Evidence has accumulated to indicate that peripheral blood mononuclear cells (PBMC) incubated with lipopolysaccharide (LPS) stimulate synthesis or release of pyrogenic cytokines including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α). These cytokines act targets in the periphery with generation of stimulatory signals directed to the brain [1, 2]. Consequently, an excessive accumulation of prostaglandin E2 inside the brain affects thermoregulatory centers in the hypothalamus and results in fever genesis.

Other lines of evidence have shown that nuclear factor-κB (NF-κB) plays an important role in inflammatory responses through the regulation of genes encoding proinflammatory cytokines and inducible enzymes such as inducible nitric oxide synthase and cyclooxygenase [3–5]. The synthesis of cytokines, such as TNF-α, IL-1β, and IL-6, is mediated by NF-κB.
NF-κB is an appropriate target for the treatment of septic shock because NF-κB–activated gene products play an important role in the pathogenesis of sepsis. To our knowledge, however, this has not been paid much attention to the possible involvement of NF-κB mechanisms in the pathogenesis of pyrogenic fever.

Therefore, in the present study, we determined to assess whether an inhibition of NF-κB mechanisms attenuates pyrogenic cytokines synthesis or release from PBMC and results in antipyresis. At first, experiments were carried out to assess the pyrogenic response in rabbits to the intravenous injection of supernatant fluids obtained from human PBMC incubated with LPS alone or LPS plus NF-κB inhibitors, including pyrrolidine dithiocarbamate (PDTC) [6, 7], curcumin (Cur) [8], sodium pyrithione (Pyri) [9], or N-acetyl-L-cysteine (NAC) [10]. Second, we assessed the effects of pretreatment of rabbits with an intravenous dose of either PDTC, Cur, Pyri, or NAC on both the fever and/or the levels of IL-1β, IL-6, and TNF-α in the serum of rabbits after an intravenous administration of LPS. Third, the effects of pretreatment with an intravenous dose of anti–IL-1β, anti–IL-6, or anti–TNF-α monoclonal antibody on the pyrogenic responses to an intravenous injection of LPS were also assessed in rabbits.

**METHODS**

**Preparation of PBMC.** Human PBMC was obtained from freshly collected buffy coat fraction from healthy donors at the Tainan Blood Bank Center (Tainan City, Taiwan, ROC). It was isolated by centrifugation over a Ficoll-Paque (Famacia, Uppsala, Sweden) density gradient at 400×g for 30 min at room temperature in a Sorvall RT6000B (Du Pont, DE) [3]. The cells collected at the interface were washed thrice with serum-free RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) and subsequently resuspended in AIM-V medium (GIBCO BRL) containing 100 μg/ml of penicillin and 100 μg/ml of streptomycin. The PBMC at a concentration of 1×10^7 cells/ml was incubated with different concentrations of tested agents in a 37°C incubator. After incubation, the PBMC supernatant was harvested by centrifugation (1,200 rpm) and stored at −80°C in a freezer.

**Animals and pyrogen assay.** Adult male New Zealand white rabbits, weighing between 2.0 and 3.1 kg were used. The pyrogen assay was carried out by using unanesthetized animals restrained in rabbit stocks. Between experiments the animals were housed individually at the ambient temperature of 22±1°C with a 12-h light–dark cycle and the lights being switched on at 0600 h. Animal chow and water were allowed ad libitum. Experiments were conducted 0900 and 1700 h, each animal being used at an interval of no less than 13 d. Throughout the experiment, colonic temperatures (T_co) were measured every minute with a copper constantan thermocouple connected to a thermometer (HR1300, Yokogawa, Tokyo, Japan). The colonic temperature of each animal was allowed to stabilize for at least 90 min before any injections. Only animals whose body temperatures were stable and in the range of 38.2 to 39.5°C were used to determine the effect of the application of NF-κB inhibitors application on the febrile response induced by the supernatant fluids obtained from LPS-treated PBMC.

