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Caffeine metabolites are inhibitors of the nuclear enzyme poly(ADP-ribose)polymerase-1 at physiological concentrations

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ABSTRACT

The activity of the nuclear enzyme poly(ADP-ribose)polymerase-1 (E.C.2.4.2.30), which is highly activated by DNA strand breaks, is associated with the pathophysiology of both acute as well as chronic inflammatory diseases. PARP-1 overactivation and the subsequent extensive turnover of its substrate NAD^+ put a large demand on mitochondrial ATP-production. Furthermore, due to its reported role in NF-κB and AP-1 mediated production of pro-inflammatory cytokines, PARP-1 is considered an interesting target in the treatment of these diseases.

In this study the PARP-1 inhibiting capacity of caffeine and several metabolites as well as other (methyl)xanthines was tested using an ELISA-assay with purified human PARP-1. Caffeine itself showed only weak PARP-1 inhibiting activity, whereas the caffeine metabolites 1,7-dimethylxanthine, 3-methylxanthine and 1-methylxanthine, as well as theobromine and theophylline showed significant PARP-1 inhibiting activity. Further evaluation of these compounds in H_2O_2-treated A549 lung epithelial and RF24 vascular endothelial cells revealed that the decrease in NAD^+-levels as well as the formation of the poly(ADP-ribose)polymer was significantly prevented by the major caffeine metabolite 1,7-dimethylxanthine. Furthermore, H_2O_2-induced necrosis could be prevented by a high dose of 1,7-dimethylxanthine. Finally, antioxidant effects of the methylxanthines could be ruled out with ESR and measurement of the TEAC.

Concluding, caffeine metabolites are inhibitors of PARP-1 and the major caffeine metabolite 1,7-dimethylxanthine has significant PARP-1 inhibiting activity in cultured epithelial and endothelial cells at physiological concentrations. This inhibition could have important implications for nutritional treatment of acute and chronic inflammatory pathologies, like prevention of ischemia-reperfusion injury or vascular complications in diabetes.

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1. Introduction

Poly(ADP-ribose)polymerase-1 (PARP-1, E.C. 2.4.2.30), a nuclear enzyme present in eukaryotes, is highly activated upon induction of DNA strand breaks and activation results in the hydrolysis of NAD^+ to form poly(ADP-ribose)polymers and nicotinamide. PARP-1 has various physiological functions; it is involved in base excision repair (BER), which was indicated by delayed repair in PARP-1 knockout mice and by interaction of PARP-1 with other members of the BER-machinery like XRCC1 and DNA polymerase β [1,2]. PARP-1 is also a regulator of transcriptional activity, for instance, PARP-1 is reported to play a role in NF-κB and AP-1 mediated production of pro-inflammatory cytokines [3–9].

The activity of PARP-1 is associated with the pathophysiology of acute inflammatory diseases like stroke and ischemia-reperfusion and also with chronic inflammation in diabetes and with pulmonary diseases such as chronic obstructive pulmonary disease (COPD) [10–13]. PARP-1 overactivation, as a result of ROS-induced DNA damage, and the subsequent extensive turnover of its substrate NAD^+ puts a large demand on mitochondrial ATP-production and cellular energy status [14]. This can result in an energy crisis and lead to a necrotic cell death [15]. PARP-1 is therefore considered an interesting target in the treatment of these diseases. Many potent synthetic PARP-1 inhibitors have been developed during the last years, but several studies showed that food components also have PARP-1 inhibiting activity [16–20]. These involve the methylxanthine theophylline (1,3-dimethylxanthine, 13X), which has been reported to have PARP-1 inhibiting activity [21]. Theophylline is one of the major metabolites of caffeine (1,3,7-trimethylxanthine), which is present in cocoa and beverages like coffee, tea and cola, and is extensively metabolized in the human body after oral intake. Caffeine is demethylated by the hepatic enzyme cytochrome P4501A2 (CYP1A2) to its major metabolites 1,7-dimethylxanthine (paraxanthine, 17X), theobromine (3,7-dimethylxanthine, 37X) and theophylline. Further demethylation of 1,7-dimethylxanthine by CYP1A2 results mainly in the formation of 1-methylxanthine (1X) and 1-methyl uric acid (1 U). 3-Methylxanthine (3X) is formed after demethylation of theobromine and theophylline [22,23].

The aim of this study was to test caffeine and several metabolites as well as other (methyl)xanthines (Fig. 1) as possible inhibitors of PARP-1. First, the PARP-1 inhibitory activity of the compounds was tested using an assay with the purified enzyme. Second, the effect of the PARP-1 inhibitors on hydrogen peroxide (H_2O_2)-induced NAD^+-depletion and necrosis was tested in vitro in cultured pulmonary epithelial and vascular endothelial cells.

