Mechanisms of modulation of cytokine release by human cord blood monocytes exposed to high concentrations of caffeine

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BACKGROUND: Serum caffeine concentrations >20 µg/ml (100 µmol/l) in infants treated for apnea of prematurity increases TNF-α and decreases IL-10, changes that perhaps are linked to comorbidities. We hypothesize that this proinflammatory cytokine profile may be linked to differential binding of caffeine to adenosine receptor subtypes (AR), inhibition of phosphodiesterases (PDEs), and modulation of toll-like receptors (TLR).

METHODS: Lipopolysaccharide-activated cord blood monocytes (CBM) from 19 infants were exposed to caffeine (0–200 µmol/l) with or without previous exposure to A_1R, A_3R, or PDE IV antagonists to determine changes in dose–response curves. Cytokines levels (enzyme-linked immunosorbent assay (ELISA)), intracellular cyclic adenosine monophosphate (cAMP) accumulation (enzyme immunoassay (EIA)), and TLR gene expression (real time qRT PCR) were measured.

RESULTS: Caffeine at ≤100 µmol/l decreased TNF-α levels (~25%, $P = 0.01$) and cAMP. All caffeine concentrations decreased IL-10 levels (17–35%, $P < 0.01$). A_1R, A_3R, and PDE blockades decreased TNF-α (31, 21, and 88%, $P \leq 0.01$), but not IL-10. Caffeine further decreased TNF-α following A_3R and PDE blockades. Caffeine concentrations directly correlated to TLR4 gene expression ($r = 0.84$; $P < 0.001$).

CONCLUSION: Neither A_3R, nor PDE blockades are involved in caffeine's modulation of cytokine release by CBM at any concentration. Besides A_1R blockade, caffeine's upregulation of TLR4 may promote inflammation at high concentrations.

Adenosine binding to any of the four 7-transmembrane spanning G-protein-coupled receptors, A_1R, $A_{2a}R$, $A_{2b}R$, and A_3R, modulates inflammation (1,2). Caffeine, a non-specific adenosine receptor (AR) antagonist, is used to treat apnea in premature infants and at concentration of 50 µmol/l in culture (equivalent to 10 µg/ ml in serum) increases intracellular cyclic adenosine monophosphate (cAMP) accumulation and attenuates TNF-α secretion by blocking A_1R on Lipopolysaccharide (LPS)-activated human cord blood monocytes (CBM) (3). Although this mechanism may be operative in the decreased incidence of bronchopulmonary dysplasia (BPD) and neurodevelopmental disabilities observed in infants treated with caffeine citrate (4,5) and in animal models (6), decrease in the anti-inflammatory cytokine, IL-10, along with increase in TNF-α in tracheal aspirates and peripheral blood in preterm infants who have serum caffeine levels ≥ 20 µg/ml (equivalent to 100 µmol/l in culture) raise concerns (7). The mechanisms explaining caffeine's polar opposite effects in the inflammatory cascade are still unclear, but highly relevant in the design of new strategies to prevent morbidities related to chronic inflammation, like BPD, in premature infants (8).

In general terms, ARs are either negatively (A_1R and likely A_3R) or positively (A_2Rs) coupled to adenylyl cyclase, decreasing or increasing intracellular cAMP levels, respectively (1,9). Changes in cAMP inversely modulate the expression of transcription factors and their final products, cytokines, and chemokines, via protein kinase A (PKA)-mediated pathways (10–13). Caffeine demonstrates the highest affinity for A_1R and the lowest affinity for $A_{2b}R$, which increases or decreases cAMP levels, respectively. At concentrations at least 40 times higher than those needed to antagonize A_1Rs, caffeine also inhibits phosphodiesterase (PDE) activity (14), which further increases cAMP accumulation. Additionally, activation of ARs antagonize inflammatory cascades activated by toll-like receptors (TLRs) on mononuclear cells (15,16) as shown by the failure of TLR4 agonists to induce TNF-α release following pretreatment with $A_{2a}R$ agonists (17). Although caffeine may inhibit TLR-mediated inflammatory cascades in macrophages by suppressing calcium mobilization (18), it may also trigger inflammation by preventing the AR-mediated antagonism of TLRs and perhaps by changing their expression (19). Hence, we hypothesize that the proinflammatory cytokine profile observed with high serum caffeine levels (>20 µg/ml, equivalent to > 100 µmol/l in culture) in premature infant at risk for BPD (7) may be linked to differential binding of caffeine to AR subtypes, inhibition of PDEs, and modulation of other components of the inflammatory cascade, such as TLRs.

RESULTS

Cord blood from 19 neonates (mean gestational age ± SD = 39.6 ± 1.2 wk; birth weight = 3,286 ± 505 g; **Table 1**) was used

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Table 1. Demographics and serum caffeine levels

Number of subjects	19
Gestational age (mean ± SD)	39 4/7 ± 1.2 wk
Birth weight (mean ± SD)	3,286 ± 505 g
Gender	73% male
Race	78% AA; 11% C
Apgar 1 min (median, range)	8 (6–9)
Apgar 5 min (median, range)	9 (8, 9)
Delivery mode	21% C/S
Caffeine level (mean ± SD)	0.67 ± 1.12 μg/ml

AA, African American; Apgar, Apgar score; C, Caucasian; C/S, cesarean section.

for experiments in culture. Cord blood serum caffeine levels (mean ± SD = 0.67 ± 1.12 μg/ml, Table 1) were below the therapeutic range.

