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Long-term continuous exposure to static magnetic field reduces popolysaccharide-induced cytotoxicity of fibroblasts

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Abstract

Purposes: Lipopolysaccharide (LPS) is one of the major substances initiating the immune host response in microbial infections that results in cytotoxicity. In terms of treatment of the immune response, research has been conducted on physical environments that can reduce LPS-induced damage. In this experiment, a long-term continuous static magnetic field (SMF) was used as a physical resource to reduce LPS-induced immune host response.

Materials and methods: Cultured fibroblasts were challenged with LPS to initiate an inflammatory reaction. Cell viability and various proinflammatory cytokine levels were detected and compared between SMF and sham-exposed groups.

Results: Our *in vitro* study revealed that, with LPS challenge, fibroblasts continuously exposed to a 0.4-T SMF for 12 h demonstrated higher cell viability compared to unexposed analogs. From cytokine test, the levels of LPS-induced interleukin-1β (IL-1β) in the SMF-exposed groups were significantly lower relative to their unexposed counterparts ($p < 0.05$). By contrast, SMF exposure tended to increase the level of LPS-induced IL-1 receptor antagonist (IL-1Ra) and IL-6.

Conclusions: Our results suggest that SMF stimulation inhibits LPS-induced cytotoxicity through reduction of proinflammatory cytokines and increase in anti-inflammatory cytokines of NIH-3T3 cells.

Keywords: *Anti-inflammatory effects, antioxidants, bacteria, E-M fields, immunology*

Introduction

Clinical observations have indicated that the mortality rate from septic shock has increased from 28–50% in the past few decades (Natanson et al. 1998, Wang et al. 2003). Given this reduction in an already low survival rate, investigation into the treatment and prevention of bacterial infections is increasingly important. Traditionally, administration of antibiotics has been the major treatment strategy for bacterial infections; however, the risk of antibacterial resistance has been widely stated (Bernstein et al. 2006).

When bacteria are phagocytosed by macrophages, they are degraded in phagosomes. After the fragments of the digested microbes are released, they become cytokine-releasing activators of host cells. The Gram-negative bacterial cell wall component, lipopolysaccharide (LPS), is the major cause of multiple organ dysfunction syndrome and periodontitis (Page 1991, Agarwal et al. 1995). It also triggers the release of inflammatory cytokines by host cells, as mentioned above (Hermann et al. 2002, Mathiak et al. 2003). It is now well known that the co-receptor of LPS formed by the Toll-like receptor 4 (TLR4) and CD14 is the binding site for signaling LPS-induced cytotoxicity (Dziarski et al. 1998, Jiang et al. 2000).

Endotoxin tolerance is a phenomenon of decreasing immune response during challenge by lethal endotoxins (such as LPS) after repeated low-level administration (Greisman et al. 1996, West & Heagy 2002). Although the subject of years of investigation, clinical applications of endotoxin tolerance for microbial infections remain unavailable because it is very difficult to accurately control the location and

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concentration of the low-level LPS injection. Thus, a new method is needed with the same attenuation effects on inflammatory damage as endotoxin tolerance without the requirement for low-concentration endotoxin injection.

Further, it is known that the physiological behavior of many tissues changes in response to variations in mechanical force (Mullender et al. 2004, Wang & Thampatty 2006). The major cellular component involved in this mechanotransduction mechanism is the cell membrane (Wang et al. 1993). It has also been reported that a static magnetic field (SMF) changes the structure of cell membranes, affecting ion channels and the avidity between ligands and their receptors (Pacini et al. 1999, Sakurai et al. 1999).

An SMF is one type of magnetic field used in a variety of clinical practice, especially oral dentistry (Darendeliler et al. 1995, Riley et al. 2001), and for pain relief (Eccles 2005). Recently, numerous investigations have focused on the effects of continuous SMF on inflammatory responses in animals (Weinberger et al. 1996) and on cytokine release by human peripheral blood mononuclear cells (Aldinucci et al. 2003). However, the effects of SMF on proinflammatory cytokines remained unknown until recently. Salerno et al. (1999) investigated the effects of 0.5-T static magnetic fields on the expression of activation markers and interleukin release in human peripheral blood mononuclear cells (PBMC). Their results show, for the first time, that exposure to the SMF of a commercially available 0.5-T MRI unit may induce modifications in the release of some interleukin in PBMC.

