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Inhibition of inflammatory responses by FC-77, a perfluorochemical, in lipopolysaccharide-treated RAW 264.7 macrophages

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Abstract Objective: This study tested whether FC-77, a perfluorochemical, inhibits inflammatory responses in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages. The possible anti-inflammatory mechanisms involved were also investigated. **Methods:** RAW 264.7 macrophages were simultaneously incubated with pure FC-77 at final volume of 10% or 30% (v/v) and LPS (1 µg/ml) for 12 or 24 h on a mechanical shaker. In some tests FC-77 was added after cells stimulated by LPS for 12 h. Then the levels of prostaglandin E₂ (PGE₂), tumor necrosis factor α (TNF-α), and interleukins (IL)-1β, -6, and -10 were measured in medium. Alterations in cyclooxygenase (COX) 2 expression and nuclear transcription factor (NF) κB activation in cells were also evaluated. **Results:** Pre- or posttreatment with FC-77 dose-dependently reduced the LPS-induced TNF-α, IL-1β, and IL-6

formation and enhanced IL-10 production compared to LPS-stimulated alone cells. FC-77 also attenuated the LPS-induced PGE₂ formation accompanied by suppressing COX-2 expression, the degradation of cytosolic inhibitor of κB-α and NF-κB activation. **Conclusions:** FC-77 inhibits inflammatory responses in LPS-stimulated macrophages by a mechanism that involves the attenuation of NF-κB dependent induction of COX-2/PGE₂ pathway and the pro-/anti-inflammatory cytokine ratio.

Keywords Perfluorochemicals · Prostaglandin E₂ · Cyclooxygenase 2 · Macrophages · Cytokines

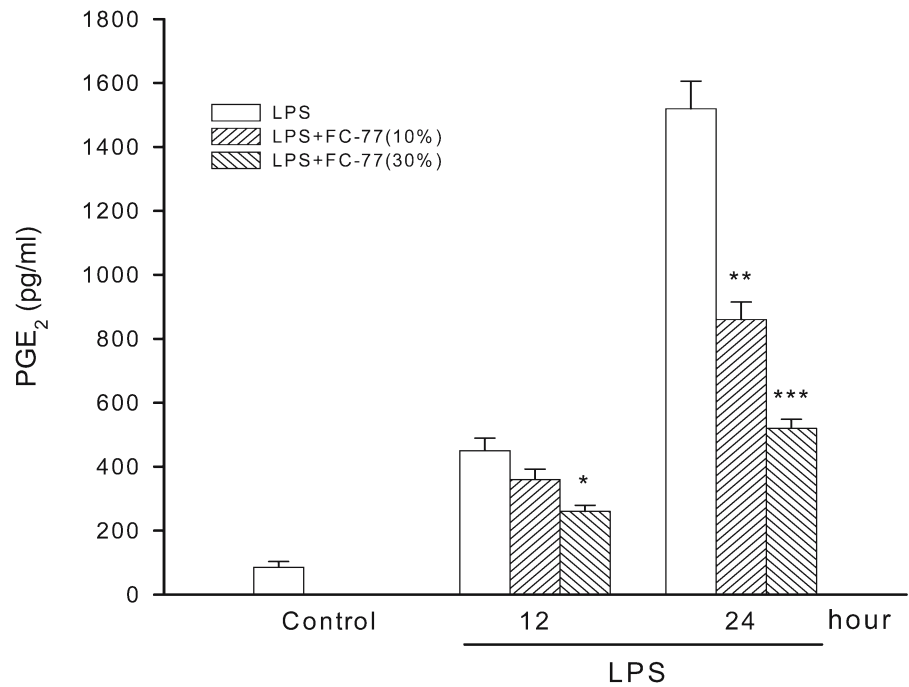
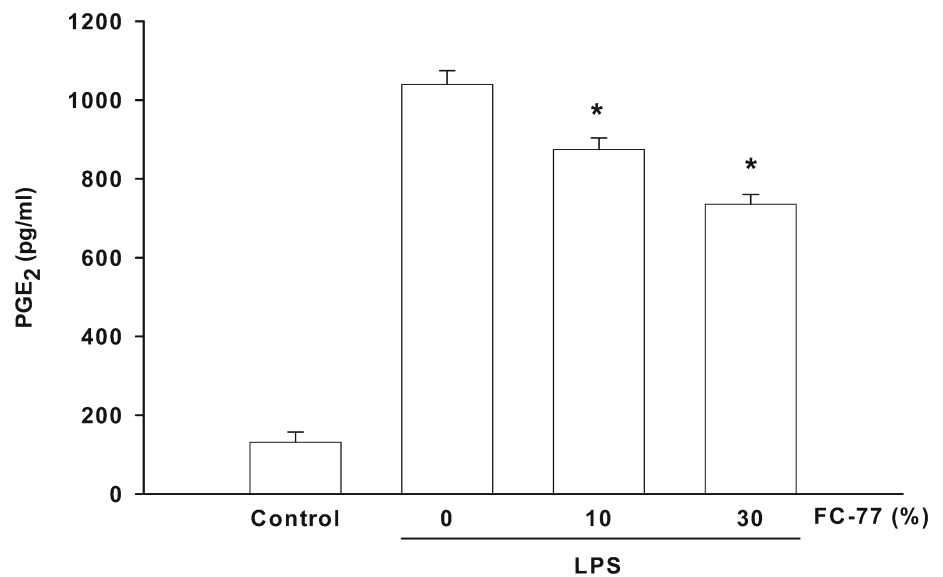
Introduction

