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ORIGINAL INVESTIGATION

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4-Caffeoyl-1,5-quinide in roasted coffee inhibits [^{3}H]naloxone binding and reverses anti-nociceptive effects of morphine in mice

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Abstract *Rationale:* Cinnamoylquinides are formed from the corresponding chlorogenic acids during coffee roasting. Instant coffee has been shown to displace binding of the mu opioid receptor antagonist, [^{3}H]naloxone, but the putative active agent, feruloylquinide, has not been characterized. *Objectives:* The goal was to identify the active agent(s) in coffee by measuring the binding affinity of individual cinnamoyl-1,5-quinides to the human mu opioid receptor, and determine the effects of these compounds on morphine-induced anti-nociceptive behavior in mice. *Methods:* Cinnamoyl-1,5-quinides in extracts of decaffeinated instant coffee were quantified by reverse-phase HPLC comparisons with synthetic samples of 3-coumaroyl-1,5-quinide and 4-coumaroyl-1,5-quinide, 3-caffeoyl-1,5-quinide and 4-caffeoyl-1,5-quinide (4-CQL) 3-feruloyl-1,5-quinide and 4-feruloyl-1,5-quinides and 3,4-dicaffeoyl-1,5-quinide (DICAQ). Affinities of the cinnamoyl-1,5-quinides and decaffeinated instant coffee extract were determined by displacement of [^{3}H]naloxone binding in cultured HEK-MOR cells. Inhibition of the anti-nociceptive activity of morphine (1 mg/kg IP) was determined in C57BL/6J mice using the hot plate test at 52°C. *Results:* Extract of decaffeinated instant coffee produced a displacement K_i of 42±16 mg/l, while the K_i of a synthetic sample of 4-CQL was 4.4±0.4 μM. Compounds with a cinnamoyl substituent in the 4-position of the quinide, i.e. 4-CQL, DICAQ, 3,4-diferuloyl-1,5-quinide, and 3,4-dicoumaroyl-1,5-quinide, had affinities for the mu opioid receptor in the low micromolar range. In the hot plate test, coffee extract, containing 0.78% of 4-CQL, reversed the anti-nociceptive effect of morphine at 10 mg/kg IP. Two cinnamoyl-1,5-quinides found in roasted coffee, DICAQ, and 4-CQL, were active at 1 and 0.1 mg/kg IP, respectively. *Conclusions:* These results suggest that the previously reported anti-opioid activity of instant coffee is caused primarily by the presence of 4-CQL, and to lesser extent by other cinnamoyl-1,5-quinides.

Keywords Coffee · Mu opioid receptor · Hot plate · Mouse · Morphine · Naloxone · Chlorogenic acid lactones

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Introduction

Since the introduction of coffee to Western culture 300 years ago, coffee has replaced alcohol as the most widely consumed psychoactive beverage (James 1998). Numerous studies have addressed the potential health risks and benefits from coffee consumption (Schilter et al. 2001). Epidemiological studies have focused on the relationship between coffee and cancers of the colon, pancreas, bladder, kidney, prostate, breast, and ovaries. Some early studies found weak associations with coffee intake and cancer. However, subsequent studies with better control for confounding factors, such as smoking and dietary habits, found no adverse relationship (Schilter et al. 2001). In contrast, several studies found that coffee has a protective effect against disease. Similar conclusions have been drawn from studies of the relationship between coffee and cardiovascular disease, despite the fact that coffee causes elevated plasma levels of homocysteine. One exception is drinking boiled coffee without filtering that results in the consumption of cholesterol-raising diter-

penes, which may cause increased heart disease (Ugert and Katan 1997). Further, high caffeine intake from coffee (500 mg/day) has been shown to cause 11% delay in time of conception in fertile European women (Bolumar et al. 1997). The mechanism is unknown but could be associated with a moderate loss of calcium after drinking coffee without milk (Schilter et al. 2001).

The beneficial effects of coffee are usually attributed to its content of caffeine (James 1998; Fredholm et al. 1999). For example, caffeine potentiates the anti-nociceptive effect of non-steroidal anti-inflammatory agents and is often used as an adjuvant in pain medication (Granados-Soto and Castaneda-Hernandez 1999). Recent studies have indicated that patients suffering from chronic back pain consume twice the amount of coffee compared to healthy individuals (McPartland and Mitchell 1997), suggesting an association between coffee consumption and pain relief. Such effects could be the result of constituents in coffee other than caffeine. Coffee is unique among plant-derived beverages, because of the presence of *p*-hydroxycinnamoylquinides (quinolactones), which are formed in the roasting process by thermal dehydration of chlorogenic acids (Clifford 1985, 2000). Cinnamoylquinides are neutral, lipophilic compounds with capacity to enter the brain and, therefore, potentially active in the CNS. Recently, we have shown that some cinnamoyl-1,5-quinides in coffee are inhibitors of the adenosine transporter as demonstrated by their ability to displace [^{3}H]-4-nitrobenzyl-6-thioinosine binding to the human adenosine transporter in cultured cell homogenates (de Paulis et al. 2002). Further, instant coffee displaced [^{3}H] naloxone binding in rat brain homogenates (Boublik et al. 1983). Although the active anti-opioid agent was never identified, mass spectroscopic evidence suggested one or several structural isomers of feruloyl-1,5-quinide (Wynne et al. 1987). These studies have suggested that roasted coffee contain constituents with psychoactive properties, other than caffeine. Based on the results of Boublik and Wynne, we sought to identify cinnamoylquinides in coffee that might be responsible for the anti-opioid activity. The goal was to measure the in vitro binding affinity of cinnamoyl-1,5-quinides in cultured cells expressing the human mu opioid receptor and to determine whether cinnamoyl-1,5-quinides can modulate the anti-nociceptive effects of morphine in vivo in mice.