In this experiment, therefore, long-term continuous static magnetic fields were used as a physical resource to attenuate LPS-induced immune host response.

Materials and methods

Cell culture

In this study, NIH-3T3 fibroblast cells (American Type Culture Collection 60008) were utilized for all *in vitro* tests. The cells were maintained in Dulbecco's modified Eagle's medium (HyClone, South Logan, UT, USA), supplemented with L-glutamine (4 mM), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (HyClone, South Logan, UT, USA). Cells were seeded in Petri dishes (Nunclon, Nunc, Rochester, NY, USA) at a density of 10,000 cells/ml. Cultures were incubated in 5% CO_2 at 37°C and 100% humidity. Before confluence, the cell monolayer was washed three times with phosphate-buffered saline (PBS), and detached with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) for 10 min at 37°C. The cell suspensions were centrifuged at 2000 ***g*** for 5 min at 25°C, and the supernatant was discarded. The pellet was then resuspended in culture medium for subculture.

SMF exposure

The cultured 3T3 cells were divided into control and SMF-exposed groups and then incubated inside two identical incubators (RCO3000TABB, Revco, Asheville, NC, USA). A neodymium (Nd2Fe14B) magnet with a flux density of 0.4 T was used to produce the SMF. The average surface magnetic flux density (Figure 1) was monitored using a Gauss meter (Model 5070, FW BELL, Orlando, FL, USA). For all exposure experiments, four 3.5-cm culture dishes were placed directly on the north surface of the permanent magnet, with unmagnetized neodymium disks placed in a second identical incubator for the controls. The permanent magnets produced ununiform magnetic fields over their surface, with the highest value at edge and the lowest value at the centre of the magnets (Figure 1). Though tested cells in the same Petri dish were exposed to different flux densities, the exposed conditions are the same for the four Petri dishes because the distributions of magnetic flux densities are symmetrical according to the centre of the magnet.

The control cells were placed on the nonmagnetic disks at the same time as their experimental counterparts. The background flux density in the control incubator was no greater than the natural magnetic field of the earth (0.05 mT). For all experiments, the

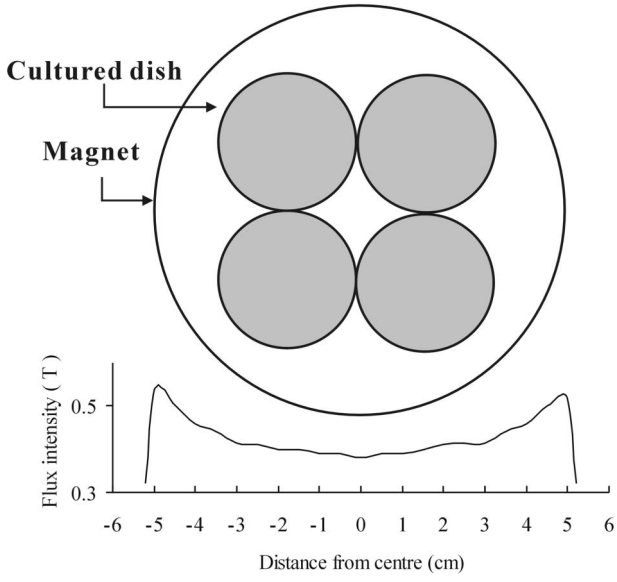

Figure 1. Schematic representation showing the relative size of the Petri dishes and magnets used in this study. In the lower part of the diagram, the solid line shows the distribution of magnetic field intensity over the upper surface of the magnet.

cells were incubated in an unexposed environment for 24 h after plating onto culture dishes. The SMF-exposure cells were then placed onto the magnet, with this defined as time point 0 h for all tests.

LPS challenge

A series of assays was conducted to test the effects of the SMF on LPS-induced cytotoxicity and inflammatory cytokine expression. Before the assay, the cells were kept in serum-free medium for 12 h to starve them. Then, the cells were washed twice with ice-cold PBS, and incubated with three commercially available LPS variants derived from *Escherichia coli* (Ec), *Pseudomonas aeruginosa* (Pa) and *Serratia marcescens* (Sm) (Sigma, St Louis, MO, USA), at serial concentrations (range 0 – 160 mg/ml) for 12 h. At observation time, cell viability was tested using the MTT method. Briefly, test cells were incubated with a tetrazolium salt (MTT) according to the supplier's instructions (MTT kit, Roche Applied Science, Mannheim, Germany). After adding the colorometric substrate for 4 h, viable cells converted the MTT salt to a water-insoluble formazan dye. After solubilizing the formazan dye with 500-μl Dimethyl sulfoxide (DMSO) for 5 min, the dye was quantitated using a microplate reader (Model 2020, Anthos Labtec Instruments, Eugendorf , Wals, Austria) at 570/690 nm, with absorbance directly correlated to cell number.

