J. Pharm. Pharmacol. 1990, 42: 842–845
Received May 2, 1990

Effect of Anti-inflammatory Drugs on the Cardiotoxin-induced Hind-paw Oedema in Rats

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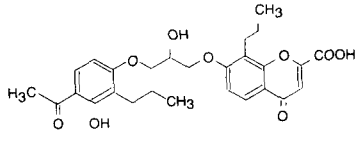
Abstract—Cardiotoxin, isolated from *Naja naja atra* venom, induced rat hind-paw oedema. This effect was suppressed by the pretreatment with dexamethasone or BW 755C, or subplantar co-injection with FPL 55712. Pretreatment with aspirin alone did not affect this response, while a significant reduction of cardiotoxin-induced paw oedema was achieved with aspirin in combination with diphenhydramine and methysergide. Subplantar co-injection of PAF antagonist, BN 52021 or L 652731, with cardiotoxin had no effect on paw oedema, whereas superoxide dismutase/catalase reduced this oedematous response. Cardiotoxin-induced paw oedema was also suppressed by pretreating the rats with isoprenaline. Pretreatment with rat anti-platelet plasma, which greatly reduced peripheral platelet count, did not affect cardiotoxin-induced paw oedema. Cardiotoxin did not trigger platelet aggregation or release reaction either in platelet-rich plasma or in washed platelet suspension. The oedematous response after subplantar co-injection of cardiotoxin with basic or acidic phospholipase A_2 appeared to be only an additive effect. These results suggest that arachidonate metabolites, in which leukotrienes are most important, participated in cardiotoxin-induced paw oedema. Superoxide radical was also involved, while PAF and platelets showed little influence in this oedema effect.

Local swelling, oedema and severe pain are often the paramount early features of cobra snakebites (Reid 1964; Campbell 1979). Crude *Naja naja* venom produces rat paw oedema (Bonta & de Vos 1965). Cardiotoxins are constituents of cobra venoms (Larsen & Wolff 1968; Slotta & Vick 1968) which, together with neurotoxins, contribute to the main toxicity of the venoms. The local irritant action of cobra venom has been attributed to cardiotoxin in the venom. Instillation of cardiotoxin into the rabbit eye caused congestion of the conjunctiva (Lee et al 1968). Local irritant action of cardiotoxin was also demonstrated by the rat paw oedema test (Lee et al 1968; Wang & Teng 1989). Cardiotoxin was as active as crude cobra venom in inducing histamine release from mast cells (Damerau et al 1975; Wang & Teng 1989). Thus, cardiotoxin appears the main inflammatory principle in cobra venom. Wang & Teng (1989) also reported that mast cells and PMN leukocytes were involved in the cardiotoxin-induced paw oedema, and that inflammatory mediators such as histamine, 5-hydroxytryptamine and kinins were supplied directly or indirectly by mast cells. We have examined the role of platelets and mediators such as leukotrienes and prostaglandins, superoxide radical and PAF in cardiotoxin-induced rat paw oedema.

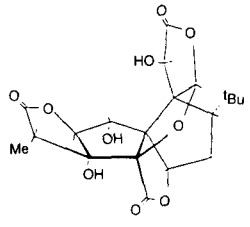
Materials and Methods

Materials

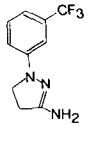
Cardiotoxin was isolated from *Naja naja atra* venom as described by Lee et al (1968). Diphenhydramine, dexamethasone, superoxide dismutase, catalase, isoprenaline, bovine serum albumin (BSA) were obtained from Sigma Chem. Co., USA. Lysine-aspirin was obtained from China Chem. Pharmaceutical Co., Taiwan. BW 755C was a gift from Wellcome Research, UK. FPL 55712 was kindly supplied by Fisons Pharmaceutical Division, UK. BN 52021 was a generous gift from Dr P. Braquet of Institut Henri Beaufour, France. L 652731 was provided from Merck, Sharp & Dohme Inc., USA. Methysergide was a gift from Sandoz Pharmaceuticals Ltd, Switzerland. Structures of drugs used in this study are shown below.



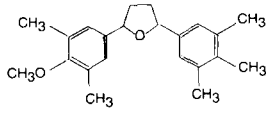
FPL 55712



BN 52021



BW 755C



L 652731

Rat hind-paw oedema

Wistar rats (180–220 g) were used. Hind-paw oedema was produced by a single subplantar injection of 0·1 mL irritant in 0·05 M phosphate buffered saline (PBS, pH 7·4) or an equal

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volume of PBS into the right and left hind-paw, respectively. The volumes of both hind-paws of each rat were measured with a plethysmometer at the beginning and at different periods after induction of oedema. Percent hind-paw swelling was calculated as the change in volume of the right paw minus the change in volume of the left paw. The data were also analysed to compare the area under the time-course curve (AUC).

