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Simultaneous Determination of Alteration of a Variety of Macrophage Functions Related to Natural Immunity Following Treatment with a DP Receptor Agonist

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[Received on April 25, 2002 ; Accepted on August 19, 2002]

Key words : PGD₂/macrophage/TNF- α /natural immunity

Abstract : Prostaglandin D₂ (PGD₂) acts via the adenylyl cyclase-coupled receptor for PGD₂ (DP receptor). Here we present evidence that BW245C, a DP receptor agonist, modulates macrophage functions related to natural immunity. BW245C inhibited macrophage chemotaxis at concentrations of 0.1 to 10 μ M and phagocytosis of *Escherichia coli* by macrophages at a concentration of 10 μ M. In addition, BW245C inhibited the production of superoxide anions by PMA-stimulated macrophages at concentrations of 0.1 to 10 μ M and nitrite production by LPS-stimulated macrophages at a concentration of 10 μ M. In contrast, BW245C potentiated the production of TNF- α , a pro-inflammatory cytokine, by LPS-stimulated macrophages at concentrations of 1 to 10 μ M. These results suggest that PGD₂ may modulate macrophage functions related to natural immunity via the DP receptor.

抄録 : プロスタグランジン D₂ (PGD₂) は、その受容体である DP 受容体を介して作用することが知られている。今回われわれは、DP 受容体アゴニストである BW245C が、自然免疫系において重要な機能的役割を演じるマクロファージの機能に対して影響を与えることを見いだした。BW245C はマクロファージ遊走能に対しては、0.1 から 10 μ M において抑制したが、貪食能に対しては 10 μ M においてのみ抑制が認められた。また、BW245C は PMA 刺激条件下でのスーパーオキシド産生能を 0.1 から 10 μ M において抑制したのに対して、LPS 刺激条件下での NO 産生能は 10 μ M においてのみ抑制がみられた。さらに、炎症性サイトカインである TNF- α に対しては、LPS 刺激条件下において BW245C は 1 および 10 μ M において増強効果を示した。以上の結果より、PGD₂ はその受容体を介してマクロファージ機能に影響を与える可能性が示唆される。

Introduction

Periodontitis is a bacterially induced chronic inflammatory disease that is the major cause for tooth loss in the adult population. The current disease paradigm for the manifestation of periodontitis involves both a bacterial and a natural host defense system. The inflammatory response is a fundamental component of the natural immunity system. Macro-

phages are commonly the first cells of the immune system to encounter invaders such as bacteria. The macrophage response to infection is initiated by adhesion to vascular endothelial cells, and progresses to directed cell migration into the extravascular tissue. Macrophages result in phagocytosis and intracellular killing of the invading microorganisms by the generation of bactericidal reactive oxygen species derived from the superoxide anion. Indeed, macrophages are essential for controlling most infections, and are

mediators of inflammation.

Prostaglandin D₂ (PGD₂) is generated from arachidonic acid in a number of tissues by the sequential actions of prostaglandin H synthase and prostaglandin D synthase. PGD₂ has been implicated in both central and peripheral physiological events. Peripherally, a large number of reports have focused on allergic disorders, platelet aggregation, vasodilation, pulmonary constriction, and bronchoconstriction^{1,2}. PGD₂ is also a precursor of 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (dPGJ₂). dPGJ₂ is a natural ligand for the peroxisome proliferator-activated receptor γ (PPAR γ), a nuclear receptor implicated in adipocyte differentiation and glucose homeostasis³⁻⁵. Recent studies documented the role of dPGJ₂, a potent PPAR γ ligand, as an anti-inflammatory agent^{6,7}. In fact, we have previously demonstrated that dPGJ₂ markedly inhibits several kind of functions in macrophages^{8,9}. However, little attention has been paid to the effect of PGD₂ on macrophage functions, although a recent study showed the possibility that PGD₂ in addition to dPGJ₂ may have anti-inflammatory properties¹⁰. These previous findings prompted us to examine the possible effects of PGD₂ on inflammatory responses. For this purpose, in this study, we simultaneously examined the effect of BW245C, an agonist of prostanoid DP receptor, on chemotaxis, phagocytosis, superoxide anion production, nitric oxide production and TNF- α production by macrophages.

Materials and Methods

1. Materials

BW245C was obtained from Cayman Chemical (Ann Arbor, MI). Fluorescein-conjugated *Escherichia coli* bioparticles was purchased from Molecular Probes (Eugene, OA).

