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STEROID-LIKE ANTI-INFLAMMATORY EFFECT OF SUPEROXIDE DISMUTASE IN SEROTONIN-, HISTAMINE- AND KININ-INDUCED EDEMATA OF MICE: EXISTENCE OF VASCULAR PERMEABILITY REGULATING PROTEIN(S)

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Abstract—Anti-inflammatory drugs (0.25–30 mg/kg), protein synthesis inhibitors or anti-inducer steroid were administered subcutaneously to mice at time 0–6 hr before the initiation of foot edema by chemical mediators. Dexamethasone (1 mg/kg) inhibited serotonin-, histamine-, bradykinin- and kallikrein-induced foot edemata 45–60% at 3–6 hr. More than 1 hr of lag time was required for inhibitory effect. SOD (5 mg/kg) began to inhibit the edema as early as 30 min and reached a maximum at 2–3 hr (40–50%) followed by gradual decrease of inhibition. Any of these four models of inflammation was not inhibited by indomethacin (5 mg/kg) through 0–6 hr, proving that these models are insensitive to the inhibitors of prostaglandin synthesis. Cyclohexamide (3 mg/kg), puromycin (8 mg/kg) or actinomycin D (2 mg/kg) reversed the inhibition attained by dexamethasone or SOD. Moreover, the protein synthesis inhibitors and actinomycin D by themselves increase the moderately induced paw swellings of normal mice. Intravenously administered SOD showed the same time course of inhibition as in the subcutaneous experiment by serotonin edema. As the half-life of SOD is only 6 min in the blood stream, this result suggests also the indirect effect of SOD. SOD may not work to release certain anti-inflammatory factor(s) from the granules like lysosomes, because the repeatedly administered SOD was as effective as first injection. Stimulation of adrenals by SOD is exclusive from the rapidity of SOD effect. Progesterone (non-anti-inflammatory steroid which occupies glucocorticoid receptor) diminish the inhibition by dexamethasone, but did not modify the inhibition by SOD. This means that the effect of SOD is not mediated by glucocorticoid receptor. Basing on these observations, the existence of a natural anti-inflammatory protein (X-protein) is supposed. This protein may be perpetually synthesized *in vivo* to regulate endothelial cell contraction. SOD is possible to prevent the degradation or inactivation of X-protein by active oxygen radicals, and the accumulation of this protein may diminish the vascular permeability. It is possible that X-protein and steroid-induced protein are the same.

The anti-inflammatory action of SOD (superoxide dismutase: an enzyme which removes specifically superoxide radical by dismutation) has been reported in carrageenan foot edema [1], rat adjuvant arthritis [2], reversed Arthus reaction of rats [2] and in human rheumatism [3]. Synovial fluid hyaluronate degradation [4] and leukocyte chemotaxis [5] were also inhibited by SOD. Guinea-pig peritoneal macrophages cultured with BCG-sensitized lymphocytes and antigen PPD (purified protein derivatives) increased active oxygen radical production which was inhibited by the addition of SOD or D-mannitol in the culture medium [6]. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibited the superoxide production of oil-induced guinea-pig peritoneal macrophages [7] or of non-stimulated peritoneal cells [8].

Dexamethasone was neither the inhibitor of superoxide production by macrophages, nor the scavenger of superoxide radical. SOD can be an anti-inflammatory drug by its capacity to remove directly the superoxide radical at the inflamed site. The effect of SOD is also assumed to be mediated by the formation of chloroform-extractable chemotactic lipid peroxide [5].

It is attempted here to find another mechanism of active oxygen radicals in the inflammation model which is sensitive to glucocorticoid and not sensitive to NSAIDs. As serotonin-, histamine- and kinin-induced paw edemata of mice are generally believed to be insensitive to indomethacin (a typical NSAID), SOD was tested in these inflammation models. Concerning serotonin-induced edema, a certain anti-inflammatory protein synthesis *in vivo* is reported by Tsurufuji *et al.* [9] as an essential step for inhibition of edema by dexamethasone. Recently, glucocorticoid-induced phospholipase A_2 inhibitory protein (macrocortin; claimed as anti-inflammatory protein) has been isolated [10]. Paw swelling induced by serotonin, histamine or kinin, is principally dependent on increase of vascular permeability, and the participation of prostaglandins is exclusive (lack of inhibitory effect by indomethacin). Possible increase of vascular permeability by active oxygen radicals is reported by Björk *et al.* [11]. They applied hypoxanthine and xanthine oxidase system on the hamster cheek pouch. Microvascular permeability was measured by the leakage of injected macromolecule (FITC-dextrane 150,000). Hydroxyl radical (·OH) or singlet oxygen ($^1O_2$) which are produced

by this system, was suggested to increase vascular permeability. These radicals can be generated from superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) on the surface of activated leukocytes locating at the inflamed site.

