The role of cyclic AMP on arginase activity by a murine macrophage cell line (RAW264.7) stimulated with lipopolysaccharide from *Actinobacillus actinomycetemcomitans*

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Abstract

The aim of the present study was to determine the role of cAMP on arginase activity in murine macrophage cell line (RAW264.7 cells) stimulated with lipolysaccharide (LPS) from *Actinobacillus actinomycetemcomitans* (LPS-Aa) or LPS from *Escherichia coli* (LPS-Ec) as a control. The cells were treated with LPS-Aa or LPS from *Escherichia coli* (LPS-Ec) for 24h. The effect of dibutyryl cAMP, 8-Bromo cGMP, forskolin and cycloheximide on arginase activity in LPS-Aa-stimulated RAW264.7 cells were also determined. Arginase activity in LPS-AA-stimulated cells in the presence of phosphodiesterase (PDE) and protein kinase A (PKA) inhibitors was assessed. The results showed that LPS-Aa stimulated arginase activity in RAW264.7 cells in a dose-dependent manner, but was less potent than LPS-Ec. Arginase activity in LPS-Aa-stimulated RAW264.7 cells was enhanced in the presence of cAMP or forskolin but not cGMP analogue. Cycloheximide blocked arginase activity in the cells in the presence of cAMP analoque or forskolin with or without LPS-Aa. A PDE 4 inhibitor (rolipram) augmented arginase activity higher than PDE3 inhibitor in LPS-Aa-stimulated cells. The effect of cAMP analogue or forskolin on arginase activity in the cells in the presence or absence of LPS-Aa was blocked by PKA inhibitor. The results of the present study suggest that arginase activity in LPS-Aa-stimulated RAW264.7 cells may be dependent on the cAMP-PKA pathway and also under regulation of cAMP-specific phosphodiesterase isoform.

Introduction

Arginase is an intracellular enzyme that catalizes the conversion of L-arginine to L-ornithine and urea. Two isoforms of this enzyme, i.e., arginase I (the hepatic isoform) and arginase II (the extrahepatic isoform) are known to exist (1). Arginase I, a cytosolic form, is believed to play a role in ureagenesis, whereas arginase II, a mitochondrial form, is thought to play in biosynthesis of polyamines and inflammation (1). Arginase may also play a crucial role in wound healing by enhancing fibroblast proliferation and collagen production as well as in the immune system by regulating, for example, T cell proliferation (2,3). Since this enzyme utilizes L-arginine, it may compete with nitric oxide synthase (NOS), an enzyme that catalizes a reaction of L-arginine to nitrix oxide (NO). Indeed, increased NO production by activated macrophages inhibited the activity of arginase and vice versa (4-6), suggesting that NOS and arginase activity are reciprocally modulated. The arginase activity of macrophages was up-regulated by bacterial lipopolysaccharide (LPS) and cytokines such as interleukin-4 (IL-4) and IL-10 but down-regulated by IFN-γ (7-9), suggesting that arginase activity may be up-regulated by activated Th2 cells, but down-regulated by activated Th1 cells. However, despite being studied for more than two decades, the exact signal transduction leading to arginase activity remains scanty. Previous studies indicated that arginase activity in macrophages and human colon adenocarcinoma cells stimulated by LPS was significantly increased in the presence of cyclic AMP analogue, forskolin, an adenylate cyclase activator, or phosphodiesterase inhibitors, suggesting the activation of cAMP pathway in arginase activity (10-13). Further studies revealed that cAMP-mediated arginase gene activation involves the activation of protein kinase A and appropriate transcription factors such as CREB (10,12-14).

Actinobacillus actinomycetemcomitans (Aa), a gram negative oral bacterium, plays a crucial role in the immunopathogenesis of periodontal disease, since this bacterium is able to induce both the host immune response and tissue destruction (15,16). We and others have shown that LPS from periodontopathogens such as Aa, *Prevotella intermedia* and *Porphyromonas gingivalis* induces NO production by murine macrophages and splenic macrophages (17-21). Of interest, NO production by these cells stimulated with LPS-Aa was regulated by different cytokines; thus, IFN-γ and IL-12 up-regulated but IL-4 suppressed NO production (20,21). We speculated that IL-4 may increase but IFN-γ and IL-12 suppress arginase activities in LPS-Aa-activated murine macrophages (20,21). Therefore, the aim of the present study was to determine whether or not arginase activity could be induced by in RAW264.7 cells stimulated with LPS-Aa and was regulated by the involvement of cAMP pathway.