Baseline Changes in cAMP and Cytokine mRNA and Protein Levels

Neither caffeine alone nor LPS alone increased intracellular cAMP accumulation in CBM by 24 h. In contrast, caffeine treatment followed by LPS-activation increase cAMP accumulation by four- to sixfold ($P < 0.01$ vs. LPS-exposed, Figure 1a). Consistent with these findings, TNF-α gene expression decreased by 60% following caffeine exposure at concentrations of 50 or 100 μmol/l, but at higher concentrations (200 μmol/l) did not decrease TNF-α transcription any longer. Conversely, all tested caffeine concentrations decreased IL-10 gene expression by 25 to 60% ($P < 0.05$ in all cases, Figure 1b,c). LPS exposure increased TNF-α and IL-10 release by CBM ($P < 0.001$ vs. no LPS) and caffeine modulated those responses ($P = 0.01$ and $P < 0.001$, respectively). Caffeine at 50 and 100 μmol/l decreased TNF-α levels by 25% (IQR −67 to −5%, $P = 0.01$) and 24% (IQR −30 to −2%, $P = 0.02$); respectively, while at 200 μmol/l did not produce changes (Figure 1d). Conversely, caffeine at 50, 100, and 200 μmol/l decreased IL-10 by 17% (IQR 34 to 8%, $P = 0.006$), 27% (IQR 34 to 21%, $P = 0.003$), and 35% (IQR 46 to 25%, $P = 0.003$), respectively (Figure 1e).

The Differential Role of A1R and A3R in the Modulation of Cytokines by Caffeine

DPCPX, a specific A_1R antagonist (10 nmol/l), decreased TNF-α secretion by 31% from baseline (IQR −60 to −6%, $P = 0.01$). The addition of caffeine at 50 and 100 μmol/l did not induce further TNF-α decrease. However, caffeine at 200 μmol/l still significantly decreased TNF-α in CBM when pretreated with DPCPX, unlike in those not pretreated ($P = 0.001$, discontinuous line) (Figure 1d). A_1R blockade did not modify the effect of caffeine on IL-10 at any concentration (Figure 1e).

In LPS-activated CBM, exposure to caffeine alone (Figure 1a) or combined with MRS1220 (A_3R antagonist, 10 nmol/l) significantly increased intracellular cAMP levels (Figure 1f). MRS1220 alone decreased TNF-α secretion by 21% (−45 to −13%; $P = 0.008$), while the addition of caffeine at 50 and 100 μmol/l produced an additional decrease of 40% (IQR −60 to −16) and 39% (IQR −62 to −15), respectively ($P = 0.001$ in all cases vs. MRS1220 alone) with no additional changes induced by caffeine at 200 μmol/l (Figure 1g). In contrast, IL-10 was not significantly modulated by A_3R blockade (Figure 1h). While MRS1220 inhibited TNF-α gene expression by 65% (IQR −72 to −10; $P = 0.02$), it did not change IL-10 gene expression. In contrast, caffeine at 50 μmol/l added to MRS1220 inhibited gene expression of both, TNF-α and IL-10 (Figure 1i).

The Effect of Caffeine in Cytokines Is Independent of PDE IV Blockade

RO-201724 (PDE IV inhibitor, 10 μmol/l) increased intracellular cAMP levels by 134% (20 to 476; $P = 0.02$), while the combined treatment with caffeine (50 μmol/l) increased cAMP levels by 283% (167 to 535; $P = 0.02$; Figure 2a). In CBM, pretreatment with RO-201724 produced an 88% (IQR −90 to −84) decrease in the release of TNF-α ($P < 0.001$ vs. no pretreatment). In the presence of RO-201724, the addition of caffeine at 50, 100, and 200 μmol/l further decreased TNF-α release by CBM by 24% (IQR −49 to −8); 19% (IQR −39 to −5) and 21% (IQR −44 to −20) ($P < 0.001$, Figure 2b). On the other hand, PDE IV inhibition alone did not modify IL-10 release by CBM; however, in the presence of caffeine at 100 or 200 μmol/l, IL-10 levels were 39 and 46% lower than with caffeine exposure alone ($P < 0.01$). Caffeine, at all concentrations, decreased IL-10 secretion regardless of PDE inhibition ($P < 0.01$, Figure 2c).

Unlike IL-10, TNF-α Modulation by Caffeine Depends on PKA Activity

To determine the role of cAMP-dependent PKA in the pathway inhibiting the transcription of TNF-α and IL-10 in response to caffeine, CBM were pretreated with RP-cAMPs (50 μmol/l), a PKA inhibitor, prior to caffeine and LPS exposure. Following pretreatment with RP-cAMPs, intracellular cAMP accumulation increased by 200-fold (Figure 2d) in CBM. Despite inducing high intracellular cAMP levels, RP-cAMP blocked the decrease in TNF-α induced by caffeine (Figure 2e). In contrast, RP-cAMPs did not modify the effect produced by caffeine on IL-10 secretion (Figure 2f).

Caffeine Alters TLR Transcription

While TLR3 mRNA was not detected in CBM by real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR), TLR1, TLR2, and TLR4 gene expression was detected and modified by caffeine in a concentration-dependent manner. Caffeine at 50 and 100 μmol/l downregulated the expression of TLR1 by 65% (83 to 37%; $P = 0.02$) and 55% (75 to 23%; $P = 0.04$), and TLR2 by 75% (90 to 57%; $P = 0.02$) and 74% (83 to 33%; $P = 0.04$); respectively (Figure 3a,b). Caffeine at 200 μmol/l did not alter TLR1 or TLR2 mRNA levels. TLR4 gene expression was directly correlated with the concentration of caffeine ($r = 0.84$; $P < 0.001$; Spearman's correlation). While TLR4 gene expression was unchanged in CBM exposed to 50 μmol/l of caffeine, it was upregulated by 2.6-fold (2.2 to 4.2; $P = 0.02$) when exposed to 200 μmol/l (Figure 3c).

