The Regulation of Procalcitonin Production in Human Peripheral Blood Mononuclear Cells

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말초혈액 단핵구 세포에서 프로칼시포닌 생성의 조절

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Background : Procalcitonin (PCT) is a new marker of severe systemic bacterial infection. PCT consists of fragments katacalcin and calcitonin, which are precursors of calcitonin in thyroid. The source and role of PCT in pathogenesis of sepsis remains clarified. This study was focused on which subsets of peripheral blood mononuclear cells (PBMC) can induce PCT when they are stimulated with endotoxin or phorbol myristate acetate (PMA), and how the PCT production is controlled.
Materials and Methods : PBMC were isolated and incubated overnight in each media containing 1 ug/mL lipopolysaccharide (LPS), or 5 ng/mL PMA. Intracellular PCT was detected using fluorescein isothiocyanate (FITC) labeled anti-katacalcin antibody (Ab). Monocytes and lymphocytes were identified by phycoerythrin-conjugated CD14 Ab and CyChrome-conjugated CD3 Ab, respectively. Ten micrograms of soluble TNF receptor (sTNFR) were pretreated in PBMC 1 hr prior to adding the stimuli. Then, PBMC were analyzed using a flow cytometer.
Results : LPS increased intracellular PCT from 10.0 % to 27.2% in CD14-positive monocytes from healthy donors, but PMA induced more PCT production from 10.0% to 40.8%. (one representative, n=8). For CD3-p ositive lymphocytes, LPS did not stimulate PCT, but PMA increased PCT production by 2.35 fold (P<0.05, n=8). In the PBMC from the same donor, sTNFR highly decreased LPS-stimulated PCT (control 10.0%, LPS 27.2%, sTNFR 12.3%), but it did not significantly affect PMA-stimulated PCT. For sepsis patients, PMA stimulated more PCT than LPS did and PCT was more expressed compared with healthy donors.
Conclusion : The PCT was produced in both monocytes and lymphocytes. PMA stimulated more PCT production than LPS did. The LPS-induced PCT production is partly mediated through TNF-α production.

Key Words : PBMC, Procalcitonin, LPS, TNF-α

INTRODUCTION

Serum procalcitonin (PCT), a 13-kDa 116-amino acids, precursor of calcitonin, which consists of the fragment katacalcin(KT) and calcitonin(CT) and an N-terminal residue (1,2).

PCT is produced by C-cells of the thyroid gland or neuroendocrine cells in the lung or intestine.

PCT has been suggested as a specific marker of bacterial infection with systemic manifestation (3-6). A recent study showed that serum PCT levels are significantly elevated in patients with sepsis, and PCT is closely associated with the prognosis of severe sepsis (7,8). It is also known that sepsis in animal

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study is associated with increase of PCT level (9). Furthermore, there are many evidences that PCT is a proinflammatory mediator as well as an inflammatory marker (10, 11).

The source and role of PCT in pathogenesis of sepsis remains clarified. The purpose of this study is to investigate the ability of PBMC to make PCT when they are stimulated with endotoxin (lipopolysaccharide, LPS) or potent non-specific stimuli such as phorbol myristate acetate (PMA). Also, we worked on the pathway involved in PCT production in PBMC by LPS and PMA. PCT has been reported to have an anti-inflammatory capacity (12). In contrast, the mortality was augmented after IV PCT administration in animal (13). The role of PCT in sepsis should be clarified in this study.

MATERIALS AND METHODS

1. Isolation and infection of PBMC

Thirty-milliliter aliquots of plateletpheresis residual were layered over 20 mL of Histopaque 1077 (Sigma, St. Louis, MO) in 50 mL polypropylene tubes (BD Falcon, Franklin Lakes, NJ) and centrifuged at room temperature for 30 min at 400×g (14). The PBMC layer was collected and washed twice in calcium- and magnesium-free phosphate buffered saline (Life Technologies).