Materials and Methods

Preparation of *A. actinomycetemcomitans* lipopolysaccharide

A. actinomycetemcomitans Y4 (serotype b) was grown in Todd-Hewitt broth supplemented with 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI) anaerobically as described elsewhere (22). LPS from this periodontopathic bacterium was isolated using a hot phenol-water extract, treated with nuclease (Sigma, St. Louis, MO), and then lyophilized as previously described (20).

Cell cultures

A murine macrophage cell line (RAW 264.7), a kind gift from Dr. T. Sizfizul (School of Biology, Universiti Sains Malaysia), was grown in Dulbecco's modified eagle medium supplemented with 5% foetal calf serum and 1% penicillin-

streptomycin (Sigma). After confluence, cells were harvested and washed 3 times, and then viable cells were counted. Two hundred microliters of culture medium containing 2 X 10^5 cells was cultured in an incubator in 5% CO2 at 37°C for 24h and stimulated with various concentrations of LPS-*A. actinomycetemcomitans* (LPS-Aa). As a control, the cell cultures were stimulated with LPS from *Escherichia coli* (LPS-Ec) (Sigma). The cultures of cell alone were used as a negative control. Dibutyryl cAMP, 8-bromo cGMP or forskolin (Sigma) at various concentrations was added in the cell cultures with or without the presence of 10μg of LPS-Aa. In other experiments, various concentration of cycloheximide (Sigma) was added in the cell cultures in the presence of LPS-Aa (10μg) alone and/or dibutyryl cAMP (10 μM) or forskolin (10 μM). To determine the involvement of phosphodiesterase (PDE), various concentration of siguazodan (a PDE3 inhibitor) or rolipram (a PDE4 inhibitor) was added the cell cultures stimulated with 10μg of LPS-Aa. PDE inhibitors were purchased from Sigma. KT5720, a PKA inhibitor, at various concentration was added in the cultures stimulated with or without 10μg of LPS-Aa in the presence of dibutyryl cAMP (10 μM) or forskolin (10 μM). All experiments were repeated 3 times, each consisting of triplicate cultures.

Measurement of arginase activity

Arginase activity in cell lysate was determined by a colorimetric assay as previously described (8). Briefly, cells were lysed with 50 μl of 0.1% Triton X-100 and shaken for 30 minutes. The cell lysate was then added with 50 μl of 25 mM Tris-Hcl (pH 7.5). To 25 μl of this lysate, 5 μl of 10 mM $MnCl_2$ was added and the mixture was heated for 10 minutes at 56°C. After adding 50 μl of 0.5 M L-arginine (pH 9.7), the mixture was incubated for 1 hour at 37°C. The reaction was stopped with 450 μl of acid solution mixture (1 H_2SO_4: 3 H_3PO_4 : 7 H_2O). The color was developed by adding 20 μl of 9% α-isonitropropiophenone (dissolved in 100% ethanol), heated for

45 minutes at 100°C, and then read at 540 nm by μQuant spectrophotometer (Biotek-Instrument Inc., Vermont, USA). Arginase activity was calculated using arginase activity index (AAI) with the following formula (9):

Arginase activity index = (test arginase activity) X 100/media only arginase

Arginase from cultured cells alone (negative control) was assigned an activity index value of 100.

Statistical analysis

The data were analyzed by a one-way analysis of variance using the SPSS statistical package (SPSS Co., Chicago, USA).

Results

Arginase activity by murine macrophages

When murine macrophages (RAW264.7 cells) were stimulated with increased concentration of LPS-Ec, the levels of arginase activity were also augmented ($p<0.05$) (Fig.1). Similarly, LPS-Aa-stimulated RAW264.7 cells produced detectable arginase activity ($p<0.05$). At any given LPS concentration, the levels of arginase activity in LPS-Aa–stimulated RAW264.7 cells were significantly lower than those in LPS-Ec-stimulated cells ($p<0.05$).

The effect of cAMP and cGMP analogue

When cells were stimulated with LPS-Aa in the presence of dibutyryl cAMP, arginase activity was increased significantly as compared with cells stimulated with LPS-Aa only ($p<0.05$) (Fig.2). Dibutyryl cAMP alone was able to augment arginase activity in

RAW 264.7 cells albeit its levels were lower than those by cells stimulated with LPS-Aa and this cAMP analogue ($p<0.05$). In contrast, arginase activity in cells stimulated with LPS-Aa in the presence of cGMP analogue was unaltered ($p>0.05$) (Fig. 2). cGMP analogue alone failed to induce arginase activity in RAW264.7 cells ($p>0.05$).

