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Caffeine Modulates TNF-α Production by Cord Blood Monocytes: The Role of Adenosine Receptors

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ABSTRACT: Caffeine, a nonspecific adenosine receptor (AR) antagonist is widely used to treat apnea of prematurity. Because adenosine modulates multiple biologic processes including inflammation, we hypothesized that AR blockade by caffeine would increase cytokine release from neonatal monocytes. Using cord blood monocytes (CBM), we investigated 1) the changes in AR mRNA profile by real time quantitative reverse-transcription polymerase-chain-reaction (qRT-PCR) and protein expression (western blot) after *in vitro* culture, caffeine or lipopolysaccharide (LPS) exposure, and 2) the modulation of cytokine release and cyclic adenosine monophosphate (cAMP) production by enzyme-linked immunosorbent assay (ELISA) induced by caffeine and specific AR antagonists: DPCPX(A_1R), ZM241385(A_{2a}R), MRS1754(A_{2b}R), and MRS1220(A_3R). After 48 h in culture, A_{2a}R and A_{2b}R gene expression increased 1.9 ($p = 0.04$) and 2.5-fold ($p = 0.003$), respectively. A_1R protein expression directly correlated with increasing LPS concentrations ($p = 0.01$), with minimal expression preexposure. Only caffeine (50 μM) and DPCPX (10 nM) decreased tumor necrosis factor-alpha (TNF-α) release from LPS activated-CBM by 20 and 25% ($p = 0.01$) and TNF-α gene expression by 30 and 50%, respectively, in conjunction with a $\geq$2-fold increase in cAMP ($p < 0.05$). AR blockade did not modulate other measured cytokines. The induction of A_1R after LPS exposure suggests an important role of this receptor in the control of inflammation in neonates. Our findings also suggest that caffeine, *via* A_1R blockade, increases cAMP production and inhibits pretranscriptional TNF-α production by CBM. (***Pediatr Res* 65: 203–208, 2009**)

Caffeine (1,3,11 trimethylxantine) is a stimulant widely used in neonatology to treat apnea of prematurity (1). At therapeutic range (5 to 15 μg/mL), caffeine blocks A_1 and A_{2a} adenosine receptors (ARs) stimulating ventilation (2–4). Recently, caffeine has also been linked to a decrease in the incidence of bronchopulmonary dysplasia and cerebral palsy in extremely premature infants (5,6), although the mechanisms explaining these findings have not been elucidated.

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The natural ligand for ARs, adenosine, has a crucial role in multiple biologic processes including inflammation (7,8). The increase in tumor necrosis factor-alpha (TNF-α) release by adult peripheral blood monocytes (PBM) in response to lipopolysaccharide (LPS) exposure can be abolished by pretreatment with A_{2a}R agonists (9,10). Adenosine binding to A_1R (11,12) and A_3R (13,14) also modulates TNF-α release from adult monocytes, whereas A_{2b}R appears to have little effect (10).

Little is known about AR expression on neonatal monocytes and the role of caffeine in modulating cytokine release. We hypothesized that caffeine blockade of ARs on neonatal monocytes would increase the release of cytokines in response to LPS. To test this hypothesis, we used cord blood monocytes (CBM) from full-term infants to 1) characterize the changes in AR mRNA profile and protein expression after 48 h in culture, exposure to caffeine and activation with LPS; and 2) determine the effect of AR blockade by caffeine on the release of pro- and antiinflammatory cytokines with indirect confirmation of AR function *via* determination of changes in intracellular cyclic adenosine monophosphate (cAMP) levels and cytokine gene expression.

METHODS

Subjects. This study complied with the Guidelines for Human Experimentation from the United States Department of Health and Human Services and received approval of The Johns Hopkins Medicine Institutional Review Board (NA_00002034). Informed consent was obtained from parents before inclusion in this study.

CBM from full-term infants ($\geq$37 wk gestation at birth) and PBM from adult volunteers were used for experiments. 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 $\leq$10th percentile for gestational age), and suspected viral infection (based on maternal serological or clinical findings). Infants who subsequently received antibiotics or had illness sufficient to be admitted to the neonatal intensive care unit (NICU) were removed from analysis. Healthy adult volunteers, who were not using theophylline, were included in the study. Caffeine levels were obtained on all blood samples.