Materials and methods

Drugs

3-Coumaroyl-1,5-quinide, 4-coumaroyl-1,5-quinide, 3-caffeoyl-1,5-quinide (3-CQL), 4-caffeoyl-1,5-quinide (4-CQL), 3-feruloyl-1,5-quinide (3-FQL), 4-feruloyl-1,5-quinide (4-FQL), 3,4-dicoumaroyl-1,5-quinide (DICOQ), 3,4-dicaffeoyl-1,5-quinide (DICAQ), and 3,4-diferuloyl-1,5-quinide (DIFEQ) were synthesized from quinic acid and the corresponding 4-hydroxycinnamic acids using the method of Wynne et al. (1985), as described by Huynh-Ba (1995). Starting cinnamic acids and reagents for the syntheses, 5-caffeoylquinic acid (chlorogenic acid), naloxone hydrochloride, and morphine hemisulfate pentahydrate were obtained from Sigma-Aldrich, Milwaukee, Wisc., USA. Tritiated naloxone with a specific radioactivity of 63 Ci/mmol was obtained from PerkinElmer Life (NEN), Boston, Mass., USA. Because chronic administration of caffeine has been shown to increase the potency of morphine (Ahlijanian and Takemori 1986), we used decaffeinated instant coffee, which contains 3% of the caffeine found in regular coffee, according to the manufacturer. In order to increase the amount of cinnamoyl-1,5-quinides in coffee, water soluble constituents of instant coffee were removed by ethyl acetate extraction as follows. A sample of commercial decaffeinated instant coffee (5 g) was dissolved in water (100 ml) and adjusted to pH 4 with 2 N hydrochloric acid (HCl). The lipophilic cinnamoyl-1,5-quinides were extracted with ethyl acetate (2×200 ml). Foaming was suppressed by addition of 2 ml ethanol. The combined extract was washed with water (2×100 ml). Drying and evaporation of the solvent gave 0.50±0.05 g of residual oil (10% of the original weight). For binding studies, the extract (0.5 g) was dissolved in 5 ml 50% aqueous dimethylsulfoxide (DMSO) and diluted with assay buffer to 10 mg/ml. For behavioral studies, the coffee extract (0.5 g) was dissolved in 50% aqueous Tween-80 (4 ml, Sigma) and diluted, first with water (16 ml) and then with 10% Tween-80 to 10 mg/ml. In order to compare the content of the extracts with those of normally prepared instant coffee, we analyzed regular and decaffeinated samples by HPLC. Decaffeinated instant coffee 0.5 g was dissolved in 100 ml water and the brown solution was clarified using Carrez reagents, according to Trugo and Macrae (1984a) as follows. The coffee solution was treated with 1 ml each of 0.3 M $K_2Fe(CN)_6$ and 1 M $Zn(OAc)_2$, shaken and let stand for 10 min, followed by filtration of the precipitated salts (Whatman No. 1).

HPLC analysis

The method of Trugo and Macrae (1984b) for analyzing chlorogenic acids was used. The coffee preparations were diluted 1000-fold with 40% aqueous methanol. A sample (5 µl) was injected into a reverse-phase HPLC column (Rexchrom ODS C18, 4.6×250 mm; Regis, Norton Grove, Ill., USA) using a manual injector (Rheodyne) equipped with a 5 µl loop. Mobile phase: 20% methanol in 10 mM citric acid (adjusted to pH 2.5 with 2N HCl). A linear gradient of 20–65% methanol from 0 to 60 min was used at a combined flow rate of 1 ml/min. The presence of cinnamoyl-1,5-quinides was detected with a UV spectrophotometer operating at 325 nm (Waters Model 486, Millipore Corp., Billerica, Mass., USA). The content of each compound in the coffee extract was calculated by comparing the area of each peak with that of 0.05 µg of the appropriate standard treated the same way. Data were

recorded using Easy Chrom Elite software (ESA, Chelmsford, Mass., USA).