2. Flow cytometry for procalcitonin

Uninfected PBMC at 2×10^6 per mL were incubated in 1.0-mL RPMI medium (Gibco, USA)(control) or in medium containing 1 μg/mL LPS (Sigma) or in medium containing 5 ng/mL PMA (Sigma) in 15 mL-conical tubes with a cap. For the inhibition experiment, the cells were incubated with the media including 10 ug/mL soluble TNF receptor (sTNFR), or with 5 nM IL-18 (R&D, USA) for 1 hour, respectively. The cell-containing tubes were cultured under the 37℃, 5% CO_2 incubator overnight. Next day, brefeldin A (BD Pharmingen), a protein secretion inhibitor was added to each tube with 1 μL per 1 mL cells, and left for 6 hours. A cold EDTA was put into each tube, vortexed and washed with PBS and decanted. The cells in each tube were permeabilized by 1 mL FACS Permeabilizing solution (BD biosciences). For staining, two monoclonal antibodies to katacalcin and calcitonin (A gift from Dr. J. Struck, BRAHMS) were bound to FITC according to the protocol (15). One of the control aliquots and one of the stimuli-exposed aliquots received Phycoerythrin-conjugated CD14 antibodies, FITC-conjugated two MAb to katacalcin and calcitonin. Two-color flow cytometric analysis was performed. The second control aliquot received isotype control goat IgG Abs (BD) to FITC-katacalcin. The tubes were incubated in the dark for 1 hour at room temperature. After washing the cells, PBMC were then analyzed using a flow cytometer (BD).

3. Flow cytometry for sepsis patients.

The PBMC from patients with septic shock were isolated on admission day. The cells were incubated and the intracellular PCT were detected following the same method described above.

4. Measurements of PCT

Serum levels of PCT in whole blood or U937 cells were measured using an automated immunoluminometric assay (LUMI test : Brahms Diagnostica, Berlin, Germany). Two antigen specific monoclonal antibodies were used, one of which binds the C-terminal region (Katacalcin) and the other, which is fluorescent labeled with acridinium ester, binds calcitonin. The inner surface of the tube is pre-coated with katacalcin antibody, which binds to PCT in the patient sample, and this in turn binds to the luminescent labeled antibody creating a 'sandwich complexes'. The intensity of the signal is measured using a luminometer. The lower limit of PCT is 0.08 ng/mL.

RESULTS

The PBMC were incubated overnight in the absence or presence of LPS or PMA. As shown in Figure 1A, the percentage of cells positive for KC (FITC) in CD14-positive monocytes in the absence of LPS (control) was 10.0%. PBMC incubated in the presence of LPS increased the percentage of KC-positive cells to 27.2%, a 63% increase. In Figure 1B, the percentage of