Isolation of cord blood monocytes. Full-term cord blood and adult peripheral blood were collected in EDTA for monocyte isolation and in no-additive tubes for caffeine level determination (BD Biosciences, Franklin Lakes, NJ). All blood samples were centrifuged at 2400$\times$ g for 10 min at 25°C, cellular portion was reconstituted in DPBS (pH 7.4; Mediatech,

Abbreviations: AR, adenosine receptor; **CBM,** cord blood monocytes; **IQR,** interquartile range; **LPS,** lipopolysaccharide; **PBM,** peripheral blood monocytes

Inc., Herndon, VA) and monocytes were isolated using Ficoll-Hypaque gradient (GE Healthcare Biosciences AB, Uppsala, Sweden). Monocytes were then washed and reconstituted in 4 × RPMI 1640 media containing 8% (vol/vol) [Delta]H human AB serum, penicillin/streptomycin (400 IU/mL/400 μg/mL), and 8 mM L-glutamine (Sigma Chemical,Aldrich, St. Louis, MO). Viable monocytes were used in experiments as outlined below.

Real time quantitative reverse-transcription polymerase-chain reaction. Total RNA was extracted from monocytes to determine changes in 1) A_1R, $A_{2a}R$, $A_{2b}R$, and A_3R gene expression after 48 h in culture (37°C/5% CO_2), caffeine exposure (50 μM) and LPS activation (0, 100, and 200 ng/mL), and 2) TNF-α gene expression after caffeine and DPCPX (10 nM) treatments alone and combined. PureLink Micro-to-midi total RNA purification system (Invitrogen, Carlsbad, CA) was used according to specifications. Approximately 1 μg of total RNA was used for generation of complementary DNA (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 quantitative reverse-transcription polymerase-chain-reaction (qRT-PCR) using 300 nM concentration of primers [Table 1; (15–19)]. SYBR Green Supermix (BioRad) was used for signal detection by MyIQ PCR Thermocycler (BioRad). Two different amplification protocols were used 1) for ARs: 40 cycles of 15 s at 95.0°C, 1 min at 60.0°C, and 30 s at 72.0°C; and 2) for TNF-α: 40 cycles of 1 min at 94.0°C, 1 min at 60.0°C, and 2 min at 72.0°C. The fold difference in gene expression was corrected to GAPDH (human glyceraldehyde phosphate dehydrogenase; reference gene) using the Pfaffl method (20). Melting curves were used to ascertain purity of PCR products, which were also visualized by 1.3% gel electrophoresis.

Western blot analysis. Changes in A_1R and $A_{2a}R$ protein expression on CBM after 24 h LPS exposure (0, 100, and 200 ng/mL) were determined using western blot. Protein was extracted by manually homogenizing monocytes followed by ice-cold ethanol precipitation. Protein pellet was reconstituted in 0.01 M PBS (pH 7.4; Quality Biologic, Gaithersburg, MD) and concentration was determined using the Bradford method. Twenty five microgram of protein were diluted in loading buffer containing 20% (wt/vol) glycerol and loaded on to 12% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, stained with Ponceau S, blocked with 2.5% nonfat dry milk with 0.1% Tween 20 in 50 mM Tris buffered saline (pH 7.4), and consecutively incubated overnight at 4°C with polyclonal rabbit anti-A_1R (Sigma Chemical, Aldrich), or monoclonal mouse anti-$A_{2a}R$ antibodies (Upstate, Lake placid, NY) both at 1:1000, or mouse anti-β-actin MAb (Sigma Chemical, Aldrich) at 1:20,000. The membrane was then washed with milk, exposed to goat anti-rabbit or anti-mouse antibodies (Bio-Rad) at 1:10,000 for 1 h and then developed with enhanced chemiluminescence using SuperSignal kit (Thermo Scientific, Rockford, IL). To quantify protein immunoreactivity, films were scanned using Adobe Photoshop, and optical density (OD) was determined with IP Lab Gel H software adjusting for background (ΔOD). β-actin was used for protein loading correction. Protein levels are expressed as relative OD measurements (arbitrary density units; ADU).

