as a negative-control serum did not stain untreated or pretreated RAW 264.7 cells in response to LPS positively.

Inhibition of LPS-induced Akt activation by 5-FU

Nuclear factor (NF)-κB activation is regulated by a series of signal molecules and the Akt signal molecule is known to activate NF-κB via IκB kinase (IKK) activation [14]. The effect of 5-FU on Akt phosphorylation was examined in order to clarify the involvement of Akt in the inhibition of LPS-induced NF-κB activation by 5-FU (Fig. 6). RAW 264.7 cells were pretreated with 5-FU and stimulated with LPS for 30 min and 1 h. LPS clearly induced the phosphorylation of Akt whereas 5-FU almost completely inhibited LPS-induced Akt phosphorylation. 5-FU alone did not affect Akt expression in RAW 264.7 cells.

No inhibition of a series of mitogen-activated protein kinases by 5-FU

Lipopolysaccharide is known to activate a series of mitogen-activated protein (MAP) kinases, such as Erk1/2, p38 and JNK/SAPK as well as NF-κB for NO production in macrophages [15, 16]. The possibility was raised that 5-FU might also inhibit LPS-induced NO production through down-regulating MAP kinases. The effect of 5-FU on the activation of a series of MAP

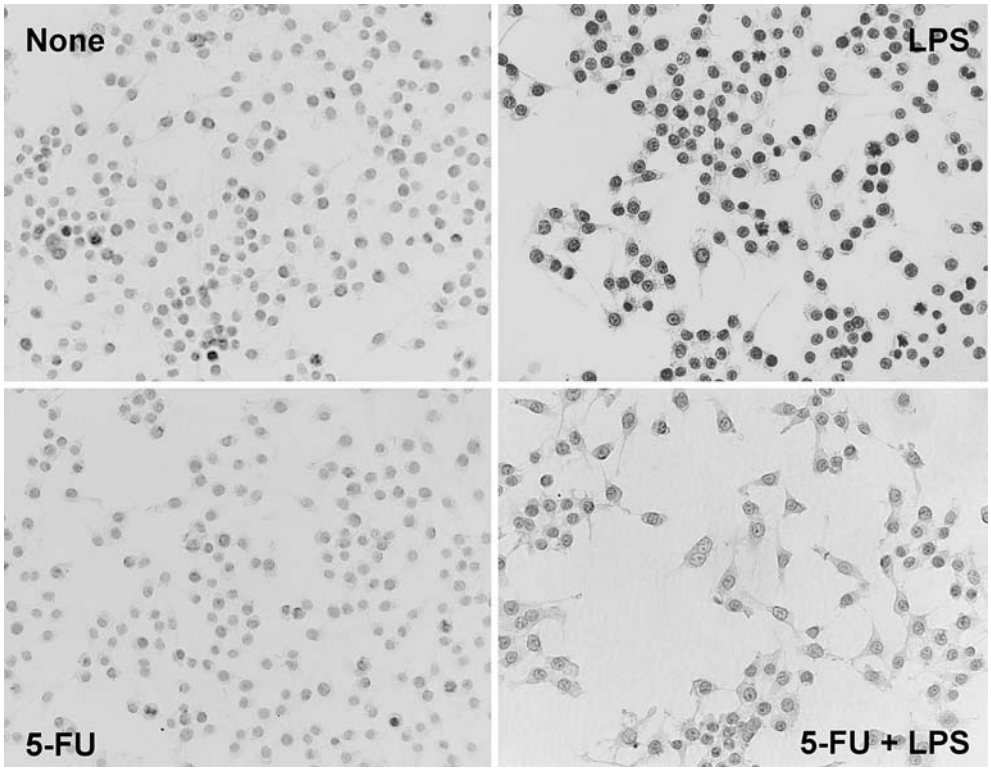

Fig. 5 Effect of 5-fluorouracil (5-FU) on lipopolysaccharide (LPS)-induced nuclear translocation of nuclear factor (NF)-κB. RAW 264.7 cells were incubated with 5-FU (500 μM) for 1 h and then incubated with LPS (1 μg/ml) for 1 h. Nuclear translocation of NF-κB was detected by immunohistochemical staining

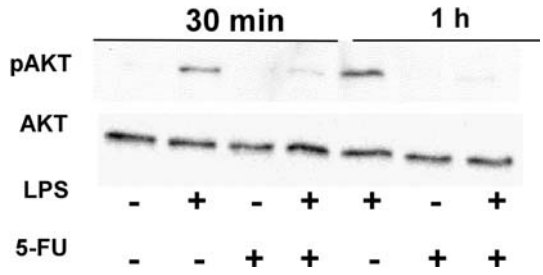

Fig. 6 Effect of 5-fluorouracil (5-FU) on lipopolysaccharide (LPS)-induced Akt phosphorylation. RAW 264.7 cells were incubated with 5-FU (500 μM) for 1 h and then stimulated with LPS (1 μg/ml) for 30 min or 1 h. Akt and the phosphorylated form (pAkt) 30 min or 1 h after LPS stimulation were detected by immunoblotting with an antibody to Akt or pAkt. One of three independent experiments is shown

kinases was examined by immunoblotting (Fig. 7). 5-FU-pretreated RAW 264.7 cells were stimulated with LPS for 30 min and 1 h. LPS rapidly phosphorylated all MAP kinase within 30 min after the stimulation. 5-FU did not influence on the LPS-induced phosphorylation of Erk1/2, p38 and SAPK/JNK.