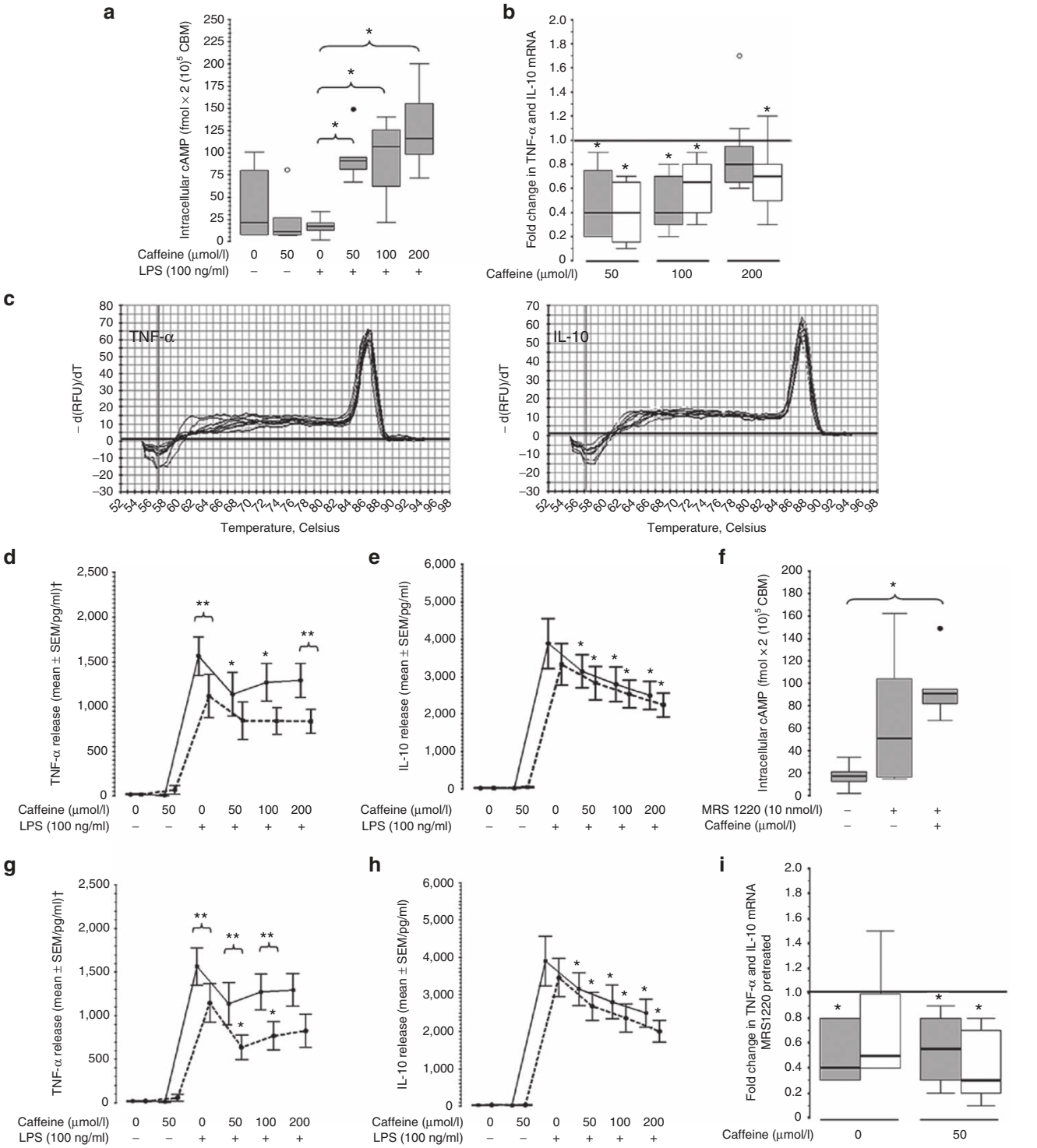

Figure 1. The differential role of A1R and A3R in the modulation of cytokines by caffeine. Effect of caffeine at 50, 100, and 200 μmol/l in (**a**) intracellular cAMP accumulation (fmol/2 $(10)^5$ cord blood monocytes (CBM); $n = 7$). Box and whiskers plot representing median and IQR. *$P < 0.05$ (Wilcoxon rank test); ○, outliers; ●, extremes. (**b**) Fold-change in TNF-α (gray) and IL-10 (white) gene expression from reference line (at 1, caffeine at 0 μmol/l; $n = 9$). Box and whiskers plot representing median and IQR. *$P < 0.05$ (Wilcoxon rank test); ○, outliers; ●, extremes. (**c**) Melting curves for TNF-α and IL-10 PCR products. (**d**) TNF-α and (**e**) IL-10 release from CBM following caffeine exposure (0, 50, 100, 200 μmol/l) alone (continuous line) or combined (dashed line) with DPCPX (A_1R antagonist) pretreatment. Mean ± SEM ($n = 11$). *$P < 0.01$ vs. LPS-activated CBM exposed to 0 μmol/l of caffeine (Bonferroni). **$P < 0.01$ vs. pretreatment counterpart. †overall $P = 0.02$ (two-way repeat measures ANOVA). (**f**) Intracellular cAMP concentration (fmol/2 $(10)^5$ CBM; $n = 6$) following caffeine (50 μmol/l) and MRS1220 alone and combined. Box and whiskers plot representing median and IQR. *$P < 0.05$ (Wilcoxon rank test); ○, outliers; ●, extremes. (**g**) TNF-α and (**h**) IL-10 release from CBM curve in response to caffeine (0, 50, 100, 200 μmol/l) alone (continuous) or combined (dashed) with MRS1220 (A_3R antagonist) pretreatment. Mean ± SEM ($n = 11$). *$P < 0.01$ vs. LPS-activated CBM exposed to 0 μmol/l of caffeine (Bonferroni). **$P < 0.01$ vs. pretreatment counterpart. †Overall $P = 0.002$ (two-way repeat measures ANOVA). (**i**) Fold change of TNF-α (gray) and IL-10 (white) gene expression after MRS1220 pretreatment with and without caffeine (50 μmol/l; $n = 6$). *$P < 0.05$ (Wilcoxon rank test) vs. reference line (at 1, no pretreatment).