Enzyme-linked immunosorbent assay. Caffeine and specific AR antagonists [Table 2; (4,21–24)] were used to investigate the role of ARs in modulating cytokine release. Concentrations were based on IC_{50} and K_i to maximize specificity to each AR. A total of 50 μM of caffeine was within therapeutic range for neonates treated for apnea of prematurity (5–15 μg/mL). Caffeine was prepared in acidic water (citric acid based, pH 4.7) and titrated to pH 7.3 before use. All other antagonists were reconstituted in DMSO (DMSO, cell-culture-concentration: 2.7×10^{-6} g/mL). ZM241385 was purchased from Tocris Bioscience (Ellisville, MO), and all other drugs were purchased from Sigma Chemical. Monocytes were exposed to caffeine, ZM241385, MRS1754, or MRS1220 in initial experiments and to caffeine and DPCPX alone and combined in additional experiments. After exposure to these conditions for 75 min (37°C/5% CO_2), monocytes were activated using *Escherichia coli* K235 LPS (100 ng/mL; Sigma Chemical, Aldrich). After 24 h, supernatants were recovered for subsequent determination of cytokine (TNF-α, IL-1β, IL-6, and IL-10) concentrations by enzyme-linked immunosorbent assay (ELISA) using LINCOplex Multiplex kits (Millipore, Billerica, MA) according to manufacturer's protocol and concentrations were calculated using the Luminex detection system (Millipore).

Intracellular cAMP levels were measured to confirm functionality of ARs during exposure to caffeine and/or DPCPX. After exposure, monocytes were lysed using 2.5% dodecyltrimethylammonium bromide in assay buffer (pH 5.8; 0.05 M sodium acetate buffer and 0.02% BSA). Intracellular cAMP levels were measured using the Amersham cAMP EIA System (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and results were reported as percentage change from baseline (no-treatment) corrected for $2 \times (10)^5$ viable monocytes.

Statistical analysis. Because of the non-normal distribution of the data, nonparametric statistics, including Wilcoxon signed rank test, Mann-Whitney test, Friedman analysis of variance (ANOVA), and Spearman Rank correlation were used. Results are reported as median with interquartile range (IQR, 25 to 75th percentile) and, in most cases, represented as box-and-whisker plots with outliers (boxes symbolize IQR). Additional analysis of the data, including correlations and multilevel analysis were performed to investigate the persistency of the effect attributed to caffeine on TNF-α release after correcting for the influence of other measured cytokines (supplemental material online, www.pedresearch.org). Significance was assigned by $p < 0.05$. SPSS 14.0 software was used.

Table 1. *Primers for real time qRT-PCR (15–19)*

Gene	Direction	Sequence	PCR-product
A_1R	S	5′-CACCTTCTGCTTCATCGTGTC-3′	308-bp
	AS	5′-AGCCAAACATAGGGGTCAGTC-3′	
$A_{2a}R$	S	5′-AACCTGCAGAACGTCACCAA-3′	245-bp
	AS	5′-GTCACCAAGCCATTGTACCG-3′	
$A_{2b}R$	S	5′-GTCATTGCTGTCCTCTGG-3′	298-bp
	AS	5′-TCCTCGAGTGGTCCATCAG-3′	
A_3R	S	5′-ACCAGCTCATTGTCACTATG-3′	311-bp
	AS	5′-ACTTCTCCTCCTTTTGGTCA-3′	
TNF-α	S	5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3′	444-bp
	AS	5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′	
GAPDH	S	5′-AACAGCGACACCCACTCCTC-3′	258-bp
	AS	5′-GGAGGGGAGATTCAGTGTGGT-3′	

bp, base pair; GADPH, glyceraldehyde phosphate dehydrogenase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-alpha; S, sense; AS, antisense.

Table 2. *Adenosine receptor antagonists (4,21–24)*

AR Antagonist	Synonym	Concentration	Target receptor
Caffeine	1,3,7-Trimethylxanthine	50 μM	A_1, A_{2a}, A_{2b}
DPCPX	1,3-Dipropyl-8cyclopentylxanthine	10 μM	A_1
ZM241385	4-(2-[7-Amino-2-[2-furyl][1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-yl-amino]ethyl) phenol	10 μM	A_{2a}
MRS1754	8-[4-[((4 Cyanophenyl)carbamoylmethyl)oxy]phenyl]- 1,3-di(n-propyl)xanthine	10 μM	A_{2b}
MRS1220	9-Chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo[1,5-c] quinazoline	1 μM	A_3

RESULTS

Cord blood from 28 neonates (mean gestational age ± SD = 39 2/7 ± 1.3 wk; birth weight = 3312 ± 513 g; Table 3) and peripheral blood from eight adult volunteers (age = 40.9 ± 9.9 y) were used for *in vitro* experiments. Serum caffeine concentrations were below the therapeutic range (mean ± SD = 1.2 ± 1.4 μg/mL for neonates and 2.4 ± 2 μg/mL for adults) and were not statistically different between age groups.