2. Isolation of macrophages from peritoneum

The protocol employed here meets the guidelines of the Japanese Society for Pharmacology. All efforts were made to minimize animal suffering and to reduce the number of animals used. Macrophages were isolated by peritoneal lavage from adult male Wistar rats weighing 200–250 g, 4 days after the injection of

20 ml of 1% glycogen. Lavage was performed by washing the peritoneal cavity with PBS supplemented with 20 U/ml heparin and 1 mM EDTA. Care was taken not to cause internal bleeding while collecting macrophages in the exudate¹¹. For the chemotaxis assay, the macrophages were allowed to adhere to plastic dishes for 2 h. They were then resuspended in DMEM. For the other assays, macrophages in DMEM were used. The purity of the adherent cells exceeded 95% as determined by immunostaining with an anti-ED2 antibody (a macrophage marker).

3. Chemotaxis assay

The chemotaxis assay was performed as previously described¹² with minor modifications¹³. In brief, zymosan-activated serum, prepared by a method reported elsewhere¹⁴, was diluted to 5% in medium and placed in the lower compartment of 96-well microchemotaxis chambers as the stimulant. A polycarbonate filter with 5- μ m pores was placed on the wells. Experiments were initiated by adding the mixture containing macrophages (100 μ l, 2×10^5 per well) with BW245C to the upper compartment of the chamber. After 90 min, the cells on the filter were fixed with methanol and stained with Diff-Quick. The upper side of the filter was then scraped free of cells. The number of cells that migrated to the lower side was determined by measuring optical densities at 595 nm.

4. Phagocytosis assay

The phagocytosis assay was performed as previously described¹⁵ with minor modifications¹⁶. In brief, macrophages (1×10^5 per well) were allowed to adhere for 2 h and were then incubated with BW245C and fluorescein-conjugated *E. coli* (K-12) bioparticles at 1×10^6 per well. After incubation for 1 h, extracellular fluorescence was quenched by adding 25 μ g trypan blue in 13 mM citrate buffer (pH 4.4). The dye was removed after 1 min and the fluorescence intensity was determined.

5. Superoxide anion production assay

Superoxide anion production was detected by a method previously described¹⁷ with minor modifications¹⁸. In brief, macrophages (1×10^5 per well) were

allowed to adhere for 2 h and were then incubated with BW245C containing PMA at 200 nM and ferricytochrome c at 160 μ M. Superoxide anion production was assessed by the inhibition of ferricytochrome c reduction using 600 U/ml superoxide dismutase. The absorbance at a wavelength of 550 nm was measured at different times after incubation.

6. Nitrite production assay

The concentrations of nitrite in the culture supernatants were determined by a microplate assay. In brief, macrophages (1×10^5 per well) were allowed to adhere for 2 h and were then incubated with BW245C and LPS at 10 μ g/ml for different times. After the supernatant was collected from the wells, the nitrite concentration was measured by commercially available Griess Reagent System (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid, Promega; Madison, WI) for 10 min. The absorbance at a wavelength of 550 nm was measured. Nitrite concentrations were determined by using sodium nitrite as a standard.

7. ELISA assay

The TNF- α production assay was performed as previously described⁸⁾ with minor modifications¹⁹⁾. In brief, macrophages (1×10^6 per well) were allowed to adhere to 24-well plates for 2 h and were then incubated with BW245C and LPS at 2 ng/ml for 20 h. After the supernatant was collected from the wells, the concentration of rat TNF- α was measured by commercially available ELISA (BioSource International, Camarillo, CA).

8. Data analyses

The results were all expressed as the mean $\pm$ S.E. and the statistical significance was determined by the unpaired-t-test followed by estimation of the least significant difference.

Results

1. BW245C inhibits chemotaxis toward zymosan-activated serum

When the macrophages and BW245C were placed in

Fig. 1 BW245C inhibits macrophage chemotaxis towards zymosan-activated serum. Macrophages and BW245C were placed in the upper well, with zymosan-activated serum in the lower well, followed by incubation for 90 min. The data from four individual animals are shown. * $p < 0.05$; ** $p < 0.01$, when compared with untreated macrophages.

the upper well, with zymosan-activated serum in the lower well as a stimulant, BW245C markedly inhibited macrophage chemotaxis at a concentration range of 0.1 to 10 μ M (Fig. 1).

2. BW245C inhibits phagocytosis of *E. coli*

When determined under the conditions of the simultaneous addition of BW245C and *E. coli*, the addition of 10 μ M of BW245C inhibited the phagocytosis of *E. coli* by macrophages, with concentrations below 1 μ M being unchanged (Fig. 2, left panel). In contrast, pretreatment of macrophages with BW245C at a concentration range of 0.01 to 10 μ M did not alter the phagocytosis of *E. coli*. However, pretreatment with BW245C at 50 μ M significantly suppressed phagocytosis (Fig. 2, right panel).