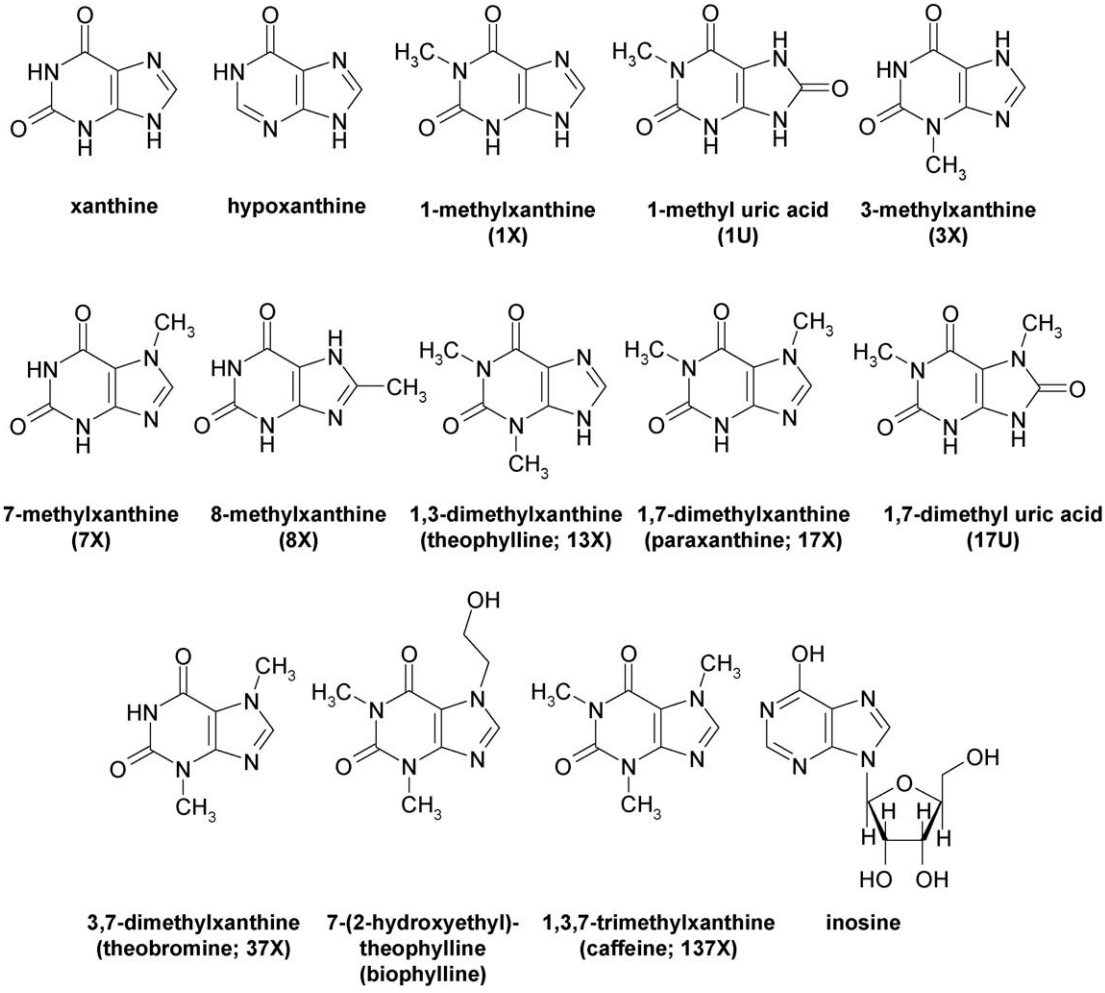

Fig. 1 – Molecular structure of the tested compounds.

2. Material and methods

2.1. Materials

Caffeine, xanthine, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 8-methylxanthine, 1,7-dimethylxanthine, 1-methyluric acid, 1,7-dimethyluric acid, theophylline, theobromine, hypoxanthine, inosine, tween 20, acetic acid, 3,3′,5,5′-tetramethylbenzidine, H_2O_2, bovine serum albumine (BSA), heparin, Dulbecco's modified Eagle's medium (DMEM), propidium iodide, trolox and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were all obtained from Sigma (St. Louis, MO, USA). Biofylline, β-NAD^+ and 1,4-dithiothreitol (DTT) were obtained from MP Biomedicals (Irvine, CA, USA). Human rPARP-1 and biotinylated NAD^+ were purchased from Trevigen (Gaithersburg, MD, USA). F-12K nutrient mixture (Kaighn's modification), fetal bovine serum, glutamate, trypsin, penicillin/streptomycin and HBSS were all obtained from Invitrogen Life Technologies (Grand Island, NY, USA). Peroxidase-labeled streptavidin was purchased from Zymed (San Francisco, CA, USA). DMPO and $FeSO_4$ were obtained from Merck (Darmstadt, Germany). Polyvinylchloride microtiter plates and endothelial cell growth supplement were obtained from BD Biosciences (San Jose, CA, USA). 2,2′-Azino-bis(2-amidinopropane) dihydrochloride (ABAP) was obtained from Brunschwig Chemicals (Amsterdam, The Netherlands). The 10H hybridoma was kindly provided by Dr. M. Miwa, via Riken Cell Bank, Tsukuba Institute BioResource Center (Ibaraki, Japan). The cell supernatant containing mouse monoclonal anti-PAR polymer antibody 10H was produced by Dr. W. Buurman (University of Maastricht, Maastricht, The Netherlands). Polyclonal goat anti-mouse immunoglobulin/FITC and fluorescent mounting medium were obtained from DAKO (Glostrup, Denmark). The ApoGlow kit was obtained from Cambrex (Verviers, Belgium).