Many studies have reported that partial liquid ventilation with perfluorochemical (PFC) improves gas exchange and pulmonary function in several models of lung injury [1, 2]. Using perflubron, a type of PFC, in partial liquid ventilation could reduce the inflammatory responses of trauma patients [3]. In addition, perflubron also suppresses the production of reactive oxygen species (ROS) [4] and malondialdehyde, an indication of lipid peroxidation, as well as pro-inflammatory cytokines [5, 6], suggesting that perflubron exerts an anti-inflammatory effect. How-

ever, perflubron is not available worldwide. FC-77, another type of PFC, has relatively higher vapor pressure and lower viscosity and less radiodensity than perflubron. Theoretically FC-77 may evaporate from the lungs faster than perflubron after finishing liquid ventilation, which may be beneficial for faster recovery after liquid assisted ventilation [7]. However, whether FC-77 has an anti-inflammatory effect is still unknown.

Overproduction of prostaglandin E₂ (PGE₂) derived from inducible cyclooxygenase (COX) 2 induced by lipopolysaccharide (LPS) and/or proinflammatory cytokines has been implicated in the pathogenesis of in-

Fig. 1 Effect of FC-77 on PGE₂ production in LPS-stimulated RAW 264.7 macrophages. After incubation of FC-77 with cells in response to LPS (1 µg/ml) for 12 or 24 h the PGE₂ levels in the medium were measured (A). After cells were stimulated with LPS (1 µg/ml) for 12 h, fresh medium containing FC-77 and cycloheximide (4 µM) was added, and the levels of PGE₂ in medium were measured after a further 12 h incubation (B). The cells cultured with DMEM alone acted as control group. Values of each group are expressed as mean ±SEM (*n*=6, each group). **p*<0.05, ***p*<0.01, ****p*<0.001 vs. respective LPS-stimulated alone cells

A**B**

flammatory diseases [8, 9]. Elevated proinflammatory cytokine release by alveolar macrophages seems to be correlated with poor outcome in patients with acute respiratory disease syndrome [10], suggesting that activated macrophages are a major source of inflammatory mediators. The present study examined whether the anti-inflammatory activity of FC-77 is associated with the suppression of COX-2/PGE₂ pathway and cytokine formation in LPS-stimulated RAW 264.7 macrophages. In addition, the possible molecular mechanisms involved were also investigated.

