

Modulation of 3',5'-cyclic AMP homeostasis in human platelets by coffee and individual coffee constituents

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Abstract

3',5'-Cyclic AMP (cAMP) is one of the most important second messengers in mammalian cells, mediating a multitude of diverse cellular signalling responses. Its homeostasis is primarily regulated by adenylate cyclases and phosphodiesterases (PDE), the activities of which are partially dependent on the downstream events of adenosine receptor signalling. The present study was conducted to determine whether coffee constituents other than caffeine can influence the homeostasis of intracellular cAMP *in vitro* and *in vivo* by evaluating the effects of selected constituents present in coffee, coffee brews and coffee extracts on platelet PDE activity. In addition, to evaluate the potential effects of these constituents on platelet cAMP concentrations and PDE activity in humans, a 7-week pilot intervention study with eight subjects was conducted. The subjects consumed a regular commercial coffee and a low-caffeine coffee at a rate of 750 ml/d for 2 weeks each. The *in vivo* results revealed a highly significant inhibition of PDE activity ($P < 0.001$) after coffee intervention that was not directly dependent on the caffeine content of coffee. Although our *in vitro* and *in vivo* findings suggest that caffeine plays some role in the modulation of platelet cAMP status, other natural and roasting-associated compounds such as pyrazines and other currently unidentified species also appear to contribute significantly. In conclusion, moderate consumption of coffee can modulate platelet PDE activity and cAMP concentrations in humans, which may contribute to the putative beneficial health effects of coffee. Further detailed mechanistic investigations will be required to substantiate these beneficial effects and to elucidate the underlying mechanisms.

Key words: Coffee: 3',5'-Cyclic AMP: Phosphodiesterases: Caffeine

Coffee is one of the most widely consumed beverages in the world and has been linked to certain beneficial effects on human health. Epidemiological evidence suggests coffee consumption to be associated with the prevention or delay of degenerative diseases including type 2 diabetes mellitus, Parkinson's and Alzheimer's diseases, CVD and cancer^(1–5). These beneficial effects have been attributed in part to the antioxidant activity of coffee^(6–8) and to the activity of caffeine as an adenosine receptor (AR) antagonist⁽⁹⁾. Coffee consumption has also been reported to inhibit platelet aggregation⁽¹⁰⁾, and regular coffee drinking is correlated with moderate reductions in body weight^(7,11,12), which may further contribute to these beneficial effects.

Roasted coffee beans contain thousands of constituents, including lipids, proteins, carbohydrates, vitamins and minerals. However, there has been relatively little work on the activity-guided characterisation of the compounds

responsible for the beneficial health effects of coffee consumption, and much work remains to be done in this area. Although the positive effects of coffee consumption can be partially attributed to its caffeine content^(1,13), recent investigations have shown that some other coffee constituents may also contribute to the observed diverse biological effects in humans^(12–16).

The average caffeine consumption by adult humans varies between different cultures and nations, ranging from 80 to 400 mg/person per d⁽¹⁷⁾. Caffeine elicits a diverse number of physiological responses, including increased vigilance, decreased psychomotor reaction time, and increased sleep latency and waking time. It may also influence intellectual performance⁽¹⁸⁾. In addition, it has been reported to cause relaxation of smooth muscles⁽¹⁹⁾, to enhance gastric secretion and the release of catecholamines, and to increase metabolic activity in the brain⁽¹⁹⁾. It is generally accepted that the main

Abbreviations: AB, Arabica Brazil; ADA, adenosine deaminase; AR, adenosine receptor; BC, blood collection; cAMP, 3',5'-cyclic AMP; CQA, 5-O-caffeoylquinic acid; NMP, N-methylpyridinium; PDE, phosphodiesterases.

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mechanisms of action underpinning the manifold biological effects of caffeine are related to its AR antagonism. This activity may also affect the expression and/or activity of 3',5'-cyclic AMP (cAMP) phosphodiesterases (PDE) and adenylate cyclases⁽²⁰⁾. Caffeine may also directly inhibit the activity of PDE and thus increase the abundance of cAMP. These, in turn, can have a multitude of downstream consequences, including the modulation of catecholamine-mediated effects⁽²¹⁾. However, at the blood concentrations resulting from normal coffee consumption, it is generally accepted that caffeine will primarily act as an AR antagonist. The same applies to its main metabolite, paraxanthine, which has been shown to inhibit the effects of endogenous adenosine on diverse physiological processes^(19,20,22).

AR are associated with intracellular pathways that contribute to various physiological processes, including the modulation of cAMP homeostasis, phospholipase C activity and mitogen-activated protein kinases⁽²³⁾. Therefore, any tissue that expresses these receptors may respond in a specific way to caffeine-mediated AR inhibition. Individual tissues may express several AR subtypes, resulting in a vast array of potential primary and secondary responses⁽²⁴⁾. Targeted manipulation of the activity of specific AR could have a range of downstream effects, including the modulation of cyclic nucleotide signalling by changing the concentrations of cAMP and 3',5'-cyclic GMP. The homeostasis of cAMP is regulated by adjusting the relative rates of its formation through adenylyl cyclases and its degradation by PDE. The PDE are a family of enzymes that hydrolyse the phosphodiester bond in the second messenger molecules cAMP and 3',5'-cyclic GMP, thereby converting them to the corresponding 5'-monoesters. Although both cyclic nucleotides can be transported out of the cell slowly, it appears that PDE activity is the primary means of rapidly reducing their concentrations within cells and thereby terminating cAMP-mediated signalling⁽²⁵⁾.

Many different cellular responses are mediated by cAMP. These include the modulation of gene expression and enzyme activity, especially that of protein kinase A and its downstream targets. The physiological consequences of changes in cAMP concentrations include the modulation of blood platelet function and heart rate, glycogen and fat homeostasis, and cortisol secretion and the effects on catecholamine signalling^(21,26).

The present study was conducted to determine whether coffee constituents other than caffeine can affect the homeostasis of intracellular cyclic nucleotides *in vitro* and *in vivo*. The effects of selected coffee constituents, extracts and brews on key elements involved in downstream AR-mediated signalling pathways were investigated. In addition, the *in vivo* effects of coffee consumption on these intracellular and extracellular key elements were investigated in a pilot human intervention study.