2.2. PARP-1 inhibition ELISA

The capacity of the compounds to inhibit PARP-1 was first determined using an inhibition assay, as described by Decker et al. [24] and Brown and Marala [25] with minor modifications. In short, human rPARP was incubated with a reaction mixture containing 50 μM β-NAD^+ (10% biotinylated β-NAD^+, 90% unlabelled β-NAD^+), 1 mM DTT and 1.25 μg/ml nicked DNA. Nicked DNA was prepared as follows, according to the method of Aposhian et al. [26] with some minor modifications: Calf thymus DNA was incubated with 10 ng/ml DNase I at 37 °C during 40 min. The length of the DNA-fragments (500 bp) was estimated using gel electrophoresis.

To determine the optimal substrate concentration and incubation temperature for the PARP-1 assay, human rPARP-1 was incubated with various concentrations β-NAD^+ (0–150 μM; 10% biotinylated β-NAD^+, 90% unlabelled β-NAD^+) at two different temperatures (4–37 °C; Fig. 2). Based on this optimization, rPARP-1 was incubated with 50 μM β-NAD^+ at 4 °C. Using these conditions, 100 μM 3-aminobenzamide and nicotinamide were used as positive controls and inhibited the enzyme 85.1 ± 0.9 and 73.5 ± 3.6%, respectively. To determine background activity, the enzyme was also incubated with a reaction mixture without β-NAD^+.

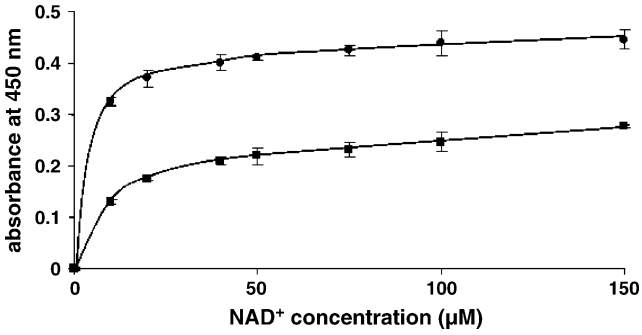

Fig. 2 – PARP-1 activity expressed as absorbance at 450 nm after incubating 400 ng/ml human rPARP-1 for 1 h in the presence of various concentrations β-NAD^+, 1 mM DTT and 1.25 μg/ml nicked DNA at 4 °C (●) and 37 °C (■).

After incubations with the reaction mixture, the plates were washed and the formation of poly(ADP-ribose)-polymers was detected after a 1 h incubation at room temperature with peroxidase-labeled streptavidin, followed by a 15 min incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of H_2O_2 at 37 °C. This reaction was stopped by adding 0.75 M HCl and the absorbance was measured at 450 nm. PARP-1 inhibition of several compounds was evaluated by addition of these compounds to the reaction mixture. Measurements were done in three-fold.

2.3. Cell culture

A549 human lung epithelial cells were grown at 37 °C in a humidified 5% CO_2 atmosphere and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, 10% fetal bovine serum (FBS), 2 mM glutamine and 50 units/ml of penicillin and 50 μg/ml of streptomycin. RF24 human vascular endothelial cells were grown at 37 °C in a humidified 5% CO_2 atmosphere in gelatine-coated culture flasks and were cultured in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% fetal bovine serum, 0.1 mg/ml heparin and 50 units/ml of penicillin and 50 μg/ml of streptomycin. During culturing at low densities, the medium was supplemented with 0.015 mg/ml endothelial cell growth supplement.

2.4. Cellular NAD^+ assay

Cells were plated in a 96-well plate (approximately 3×10^4 cells/well) and cultured for 24 h before exposure. H_2O_2 was used to induce DNA strand breaks, activate PARP-1 and deplete cellular NAD^+ levels. To determine the optimal H_2O_2-concentration and incubation period for the cells, cells were first treated with different concentrations H_2O_2 (0–300 μM) for different periods of time (0–60 min), as previously described [21]. Maximal decreases in NAD^+-levels in both A549 and RF24 cells were reached after treatment with 300 μM H_2O_2 during 30 min. The PARP-1 inhibitor 3-aminobenzamide strongly prevented the decrease in NAD^+-levels and served in all the experiments as a positive control. During the