Cell culture

HEK-MOR cells (courtesy of Dr. Tim Hales, George Washington University, Washington D.C., USA) expressing the human mu-1 receptor were grown in high glucose Dulbecco's modified Eagle's medium supplemented with $NaHCO_3$ (40 mM), 10% fetal bovine serum (Gibco, Grand Island, N.Y., USA), 400 mg/l geneticin, and 0.1 mM hypoxanthine. The cells were cultured at 37°C in tissue culture flasks (75 cm^2, Falcon) under an atmosphere of CO_2/air (5/95, v/v). When reaching confluency (70–80%), the cells were trypsinized and harvested by centrifugation. Harvested cells were re-suspended in approximately 30 times the pellet volume in 50 mM Tris buffer at 4°C, the cell membranes were disrupted by Polytron homogenization (12,500 rpm for 25 s), and the suspension was centrifuged at 60,000*g* for 60 min. The supernatant was discarded and the pellet was diluted to 0.2–0.5 mg protein/ml in 50 mM Tris buffer. Protein content was measured by the Lowry phenol method using bovine serum albumin (Sigma) as standard.

Mu opioid receptor binding

The method of Pert and Snyder (1974) was used. The cell membranes in final concentration of 0.03–0.10 mg protein/ml were incubated with [^{3}H]naloxone at 2 nM concentration in 50 mM Tris buffer (pH 7.5) with and without 50 mM NaCl at 4°C for 1.5 h in a total volume of 1.0 ml. Non-specific binding was defined with 10 μM naloxone. Each determination was carried out in triplicate. Bound and free [^{3}H]naloxone were separated by vacuum filtration through fiberglass filters (Schleicher & Schuell, Keene, N.H., USA) that were presoaked with 0.3% polyethylenimine (Sigma) for 60 min, using a Brandel M-24R cell harvester. The filters were washed three times for 10 s with ice-cold 50 mM Tris buffer and placed in 20 ml vials containing 10 ml scintillation fluid (Cytoscint; ICN). After shaking the vials for 30 min, beta spectrometry was performed using a Beckman L5801 instrument at 47% counting efficiency. IC_{50} values and Hill slopes (n_H) were calculated from log-logit analysis of competition binding data. K_i values of the competing ligands were calculated from IC_{50} values using the Cheng–Prusoff equation, $K_i = IC_{50}/(1+L/K_D)$, where L is the concentration of [^{3}H]naloxone and K_D is its equilibrium dissociation constant obtained from Scatchard analysis of saturation binding. Data are expressed as mean±SEM.

Hot plate nociception

Eight-month-old male and female mice (C57BL/6J, Bar Harbor, Maine, USA) were placed on a hot plate maintained at 52°C (Model 35D; IITC Corp., Woodland Hills, Calif., USA). Animals were confined to the hot surface area by a plastic cylinder measuring 15 cm in diameter and 12.5 cm height. Five behaviors were considered to be responses to thermal stimulation: paw licking, lifting, or shaking, animal jumping or retreating (Mogil et al. 1996; Plone et al. 1996; King et al. 2003). The latency for the animal to display any of these behaviors was measured with a stopwatch to one-tenth of a second with 30 s maximum. Test compounds were dissolved in 10% Tween-80 (20 mg/ml) with the exception of morphine, which was dissolved in saline. Dose–response studies were conducted by administering either cinnamoylquinides alone 15 min prior to testing or morphine (1 mg/kg IP) 30 min and cinnamoylquinides 15 min prior to testing in groups of six to ten animals per dose. Time-course studies were conducted by testing the animals 15, 30, 60, and 90 min after treatment. The study was approved by Vanderbilt Institutional Animal Care and Use Committee and NIH principals of laboratory animal care were complied with.

Table 1 Displacement of [^{3}H]naloxone binding in cultured HEK-MOR cell homogenates by cinnamoyl-1,5-quinides and their abundance in extracts of decaffeinated instant coffee

Compound	Abundance[a](%)	Affinity (K_i μM)	Hill slope	n[b]
4-Caffeoylquinide (4-CQL)	0.78	4.4±0.4	1.01±0.04	3
3-Caffeoylquinide (3-CQL)	1.48	105±12	0.66±0.07	3
3,4-Dicaffeoylquinide (DICAQ)	0.06	15.1±3.4	1.00±0.05	8
4-Feruloylquinide (4-FQL)	0.16	90±21	1.07±0.12	3
3-Feruloylquinide (3-FQL)	0.32	41±8	1.09±0.08	3
3,4-Diferuloylquinide (DIFEQ)	<0.02	11.9±2.0	1.26±0.09	5
3,4-Dicoumaroylquinide (DICOQ)	<0.02	3.9±0.8	1.28±0.09	5
Instant coffee extract (decaffeinated)		42±16[c]	0.98±0.07	6

[a]Amounts of cinnamoyl-1,5-quinides in extracts of decaffeinated instant coffee used in behavioral experiments as measured by reverse-phase HPLC
[b]Number of experiments, performed in triplicate
[c]K_i value in mg/l

