Effect of colchicine on DFP induced serum β-glucuronidase and ^{14}C leucine incorporation

Zero time	10 min	Serum β-glucuronidase[a]	CPM in serum[b]	CPM in liver[c]
^{14}C Leu[d]	DFP[e]	4.9 ± 0.84	9117 ± 1483	1011 ± 35
^{14}C Leu + colchicine[f]	DFP	4.8 ± 0.85	5619 ± 442	1423 ± 144
^{14}C Leu	Saline[g]	0.10 ± 0.05	8590 ± 1291	936 ± 87
^{14}C Leu + colchicine	Saline	0.07 ± 0.02	5845 ± 946	1383 ± 271

[a] μmoles phenolphthalein released/h/ml at 70 min. [b] TCA precipitable counts in 50 μl aliquots, 70 min. [c] TCA precipitable counts in 50 μl aliquots of 1 g homogenized liver diluted in 20 ml buffer. [d] 25 μCi via femoral vein. [e] 1.25 mg/kg, i.p. in 0.25 ml saline. [f] 40 μmoles/200-g-rat, via femoral vein. [g] 0.25 ml, i.p. Data are means ± SD.

(Sigma Chemical, St. Louis, MO.) (40 μmoles). At 10 min, DFP (1.25 mg/kg) or saline (0.25 ml) was injected i.p. At 70 min, the animals were sacrificed by cardiac puncture and 50 μl of serum was added to 1 ml of 5% trichloroacetic acid (TCA) and centrifuged for 1 min in a Beckman microfuge. The TCA insoluble pellets were washed twice with 5% TCA and resuspended in 1 ml 0.1 N NaOH and 9 ml Biofluor (New England Nuclear, Boston, Mass.) for counting. An additional 25 μl serum sample was used to determine serum β-glucuronidase activity[7]. Liver protein synthesis was evaluated by measuring incorporation of ^{14}C-leucine into the TCA insoluble pellet from a 50 μl aliquot of liver homogenate. The homogenate was prepared, using a polytron homogenizer from 1 g of liver in 20 ml of buffer. An aliquot of the homogenate was treated identically to the serum sample just described.

As can be seen in the table, colchicine treatment significantly impaired the appearance of TCA-precipitable counts in the serum and led to the build-up of TCA-precipitable counts in the liver. Calculations on individual animals indicate that total incorporation of ^{14}C-leucine into TCA insoluble material was not adversely affected, but that these doses of colchicine inhibited the secretion of newly synthesized protein. This is in marked contrast to the DFP induced secretion of β-glucuronidase into the serum of colchicine treated rats which was unaffected by the massive colchicine dose utilized. This indicates a clear distinction between the DFP induced secretion of β-glucuronidase and the bulk of the protein synthesized and secreted into the serum by hepatocytes[1-4]. An explanation as to why the secretion of liver microsomal β-glucuronidase is apparently unique in its microtubule independence is not readily apparent. Induced secretion of stored or slowly turning over cellular products from other cells has been shown to be inhibitable by colchicine[8], so it is unlikely that these properties alone would lead to microtubule independence. However, in the liver this has not been evaluated and, indeed, this finding could represent a fundamental distinction between intercellular packaging and secretion of proteins that are normally stored by the hepatocyte (β-glucuronidase) and those proteins that are readily secreted after synthesis. The lack of susceptibility to colchicine or vincristine (not shown) inhibition can be interpreted in one of two ways. First, it is possible that the secretion of microsomal β-glucuronidase occurs by a mechanism truly independent of microtubules. Alternatively, the secretion may be microtubule dependent, but involve a pool of microtubular structures that are not available for interaction with colchicine or vincristine. Experiments to date do not rule out either interpretation. However, the existence of an extensive pool of colchicine insensitive microtubular structures is consistent with the findings of Redman et al.[1]. The experiments presented here provide an indication that the control of protein secretion from the liver may be even more complex than originally envisioned.

7 P. Stahl and O. Touster, J. biol. Chem. *246*, 5398 (1971).
8 R. B. Zurier, G. Weissman, S. Hoffstein, S. Kammerman and H. H. Tai, J. clin. Invest. *53*, 297 (1974).

Lead potentiation of endotoxin lethality in rats: Lack of effect of kininase inhibition

S. L. Gartner[1,2]

Department of Experimental Medicine, Naval Medical Research Institute, Bethesda (Maryland 20014, USA),
12 April 1977

Summary. Although lead and SQ_{20881} are potent in vitro inhibitors of kininase II activity, SQ_{20881} does not alter the sensitivity of rats to endotoxin. These results indicate that marked changes in plasma kininase activity do not contribute to endotoxin morbidity and that kininase inhibition is not the mechanism whereby lead ions sensitize rats to endotoxin.

