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Potentiation of the Anti-Inflammatory and Analgesic Activity of Aspirin by Caffeine in the Rat (39257)

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(Introduced by C. H. Ellis)

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Although the results of several clinical studies (1, 2) have indicated that caffeine has a potentiating effect on the analgesia produced by aspirin, this effect has not been demonstrated in animals,. Lack of corroborative data stems from the difficulties associated with the measurement of mild analgesia in laboratory animals. Recently, we reported (3) the development of three mild analgesic assays which were based on the hyperalgesic response of rats to pressure applied to their inflamed hindlimbs. These assays circumvent the subjectivity involved in the evaluation of analgesia in animals by producing a painful response which is readily quantitated. Beaver (4) has suggested that the mild analgesia produced by aspirin-like drugs may be the result of their anti-inflammatory action and not a consequence of central nervous system activity. Using the hyperalgesic assays, we found, as Beaver suggested, that the analgesia produced by aspirin is a result of its anti-inflammatory activity (3). We now report that caffeine potentiates the acute anti-inflammatory activity of aspirin and that this potentiation may be translated into enhanced analgesia. This potentiative effect was not mediated through enhanced prostaglandin synthetase inhibition or changes in the pharmacokinetics of aspirin.

Materials and methods. Anti-inflammatory tests. Acute anti-inflammatory activity was assayed in the carrageenan hindlimb (5, 6) and pleural (7, 8) models of inflammation. Male Sprague-Dawley rats, 180 ± 20 g obtained from ARS/Sprague-Dawley, Madison, Wisconsin, were used in both assays. In the pleurisy assay, the 3-hr exudate was aspirated and the number of inflammatory cells and edema volume was determined quantitatively (9). Each drug was suspended in a 0.50% medium viscosity carboxymethylcellulose gum (Hercules, Inc., Delaware) and was administered by gavage 30 min prior to the intrapleural injection of carrageenan. The carrageenan rat hindlimb assay was performed as previously described (10). Drugs were prepared as indicated in the pleurisy studies but were administered by gavage 1 hr prior to the subplantar injection of carrageenan. In both carrageenan assays, the ED_{50}'s ± SE were calculated graphically (11). Adjuvant arthritis was induced in 160–190 g Lewis female rats by the intradermal injection of a 1% suspension of heat-killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, Michigan) into the base of the tail. Drugs were incorporated into a ground meal diet and administered for 14 days, beginning on the day of adjuvant injection. The average arthritic joint score (12) for each experimental group was determined under blind conditions on the 14th day after sensitization, and the percentage reduction of the score produced by the drugs was calculated.

Mild analgesic tests. Female Sprague-Dawley rats, 135–165 g were used in the hyperalgesic assays. An F-shaped hydraulic clamp, driven by pneumatic pressure was manipulated to apply a constant, predetermined force to the irritant-injected rat hindlimb. The jaws of the clamp were positioned above and below the inflamed hindlimb and then were activated by a foot pedal. Depression of the foot pedal also activated an electric stopwatch which stopped when the pedal was released. The algesia was quantitated in terms of the time in which a flight response or vocalization was detected. The time in tenths of a second in which a response was obtained was converted to an arbitrary score of zero to four. The observer

was blind to the drug treatment of each animal. In the trypsin hyperalgesic assay (3), 0.10 ml of a 0.10% solution of trypsin in pyrogen-free water was injected into one hindlimb. Drugs were administered by gavage 30 min later. Half an hour after the drug administration a 6.0 kg force was applied to the hindlimb and the algesic response was quantitated. In the carrageenan hyperalgesic assay drugs were administered 30 min after the hindlimb injection of 0.10 ml of a 0.05% suspension of carrageenan. Then, 2.5 hr later a 3.5-kg force was applied and the algesia was quantitated.