Materials and methods

Chemicals

5-O-Caffeoylquinic acid (CQA), caffeic acid, methanol, AMP, cAMP, benzamidine, paraxanthine, caffeine, K₃PO₄, KOH,

bovine serum albumin, IBMX (3-isobutyl-1-methylxanthine), Forskolin, 2-isobutyl-3-methoxypyrazine, 2-ethyl-3,5(6)-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine (2,3-DE-5-MP), 2,3,5,6-tetramethylpyrazine and Milrinone were obtained from Sigma-Aldrich. Leupeptin, pepstatin A, phenylmethylsulphonyl fluoride and pyridine were purchased from Alexis. Rolipram and Zaprinast were obtained from Calbiochem. All organic solvents and other chemicals were of analytical grade and met the standards required for cell-culture experiments. Radiolabelled cAMP ([2,8-³H]-adenosine 3',5'-cyclic phosphate ammonium salt; 9.25 MBq/ml) was obtained from Hartmann Analytik and [1',2',3',4',5'-¹³C₅]adenosine from Omicron Biochemicals, Inc.

Isolation of platelets

For *in vitro* experiments, platelet concentrates were obtained from the Red Cross Blood Transfusion Service (DRK Blutspendedienst Baden-Wuerttemberg/Hessen). Each platelet concentrate was a suspension of human platelets (pooled from four donors) in 255 ml of a platelet additive solution and CPD (citrate-phosphate-dextrose) plasma, containing approximately 2.0–4.5 × 10¹¹ thrombocytes/preparation. Concentrates were stored for at most 5 d at 25°C under constant agitation. Platelets were isolated from each concentrate by decanting 50 ml of this suspension into 50 ml tubes, adding EDTA to a final concentration of 5 mM, mixing thoroughly and then centrifuging the resulting suspension at 1500g for 15 min at room temperature. The supernatant was discarded and the pellet resuspended in Run III buffer with the following composition (values in parentheses indicate concentrations in mM): Tris/HCl, pH 7.4 (50); MgCl₂ × 6H₂O (10); EDTA (0.1); benzamidine (5); pepstatin A (1); leupeptin (1); soybean trypsin inhibitor (0.5); phenylmethylsulphonyl fluoride (0.5); β-mercaptoethanol (0.5). The volume of the resulting suspension was made equal to that of the original concentrate.

For *in vivo* experiments, platelets were isolated from platelet-rich plasma by centrifugation at 800g for 15 min at room temperature to yield a platelet pellet. The supernatant was aspirated and stored for the adenosine deaminase (ADA) experiments. The pellet was gently resuspended in Run III buffer for the PDE activity assay or in stimulation buffer for the cAMP assay. If platelet clumps were observed at this stage, inadvertent platelet activation was assumed to have occurred and the preparation was discarded.

Preparation and analysis of coffee extracts for *in vitro* studies

A total of four coffee extracts were examined. Extracts AB1 and AB2 were prepared from a single batch of green coffee beans (Arabica Brazil) roasted in a fluidised bed roaster (RFB Neuhaus Neotec, Institute of Thermal Separation Processes, Technische Universität Hamburg-Harburg). Coffee brews were prepared from ground coffee beans as described by Lang *et al.*⁽²⁷⁾. Briefly, 48 g of coffee powder were placed onto the filter of a conventional coffee machine and extracted with 900 ml of (boiling) water; the resulting coffee brews were

immediately cooled (ice bath) and freeze-dried *in vacuo* (lyophilised) to generate extracts AB1 and AB2. The *N*-methylpyridinium (NMP) and chlorogenic acid (CGA) contents of the extracts were quantified as described in the literature^(28–30). The caffeine contents of the brewed AB1 and AB2 coffees were 737.2 and 610.9 mg/l, respectively. Extract AB1 represents a light roast (2 min roasting time) and thus had a high CGA content (1606.5 mg/l) with a low NMP content (5.4 mg/l). Extract AB2 represents a dark roast (5 min roasting time) and thus had a relatively low CGA content (185.1 mg/l) and a high NMP content (71.7 mg/l)^(6,14).

In addition to extracts AB1 and AB2, two other ground coffee extracts were examined. The commercial and low-caffeine coffee extracts were derived from brews generated using pre-packaged coffee pads, which contained ground coffee and an individual filter. Each pad contained 7.8 (sd 0.2) g of coffee and yielded 110 to 130 ml of coffee brew. These brews were immediately cooled (ice bath) and lyophilised, giving 14 g lyophilisate/l brew for the low-caffeine coffee and 16 g lyophilisate/l brew for the commercial coffee. The CGA contents of these extracts (i.e. the sums of their 3-, 4- and 5-CQA contents) were quantified as described previously⁽³⁰⁾. The caffeine contents of the commercial and low-caffeine coffee brews were 618.1 and 178.9 mg/l, respectively.

Human blood platelet 3',5'-cyclic AMP phosphodiesterase activity assay

After isolation, the platelets were resuspended in Tris buffer (50 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂, 0.1 mM-EDTA, 5 mM-benzamidine, 0.5 mM-β-mercaptoethanol and a protease inhibitor mix) and lysed in a Wheaton glass tissue grinder, using a 'tight' pistil for forty strokes. After centrifugation (100 000 g at 4°C for 40 min), the supernatant (consisting of the so-called cytosolic fraction) was separated and used directly for the PDE assay. PDE activity was determined with a slight modification of the procedure reported by Poch⁽³¹⁾, using 1 μM of cAMP (substrate). Briefly, 50 μl of the supernatant and 50 μl of Run III buffer with or without a PDE modulator were incubated at 37°C with 50 μl [³H]cAMP mix (30 mM-Tris/HCl, pH 7.4, 9 mM-MgCl₂, 3 mM-5'-AMP, 3 μM cAMP and 0.481 MBq [³H]cAMP/5 ml). The enzyme reaction was stopped at a maximal cAMP turnover of 20% by adding 250 μl of 0.266 M-ZnSO₄ on ice. [³H]5'-AMP was then precipitated by adding 250 μl of 0.266 M-Ba(OH)₂. The tubes were centrifuged at 20 000 g, and the supernatant containing the non-hydrolysed [³H]5'-cAMP was subjected to liquid scintillation counting. Typing for PDE isoenzyme activity in platelet concentrates and platelet-rich plasma from the intervention study subjects was achieved with the aid of specific inhibitors, namely PDE3-specific Milrinone (CAS: 78 415-72-2), PDE4-specific Rolipram (CAS: 61 413-54-5) and PDE5-specific Zaprinas (CAS: 37 762-06-4). PDE1 typing was performed using Ca²⁺/calmodulin as a specific stimulator. These compounds were used at final concentrations of 10 μM (Milrinone and Zaprinas), 50 μM (Rolipram) and 0.2 μM (Ca²⁺/calmodulin). For each blood sample, three independent measurements were performed. The inter-assay variability was determined to be 9.6%.