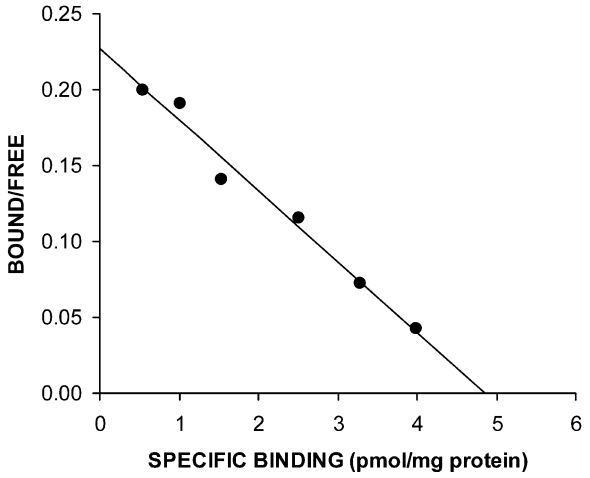

Fig. 1 Scatchard analysis of specific saturation binding of [^{3}H] naloxone binding in cultured HEK-MOR cell homogenates. Specific radioactivity was 63 Ci/mmol. The resulting equilibrium dissociation constant K_D was 2.19 nM and the receptor density B_{max} was 4.88 pmol/mg protein

Data analysis

Hot plate studies were compared using between subjects design, except the time-course study, which was compared within subjects. Two-group analyses were conducted using Student *t*-tests. Experiments with three or more groups were analyzed using factorial analysis of variance (ANOVA). Within-subjects data were analyzed using repeated measures ANOVA (RMANOVA). Follow-up tests were conducted using Bonferroni-Dunn corrected *t*-tests, in which the α level is adjusted for the number of comparisons made, and only between groups for which we had a priori hypotheses. Significance was defined as $P<0.05$.

Results

HPLC analysis of instant coffee

The total amount of chlorogenic acids and cinnamoyl-1,5-quinides in the coffee extracts used in this study were 8.2 and 2.9%, respectively. Extraction of instant coffee with ethyl acetate and washing with water caused a fourfold increased concentration of total chlorogenic acids in the extracts from 2.1% and a tenfold increase in total cinnamoyl-1,5-quinides from 0.29% in the original coffee preparations. The increases in individual cinnamoyl-1,5-quinide concentrations in the extracts were also approximately 10-fold, with 3-CQL having the highest concentration at 1.5% (Table 1).

Mu opioid receptor binding

Scatchard analysis of [^{3}H]naloxone binding in cultured HEK-MOR cell preparation showed a K_D of 2.5±0.3 nM and a B_{max} of 4.9 pmol/mg protein (Fig. 1). Affinity constants of cinnamoyl-1,5-quinides for displacing 2 nM [^{3}H]naloxone binding in the HEK-MOR cell homogenate is shown in Table 1, together with their respective amounts in extracts of decaffeinated instant coffee. These binding experiments have confirmed that the feruloyl-1,5-quinides 3-FQL, and 4-FQL have moderate affinities with K_i 41 and 90 μM, respectively, in displacement of [^{3}H]naloxone binding to the human mu opioid receptor. In addition, we

Table 2 Effects of morphine or 3,4-dicinnamoyl-1,5-quinides alone on hot plate detection latencies in mice

Compound	Dose (mg/kg IP)	Latency (s)[a]	*n*[b]
Saline + morphine	0	11.68±1.00	10
	0.5	16.06±1.41	10
	1.0	20.09±1.42	10
	1.5	20.00±2.12	10
	2.0	19.68±1.38	10
10% Tween-80 + DIFEQ[c]	0	14.10±0.87	6
	10	12.88±1.51	6
	20	12.02±1.73	6
	30	14.10±2.12	6
	50	13.22±2.03	6
	100	9.23±0.97	7
10% Tween-80 + DICAQ[c]	0	8.81±0.60	8
	10	10.53±1.12	10
	30	8.67±0.76	10
	50	9.41±0.77	8

[a]Mean latency±SEM to exhibit one of five different responses (paw licking, lifting, or shaking, animal jumping or retreating) to hot plate at 52°C
[b]Number of animals per dose
[c]Latency measured 15 min after administration of dicinnamoylquinides

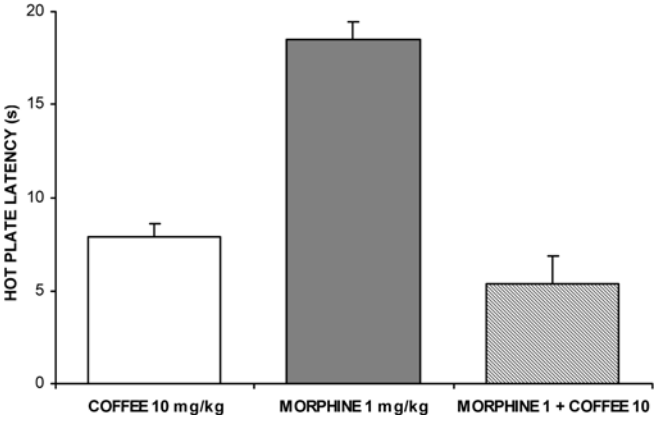

Fig. 2 Reversal of the anti-nociceptive effects of morphine in C57BL/6J mice after administration of decaffeinated instant coffee extract (10 mg/kg IP) containing 2.9% total cinnamoyl-1,5-quinides. Latency was measured as the time for each animal to exhibit any of five responses to the hot plate at 52°C (paw licking, lifting, or shaking, animal jumping or retreating). Coffee by itself was not significantly different (P=0.10) from the average effect of vehicle (10% Tween-80). Morphine (1 mg/kg IP) was given 15 min prior to treatment. The bars represent mean±SEM (n=8)