Materials and methods

Reagents

The FC-77 (chemical formula: C₈F₁₈, with a purity 100%) was purchased from 3M company (St. Paul, Minn., USA). Antibodies of COX-2 and κ B- α (Transduction Lab., Lexington, Ky., USA) as well as α -tubulin antibody (Santa Cruz, San Francisco, Calif., USA) were used in this study. Enzyme-linked immunosorbent assay kits including PGE₂, tumor necrosis factor (TNF) α , interleukin (IL) 6, IL-1 β , and IL-10 were purchased from Biosource International (Camarillo, Calif., USA). *Escherichia coli* derived LPS (*E. coli* serotype 055:B5) was purchased from Sigma Chemical (St. Louis, Mo., USA).

Cell culture

To prepare the tested solution of FC-77 (10% and 30%, v/v) pure FC-77 (100%) was mixed with Dulbecco's modified Eagle's medium (DMEM; v/v=1:9 or 3:7, respectively). Macrophages (1 \times 10⁶ cells) were cultured in 24-well plates with a total volume of 600 μ l FC-77 tested solution (10% or 30%, v/v) per well in the presence or absence of LPS (1 μ g/ml). As FC-77 is not miscible with the medium, cells incubated with FC-77 were shaken by using a mechanical shaker with gently agitation at 60 rpm [6]. After incubation for 12 or 24 h the medium was collected and centrifuged to obtain the FC-77 free supernatants for the following PGE₂ and cytokine assays, and cells washed twice with PBS buffer to remove residual FC-77 were collected for western blot assay. To further evaluate whether FC-77 has a therapeutic effect, after cells stimulated with LPS (1 μ g/ml) for 12 h, fresh medium containing FC-77 (10% or 30%, v/v) and cycloheximide (4 μ M) was added and the PGE₂ and cytokine levels of medium were measured after a further 12 h incubation. The cells cultured with DMEM alone acted as control group.

Western blot analysis of COX-2

The COX-2 western blotting assay of cells was determined according to our previously described method [11]. Expression of COX-2 protein was detected by enhanced chemiluminescence reagent (Amersham, Buckinghamshire, UK) and quantified by densitometry and normalized with respect to α -tubulin using Image-pro plus software.

κ B- α western blotting

After incubation with LPS for 1 h in the presence or absence of FC-77 the protein of cells was extracted and separated on 10% sodium

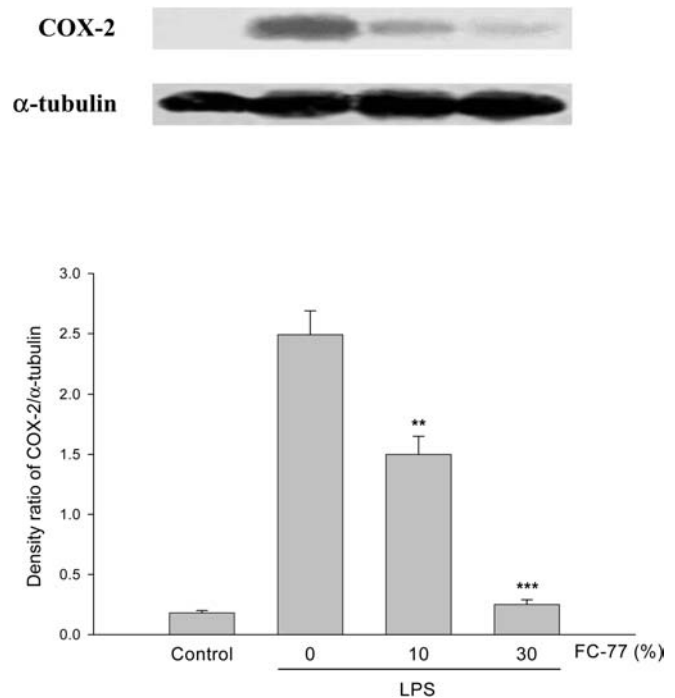


Fig. 2 Effect of FC-77 on COX-2 protein expression in LPS-stimulated RAW 264.7 macrophages. After incubation of FC-77 with cells in response to LPS (1 μ g/ml) for 24 h the cells were collected for COX-2 protein assay. The cells cultured with DMEM alone acted as control group. Western blotting is representative of five similar experiments. Values of each group are expressed as mean \pm SEM. ** p <0.01, *** p <0.001 vs. LPS-stimulated alone cells

dodecylsulfate polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass., USA). Membranes incubated with 5% (w/v) skim milk, the inhibitor (I) of κ B- α and anti- α -tubulin antibody were then added. The relative protein expression was quantified by densitometry as described above.