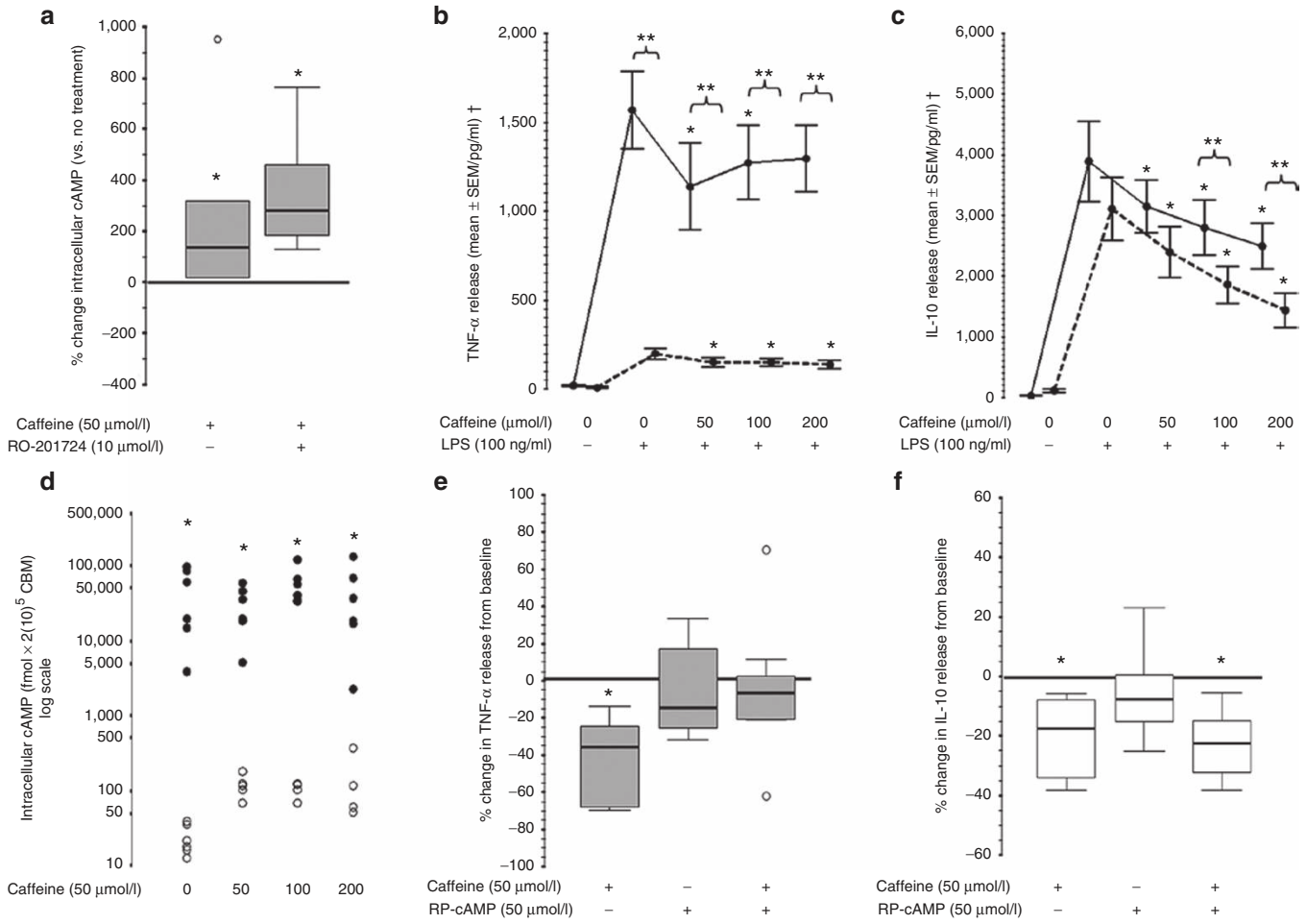

Figure 2. The role of PDE IV blockade and PKA mediation in the effect of caffeine in TNF-α and IL-10. (**a**) Intracellular cAMP accumulation (fmol/2 (10)^5 cord blood monocytes (CBM); $n = 6$) following RO-201724 (PDE IV inhibitor, 10 µmol/l) alone and combined with caffeine (50 µmol/l). Box and whiskers plot representing median and IQR. *$P < 0.05$ (Wilcoxon rank test). ○, outliers. (**b**) TNF-α and (**c**) IL-10 release from CBM curve in response to caffeine (0, 50, 100, 200 µmol/l) alone (continuous line) or combined (dashed line) with RO-201724 pretreatment. Mean ± SEM ($n = 11$). *$P < 0.01$ vs. LPS-activated CBM exposed to 0 µmol/l of caffeine (Bonferroni). **$P < 0.05$ vs. pretreatment counterpart. †Overall $P < 0.05$ (two-way repeat measures ANOVA). (**d**) Intracellular cAMP accumulation (fmol/2 (10)^5 CBM; log scale; $n = 6$) following caffeine exposure (0, 50, 100, 200 µmol/l) alone (○) and combined (●) with RP-cAMPs (PKA inhibitor; 50 µmol/l) pretreatment. *$P < 0.05$ (Wilcoxon rank test). (**e**) TNF-α (gray) and (**f**) IL-10 (white) release from CBM curve in response to RP-cAMP and/or caffeine ($n = 7$). *$P < 0.05$ (Wilcoxon rank test) vs. no treatments. ○, outliers.

DISCUSSION

Caffeine at 50 µmol/l (equivalent to 10 µg/ml in serum) decreases TNF-α secretion by CBM via A_1R blockade (3), but *in-vivo* data suggest that higher serum caffeine concentrations are associated higher TNF-α, IL-8, IL-6 and lower IL-10, suggesting a proinflammatory profile potentially linked to BPD and other comorbidities (7). The mechanisms may involve not only nonspecific ARs blockade, but also inhibition of PDE IV and alteration in TLR expression. Using a culture treatment paradigm modeled to replicate the impaired hepatic metabolism of caffeine in neonates and the relevant time and concentrations of caffeine in infants receiving daily doses to achieve serum concentrations between 10 to 20 µg/ml or higher, we show that caffeine at concentrations of 50 and perhaps 100 µmol/l (equivalent to 10 and 20 µg/ml, respectively) decrease TNF-α and IL-10 secretions by CBM, while at concentrations greater than 100 µmol/l only decrease IL-10 secretion and gene expression. The blockade of A_1R, but not A_3R, is operative in the effect of caffeine on TNF-α secretion via a PKA-mediated mechanism, while neither of these receptors is involved in the effects on IL-10. PDE IV inhibition does not play a significant role in caffeine's immunomodulatory effects. Additionally, caffeine may promote the activation of TLR-mediated inflammatory pathways relevant to sick neonates, by upregulating TLR 4 in a concentration-dependent manner.

A_1R expression is upregulated on CBM following LPS exposure (a TLR4 agonist), suggesting the important role of this receptor in the control of TLR-mediated inflammation in neonates (3). A_1R blockade or $A_{2a}R$ activation produces an equally significant increase in cAMP accumulation and decrease in TNF-α production by adult monocytes (20). In accordance with these findings, our results using CBM suggest that low caffeine concentrations (50 µmol/l—equivalent to 10 µg/ml in serum) preferentially block A_1R, probably allowing adenosine to bind to $A_{2a}R$ (Gαs-coupled), thereby increasing cAMP production (1). At increasing