3. BW245C inhibits superoxide anion production

The addition of increasing concentrations of BW245C markedly blocked the production of superoxide anion by macrophages stimulated with PMA at 1 h after incubation (Fig. 3). This inhibition of superoxide anion production by BW245C persisted for at least 4 h. However, the lowest concentration of BW245C did not affect the production.

Fig. 2 BW245C inhibits the phagocytosis of *E. coli* by macrophages. Macrophages were allowed to adhere to plates for 2 h. Adherent macrophages were incubated with BW245C and *E. coli* for 1 h (left panel). Adherent macrophages were preincubated with BW245C for 1 h, followed by incubation with *E. coli* for 1 h (right panel). The data from three individual animals are shown. * $p < 0.05$, when compared with untreated macrophages.

Fig. 3 BW245C inhibits the production of superoxide anion by macrophages. Macrophages were allowed to adhere to plates for 2 h. Adherent macrophages were incubated with BW245C containing PMA at 200 nM and ferricytochrome c at 160 μM. The data from six individual animals are shown. * $p < 0.05$, when compared with macrophages treated with PMA alone.

4. BW245C inhibits nitrite production

The addition of BW245C at 10 μM significantly inhibited nitrite production by macrophages stimulated with LPS at 3.5 h after incubation, with concentrations below 1 μM being unchanged (Table 1). After incubation for 24 h, however, BW245C did not affect

the nitrite production at concentrations of up to 10 μM. When macrophages were pretreated with BW245C for 24 h, followed by incubation with LPS for 24 h, furthermore, BW245C was not found to affect the production (Table 1, right column).

5. BW245C potentiates TNF-α production

The addition of increasing concentrations of BW245C in the presence of LPS markedly potentiated the production of TNF-α as compared with macrophages in the absence of LPS (Fig. 4). In addition, BW245C at concentrations above 1 μM significantly potentiated the LPS-stimulated TNF-α production. However, BW245C at 10 μM induced less potent potentiation than at 1 μM. In contrast, BW245C at concentrations of up to 10 μM alone did not alter the production of TNF-α by macrophages in the absence of LPS (data not shown).

Discussion

Chemotaxis plays an important role in the development of inflammatory diseases^{20,21}. It requires the degradation of basal laminae and interstitial stroma, and the processes of chemotaxis involve matrix metalloproteinases, a family of endopeptidases capable of degrading extracellular matrix compo-

Table 1 BW245C inhibits the production of nitrite by macrophages

	Nitrite production (μM)		
	3.5 h	24 h	24 h+24 h
BW245C (μM)			
0	7.59 $\pm$ 1.8	37.3 $\pm$ 0.04	17.6 $\pm$ 0.29
0.01	7.37 $\pm$ 0.6	36.1 $\pm$ 0.26	17.0 $\pm$ 0.74
0.1	7.54 $\pm$ 0.6	36.1 $\pm$ 0.11	18.3 $\pm$ 0.26
1	7.30 $\pm$ 1.0	36.2 $\pm$ 0.12	18.1 $\pm$ 0.31
10	6.87 $\pm$ 0.7**	36.4 $\pm$ 0.18	17.8 $\pm$ 0.63

Macrophages were allowed to adhere to plates for 2 h. Adherent macrophages were incubated with BW245C containing LPS at 10 $\mu\text{g/ml}$ for 3.5 or 24 h (left and middle column). Adherent macrophages were preincubated with BW245C for 24 h, followed by incubation with LPS at 10 $\mu\text{g/ml}$ for 24 h (right column). The data from 3 to 5 individual animals are shown. ** $p < 0.01$, when compared with macrophages treated with LPS alone.

nents²²⁻²⁴). Several studies have reported that phosphoinositide 3-kinase is associated with chemotaxis²⁵), chemoattractant receptors that can activate at least both class 1 A and class 1 B phosphoinositide 3-kinase. In addition, recent studies in transgenic mice found that the chemoattractant-induced formation of 3'-phosphorylated lipids, the activation of protein kinase B (Akt), and chemotaxis depended entirely on p110 γ , the only known phosphoinositide 3-kinase of class 1 B²⁵). However, there are no reports that PGD₂ and/or DP receptor agonist can modulate matrix metalloproteinases or phosphoinositide 3-kinase in any cell types. The exact molecular mechanisms underlying the suppression of chemotaxis by BW245C, therefore, still remain to be elucidated in future studies.