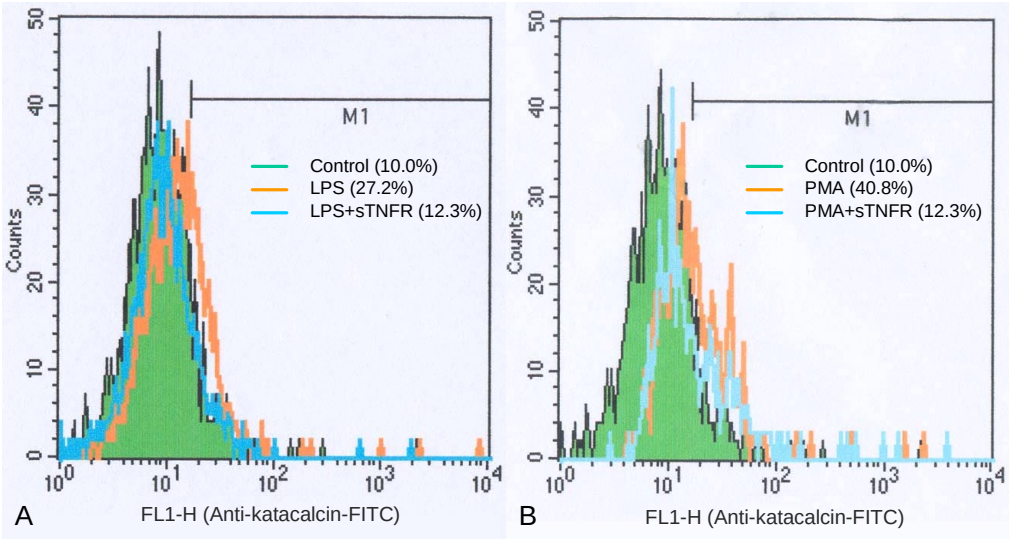

Figure 1. Flow cytometric analysis of the intracellular content of PCT components Katacalcin (KC) in human PBMC. Data are representative results obtained in 8 separate experiments.
(A) Mononuclear cells were incubated with media alone (control) or LPS overnight. sTNFR were pre-incubated 1hr before LPS was added into cells. LPS stimulated intracellular PCT production from 10% to 27.2% in CD14+ cells. sTNFR decreased LPS-stimulated KC from 27.2% to 12.3%
(B) The cells from the same donor as A were incubated with PMA. PMA induced more PCT production in CD14+ cells from 10.0% to 40.8%. sTNFR did not significantly affect PMA-stimulated PCT.

KC (FITC)-positive cells in monocytes in the presence of PMA was increased to 40.8%, a 75.5% increase compared with the same control as Figure 1A. However, 5 nM IL-18 did not significantly increase PCT production in PBMC, respectively (data not shown).

It was shown that TNF-α increased 2-5 hours before PCT appeared in the blood by endotoxin administration (16). Procalcitonin production could occur through TNF-α induction. Therefore, we examined the possibility that LPS-induced TNF-α production may account in part, for the PCT expression. Soluble TNFR was pre-incubated 1 hour before the stimuli including LPS and PMA were added to the cells. As shown in Figure 1A, sTNFR reduced PCT production by LPS from 27.2% to 12.3%, a 86.6% inhibition compared with 10.0% of the control. However, Figure 1B showed that sTNFR slightly reduced PCT production by PMA from 40.8% to 34.5%, a 20.5% inhibition.

In the same sets of cells as in the Figure 1, the PBMC were incubated overnight in the absence or presence of LPS or PMA. As shown in Figure 2, the cells positive for KC (FITC) in CD3-positive lymphocytes in the presence of LPS showed 1.24±0.25 fold-increase compared with the control. In contrast, PMA significantly increased PCT production up to 2.35±1.05 fold in lymphocytes compared with the control ($P<$ 0.05).

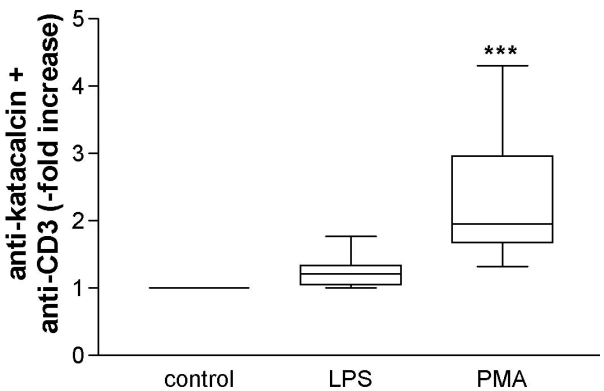

Figure 2. Flow cytometric analysis of PBMC showing PCT increase in PMA-stimulated CD3+ lymphocytes (n=8). Incubated PBMC were analyzed with anti-CD3-Cyc and anti-Katacalcin-FITC. LPS did not significantly induce PCT production. In contrast, PMA significantly increased PCT secretion by 2.35±1.05 fold (***P<0.05).