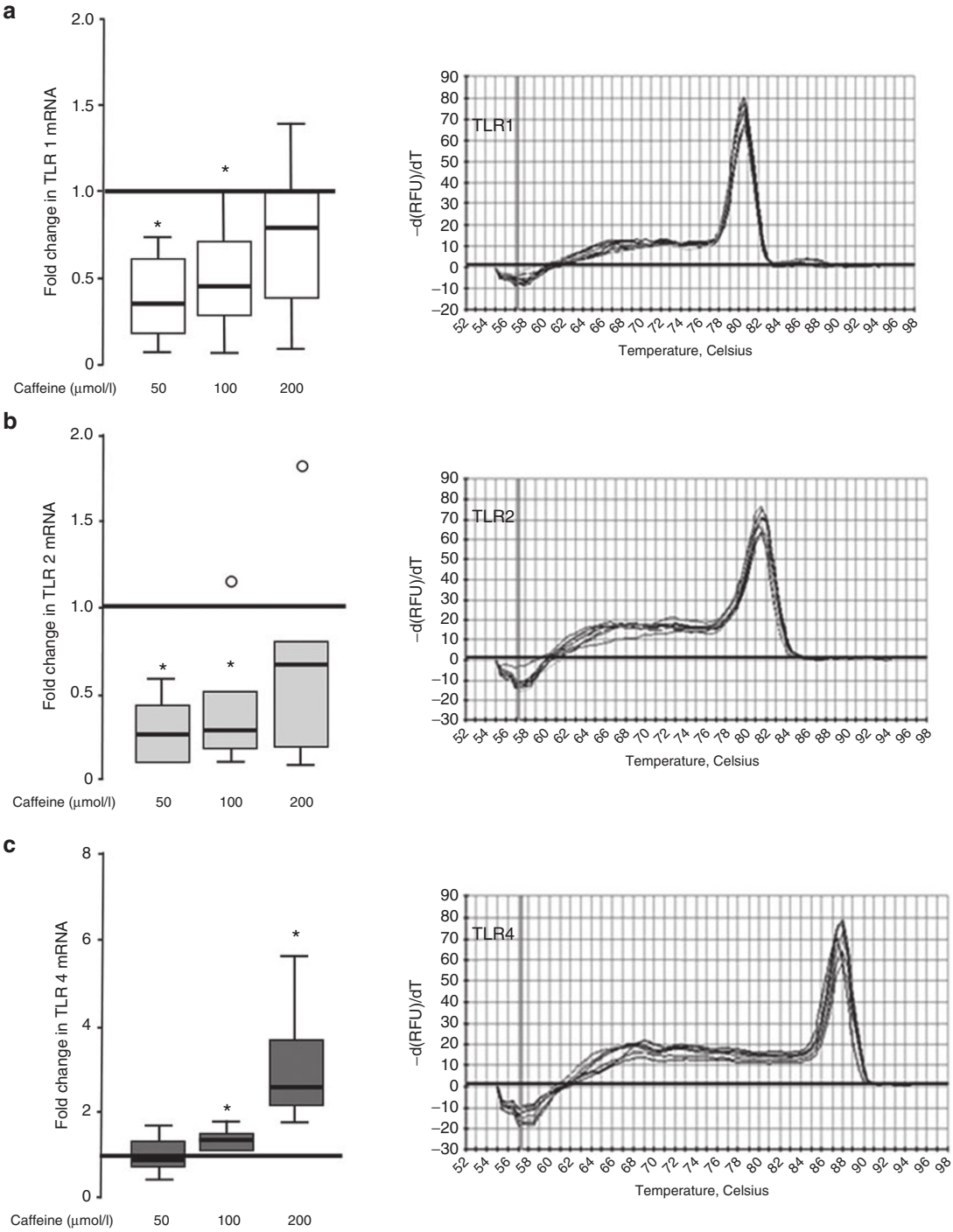

Figure 3. Caffeine alters TLR transcription. Fold change in (**a**) TLR1; (**b**) TLR2, and (**c**) TLR4 gene expression induced by caffeine at 50, 100, and 200 μmol/l ($n = 7$). *$P < 0.05$ (Wilcoxon rank test) vs. reference line (at 1, caffeine at 0 μmol/l). ○, outliers. Melting curves for polymerase chain reaction products are shown for each of the TLR subtypes.

concentrations, this preferential A_1R blockade by caffeine may be lost.

A_3R is highly expressed in inflammatory tissues and mononuclear cells (3,21), but the interaction with adenylyl cyclase (protein G_i or G_q) varies by cellular type (1,20). In either case, the net effect of A_3R blockade is cAMP accumulation and decrease cytokine expression. Similar to the effect described in adult peripheral blood monocytes (20), our results show that A_3R blockade (MRS1220) increases cAMP accumulation and decreases TNF-α production. Caffeine significantly intensifies the effect produced by MRS1220 alone, suggesting caffeine effects are independent of A_3R blockade in CBM.

Xanthines also decrease TNF-α and IL-10 release from mononuclear cells via PDE inhibition and cAMP accumulation (14,22–24). However, because caffeine has a considerably higher IC50 for PDE inhibition than for cytokine modulation

Table 2. List of antagonists and inhibitors

Antagonist	Synonym	Dose (s)	Target
Caffeine	1,3,7-Trimethylxanthine	50, 100, 200 μmol/l	$A_1 > A_2 > A_3$
DPCPX	1,3-Dipropyl-8cyclopentylxanthine	10 nmol/l	A_1
MRS1220	9-Chloro-2-(2-furanyl)-5-(phenylacetyl amino)- [1,2,4]triazolo[1,5-c] quinazoline	10 nmol/l	A_3
RO-201724	4- (3- Butoxy- 4- methoxybenzyl)- 2- imidazolidinone	10 μmol/l	PDE IV
RP-cAMPs	Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate	50 μmol/l	cAMP-PKA

A_1, A_{2a}, A_{2b}, A_3, adenosine receptor subtypes; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase; PKA, protein kinase A.