Effect of Forskolin

Forskolin, an adenylate cyclase activator, is known to increase the levels of cAMP (26). The present study showed that adding increasingly concentration of forskolin was paralleled with increased arginase activity in RAW264.7 cells with or without the presence of LPS-Aa ($p<0.05$) (Fig. 3), suggesting that increased arginase activity may be associated with increased cAMP activity in LPS-Aa-stimulated RAW264.7 cells.

Effect of cycloheximide

As seen in Fig. 4, cycloheximide reduced arginase activity in the cell cultures stimulated with LPS-Aa in the presence of dibutyryl cAMP or forskolin ($p<0.05$). Arginase activity in RAW264.7 cells in the presence of cAMP analogue or forskolin alone was reduced by cycloheximide ($p<0.05$).

Effect of phosphodiesterase inhibitors

In the presence of siguazodan (PDE3 inhibitor) or rolipram (PDE4 inhibitor), arginase activity was significantly enhanced in LPS-Aa-stimulated RAW264.7 cells as compared with that in cells stimulated with LPA-Aa alone ($p<0.05$) (Fig. 6). Comparing between the effect of siguazodan and rolipram, the levels of arginase activity in LPA-Aa-stimulated RAW264.7 cells in the presence of rolipram was higher than those in the presence of siguazodan, particularly after adding the drugs at concentration of 10 μM and 100 μM ($p<0.05$).

Effect of a protein kinase A inhibitor

Arginase activity in RAW264.7 cells in the presence of LPS-Aa and dibutyryl cAMP or forskolin could be significantly reduced by KT5720, an PKA inhibitor, in a dose dependent fashion ($p<0.05$) (Fig. 7). Similarly, increased concentration of PKA inhibitor added in cAMP analogue- or forskolin-activated RAW264.7 cell cultures was associated with steadily decreased arginase activity ($p<0.05$).

Discussion

Our previous study showed that LPS-Aa stimulates the production of NO by RAW264.7 cells and splenic spleen cells (20,21). The present study showed that LPS-Aa is also able to stimulate arginase activity by these cells. Altogether, it seems plausible that LPS-Aa is capable to stimulating NO production and arginase activity by murine macrophages, concurrently. Support can be drawn from the fact that LPS-Ec activates both inducible nitric oxide synthase and arginase in RAW264.7 cells by

different transcriptional mechanisms (24). It should be noted, however, that LPS-Aa at any given concentration induced significantly lower arginase activity than LPS-Ec, indicating that LPS-Aa may be less potent than LPS-Ec in inducing arginase activity in murine macrophages.

The next experiments were to delineate whether or not arginase activity in LPS-Aa-stimulated murine macrophages was under control of cAMP and/or cGMP pathway. The results of the present study revealed that cAMP but not cGMP analogue augmented arginase activity in LPS-Aa-stimulated RAW264.7 cells, suggesting that LPS-Aa may utilize the cAMP but not cGMP, pathway to activate arginase activity in RAW264.7 cells. These results are in accordance with previous reports showing that LPS from enteric bacteria stimulates arginase activity in murine macrophages and human colon carcinoma cells in a cAMP dependent mechanism (10-13). Of interest, cAMP analoque alone was able to stimulate arginase activity in RAW264.7 cells as seen in the present study. Altogether, it seems to suggest that LPS-Aa may amplify arginase activity in cAMP analogue-activated RAW264.7 cells. The exact mechanisms by which LPS-Aa promoted arginase activity in cAMP-activated RAW264.7 cells as seen in the present study is not clear. Morris and colleagues demonstrated that cAMP alone induces high expression of arginase I, whereas bacterial LPS stimulates the expression of arginase II in RAW264.7 cells (10). In this study, increased expression of both arginase I and II could be observed when the cells were stimulated with both LPS and cAMP analogue. If so, one may assume that stimulatory effect of both LPS-Aa and cAMP on arginase activity in RAW264.7 as seen in the present study may be due to increased expression of both arginase I and II. However, this notion remains to be investigated further, since the present study did not asses the expression of arginase isoforms.