Determination of protein concentrations

Protein concentrations were determined according to the method of Bradford⁽³²⁾ using bovine serum albumin as a standard.

Human intervention study design

A human intervention study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local Ethics Committee of the Medical Association of Rhineland-Palatinate, Mainz, Germany (no. 837.380.10 (7389)). A total of ten healthy subjects (seven males and three females) were recruited from within the University of Kaiserslautern, Germany. The inclusion criteria were as follows: age 20–44 years; no known active ongoing disease (apparent good health); non-smoking status; average coffee consumption. The exclusion criteria were as follows: drug treatment (including sympathomimetic drugs, α- or β-adrenergic receptor blockers, theophylline and any anti-hypertensive medication); participation in any sport at a high level; metabolic disorders/diseases; a BMI ≥ 28 (kg/m²). Written informed consent was obtained from all subjects. Of the original ten volunteers, two dropped out of the study for private reasons and eight (six males and two females) completed the intervention study.

Throughout the study, the subjects were asked to refrain from consuming any coffee other than the study brews, tea, cola drinks, energy drinks or any other caffeine-containing drinks or medications. A written list of drinks to be avoided was provided to the subjects at the time of screening.

In this study, two different coffees were used: a commercial blend of 100% Arabica coffee and a low-caffeine coffee (a 3:1 mixture of decaffeinated Arabica Brazil and non-decaffeinated Arabica coffee). As shown in Fig. 1, the short-term intervention was a 7-week human study that was divided into five phases. During a 1-week initial washout (first washout) phase, the subjects consumed 750 ml of water daily. In the first coffee phase, they consumed 750 ml of a regular coffee brew (prepared from coffee pads) daily (eight coffee pads, with/without sugar in three equal portions: morning, noon-time and afternoon). In the second washout phase, the coffee brew was replaced with an equivalent volume of water (750 ml/d). In the second coffee phase, the subjects consumed 750 ml of low-caffeine coffee every day, followed by a final washout phase during which they again consumed an equivalent volume of water (750 ml) daily instead of coffee. Blood collection (BC) was performed at the beginning and at the end of each study week (1st BC to 8th BC). Anthropometric measurements were performed in the morning before breakfast, at the beginning and end of each study phase: the subjects' body weights were determined using a medical scale (Seca delta 707; Seca) and their heights were measured using a Seca 206 measuring tape (Seca). The subjects had a mean BMI of 23 (sd 2.3) kg/m² and an average age of 29 (sd 9) years.

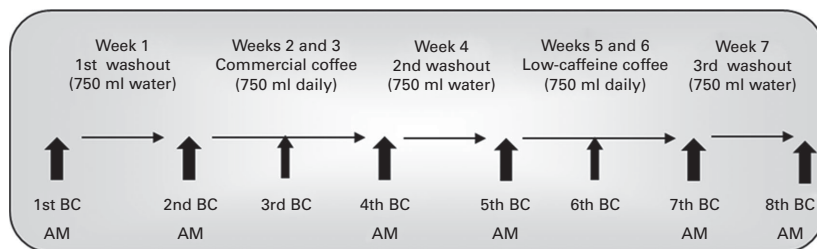


Fig. 1. Study design for the short-term human intervention. Blood samples were collected at eight time points (↑). A commercial coffee was consumed during the first coffee phase (weeks 2 and 3). During the second intervention phase of the study (weeks 5 and 6), a low-caffeine coffee was consumed by the subjects. BC, blood collection; AM, anthropometric measurements.

Quantification of adenosine concentrations in blood samples

Adenosine concentrations in blood samples were quantified by means of HPLC–electrospray ionisation (ESI)–MS/MS by a minor modification of the procedure described by Doležalová *et al.*⁽³³⁾. Blood samples were deproteinised with an equal volume of 1 M-perchloric acid immediately after collection to prevent potential enzymatic degradation or production of adenosine. The samples were then cooled in ice-cold water and centrifuged (4000 *g* for 5 min at 4°C). The clear supernatant (300 µl) was transferred to a test-tube and 20 µl of an internal standard containing 1.6 µM [1',2',3',4',5'-¹³C₅]adenosine were added. The solution was neutralised by adding 20 µl of buffer containing 2.5 M-K₃PO₄ and 1.3 M-KOH. After a second brief centrifugation step (4000 *g* for 5 s), the potassium perchlorate precipitate was removed. The supernatant was then diluted with 250 µl of redistilled water and transferred to a preconditioned StrataX 33u polymeric reversed-phase solid-phase extraction column (30 mg/ml; Phenomenex). After the sample had passed through the column, it was rinsed with 1.5 ml of re-distilled water. Adenosine was then eluted with 2 × 500 µl of methanol.

The eluate was concentrated to a volume of about 100 µl using a vacuum centrifuge and then diluted to a ratio of 1:4 with 0.02% acetic acid. Aliquots (10 µl) of the resulting solution were analysed by HPLC–ESI–MS/MS. Chromatographic separation of adenosine was performed on an Aqua C18 column (250 × 4.6 mm, 5 µm; Phenomenex), using an isocratic solvent system of 0.1% formic acid in water (v/v) and methanol (85:15, v/v) at a flow rate of 0.5 ml/min.