(23), serum concentrations 25 times higher than those achieved during standard treatment for apnea of prematurity would be required to elicit PDE inhibition *in vivo* (4,14). Here, we show that although RO-201724 decreases TNF-α secretion by CBM, caffeine further decreases this cytokine suggesting that PDE inhibition does not explain the effect of caffeine on TNF-α. In contrast, the inhibition of PDE IV and the subsequent increase in cAMP accumulation does not significantly modify IL-10 levels, suggesting that the pathway mediating the effect of caffeine in IL-10 is not primarily cAMP-dependent.

Adult monocytes express TLR1, TLR2, and TLR4, among many others; however, the expression profile on CBM has not been well defined. Contrary to TLR2 expression on CBM, TLR4 directly correlates with gestational age (25,26), and further expression is induced by LPS (27). Because LPS is a TLR4 agonist and caffeine mediates the inhibition of LPS-induced TNF-α production by CBM, we hypothesized that changes in TLR expression may account for some of caffeine immunomodulatory effects. Here, we report the direct correlation between caffeine concentrations and TLR4 expression, as well as the inhibitory effect of caffeine at 50 and 100 μmol/l on TLR1 and TLR2 expression. In LPS-activated CBM, the increase TLR4 expression induced by caffeine at 200 μmol/l, may upregulate TNF-α expression via MyD88-NFkB pathway (10,13). The implications of TLR1 and TLR2 downregulation induced by caffeine remain unexplored but this novel mechanism may explain some of the anti-inflammatory properties derived from clinical studies demonstrating decrease incidence of BPD (4).

The pathways mediated by TLR-NFκB (21,28) and cAMP-PKA (10,20) are the main biochemical cascades regulating TNF-α production by adult monocytes. NFκB, a transcription factor for TNF-α, also upregulates A_2R expression following LPS exposure as a delayed feedback attenuating persistent inflammation (29). The decrease in TNF-α production following AR activation can be abolished by the blockade of PKA (20). Similarly, we show that caffeine inhibitory effect on TNF-α transcription in CBM is suppressed by RP-cAMPs, suggesting that this process is PKA mediated.