Enzyme assays. Prostaglandin synthetase activity of washed human platelets and white blood cells was assayed using a column chromatographic procedure (13). Other tissue homogenates were incubated with the following components: 10^{-5} *M* [^{14}C]arachidonic acid; 0.5 m*M* epinephrine; 0.5 m*M* reduced glutathione, and 0.1 *M* Tris-chloride. Extract concentrations were adjusted so that less than 10% of the substrate was consumed. After a 10-min incubation at 37°, 5 μg each of $PGF_2\alpha$ and PGE_2 were added to each assay tube as internal chromatographic standards. The mixtures were acidified with 50 μl of 2 *N* HCl and extracted with 6 ml of ethyl acetate. A 3-ml portion of the organic extract was dried in vacuum at room temperature, resuspended in 50 μl of ethyl acetate, and chromatographed on silica gel G thin-layer plates. These were developed with two solvent systems in the same dimension: (1) hexane/ether/acetic acid, 70/30/1 (v/v), and (2) ethyl acetate/methanol/water, 80/20/50 (v/v), organic layer. Dried plates were lightly sprayed with 10% ethanolic phosphomolybdate and warmed until blue bands corresponding to the prostaglandins appeared. These bands were scraped from the plates and counted in 10 ml of a scintillation mixture, which consisted of 4 g of Omnifluor per liter of toluene/Triton X-100 (2/1, v/v).

Guinea pig ilea, obtained from fasted animals, were immediately homogenized in a Waring Blender in 2.5 vol of cold 0.1 *M* Tris-chloride, pH 8, and centrifuged at 5000*g* and 4° for 10 min. The supernate (0.5 to 0.8 ml/assay) was used on the same day. Microsomal fractions were also prepared by centrifuging this supernate at 100,000*g*. Pellets were resuspended in a small volume of 0.1 *M* Tris-chloride, pH 8, and stored frozen at −20°. Microsomal extracts of bovine seminal vesicles (Pel-Freez Corp., Rogers, Arkansas) were prepared in the same way.

Plasma salicylate levels. Plasma was analyzed for total salicylate by a modification of the method of Stevenson (14). A dilution of plasma in 0.5% acetic acid was extracted with freshly distilled dibutyl ether containing 0.4% malonic acid. The organic phase was washed with 1.0 *M* phosphate buffer, pH 7.4, and the salicylate was measured in the aqueous phase by its fluorescence (ex. 305 nm, em. 400 nm). The limit of detection was typically 0.2 μg/ml with deviations of approximately 5% between duplicates.

Results: Anti-Inflammatory studies. Caffeine inhibited the development of edema in the carrageenan hindlimb and pleural assays producing ED_{50}'s of 14 ± 2.2 and 21 ± 5.5 mg/kg, respectively. Caffeine was inactive at 10 mg/kg against the pleural exudate volume and at 5 mg/kg against the hindlimb volume. These low ineffective dose levels were used in attempts to potentiate the activity of various anti-inflammatory drugs (Table I). The pleural edema volume ED_{50} produced by aspirin decreased from 31 ± 7.0 to 12 ± 4.8 mg/kg when caffeine at 10 mg/kg was given with aspirin. In the carrageenan hindlimb edema assay the addition of 5 mg/kg of caffeine resulted in a significant reduction of the second phase ED_{50} of aspirin from 52 ± 16.0 to 12 ± 2.9 mg/kg. The oral efficacy of indomethacin and phenylbutazone was increased more than threefold by caffeine in both carrageenan assays (Table I). However, caffeine did not enhance the anti-inflammatory activity of the steroidal anti-inflammatory drug hydrocortisone or the nonsteroid sodium salicylate in these carageenan assays.

Additional carrageenan pleurisy studies revealed that caffeine itself inhibited the number of neutrophils mobilized in 3 hr. At 37 ± 17.5 mg/kg caffeine reduced the neutrophil count 50%. However, the low doses of caffeine used in the above potentiation studies had no effect on the number of neutrophils mobilized. In those cases where caffeine potentiated the anti-edematous ac-