Detection was achieved with a triple-quadrupole tandem mass spectrometer API 3200™ (Applied Biosystems) using positive ESI. The ESI settings were as follows: spray capillary voltage, 5.5 kV; curtain gas, N₂ (+390°C at 10 psi); GS1, 40 psi; GS2, 80 psi; electron multiplier voltage, 2.1 kV. The main characteristic fragmentation ion derived from the molecular ion, (M + H)⁺, of each compound was analysed in the multiple reaction mode. The ion transitions considered were *m/z* 273 → *m/z* 136 for [1',2',3',4',5'-¹³C₅]adenosine and *m/z* 268 → *m/z* 136 for adenosine, with both species having retention times of 8.4 min. Adenosine was quantified by comparing its peak area with that of the isotopically labelled standard. The correlation coefficient for the relationship between the standard and analyte peak areas was *R* 0.998. The method had a limit of quantification of 61.1 fmol and

a limit of detection of 30.6 fmol. Experiments carried out using spiked samples revealed a recovery of 102.7 (SD 12.8)%.

Determination of plasma adenosine deaminase activity in the plasma

The activity of ADA in the plasma of subjects was determined using a commercial assay kit obtained from Diazyme Laboratories. This assay is based on the enzymatic deamination of adenosine to inosine, which is converted into hypoxanthine by purine nucleoside phosphorylase. Hypoxanthine is then converted to uric acid and H₂O₂ by xanthine oxidase. The H₂O₂ generated mediates the oxidative dye coupling of *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-3-methylaniline and 4-aminoantipyrine in the presence of peroxidase and can be quantified by monitoring the reaction's progress at a wavelength of 550 nm.

Determination of intracellular 3',5'-cyclic AMP concentrations

Platelets were isolated as described above. The concentrations of cAMP were determined using the PerkinElmer LANCE® Kit (Perkin-Elmer LAS GmbH) according to the manufacturer's protocol.

Statistical analysis

Results are expressed as arithmetic means and standard deviations or medians and interquartile ranges (the range between the lower quartile, Q1, and the upper quartile, Q3). The Anderson–Darling test was used to determine whether the data were normally distributed. The significance of differences was determined using ANOVA for repeated measures followed by Student's paired *t* test for parametric data.

Results

The effects of selected coffee constituents, extracts and brews on key elements of the cAMP signalling pathway in platelets were studied *in vitro*. To verify these *in vitro* observations, a pilot human intervention study investigating the *in vivo* effects of coffee consumption was carried out in eight healthy subjects (six males and two females) by monitoring platelet PDE activity and cAMP concentrations as well as plasma adenosine concentrations and ADA activity.

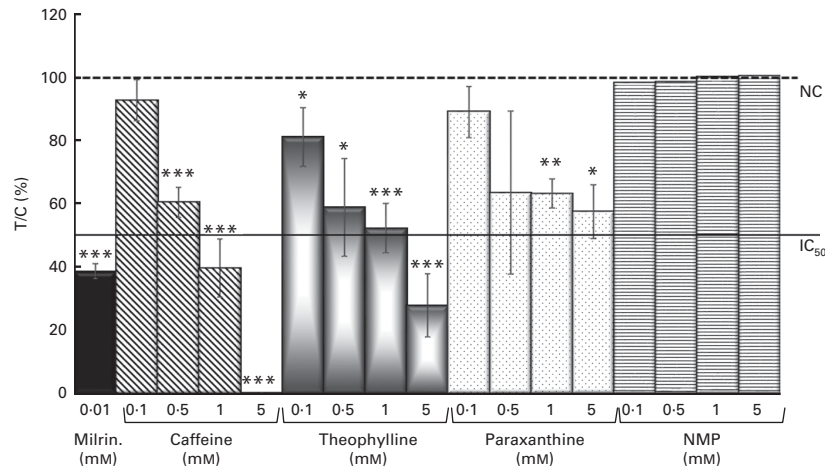


Fig. 2. Modulation of 3',5'-cyclic AMP-phosphodiesterase activity in platelet lysates by selected coffee constituents (caffeine, theophylline, paraxanthine and *N*-methylpyridinium (NMP)) and caffeine metabolites. Lysates were directly incubated with various concentrations of NMP, caffeine, theophylline and paraxanthine (0.1–5 mM). Values are means of five independent experiments (triplicates), with their standard deviations represented by vertical bars. Data were normalised to protein content and expressed as a percentage of the negative control (NC). Mean values were significantly different from that of the NC: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t* test). Positive control: Milrinone (Milrin., 10 μ M); NC: 1% dimethyl sulphoxide. The reported IC_{50} values (representing half-maximal inhibitory concentrations) are the means of the IC_{50} values from the triplicate experiments, each of which was calculated by linear regression. T/C (%): test over control, i.e. the observed result as a percentage of the NC (solvent) result.

In vitro phosphodiesterase experiments in platelet cell lysates

Platelets are known to express three PDE isoforms – PDE2, PDE3 and PDE5⁽³⁴⁾ – that contribute to the regulation of intracellular cAMP/3',5'-cyclic GMP homeostasis and platelet function. To evaluate the effects of coffee constituents on cAMP hydrolysis *in vitro*, platelets were isolated from platelet-rich plasma and platelet concentrates. Platelet lysates were incubated with test compounds and coffee extracts and their cAMP-hydrolysing activity was determined. The PDE isoenzyme pattern in each batch of the platelets was characterised by incubation with the inhibitors/modulators Rolipram (PDE4 inhibitor), Milrinone (PDE3 inhibitor), Zaprinast (PDE5 inhibitor) and Ca^{2+} /calmodulin (PDE1 stimulator). The selective PDE3 inhibitor Milrinone decreased the total cAMP-hydrolysing activity by about 60% in all the tested platelet lysates, indicating that PDE3 is the primary platelet isoenzyme. The other PDE modulators had no detectable effects or only marginal effects (data not shown). Rolipram (50 μ M) reduced platelet cAMP-hydrolysing activity by about 12% and Zaprinast (10 μ M) caused a reduction of about 20%. The addition of Ca^{2+} /calmodulin (0.2 μ M) did not induce significant additional cAMP hydrolysis.

The effects of individual coffee constituents and coffee extracts on PDE activity in platelet lysates are shown in Figs. 2–5. The coffee constituents tested had varying inhibitory effects on PDE activity in platelets. However, NMP did not exhibit any activity. Caffeine (half-maximal inhibitory concentration, $IC_{50} = 0.7$ (SD 0.1) mM) and theophylline ($IC_{50} = 0.9$ (SD 0.1) mM) exhibited pronounced effects, but the mammalian caffeine metabolite paraxanthine was clearly less active (Fig. 2). The tested pyrazines had PDE-inhibiting effects comparable to that of caffeine (Fig. 3), with 2,3-DE-5-MP being the most potent ($IC_{50} = 0.4$ (SD 0.1) mM).

