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Is There a Relation between Adenosine and Caffeines' Mechanisms of Action and Toll-Like Receptor-4 (TLR-4)?

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Abstract

Previous studies showed that endogenous adenosine, an anti-inflammatory agent, was released at sites of injury and inflammation thereby decreasing the excessive production of pro-inflammatory cytokines. Caffeine, a non-specific adenosine blocker, has been reported in several studies to have opposing immune-modulatory effects. In this study, the effects of caffeine and adenosine on TLR-4 in promoting or decreasing the production of TNF-*α* and IL-12 by LPS-stimulated monocytes were investigated. Monocytes were isolated using Pluribead® kit from pooled blood obtained from ten volunteers. The monocytes were then incubated for 24 hours with Lipopolysaccharide (pLPS) extracted from *Escherichia coli* (aTLR-4 ligand activator), adenosine, caffeine and LPS extracted from *Rhodobacter sphaeroides* (LPS-RS, a TLR-4 ligand blocker), each alone or in different combinations. Later, the levels of pro-inflammatory cytokines TNF*α* and IL-12 were assessed in supernatants using an Enzyme Linked Immuno Assay (ELISA). Caffeine and adenosine significantly reduced the amount of TNF*α* and IL-12 produced by LPS-stimulated monocytes. Regarding non-stimulated and LPS-RS blocked monocytes, the presence of adenosine and caffeine significantly decreased TNF*α* levels produced by these cells but had little or non-significant effect on the levels of IL-12. In conclusion, both caffeine and adenosine blocked the production of the pro-inflammatory cytokines by pLPS-stimulated-monocytes. TLR-4 did not appear to be involved in the signaling pathway of caffeine and adenosine since blocking of TLR-4 did not abolish the effects of adenosine and caffeine on production of cytokines, in particular TNF-*α*.

Keywords

TLR-4, Adenosine, Caffeine, LPS-RS, Cytokines

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1. Introduction

Pathogenic Lipopolysaccharide (pLPS) containing six fatty acyl groups is a constituent of the cell wall of pathogenic Gram-negative bacteria such as *Escherichia coli* [1] [2]. Binding of pLPS to Toll-Like Receptor-4 (TLR-4), results in the activation of two signaling pathways that lead to the production of pro-inflammatory cytokines, such as IL-12 and TNF-*α*. Cytokine release results in the initiation of an inflammatory cascade aimed at elimination of the invading pathogen [3]-[5]. On the other hand, LPS containing 4 or 5 fatty acyl groups such as the LPS of the photosynthetic bacterium, *Rhodobacter sphaeroides* (LPS-RS), which binds to TLR-4, does not activate the signaling pathways and block pLPS from doing so [6] [7].

Adenosine, an endogenous anti-inflammatory agent, is normally present at low concentrations in the body as it is produced by almost all cells as a by-product of metabolism [8]. However, during inflammation, stress, or tissue injury, adenosine is released in high amounts reaching 10 - 100 folds in an attempt to control the inflammatory response [8]. Referred to as "retaliatory metabolite", adenosine exerts its anti-inflammatory role by binding to its receptors (A1, A2A, A2B, and A3) present on different immune cells [9]. While the activation of different adenosine receptors results in opposing immune-modulatory effects, it is shown that during stress, adenosine binds and activates A2A receptors which are anti-inflammatory in nature [8]. Previous studies demonstrated that binding of adenosine to its A2A receptors specifically, decreased TNF-*α* and IL-12 production in pLPS-stimulated monocytes and neutrophils, and thus suppressed inflammation [10]-[12].

On the other hand, caffeine, the widely consumed psychostimulant, is a non-specific adenosine antagonist which blocks all adenosine receptors [13]. Despite being a weak phosphor-diesterase (PDE) blocker, it is believed that at normal physiological concentrations the major effects of caffeine are exerted via adenosine receptors inhibition and not PDE blockage [13]. Being a blocker of an anti-inflammatory molecule, the role of caffeine on immunity had been assessed before. However, previous studies concerning caffeine and immunity had been controversial; while most studies showed that caffeine decreased cytokines production during inflammation and tissue injury [14] [15], other studies showed that caffeine either worsens inflammation or plays no significant role at all [16]-[18]. One of the previous studies indicating a possible anti-inflammatory role of caffeine suggested that caffeine reduced cytokine levels by increasing endogenous cAMP levels [14] [15]. However, other studies argued that the elevation in cAMP was due to phosphor-diesterase (PDE) inhibition which was unlikely to occur at normal physiological concentrations of caffeine [13]. Another study by Verani *et al*. reported that caffeine upregulated the anti-inflammatory A2A receptors and thus decreased inflammation [19].

The aims of this study are to determine the effect of both caffeine and adenosine on pLPS-stimulated monocytes and to assess whether TLR-4, the LPS receptor, is implicated in the signaling of either adenosine or caffeine.