MATERIALS AND METHODS

Animals

Male ICR mice (25–30 g) were obtained from the Sizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan).

Assay

The method mainly followed that of Tsurufuji *et al.* [9]. Indicated doses of irritants, as 5 μl saline solution, were injected into the plantar surface of the right hind paw. An equal volume of saline was injected into the left hind paw. Doses of irritants were presented as irritant molecules themselves excluding salts. The thicknesses of both hind paws were measured with Dial Thickness Gauge (KG-1, Citizen Watch Co., Tokyo) at 12 min for serotonin and histamine, 6 min for bradykinin and 15 min for kallikrein. The difference of thickness between right and left hind paws was taken as inflammatory index. Drug (in 0.5 ml) was injected subcutaneously in the abdomen or intravenously in the tail at the time indicated before the irritants.

Cycloheximide (3 mg/kg), puromycin·2HCl (8 mg/kg) or actinomycin D (2 mg/kg) was injected subcutaneously in the abdomen apart from drug every 1.5 hr beginning at 0 hr except Table 3. Progesterone treatment was conducted by s.c. injection at 1 hr before administration of dexamethasone or SOD.

Chemicals

Serotonin creatinine sulfate·H_2O, cycloheximide, copper acetate, D-mannitol and benzoic acid were purchased from Nakarai Chemical Co. (Kyoto). Bradykinin·3 acetates were obtained from Japan Protein Research Development Assoc. Kallikrein (kininogenase, pancreatic 5 units/mg protein) and SOD (bovine Cu,Zn-superoxide dismutase, lyophilized powder, 3,000 units/mg protein) are the products of Sigma Co. Dexamethasone (Decadoron, Japan-Merck Co., 4 mg/ml phosphate ester) is for injection. Indomethacin (August Brandes Co.) was dissolved in a small volume of 1 N NaOH before saline addition and finally the pH of the solution was adjusted to pH 7.0 with 0.1 N HCl. Progesterone (Proge hormone, 10 mg/ml sesame oil) was obtained from Mochida Seiyaku Co. (Tokyo). Puromycin (dihydrochloride) and actinomycin D (free of salt) are the products of Makor Chem. Co. (Jerusalem). Catalase (Type 2, 260,000 units/ml 30% glycerol and 10% ethanol, from beef liver) was obtained from Boehringer Mannheim Co. Bovine serum albumin (BSA) was a product of Armour Pharmaceutical Co. Heat-inactivation of SOD was performed by heating the solution to 100° for 30 min.

RESULTS

1. *Preliminary experiments*

After testing various doses of serotonin (0.02–1.2 μg/paw), the dose of 0.3 μg/paw was adopted as the minimum dose to induce almost complete swelling at 12 min. Paw swelling was maximum between 10 and 15 min. The time-dependent curve of swelling in dexamethasone (1 mg/kg, 3 hr) or SOD (5 mg/kg, 2 hr) treated mouse, paralleled that of control, which offered also the reason to determine the inhibitory capacity of drug at 12 min. Saline injection made very slight paw swelling after 10 min. The dose for inducing moderate paw swelling was settled as 0.05 μg serotonin/paw. Moderate swelling model was necessary to detect the stimulatory effect by protein synthesis inhibitors. Histamine edema was measured at 12 min with doses of 6–120 μg/paw and fixed as 30 μg/paw for extensive swelling and 5 μg/paw for moderate swelling. Bradykinin was tested in the range of 2.5–20 μg/paw and the condition for extensive swelling was set as 10 μg/paw at 6 min. Kallikrein edema was measured at 15 min with doses of 1–100 μg/paw and settled as 3 μg/paw and 1 μg/paw for extensive and moderate swellings, respectively. The inhibition of kallikrein-induced edema by a high dose of indomethacin (30 mg/kg, at 4 hr) was in an exceptional mode. Paw swelling of indomethacin-treated mice was suppressed at 15 min, but increased up to the control level at 30–90 min. This observation, as well as the requirement of lag time for the appearance of inhibitory effect by indomethacin, may explain the contradictory data reported previously for the effect of indomethacin- on kinin-induced edema.