NF- κ B activation assay

After incubation with LPS for 1 h in the presence or absence of FC-77 the nuclear protein fractions of cells were separated. Then nuclear transcription factor (NF) κ B kit (BD Biosciences Clontech, Palo Alto, Calif., USA) was used to determine the activation of NF- κ B.

Cell viability assay

Cell respiration, an indicator of cell viability, assessed by the mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide to formazan, was quantified spectrophotometrically by measurement at OD₅₅₀.

Statistical analysis

Data are expressed as means \pm SEM. All results were analyzed by one-way analysis of variance followed by a multiple comparison test (Scheffe's test). A p value less than 0.05 was considered statistically significant.

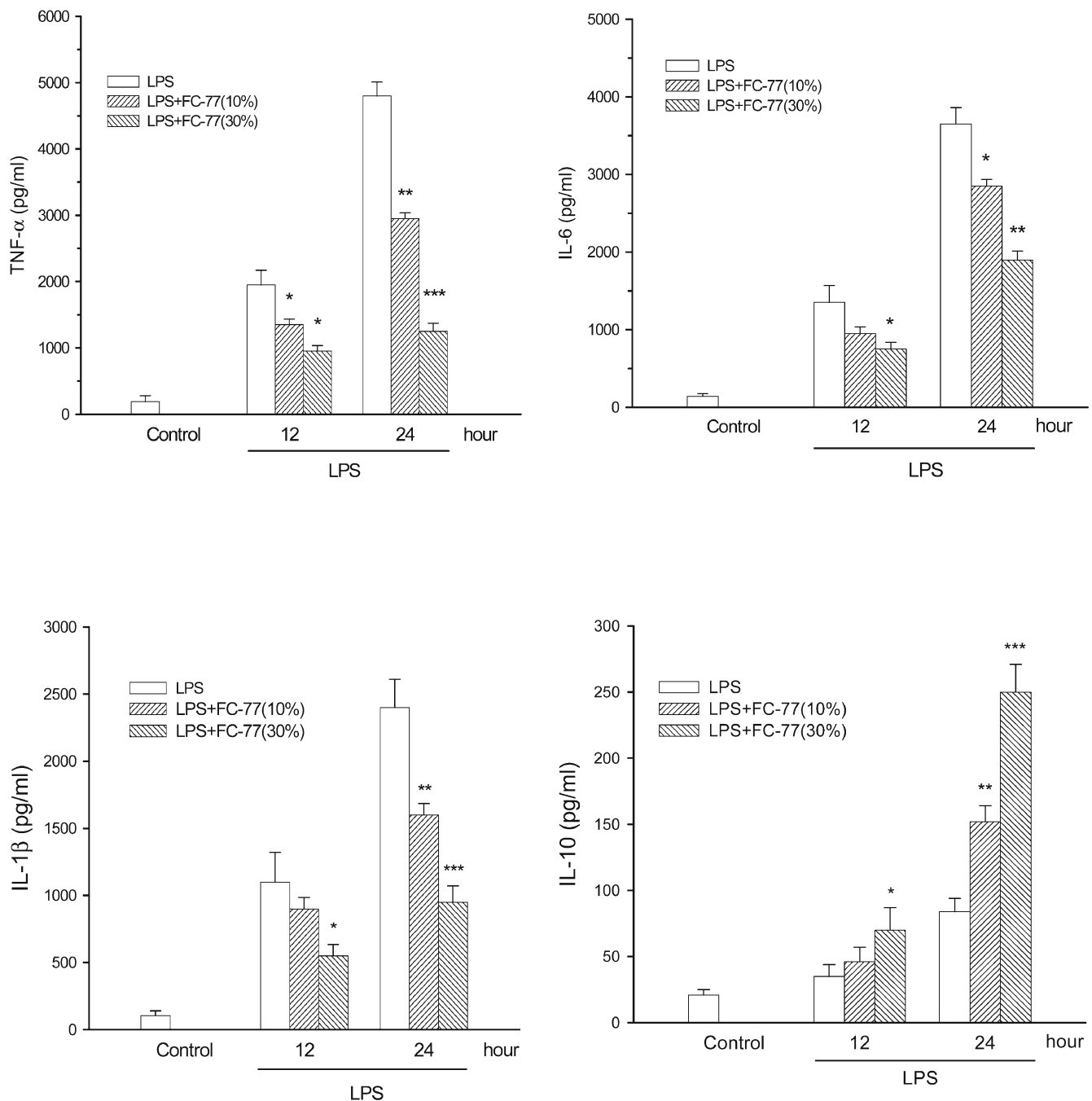


Fig. 3 Effect of simultaneous treatment with FC-77 on TNF- α , IL-1 β , IL-6, and IL-10 production in LPS-stimulated RAW 264.7 macrophages. After incubation of cells with LPS for 12 or 24 h in the presence or absence of FC-77 the concentrations of TNF- α , IL-

1 β , IL-6, and IL-10 in the medium were measured. The cells cultured with DMEM alone acted as control group. Values of each group are expressed as mean \pm SEM ($n=6$, each group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. respective LPS-stimulated alone cells

Results

FC-77 (10% or 30%, v/v) added simultaneously with LPS for 12 or 24 h (Fig. 1A) or added into cells stimulated by LPS for 12 h (Fig. 1B) resulted in a dose-dependent in-

hibition in the LPS-induced PGE₂ production compared to that of LPS-stimulated alone cells.

Incubation with FC-77 for 24 h significantly inhibited the LPS-induced COX-2 protein expression (Fig. 2) compared to that of LPS-stimulated alone cells.