Inflammatory responses assay

In this study, the 50% toxicity concentration, TC_{50}, was defined as that which caused cells to grow around 50% more than the control group. In the further inflammatory response assay, both the SMF and sham-exposure cells were subjected to the LPS treatments at their respective TC_{50}.

The effects of SMF on expression of inflammatory cytokine (IL-1α, IL-1β, IL-1Ra and IL-6) by the LPS challenged cells were determined using an Enzyme-Linked Immunosorbent Assay (ELISA, R & D System Inc., Minneapolis, MN, USA). Further, to test the differences in growth with and without SMF treatment, cell viability was detected using the MTT method as mentioned above. Moreover, to compare the morphologies of the NIH-3T3 cells in each experimental group, cell images were recorded before the cell number assay. For each group, four samples were prepared and five areas within a sample were examined.

Statistical analysis

All data measured are presented as mean $\pm$ standard deviation (SD) for four samples. For all assays, the Student's *t*-test was used to assess the differences between the control and SMF-exposed cells, with $p < 0.05$ considered statistically significant for all tests.

Results

LPS-induced cytotoxicity assay revealed that NIH-3T3 cells challenged with the various LPS types showed a trend to decreasing cell viability (Figure 2). When the Ec, Pa and Sm LPS concentrations reached 40, 10 and 30 mg/ml, the detected optical density (0.101 ± 0.016, 0.104 ± 0.003 and 0.111 ± 0.014, respectively) was almost half that of the control counterparts. Thus, in the following experiments, the TC_{50} was defined as 40, 10 and 30 mg/ml for Ec, Pa and Sm, respectively.

We found that the SMF inhibited LPS-induced cytotoxicity and proinflammatory cytokine release. An example of the morphological changes in the test cells treated with Ec-LPS is presented in Figure 3. The untreated control cells were evenly distributed and appeared to form a relatively compact, continuous monolayer (Figure 3A). When cells were exposed to the SMF, no obvious changes in morphology were observed in the controls (Figure 3B). As a result of LPS treatment, however, the number of NIH- 3T3 cells decreased, and abundant cellular debris was noted in the medium. By contrast, the morphology of the LPS-challenged cells changed from polygonal to more-rounded relatively thin shapes (Figure 3C). Interestingly, when LPS-challenged cells were cotreated with 0.4-T SMF, a greater number of viable cells and less debris were observed compared to the unexposed cultures (Figure 3D). Similar results were also revealed in the Pa and Sm-LPS experiments. Statistically significant differences in cell viability were not demonstrated when cells were exposed to the SMF without LPS treatment (Figure 4). When Ec, Pa or Sm-derived LPS was added to the serum-free medium, the detected optical densities decreased significantly to 47.7, 52.7 and 51.4% of the control values, respectively. However, SMF exposure significantly reduced LPS-induced cytotoxicity ($p < 0.05$). With Ec, Pa and Sm LPS treatment, the optical densities of the SMF-exposed cells were 1.35, 1.35 and 1.22-fold higher, respectively, than the unexposed cultures ($p < 0.05$).

The effects of the SMF on LPS-induced proinflammatory cytokine production were tested by co-culturing the cells with the three types of LPS for 12 h. The LPS treatments significantly increased IL-1β and IL-6 production, (Figure 5A, 5B), however, significant effects on the production of IL-1α and IL-1Ra were not demonstrated (Figure 5C, 5D). The effects of SMF on LPS-induced cytokine release