2. *Lag time for drug effect*

Inhibition of serotonin-induced foot edema by dexamethasone (1 mg/kg) appeared after a 1-hr latent period (Fig. 1) which is required for protein synthesis [9]. Subcutaneously administered SOD (5 mg/kg) resulted in a different mode of time dependency for its inhibitory effect. SOD did not inhibit the edema at 15 min (the time of measurement of swelling was 15 + 12 = 27 min), but began to inhibit at 30 min. The maximum inhibition was

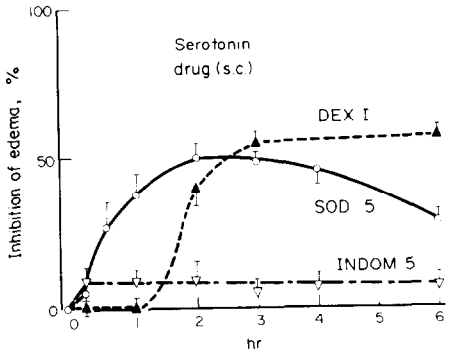

Fig. 1. Inhibition of 0.3 μg/paw serotonin-induced foot edema by subcutaneously administered SOD, 5 mg/kg (○); dexamethasone (DEX), 1 mg/kg (▲); and indomethacin (INDOM), 5 mg/kg (▽). Edema was measured at 12 min after serotonin injection. Saline injected left hind paw was used to obtain a basal value. Inhibition was calculated by finding the percentage of swelling by drug treatment over control. Time axis shows the interval between drug and serotonin injection time. Vertical lines represent the S.E.M. of 3–7 experiments (15–35 mice).

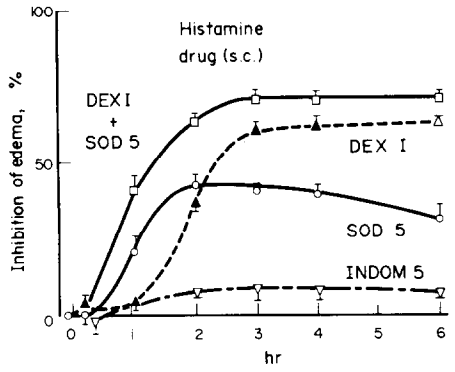

Fig. 2. Inhibition of 30 μg/paw histamine-induced foot edema by subcutaneously administered SOD, 5 mg/kg (○); dexamethasone, 1 mg/kg (▲); SOD, 5 mg/kg + dexamethasone, 1 mg/kg (□); and indomethacin, 5 mg/kg (▽). Edema was measured at 12 min after histamine injection. Vertical lines represent the S.E.M. of 3 experiments (12 mice).

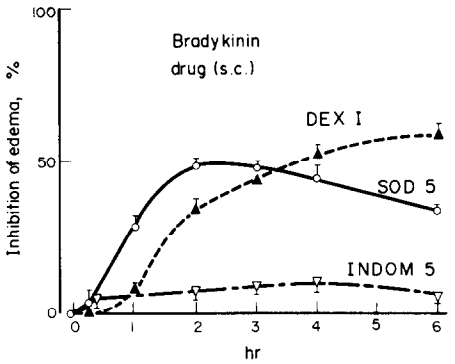

Fig. 3. Inhibition of 10 μg/paw bradykinin-induced foot edema by subcutaneously administered SOD, 5 mg/kg (○); dexamethasone, 1 mg/kg (▲); and indomethacin, 5 mg/kg (▽). Edema was measured at 6 min after bradykinin injection. Vertical lines represent the S.E.M. of 3–5 experiments (12–20 mice).

observed at 2–3 hr. Supposing that this time lag may be derived from the time for arrival of subcutaneously administered SOD at the inflamed site, SOD (5 mg/kg) was intravenously injected. The time course (1–6 hr) was exactly as for s.c. injection. Intravenously given SOD must make its effect more rapidly than subcutaeously injected SOD if the retardation of effect depends on the transporting time of SOD. Intravenously administered dexamethasone (1 mg/kg) showed also the same time course as for the subcutaneous case. The lag time for the appearance of inhibition by SOD is very short, but is related to protein synthesis as directly demonstrated later.

Dose-dependent inhibitions of these two drugs on serotonin edema were as follows (mean of 3–4 experiments, namely of 12–16 mice for one dose): dexamethasone (at 3 hr)—30 mg/kg (65%), 5 mg/kg (62%), 1 mg/kg (55%) and 0.2 mg/kg (30%); SOD (at 2 hr)—30 mg/kg (55%), 15 mg/kg (53%), 5 mg/kg (50%) and 2.5 mg/kg (15%). Inhibitions by indomethacin (5 mg/kg) never exceeded 10% (Fig. 1). Even 30 mg/kg was non-inhibitory in serotonin edema.

Inhibitions of histamine-induced foot edema by subcutaneously injected dexamethasone (1 mg/kg) and SOD (5 mg/kg) were very similiar to those obtained with serotonin edema (Fig. 2). Simultaneous injection of dexamethasone and SOD seemed to suppress the histamine edema in an additive manner. This was true also in serotonin edema (two experiments, eight mice for one point, data not presented). SOD must not be competitive with dexamethasone for inducing anti-inflammatory protein. Indomethacin (5, 15 and 30 mg/kg) was again non-inhibitory. Dose dependencies to inhibit histamine-induced edema were determined (3–4 experiments, 12–16 mice): dexamethasone (at 3 hr)—30 mg/kg (67%), 5 mg/kg (63%), 1 mg/kg (61%) and 0.25 mg/kg (41%); SOD (at 2 hr)—30 mg/kg (46%), 15 mg/kg (45%) 5 mg/kg (42%) and 2.5 mg/kg (26%).