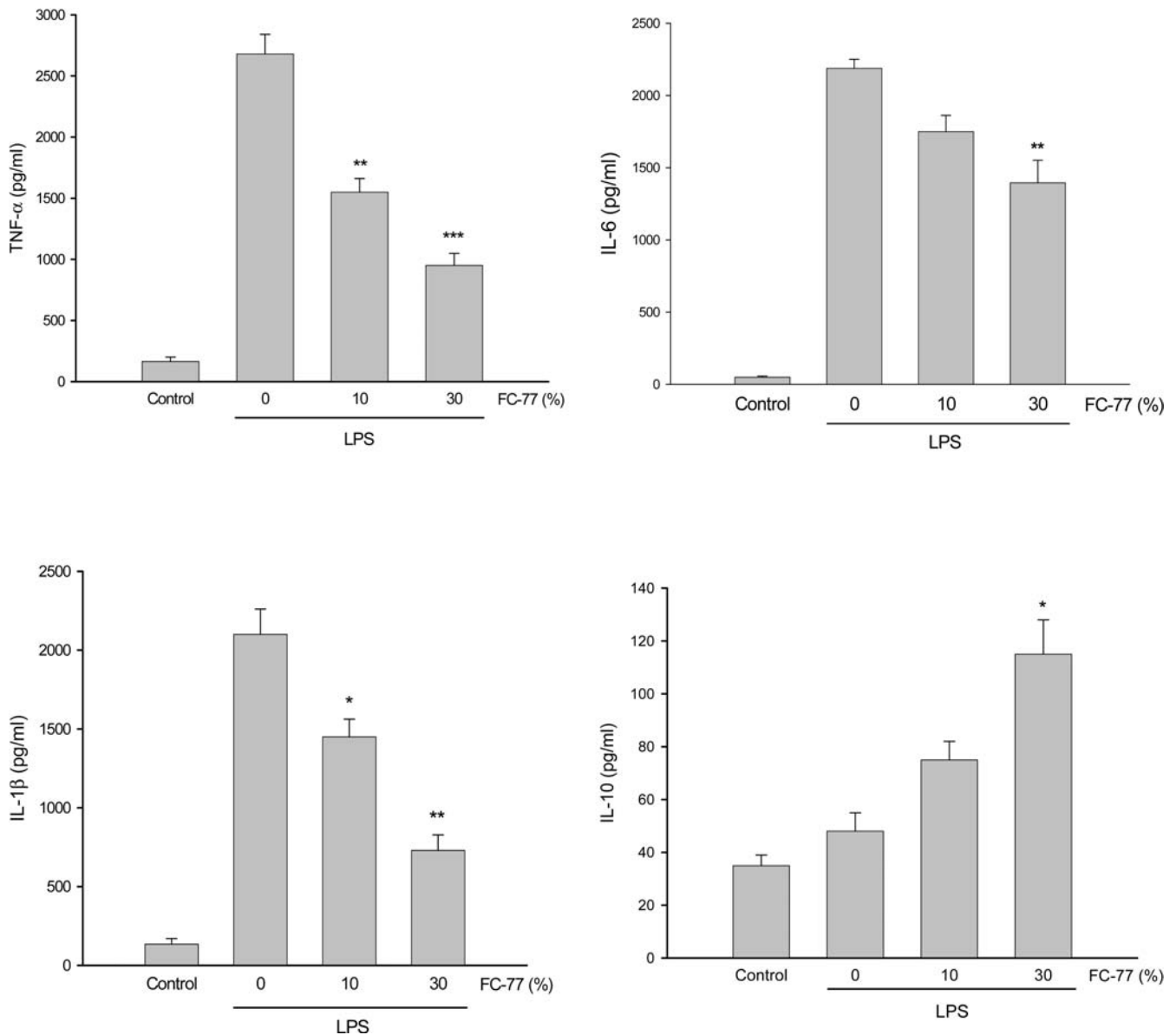


Fig. 4 Effect of posttreatment with FC-77 on TNF- α , IL-1 β , IL-6, and IL-10 production in LPS-stimulated RAW 264.7 macrophages. After cells were stimulated with LPS (1 μ g/ml) for 12 h, fresh medium containing FC-77 and cycloheximide (4 μ M) was added, and the levels of TNF- α , IL-1 β , IL-6, and IL-10 in medium were

measured after a further 12-h incubation. The cells cultured with DMEM alone acted as control group. Values of each group are expressed as mean \pm SEM ($n=6$, each group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. LPS-stimulated alone cells

FC-77 added simultaneously with LPS for 12 or 24 h (Fig. 3) or added into cells stimulated by LPS for 12 h (Fig. 4) caused a dose-dependent inhibition in TNF- α , IL-1 β , and IL-6 formation but markedly enhanced the IL-10 formation compared to that of LPS-stimulated alone cells.

Treatment with LPS for 1 h resulted in a marked degradation of cytosolic I κ B- α . FC-77 added simultaneously with LPS for 1 h prevented the cytosolic I κ B- α degradation compared to that of LPS-stimulated alone cells (Fig. 5).

When cells were treated with FC-77 for 1 h, the LPS-induced formation of p50 and p65 NF- κ B specific DNA-protein complex in nuclear extracts was dose-dependently inhibited (Fig. 6). In unstimulated cells FC-77 itself did not affect these mediators tested, including the basal PGE₂ and cytokines formation as well as COX-2, I κ B- α protein expression, and the activation of NF- κ B (data not shown).