experiments, cells were preexposed to the methylxanthines for 15 min and subsequently exposed to 300 μM H_2O_2 and the methylxanthines for 30 min. The maximal DMSO-concentration was 0.1% and was also used as a control. After the exposure, the cells were lysed in ethanol (50 μl/well) and stored at −80 °C. During this lysis, 20 mM isonicotinic acid hydrazine was used to inhibit NAD-glycohydrolase and to prevent NAD^+-hydrolysis [27]. After lysing the cells, intracellular NAD^+ was determined using the NAD^+-cycling method, as described by Jacobson and Jacobson [28] with minor modifications. After thawing and lysis of the cells, 100 μl reaction mixture without ethanol was added to the wells. The final reaction mixture in the microtiter plate contained 2.86 M ethanol, 114 mM bicine, 4.8 mM EDTA, 0.95 mg/ml BSA, 47.6 mg/ml alcohol dehydrogenase, 1.9 mM phenazine ethosulfate and 0.48 mM MTT. The reaction was measured in time spectrophotometrically at 540 nm at 37 °C during 15 min. A standard curve (0–1.5 μM) was included to calculate the NAD^+-levels in the cells. The calculated NAD^+-levels for each experiment were average values of at least four measurements.

2.5. Immunofluorescent staining of PAR-polymers

To determine the effect of 1,7-dimethylxanthine on the formation of the PAR-polymer, A549 cells (approximately 0.8×10^6 cells/well in 6-well plate) were 15 min pretreated with 1,7-dimethylxanthine and thereafter treated with H_2O_2 for 5 min. After the incubation, the cells were trypsinized, washed with PBS and fixed in methanol. After fixation, cells were stained as described by Hageman et al. [12] with some modifications. Fixed cells were washed with 0.1% BSA in PBS and incubated with 100 μl mouse monoclonal anti-PAR polymer antibody 10H for 1 h at room temperature. After washing with 0.1% BSA, the cells were incubated with 100 μl polyclonal goat anti-mouse immunoglobulin/FITC for 1 h at room temperature. After washing again with 0.1% BSA, the cells were incubated for 15 min with 200 μl, 20 μg/ml propidium iodide. Cells were transferred to slides and were mounted with fluorescent mounting medium. At least 100 cells per slide were evaluated for the presence of PAR polymers in their nucleus using fluorescence microscopy and Lucia GF 4.80 software. Subsequently, the percentage of PAR polymer positive cells was calculated.

2.6. Measurement of the ADP:ATP ratio

To determine the effect of 1,7-dimethylxanthine on H_2O_2-induced cell death, A549 and RF24 cells (approximately 3×10^4 cells/well in 96-well plate) were 30 min pretreated with 1,7-dimethylxanthine and thereafter treated with H_2O_2 for 8 h. After 1 h incubation with H_2O_2 at 37 °C, FBS was supplied to a 10% final concentration and cells were incubated for additional 7 h. After the incubation, the ADP:ATP ratio was determined using the ApoGlow kit. The assay is based on the bioluminescent measurement of the adenylate nucleotides and can be used to determine the mode of cell death [29]. After 8 h incubation, cells were lysed and processed for ATP and ADP measurements according to the instruction manual.

2.7. Electron spin resonance spectroscopy

Electron spin resonance (ESR) studies were performed at room temperature using a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12 kW power supply operating at X band frequencies. The following instrument conditions were used: scan range, 60 G; center magnetic field, 3490 G; modulation amplitude, 1.0 G; microwave power, 50 mW; receiver gain, 10^5; modulation frequency, 100 KHz; microwave frequency, 9.85 GHz; time constant, 40.96 ms; scan time, 20.97 s; number of scans, 25. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was used for trapping hydroxyl radicals and was purified in nitrogen flushed Milli-Q water by mixing with 30 mg/ml charcoal during 20 min at 35 °C. This procedure was used three times to remove background ESR signals. Finally, the concentration of DMPO was determined spectrophotometrically at 234 nm. Incubation mixtures (total volume 200 μl) consisted of $FeSO_4$ (0.5 mM), H_2O_2 (0.55 mM) and DMPO (100 mM) in the absence or presence of methylxanthines (100 μM). Quercetin was used as a positive control. ESR spectra were recorded using 100 μl capillary glass tubes. Quantitation of the spectra (in arbitrary units) was performed by peak integration using the WIN-EPR spectrum manipulation program.

2.8. TEAC assay

The TEAC assay (Trolox Equivalent Antioxidant Capacity assay) was used to test the total radical scavenging capacity of the compounds. This assay is based on the ability of a compound to scavenge the stable ABTS radical ($ABTS^{\bullet+}$). The blue–green $ABTS^{\bullet+}$ was produced through the reaction between 0.36 mM ABTS and 1.84 mM ABAP in 145 mM sodium phosphate buffer, pH 7.4, at 70 °C until the absorption of the solution reached 0.70 ± 0.02 at 734 nm. In the assay, 50 μl of the tested compounds was added to 950 μl of the $ABTS^{\bullet+}$ solution. The reduction in absorbance at 734 nm was measured in time for 5 min and was compared to a blank where 50 μl of the solvent was added to 950 μl of $ABTS^{\bullet+}$ solution. A calibration curve was constructed using different concentrations of the synthetic antioxidant trolox (0–20 μM). Uric acid was used as a positive control. The TEAC value of the methylxanthines was defined as the concentration (mM) of trolox having an antioxidant capacity equivalent to 1 mM methylxanthine.