Figure 3 shows the result with bradykinin edema. Dexamethasone (1 mg/kg) seemed to be a little retarded for manifesting its maximum inhibition. SOD (5 mg/kg) inhibited just as in serotonin or histamine edema. Dose dependencies tested with four experiments (16 mice) were as follows: dexamethasone (at 4 hr)—30 mg/kg (65%), 5 mg/kg (55%), 1 mg/kg (52%) and 0.25 mg/kg (33%); SOD (at 2 hr)—30 mg/kg (56%), 15 mg/kg (52%), 5 mg/kg (49%) and 2.5 mg/kg (24%). Indomethacin (5 mg/kg) was not inhibitory, but very high dose of indomethacin (30 mg/kg) showed about 40% inhibition only at 3–4 hr. This inhibition may have no significance for the pharmacological action of this drug.

Figure 4 is the result with kallikrein edema. Inhibitions by dexamethasone and SOD were very similar to that of bradykinin-induced edema. Dose dependencies were as follows (four experiments, 16 mice for one dose): dexamethasone (at 4 hr)—30 mg/kg (61%), 15 mg/kg (50%), 1 mg/kg (46%)

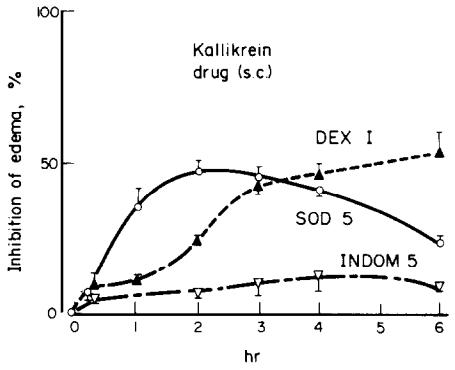

Fig. 4. Inhibition of 3 μg/paw kallikrein-induced foot edema by subcutaneously administered SOD, 5 mg/kg (○); dexamethasone, 1 mg/kg, (▲); and indomethacin, 5 mg/kg (▽). Edema was measured at 15 min after kallikrein injection. Vertical lines represent the S.E.M. of 3–5 experiments (12–20 mice).

Table 1. Inhibitions of paw swellings by oxygen radical scavengers, inactivated SOD, BSA (bovine serum albumin) and copper salt

Drug	Dose	Edema inhibition % at 2 hr			
		Serotonin	Histamine	Bradykinin	Kallikrein
SOD	15 mg/kg	47 ± 3	46 ± 3	51 ± 1	55 ± 4
Inactivated SOD	15 mg/kg	2 ± 2	7 ± 2	13 ± 4	7 ± 2
BSA	30 mg/kg	11 ± 4	—	—	—
Cu-acetate	4.4 mg/kg*	6 ± 3	—	4 ± 2	14 ± 3
Catalase	4.3×10^5 U/ml	3 ± 5	2 ± 1	9 ± 1	14 ± 2
D-Mannitol	300 mg/kg	1 ± 4	6 ± 3	6 ± 2	13 ± 5
Benzoic acid	150 mg/kg	—	—	59 ± 3	53 ± 5
Benzoic acid	75 mg/kg	—	—	44 ± 1	23 ± 3

Inhibition was presented as mean ± S.E.M. of 3–4 experiments (12–16 mice). Doses of irritants were as in Figs. 1–4.

* This dose of copper corresponds to the copper content of 300 mg/kg SOD. High doses of SOD were used as a standard to demonstrate clearly the inactivation effect.

and 0.25 mg/kg (26%); SOD (at 2 hr)—30 mg/kg (47%), 15 mg/kg (52%), 5 mg/kg (47%) and 2.5 mg/kg (22%). The dose of indomethacin (30 mg/kg) which inhibited 34% of kallikrein edema at 4 hr, was too high to discuss the anti-inflammatory action. Moreover, the intrinsic effect of indomethacin must be far less than 34% which was determined at 15 min after kallikrein injection, because the inhibition by this dose of indomethacin was not noted after 30 min.

3. *Effect of active oxygen scavengers*

Heat-inactivated SOD did not inhibit the paw swelling induced by any of four irritants (Table 1). As SOD contains copper which is essential for its enzymatic activity, high dose of copper acetate was tested. Copper did not inhibit the edema of three irritant models.

Common protein (bovine serum albumin) was naturally non-suppressive. It is clear that the enzymatic activity of SOD is required to suppress the edemata. This suggests the possible participation of superoxide or certain active oxygen radical(s) derived from it.