The coffee extracts exhibited different PDE inhibition profiles in platelet lysates (Fig. 4), indicating that the degree of roasting has some effect on biological activity. The dark roast (AB2) had an IC_{50} value of 0.25 (SD 0.09) mg/ml and was a more potent inhibitor than the light roast (AB1), which had an IC_{50} of 0.5 (SD 0.1) mg/ml. As both extracts had similar caffeine contents (AB1: 52.5 mg/g; AB2: 40.0 mg/g), the degree of roasting clearly has additional effects on PDE activity inhibition. This conclusion is corroborated by the observation that extracts of commercial coffee (47.9 mg caffeine/g extract) and low-caffeine coffee (21.1 mg caffeine/g extract) had similar effects on platelet PDE activity (IC_{50} 4.1 (SD 0.5) and 4.2 (SD 1.6) mg/ml, respectively). It therefore seems that coffee constituents formed during the roasting process have a significant impact on the observed effects.

The CGA contents (i.e. the sum of the 3-, 4- and 5-CQA contents) of extracts AB1 and AB2 were 114 and 12.1 mg/g, respectively, while those of the commercial and low-caffeine coffees were 36.2 and 40.9 mg/g, respectively. Similarly, AB1 and AB2 brews differed significantly in their CGA contents (AB1: 1606.5 mg/l; AB2: 185.1 mg/l). However, the commercial and low-caffeine coffees had the same CGA contents (448 mg/l brew). In preliminary experiments (Fig. 5), 5-CQA and caffeic acid were found to exhibit rather potent PDE-inhibitory activity, with IC_{50} values of 0.49 (SD 0.01) and 0.48 (SD 0.09) mM, respectively. Further investigations are ongoing to extend these findings over a wider range of concentrations and substances. Overall, these *in vitro* results indicate that a range of non-caffeine coffee constituents may exhibit caffeine-like PDE-inhibitory activity in human platelet lysates, including pyrazines and certain polyphenols.

Platelet phosphodiesterase activity in vivo

Platelet PDE activity was evaluated in a human intervention study. Platelets were isolated from peripheral venous blood

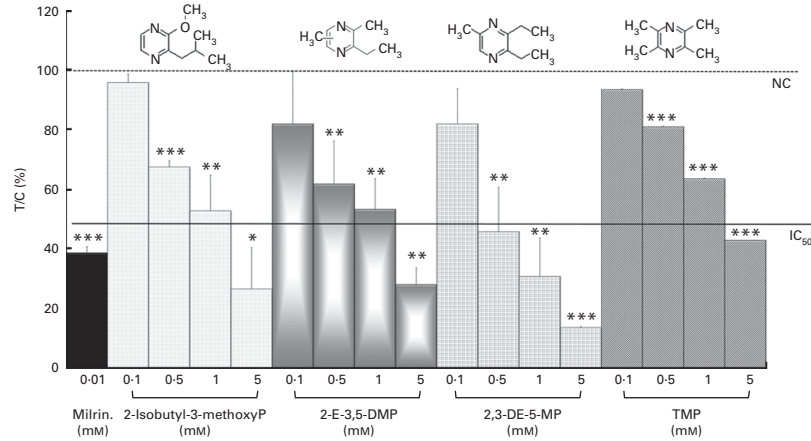


Fig. 3. Modulation of 3',5'-cyclic AMP-phosphodiesterase activity in platelet lysates by selected pyrazines (2-isobutyl-3-methoxypyrazine (2-isobutyl-3-methoxyP), 2-ethyl-3,5(6)-dimethylpyrazine (2-E-3,5(6)-DMP), 2,3-diethyl-5-methylpyrazine (2,3-DE-5-MP) and 2,3,5,6-tetramethylpyrazine (TMP)). Lysates were directly incubated with various concentrations of pyrazines (0.1–5 mM). Values are means of five independent experiments (triplicates), with their standard deviations represented by vertical bars. Data were normalised to protein content and expressed as a percentage of the negative control (NC). Mean values were significantly different from that of the NC: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t* test). Positive control: Milrinone (Milrin., 10 μ M); NC: 1% dimethyl sulphoxide. The reported IC_{50} values (representing half-maximal inhibitory concentrations) are the means of the IC_{50} values from the triplicate experiments, each of which was calculated by linear regression. T/C (%): test over control, i.e. the observed result as a percentage of the NC (solvent) result.

platelet-rich plasma samples collected from the subjects as described in the 'Materials and methods' section. The results of cAMP-hydrolysing activity assays using platelets from the eight subjects (three independent measurements per blood sample) are shown in Fig. 6. PDE activity increased significantly from an initial value of 38.9 (SD 9.3) (1st BC) to 72.7 (SD 6.6) pmol cAMP/min \times mg protein after the first washout phase (2nd BC). During the subsequent 2-week intervention with commercial coffee (3rd BC and 4th BC), a highly significant inhibition of PDE activity was observed (6.3 (SD 1.5) pmol cAMP/min \times mg protein). This effect persisted into the next washout phase (second washout, 5th BC). Platelet PDE activity then returned to levels observed in the first washout phase (2nd BC) over the following 2-week intervention with the low-caffeine coffee (71.8 (SD 6.5) pmol cAMP/min \times mg

protein, 7th BC). A further slight increase in PDE activity was observed in the 2nd week of the low-caffeine coffee intervention (7th BC). This increase persisted through the final washout phase, lasting until the end of week 7 (8th BC).