3. Results

3.1. Enzyme assay

To assess the effect of the methylxanthines on PARP-1 activity, purified rPARP-1 was incubated with various methylxanthines. In Table 1 data are shown of PARP-1 inhibition by the various methylxanthines (100 μM). These results reveal 1,7-dimethylxanthine to be a very potent inhibitor of PARP-1 ($68.5 \pm 1.2\%$). The 1-, 3-, 7-, 1,3- and 3,7-methylated xanthines (1-methylxanthine, 3-methylxanthine, 7-methylxanthine, theophylline and theobromine, respectively) moderately

Table 1 – Percentage PARP-1 inhibition and IC_{50} values of the tested compounds

Compound	% inhibition (100 μm)	IC_{50} (μM)
Xanthine	n.i.	n.d.
Hypoxanthine	16.2 ± 6.2	>200
1-Methylxanthine	46.2 ± 0.0	145.0 ± 2.8
1-Methyl uric acid	n.i.	n.d.
3-Methylxanthine	49.6 ± 4.3	115.2 ± 11.2
7-Methylxanthine	24.5 ± 4.2	172.3 ± 3.4
8-Methylxanthine	18.5 ± 4.1	>200
Theophylline	43.2 ± 2.0	194.8 ± 23.3
1,7-Dimethylxanthine	68.5 ± 1.2	15.0 ± 0.97
1,7-Dimethyl uric acid	n.i.	n.d.
Theobromine	40.5 ± 1.3	160.2 ± 0.5
Biophylline	10.8 ± 5.5	>200
Caffeine	8.0 ± 4.7	>200
Inosine	n.i.	n.d.

n.i. = no inhibition. n.d. = not determined. PARP-1 activity was measured after incubating 400 ng/ml human rPARP-1 for 1 h in the presence of 50 μM β-NAD^+, 1 mM DTT and 1.25 μg/ml nicked DNA at 4 °C. Values are presented as average ± S.E. of two replicate experiments with $n = 3$ measurements.

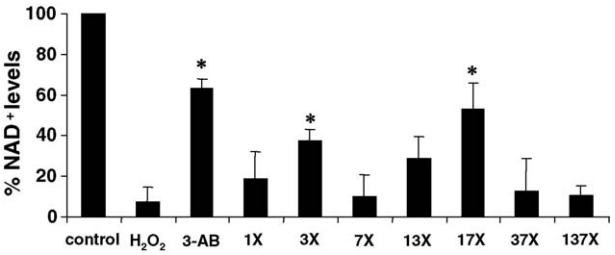

Fig. 3 – NAD^+ levels in A549 human pulmonary epithelial cells after 30 min incubation with 300 μM H_2O_2 with or without 15 min pretreatment with 100 μM of the indicated methylxanthines or 3-aminobenzamide (3-AB). Values are expressed as % of control incubation (without H_2O_2) and are average ± S.E. of at least two replicate experiments with $n = 4$ measurements. * indicates a statistically significant difference according to Student's t-test ($P < 0.05$) as compared to incubation with H_2O_2 without methylxanthines.

inhibited PARP-1. Xanthine, 1-methyl uric acid, 1,7-dimethyl uric acid and inosine showed no inhibition at all. The parent compound 1,3,7-trimethylxanthine (caffeine) showed only a very weak inhibition. For methylxanthines that inhibited PARP-1 activity more than 50% at the tested concentrations, an IC_{50} was determined (Table 1). The strongest PARP-1 inhibitor was found to be 1,7-dimethylxanthine with an IC_{50} of 15.0 μM. Other IC_{50} were 115.2, 145.0, 160.2, 172.3 and 194.8 μM for 3-methylxanthine, 1-methylxanthine, theobromine, 7-methylxanthine and theophylline, respectively.

3.2. NAD^+-levels

Methylxanthines, which showed PARP-1 inhibition in the enzyme-assay ($IC_{50} < 200$ μM), were also screened for their effect on NAD^+-levels in H_2O_2 treated A549 cells. Also, the parent compound caffeine was further evaluated in the A549 cells. The H_2O_2-induced depletion of the intracellular NAD^+-stores was used as a measure of PARP-1 activation. In the A549 cells, pre-incubation with 100 μM 3-methylxanthine and 1,7-dimethylxanthine resulted in significantly higher NAD^+-levels, when compared to cells treated with 300 μM H_2O_2 (Fig. 3). To further evaluate the PARP-1 inhibiting activity of 1,7-dimethylxanthine, both A549 and RF24 cells were exposed for 30 min to 300 μM H_2O_2 after pre-incubating for 15 min with different concentrations 1,7-dimethylxanthine (Fig. 4). In these cells, 1,7-dimethylxanthine significantly prevented the H_2O_2-induced decrease in NAD^+-levels in a dose-dependent manner.