As indomethacin was not inhibitory, the effect of SOD may not be mediated by prostaglandins or lipoxygenase products. Direct participation of hydrogen peroxide is not possible, because even a high dose of catalase was not suppressive in any model of four inflammations. A typical hydroxyl radical scavenger, D-mannitol, had no effect, but another scavenger, benzoic acid, inhibited the paw swellings induced by kinins. Involvement of hydroxyl radical cannot be overlooked. The data presented in Table 1 were measured at 2 hr after drug administration, and nearly the same result was also obtained at 3 hr.

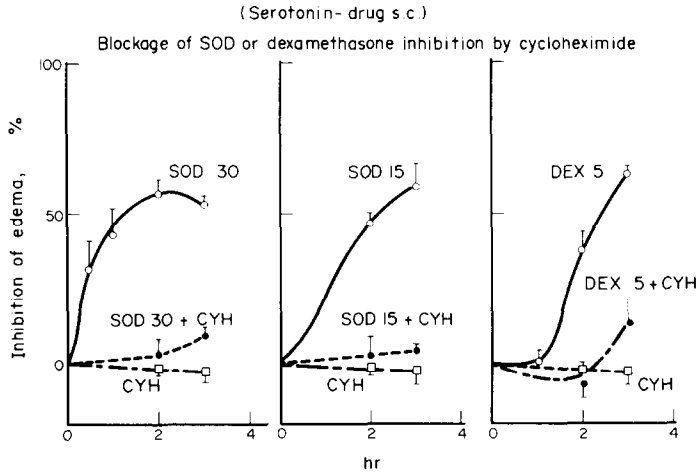

Fig. 5. Reversion of SOD and dexamethasone inhibitions by cycloheximide in 0.3 μg/paw serotonin-induced foot edema. The drug was administered at 0 time. Cycloheximide, 3 mg/kg, was given subcutaneously in another part of the abdomen, separated from the drug-injected site at 0, 1.5 and 3 hr. Drug only (○), cycloheximide (CyH) (□) and drug + cycloheximide (●). SOD is 30 or 15 mg/kg and dexamethasone is 5 mg/kg. Vertical lines represent the S.E.M. of 3–4 experiments (15–20 mice). High doses of drugs were used to demonstrate clearly the effects of cycloheximide.

Table 2. Effects of dexamethasone and SOD on paw swellings modified by protein synthesis inhibitors

Serotonin	Treatment	Paw swelling ($\times 10^{-2}$ mm) 1 hr	2 hr	4 hr
0.3 μg/paw	Saline	83 ± 4	80 ± 3	85 ± 4
	Dexamethasone	80 ± 2	42 ± 3	36 ± 4
	SOD	44 ± 4	43 ± 2	32 ± 5
Non-injected control	Cycloheximide	84 ± 4	80 ± 3	78 ± 4
	Cycloheximide + dexamethasone	86 ± 3	86 ± 3	70 ± 5
$80 \pm 2 \times 10^{-2}$ mm	Cycloheximide + SOD	81 ± 4	78 ± 5	72 ± 1
	Puromycin	101 ± 2	89 ± 1	93 ± 4
	Puromycin + dexamethasone	91 ± 4	89 ± 6	60 ± 3
	Puromycin + SOD	101 ± 2	93 ± 3	83 ± 1
0.05 μg/paw	Saline	50 ± 4	48 ± 2	45 ± 4
	Dexamethasone	48 ± 2	24 ± 3	24 ± 2
	SOD	36 ± 2	28 ± 1	25 ± 6
Non-injected control	Cycloheximide	68 ± 1	67 ± 4	70 ± 5
	Cycloheximide + dexamethasone	59 ± 3	55 ± 2	44 ± 3
$48 \pm 2 \times 10^{-2}$ mm	Cycloheximide + SOD	58 ± 2	54 ± 6	62 ± 3
	Puromycin	55 ± 3	60 ± 2	60 ± 1
	Puromycin + dexamethasone	54 ± 3	50 ± 2	38 ± 1
	Puromycin + SOD	55 ± 6	58 ± 3	55 ± 2

This experiment was performed independently from that of Fig. 5. Paw edema was induced by 0.3 μg serotonin/paw (extensive swelling) or by 0.05 μg/paw (moderate swelling). Dexamethasone (1 mg/kg) or SOD (5 mg/kg) was injected subcutaneously at 1, 2 or 4 hr before serotonin. Cycloheximide (3 mg/kg) or puromycin (8 mg/kg) was administered subcutaneously every 1.5 hr. Paw swelling was presented as mean ± S.E.M. of 3–4 experiments (12–16 mice).