In this study, BW245C at 10 μM inhibited phagocytosis under the conditions employed in the simultaneous addition of BW245C and *E. coli*, but not under the conditions employed in preincubation with BW245C, followed by the addition of *E. coli*. Moreover, BW245C at 50 μM inhibited phagocytosis under the conditions employed in preincubation. These data argue in favor of the idea that BW245C may act transiently, and the action time of BW245C may be

Fig. 4 BW245C potentiates the production of TNF- α by macrophages in response to LPS. Macrophages were allowed to adhere to plates for 2 h. Adherent macrophages were incubated with BW245C and LPS at 2 ng/ml for 20 h. The data from three individual animals are shown. * $p < 0.05$; ** $p < 0.01$, when compared with untreated macrophages. # $p < 0.05$, when compared with macrophages treated with LPS alone.

short.

This study shows that BW245C inhibits superoxide anion production by PMA-stimulated macrophages. PMA activates macrophages via the activation of protein kinase C and the subsequent phosphorylation of the cytosolic factor p47^{phox} to produce superoxide anions²⁶⁻³⁰). On the other hand, the DP receptor is coupled positively to adenylyl cyclase through G_s, resulting in an increase in the intracellular concentration of cAMP. Recent findings have indicated that dibutyryl-cAMP, 8-bromo-cAMP (cell permeable cAMP analogs), forskolin (an adenylyl-cyclase activator), and Ro 20-1724 (a phosphodiesterase inhibitor), but not 8-bromo-cGMP (cell permeable cGMP analogs) can inhibit superoxide anion production by PMA-stimulated macrophages³¹). In addition, a previous study showed that cAMP down-regulates PKC activation³²). These previous findings all support the present proposal that BW245C may inhibit superoxide anion production via the down-regulation of PKC by increased cAMP.

BW245C resulted in the less potent inhibition of nitrite production than that of superoxide anion

production. The inhibition of nitrite production by BW245C did not appear in the conditions employed in both the long incubation time and preincubation. We used LPS as a stimulant in the nitrite production assay, because LPS can produce nitric oxide, followed by nitrite via the up-regulation of inducible nitric oxide synthase (iNOS). In the superoxide anion production assay, we used PMA as a stimulant. Our observations suggest that the DP receptor signal may selectively interact with the PKC pathway by PMA rather than the LPS signal pathway.

An interesting finding of this study is that BW245C potentiates the production of TNF- α by LPS-stimulated macrophages. A previous study clearly demonstrated that pentoxifylline, which increases intracellular cAMP by inhibiting phosphodiesterase, inhibits the production of TNF- α by monocytes treated with both PMA and phytohemagglutinin^{33–36}. Another study showed that elevated cAMP inhibits the LPS-stimulated pathway in monocytes³⁷. Again, the DP receptor potentiates adenylyl cyclase. Therefore, it is unlikely that increased cAMP through the activation of DP receptor is associated with the further-potential of TNF- α production by LPS-stimulated macrophages. There have been several reports that the TNF- α gene acts constitutively in macrophages and the regulation of TNF- α expression in LPS-stimulated macrophages occurs posttranscriptionally^{38,39}. Therefore, the possibility that activation of the DP receptor by BW245C may affect the transcription and/or post-transcription modulation of TNF- α mRNA cannot be ruled out.

On the other hand, PGD₂ and dPGJ₂ levels were increased in carrageenin-induced pleurisy in rats concomitant with the resolution of inflammation⁴⁰. In this model, the application of PGD₂ and dPGJ₂ can aid the resolution of inflammation⁴⁰. This previous study suggested the possibility that PGD₂ and dPGJ₂ may have anti-inflammatory properties. It is not clear how PGD₂ can aid the resolution of inflammation, however, although it was found that dPGJ₂ acts as an anti-inflammatory via PPAR γ . Furthermore, PGD₂ synthase, which is required for dPGJ₂ synthesis, is predominantly expressed in macrophages⁴¹. The question of whether PGD₂ actively acts as an anti-

inflammatory following enzymatic conversion into dPGJ₂, or PGD₂ directly acts as an anti-inflammatory via activation of DP receptor remains to be addressed before drawing any conclusions. It thus appears that BW245C clearly inhibited chemotaxis, phagocytosis, superoxide anion production and nitrite production, and potentiated TNF- α production by macrophages. The action mechanism of potentiation of BW245C on TNF- α production by macrophages is currently under investigation.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (13671957) from the Japan Society for the Promotion of Science of Japan.

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