2. Materials and Methods

2.1. Reagents

LPS extracted from *Escherichia coli* 111:B4 strain (Invivogen 3950 sorrento Valley Blvd., San Diego CA-92121-USA), adenosine (Sigma, 3050 Spruce Street, Saint Louis, USA), caffeine (Sigma, 3050 Spruce Street, Saint Louis, USA), and LPS extracted from *Rhodobacter sphaeroides* (Invivogen, 3950 Sorrento Valley Blvd., San Diego CA-92121-USA).

2.2. Blood Specimens and Isolation of Monocytes

Human monocytes were isolated from blood withdrawn from 10 informed healthy volunteers (age > 18 years old) using vacutainers containing an anticoagulant. Blood specimens were pooled and monocytes were separated using the PluriBeads® M-kit (PluriSelect Life Science, Leipzig, Germany) (viability, >95%). Collected cells were then cultured in 0.5 ml RPMI medium containing 1% pen-strep, 1% L-glutamine, and 10% heat-inactivated FBS at a concentration of 2×10^5 cells/ml.

Recruitment of volunteers was approved by the Institutional Review Board of American University of Beirut (IRB-AUB). Volunteers refrained from caffeine consumption for at least 48 hours before blood withdrawal to insure that previous high plasma caffeine levels could not interfere with the purpose of the study.

2.3. Culture of Monocytes

Monocyte culture was performed in a 48 well plate. Cells were incubated with the different reagents at 37˚C and

5% CO_2 for 24 hours according to the protocol indicated in **Table 1**. Each well contained 2×10^5 monocytes/ml and were incubated with 2 µl of 0.1 µg/ml pLPS extracted from *Escherichia coli* 111:B4 strain, 381 µl of 100M adenosine, 121 µl of 100 M caffeine and 10 µl of 1 µg/ml LPS-RS extracted from *Rhodobacter sphaeroides*, each alone or in different combinations in 0.5 ml culture media (**Table 1**). LPS-RS (TLR-4 antagonist) and caffeine (adenosine antagonist) were added to the cells before the other reagents at time t = −2 hours and t = −1 hour respectively. Following incubation for 24 hours, cytokine levels in supernatants were determined.

2.4. TNF-*α* and IL-12 Levels

TNF-*α* and IL-12, in supernatants were determined using ELISA (Abcam TNF alpha Human ELISA kit (Ab46087) and Abcam IL-12 human ELISA kit; (Abcam company, Moscow, Russia) according to the manufacturer's instructions.

2.5. Statistical Analysis

The unpaired student T-test was implemented to assess the sample variations between groups using the Graphpad online software. Results were considered to be statistically significant when p value was <0.05.

3. Results

3.1. Effect of Adenosine on pLPS-Stimulated Monocytes

The addition of adenosine to pLPS-stimulated monocytes resulted in a significant six fold decrease in the level of TNF-*α* (p = 0.0024) and one-and-a-half fold decrease in IL-12 (p = 0.023) levels as compared to LPS-stimulated monocytes alone (**Figure 1** and **Figure 2**).

3.2. Effect of Adenosine on TLR-4

To assess the effect of adenosine on TLR-4, adenosine was added to non-stimulated monocytes and to LPS-RS blocked monocytes. This resulted in a significant decrease in TNF-*α* levels by four folds (p = 0.0003) and two-and-half fold (0.0011) as compared to non-stimulated monocytes and LPS-RS blocked monocytes respectively. However, no significant reduction in IL-12 levels was observed (p > 0.05) (**Figure 1** and **Figure 2**).

Table 1. Culture and treatment of monocytes.

Wells	Monocytes 2×10^5 cells/ml in 0.5 ml culture media			
	pLPS (0.01 µg/ml*) T = 0 hr	LPS-RS (0.1 µg/ml) T = −2 hr	Adenosine (100 M) T = 0 hr	Caffeine (100 M) T = −1 hr
Well 1	--	--	--	--
Well 2	+	--	--	--
Well 3	--	+	--	--
Well 4	+	+	--	--
Well 5	--	--	+	--
Well 6	--	--	+	--
Well 7	--	--	+	+
Well 8	+	--	--	+
Well 9	+	--	+	--
Well 10	+	--	+	+
Well 11	--	+	--	+
Well 12	--	+	+	--
Well 13	--	+	+	+

*Concentrations used were: 2 µl of 0.1 µg/ml pLPS extracted from *Escherichia coli* 111:B4 strain, 381 µl of 100 M adenosine, 121 µl of 100 M caffeine and 10 µl of 1 µg/ml LPS-RS extracted from *Rhodobacter sphaeroides*.