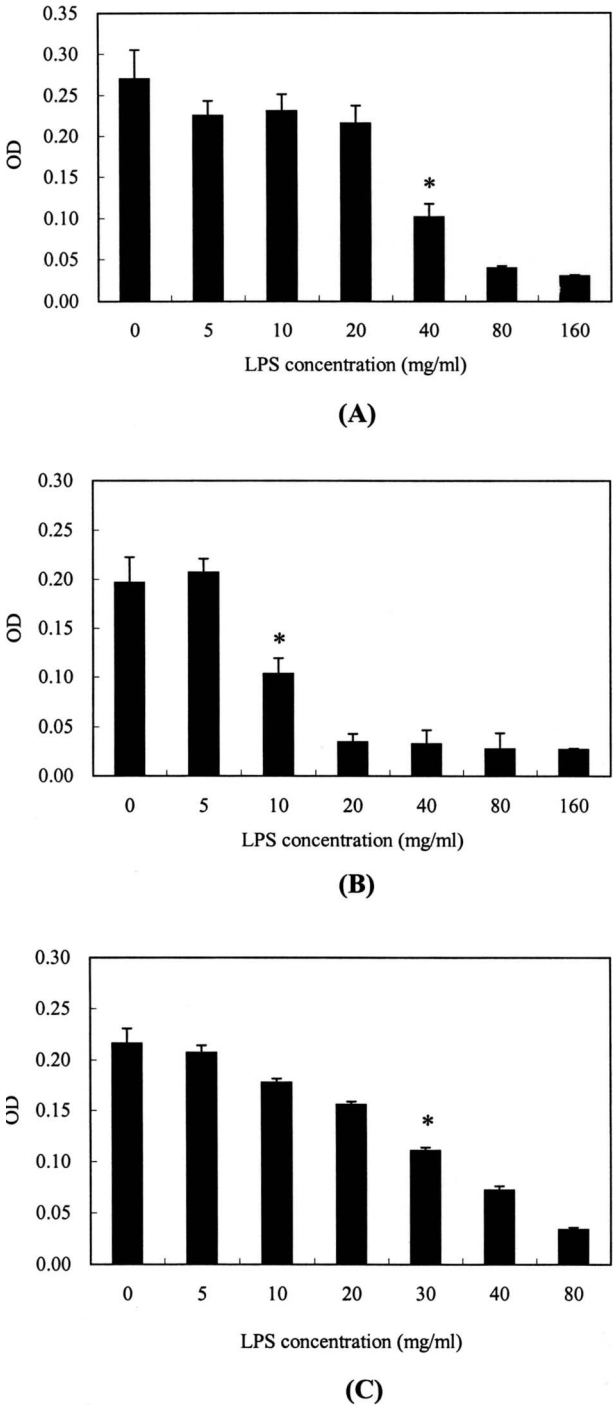

Figure 2. Cytotoxic effects of Ec (A), Pa (B) and Sm (C) – drived LPS on NIH-3T3 cells at different concentrations; LPS concentration is inversely related to cell viability. The star signs denote the TC_{50} concentrations used for the other experiments. Error bars indicate the standard deviation of the mean for $n = 6$ independent experiments.

varied between the four cytokines. IL-1β release from SMF-exposed cells remained at low levels, without significant changes demonstrated in any of the tests. In contrast, when cells were exposed to SMF, the detected IL-1β significantly decreased in

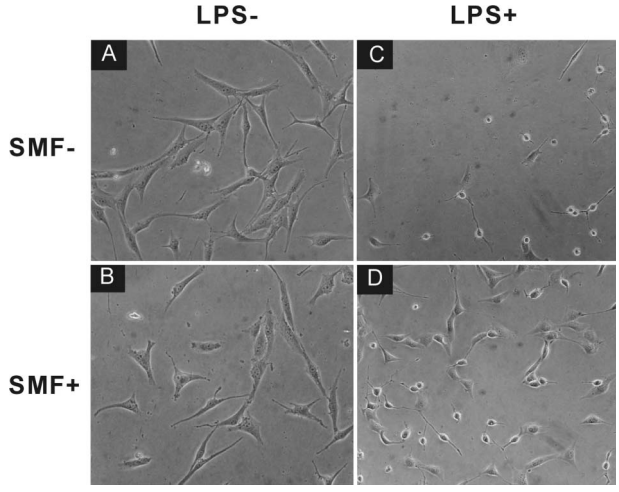

Figure 3. Microscopy of SMF-treated and untreated NIH-3T3 cells. (A) Untreated cells are characterized by a relatively abundant, continuous monolayer; (B) No obvious changes in morphology comparing control cells to the SMF-exposed analogs; (C) Morphologically, the lipopolysaccharide (LPS)-treated cells changed from polygonal to a relatively round shape; (D) The SMF tended to reduce LPS-induced cytotoxicity (magnification $\times 100$).