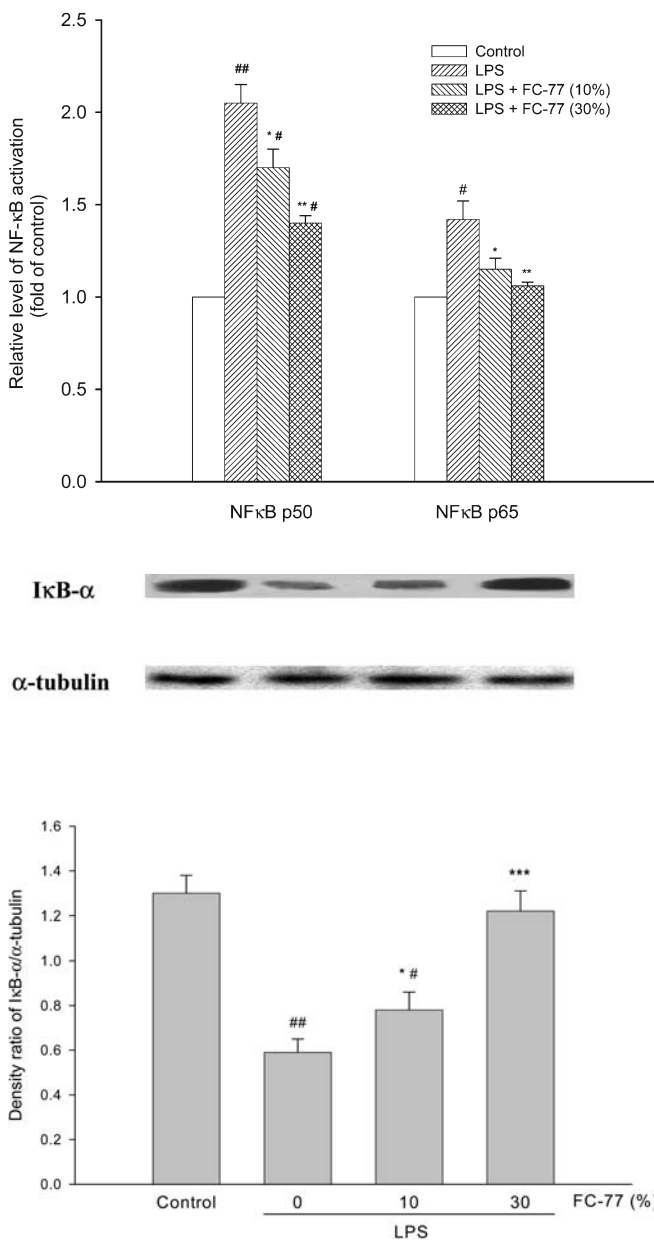


Fig. 5 Effect of FC-77 on the cytosolic IκB-α degradation in LPS-stimulated RAW 264.7 macrophages. After incubation with LPS for 1 h in the presence or absence of FC-77 the cytosolic protein of cells was collected for IκB-α protein assay by western blotting. Western blotting is representative of five similar experiments. The cells cultured with DMEM alone acted as control group. Values of each group are expressed as mean \pm SEM ($n=5$, each group). * $p<0.05$, *** $p<0.001$ vs. LPS-stimulated alone cells; # $p<0.01$, ## $p<0.001$ vs. control cells

FC-77 did not significantly diminish cell respiration at the solution studied (10% or 30%, v/v), with cell viability more than 95% of controls.

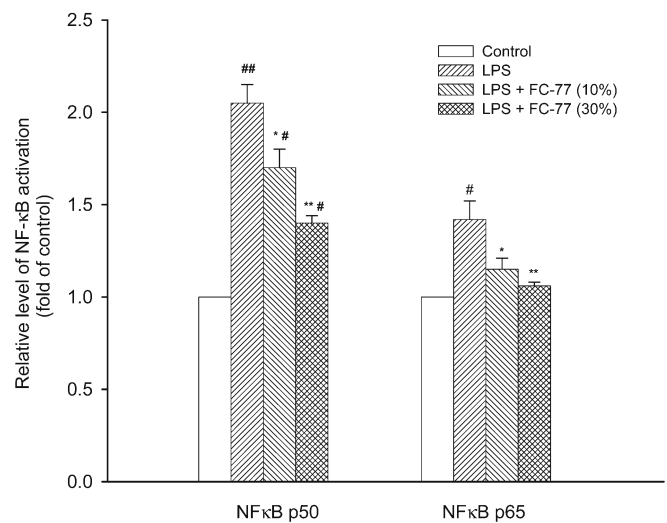


Fig. 6 Effect of FC-77 on nuclear NF-κB activation in LPS-stimulated RAW 264.7 macrophages. After incubation with LPS for 1 h in the presence or absence of FC-77 the nuclear extracts were prepared for assaying the formation of p50 and p65 NF-κB specific DNA-protein complex by enzyme-linked immunosorbent assay. The cells cultured with DMEM alone acted as control group. Values of each group are expressed as mean \pm SEM ($n=5$, each group). * $p<0.05$, ** $p<0.01$ vs. LPS-stimulated alone cells; # $p<0.01$, ## $p<0.001$ vs. control cells