The PCT response to LPS and PMA in the sepsis patients (n=4) were compared with the healthy donors (n=8) (Figure 3). The increase of KC-positive PCT content of CD14-positive monocytes after stimulation of LPS ranged 1.25 to 4.3-fold in cells from healthy donors. In the septic shock patients, PCT increase induced by LPS ranged 0.96 to 2.5-fold. However, PCT

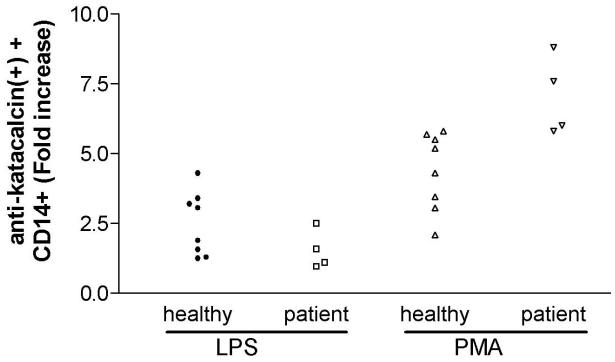

Figure 3. Flow cytometric analysis in PBMC from healthy donor and patients with septic shock (n=8, and n=4, respectively). The Katacalcin-positive cells out of CD14+ monocytes were compared between healthy subjects and the patients. The PCT increase was described in fold-increase compared with control cells. In healthy donor, LPS-stimulated PCT increase ranged from 1.25 to 4.3-fold, but the PCT production rather tended to decrease in sepsis patients. In contrast, the PMA-stimulated PCT increase was magnified in patients ranging 2.0 to 5.8-fold compared to healthy donors, ranging 5.8 to 8.8-fold.

response to the PMA was magnified in septic shock patients. The KC-positive PCT in monocytes after PMA stimulation increased to 2.1 to 5.7-fold in cells from healthy donors. In contrast, PMA-induced PCT increase of sepsis patients were remarkable ranging from 5.8 to 8.8-fold.

To verify that monocytes are the source of PCT, the human monocytic cell line U937 cells were used for PCT measurement. After overnight culture of cells after stimulation of LPS or PMA, PCT content was measured in U937 cells. PCT was not detected using LUMI test. In the whole blood after stimulation of LPS or PMA, PCT was not measured, either.

DISCUSSION

PCT has been suggested as a specific marker of bacterial infection with systemic manifestation (3, 5, 6, 17). Several reports showed that PCT is a mediator of sepsis, which causes a sepsis and simultaneously presents during the clinical expression of sepsis (18, 19).

PCT has been also reported to have anti-inflammatory capacity (12, 20). In contrast, it has been reported that the mortality was augmented after PCT administration in animals (13). Therefore, the role of PCT in sepsis is not clear.

The source of PCT has been known as lung and intestine, but the information about other source of PCT is limited. In this study, we showed that PCT was produced both in monocytes and lymphocytes, although its production in lymphocytes was restricted only by PMA. This suggests that the regulation of PCT production is differently controlled by the type of stimuli. LPS stimulated-PCT production in monocytes that we observed was consistent with the previous reports (21, 22). Furthermore, we found that a mitogen, PMA stimulation leads to the induction of PCT synthesis, although IL-18 did not induce PCT production in PBMC. IL-18 is one of the pro-inflammatory cytokine and stimulated by LPS. However, IL-18 could not contribute to a pathway of PCT induction by LPS.

The action of LPS to PBMC could occur probably through CD14 receptor. This pathway could go to stimulate TNF and then it stimulates PCT production. As it was expected, sTNFR blocked PCT production by LPS, suggesting that PCT production is mediated through TNF-α pathway, in part. This is consistent with previous reports that monoclonal Ab against TNF partly decreased PCT induced by *Staphylococcus aureus* (22). Therefore, the pathway of PCT production in the bacterial infection was proved that TNF pathway is partly involved, regardless of gram positive bacteria or gram negative bacteria. However, PMA-induced pathway was not blocked via sTNFR. PMA is a nonspecific stimuli, mitogen, and well known to be a protein kinase C activator. It is possible that PMA-induced PCT production is related to the signaling cascade such as MAP kinase through PKC, which should be confirmed in further experiments. The documentation of novel pathway to make PCT by LPS or PMA can be employed for the intervention tool in sepsis.