Lead ions markedly sensitize rats to small quantities of endotoxin and elucidation of the mechanism of this synergism may clarify the toxic action of bacterial lipopolysaccharides. This report examines the hypothesis that one role of lead may be to inhibit a kininase enzyme which is responsible for degrading the hypotensive peptide, bradykinin. In this regard, endotoxin treated animals do have increased levels of circulating bradykinin[3] which may be the result of impaired kinin degradation as well as endotoxin activation of Hageman Factor and subsequent kallikrein formation. Under normal conditions,

1 This investigation was supported by the Naval Medical Research and Development Command, NNMC, Department of the Navy, Research Task No. MR041.20.01.0435. The opinions and assertions contained herein are the private ones of the author, and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.
2 The experiments reported herein were conducted according to the principles set forth in the 'Guide for the Care and Use of Laboratory Animals', Institute of Laboratory Animal Resources, National Research Council, DHEW Pub. No. (NIH) 74-23.
3 V. M. Sardesai and J. C. Rosenberg, J. Trauma *14*, 945 (1944).

bradykinin is rapidly metabolized by a number of enzymes, including kininase II present in plasma and lung. Kininase II is inhibited by lead ions[4]. Furthermore, since kininase II is thought to be identical with angiotensin converting enzyme[5], the same enzyme may be responsible for inactivating bradykinin, a vasodilator, and also forming angiotensin II, a potent vasoconstrictor. Certainly an imbalance in these vasoactive peptides could contribute to the marked changes in microvascular circulation and poor tissue perfusion which are present during endotoxemia.

Recent studies on the localization of kininase II suggest that this enzyme is associated with the plasma membranes of endothelial cells, enabling easy access to substrates circulating in the bloodstream[6]. Similarly, this location might very well expose the active site of kininase II to inhibition by i.v. injections of lead. Therefore, experiments were designed to assay the potency of known kininase inhibitors, including lead and the nonapeptide, SQ_{20881}, and determine whether kininase inhibition is responsible for the sensitization of lead-treated rats to endotoxin.

Materials and methods. Kininase II activity was measured by the method of Cushman and Chung[7] using hippuryl-histidyl leucine as substrate. Incubation time was 20 min and assays were performed in triplicate using heparinized plasma or lung tissue as the source of enzyme. Blood was obtained from rats by cardiac puncture and centrifuged at 3000 × g for 10 min to remove cells. Lung tissue (0.5 g per 10 ml water) was dispersed using a Polytron homogenizer (Brinkman Instrument Company). Protein was determined by the Lowry method[8]. The results are expressed as μmoles hippuric acid formed per mg protein. Adult, male Sprague-Dawley rats weighing between 180 and 220 g were used. Substances were injected separately in 0.5 ml via the femoral vein. Endotoxin and SQ_{20881} were dissolved in phosphate buffered saline (PBS) and lead acetate was dissolved in distilled water. *Serratia marcescens* endotoxin (Lot No. 504314), prepared by the Boivin method, was obtained from Difco Laboratories.

Results and discussion. Table 1 shows the effect of SQ_{20881} and lead acetate on kininase II activity. It can be seen that the in vitro addition of SQ_{20881} completely blocks kininase activity in both plasma and lung. The concentra-

Table 1. Inhibition of kinase II activity by SQ_{20881} and lead acetate added in vitro

Incubation conditions	Kininase II specific activity*	Percent of control
Plasma, control	0.0398	100
+ SQ_{20881}	0	0
+ lead acetate	0.0157	39
Lung, control	0.669	100
+ SQ_{20881}	0	0
+ lead acetate	0.130	19

*These data are expressed as μmoles hippuric acid formed per mg protein. The final concentration of SQ_{20881} was 0.04 mg/ml and lead acetate, 1 mg/ml.