Discussion

Proinflammatory cytokines including TNF- α , IL-6, IL-1 β , and TNF- α increase greatly after exposure to LPS, which in turn induces COX-2 expression [12]. Thus inhibition of these proinflammatory cytokines formation by FC-77 may account for the reduction in COX-2/PGE₂ pathway. Although we found that FC-77 inhibits IL-6 formation, the mechanisms involved are not well understood. Previous studies have reported that the effects of PGE₂ on IL-6 and TNF- α formation are contrary, inducing IL-6 formation but inhibiting TNF- α production [13, 14]. Recent study further demonstrated that treatment of anti-TNF antibody abolishes the LPS-induced IL-6 production in vitro and in vivo while PGE₂ does not contribute significantly to LPS-induced IL-6 generation [15]. Accordingly, we proposed that FC-77 inhibited LPS-induced IL-6 formation is mainly secondary to the suppression of TNF- α synthesis. Under inflammatory conditions enhanced ROS formation may cause the release of TNF- α and induction of COX-2, and treatment with antioxidants reverses these inductions [16]. Our unpublished data show that FC-77 dose-dependently inhibits the LPS-induced hydrogen peroxide formation in RAW264.7 macrophages. Therefore the antioxidant activity of FC-77 may at least in part contribute to the inhibition in proinflammatory cytokines formation.

A novel finding in this study was that FC-77 also increases the production of IL-10, which differs from the

effect of perflubron [3]. IL-10 is a pleiotropic cytokine that controls inflammatory process by suppressing the production of proinflammatory cytokines, ROS, and PGE₂ formation [17, 18, 19]. However, PGE₂ also can induce IL-10 release in macrophages [20]. Furthermore, administration of antioxidant or inhibitor of nitric oxide synthase (NOS) decreases the LPS-induced TNF- α and IL-1 β generation but enhances IL-10 formation [21, 22], suggesting that the regulation of IL-10 is very complex. Our preliminary data demonstrate that FC-77 significantly inhibits NO formation by suppressing inducible NOS expression in LPS-stimulated RAW264.7 macrophages. Thus the attenuation of ROS and NO production by FC-77 may contribute to the enhancement of IL-10 formation, which in turn leads to a decrease in the proinflammatory/anti-inflammatory cytokine ratio. Furthermore, pre- or posttreatment with FC-77 inhibited LPS-induced PGE₂ and proinflammatory cytokine formation but increased IL-10 production, indicating that FC-77 may be a potential preventive and therapeutic agent for inflammatory diseases.

To further investigate the molecular mechanism involved we evaluated the alteration in NF- κ B activation. NF- κ B, a p50/p65 heterodimer, is a key nuclear tran-

scription factor for regulating induction of COX-2 and proinflammatory cytokines [23]. Once activated by LPS, the I κ B- α /NF- κ B complex in cytoplasm is dissociated, causing the translocation of NF- κ B into nucleus and in turn activating gene transcription. Our results indicate that the activation of NF- κ B is inhibited by FC-77 by preventing the degradation of I κ B- α in cytoplasm, which is similar to that of perflubron [24]. Other possible mechanisms reported by previous studies, such as a reduction in phagocyte function due to modification of cell membrane receptor-ligand binding [4] or altered membrane fluidity [25] by PFCs, may be also involved in the anti-inflammatory activity of FC-77.

In conclusion, we provide a novel mechanism to explain the anti-inflammatory activity of FC-77 by suppressing COX-2/PGE₂ pathway, which may be associated with the attenuation of proinflammatory/anti-inflammatory cytokine ratio and NF- κ B activation in LPS-stimulated RAW 264.7 macrophages.

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