all experimental groups compared to the unexposed cells ($p < 0.05$). However, IL-1Ra production in the SMF-exposed cells, compared to sham-exposed groups, was significantly increased (1.88, 1.99 and 1.74-fold) for the Ec, Pa and Sm-treated groups, respectively. Similar results are found in IL-6 detection. Cells exposed to SMF produced significantly larger quantities of IL-6 (170.1 ± 25.3 and 240.4 ± 29.6 pg/ml in Ec and Sm tests, respectively) compared to those of their unexposed counterparts (132.2 ± 13.6 and 166.7 ± 15.2 pg/ml in Ec and Sm co-cultured tests, respectively). For each type of LPS, SMF treatment revealed no significant effect on IL-1α production in the test cells.

Discussion

LPS-induced cytotoxicity assay of NIH-3T3 cells challenged with LPS revealed decreasing trends in cell density (Figure 2). The TC_{50} of the lipopolysaccharides from the three studied bacterial sources was different. This variation can be attributed to the structure of the specific LPS molecules. Previous study has demonstrated that LPS purified from various bacterial sources is distinguished by differences in the structure of lipid A as well as the polysaccharide chain (Agarwal et al. 1995).

Cytokines, such as IL-1α and IL-1β can exert strong inflammatory effects during bacterial infection (Agarwal et al. 1995, Hermann et al. 2002, Mathiak et al. 2003). These factors, which are expressed in the infection period, induce severe inflammatory responses. In this study, 12-h continuous SMF

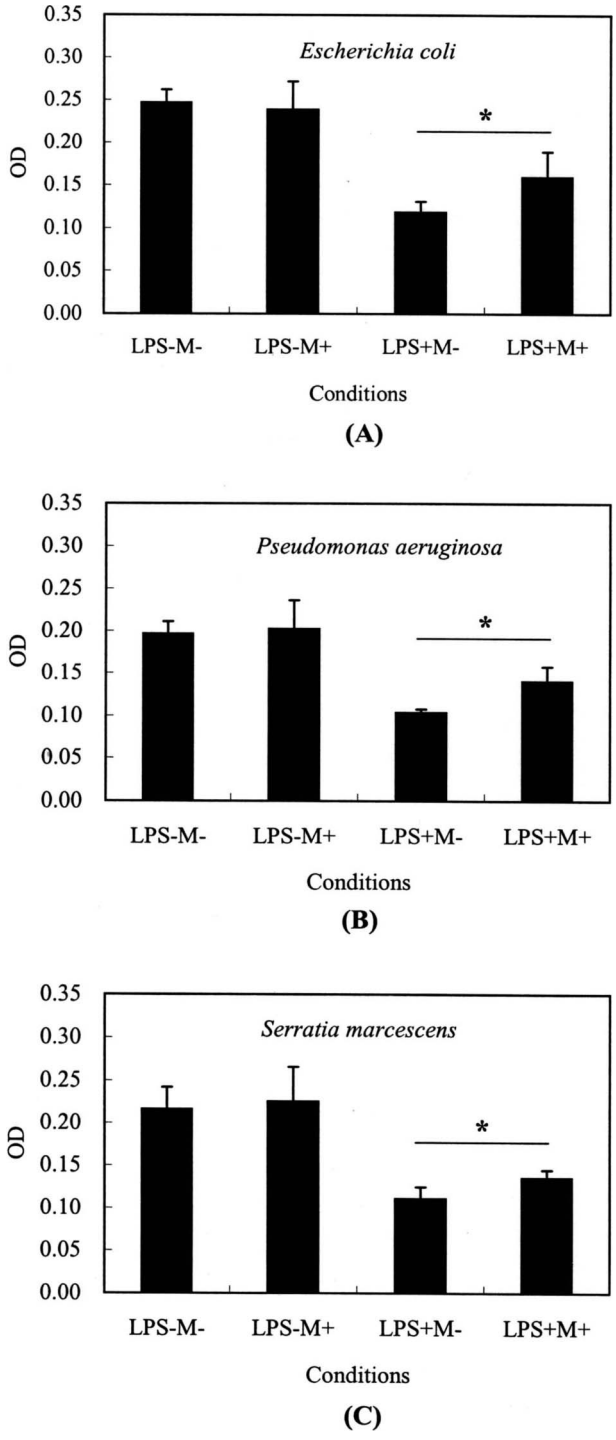

Figure 4. Cytotoxic effects of Ec (A), Pa (B) and Sm (C)-derived LPS on NIH-3T3 cells were significantly reduced with long-term continuous exposure to static magnetic field. (M, SMF exposure, error bars indicate the standard deviation of the mean for $n = 4$ independent experiments; $^{*}p < 0.05$).