4. *Reversion of edema inhibition by protein synthesis inhibitors*

Cycloheximide (3 mg/kg) reversed the inhibitions by dexamethasone (5 mg/kg) and by SOD (30 and 15 mg/kg) (Fig. 5). High doses of drugs were adopted in this experiment of serotonin edema to assure the potency of cycloheximide. This dose of cycloheximide inhibits more than 90% of [^{3}H]leucine incorporation into total cellular proteins of rat kidney at 1–4 hr after injection [12]. The same reversive effect of cycloheximide was observed also in histamine-, bradykinin- and kallikrein-induced edemata (data not presented). SOD might work as a stimulant of natural anti-inflammatory protein production or an inhibitor of its degradation. Chemical inactivation of SOD by contact with cycloheximide in the body of mice is not possible. Equal volumes of SOD (4×10^{-5} M—concentration used for 30 mg/kg) and cycloheximide (6.4×10^{-2} M—concentration used for 3 mg/kg test) were incubated together for 90 min

Table 3. Increases of paw swelling in non-treated mice by s.c. injections of protein synthesis inhibitors

Irritant	Protein synthesis inhibitor	Paw swelling ($\times 10^{-2}$ mm) 15 min	1 hr	4 hr
Serotonin	Saline	45 ± 3	50 ± 4	45 ± 4
0.05 μg/paw	Cycloheximide	58 ± 4	68 ± 1	47 ± 2
Control	Puromycin	67 ± 1	55 ± 3	51 ± 1
48 ± 2	Actinomycin D	62 ± 2	57 ± 2	47 ± 3
Histamine	Saline	43 ± 4	46 ± 5	47 ± 2
5 μg/paw	Cycloheximide	53 ± 1	62 ± 2	48 ± 5
Control	Puromycin	67 ± 1	69 ± 2	50 ± 1
45 ± 2	Actinomycin D	64 ± 2	77 ± 3	53 ± 4
Kallikrein	Saline	29 ± 3	32 ± 1	34 ± 2
1 μg/paw	Cycloheximide	34 ± 1	48 ± 2	34 ± 4
Control	Puromycin	40 ± 2	41 ± 3	33 ± 3
32 ± 1	Actinomycin D	38 ± 1	41 ± 1	30 ± 3

Moderate swelling-inducing doses of each irritant were administered just after a single injection of protein synthesis inhibitor. Paw swelling was presented as mean ± S.E.M. of 3–4 experiments (12–16 mice). All inhibitors at the concentration used augumented the paw swelling at 15 min and 1 hr, but were non-effective at 4 hr.

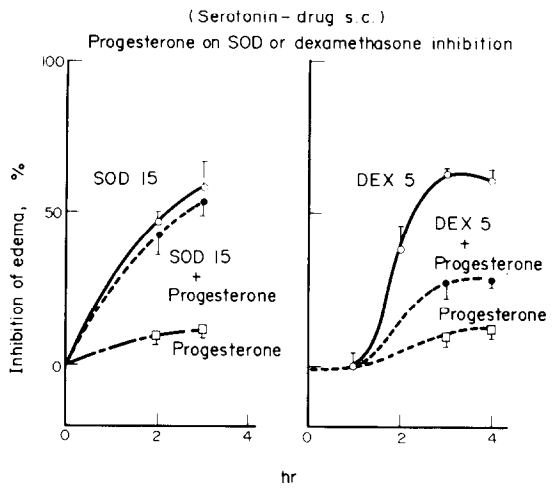

Fig. 6. Effect of progesterone (anti-inducer steroid) against the inhibition by drugs on 0.3 μg/paw serotonin edema. Progesterone, 200 mg/kg (□), was injected subcutaneously 1 hr before drug at 0 time. SOD, 15 mg/kg, or dexamethasone, 5 mg only (○), and combined test with progesterone (●). Vertical lines represent the S.E.M. of 3–4 experiments (15–20 mice).

at 37°, and SOD activity was measured with xanthine oxidase plus lactate dehydrogenase–NADH system [7, 13]. No decrease of SOD activity was observed compared to non-incubated SOD plus cycloheximide or incubated SOD alone.

Cycloheximide itself showed little stimulation of paw swelling in Fig. 5, but the increasing effect by drug was difficult to observe with an extensively inflamed condition. The upper part of Table 2 shows reversion of inhibition attained by dexamethasone or SOD using puromycin or cycloheximide. Puromycin itself also increased slightly the edema induced by 0.3 μg/paw (extensive inflammation).