Preparation of anti-platelet plasma

Anti-platelet plasma was prepared as described by Lefort & Vargaftig (1978). Rat platelets were washed with saline and freeze-thawed five times. After centrifugation, the platelets were mixed with Freund's complete adjuvant and injected into all four foot pads of rabbit. The 2nd and 3rd immunizations were carried out at 15 day intervals. Seven days after the last immunization, blood was collected and plasma separated. After heating at 56°C for 30 min, this plasma was lyophilized and stored at −70°C. The activity of anti-platelet plasma was estimated by measuring the platelet lysis effect in rat platelet-rich plasma.

Depletion of platelets

Wistar rats (180–220 g) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.p.). Anti-platelet plasma was given by the i.v. infusion for 30 min. One hour later the 2nd infusion was made. The animals were used for hind-paw oedema experiments 30 min after the last infusion of anti-platelet plasma.

Platelet isolation

Blood was withdrawn from Wistar rats, mixed with sodium citrate (3·8%) (1 : 14 to blood), centrifuged for 10 min at 120 *g* and room temperature (22°C) and the supernatant obtained as platelet-rich plasma. This was mixed with EDTA to a final concentration of 6 mM then centrifuged at 500 *g* for 10 min. The pellets were washed twice with Tyrode solution (Ca^{2+}-free) with EDTA 2 mM, BSA 3·5 mg mL^{-1} and suspended in Tyrode solution of the following composition (mM): NaCl 136·8, KCl 2·8, $NaHCO_3$ 11·9, $MgCl_2$ 1·1, NaH_2PO_4 0·33, $CaCl_2$ 1·0, dextrose 11·2 and BSA 3·5 mg mL^{-1}.

Platelet aggregation and ATP release reaction

Aggregation was measured by a turbidimetric method (O'Brien 1962; Born & Cross 1963). ATP released from the platelets was detected by the bioluminescence method of DeLuca & McElory (1978). Both the aggregation and the release reaction were simultaneously and continuously measured by a Lumi-aggregometer (Model 1020, Payton, Canada).

Statistical evaluation

The results are expressed as the means ± s.e.m. of the indicated number of experiments and Student's *t*-test was used for the statistical evaluation. *P* values < 0.05 were considered to be significant.

Results

Cardiotoxin-induced rat hind-paw oedema was not affected by aspirin (180 mg kg^{-1} s.c.) pretreatment alone, while the paw swelling occurring after 3 h was significantly reduced by aspirin in combination with diphenhydramine and methysergide (each 10 mg kg^{-1} s.c.) (Fig. 1). The response was suppressed to 61% of the control value as calculated from the AUC. Pretreatment with dexamethasone (1 mg kg^{-1} s.c.) or BW 755C (50 mg kg^{-1} i.p.) suppressed cardiotoxin-induced the oedema to 68 and 37%, respectively, of the control value (Table 1). Subplantar co-injection of FPL 55712 (100 μM) with cardiotoxin reduced the oedema to 53% of the control value.

The oedema caused by cardiotoxin was also suppressed by about 30% by subplantar co-injection with superoxide dismutase/catalase (each 500 units), while neither BN 52021 (50 μg) nor L 652731 (20 μg) was effective in this respect.

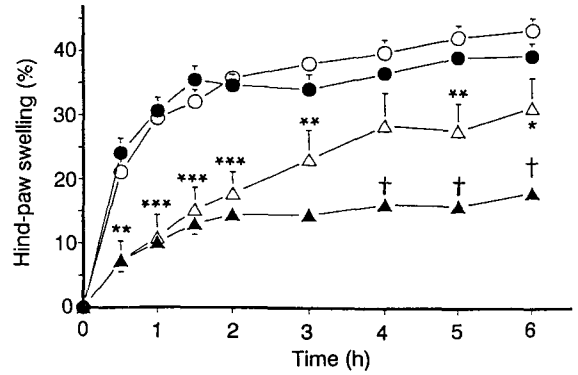

FIG. 1. Effect of aspirin, diphenhydramine and methysergide on the hind-paw oedema of rats caused by cardiotoxin. In the control group, subplantar injection of cardiotoxin (15 μg) in the absence of other drugs (○). Aspirin (180 mg kg^{-1} s.c.) (●), diphenhydramine/methysergide (each 10 mg kg^{-1} s.c.) (△), or aspirin in combination with diphenhydramine/methysergide (▲) was given 1 h before cardiotoxin injection in the paw. Values are expressed as the means ± s.e.m. of 5–7 experiments. Statistically significant differences from the corresponding control values are noted as $^{**}P < 0.01$, $^{***}P < 0.001$ and from the corresponding values in (△) group are noted as $\dagger P < 0.05$.