Effect of culture and caffeine on AR gene expression in CBM. A_1R gene expression was undetected in full-term CBM whereas PCR products of alternative splicing variants expressed at baseline, and after 48 h in culture in adult PBM, showing alternative splicing products. In CBM, after 48 h in culture, $A_{2a}R$ mRNA expression increased 1.9-fold (IQR 0.5–11.8; $p = 0.04$) and $A_{2b}R$ mRNA increased 2.5-fold (IQR 1.6–3.5, $p = 0.003$). As a comparison, culture conditions did not change $A_{2a}R$ mRNA expression but did increase $A_{2b}R$ mRNA expression by 2.3-fold (IQR 1.3–7.2, $p = 0.03$) in adult PBM (Fig. 1). *In vitro* exposure to caffeine did not further modify the gene expression of any AR subtype.

Effect of LPS exposure on AR expression in CBM. Although A_1R mRNA was not detected in CBM at baseline by real time qRT-PCR (Fig. 1*A*), 24 h exposure to LPS (100 ng/mL) induced the expression of this gene in 67% ($n = 8/12$) of the subjects with no further induction at higher LPS concentration (200 ng/mL). Electrophoresis (Fig. 2*A*) showed A_1R PCR products at 308-bp, which corresponds to the two recognized transcript variants (NM_000674.2 and NM_001048230.1; NCBI database). Nontranslated products of alternative splicing are shown at 455-bp (Alternative Splicing and Transcript Diversity Database; www.ebi.ac.uk/astd). LPS exposure also increased $A_{2a}R$ mRNA expression by 4.2-fold (IQR 3.6–12.2; $p = 0.02$; Fig. 2*B*).

Western blot analysis showed that A_1R and $A_{2a}R$ protein expression directly correlated with LPS concentrations to which CBM were exposed ($r = 0.64$, $p = 0.01$ and $r = 0.71$, $p = 0.001$, respectively). LPS at 100 and 200 ng/mL, increased A_1R protein by 35% (IQR 18 to 360%; $p = 0.03$) and 100% (IQR 41 to 785%; $p = 0.03$), respectively ($p = 0.002$, Friedman ANOVA; Fig. 2*C* and *D*) and increased $A_{2a}R$ protein by 174% (IQR 16 to 455%; $p = 0.02$) and 230% (IQR 36 to 621%; $p = 0.02$), respectively, *versus* no LPS exposure ($p = 0.006$, Friedman ANOVA).

Effect of AR antagonists on cytokine release from LPS-activated monocytes. Exposure to LPS significantly increased the release of all measured cytokines (TNF-α, IL-1β, IL-6, and IL-10) from CBM and adult PBM compared with control conditions ($p < 0.05$). In response to LPS, cytokine release did not differ between neonates and adults, except for the antiinflammatory cytokine, IL-10, which showed 80% (IQR 69 to 96%, $p = 0.04$) less release from CBM compared with adults PBM (Fig. 3). Mode of delivery did not influence baseline cytokine levels or response to antagonists.

In LPS-activated CBM, caffeine (a nonspecific AR antagonist) down-regulated TNF-α release by 20% from baseline (IQR −40 to −9%, $p = 0.015$) whereas $A_{2a}R$, $A_{2b}R$, and A_3R specific antagonists did not produce an effect. In experiments using adult PBM, there was a trend toward an increase in TNF-α release from baseline after exposure to all AR antagonists with MRS1220 (A_3R antagonist) reaching significance ($p = 0.03$). The differences in TNF-α release between CBM and adult PBM after caffeine and ZM241385 reached significance ($p = 0.02$ and 0.03 for each treatment, respectively; Fig. 4*A*). Caffeine and specific antagonists did not affect IL-1β, IL-6, and IL-10 release from monocytes in either age group (data not shown). Because the observed decrease in TNF-α release by CBM following caffeine exposure (Fig. 4*A*) can result from 1) the blockade of ARs and/or 2) the interaction with other cytokines, we determined the influence of these two factors using a multilevel model. Correcting for interactions with other cytokines, the model supported our initial findings associating caffeine with down-regulation of TNF-α release (−56%, $\beta = -303 \pm 66$ pg/mL; $p = 0.01$). The mechanism behind this effect was explored in the next series of experiments.