Discussion

In the present study, we demonstrate that 5-FU prevents LPS-induced NO production in RAW 264.7 cells via inactivation of Akt-dependent NF-κB signal pathway. The possibility was excluded that 5-FU inhibits the NO production via its cytotoxic action on RAW 264.7 cells. The present study clearly showed that 5-FU inhibits the expression of iNOS mRNA and protein in LPS-stimulated RAW 264.7 cells, suggesting the involvement of an early event, such as signal transduction in LPS stimulation. In fact, 5-FU inhibits the activation and nuclear translocation of NF-κB in LPS-stimulated RAW 264.7 cells. Moreover, 5-FU inhibits the phosphorylation of Akt that regulates NF-κB activation as an upstream molecule through IKK activation [14], although the mechanism of the inhibition by 5-FU is still unclear. Collectively, 5-FU inhibits NF-κB activation through preventing Akt activation, and the failure of NF-κB activation results in attenuated expression of iNOS protein and subsequent NO production.

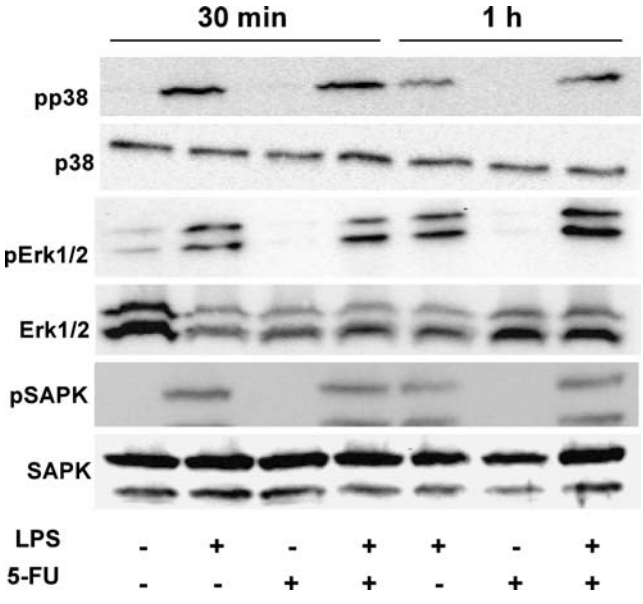

Fig. 7 Effect of 5-fluorouracil (5-FU) on lipopolysaccharide (LPS)-induced phosphorylation of a series of mitogen-activated protein kinases. RAW 264.7 cells were incubated with 5-FU (500 μM) for 1 h and then stimulated with LPS (1 μg/ml) for 30 min or 1 h. Erk1/2, p38, SAPK and their phosphorylated forms (pp38, pErk1/2, pSAPK) 30 min or 1 h after LPS stimulation were detected by immunoblotting with an antibody to Erk1/2, p38, SAPK or their phosphorylated forms

5-Fluorouracil is shown to inhibit NO production in LPS-stimulated RAW 264.7 cells. This indicates that 5-FU inhibits high levels of NO output in activated macrophages. High levels of NO generated by activated macrophages play an important role on anti-tumor activity in tumor immunity [2]. Therefore, 5-FU is very possible to attenuate anti-tumor activity of activated macrophages. This is the first report on an inhibitory action of 5-FU on NO production in activated macrophages. By the way, NO generated by cancer cells is involved in the carcinogenesis, growth, invasion and metastasis of solid tumor cells [17, 18]. There are several reports concerning the inhibitory action of 5-FU on NO production in cancer cells [5, 6]. On the other hand, 5-FU enhances NO production in gastric cancer cells [7] and liver carcinoma cells [8]. The effect of 5-FU on NO production in the microenvironment together with cancer cells and activated macrophages would be very complicated and difficult to interpret.

Akt can exert a positive effect on NF-κB function by phosphorylation and activation of IKK, and subsequent phosphorylation and degradation of IκB [14]. When 5-FU inhibits Akt phosphorylation, IKK-α and β, the downstream molecules of Akt, remain intact and IκB continues to be inactive. Thus, NF-κB is stabilized and its activation is inhibited. Jung et al. [6] reported that 5-FU inhibits NO production through the inactivation of IKK in stomach cancer cells. Further, Azuma et al. reported that 5-FU suppression of NF-κB is mediated by the inhibition of IKK in human salivary gland cancer cells [19]. Their findings are reasonable since Akt is an upstream molecule of IKK and 5-FU inhibits the Akt phosphorylation. Furthermore, it is certain that 5-FU exclusively inhibits NF-κB activation without the involvement of a series of MAP kinases since the Akt molecule does not trigger activation of MAP kinases. The inhibitory action

of 5-FU on Akt function is of particular interest since Akt plays an important role on human-cancer progression [20, 21] and Akt is associated with increased resistance to multiple chemotherapeutic agents [22, 23]. 5-FU is also reported to suppress NF-κB activity and induce apoptosis in human salivary gland cancer cells. The relationship of 5-FU-mediated Akt/NF-κB inhibition with cancer progression, and drug resistance and sensitivity must wait for further characterization.

It is of particular interest that 5-FU does not inhibit the production of tumor necrosis factor (TNF)-α at the protein and mRNA levels in LPS-stimulated RAW 264.7 cells (data not shown). LPS-induced TNF-α production is also mediated by NF-κB activation [24, 25]. TNF-α and NO production might depend on Akt-independent and -dependent NF-κB activation, respectively. The hypothetical idea is likely since TNF-α production is largely mediated by MyD88-dependent NF-κB activation and NO production is mediated by MyD88-independent pathway followed by the expression of interferon-β which activates phosphoinositide 3-K/Akt signaling [26–28]. However, the precise mechanism of the phenomenon is still a matter for speculation.

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