**Reagents.** All drugs were purchased from Sigma (St. Louis, MO, USA). The drug solutions were prepared in pyrogen-free glassware that was heated at 121°C for 5 h before use. All solutions were passed through 0.22 μm filters (Millipore). LPS (E. coli serotype 0127: B8) was purchased from Sigma Chem. Co. and made up in 0.9% NaCl solution. Pyrrolidine dithiocarbamate (PDTC) and sodium pyrithione (Pyri) were dissolved in pyrogen-free sterile saline solution on the day preceding the experiment. N-acetyl-L-cysteine (NAC) was dissolved in saline and adjusted to pH 7.4 by the addition of 1 N NaOH. Curcumin (Cur) purchased from Sigma was dissolved in absolute ethanol. All the experimental culture media used were serum free AIM-V (GIBCO BRL) containing 50 μg of gentamicin per ml. Monoclonal mouse antimouse (anti-h) interleukin-1β (anti-IL-1β), anti-h TNF-α (anti-TNF-α), and anti-h IL-6 (anti-IL-6) were obtained from R & D (Minneapolis, MN, USA). A type-specific matched mouse immunoglobulin (IgGl) control monoclonal antibody (MAB) was purchased from Chemicon International, Inc. (Temecula, CA, USA).

**Determination of TNF-α, IL-1β, and IL-6 in supernatant fluids.** PBMC were cultured at a density of 1×10^7 cells/ml in 6-well flat-bottom plates (Nun, Brand Products, Denmark) stimulated with LPS (0.1 μg/ml) alone or cocultured with NF-κB inhibitor PDTC (2 mg/ml), Pyri (15 μg/ml), Cur (37 μg/ml), or NAC (5 mg/ml) for 24 h. At the end of the experiment, the culture supernatants were collected and stored at −80°C before an analysis for secreted cytokines. The amounts of the cytokines TNF-α, IL-6, and IL-1β in the culture supernatants were determined by using double-antibody sandwich ELISA (R & D Systems) according to the manufacturer’s instructions. Recombinant human IL-1β (16 to 1,000 pg/ml), TNF-α (8 to 500 pg/ml), or IL-6 (5 to 300 pg/ml) represented the standards for calibration, and the detection limit of all...
assays was 20 pg/ml.

**TNF-α assay of rabbit’s serum.** For measurement of serum cytokines, 5 ml of blood was withdrawn from the marginal ear vein of each rabbit. The blood samples were centrifuged at 1,400×g for 15 min at 4°C. The serum was collected in polyethylene tubes and stored at −70°C until the cytokine assay. TNF-α activity in serum samples was measured by an *in vitro* cytotoxicity assay with TNF-sensitive L.P3 cells, as previously described [11] with slight modifications. Briefly, 2.5×10⁴ cells were plated in 96-well microplates (Nunc, Roskilde, Denmark) in RPMI 1640 (GIBCO BRL) containing 10% fetal bovine serum (FBS) (GIBCO BRL) and incubated in a humidified atmosphere of 5% CO₂ at 37°C for 4 h. After incubation, samples (100 μl) in a series of dilutions, or recombinant human TNF-α (R & D) as an internal reference, were added to the well, followed by the addition of 50 μl of actinomycin D (Sigma) at a final concentration of 1.6 μg/ml. After 24 h of incubation, the cells were washed with saline, stained with 0.05% crystal violet for 30 min, and eluted with 50% ethanol in 0.1% acetic acid solution. The microplates were read at 590 nm on a Multiskan photometer (MR 5000; Dynatech, McLean, VA, USA). The sensitivity of TNF-α assay was 0.3 U/ml.

**IL-1β bioassay of rabbit serum.** IL-1β was measured with the IL-1-dependent murine T-cell line D10N4M as previously described [12, 13]. Briefly, the D10N4M cells were maintained in RPMI 1640 (GIBCO BRL) with 10% FBS (GIBCO BRL), recombinant IL-1β (40 pg/ml; R & D), recombinant IL-2 (20 ng/ml; R & D), 50 μM 2-mercaptoethanol (Serva, Heidelberg, Germany), and concanavalin A (3 μg/ml) (Sigma) and were fed every 3 d before being assayed. The serially diluted rabbit serum samples, or recombinant human IL-1β (50 μl) as an internal reference, were added to each well of microplates (Nunc), followed by the addition of 50 μl of washed D10N4M cells (2×10⁵/ml). After 72 h of incubation, the cells were pulsed with 0.5 μCi of [³H]thymidine (6.7 Ci/mmol) (Dupont NEN, Boston, MA, USA) per well for 4 h. The cells were harvested on glass fiber filters with an automatic cell harvester (Cambridge). The radioactivity incorporated was assayed in a liquid scintillation counter (LS 5000 TA; Beckman). The sensitivity of this assay is 1 pg/ml.