TABLE I. EFFECT OF CAFFEINE ON THE ANTI-INFLAMMATORY ACTIVITY OF VARIOUS DRUGS

Drug(s)	Carrageenan hindlimb edema ED_{50}[a] (mg/kg, p.o.) 2nd Phase	Carrageenan pleurisy ED_{50}[b] (mg/kg, p.o.)
Caffeine	14 ± 2.2	21 ± 5.5
Aspirin	52 ± 16.0	31 ± 7.0
Aspirin + caffeine	12 ± 2.9 (5)[c]	12 ± 4.8 (10)
Sodium salicylate	80 ± 45.0	88 ± 56.0
Sodium salicylate + caffeine	68 ± 15.4 (5)	Inactive at 30 (10)
Phenylbutazone	35 ± 15.3	60 ± 40.7
Phenylbutazone + caffeine	<10 (5)	~20 (10)
Indomethacin	2.3 ± 1.10	1.7 ± 0.66
Indomethacin + caffeine	<0.7 (5)	<0.7 (10)
Hydrocortisone	24 ± 6.0	14 ± 10.2
Hydrocortisone + caffeine	Inactive at 8 (5)	11 ± 7.4 (10)

[a] ED_{50} represents the dose in milligrams per kilogram which reduced the 2nd phase volume 50% relative to solvent-fed control rats.

[b] ED_{50} represents the dose in milligrams per kilogram which reduced the 3-hr exudate volume 50% relative to solvent-fed control rats.

[c] Numbers in parentheses indicate the dose of caffeine in mg/kg, p.o. There were at least five rats at each dose level.

tivity of drugs there was no reduction in the number of neutrophils mobilized.

Caffeine failed to enhance the anti-inflammatory activity of aspirin in the adjuvant arthritis assay. The addition of 50 to 250 mg/kg/day of caffeine to the diet did not significantly increase the weak activity of a 100 mg/kg/day dose of aspirin.

Analgesic studies. Caffeine did not exert any analgesic activity in the trypsin or carrageenan hyperalgesic tests. However, when caffeine was added to aspirin and tested in the carrageenan hyperalgesic assay, the ED_{50} of aspirin decreased from 70 ± 5.7 to 18 ± 4.7 mg/kg. Aspirin was inactive in the trypsin hyperalgesic test at 180 mg/kg. Adding caffeine to the aspirin failed to elicit any inhibitory activity in this assay.

Prostaglandin synthetase studies. Caffeine did not enhance the inhibition produced by aspirin of prostaglandin synthesis by bovine seminal vesicle enzyme, washed human WBC/platelet enzyme, fresh guinea pig ileum homogenates, or the guinea pig ileum microsomal preparation (Table II). Neither did it affect the diarrhea induced by arachidonic acid in the mouse (15).

Plasma salicylate levels. Plasma salicylate levels measured at various times after the administration of aspirin and caffeine are shown in Table III. Caffeine did not significantly alter the absorption rate, time to achieve peak plasma level of salicylate, or the elimination of salicylate from the plasma in an 8-hr period.

Discussion. Caffeine has been found to potentiate the acute anti-inflammatory activity of aspirin. This potentiated inhibition was confined to the edematous component of the inflammation as the number of neutrophils mobilized in response to the intrapleural injection of carrageenan was the same in normal animals and in aspirin-treated animals that did or did not receive low doses (5–10 mg/kg) of caffeine. It was necessary to use these low doses of caffeine as moderate doses (15–45 mg/kg) of this xanthine derivative inhibited both the cellular mobilization and edematous component of the acute carrageenan inflammation.

The carrageenan hyperalgesic assay was designed in such a way that anti-inflammatory drugs are able to decrease the inflammation and concomitant hyperalgesia. This results in lower sensitivity to pressure and an "apparent" analgesic effect. Aspirin is active in this test and its "apparent" analgesic activity is potentiated threefold by caffeine. In the trypsin hyperalgesic test each drug is given after hyperalgesia has developed and only 30 min are allowed between drug administration and application of the algesic force. The edema volume and concomitant hyperalgesia resulting from the subplantar injection of trypsin are not inhibited by anti-inflammatory drugs (19). In-