3.3. PAR-polymer formation

To further confirm the PARP-1 inhibiting activity of 1,7-dimethylxanthine, A549 cells were exposed to 300 μM H_2O_2 for 5 min after pre-incubating for 15 min with 1,7-dimethylxanthine (Fig. 5). A significantly decreased number of PAR-polymer positive cells was observed after incubation with 100 μM 1,7-dimethylxanthine, confirming the results of the enzyme assay and the NAD^+-measurements.

3.4. ADP:ATP ratio

To investigate the effect of 1,7-dimethylxanthine on H_2O_2-induced cell death, the ADP:ATP ratio was determined. ADP:ATP ratios for cells undergoing apoptosis are expected to be higher than control values but below 1, while much higher values characterize necrotic cells [29]. Incubation of both the epithelial and the endothelial cells with 10 mM H_2O_2 increased the ADP:ATP ratio to values of 2.1 ± 0.4 and 9.1 ± 0.8 respectively, indicating necrotic cell death. Treatment of the cells with lower concentrations H_2O_2 failed to increase the

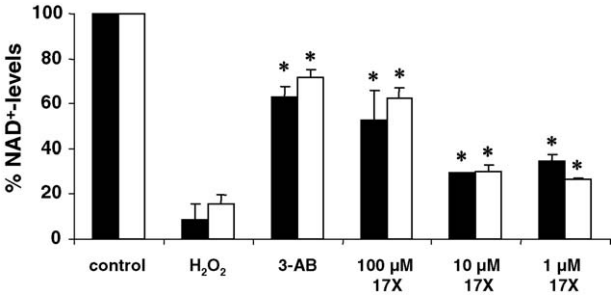

Fig. 4 – NAD^+ levels in A549 human pulmonary epithelial (black bars) and RF24 human vascular endothelial cells (white bars) after 30 min incubation with 300 μM H_2O_2 with or without 15 min pretreatment with 100, 10 and 1 μM 1,7-dimethylxanthine or 100 μM 3-aminobenzamide (3-AB). Values are expressed as % of control incubation (without H_2O_2) and are average ± S.E. of at least two replicate experiments with $n = 4$ measurements. * indicates a statistically significant difference according to Student's t-test ($P < 0.05$) as compared to incubation with H_2O_2 without 1,7-dimethylxanthine.

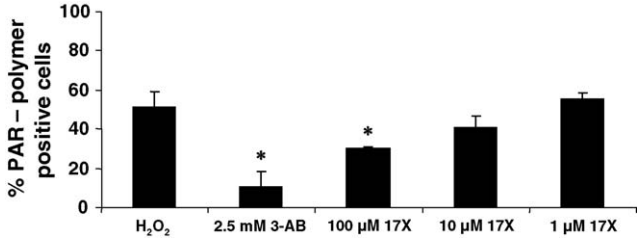

Fig. 5 – Percentage of PAR-polymer positive A549 cells after 5 min incubation with 300 μM H_2O_2 with or without 15 min pretreatment with 100, 10 and 1 μM 1,7-dimethylxanthine or 2.5 mM 3-aminobenzamide (3-AB). Results are average ± S.E. of two replicate experiments with $n = 2$ measurements. * indicates a statistically significant difference according to Student's t-test ($P < 0.05$) as compared to incubation with H_2O_2 without 1,7-dimethylxanthine.

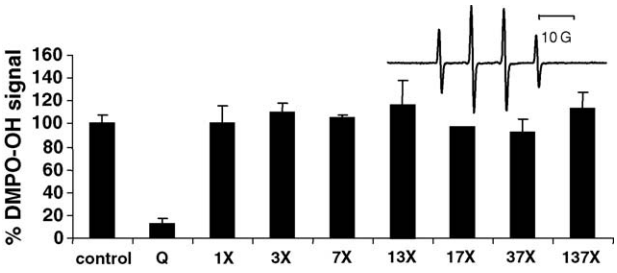

Fig. 7 – Hydroxyl radicals, generated by $FeSO_4$ and H_2O_2, are not scavenged by 100 μM methylxanthines. Values are expressed as % of control ($FeSO_4$+ H_2O_2 + DMPO) and are presented as average ± S.E. of duplicate measurements. Q = quercetin (100 μM). Insert: ESR spectrum for the hydroxyl radical spin adduct (DMPO-OH).

ADP:ATP ratio. Pretreatment of epithelial and endothelial cells with 1 mM 1,7-dimethylxanthine reduced necrosis, as shown by a statistically significant lower ADP:ATP ratio of 0.6 ± 0.4 and 2.0 ± 0.8, respectively (Fig. 6).