Table 1. Effect of anti-inflammatory drugs on cardiotoxin-induced paw oedema.

Drugs[a]	Oedema[b] (AUC)	N[c]
Control	209·4 ± 7·6	7
(1) Interference with eicosanoid pathway		
aspirin (180 mg kg^{-1} s.c.)	200·8 ± 8·8	5
FPL 55712 (100 μM)	111·5 ± 15·0***[d]	5
BW 755C (50 mg kg^{-1} i.p.)	77·8 ± 6·5***	5
dexamethasone (1 mg kg^{-1} s.c.)	143·4 ± 13·5***	5
(2) Removal of superoxide radical superoxide dismutase/catalase (each 500 units)	140·4 ± 12·3***	5
(3) PAF antagonist		
BN 52021 (50 μg)	228·5 ± 16·7	5
L 652731 (20 μg)	216·0 ± 21·6	4
(4) β-Adrenoceptor agonist		
isoprenaline (1·5 mg kg^{-1} s.c.)	129·4 ± 8·7***	5

[a] Dexamethasone was given for 2 days, aspirin was given 1 h, whereas BW 755C or isoprenaline was given 30 min before subplantar injection of cardiotoxin (15 μg). PFL 55712, superoxide dismutase/catalase, BN 52021 or L 652731 was co-injected with cardiotoxin. [b] Responses are presented as the areas under the curves measured in the 6 h period after subplantar injection of cardiotoxin (15 μg). [c] Number of animals. [d] Treatment vs control $^{***}P < 0.001$.

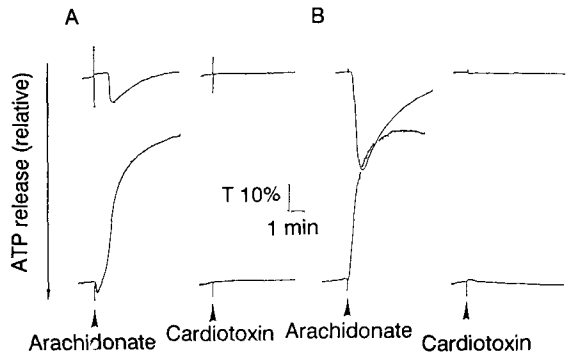

FIG. 2. Aggregation and release reaction of rat platelet-rich plasma (A) and washed platelet suspension (B) induced by sodium arachidonate (100 μM) and cardiotoxin (150 μg mL^{-1}). Similar results were obtained in three other experiments.

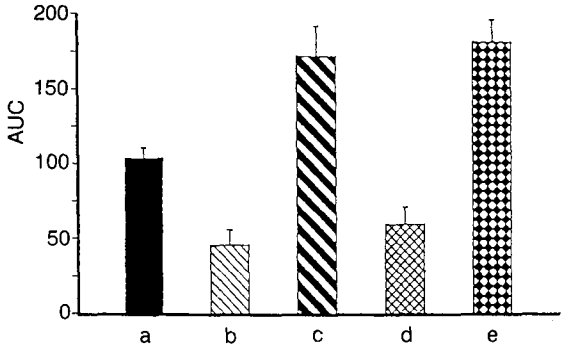

FIG. 3. Additive effect of cardiotoxin on the hind-paw oedema caused by TMVPLA$_2$ II and NNAVPLA$_2$. Responses are presented as the areas under the curves measured in the 6 h period after the subplantar injection of cardiotoxin 5 μg (a); TMVPLA$_2$ II 1 μg (b); NNAVPLA$_2$ 5 μg (d); cardiotoxin plus TMVPLA$_2$ II (c); cardiotoxin plus NNAVPLA$_2$ (e). Values are expressed as the means $\pm$ s.e.m. of 5–9 experiments.

Isoprenaline (1·5 mg kg^{-1} s.c.) pretreatment reduced the oedema to 61% of the control value.

The platelet count in peripheral blood was decreased to less than 4% of the control value after i.v. infusion of anti-platelet plasma. Under this condition, cardiotoxin-induced oedema was unaffected as calculated from the AUC measured in the 1, 3 and 6 h periods after induction of oedema.