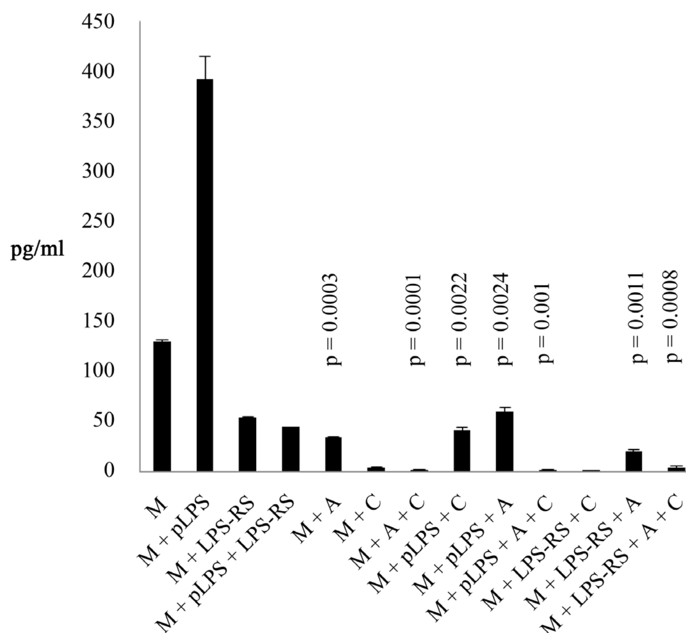

Figure 1. TNF-*α* levels in supernatants determined by ELISA. Results after 24 hrs incubation of monocytes with the presence or absence of 2 µl of 0.1 µg/ml pLPS extracted from *Escherichia coli* 111:B4 strain, 381 µl of 100 M adenosine, 121 µl of 100 M caffeine and 10 µl of 1 µg/ml LPS-RS extracted from *Rhodobacter sphaeroides*. After incubation, supernatant was used to assess the TNF-*α* level in each well. M = monocytes, pLPS = *E. coli* Lipopolysaccharide, LPS-RS = *Rh. sphaeroides* Lipopolysaccharide, A = adenosine and C = caffeine. Only significant p values (<0.5) are displayed on figure.

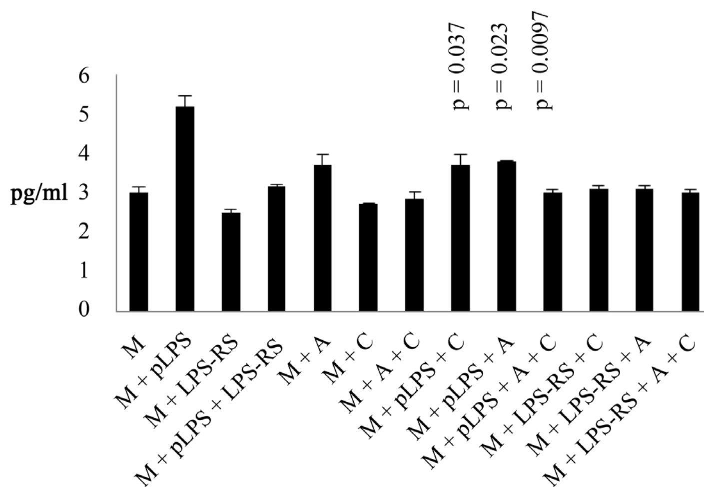

Figure 2. IL-12 levels in supernatants determined by ELISA. Results after 24 hrs incubation of Monocytes with the presence or absence of 2 µl of 0.1 µg/ml pLPS extracted from *Escherichia coli* 111:B4 strain, 381 µl of 100 M adenosine, 121 µl of 100 M caffeine and 10 µl of 1 µg/ ml LPS-RS extracted from *Rhodobacter sphaeroides*. After incubation, supernatant was used to assess the TNF-*α* level in each well. M = monocytes, pLPS = *E. coli* Lipopolysaccharide, LPS-RS = *Rh. sphaeroides* Lipopolysaccharide, A = adenosine and C = caffeine. Only significant p values (<0.5) are displayed on figure.

3.3. Effect of Caffeine on pLPS Stimulated Monocytes

This resulted in a significant nine fold decrease in the level of TNF-*α* (p = 0.0022) and a significant one-and-a-half fold decrease in the level of IL-12 (p = 0.037) as compared to LPS-stimulated monocytes alone.

3.4. Effect of Caffeine on TLR-4

When caffeine was added to non-stimulated monocytes or LPS-RS blocked monocytes, it resulted in a significant decrease in the levels of TNF-*α* by twenty nine fold and fifty fold respectively (p = 0.0001, and p = 0.0001 respectively) but IL-12 levels were not affected significantly (p > 0.05). Moreover, it is worth noting that in all scenarios caffeine was decreasing TNF-*α* levels more than adenosine.