3.5. *Electron spin resonance spectroscopy*

To rule out antioxidant effects of the methylxanthines, which were tested in the A549 and RF24 cells, scavenging of hydroxyl radicals was measured using ESR spectroscopy. First, hydroxyl radicals were generated by H_2O_2 in the presence of $FeSO_4$. In combination with the spin-trap DMPO, stable DMPO-OH adducts were formed. Addition of the methylxanthines (100 μM) did not decrease the observed DMPO-OH signal (Fig. 7).

3.6. *TEAC assay*

The total radical scavenging capacity of the methylxanthines was assessed using the TEAC assay. In Table 2 TEAC values of the methylxanthines are presented. The TEAC values are very low and negligible, which indicates that these methylxanthines do not have significant antioxidant effects.

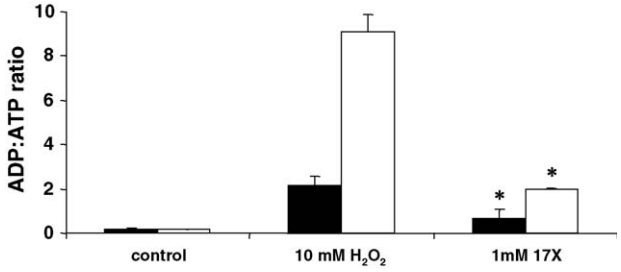

Fig. 6 – ADP:ATP ratio in A549 (black bars) and RF24 (white bars) cells after 8 h incubation with 10 mM H_2O_2 with or without 30 min pretreatment with 1 mM 1,7-dimethylxanthine. Results are average ± S.E. of three measurements. * indicates a statistically significant difference according to Student's t-test ($P < 0.05$) as compared to incubation with H_2O_2 without 1,7-dimethylxanthine.

4. Discussion

The present study revealed that the major caffeine metabolite 1,7-dimethylxanthine is a PARP-1 inhibitor and is more potent than the parent compound caffeine. The potency of caffeine, caffeine metabolites and other methylxanthines to inhibit PARP-1 was investigated using purified human rPARP-1 and cultured pulmonary epithelial and vascular endothelial cells. A previous study already reported that the methylxanthine theophylline can inhibit PARP-1 [21]. Results from our enzyme studies have shown that the major caffeine metabolite 1,7-dimethylxanthine is even more potent in inhibiting PARP-1. The parent compound caffeine showed only very weak inhibition in spite of its application as a PARP-1 inhibitor [30,31]. Other methylxanthines tested in this study, such as 1-methylxanthine and 3-methylxanthine, also inhibited the purified enzyme showing decreased formation of the PAR-polymer, indicating a decreased consumption of the substrate NAD^+. This decreased depletion of NAD^+ was also observed in cultured epithelial and endothelial cells treated with a high concentration of H_2O_2. In both cell lines, the inhibiting activity of 1,7-dimethylxanthine was demonstrated by higher

Table 2 – TEAC values for the methylxanthines

Compound	TEAC
1-Methylxanthine	0.14 ± 0.02
3-Methylxanthine	0.06 ± 0.01
7-Methylxanthine	0.05 ± 0.01
Theophylline	0.06 ± 0.01
1,7-Dimethylxanthine	0.05 ± 0.01
Theobromine	0.05 ± 0.02
Caffeine	0.05 ± 0.01
Uric acid	1.06 ± 0.02

Values are expressed as mM trolox equivalents for 1 mM methylxanthine and are presented as average ± S.E. of two duplicate measurements.

NAD^+-levels upon treatment with H_2O_2 in presence of this methylxanthine. Also, a significantly reduced number of PAR-polymer positive cells was detected after pre-incubation with 1,7-dimethylxanthine. Results from these cell studies confirm that PARP-1 is significantly inhibited by the major caffeine metabolite 1,7-dimethylxanthine. Also, the caffeine metabolite 3-methylxanthine prevented the decline in NAD^+-levels in the A549 cells.

The presence of one or two methyl groups appears important for inhibition of the purified PARP-1 enzyme. The dimethylxanthine 1,7-dimethylxanthine clearly inhibited PARP-1 (IC_{50}: 15.0 μM). Also 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) inhibited PARP-1 (IC_{50}: 145.0, 115.2, 172.3, 184.8 and 160.2 μM respectively), in contrast to xanthine which did not show any PARP-1 inhibition. However, this inhibition appeared not dependent solely on the number or the position of the methyl groups. 1-Methylxanthine and 3-methylxanthine showed stronger inhibition when compared to the dimethylxanthines theophylline and theobromine. 1,7-Dimethylxanthine could inhibit the enzyme stronger than theophylline (1,3-dimethylxanthine), while 7-methylxanthine was less potent in inhibiting PARP-1 as compared to 3-methylxanthine. Furthermore, the trimethylxanthine caffeine showed very weak inhibition. The metabolites of 1-methylxanthine and 1,7-dimethylxanthine, 1-methyl uric acid and 1,7-dimethyl uric acid respectively, showed no inhibiting activity. Application of a Free–Wilson analysis could not indicate a strong association between specific methyl groups and inhibiting activity. In conclusion, no obvious structure-activity relationship with regard to the position of the methyl groups and PARP-1 inhibiting effects could be observed. The presence of a methyl group clearly increases the inhibiting potency of the xanthines, but the number of methyl groups also appears to be a limiting factor.