**RESULTS**

**Cytokine production in LPS-treated PBMC.** As shown in Table 1, the levels of IL-1β, IL-6, and TNF-α in the supernatant fluids obtained from the PBMC treated with LPS (0.1 μg/ml) alone or with Cur (37 μg/ml), PDTC (2 mg/ml), Pyri (15 μg/ml), or NAC (5 mg/ml) for 24 h were assayed. It was found that the LPS stimulated human PBMC to produce high levels of IL-1β, IL-6, and TNF-α. The increased levels of all these cytokines after LPS treatment were significantly reduced by PDTC, pyrithione, Cur or NAC (Table 1). Pretreatment with Pyri at 15 μg/ml significantly reduced the levels of IL-1β and IL-6, but it had no effect on the TNF-α level in the supernatant fluids.

**The fever induced by the supernatant fluids from LPS-treated PBMC.** To determine whether LPS can act through an NF-κB mechanism in human PBMC to induce a pyrogenic response, the supernatant fluids obtained from PBMC (107 cells/ml) treated for 24 h with LPS (0.1 μg/ml) alone or plus Cur (37 μg/ml), PDTC (2 mg/ml), Pyri (15 μg/ml), or NAC (5 mg/ml) were given intravenously to the rabbits. An intravenous in-
Injection of the supernatant fluids (1 ml/kg of body weight) obtained from LPS-treated PBMC produced a short latency and monophasic fever. The colonic temperature began to rise at 5 to 10 min, peaked at 50 min, and returned to the preinjection level at 4 h. As shown in Fig. 1, pretreatment with any one of these NF-κB inhibitors inhibited the fever induced by an intravenous administration of the supernatant fluids. However, intravenous administration of LPS (0.1 μg/ml/kg), PDTC (2 mg/ml/kg), Pyri (15 μg/ml/kg), NAC (5 mg/ml/kg), or Cur (37 μg/ml/kg) produced an insignificant effect on basal Tco in rabbits.

**NF-κB inhibitors on LPS-induced fever**

The colonic temperatures of rabbits started to rise at 0.5 h, peaked at 1.5 h, and returned to the preinjection level at about 5 h after an intravenous dose of 2 μg/kg of LPS. The LPS-induced fever was significantly attenuated by the pretreatment of rabbits with Pyri (10 mg/kg, I.V.), PDTC (2 mg/kg, I.V.), NAC (20 mg/kg, I.V.) or curcumin (10 mg/kg, I.P.) 1 h before LPS (2 μg/kg, I.V.) (Fig. 2).

**Cytokine production and fever**

The intravenous administration of LPS (0.5, 1.0, and 2 μg/kg) induced dose-dependent fever in rabbits (Fig. 3). Colonic temperatures started to rise at 30 min and reached the peak level at 1–2 h after LPS injection. Body temperature returned to the preinjection level at 4 h. In parallel with the colonic temperature changes, the concentrations of IL-1β, TNF-α, and IL-6 (Fig. 3) in serum also started to rise at 30 min and reached peak level at 1 h. The levels of these cytokines in serum returned to the preinjection values at 3 h.

**Antipyresis with PDTC**

An I.V. dose of PDTC (2 to 8 mg/ml/kg; 1 h before
LPS injection), though showing no effect on basal colonic temperature or concentrations of IL-1β, TNF-α, and IL-6 in serum, did attenuate the LPS-induced fever as well as the increased concentrations of IL-1β, TNF-α, or IL-6 in serum (Fig. 4).

Antipyresis with MAb to IL-1β, IL-6, or TNF-α
An I.V. dose of MAb to IL-1β (2 μg/kg), IL-6 (2 μg/kg), or TNF-α (2 μg/kg) 1h before LPS (2 μg/kg, I.V.) injection, though showing no effect on basal colonic temperature, did attenuate the LPS-induced fever (Table 2). The antipyretic effects exerted by anti–IL-1β monoclonal antibody were greater than those exerted by anti–IL-6 or anti–TNF-α monoclonal antibodies (p<0.05, ANOVA followed by Student-Newman-Keuls’ test).

DISCUSSION
In the present results, when incubating the LPS with the human PBMC, we observed that LPS was able to stimulate the synthesis or release of IL-1β, IL-6, or TNF-α in the supernatant fluids. While the supernatant fluids obtained from the LPS-treated human PBMC were being intravenously injected into the rabbits, the febrile responses were correlated well with the concentrations of these cytokines in the supernatant fluids. When an NF-κB inhibitor such as curcumin, pyrrolidine dithiocarbamate, pyrithione, or NAC was being added into the LPS-human PBC incubation, the levels of these cytokines in the supernatant fluids and the febrile responses to intravenous injection of the supernatant fluids were greatly reduced. Furthermore, it was found that pretreatment with an