The involvement of cAMP pathway in generating arginase activity in LPA-Aa-stimulated RAW264.7 cells was also supported by the fact that forskolin with or

without the presence of LPS-Aa increased arginase activity in these cells. Using rabbit alveolar macrophages, arginase activity was up-regulated when the cells were stimulated with forskolin and LPS from enteric bacteria (12). In this study forskolin alone was able to activate arginase activity. If so, it seems to suggest, yet again, that LPS-Aa may amplify the stimulatory effect of forskolin on arginase activity. Forskolin-mediated adenylate cyclase activation leads to activate G protein $G_s\alpha$ subunit which may in turn alter intracellular cAMP levels (25), thereby enhancing arginase activity. However, whether or not the ability of both forskolin and LPS-Aa to up-regulate arginase activity in RAW264.7 cells as seen in the present study may involve G protein activation needs to be further clarified.

The fact that cycloheximide prevented arginase activity in RAW264.7 cells activated by LPS-Aa in the presence of cAMP analogue or forskolin as seen in the present study indicates that up-regulation of arginase activity by the cAMP pathways requires an intact protein synthesis in such that this pathway may regulate the de novo synthesis of argianse proteins. These results are in the line with a previous report (12).

Phosphodiesterase (PDE), a family of proteins consisting of 11 isoforms, catalyzes the hydrolysis of cAMP to inactive 5′ -adenosine monophosphate, thereby reducing the levels of intracellular cAMP (26). Therefore, increased levels of cAMP may be achieved by reducing the activation of PDE by using specific PDE isoform inhibitors. Indeed, the present study showed that siguazodan and rolipram, PDE3 arnd PDE4 inhibitor respectively, enhanced arginase activity in LPS-Aa-stimulated RAW264.7 cells, suggesting that inhibition of either PDE3 and PDE4 activation may result in increased levels of cAMP which may in turn enhance arginase activity in LPS-Aa-stimulated RAW264.7 cells. Of interest, the results of the present study revealed that inhibition of PDE4 by rolipram induced higher levels of arginase activity than that of PDE3 by siguazodan in LPS-Aa-stimulated RAW264.7 cells. These

results suggest that PDE4 may play a predominant role over PDE3 in regulating cAMP levels following activation of RAW264.7 cells by LPS-Aa. Similarly, Hammermann and colleagues also demonstrated that inhibition of PDE4 by rolipram resulted in higher levels of arginase activity in rabbit alveolar macrophages than that of PDE3 by siguazodan (12). However, the exact reason to explain the results of the present study is unclear. One possibility is that differential levels of PDE3 and 4 in RAW264.7 cells may account the different levels of arginase activity in the cells in the presence of LPA-Aa and either PDE3 or PDE4 inhibitor as seen in the present study. Despite the fact that PDE1,PDE3, PDE4 are expressed by macrophages, the latter one is a major PDE isoform in these cells (26,27). That the levels of cAMP after PDE4 inhibition may be higher than those after PDE3 inhibition in activated macrophages is therefore obvious.

Activation of adenylyl cyclase leads to increase the levels of intracellular cAMP which in turn binds to regulatory (R) subunits of protein kinase A (PKA), thereby inducing dissociation of the holoenzymes and subsequent phosphorylation of key substrates (28). Hence, PKA is believed to be the most important effector of cAMP pathway. Indeed, previous studies indicated the involvement of PKA in arginase activity (12,14). The results of the present study support these previous reports, since in the presence of protein kinase A (PKA) inhibitor, arginase activity was reduced in RAW264.7 cells in the presence of cAMP analogue or forskolin with or without LPS-Aa. Therefore, one may speculate that PKA is a part of downstream signal which mediates the cAMP-dependent up-regulation of arginase activity in LPS-Aa-stimulated RAW264.7 cells.

In conclusion, the present study showed that LPA-Aa stimulates arginase activity in a murine macrophage cell line (RAW264.7 cells). The arginase activity in LPS-Aa-stimulated 264.7 cells was up-regulated by cAMP, but not cGMP analogue and forskolin. Inhibition of PDE4 activation induced higher levels of arginase activity

than that of PDE3 in RAW264.7 cells in the presence of LPS-Aa and/or cAMP analogue and forskolin. KT5729, a PKA inhibitor, reduced arginase activity in these cells stimulated with LPS in the presence of cAMP analogue or forskollin. These results suggest, therefore, that LPS-Aa may stimulate arginase activity in murine macrophages in a cAMP-PKA dependent pathway.

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Legend of figures

Fig. 1.The mean and standard deviation of arginase activity in RAW264.7 cells stimulated with LPS from *E. coli* (LPS-Ec) and *A. actinomycetemcomitans* (LPS-Aa). The arginase activity index of cells alone was set at 100. AAI = arginase activity index.