Adenosine decreases TNF-α and increases IL-10 production by monocytes, although the specific signaling cascade remains unknown (30,31). AR activation induces accumulation of the IL-10 transcription factor C/EBPβ (enhancing-binding protein) (32). Transient MAPK-mediated phosphorylation of histone H3 in the IL-10 promoter may facilitate C/EBPβ binding via a cAMP-PKA independent pathway (33,34). Here, we show that caffeine produces a transcriptional downregulation of IL-10, which is not mediated by A_1R or A_3R blockade and unlike TNF-α is PKA independent. We speculate that the modulation of A_2R expression or function is involved in the effect of caffeine on IL-10 and the mechanisms may include: (i) the prevention of LPS-induced upregulation of A_2R expression by caffeine (3), which by decreasing A_2R availability, decreases transcription factors (C/EBPβ) and IL-10 production; and (ii) the nonspecific blockade of $A_{2a}R$, as well as A_1R by caffeine, which at increasing concentrations further decrease IL-10 and increase TNF-α production, as observed in our experiments. The effects of caffeine in other steps of the IL-10 signaling pathway are still unexplored.

In conclusion, caffeine decreases the release of TNF-α and IL-10 via distinct mechanisms. A_1R, but not A_3R, is operative in the effect of caffeine on TNF-α, while neither of these receptors modulates IL-10 production by CBM. Similarly, caffeine's immunomodulatory effects are not primarily produced by PDE inhibition. The mechanism responsible for the decrease of IL-10 produced by caffeine is still unclear, but is potentially related to the modulation of A_2R expression and/or function. Here, we also describe for the first time the inhibition of TLR1 and TLR2, and the induction of TLR4 expression on LPS-activated CBM exposed to caffeine, which extends our understanding about the immunomodulatory effects of this drug in the newborn population. The understanding of the distinct effects of caffeine on cytokines is relevant in the context of the potential side effects of its use in the clinical setting in a population at high risk for BPD and other morbidities linked to inflammation.

METHODS

Subjects

This study complied with the Guidelines for Human Experimentation from the US Department of Health and Human Services and received approval from the Johns Hopkins Medicine Institutional Review Board (NA_00002034). The work has been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki)* (35). Only cord blood of subjects whose parents provided informed consent for the study was considered for experiments. CBM were isolated from full-term infants (≥ 37 wk gestation). Cord blood was collected after repeat cesarean section without labor or vaginal delivery without evidence of chorioamnionitis. We excluded births with known genetic disorder, intrauterine growth restriction or small for gestational age (birth weight ≤ 10th percentile for gestational age), and suspected viral infection (based on serological or clinical findings). Infants who subsequently received antibiotics or had illness

Table 3. Primers for real-time qRT-PCR

Gene	Direction	Sequence (5′-3′)	Product	UniSTS
TNF-α	S	CACTAAGAATTCAAACTGGGGC	166 bp	28,864
	AS	GAGGAAGGCCTAAGGTCCAC		
IL-10	S	ACCTGGGTTGCCAAGCCTTGTC	158 bp	Ref. (39)[a]
	AS	AAATCGATGACAGCGCCGTAGC		
TLR1	S	CACATCAAGTGAAAAATATTCCTCC	151 bp	24,274
	AS	TAAATGGTGAACTGCGACCC		
TLR2	S	CTACTGGGTGGAGAACCTTATGGT	76 bp	1,092
	AS	CCGCTTATGAAGACACAACTTGA		
TLR3	S	TCCTAGAAGAGATGTAATTG	169 bp	66,831
	AS	CCCAAAAACTCTGTACATTA		
TLR4	S	GTTTCTGAGCAGTCGTGCAG	172 bp	480,158
	AS	CAGGGCTTTTCTGAGTCGTC		
GAPDH	S	AACAGCGACACCCACTCCTC	258 bp	270,428
	AS	GGAGGGGAGATTCAGTGTGGT		

[a]Not listed at UniSTS.
AS, antisense; bp, base pair; GADPH, glyceraldehyde phosphate dehydrogenase; IL-10, interleukin-10; qRT-PCR, quantitative reverse-transcription polymerase-chain reaction; S, sense; TLR, Toll-like receptor; TNF, tumor necrosis factor.

sufficient to be admitted to the neonatal intensive care unit were removed from final experiments. Serum caffeine levels were obtained on all blood samples and those with levels > 4 μg/ml were removed from final analysis.

CBM Isolation

Cord blood was collected in ethylenediaminetetraacetic acid and in no-additive tubes for monocyte isolation and caffeine level determination, respectively (BD Biosciences, Franklin Lakes, NJ). Blood samples were centrifuged at 2,400 × g for 10 min at 25 °C, cellular portion was reconstituted in Dubelcco's phosphate-buffered saline (pH 7.4; Mediatech, Herndon,VA), and CBM were isolated using Ficoll-Hypaque gradient (GE Healthcare Biosciences AB, Uppsala, Sweden). CBM were then washed and reconstituted in 4× RPMI 1640 media containing 8% (v/v) human AB serum, penicillin/streptomycin (400 IU/ml/400 μg/ml), and 8 mmol/l L-glutamine (Sigma-Aldrich, St. Louis, MO). Cell count and viability was determined using ammonium-chloride-potassium lysing buffer (Quality Biological, Gaithersburg, MD) and the trypan blue exclusion method (Gibco, Grand Island,NY), respectively (36). Viable CBM were cultured and used in experiments, as outlined below.