found that the more abundant 4-CQL has 10 times higher affinity for the MOR-1 binding site than either 3-FQL or 4-FQL with a K_i of 4.4 μM (Table 1). The compound with highest affinity was DICOQ with K_i 3.9 μM. Extracts of decaffeinated instant coffee showed a K_i value of 42 ±16 mg/l (n=6), respectively. Hill slopes were equal to unity, indicating binding to a single class of receptors. Both caffeine and 5-caffeoylquinic acid (chlorogenic acid) at 100 μM concentration displaced less than 10% binding. Opiate receptor antagonists can be distinguished from agonists by the sodium dependency of the latter (Pert and Snyder 1974). In the presence of high sodium concentration the affinities of full and partial agonists decrease, while those of antagonists remain unaffected or even increase. The affinities of DICAQ in the absence and presence of 50 mM sodium chloride were 41±6 and 126 ±23 μM (n=2), respectively. The affinities of naloxone in the absence and presence of 50 mM sodium chloride were 0.0037 and 0.0029 μM (n=1), respectively. This confirms that naloxone is an antagonist at the mu opioid receptor, as expected, while suggesting that DICAQ is a partial agonist. No significant effect of sodium was seen with coffee extract (K_i 4.7 and 5.5 mg/l, respectively).

Nociceptive studies

Hot plate latencies of saline were not different from pooled data of latencies under 10% Tween-80, i.e. 11.68±1.00 s (n=10), and 10.07±0.54 s (n=42), respectively [t(42)=1.7, P=0.10]. Using increasing doses of morphine, latency to exhibit any of the five signs of nociceptive behavior increased significantly to 20.09±1.42 s (n=10) with 1 mg/kg IP of morphine [F(4,45)=6.3, P=0.004]. The latency did not increase further with 1.5 or 2 mg/kg [t(18)=0.2, P=0.84] (Table 2). When 10 mg/kg IP of coffee extract was administered alone (Fig. 2), hot plate latency was 7.90 ±0.93 s (n=8), which was not significantly different from the average latency of 10% Tween-80 [t(48)=1.7, P=0.10]. However, when coffee extract (10 mg/kg IP) was given after morphine (1 mg/kg IP), complete reversal of the anti-nociceptive effect of morphine was seen from 18.50 ±1.48 s with morphine alone to 5.36±0.45 s with morphine plus coffee extract [F(2,21)=49.8, P<0.0001]. Follow-up tests indicate that latencies under coffee extract plus morphine were significantly lower than latencies under morphine alone [t(14)=9.4, P<0.0001].