Platelet 3',5'-cyclic AMP concentrations in vivo

By the end of the first washout phase (2nd BC), platelet cAMP concentrations decreased from an initial value of 1.7 nm/mg protein (1st BC) to 0.2 nm/mg protein (Fig. 7). During the subsequent intervention with the commercial coffee, platelet cAMP concentrations increased to 1.3 nm/mg protein (4th BC). A further increase was observed after the second washout phase (5th BC). During the 1st week of the low-caffeine coffee intervention (6th BC), platelet cAMP concentrations

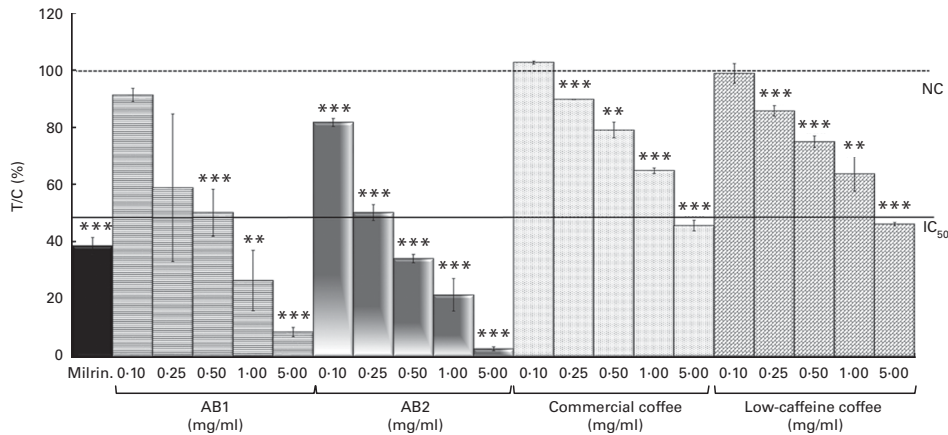


Fig. 4. Modulation of 3',5'-cyclic AMP-phosphodiesterase activity in platelet lysates by Arabica Brazil (AB) 1, AB2, commercial and low-caffeine coffee extracts. Lysates were directly incubated with various concentrations of coffee extracts (0.1–5 μ g/ml). Values are means of five independent experiments (triplicates), with their standard deviations represented by vertical bars. Data were normalised to protein content and expressed as a percentage of the negative control (NC). Mean values were significantly different from that of the NC: ** $P < 0.01$; *** $P < 0.001$ (Student's *t* test). Positive control: Milrinone (Milrin., 10 μ M); NC: 1% dimethyl sulphoxide. The reported IC_{50} values (representing half-maximal inhibitory concentrations) are the means of the IC_{50} values from the triplicate experiments, each of which was calculated by linear regression. T/C (%): test over control, i.e. the observed result as a percentage of the NC (solvent) result.

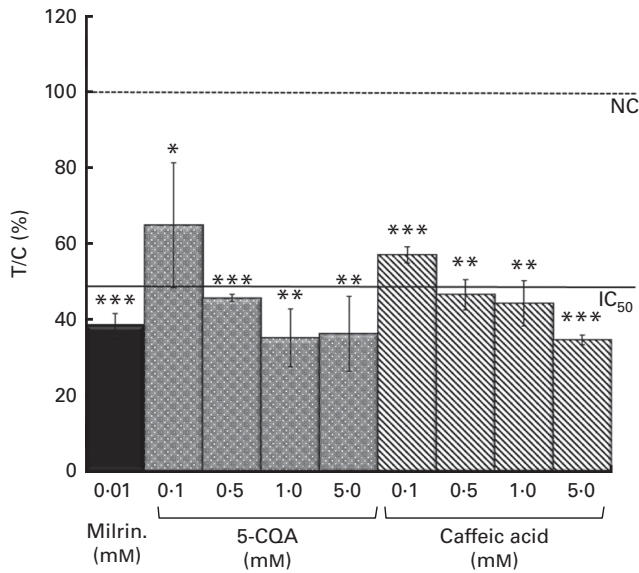


Fig. 5. Modulation of 3',5'-cyclic AMP-phosphodiesterase activity in platelet lysates by coffee polyphenols. Lysates were directly incubated with various concentrations of 5-O-caffeoylquinic acid (5-CQA) and caffeic acid (0.1–5 mM). Values are means of five independent experiments (triplicates), with their standard deviations represented by vertical bars. Data were normalised to protein content and expressed as a percentage of the negative control (NC). Mean values were significantly different from that of the NC: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's t test). Positive control: Milrinone (Milrin., 10 μM); NC: 1% dimethyl sulphoxide. The reported IC_{50} values (representing half-maximal inhibitory concentrations) are the means of the IC_{50} values from the triplicate experiments, each of which was calculated by linear regression. T/C (%): test over control, i.e. the observed result as a percentage of the NC (solvent) result.

decreased to almost the initial value (1st BC). However, after the 2nd week of the intervention (7th BC), platelet cAMP concentrations increased to a maximum of 4.5 nmol/mg protein, remaining at approximately this level during the final washout phase and until the end of the study (8th BC).

Effect of coffee consumption on plasma adenosine concentrations and adenosine deaminase activity

There was no appreciable change in plasma adenosine concentrations over the course of the study (Fig. 8). The concentrations exhibited a slight tendency to decline during the coffee intervention phases relative to the preceding washout phases. However, these changes were not significant. In addition, there was no difference between the responses to the study coffees. In addition, no changes were observed in ADA activity or uric acid concentrations (data not shown).

Discussion

In the present study, platelet cAMP concentrations and PDE activity were investigated as potential biomarkers of the biological effects of coffee consumption. Different coffee extracts and coffee constituents were studied to identify components with potential biological activity.

Caffeine has widely been used in biochemical studies to inhibit PDE until more potent (theophylline) and more

specific PDE inhibitors such as Rolipram became available⁽³⁵⁾. There is evidence that the caffeine concentrations required for PDE activity inhibition *in vitro* (which range from 100 to 1000 μM in mouse and pig cerebral cortex slices) are significantly higher than those required for AR interaction (10–100 μM)⁽³⁶⁾. With the exception of NMP, all the tested coffee constituents exhibited at least some platelet PDE-inhibitory activity. The PDE-inhibiting effect of caffeine was more pronounced than that of theophylline, while paraxanthine was only weakly active. The polyphenols 5-CQA and caffeic acid appeared to have activities similar to the tested pyrazines and caffeine. Coffee extracts also exhibited a marked PDE-inhibitory activity, indicating that inhibition is associated with the degree of roasting.