3.5. Effect of the Combination of Adenosine and Caffeine; (Figure 1 and Figure 2)

- On pLPS-stimulated monocytes; significantly decreased TNF-*α* levels by three hundred fold (p = 0.001) and IL-12 levels by about 2 fold (p = 0.0097).
- On non-stimulated monocytes; significantly decreased TNF-*α* by fifty folds (p = 0.0001). Had no significant effect on IL-12 levels (p > 0.05).
- On LPS-RS blocked monocytes; significantly decreased TNF-*α* by thirteen fold (p = 0.0008). Had no significant effect on IL-12 levels (p > 0.05).
- It is worth noting that the combination of adenosine and caffeine decreased TNF-*α* levels more than when each reagent was used alone.

4. Discussion

In the presence of Gram negative bacteria, TLR-4 recognizes pLPS and initiates an inflammatory response in an attempt to eliminate these bacteria. While inflammation is usually beneficial, excessive inflammation can result in tissue injury or sepsis. To control the inflammatory response, adenosine is released at sites of tissue injury and inflammation where it acts as an endogenous anti-inflammatory agent [8]. It has been reported that adenosine decreases TNF-*α* and IL-12 production in LPS-stimulated monocytes and thus suppresses inflammation [10]-[12].

Our results indicated that when adenosine was added to pLPS-stimulated monocytes, a significant decrease in both cytokines, IL-12 and TNF*α*, was observed. These results are consistent with previous studies demonstrating that adenosine decreases the production of pro-inflammatory cytokines in pLPS-stimulated monocytes [10] [11]. On the other hand, the addition of adenosine to LPS-RS blocked monocytes or to non-stimulated monocytes significantly decreased TNF*α* levels but had little effect on the levels of IL-12 released by these cells. Such results suggest that TLR-4 is probably not implicated in the signaling of adenosine since the blockage of TLR-4 by LPS-RS or the lack of its stimulation in non-stimulated monocytes did not prevent adenosine from exerting its anti-inflammatory role and decreasing TNF*α* levels mainly.

Caffeine, a popular psychostimulant, belongs to the methyl xanthine family of drugs [13]. It is a non-specific adenosine blocker that antagonizes all adenosine receptors. Previous studies investigating the role of caffeine in immunity were controversial. While most studies agreed that caffeine decreases inflammation [14]-[16], other studies presented evidence on caffeine increasing tissue injury, inflammation, or even playing no significant role in immunity [14] [18]. Thus, the aim was to assess the role of caffeine in promoting or decreasing inflammation in pLPS stimulated monocytes. Moreover, knowing that caffeine has no identified receptors yet, we investigated the role of TLR-4 in caffeine signaling. The results indicated that caffeine significantly decreased the levels of both TNF*α* and IL-12 produced by pLPS stimulated monocytes. These findings are in agreement with previous studies suggesting that caffeine decreases cytokines production in pLPS-stimulated monocytes and might play an anti-inflammatory role [14] [15]. The addition of caffeine to LPS-RS blocked monocytes and to non-stimulated monocytes resulted in a significant decrease in TNF*α* levels but had a little effect on the levels of IL-12 released by these cells. These results again indicate that TLR-4 might not be involved in the signaling of caffeine since a significant decrease in cytokines, in particular TNF*α*, was still observed when TLR-4 receptor was not stimulated or blocked. It is worth noting that in all cases the decreased TNF*α* level caused by caffeine was greater than that caused by adenosine. This reinforces the possibility that caffeine is an immune-modulatory molecule.

This result concurs with the report of Verani *et al*. who showed that caffeine had an anti-inflammatory effect [19]. They suggested that caffeine upregulated the adenosine anti-inflammatory A_{2a} receptors. Thus, despite previous data stating that the major effects of caffeine are due to adenosine antagonism, caffeine does not seem to block the anti-inflammatory adenosine receptors present on monocytes.

It had been noted that in LPS-RS blocked monocytes and non-stimulated monocytes, the addition of adenosine, caffeine, or both reagents decreased TNF*α* levels significantly but did not result in significant changes in IL-12 levels. This different effect can be due to the fact that TNF-*α* is usually produced in higher levels than IL-12 and thus we expect that the decrease in TNF-*α* levels, upon blockage of TLR-4 in LPS-RS blocked monocytes or the lack of its stimulation in monocytes alone, will be more pronounced than that of IL-12. Another possible explanation is that the production of IL-12 is regulated by different mechanism than that of TNF-*α* [20].

5. Conclusion

In conclusion, both caffeine and adenosine blocked the production of the pro-inflammatory cytokines by pLPS-stimulated monocytes. TLR-4 did not appear to be involved in the signaling pathway of caffeine and adenosine since blocking of TLR-4 did not abolish the effects of adenosine and caffeine on production of cytokines, in particular TNF-*α*.

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