stimulation tended to prevent increased IL-1β production (Figure 5A). These results can be compared with the microscopic observations (Figure 3) and cytotoxicity assay (Figure 4), which show that development of endotoxin tolerance in the NIH-3T3 cells occurred after 12 h of continuous 0.4-T SMF exposure.

A member of the IL-1 family, IL-1Ra is produced mainly by monocytes and macrophages after LPS stimulation (Dinarello 1998). Clinical findings indicate that administration of IL-lRa reduces pathological processes such as septic shock and inflammatory disease (Tilg et al. 1997, Ashdown et al. 2007). Therefore, it is an anti-inflammatory cytokine due to its ability to bind to the IL-1α and IL-1β (Dinarello 1998).

According to previous study, fibroblasts also respond to IL-lRa. For example, in cultured fibroblasts, PGE2 secretion due to IL-1α stimulation was inhibited by the presence of IL-1Ra (Portnoy et al. 2006). Furthermore, fibrotic lung disease could be attenuated by inhibition of IL-1β with IL-1Ra in animal models (Piguet et al. 1993). In this study, we found that long-term continuous SMF treatment increases the IL-1Ra production in LPS-challenged cells (Figure 5C). The reduction in LPS-induced cytotoxicity with SMF treatment can be attributed to the anti-inflammatory effect of SMF-induced IL-1Ra production (Figure 4).

IL-6 was initially classified as a proinflammatory cytokine, mainly because it increases in the early stage of infection. However, *in vitro* cell culture (Ulich et al. 1991) as well as animal studies (Mizuhara et al. 1994, Matthys et al. 1995) have demonstrated that IL-6 inhibits TNF-α production and increases the level of IL-1Ra (Tilg et al. 1994). In a clinical study, Steensberg et al. (2003) found that low-dose recombinant human IL-6 infusion increased plasma levels of the anti-inflammatory IL-1Ra. They concluded that physiological concentrations of IL-6 induce an anti-inflammatory rather than an inflammatory response in humans. Accordingly, it has recently been suggested that IL-6 should be classified as an anti-inflammatory cytokine (Fiers 1991, Xing et al. 1998).

In this study, long-term continuous SMF stimulation tended to increase IL-6 production (Figure 5B). The fact that IL-6 protects against LPS-induced damage (Aderka et al. 1989, Schindler et al. 1990, Barton & Jackson 1993), possibly suggests that IL-6, dependently of SMF, induces IL-1Ra and suppresses excess inflammatory response in LPS-treated cells.

Low-dose radiotherapy has been used to clinically demonstrate anti-inflammatory effects via modulation of cytokine production from inflammatory-responding cells (Rodel et al. 2007). However, due to the problem of dose-accumulation, the efficacy of long-term treatment with low-dose radiotherapy remains doubtful. As SMF does not involve ionizing radiation, this makes it potentially useful in clinical practice. Furthermore, utilization of a permanent magnet for stimulation obviates the need for an