Since the human body is exposed daily to oxygen radicals which can damage cells and tissues, treatment with hydrogen peroxide in this in vitro model is considered a physiologically relevant model of PARP-1 activation. Possible antioxidant activity of the methylxanthines, however, had to be ruled out, since the observed effects on cellular NAD^+ could be due to scavenging activity of the methylxanthines. Using ESR-spectroscopy and the TEAC assay, scavenging of (hydroxyl) radicals by the methylxanthines was determined. None of the methylxanthines tested showed any relevant radical scavenging activity at the concentrations used in the cell studies, suggesting that even at concentrations of 1 or 10 μM the observed effects on the cellular NAD^+-levels were most likely due to PARP-1 inhibition.

We showed that incubation of epithelial and endothelial cells with 10 mM H_2O_2 for 8 h induced necrotic cell death as indicated by a dramatically increased ADP:ATP ratio of approximately 2.1 ± 0.4 and 9.1 ± 0.8, respectively. Treatment of cells with 1,7-dimethylxanthine significantly reduced the ADP:ATP ratio. The H_2O_2 concentration used was rather high, but lower concentrations failed to significantly increase the ADP:ATP ratio in these cells (data not shown). After incubation with 1 mM 1,7-dimethylxanthine, necrotic cell death was reduced and, in the epithelial cells, also a shift to apoptosis could be observed, indicated by decreased ADP:ATP ratio values between control values and 1. Several studies showed that PARP-1 activation is required for a necrotic type of cell death and that PARP-1 inhibition or PARP-1 gene deletion can prevent or reduce induction of necrosis [15,32,33]. These studies indicate that inhibition of PARP-1 could have therapeutic value in pathophysiology of diseases like ischemia-reperfusion injury or stroke. Complete inhibition of PARP-1 is not considered favourable, since PARP-1 has been demonstrated to be involved in repair of DNA damage [34,35]. It is therefore thought advisable to have residual PARP-1 activity to allow for efficient repair of damaged DNA. Moderate inhibition of PARP-1 activation appears to be the best practice.

In diabetes, PARP-1 has been reported to be involved in the development of diabetic vascular dysfunction. Animal studies showed that, in PARP-1 knock-out mice or in wildtype mice treated with PARP-1 inhibitors, the endothelial dysfunction was reversed [11,20]. Also, the importance of PARP-1 in the process of beta-cell death has already been extensively studied [36]. Recently, a number of epidemiological studies showed a strong relation between intake of specific food components and risk for type 2 diabetes [37,38]. In the study of Salazar-Martinez et al. [38], long-term coffee consumption was associated with a lower risk for type 2 diabetes. In their study, also a high intake of caffeine was related to a decreased risk for type 2 diabetes [38]. They suggested that a possible mechanism for the observed inverse association could be the presence of antioxidants in coffee and their effect on glucose metabolism and insulin resistance. Since caffeine is extensively metabolized in the human body after oral intake, the formation of caffeine metabolites and the possible effect of these metabolites on the risk of diabetes should also be taken into account. Results of our study demonstrate that 1,7-dimethylxanthine, the major metabolite of caffeine, and not caffeine itself has clear PARP-1 inhibiting activity. By inhibiting PARP-1, this caffeine metabolite could reduce endothelial tissue damage and inflammatory processes, which are known features of diabetes [39,40]. Even at concentrations as low as 10 and 1 μM, 1,7-dimethylxanthine significantly prevented the H_2O_2-induced decrease in NAD^+-levels in both pulmonary epithelial and in vascular endothelial cells. These concentrations are considered to be physiological plasma levels. Tang-Liu et al. [22] reported that oral intake of caffeine in a dose representing moderate coffee intake (4 cups/day), resulted in maximal plasma levels of approximately 20 μM 1,7-dimethylxanthine lasting for several hours after intake.

In conclusion, caffeine metabolites and in particular 1,7-dimethylxanthine have PARP-1 inhibiting activity at physiological concentrations. Inhibition of PARP-1 could have important implications for the development of vascular dysfunction and inflammation, processes, which are observed in the progression of diabetes. Furthermore, PARP-1 inhibition is also shown to be relevant in other pathologies like ischemia-reperfusion, stroke and pulmonary inflammatory diseases such as COPD and asthma [12,13,32,41]. It is therefore suggested that dietary PARP-1 inhibitors like the caffeine metabolite 1,7-dimethylxanthine may contribute to treatment or prevention of vascular complications in diabetes but also other pathologies like mild ischemia-reperfusion damage and stroke.

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