To evaluate the biological relevance of these findings, it is necessary to consider the concentrations of these biologically active constituents in coffee extracts. The light roast extract (AB1) contained a much higher concentration of phenolic acids (114 mg/g) than the dark roast (AB2; 12.1 mg/g). Moreover, extract AB1 contained about 20% more caffeine than extract AB2, and yet extract AB2 was more potent in terms of inhibiting PDE activity (Fig. 4) than extract AB1. Although NMP itself does not inhibit PDE activity, it is regarded as an indicator of the abundance of roast-associated compounds such as pyrazines and melanoidins, which may substantially contribute to inhibitory effectiveness. Interestingly, the commercial and low-caffeine coffees, with similar CGA contents, also had similar effects on PDE activity, despite the caffeine content of the latter being only 20% of that of the former. This indicates that CGA may also contribute to PDE activity inhibition associated with coffee consumption. However, as extract AB2 was appreciably more active than AB1, despite its CGA content being only 40% that of extract AB1, roast-associated compounds also appear to contribute significantly to PDE activity inhibition.

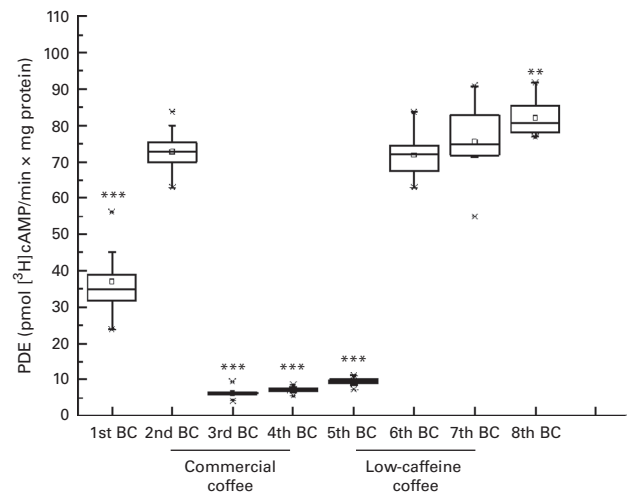


Fig. 6. Modulation of 3',5'-cyclic AMP (cAMP)-phosphodiesterase (PDE) activity in the platelets of subjects. Values are means ($n = 8$), with their standard deviations represented by vertical bars. Data were normalised to protein content and expressed in units of PDE activity (pmol cAMP/min \times mg protein). Mean values were significantly different from that observed during the first washout phase: ** $P < 0.01$; *** $P < 0.001$ (F test followed by Student's t test). BC, blood collection.

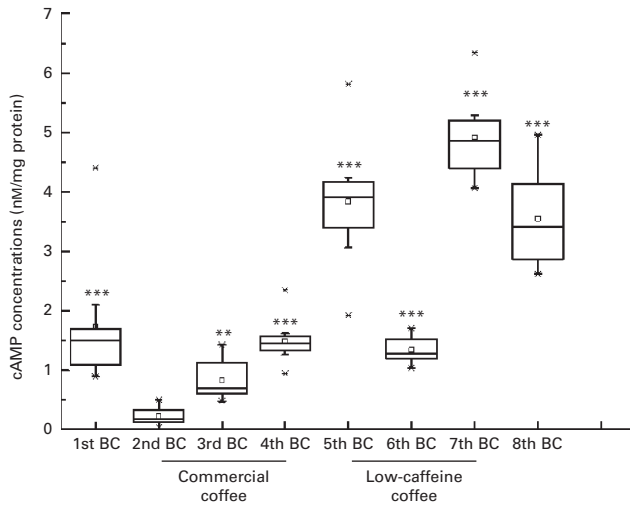


Fig. 7. Modulation of 3',5'-cyclic AMP (cAMP) concentrations in the platelets of subjects. Values are means (n 8), with their standard deviations represented by vertical bars. Data were normalised to protein content and expressed as cAMP concentrations (nmol/mg protein). Mean values were significantly different from that observed during the first washout phase: ** $P < 0.01$; *** $P < 0.001$ (F test followed by Student's t test). BC, blood collection.

The levels of coffee constituents in an individual's plasma (and in cells in which PDE activity inhibition might have significant biological effects) will depend on his or her coffee intake, caffeine content of the coffee, and individual variance concerning clearance times. The results of the present study strongly suggest the biokinetics of coffee constituents to reach inhibitory concentrations at relevant target sites, be it intracellular or at membrane receptors^(37,38). Overall, our findings strongly suggest that caffeine is not the only coffee constituent that contributes significantly to PDE activity inhibition in human platelet lysates and that roast-associated compounds apparently have particularly notable effects.

As shown in Figs. 6 and 7, the first washout (2nd BC) led to a significant increase in platelet PDE activity, which was mirrored by a strong reduction in cAMP concentrations. The subsequent 2-week intervention with the commercial coffee (3rd BC) resulted in a strong inhibition of PDE activity and a return of cAMP concentrations to about the baseline value (4th BC). After another week of washout (5th BC), PDE activity remained low, but cAMP concentrations had increased to approximately twice the baseline value. This may reflect a situation in which adenylyl cyclase became activated as a result of reduced AR antagonism due to the washout, while platelet PDE activity remained largely inhibited. During the subsequent intervention with the low-caffeine coffee, having only about one-third caffeine content of the commercial coffee, PDE activity rebounded to almost the levels observed after the first washout (6th BC). This was associated with the expected reduction in cAMP concentrations, which decreased to about the baseline value. To our surprise, PDE activity remained high at later time points (7th BC and 8th BC), but cAMP concentrations returned to the values (higher) observed before the second coffee intervention (5th BC).

We cannot offer a simple explanation for this effect. Mechanistic interpretation is hampered by the fact that the AR population expressed by the platelets and its dynamics could not be monitored. It has previously been suggested that although human platelets lose their nuclei during their differentiation from megakaryocytes, they still contain cytoplasmic mRNA and may thus retain some translation proficiency^(39,40). It has also been reported that human platelet adenosine (A_{2A}) receptors and their functions, such as the modulation of cAMP concentrations, were up-regulated in response to caffeine treatment over 1 (600 mg/d) or 2 weeks (400 mg/d). This prompted the suggestion that these effects might be induced during the differentiation of precursor cells rather than in mature platelets⁽⁴¹⁾. It has also been reported that treatment with the A_{2A} receptor agonist 2-hexynyl-5'- N -ethylcarboxamidoadenosine increased cAMP accumulation and inhibited platelet aggregation in controls, whereas caffeine consumption reduced them⁽²²⁾. We therefore cannot rule out the possibility that the AR status and function of the platelet populations examined in the pilot human intervention study may have changed between the late (7th BC and 8th BC) and earlier stages of the study and would therefore have responded differently to the various interventions at different time points.