Table 2. Effects of SQ_{20881} and lead acetate injection on plasma and lung kininase II

Enzyme source Time post-injection	Substance injected*	Kininase II specific activity**	Percent of control
Plasma 5 min	PBS	0.0185	100
5 min	SQ_{20881}	0	0
5 min	lead acetate	0.0262	142
Plasma 60 min	PBS	0.0177	100
60 min	SQ_{20881}	0.0053	30
60 min	lead acetate	0.0179	101
Lung 5 min	PBS	0.769	100
5 min	SQ_{20881}	0.817	106
5 min	lead acetate	0.692	90
Lung 60 min	PBS	0.317	100
60 min	SQ_{20881}	0.366	115
60 min	lead acetate	0.426	134

*The following substances were injected i.v. into rats: 1. Phosphate buffered saline (PBS), 0.5 ml; 2. SQ_{20881} (0.4 mg/0.5 ml PBS); and 3. lead acetate (10 mg/0.5 ml). Samples of plasma and lung were taken at 5 and 60 min post-injection, and analyzed as described in the 'methods' section. **These data are expressed as μmoles hippuric acid per mg protein.

4 C. G. Huggins, R. J. Corcoran, J. S. Gordon, H. W. Henry and J. P. John, Circulation Res. *26/27*, Suppl. 1, 93 (1970).
5 E. Ueda, T. Kobubu, H. Akutsu and T. Ito, Jap. J. Circ. *36*, 583 (1972).
6 R. L. Soffer, A. Rev. Biochem. *45*, 73 (1976).
7 D. W. Cushman and H. S. Chung, Biochem. Pharmac. *20*, 1637 (1971).
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).

Table 3. Endotoxin lethality after i.v. injection of SQ_{20881}

Treatment of animals*	No. deaths/ total No. rats injected**	Percent lethality
SQ_{20881} + endotoxin (3.3 mg)	8/11	73
PBS + endotoxin (3.3 mg)	6/11	55
SQ_{20881} + endotoxin (1.0 mg)	1/5	20
PBS + endotoxin (1.0 mg)	1/5	20
SQ_{20881} + endotoxin (200 μg)	1/11	9
PBS + endotoxin (200 μg)	1/11	9
SQ_{20881} + PBS	0/3	0

*Concentration of SQ_{20881} was 0.4 mg/0.5 ml PBS. Phosphate buffered saline (PBS), 0.5 ml. **Survival of animals was recorded 72 h post-injection.

Table 4. Effect of SQ_{20881} and bradykinin on sensitivity of rats to endotoxin

Treatment of animals*	No. deaths/ total No. injected**	Percent lethality
SQ_{20881} + bradykinin + endotoxin	5/15	33
PBS + PBS + endotoxin	9/16	56
SQ_{20881} + PBS + endotoxin	5/10	50

*SQ_{20881} and bradykinin were injected i.v. into rats immediately before endotoxin administration. These doses were given: SQ_{20881} (0.4 mg/0.5 ml) bradykinin (200 μg/0.5 ml), endotoxin (3.3 mg/0.5 ml), and phosphate buffered saline, 0.5 ml (PBS). **Survival of animals was recorded 72 h post-injection.

tion (0.04 mg/ml) of SQ_{20881} used in vitro approximates the concentration present in the blood following the i.v. injection of 0.4 mg of this inhibitor. Kininase activity was also inhibited in plasma and lung when lead acetate was added during the incubation (table 1).

Kininase II activity was also measured in plasma and lung from rats which had previously been injected with SQ_{20881} or lead acetate. Rats were killed at 5 min and 60 min post-injection and the results are shown in table 2. In this case, injected lead acetate does not inhibit kininase activity in plasma and lung. The nonapeptide, SQ_{20881}, markedly reduces kininase activity in the plasma during the first 5 min after injection and even 60 min post-injection only 30% of the control activity was detected in the plasma. In lung, however, SQ_{20881} administered in vivo did not alter kininase activity at either time period.

Additional experiments were performed to determine the effect of SQ_{20881} on the endotoxin lethality. Rats were given various doses of *S. marcescens* endotoxin, and SQ_{20881} or phosphate buffered saline. The 0.4 mg dose of SQ_{20881} used in vivo has been shown to be effective in potentiating the action of bradykinin in the rat[9]. Mortality was observed after 72 h and the results in table 3 show that the kininase inhibitor had no appreciable effect on endotoxin sensitivity. In subsequent experiments, SQ_{20881} was given 1.5 h before, and simultaneously with endotoxin. Again there was no alteration in lethality due to the SQ_{20881}. In an additional group of animals, bradykinin was injected along with the kininase inhibitor and endotoxin (table 4). Even under these conditions with very high circulating bradykinin these was no increase in endotoxin morbidity.