TABLE II. EFFECT OF CAFFEINE ON THE PROSTAGLANDIN SYNTHETASE INHIBITION PRODUCED BY ASPIRIN

Prostaglandin synthetase assay	Compound(s)	Molar concentration	Dose (mg/kg, ip)	Inhibition[c] (%)	
Bovine seminal Vesicle enzyme	Aspirin	2×10^{-4}	–	11.0	
	Caffeine	1.8×10^{-4}	–	−22.0	
	Aspirin + caffeine	$(2 \times 10^{-4}) + (1.8 + 10^{-4})$	–	−3.3	
Washed human WBC/ platelets	Aspirin	5×10^{-4}	–	24.8	
	Caffeine	1×10^{-3}	–	−9.7	
	Aspirin + caffeine	$(5 \times 10^{-4}) + (10^{-3})$	–	5.5	
				$F_{2\alpha}$	E_2
Fresh homogenate of guinea pig ileum	Aspirin	1×10^{-4}	–	66	54
	Caffeine	2×10^{-5}	–	11	−5
	Aspirin + caffeine	$(10^{-4}) + (2 \times 10^{-5})$	–	−19	−8
Microsomes of guinea pig ileum	Aspirin	1×10^{-4}	–	26	45
	Caffeine	2×10^{-5}	–	4	−10
	Aspirin + caffeine	$(10^{-4}) + (2 \times 10^{-5})$	–	−19	−8
Arachidonic acid-induced diarrhea assay in the mouse	Aspirin	–	3	83	
	Aspirin	–	1	14	
	Caffeine	–	10	0	
	Aspirin × caffeine	–	1 + 10	20	

[a] Negative sign indicates stimulation rather than inhibition.

TABLE III. EFFECT OF CAFFEINE ON THE PLASMA SALICYLATE LEVELS PRODUCED BY ASPIRIN IN THE RAT

Time after drug treatment (hr)	Plasma salicylate level[a] (μg/ml)	
	Aspirin (10 mg/kg, p.o.)	Aspirin + caffeine (10 mg/kg + 10 mg/kg, p.o.)
1	26 ± 0.9	29 ± 1.0
2	23 ± 0.6	19 ± 1.2
4	12 ± 1.0	11 ± 1.3
8	4 ± 1.1	3 ± 0.5

[a] Each value represents the mean of five rats.

terestingly, aspirin was inactive in this test. Augmenting the anti-inflammatory activity threefold with caffeine (Table I) still failed to elicit analgesia in the trypsin hyperalgesic assay. From these mild analgesic studies it may be concluded that the analgesia produced by aspirin results from its anti-inflammatory activity. This concept has been suggested in a recent review article on mild analgesics (4). Potentiation of the acute anti-inflammatory activity, therefore, can account for the potentiated analgesia produced by the combination of aspirin and caffeine.

The three drugs potentiated by caffeine (aspirin, indomethacin, and phenylbutazone) are prostaglandin synthetase inhibitors (16), whereas the two drugs not potentiated, hydrocortisone and sodium salicylate, are inactive against the synthetase. This correlation suggested that the potentiation afforded by caffeine may be the result of augmented synthetase inhibition. However, our enzyme studies, both *in vitro* and *in vivo* did not support this hypothesis.

Attempts to explain the potentiative effects of caffeine on changes in the pharmacokinetics of aspirin were also negative. None of the pharmacokinetic parameters studied were affected by the low doses of caffeine which produced potentiation.

Caffeine did not potentiate the anti-inflammatory activity of aspirin against the development of the chronic inflammation represented by adjuvant arthritis. It is possible that the duration of the potentiation may be too short to affect a chronic inflammation. No studies were directed to this point.

Summary. Caffeine has been found to potentiate the acute anti-inflammatory activity of aspirin, indomethacin, and phenylbutazone, but not the activity of sodium salicylate or hydrocortisone, in the carrageenan pleurisy or hindlimb models of inflamma-

tion in the rat. The mobilization of inflammatory cells was not affected by aspirin in the presence or absence of caffeine.

The mild analgesia produced by aspirin was confined to a hyperalgesic test in which this drug was able to reduce inflammation and concomitant hyperalgesia and thereby produce an "apparent" analgesic effect. This "apparent" analgesia produced by aspirin was potentiated by caffeine. The mechanism responsible for the potentiated anti-inflammatory and mild analgesic activity of aspirin remains unknown since caffeine did not alter the plasma salicylate levels or prostaglandin synthetase inhibition produced by aspirin.

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