Role of A_1R in the decrease of TNF-α release from CBM. Because specific $A_{2a}R$, $A_{2b}R$, and A_3R antagonists did not affect the release of TNF-α and LPS exposure induced A_1R gene and protein expression, we hypothesized that A_1R was involved in the reduction of TNF-α secretion after caffeine exposure. DPCPX, a specific A_1R antagonist (10 nM), down-

Table 3. *Demographics and serum caffeine in neonates*

Full-term infants ($n = 28$)	
Gestational age (mean ± SD)	39 2/7 ± 1.3 wk
Birth weight (mean ± SD)	3312 ± 513 g
Gender	68% male
Race	64% AA; 12% C
APGARS 1 min (median, range)	8 (7–9)
APGARS 5 min (median, range)	9 (8–9)
Delivery mode	60% SVD; 40% C/s
Caffeine level (mean ± SD)	1.2 ± 1.4 μg/ mL

AA, African American; APGARS, apgar score; C, Caucasian; C/s, cesarean section delivery; SD, standard deviation; SVD, spontaneous vaginal delivery.

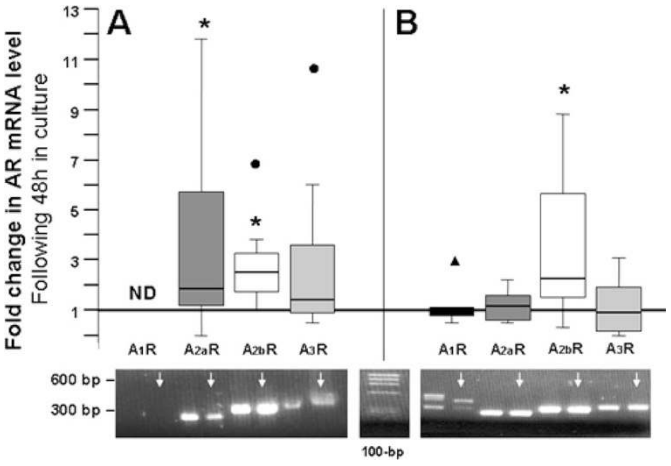

Figure 1. Fold change in AR mRNA levels after 48 h in-culture. (*A*) Neonates ($n = 12$) and (*B*) Adults ($n = 8$). *Solid line* inside boxes represents median. ▲, Extremes; ●, outliers. *$p < 0.05$ (Wilcoxon signed rank test) *versus* reference line (at 1, no change). ND, nondetected. Electrophoresis shows RT-PCR products before and after culture, *arrows* identify products after culture.