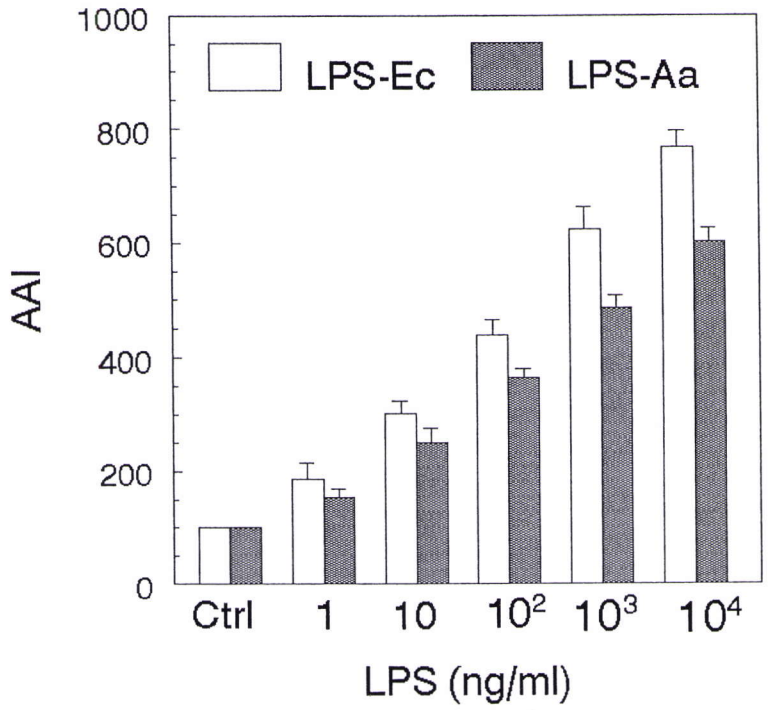
Fig. 2. Effect of dibutyryl cAMP (cAMP analogue) and 8-bromo cGMP (cGMP analogue) on the mean and standard deviation of arginase activity in LPS-Aa-stimulated RAW264.7 cells. AAI = arginase activity index

Fig. 3. Effect of forskolin, an adenylyl cyclase activator, on the mean and standard deviation of arginase activity in LPS-Aa-stimulated RAW264.7 cells. AAI = arginase activity index

Fig. 4. Effect of cycloheximide on the mean and standard deviation of arginase activity in RAW264.7 cells in the presence of cAMP analogue or forkolin with or without LPS-Aa. AAI = arginase activity index

Fig. 5. Effect of siguazodan, a PDE3 inhibitor, and rolipram, a PDE4 inhibitor, on the mean and standard deviation of arginase activity in LPS-Aa-stimulated RAW264.7 cells. AAI = arginase activity index

Fig. 6. Effect of protein kinase A (PKA) inhibitor, KT5720, on on the mean and standard deviation of arginase activity in RAW264.7 cells in the presence of cAMP analogue or forkolin with or without LPS-Aa.. AAI = arginase activity index


1000
800
600
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AAI
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10
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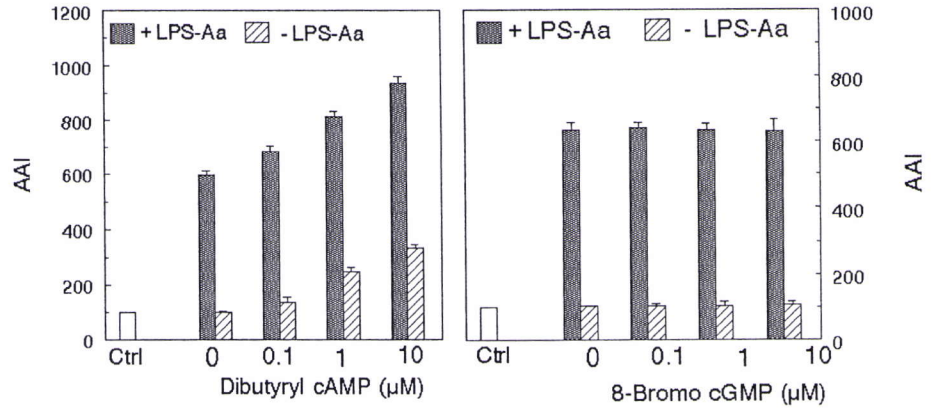
LPS (ng/ml)

Fig. 1


+LPS-Aa
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8-Bromo cGMP (μM)

Fig. 2