Next, the effects of three cinnamoyl-1,5-quinides, DIFEQ, DICAQ, and 4-CQL, on morphine-induced anti-nociception were studied in separate experiments. Neither DIFEQ nor DICAQ showed anti-nociceptive activity by themselves, when given at doses up to 50 mg/kg IP (Table 2). At the highest dose of DIFEQ (100 mg/kg IP), a significant decrease in latency was observed relative to vehicle (10% Tween-80) [t(13)=3.49, P=0.04] (Table 2). However, in this experiment, the latency for vehicle alone (14.10±0.87 s) was unusually high and significantly different from those of the other experiments [t(50) =2.15, P=0.036]. In the morphine experiments, the hot plate latencies with vehicle alone were 11.77±1.03 s (n=10), 9.94±0.63 s (n=16), and 8.57±0.86 s (n=18), respectively (Fig. 3a,b,c). Administration of morphine alone (1 mg/kg IP) resulted in each experiment in significant increase in latency to reach 17.51±0.86 s (n=17), 17.41±0.89 s (n=16), and 17.67±0.51 s (n=18), respectively. DIFEQ, DICAQ, and 4-CQL dose-dependently reversed the effect of morphine. Administration of DIFEQ significantly blocked morphine-induced increase in hotplate latency [F(5,64)=16.3, P<0.0001] (Fig. 3a). Follow-up tests showed that doses of DIFEQ 10 and 20 mg/kg did not significantly reverse morphine-induced latency [10 mg/kg: t(23)=1.72, P=0.099; 20 mg/kg: t(23) =2.5, P=0.020]. Doses of DIFEQ 30 mg/kg [t(46)=4.79, P<0.0001] and higher completely blocked the effect seen with morphine. Administration of DICAQ significantly blocked the effects of morphine on hotplate latency [F (6,71)=26.8, P<0.0001] (Fig. 3b). Follow-up tests showed that this effect was evident at every dose tested (all t-values >3.0, all P-values <0.0065). For example, a dose of 1 mg/kg DICAQ reversed morphine-induced latency [t(22) =3.0, P=0.006]. Doses of DICAQ 10 mg/kg and higher reduced the latency to levels significantly lower than that of vehicle [t(32)=3.12, P=0.004]. 4-CQL was the most potent of the three compounds in reversing the effects of morphine [F(8,77)=42.4, P<0.0001] (Fig. 3c). Follow-up tests showed that the three lowest doses of 4-CQL did not significantly reverse morphine-induced hotplate latency [0.01 mg/kg: t(24)=0.58, P=0.57; 0.03 mg/kg: t(24)=0.91, P=0.37; 0.05 mg/kg: t(24)=0.94, P=0.36]. However, doses of 0.1 mg/kg and higher completely blocked the effect of morphine on the hotplate [t(24)=4.11, P=0.0004]. Similar to DICAQ, doses of 4-CQL 0.5 mg/kg and higher produced latencies below those of control. The time course of the anti-opioid effect of DICAQ at 3 mg/kg IP showed complete inhibition that lasted for at least 60 min [F(5,35)=8.24, p<0.0001]. At 15, 30, and 60 min after treatment the latencies were 11.00±1.11, 10.76±1.00, and 11.03±0.97 s, respectively. Follow-up planned comparisons demonstrate that hot plate latencies under DICAQ were still reduced at 90 min compared to treatment with morphine plus Tween-80 (14.10±1.96 s vs 19.26±0.94 s), but this difference did not reach statistical significance [t (14)=2.4, P=0.03].

Discussion

This study demonstrates that roasted coffee contains compounds that are pharmacologically active on the human opioid receptor system. Our studies with decaffeinated coffee extracts (de Paulis and Martin 2004) have confirmed the previous results of coffee as having binding affinity to mu opioid receptors (Boublik et al. 1983). Previous studies of instant coffee did not clarify, whether the binding affinity was caused by a single compound or a mixture of similar compounds (Boublik et al. 1983; Wynne et al. 1987). Based on mass spectroscopic evidence, a positional isomer of feruloyl-1,5-quinide was