In rat platelet preparations, arachidonate (100 μM) induced aggregation and the release of ATP from platelet-rich plasma and the washed platelet suspension, while cardiotoxin (150 μg mL^{-1} did not activate platelets in these respects in these two preparations (Fig. 2).

In view of the interaction between cardiotoxin and phospholipase A$_2$ on rat hind-paw oedema, cardiotoxin co-injected with NNAVPLA$_2$, an acidic phospholipase A$_2$ from *Naja naja atra* venom (Teng et al 1987), or TMVPLA$_2$ II, a basic phospholipase A$_2$ from *Trimeresurus mucrosquamatus* venom (Teng et al 1989), did not potentiate their oedema-producing effects (Fig. 3).

Discussion

Paw oedema caused by cobra venom results from a combination of the amine phase and of the so-called prostaglandin phase (Bonta & de Vos 1965; Bonta 1969). Cardiotoxin was reported to induce histamine release from mast cells (Damerau et al 1975). Recently, Wang & Teng (1989) reported that histamine and 5-HT released from mast cells play a role in cardiotoxin-induced paw oedema. In this study, aspirin alone did not affect this oedema, but significant inhibition appeared as aspirin was injected with diphenhydramine and methysergide. The oedema was also suppressed by FPL 55712, a SRS-A antagonist (Samhoun & Piper 1986), BW 755C, a dual cyclo-oxygenase/lipoxygenase inhibitor (Higgs et al 1979) and dexamethasone. These results indicate that arachidonate metabolites act as mediators, in which leukotrienes are more important than prostaglandins, in cardiotoxin-induced paw oedema. The role of prostaglandins became prominent only when the influence of histamine and 5-HT was reduced.

PMN leukocytes are involved in the oedema (Wang & Teng 1989). The superoxide radical can be produced through a respiratory burst of PMN leukocytes under proper stimulation (Green et al 1979). In isolated PMN leukocyte suspension, cardiotoxin (150 μg mL^{-1}) induced superoxide radical formation (1·14 $\pm$ 0·15 nmol/10^6 cell). Moreover, superoxide dismutase/catalase suppressed the oedema. Thus, the superoxide radical acts as one of the mediators in the oedematous response caused by cardiotoxin.

BN 52021 and L 652731, two PAF antagonists (Braquet & Godfroid 1986), inhibited PAF-induced vasopermeation increase and carrageenan-induced rat paw oedema (Hwang et al 1985, 1986), but did not affect oedema caused by cardiotoxin. Thus PAF plays a minor role in the oedematous response. The inhibitory effect of the β-adrenoceptor agonist, isoprenaline, could be due to its direct action on vascular endothelial cells and the inhibition of mast cell degranulation (Dobbins et al 1982; Undam et al 1985).

Platelets may contribute to the process by increasing vascular permeability, attracting leukocyte and promoting blood coagulation (Henson & Ginsberg 1981). The role of platelets in cardiotoxin-induced paw oedema was investigated in addition to that of mast cells and PMN leukocytes (Wang & Teng 1989). The oedema was unaffected by i.v. pretreatment with anti-platelet plasma, which greatly reduced the platelet count in peripheral blood. In addition, cardiotoxin did not induce platelet activation in platelet-rich plasma and in washed platelet suspension. Similar results were reported in rabbit platelets (Teng et al 1984). Thus, unlike mast cells and PMN leukocytes, platelets could not be involved in a significant role in cardiotoxin-induced oedema.

Several lines of evidence indicate that cardiotoxin and phospholipase A$_2$ influence each other's action. Cardiotoxin markedly potentiated guinea-pig ileum contractility and the haemolytic activity of phospholipase A$_2$ (Condrea et al 1964; Damerau et al 1975). It also induced membrane depolarization of the rat diaphragm muscle and blockade of the axonal action potential accelerated by phospholipase A$_2$ (Chang et al 1972). In the present study, the oedematous response caused by cardiotoxin co-injected with NNAVPLA$_2$ or TMVPLA$_2$ II only appeared additive. Cardiotoxin and phospholipase A$_2$ did not influence each other's action on rat mast cells (Kaiser et al 1972; Damerau et al 1975). Therefore, cardiotoxin- and phospholipase A$_2$-induced paw oedema

probably arises through a similar mode of action, and similar inflammatory mediators are involved.

Acknowledgements

This work was supported by a research grant from the National Science Council of the Republic of China (NSC 78-0412-B002-60).

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