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**Fig. 2.** Changes in the Tco and Fl-2h in rabbits treated with saline+saline, saline+LPS (2 μg/ml/kg), PDTC (2 mg/ml/kg)+LPS (2 μg/ml/kg), Pyri (10 mg/ml/kg)+LPS (2 μg/ml/kg), NAC (20 mg/ml/kg), or Cur (10 mg/ml/kg)+LPS (2 μg/ml/kg). Rabbits were injected intravenously with saline, Cur, NAC, Pyri, or PDTC 1h before LPS administration. Points represent mean±SEM of 8 animals. † Significantly different from control values (saline+saline) (p<0.05; one-way ANOVA followed by Student-Newman-Keuls’ test). * Significantly different from control values (saline+LPS) (* p<0.05; one-way ANOVA followed by Student-Newman-Keuls’ test).

**Fig. 3.** Changes in the Tco and the serum concentrations of IL-1β, TNF-α, and IL-6 in rabbits treated with saline+saline (●), saline+LPS (2 μg/kg, i.v.) (○), saline+LPS (1 μg/kg, i.v.) (■), or saline+LPS (0.5 μg/kg, i.v.) (▲). Rabbits were injected intravenously with saline one hour before LPS administration. Points represent mean±SEM of 8 animals. † Significantly different from control values (saline+saline) (p<0.05; one-way ANOVA followed by Student-Newman-Keuls’ test).
intravenous dose of Cur, PDTC, Pyri, or NAC 1 h before intravenous administration of LPS greatly attenuated the LPS-induced fever in rabbits. The present results further demonstrated that the colonic temperatures and circulating levels of IL-1β, IL-6, and TNF-α were both increased dose-dependently following an intravenous administration of LPS in rabbits. The fever and the increased circulating levels of IL-1β, IL-6, and TNF-α produced by LPS were also decreased by PDTC (present results), Cur, Pyri or NAC (unpublished data). Apparently there is no discrepancy between human beings and rabbits in terms of pyrogenic cytokine production in response to LPS. The present results further showed that anti–IL-1β MAbs given I.V. inhibited almost completely the fever induced by I.V. LPS in vivo. The antipyretic action of MAbs to IL-1β was greater than that exerted by MAbs to TNF-α or IL-6. The data are consistent with the concept that IL-1β represents a most important mediator for fever induced by LPS [16]. Indeed, our recent results showed that the NF-κB inhibitors were effective in inhibiting the fevers induced by I.V. IL-1β (Lee, Huang, Shao, Liao, and Lin: unpublished data).

It has been shown that LPS activates NF-κB mechanisms in human PBMC [17]. Our unpublished data (Lin et al.) further showed that LPS could cause the degradation of inhibitory κB to trigger the translocation of NF-κB from the cytoplasm to the nucleus where it activates the expression of specific cellular genes encoding cytokines. Therefore, the levels of cytokines including IL-1β, IL-6, and TNF-α in supernatant fluids from LPS-stimulated human PBMC were increased following incubation. Agents that inhibit the NF-κB mechanisms such as NAC, PDTC, Cur, or Pyri can reduce the synthesis or the release of pyrogenic cytokines from PBMC. In fact, cytokines stimulated by the NF-κB can also directly activate the NF-κB mechanisms and thus establish a positive autoregulatory loop that can amplify the inflammatory reaction.

| Table 2. The effects of cytokine-specific antibody (MAb) on the pyrogenic response to an intravenous administration of LPS (2 μg/kg) in rabbits.* |
|---------------------------------|----------------|----------------|
| Treatment                               | FI (°C/5 h) (n) | F-value |
| AIM-V medium (ml/kg, i.v.)            | 0.44±0.03 (8)  | 291.558   |
| Control antibody (2 μg/kg, i.v.)      | 3.98±0.12 (8)**|           |
| +LPS (2 μg/kg)                       |               |           |
| Anti-IL-1β MAb (2 μg/kg, i.v.)       | 0.75±0.06 (8) †|           |
| +LPS (2 μg/kg)                       |               |           |
| Anti-IL-6 MAb (2 μg/kg, i.v.)        | 3.04±0.09 (8) †|           |
| +LPS (2 μg/kg)                       |               |           |
| Anti-TNF-α MAb (2 μg/kg, i.v.)       | 2.46±0.11 (8) †|           |
| +LPS (2 μg/kg)                       |               |           |

*Mean changes (±SEM) in FI. The control antibody or antibody to IL-1β, IL-6, or TNF-α was administered 1 h before the intravenous injection of LPS (2 μg/kg) in rabbits. n: number of animals tested. **Significantly different from the corresponding control value (AIM-V medium) (p<0.05, ANOVA followed by Student-Newman-Keuls’ test). †Significantly different from the corresponding control values (LPS+control antibody) (p<0.05, ANOVA followed by Student-Newman-Keuls’ test). The legends of the figures.
Blocking NF-κB Activation

[18]. In the present results, LPS may have induced the activation of NF-κB pathways in PBMC that resulted in an excess synthesis or release of pyrogenic cytokines, which can be blocked by NF-κB inhibitors.