We found that there was up-regulation of PCT by PMA in monocytes from the patients with sepsis. Previously, the experimental models of endotoxin showed both hyper-response and hypo-response to LPS in inflammatory process. In this study, LPS-exposed cells from sepsis patients showed a mildly decreased tendency of PCT production, which could be explained by the tolerance to endotoxin as explained in the previous study (23). Sepsis patients have been already exposed to the large amount of LPS and could

use up all the intracellular PCT, which could be a defense mechanism in the sepsis patients. For sepsis patients, PMA did not show the decrease of PCT production, rather showed the tendency of more prominent production of PCT than LPS did. This suggests that high PCT level in serum of septic patients could be the result of production from the cells as well as surrogate marker. Also, these different responses of the PBMC to the two stimuli used are probably associated with their different activating pathways. PMA can cause receptor-independent induction of PCT by activating enzymes such as protein kinase C. In contrast, the receptor of LPS could be exhausted due to the continuous stimuli of endotoxin, which contributes to LPS tolerance. However, the different response of PCT to LPS and PMA in sepsis patients should be further investigated. In conclusion, The PCT was produced in both monocytes and lymphocytes. PMA stimulated more PCT production than LPS. The profoundly increased PCT production was observed in cells from septic patients. The LPS-induced PCT production is partly mediated through TNF-α production. The intervention of PCT production may be considered for further study to control of sepsis.

ABSTRACT

목 적 : 프로칼시토닌은 중증 전신성 세균 감염의 새로운 표지자로 이용되고 있다. 프로칼시토닌의 구조는 감상선에서 분비되는 칼시토닌의 전구체로, 카타칼신과 칼시토닌으로 알려져 있으나, 패혈증의 발병에서 프로칼시토닌의 합성장소와 그 역할은 아직 분명하지 않다. 본 연구는 어떤 말초혈액세포가 내독소와 PMA로 자극시에 PCT 분비를 유도할 수 있는지와 어떤 기전에 의해 PCT 생성이 조절되는지를 보고자 한다.

재료 및 방법 : 말초혈액 단핵구세포를 분리하여, 각각 LPS (1 ug/mL)나 PMA (5 ng/mL)가 들어있는 배지에 하룻 밤 배양시킨 뒤 다음 날 세포내 프로칼시토닌을 FITC-항 카타칼신 항체를 이용하여 측정하였다. 단핵구와 림프구는 각각 phycoerthrin-CD14 항체와 Cychrome-CD3 항체를 이용하였다. 기전에 대한 분석실험은 여러 자극제를 말초혈액 단핵구세포에 넣기 1시간 전 수용성 TNF 항체 10 ug/mL로 세포를 미리 처리한 뒤 유세포분석기를 이용하여 분석하였다.

결 과 : LPS는 건강한 공여자에서 얻은 말초혈액 단핵구에서 대조군의 프로칼시토닌 생성을 10.0%에서 27.2%로 증가시켰고 PMA는 10.0% 에서 40.8%로 더 많은 PCT생성의 증가를 보였다(n=8 중 대표). CD3 양성 림프구에서는 LPS는 PCT생성을 시키지 못하였으나 PMA는 프로칼시토닌 생성을 2.35배까지 증가시켰다(P<0.05, n=8). 같은 공여자의 말초혈액 단핵구에서 수용성 TNF 항체는 LPS로 자극된 PCT생성을 감소(대조군 10.0%, LPS군 27.2%, 수용성 TNF 항체 12.3%)시켰으나, PMA자극으로 생성된 프로칼시토닌에는 영향을 주지 못하였다. 패혈증 환자에서는 건강한 군에 비해 PMA가 LPS보다 더 많은 PCT생성을 보였다.

결 론 : 프로칼시토닌은 말초혈액세포 중 단핵구와 림프구 모두에서 생성되었고, PMA가 LPS보다 더 많은 프로칼시토닌 생성을 유도하였다. LPS에 의한 프로칼시토닌 생성과정 중 일부는 TNF-α 생성을 매개로 한다.

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