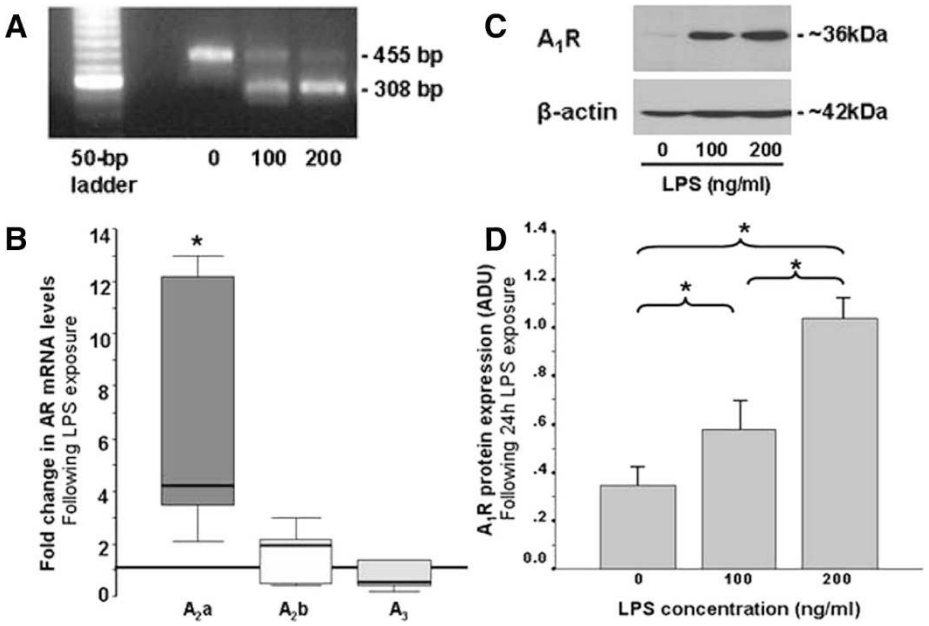

Figure 2. Effect of LPS exposure on AR gene and protein expression. (*A*) RT-PCR products for A_1R from CBM after LPS exposure (0, 100, and 200 ng/mL). Transcript variants were amplified as 308-bp products (455-bp product is result of alternative splicing). (*B*) Fold change in AR mRNA on CBM after 24 h LPS exposure at 100 ng/mL ($n = 8$). *Solid line* inside boxes represents median. *$p < 0.05$ (Wilcoxon signed rank test) *versus* reference line (at 1, no change). (*C*) Western blot showing A_1R protein at ~36 kD band in 12% SDS-PAGE, and (*D*) A_1R protein levels expressed as arbitrary density units (ADU) adjusted to β-actin. Data as mean ± SEM (ADU) after exposure to LPS at 0, 100, and 200 ng/mL ($n = 6$). *$p < 0.05$ (Wilcoxon signed rank test); overall $p = 0.001$ (Friedman ANOVA).

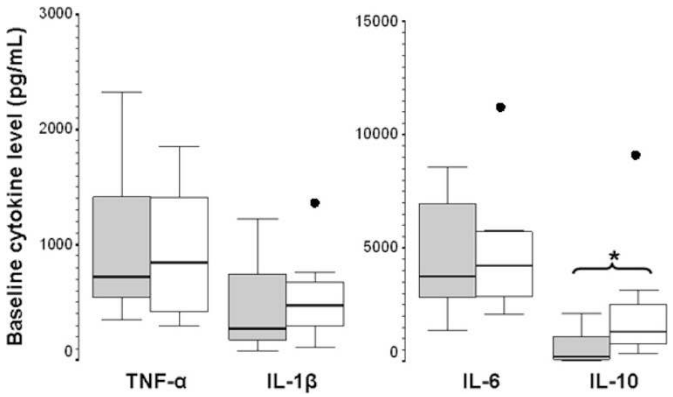

Figure 3. Cytokines (pg/mL) released from neonatal (*gray box*) and adult (*white boxes*) monocytes after exposure to LPS (100 ng/mL). *Solid line* inside boxes represents median. * $p < 0.05$ (Mann-Whitney test). ●, outliers ($n = 12$).

regulated TNF-α release by 25% from baseline (IQR −54 to −8%, $p = 0.01$) and the addition of caffeine to cells pretreated with DPCPX down-regulated TNF-α release by a total of 43% from baseline (IQR −70 to −26%, $p = 0.01$). The additional decrease in TNF-α release produced by caffeine after pretreatment with DPCPX was significant ($p = 0.03$ *versus* DPCPX alone; $p = 0.001$ by Friedman ANOVA; Fig. 4*B*).

In accordance with these findings outlined above, caffeine increased intracellular cAMP levels by 2.8-fold (IQR 2.3–3.8, $p = 0.04$) and DPCPX by 2.1-fold from baseline (IQR 1.6–2.3, $p = 0.03$). The addition of caffeine after pretreatment with DPCPX increased cAMP by 2-fold (IQR 1.7–2.2, $p = 0.04$) from baseline, similar to DPCPX alone (Fig. 4*C*). Caffeine inhibited TNF-α gene expression by 30% (IQR −10 to −40%, $p = 0.01$) whereas DPCPX inhibited the gene by 50% (IQR −40 to −50%, $p = 0.04$; Fig. 4*D*).

DISCUSSION

In this study, we determined the change in AR expression in full-term CBM compared with that on adult PBM after *in vitro* culture and exposure to caffeine or LPS. We also determined the effect of caffeine on cytokine release from CBM and indirectly confirmed the AR function by measuring the changes in cAMP production and TNF-α gene expression. We demonstrated that in contrast to adult PBM, A_1R gene and protein expression in CBM is minimal until exposure to LPS, which also up-regulates $A_{2a}R$. We showed that TNF-α release in response to LPS is similar in CBM and adult PBM, and exposure to caffeine or DPCPX (A_1R antagonist) down-regulates TNF-α release only from CBM. In accordance with these findings, we found that intracellular cAMP concentration increases and TNF-α gene expression decreases as a result of caffeine or DPCPX exposure. These data suggest that the effect of caffeine on TNF-α release was in part mediated *via* A_1R blockade.