The activation of the NF-κB transcription family plays an important role in inflammation through its ability to induce a transcription of proinflammatory genes [19]. This pathway is activated on appropriate cellular stimulation, most often by signals related to pathogens or stress. For example, LPS released by gram-negative bacteria is the most frequent cause of sepsis. The host response to LPS has been shown to involve multiple inflammatory effector mechanisms, including cytokines [20]. TNF-α is one of two particular gene products with a central role in the septic shock [21, 22]. In fact, IL-1β is mainly produced by monocytes, macrophages, endothelial cells, B cells, and activated T cells [23]. IL-1β is able to induce fever and acute phase responses and to potentiate TNF-α related tissue damage [24, 25]. Similarly, TNF-α is a potent cytokine that exerts diverse effects by stimulating a variety of cells [26]. IL-6 is a pleiotropic cytokine produced by T cells, monocytes, and macrophages [27]. Elevated levels of circulating IL-6, IL-1β, and TNF-α have been reported previously in various pathological states and to be a predictor of poor outcome in heatstroke [24, 28–31]. In fact, heatstroke syndrome is characterized by marked hyperthermia (core body temperature >40°C), several severe neurological abnormalities, and varying degrees of organ dysfunction [26]. It has been reported that hyperthermia produces hypoxic stress in the intestine and increases in intestinal permeability that might allow bacterial translocation from the gut [32, 33]. Thus it appears that pyrogenic fever shares with heatstroke as well as sepsis the similar pictures in the plasma levels of IL-1β, IL-6, and TNF-α. New therapeutic interventions aimed at limiting the activity of NF-κB, a critical transcription factor in the regulation of acute inflammation, may have a benefit effect in these pathological states [26].

Besides the role played by NF-κB in PBMC, evidence has accumulated to indicate that NF-κB is involved in brain function [34]. The circulating cytokines are thought to be transported by the blood stream to circumventricular organs such as the organum vasculosum laminae terminalis (OVLT) [34–38]. A cytokine-prostaglandin coupling pathway within the OVLT may be critically important in fever genesis [39–41]. An expression of the inhibitory factor κBα (index of the activity of NF-κB) within the brain was induced by a systemic administration of LPS, IL-1β, and TNF-α [42, 43]. Endotoxin also stimulates the IL-1 production in the rabbit OVLT accompanied by fever genesis [44]. It is very likely that besides peripheral blood mononuclear cells, the NF-κB mechanisms also exit in OVLT areas. In this study, the systemic administration of NF-κB inhibitors may have affected the NF-κB mechanisms in peripheral blood mononuclear cells and the central nervous system to inhibit the cytokine release and fever genesis. Furthermore, as mentioned before, NF-κB is also involved in the induction of the COXmRNA. Thus it is possible that the inhibition by the NF-κB inhibitor in the LPS-induced fever was to a large degree due to the inhibition of the production of PGE2.

As shown in the present results, several pyrogenic cytokines including IL-1β, TNF-α, and IL-6, may induce fever. IL-6 produced in the peripheral blood stream or the brain stem is believed to be required for the final step leading to fever [45]. However, lipopolysaccharide may act through the subdiaphragmatic vagal nerve activity or the hypothalamic–pituitary–adrenal axis and other components of the neuroendocrine system to induce febrile response [44, 45].

In conclusion, it should be mentioned that PBMCs were incubated with LPS and the NF-κB inhibitor and the supernatant fluid was injected into rabbits. Neither LPS nor the inhibitors were removed from the supernatant fluid. An injection of the supernatant fluid implies that the LPS, the inhibitor, and the produced cytokine from the cells were administered all together. However, in this study we observed that an i.v. administration of LPS (0.1 μg/ml/kg), PDTC (2 mg/ml/kg), Pyri (15 μg/ml/kg), or Cur (37 μg/ml/kg), or NAC (5 mg/ml/kg) had an insignificant effect on the basal levels of $T_{co}$. This indicates that fever response observed in the present study is unlikely to be due to the remaining residue LPS or NF-κB inhibitors in supernatant fluids.

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