This work confirms the results of previous investigators[10,11] that the nonapeptide, SQ_{20881} when added in vitro is a very effective inhibitor of kininase II activity in rat plasma and lung. Furthermore, the in vitro addition of lead acetate was shown to inhibit kininase activity in plasma and lung. The concentration of lead acetate used in vitro approximates the amount present in the blood immediately following the injection of 10 mg of lead acetate in a 200 g rat. This dose of lead acetate markedly sensitizes rats to small quantities of endotoxin[12]. It was clear, however, from the lethality data presented in this study that SQ_{20881} did not sensitize rats to endotoxin. Even the combination of kininase inhibitor and bradykinin did not effect endotoxin morbidity. These results lead to the conclusion that marked alterations in level of circulating kininase activity do not play a critical role in the lethal effects of endotoxin and that the mechanism of lead sensitization is not due to its kininase inhibiting properties.

9 A. Bianchi, D. B. Evans, M. Cobb, M. T. Peschka, T. R. Schaeffer and R. J. Laffan, Eur. J. Pharmac. *23*, 90 (1973).

10 R. Igic, E. G. Erdos, J. Yeh, K. Sorrells and T. Nakajima, Circulation Res. *30/31*, Suppl. II, 51 (1972).

11 S. Oparil, T. Koerner and J. K. O'Donoghue, Circulation Res. *34*, 19 (1974).

12 H. Selye, B. Tuchweber and L. Bertok, J. Bacteriol. *91*, 884 (1966).

Offset-induced audiogenic seizures[1]

Chia-Shong Chen and G. R. Gates[2]

Department of Psychology, Monash University, Clayton, Victoria 3168 (Australia), 21 March 1977

Summary. A relatively stereotyped seizure reaction can be triggered by the 'offset' of an intense bell sound in C57BL/6J mice. Susceptibility to this offset-induced audiogenic seizure was found to depend upon the age of the animals tested (higher in older mice) and the duration of the noise exposure (more effective with longer exposure).

When certain strains of mice are exposed to intense noise, they may show a characteristic stereotyped sequential reaction consisting of wild running, clonic seizure and in some cases tonic seizure and death. This phenomenon is widely known as audiogenic seizure. Susceptibility to audiogenic seizure may be due to the genetic background of the animals: highly seizure-prone mice are likely to seize on the first exposure to an intense noise whereas normally-resistant mice are not[3]. However, seizure susceptibility can be induced in seizure-resistant strains of mice by drug-withdrawal procedures (e.g. ethanol[4,5] or barbiturate[6] withdrawal) or by a priming procedure which consists of exposing animals to an intense noise a few days prior to testing for audiogenic seizure[7,8]. In this report we describe a different type of audiogenic seizure reaction which, to the best of our knowledge, has not been documented in the literature. The classic audiogenic seizure phenomenon described presviously refers to a seizure driven by continuous acoustic stimulation. The seizure to be described here is, instead, triggered by the offset of intense auditory stimulation.

Animals used in this experiment were C57BL/6J mice which are normally regarded as being seizure-resistant[3]. However, they can be made seizure susceptible by priming at certain ages[7]. The offset-induced audiogenic seizure was discovered when we were testing for the rate of spontaneous audiogenic seizure (i.e. animals who seize on the very first exposure to noise) in mature C57BL/6J mice. Animals of various ages were exposed to a 125–127 dB (re 0.0002 dyn/cm^2) bell sound for 60 sec or until seizure occurred. In the process of testing we noted that some animals showed a relatively stereotyped motor display after the bell was turned off. Typically, an animal would suddenly jerk backwards into an exaggerated standing posture with all 4 limbs rigidly extended. The whole body shook vigorously and the tail writhed in a lateral, reptile-like swimming motion. Before collapsing into a relatively relaxed and flattened posture, most of

1 This work was supported by the Australian Research Grants Committee fund to the first author.

2 Current address: Department of Psychology, University of Melbourne, Melbourne, Australia.

3 J. L. Fuller and F. R. Sjursen, J. Hered. *58*, 135 (1967).

4 J. L. Fuller and R. E. Wimer, in: Biology of the Laboratory Mouse, p. 609. Ed. E. L. Green. McGraw-Hill, New York 1966.

5 G. Freund and D. W. Walker, Psychopharmacologia, Berlin *22*, 45 (1971).

6 G. R. Gates and C.-S. Chen, Nature *249*, 162 (1974).

7 K. R. Henry, Science *158*, 938 (1967).

8 C.-S. Chen and G. R. Gates, Medikon *4*, 19 (1975).