AR gene expression in monocytes differs between neonates and adults. The dose-related induction of A_1R protein expression in CBM after LPS exposure could explain the baseline A_1R expression observed in adult PBM. This finding suggests that A_1R induction occurs some time after birth following exposure to infections and also implies, similar to $A_{2a}R$, a role in the modulation of inflammation (8,25).

Because of the nonspecific effect of caffeine on ARs, the identification of the particular AR subtype(s) involved in the modulation of TNF-α release is challenging. We used specific AR antagonists to evaluate the effects of endogenous adenosine. We believe that this approach is most applicable to studying the effects of caffeine, a nonspecific AR antagonist, compared with the use of agonists.

A_1R dysregulation leads to a proinflammatory state (11) and because of the high affinity of A_1R (coupled to G_i-protein) for adenosine, A_1R blockade could potentially increase cAMP not only directly but also indirectly by displacing adenosine toward $A_{2a}R$ (coupled to G_s-protein), which ultimately will down-regulate TNF-α release (26,27). A_1R is directly induced in response to infection and oxidative stress in multiple cells (28). Our experiments using DPCPX demonstrate that the

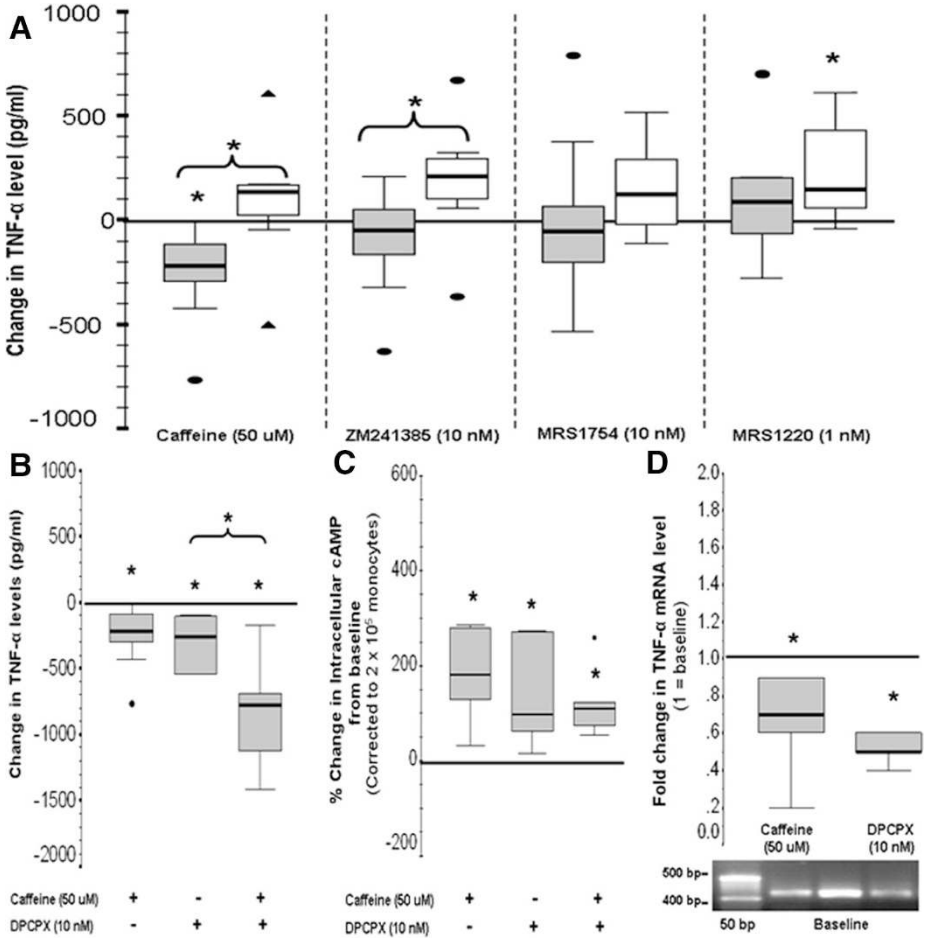

Figure 4. Effect of AR antagonists on TNF-α release. (*A*) Change from baseline in TNF-α (pg/mL) release from neonatal (*gray boxes*; $n = 12$) and adult (*white boxes*; $n = 8$) monocytes pretreated with caffeine and specific A_{2a}R, A_{2b}R, and A_3R antagonists. (*B*) Change in TNF-α (pg/mL) release ($n = 8$); (*C*) Percentage change in intracellular cAMP concentrations ($n = 6$); and (*D*) Fold change in TNF-α gene expression ($n = 8$) in LPS-activated CBM in response to caffeine and DPCPX alone and combined. Electrophoresis showed 444-bp RT-PCR product for TNF-α. Reference lines (baseline conditions), positioned at 0 (*A*, *B*, and *C*) and 1 (*D*), represent no change. Line inside boxes represents median. *$p < 0.05$ (Wilcoxon signed rank test *versus* baseline and Mann-Whitney test, between age groups). ▲, extremes; ●, outliers.