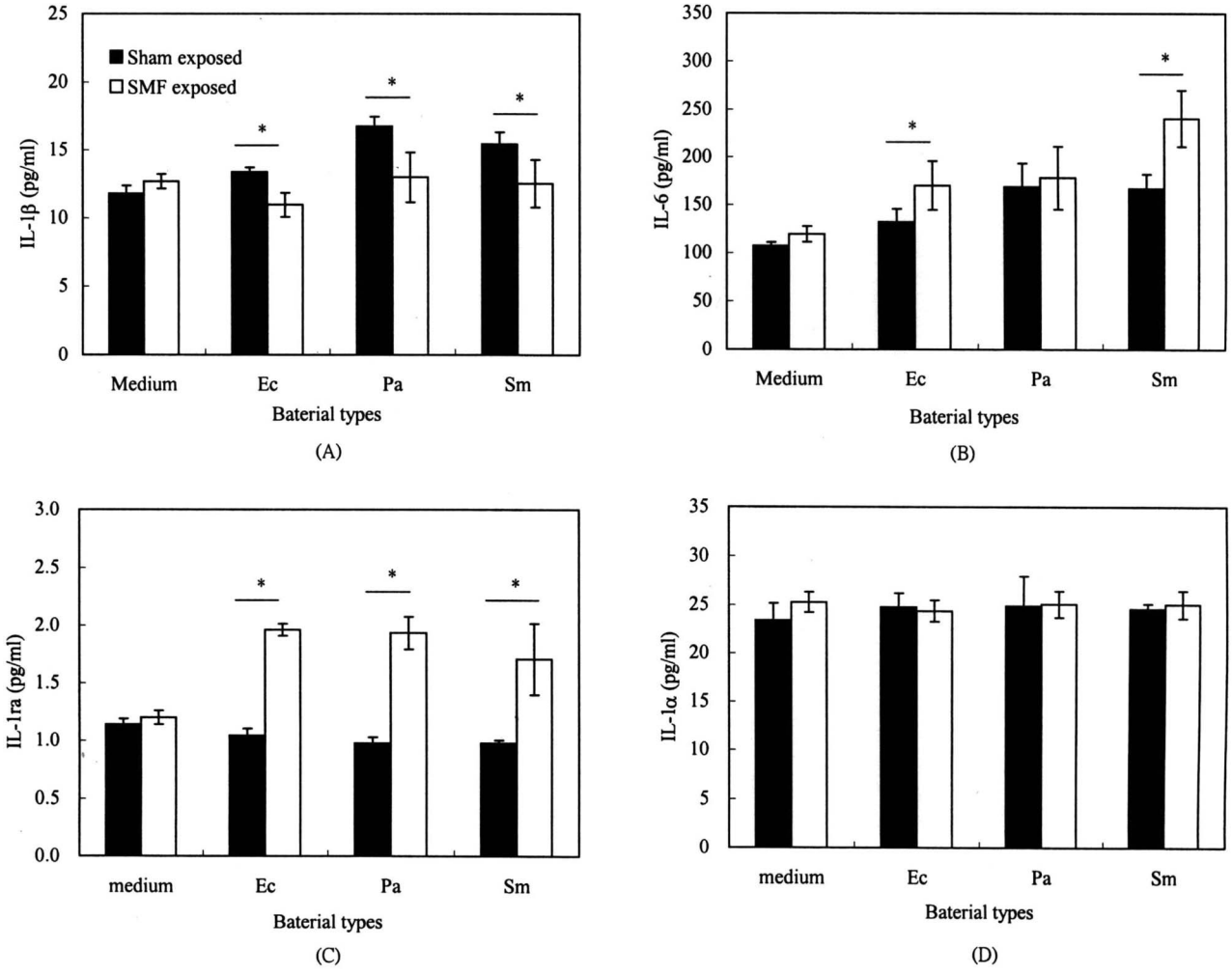

Figure 5. After 12-h SMF exposure, IL-1β (A), IL-6 (B) and IL-1Ra (C) production due to lipopolysaccharide (LPS) treatment was significantly changed by exposure to long-term continuous SMF (error bars indicate the standard deviation of the mean for $n = 4$ independent experiments; $^{*}p < 0.05$).

external energy source and the attendant leads, making SMF stimulation more suitable for long-term local healing. Although modification of proinflammatory cytokine release was not detected with short-term SMF exposure (1 – 4 h) (Salerno et al. 1999, Aldinucci et al. 2003), we found that the long-term continuous variant has an attenuation effect on immune response of LPS-challenged fibroblasts. Based on our results, it appears that the possible mechanism of this preventive effect occurs via down-regulation of proinflammatory cytokines and up-regulation of anti-inflammatory cytokines.

Phospholipids are oriented by the external magnetic fields when their flux densities exceed a certain threshold (Aoki et al. 1990, Suda & Ueno 1994) resulting in distortion of the cellular membrane and alteration of the biological properties of receptors imbedded in the membrane and, thereby, altering cellular protein expression (Feinendegen & Muhlensiepen 1987, Coots et al. 2004).

Based on these observations, it appears reasonable to suggest that SMF stimulation inhibits LPS-induced cytotoxicity by reducing the release of proinflammatory cytokines and increasing anti-inflammatory cytokine release of in NIH-3T3 cells.

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