The significant increase in platelet intracellular cAMP concentrations at the end of the second washout phase (5th BC; Fig. 7) could thus be interpreted to reflect, at least in part, the absence of the AR-antagonist caffeine in a situation of putative up-regulation of the A_{2A} receptors (stimulating adenylyl cyclase activity) in platelet precursor cells as a consequence of previous repeated coffee consumption. In addition, cAMP/protein kinase A-mediated activatory phosphorylation of PDE may also have contributed by

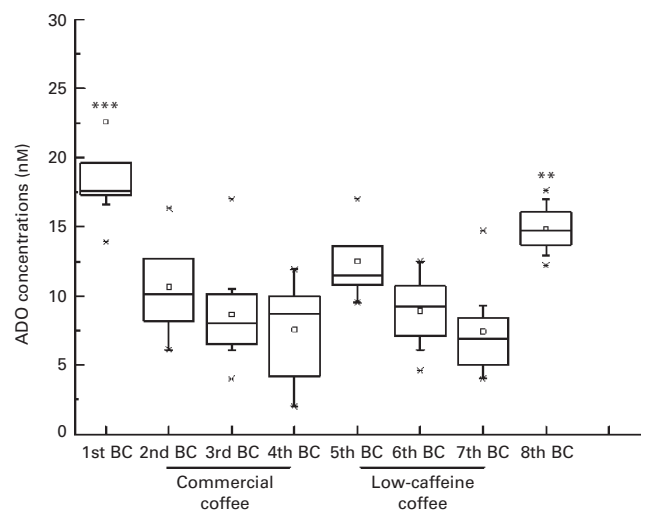


Fig. 8. Modulation of adenosine (ADO) concentrations in the plasma of subjects. Values are means (n 8), with their standard deviations represented by vertical bars. Data were expressed as ADO concentrations (nmol). Mean values were significantly different from that observed during the first washout phase: ** $P < 0.01$; *** $P < 0.001$ (F test followed by Student's t test). BC, blood collection.

inducing counterbalancing delayed cAMP synthesis⁽⁴²⁾. Similar effects of long-term caffeine treatment have been observed in mouse brain samples⁽⁴³⁾. However, these hypotheses remain to be confirmed.

Our *in vitro* findings may lead to the reasoning that to achieve plasma concentrations of caffeine and other coffee constituents required for direct PDE activity inhibition, it would be necessary to consume physiologically implausible quantities of coffee. In keeping with this reasoning, direct modulation of platelet PDE activity by caffeine due to coffee consumption is generally considered to be unlikely^(44–47). For example, it has been estimated that it would be necessary to consume near-convulsant doses of caffeine to achieve an effective direct inhibition of PDE activity *in vivo*.

Given this background, our *in vivo* findings were quite unexpected: our pilot human intervention study clearly showed that normal levels of daily coffee consumption have pronounced effects on platelet PDE activity in humans. Obviously, this novel finding will have to be confirmed in an extended human study. More importantly, the underlying cause(s) need to be elucidated. Apparently, caffeine is not the only coffee constituent that contributes to the PDE-inhibitory activity of coffee. As roast-associated compounds appear to be particularly relevant, the observed effects may be due to a combination of factors, also including those elicited by coffee phenolics. Differential biokinetics of all the relevant coffee constituents in the plasma and cellular/subcellular fractions may also contribute to reaching overall effective inhibitory concentrations in combination at relevant intracellular or membrane target sites⁽³⁶⁾. This is consistent with findings from a previous human coffee intervention study revealing coffee consumption to cause a significant increase in the conjugated phenolic acid content of platelets independent of caffeine and associated with decreased platelet aggregation⁽¹⁰⁾.

It is worth noting that several PDE inhibitors have found clinical applications as anti-platelet agents⁽³⁴⁾. Platelets are involved in the development of atherosclerotic diseases, and the reduction of platelet activity by these agents reduces the incidence and severity of these conditions. There is some evidence indicating that increased intra-platelet cAMP concentrations are associated with decreased platelet activation, aggregation and secretion. Platelets express a multitude of extracellular receptors that have varying impacts on intracellular signalling pathways via cAMP or calcium signalling. Both these species are important second messengers that play key roles in platelet activation, eventually leading to blood clotting and thrombosis. Reduced platelet aggregation is considered beneficial in the prevention of CVD.

Neither the activity of ADA nor the concentrations of adenosine in plasma samples were significantly affected by coffee consumption. The latter finding is inconsistent with findings in rats, which suggested that adenosine antagonists such as caffeine influence plasma adenosine concentrations in rats via a currently unknown mechanism and that caffeine doses corresponding to those achieved in humans consuming 3–6 cups of coffee/d are sufficient to induce these effects⁽⁴⁷⁾. The negligible effects on plasma adenosine concentrations observed in the human participants of our pilot study may

be due to the rapid and effective regulation of plasma adenosine homeostasis in humans via processes such as phosphorylation (mediated by adenosine kinase) and deamination (catalysed by ADA)^(48,49).

Conclusion

Taken together, the results of the present study provide evidence that moderate consumption of coffee may modulate platelet aggregation, a critical process in CVD and thrombosis, at least in part by modulating PDE activity and cAMP homeostasis in humans. Such effects appear to be mediated by a combination of coffee constituents and roast-associated compounds of as yet unidentified identity seemingly playing a particular role alongside caffeine and phenolics. Further detailed mechanistic investigations will be required to substantiate such beneficial health effects of coffee consumption and to elucidate the underlying mechanisms.

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The authors' contributions are as follows: G. A. M., M. B., G. E. and T. B. designed and performed the *in vivo* study; M. H. and G. A. M. established the PDE assay; G. A. M. measured cAMP concentrations and PDE activity *in vivo* and *in vitro*; G. A. M. and T. E. developed and performed the adenosine analysis; M. E. performed the ADA and uric acid measurements; G. E. and E. R. supervised G. A. M.'s work. All authors read and approved the final manuscript.

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