blockade of A_1R on LPS-activated CBM significantly down-regulates TNF-α release, *via* a pretranscriptional mechanism, to levels similar to that after caffeine exposure. These findings strongly suggest that, at least in part, A_1R blockade mediates the effect of caffeine on TNF-α release, which is also associated with concurrent changes in cAMP levels and TNF-α gene expression.

A_{2a}R agonists down-regulate TNF-α release; (10,29) however, specific A_{2a}R antagonists (ZM 241385) failed to increase TNF-α release from adult PBM (30) or CBM (our data). This inconsistency may be related to the multiple AR subtypes differentially modulating cAMP production. As demonstrated by our data, LPS-activated CBM express the full spectrum of ARs identified to date, making possible that specific A_{2a}R blockade displaces adenosine to bind to other available receptors, including A_{2b}R and A_3R, which will increase cAMP production. Studies targeting the A_{2b}R are less abundant and conclusive and most show that in response to specific A_{2b}R agonists, TNF-α is up-regulated probably as a result of the concomitant IL-19 release (10,31). In our experiments, MRS1754 (A_{2b}R antagonist) appears to have a role in down-regulating TNF-α release (multilevel analysis, supplemental material online, www.pedresearch.org), however, its effect is abolished by the influence of other cytokines (Fig. 4*A*). Because caffeine simultaneously blocks A_{2a}R and A_{2b}R and the blockade of the latter could potentially have an effect in the TNF-α release, we cannot rule out that the mechanism of caffeine could also be directly or indirectly mediated by A_{2b}R.

A_3R activation has been linked to pro- and anti-inflammatory effects, from pro- to antiinflammatory (14,26,32). Recently, A_3R has been described as positively coupled to adenylyl cyclase, thereby increasing cAMP production upon activation (33,34). Because caffeine, at 50 μM, blocks all ARs except for A_3R, we speculate that the down-regulation of TNF-α release associated with caffeine is also mediated by the indirect increase in A_3R affinity for adenosine when the other ARs are blocked. This mechanism could explain our results showing an additional decrease in TNF-α release after caffeine is added to DPCPX pretreated CBM.

The synchronized increase of intracellular cAMP levels and decrease of TNF-α gene expression after caffeine exposure suggest that the decrease in TNF-α release is likely a pretranscriptional process. Several pathways related to accumulation of intracellular cAMP such as cAMP/protein kinase A (35) and modifications in NF-κB (36) are targets of our future experiments.

Although TNF-α release is modulated by caffeine *via* AR blockade, that is not the case for all cytokines. Multiple studies using adult PBM show that IL-1β (14,35), IL-6 (29), and IL-10 (35) release from these cells are not modulated by ARs. A recent study using neonatal monocytes reported similar finding related to IL-6 (32). We have extended this finding

by also showing that IL-1β and IL-10 release are also independent of ARs in CBM.

In this article, we demonstrate the significant differences in AR mRNA profile between monocytes from neonates and adults, the induction of A_1R gene and protein expression after LPS exposure and also the suppressive effect of caffeine on TNF-α release by LPS-activated CBM *via* mechanisms involving ARs. Our data suggest that A_1R blockade is operative in mediating the effect of caffeine. Although the direct applicability of our findings to premature infants is still unclear, we believe that our results are clinically relevant to neonates exposed to caffeine either throughout gestation or postnatally. Whether the suppression of TNF-α release produced by caffeine has a beneficial effect by decreasing chronic inflammation or has a detrimental effect by increasing the risk for infection is unclear. Nevertheless, reduction in the incidence of chronic lung disease and periventricular leukomalacia in premature infants has been attributed to the use of caffeine citrate (5). Our data suggest a potential biologic mechanism to explain these clinical observations and add to the body of evidence characterizing the differences in inflammatory response between neonates and adults as it relates to ARs on monocytes.

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