Edema-enhancing effects of puromycin or cycloheximide became evident in edema induced by 0.05 μg serotonin/paw (moderate inflammation) (Table 2, lower part). Both protein synthesis inhibitors reversed the inhibitions by dexamethasone and SOD in moderately induced inflammation. Actinomycin D reversed almost completely the moderately induced serotonin edema which was suppressive at 2 and 3 hr by 1 mg/kg dexamethasone or by 15 mg/kg SOD (data not presented). Degree of paw swelling may depend on the accumulation of the natural anti-inflammatory protein. This protein is inducible by both endogenous and exogenous glucocorticoid, and accumulates through the blockage of its inactivation or degradation by active oxygen radical scavengers (SOD, etc.).

Table 3 demonstrates the increase of paw swellings induced by moderate dose of serotonin, histamine or kallikrein by single injection of protein synthesis inhibitors. Enhancing effects of these protein inhibitors were noted at 15 min and at 1 hr in normal mice (increase of 30–50%). This suggests strongly the existence of perpetual synthesis and degradation of natural anti-inflammatory protein in the normal state.

5. *Effect of progesterone*

High dose of non-anti-inflammatory steroid such as progesterone blocks inhibitory action of dexamethasone on serotonin edema [9].

The anti-inducer steroid pre-occupies the glucocorticoid receptor of the target cells which produce anti-inflammatory steroid inducible protein. Progesterone (200 mg/kg) was subcutaneously administered 1 hr before dexamethasone or SOD in Fig. 6. Inhibition of serotonin edema by dexamethasone at 3 and 4 hr was blocked about 60% by pretreatment of progesterone. Nevertheless, the inhibition by SOD was not reversed by this pretreatment. This result demonstrates that the action of SOD has no relation with the mechanism of steroid-inducible protein production.

6. *Effect of repeated administrations of SOD*

Inhibition of single administration of SOD began to decrease after 3 hr. The experiment with kallikrein-induced edema was conducted to determine whether repeated injection of SOD can prevent this decrease or not. Double injections of 15 mg/kg at 0 and 3 hr were more effective (55% at 6 hr) than single injection of 30 mg/kg at 0 hr (42% at 6 hr). Triple injections of 5 mg/kg (at 0, 2 and 4 hr) were more suppressive (51% at 6 hr) than single injection of 15 mg/kg at 0 hr (27% at 6 hr). It is not imaginable that the anti-inflammatory effect of SOD is due to the release of certain factor(s) from the granules like lysosomes. Release of a certain factor by the second stimulation is far less than that by the first in the case of degranulation, but the second or the third administration of SOD showed the same effect as the first injection. Stabilized SOD conjugate [13] may maintain the inhibitory effect of mediator-induced edemata for a long time.

DISCUSSION

Arachidonate metabolite theory for inflammation is dominant, but it seems difficult to explain the result obtained here with it. Increase of vascular permeability is common at an early stage of all kind of inflammations. Serotonin-, histamine-, bradykinin- and kallikrein-induced vascular permeability are here demonstrated to be inhibited by s.c. injection of dexamethasone and/or of SOD. Dexamethasone inhibited the paw swelling (2–6 hr). In contrast, SOD began to suppress edema as early as 30 min and attained maximum inhibition at 2–3 hr followed by gradual decrease of inhibition. Intravenously administered SOD showed the same time course of inhibition as subcutaneously injected SOD in serotonin edema, which suggests the indirect effect of SOD. Intravenous administration must manifest more early inhibition and rapid disappearance of effect if SOD is working directly. Petrone *et al.* [5] extracted a chemotactic lipid peroxide which is formed by superoxide radical. SOD inhibits the inflammation through preventing the leukocyte chemotaxis. Clinical observations suggest also the possibility of indirect effect of SOD (Orgotein). Huber *et al.* [15] noted the recovery of rheumatic patients after the disappearance of administered

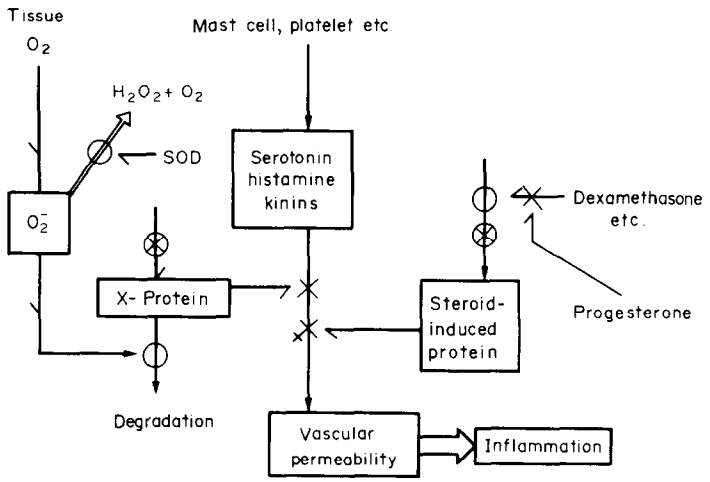

Fig. 7. Supposed participation of superoxide radical on serotonin-, histamine- or kinin-induced foot edema. (○) shows stimulation, (×) shows inhibition and ⊗ is the site of protein synthesis inhibitors. Postulated anti-inflammatory 'X-protein' is perpetually synthesized in the normal state. Continuous degradation or inactivation of X-protein requires superoxide, which can be produced in the inflamed site. The superoxide-removing effect of endogenous or administered SOD may result in less degradation of X-protein. This leads to the accumulation of X-protein and lowers the vascular permeability.