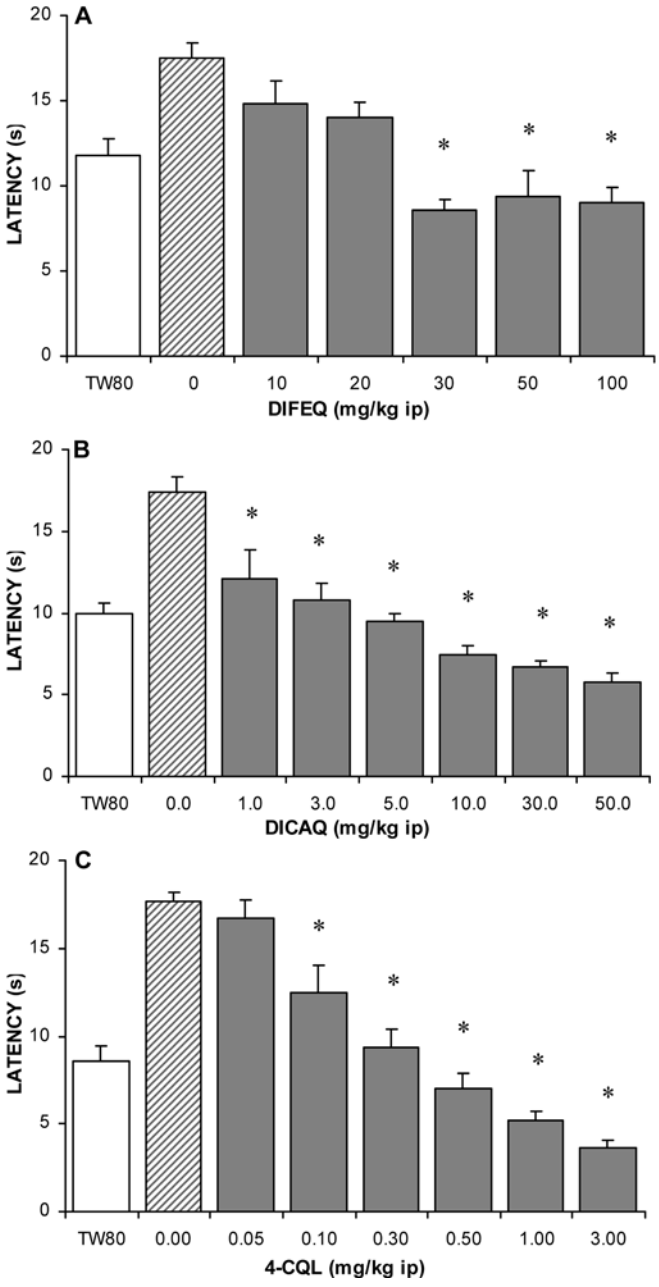

Fig. 3 Reversal of the anti-nociceptive effects of morphine in C57BL/6J mice after **a**3,4-diferuloyl-1,5-quinide (*DIFEQ*), **b** 3,4-dicaffeoyl-1,5-quinide (*DICAQ*), and **c**4-caffeoyl-1,5-quinide (*4-CQL*). Morphine (1 mg/kg IP) was given 15 min prior to cinnamoyl-1,5-quinides. The bars represent mean±SEM of each experiment (n=8). Results of morphine and vehicle (10% Tween-80) are the average of two separate experiments. Significant inhibitions (P<0.05) were seen at **a** 30 mg/kg IP, **b**1 mg/kg IP, and **c** 0.1 mg/kg IP, respectively, and denoted with an *asterisk*

proposed to be the active constituent. Caffeoyl-1,5-quinides and feruloyl-1,5-quinides are formed from their corresponding cinnamoylquinic acids by thermal dehydration in the roasting process. The latter are mixtures of mono-esters and di-esters of various 4-hydroxycinnamic acids with the aliphatic alcohols of (−)-quinic acid, i.e. (*R*,*R*,*S*,*R*)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid (Clifford 2000), and occurs naturally in plants. Because elimination of a water molecule from the six-membered ring of the quinic acid requires a *syn*-1,3-diaxial configuration of the hydroxyl and carboxyl groups, only those isomers that lack a cinnamoyl substituent in the 5-position of the quinic acid are able to form a 1,5-quinide. As a result, the most abundant quinides in roasted coffee are 3-caffeoyl-1,5-quinide (3-CQL), 4-caffeoyl-1,5-quinide (4-CQL), each present in less than 0.3% dry weight of the beans (Bennat et al. 1994; Schrader et al. 1996), and 3,4-dicaffeoyl-1,5-quinide (DICAQ). Because neither DIFEQ nor DICOQ are present in detectable amounts in roasted coffee, they should be regarded as model compounds of their close structural analog, DICAQ.

Both 4-CQL and DICOQ have considerable affinity for the human mu opioid receptor with K_i values of 4.4 and 3.9 μM, respectively (Table 1). Further, we found that the ferulic acid derivatives 3-FQL and 4-FQL are relatively weak at displacing [^{3}H]naloxone binding with K_i values of 90 and 41 μM, respectively (Table 1). In the previous study, the fraction containing the highest affinity inhibited 1 nM [^{3}H]naloxone binding by 50% at 25 mg/l (Boublik et al. 1983). Assuming that this represented an equal mixture of 3-FQL and 4-FQL, the corresponding K_i value would be 65 μM, in complete agreement with the result of this study. However, the feruloyl-1,5-quinides constituted 0.48% in the extract (Table 1) and therefore representing only approximately 0.05% in the original instant coffee. The more abundant caffeoyl derivatives 3-CQL and 4-CQL in roasted coffee (Bennat et al. 1994; Schrader et al. 1996) constituted 2.2% in the extract. A possible explanation why Boublik's study did not recognize the presence of 4-CQL in instant coffee is that they used silica gel for the purification (Wynne et al. 1987). In our hands, caffeoyl derivatives of chlorogenic acid seem to bind irreversibly to silica, presumably by the aromatic hydroxyl groups forming a chelating complex with silicon dioxide. The affinity of 4-CQL for the human mu opiate receptor was 4.4 μM, while the corresponding 3-isomer was virtually without affinity (Table 1). Comparison of the affinities with structures of the cinnamoyl-1,5-quinides suggests that a cinnamoyl group in the 4-position of the quinide is required for activity. Analysis of the coffee extract showed that the content of 4-CQL was 0.78%. Assuming that all binding affinity of the extract (42 mg/l) was the result of 4-CQL, the required K_i value of 4-CQL would have to be 0.9 μM. This fivefold discrepancy suggests that part of the binding affinity seen with instant coffee extract could have been caused by constituents other than 4-CQL, including the major, less active isomer 3-CQL, which constituted 1.48% of the extract.

The results of this study further demonstrate that cinnamoyl-1,5-quinides in instant coffee have anti-opioid properties in vivo. By using moderate thermal stimulation at 52°C (Elmer et al. 1998; Mauderli et al. 2000), dose-dependent latencies in mice can be evaluated with morphine doses as low as 0.1 mg/kg (Crain and Shen 2001). The latency in mice to display any of five behaviors that are considered natural responses to thermal stimulation (Mogil et al. 1996; King et al. 2003), of which paw

lifting or shaking have shown significant correlation to a single measurement, such as hind paw licking (Elmer et al. 1998). By using these nociceptive responses to the hot plate at 52°C and a low dose of morphine (Sung et al. 2000), we found that instant coffee extract reversed the anti-nociceptive effect of morphine. It could be argued that by including paw lifting, paw shaking or animal retreating in the measured behaviors, we might no longer record strictly nociceptive response but rather the sensory detection threshold. However, studies in rats have demonstrated that these signs of discomfort produce reliable measures of nociception in the hot plate assay, in particular when lower temperature is used as the noxious stimulus (Plone et al. 1996).