Treatment Protocol in Culture

Neonates treated with caffeine citrate for apnea of prematurity receive daily maintenance doses to achieve serum levels of 10 to 20 μg/ml. Due to immaturity of the cytochrome P-450 system in neonates, the hepatic metabolism of caffeine is limited for several weeks after birth (37,38). To model the array of caffeine's effects in cytokine levels in the blood of neonates with limited hepatic clearance, we used a single dose of caffeine in citrated buffer to achieve comparable concentrations in culture (50 to 100 μmol/l) and since some neonates achieve levels > 20 μg/ml, which may switch the cytokine expression response (7), we also tested a concentration of 200 μmol/l.

Caffeine, A_1R antagonist (DPCPX), A_2R antagonist (MRS1220), PDE IV inhibitor (RO-201724), and cAMP-dependent PKA antagonist (RP-cAMPs) (**Table 2**) were used to investigate the changes in the dose–response curves of increasing caffeine concentrations in TNF-α and IL-10 release by CBM. Caffeine was reconstituted in citric acid monohydrate based water (pH 7.3), which in solution forms caffeine citrate salts. All other antagonists were reconstituted in dimethyl sulfoxide (cell-culture-concentration: 2.7×10^{-6} g/ml). RO-201724 was purchased from Calbiochem (San Diego, CA), and all other drugs were purchased from Sigma Chemical Co (St. Louis, MO). Although caffeine is a nonspecific PDE inhibitor at high concentrations, PDE IV is the most abundant in monocytes and has the greatest cAMP degradation activity. Thus, we selected RO-201724 to target this subfamily of PDEs.

CBM were treated with DPCPX, MRS1220, RO-201724 or RP-cAMPs 1 h before exposure to caffeine at 0, 50, 100, and 200 μmol/l. Following an additional hour in culture (37 °C/5%CO_2), CBM were activated using *Escherichia coli* K235 LPS (100 ng/ml; Sigma-Aldrich). Twenty-four hours after LPS-activation, media were collected, centrifugated at 2,000 × g for 5 min, and supernatants were used to measure cytokines levels by enzyme-linked immunosorbent assay (ELISA), while CBM attached to the well were recovered using trypsin-ethylenediaminetetraacetic acid 0.05%, phenol free (Life Technology, Carlsbad, CA), evaluated for viability using Trypan-blue exclusion method as described above, and then lysed for RNA isolation and real-time RT-PCR or to measure intracellular cAMP accumulation by enzyme immunoassay (EIA).

CBM viability was assessed by their property to adhere to the plastic at 24 h after LPS-activation. LPS activated CBM were observed under the inverted microscope to evaluate the morphological appearance and the degree of debris and to ascertain good quality of the culture. If any concerns, a 1 μl aliquot of recovered CBM from each well was evaluated using the trypan blue exclusion method. No difference in cell viability was observed between treatments. However, those culture plates that show increased cell death were discarded and not used for analysis.

ELISA/EIA

TNF-α and IL-10 concentrations were measured by ELISA using multiplex kits according to manufacturer's protocol and concentrations were calculated using the Luminex detection platform (Millipore, Billerica, MA).

Intracellular cAMP levels were measured to confirm function of ARs during exposure to caffeine and other treatments. After exposure, CBM were lysed using 2.5% dodecyltrimethylammonium bromide in assay buffer (pH 5.8; 0.05 M sodium acetate buffer and 0.02% bovine serum albumin). Intracellular cAMP levels were measured using the Amersham cAMP EIA System (GE Healthcare, Little Chalfont, Buckinghamshire, UK) following manufacturer's protocol as described previously (3), and results were reported corrected for 2×10^5 viable CBM.

Real-Time qRT-PCR

Total RNA was extracted from LPS-activated CBM to determine changes in cytokines (TNF-α, IL-10) and TLRs (TLR1, TLR2, TLR3,

TLR4) gene expression following caffeine (50, 100, and 200 μmol/l vs. 0 μmol/l) and MRS1220 (10 nmol/l, specific A_3R antagonist) treatment alone and combined. RNA mini kit (Invitrogen, Carlsbad, CA) was used according to specifications. Approximately 1 μg of total RNA was used for generation of cDNA using iScript cDNA synthesis kit (BioRad, Hercules, CA). Reverse transcription protocol included 5 min at 25 °C; 30 min at 42 °C and 5 min at 85 °C. cDNA was then used to amplify target genes by real-time qRT-PCR using primers at 300 nmol/l (**Table 3**) (39). SYBR Green Supermix (BioRad) was used for signal detection by PCR Thermocycler (BioRad). The amplification protocols included 40 cycles of: 1 min at 95.0 °C, 1 min at 61.0 °C (cytokines) or 59.0 °C (TLRs), and 1 min and 15 s at 72.0 °C. GAPDH (glyceraldehyde phosphate dehydrogenase), β-actin, and G6PDH (glucose-6 phosphate dehydrogenase) were preliminarily tested to establish gene expression stability under experimental conditions using the BestKeeper approach (40). Based on these results, GAPDH was used as the reference gene to calculate fold difference in gene expression using the Pfaffl method (41) as reported previously (3). Melting curves were used to ascertain purity of PCR products.

Statistical Analysis

Nonparametric statistics including Bonferroni adjusted- Wilcoxon signed rank test and Friedman two-way ANOVA for ranks were applied. Results are reported as median with IQR (25th to 75th percentile) and represented as box-and-whisker plots with outliers (boxes symbolize IQR) for cAMP and RT-PCR data. To better represent dose–response curves for measured cytokines, these data are represented as mean ± SEM. Comparison between curves was made by two-way repeat measures ANOVA with Bonferroni correction for pair analysis. Significance was assigned by $P \leq 0.01$. Analysis was performed using IBM SPSS 18.0 software (IBM, Armonk NY).

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