Protein synthesis inhibitors can increase the vascular permeability of both normal and slightly inflamed states. Dexamethasone induces the anti-inflammatory protein which can be blocked by synthesis inhibitors. Progesterone competes with dexamethasone on glucocorticoid receptor and partially abolishes the effect of dexamethasone. There is also a possibility that hydroxyl radical or other active oxygen radicals, which can be produced from superoxide radical or hydrogen peroxide, participate in degradation of inactivation of X-protein. X-protein and steroid-induced protein could possibly be the same.

SOD in the blood and tissues. The data in this report offer the possible existence of another mechanism that SOD works indirectly through vascular permeability against inflammation. The effect of SOD may not be mediated by the stimulation of glucocorticoid receptor which produces anti-inflammatory protein, because progesterone (anti-inducer steroid which competes for glucocorticoid receptor with dexamethasone), has no influence on serotonin-induced edema inhibited by SOD.

The existence is supposed of an anti-inflammatory 'X-protein' which keeps at an appropriate level for regulating endothelial cell concentration (Fig. 7). Quinolinate phosphoribosyl transferase [16], ribonuclease [17], *t*-RNA(lysyl)ligase [17] are reported to be inactivated by superoxide radical. Enzyme reactions requiring superoxide or hydroxyl radicals are also well known [18–20]. Bacteria, immune complex or chemical mediator can activate superoxide production of leukocytes or macrophages.

Superoxide, or hydroxyl radical derived from it, may diminish the X-protein. This results in the increase of vascular permeability for inflammation development. A typical hydroxyl radical scavenger, D-mannitol had no effect in any of four inflammation models, but another scavenger, benzoic acid, inhibited bradykinin and kallikrein edemata. Inhibitions by benzoic acid of both edemata were also reversed completely by cycloheximide (data not shown).

According to Babior *et al* [21], alkyl radical-like product from D-mannitol formed by hydroxyl radical has still hydroxyl-radical-like activity, but benzoate-hydroxyl radical product has no such activity. The effect of D-mannitol must not be made important if we follow this observation. It is possible that hydroxyl radical is inactivating X-protein. Participation of other active oxygen radicals like singlet oxygen cannot be excluded at the moment. However, the concentration of superoxide radical must be most important, because all other active oxygen radicals are believed to be produced from superoxide.

Stimulation of mediator release by superoxide is already assumed by Handin *et al.* [22]. Xanthine oxidase (superoxide-generating system) induced serotonin release from human platelet.

This release was inhibited by SOD and not by catalase or D-mannitol. Pre-incubation of platelets with indomethacin had no effect. Low concentration of thrombin (0.002 U/ml) released only a little serotonin from platelets, but the addition of xanthine oxidase resulted in a 10-fold increase of serotonin release (corresponding to the effect by 0.02 U/ml thrombin without xanthine oxidase). In my experiment, however, the releasing process of serotonin or histamine is not involved, for these mediators themselves were injected into footpads. There are some reports to support the involvement of active oxygen radical in the synthesis of prostaglandins [23–25] or other lipid peroxides [26]. Many lipid peroxides are supposed to work as chemoattractants for leukocytes and macrophages [5, 27, 28]. These mechanism again can be excluded in the results obtained here, because more than 30 min is generally required for the accumulation of leukocytes at the irritant injected site. The maximum swelling by

serotonin or histamine attained as early as 10 min after the injection and 6 or 15 min were sufficient for bradykinin and kallikrein, respectively. SOD was not tested with adrenalectomized mice, but the possibility of stimulating adrenals to release corticosteroid can be excluded. Inhibition of swelling by SOD began already at 30 min compared to the retarded appearance of dexamethasone inhibition (after 2 hr). Huber *et al.* [2] observed that adrenalectomized animals respond to SOD just as normal animals do.

Repeatedly injected low doses of SOD maintained the suppressed state of edema. As the half-life of native SOD is only 6 min, it is interesting to inject the long-acting stabilized polyethylene glycol–SOD, Ficoll–SOD [13] or bovine serum albumin–SOD conjugates [29] in the inflammation models as here examined. The roles of serotonin, histamine and kinins are not limited to the inflammation, so that the existence of X-protein-like factor(s) can be supposed in other domains of physiological and pharmacological research.

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