HPLC analysis of the coffee extract showed that it contained 0.78% 4-CQL and 0.06% DICAQ (Table 1). Considering their in vivo activities in the hot plate assay, the levels of 4-CQL and DICAQ in the extract are sufficient to explain the inhibition of morphine-induced latency by 10 mg/kg decaffeinated instant coffee extract (Fig. 2). Previous attempts to validate the findings of Boublik by oral administration of instant coffee in rat concluded that the acute anti-opioid effect of decaffeinated instant coffee has little relevance for normal coffee consumption in humans (Strubelt et al. 1986). In their study, high doses of decaffeinated instant coffee (60 mg/kg IV, 150 mg/kg IP, or 2,000 mg/kg PO) had no apparent effect on morphine-induced analgesia in mice (Strubelt et al. 1986). Because the complete blockade of morphine-induced latency increase with 10 mg/kg IP extract seen in our study represents an intraperitoneal dose of the cinnamoyl-1,5-quinides present in 100 mg/kg instant coffee, our results are in contrast to the results of Strubelt et al. This discrepancy could be the result of the methods used. The dose of morphine used by Strubelt was relatively high (10 mg/kg IP) and the analgesic activity was measured by the tail flick method after administration of instant coffee (Strubelt et al. 1986). The tail flick method is qualitatively different from the hot plate method (Mogil et al. 1996) and the latencies were not measured until 60 min after IP administration of instant coffee.

We found that all three cinnamoyl-1,5-quinides were active in reversing the anti-nociceptive effect of morphine (Fig. 3). Clifford has suggested that some cinnamoyl-1,5-quinides have partial agonist activity at the mu opioid receptor (Clifford 2000). The 3-fold loss of binding affinity seen with DICAQ in the presence of sodium suggests that DICAQ may be a partial agonist. However, no evidence of opioid receptor agonism was seen with either DIFEQ or DICAQ in the hot plate test (Table 2), even though the moderate temperature of the hot plate makes the test sensitive for partial agonism (Lattanzi et al. 2002). Although DIFEQ, DICAQ, and 4-CQL have similar in vitro affinities for the mu opioid receptor, a 200-fold separation in their activities in vivo was observed. DICAQ seemed at least 20 times more potent than DIFEQ (Fig. 3a,b). In particular, 4-CQL showed remarkably high anti-morphine activity with significant inhibition at 0.10 mg/kg IP (Fig. 3c). This activity of 4-CQL seems to be 10 times that of DICAQ and is similar to the ED_{50} reported for naloxone, i.e. 0.07 mg/kg SC (McGilliard and Takemori 1978). High doses of cinnamoyl-1,5-quinides produced hot plate latencies below those of control. Although it is incorrect to define 100% inhibition of morphine-induced hot plate latency below that of the average control value (10.07±0.73 s), log-logit analyses of the dose–response data suggest a single mechanism of action with maximally observed reduction of latency of approximately 3.5 s. This hyperalgesic effect of the cinnamoyl-1,5-quinides was seen only in combination with morphine. Opioid agonist-activated increase in pain sensation is a known phenomenon, mediated by the release of *N*-methyl-D-aspartic acid (NMDA), as evidenced by its inhibition by dizocilpine or memantine (Larcher et al. 1998). Because activation of NMDA receptors facilitates pain sensation via a non-opioid mechanism, hyperalgesia occurs whenever the agonist activity of morphine or fentanyl is blocked by an opioid receptor antagonist, such as naloxone (Celerier et al. 2004). Thus, the apparent hyperalgesic responses to both DICAQ and 4-CQL in the presence of morphine could be taken as further evidence of their antagonistic activity at the mu opioid receptor.

In conclusion, the results of this study demonstrate that cinnamoyl-1,5-quinides present in coffee bind to mu opioid receptors and dose-dependently reverse the anti-nociceptive effects of morphine. We have confirmed that the ferulic acid derivatives 3-FQL and 4-FQL block the human mu opiate receptor, as previously suggested by Wynne et al. (1987), but conclude that the affinities are weak. Other cinnamoylquinides in roasted coffee, some of which are 5 times more abundant than FQL, have 10 times higher affinity in vitro than either 3-FQL or 4-FQL. In particular, the caffeic acid derivative 4-CQL, which is found in all roasted coffees, showed in vivo inhibition of morphine-induced anti-nociceptive behavior in mice with the same order of magnitude as that reported for naloxone. This suggests that the previously reported anti-opioid activity of instant coffee is caused primarily by the presence of 4-CQL and that the combined affinities of 4-CQL and other cinnamoyl-1,5-quinides in roasted coffee have the potential to occupy mu opioid receptors. However, the acute and chronic effects of these compounds from normal daily consumption of coffee remain to be determined.

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