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Inhibitory effects by anti-inflammatory drugs on enzyme release from rabbit polymorphonuclear leukocyte lysosomes*

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In 1967, Weissmann [1] reviewed the important roles of polymorphonuclear (PMN) leukocyte lysosomes in inflammation. In our previous paper [2], it was suggested that nonsteroidal anti-inflammatory drugs such as phenylbutazone had a membrane-stabilizing activity on intact lysosomes, for they inhibited the release of enzymes from rabbit PMN leukocyte lysosomes *in vitro*.

This paper deals with the influence of certain anti-inflammatory agents on the release of enzymes from the rabbit PMN leukocyte lysosomes. The method of preparation of lysosomes reported by Ignarro [3] was modified in order to obtain the intact lysosomes. Namely, heparinized Ca^{2+}-free Hanks' solution containing glucose was injected before harvesting the leukocyte suspension, and the lysosomes were liberated from the leukocytes suspended in 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4). Anti-inflammatory drugs exhibited a membrane-stabilizing activity on these intact lysosomes, which were prepared by the method described below.

Preparation of PMN leukocytes and their lysosomes. Male albino rabbits (3 to 3·5 kg) were injected intraperitoneally with 200 ml of 0·1% (w/v) glycogen in sterile saline, and 4 hr later, with 100 ml of Ca^{2+}-free Hanks' solution containing 0·1% glucose and 10 units/ml of heparin. Immediately after the latter injection, the exudate containing suspended PMN leukocytes (2–6 × 10^6 cells/ml, 150–200 ml/animal) was withdrawn from the abdominal cavity through a needle.

This suspension was stored in ice water until the next operation. The leukocyte suspension, contaminated with a small number of erythrocytes, was filtered through cheese cloth. The filtrate containing over 90 per cent PMN leukocytes was centrifuged at 250 *g* for 5 min. The cell pellet was resuspended in 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4). This suspension was centrifuged again under the same conditions.

To the cell pellet was added the same buffer or 0·34 M sucrose–0·04 M Tris–acetate buffer (pH 7·4) to adjust the leukocyte counts to 1 × 10^8/ml, followed by vigorous uptake and expulsion from a 10-ml pipette five or six times, to liberate the lysosomes from PMN leukocytes. The suspension was centrifuged at 600 *g* for 10 min to separate lysosomes from intact cells, nuclei and other cellular debris. The supernatant was mainly used as a suspension of lysosomes (600 *g* supernatant fraction), which contained mitochondria and other organelles besides the lysosomes. This lysosome suspension was further centrifuged at 8200 *g* for 15 min. To the pellet was added the same volume of 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4). This was also used as the suspension of lysosomes (600–8200 *g* fraction). These procedures were carried out as quickly as possible at a temperature of 4° or less.

Drugs and preparation of test solution. Phenylbutazone, acetylsalicylic acid, ibuprofen, indomethacin and tinoridine hydrochloride were supplied by our Laboratories. Hydrocortisone and prednisolone, which were not in salt form, were purchased from Sigma Chemical Co., Ltd. These drugs were dissolved in ethanol solution and diluted with sucrose buffer in such a way that the concentration of ethanol finally became 10% (v/v) in the test solution.

Assay procedure. Effects of test drugs on lysosome membranes were studied by determining the release of acid phosphatase or aryl sulfatase as a marker enzyme. None of the agents tested inhibited directly the activities of the marker enzymes. The test solution (200 μl) or 10% (v/v) ethanol–

* This paper corresponds to "Studies on Anti-inflammatory Agents—XXVII".

Table 1. Method of preparation of lysosomes and stabilizing activity of test drugs on lysosomes*

Drug (100 μM)	Incubation time (min)	Per cent of total acid phosphatase		
		0·25 M sucrose buffer†		0·34 M sucrose buffer†
		600 *g* Sup.‡	600–8200 *g*‡	600–8200 *g*‡
Control	0	5·2 ± 0·4§	4·3 ± 0·3	6·8 ± 0·3‖
Control	30	15·7 ± 0·5	17·0 ± 0·4	19·9 ± 0·3‖
Phenylbutazone	30	10·3 ± 0·4‖,¶ (51)	9·4 ± 0·3¶ (57)	14·1 ± 0·4‖,¶ (44)
Prednisolone	30	12·2 ± 0·4¶ (33)	13·4 ± 0·3¶ (28)	17·0 ± 0·3‖,¶ (22)
Total activity**		75·3 ± 6·8 (100%)	69·7 ± 7·6 (100%)	69·6 ± 7·0 (100%)

* Results are shown as the mean ± S.E. ($N = 4$) of two separate experiments; the numbers in parentheses show the per cent inhibition. The per cent inhibition was calculated by the following formula: $(A - B)/A \times 100$ (A, the free activity increased during the incubation of control; B, the free activity increased during the incubation of test drug).

† Preparation medium of lysosomes from PMN leukocytes.

‡ Lysosome fraction used. Sup. = supernatant fraction.

§ $P < 0.05$.

‖ $P < 0.01$ (significant) vs 600–8200 *g* fraction prepared from leukocytes in 0·25 M sucrose buffer.

¶ $P < 0.01$ (significant) vs control (30 min).

** Amount of *p*-nitrophenol formed (in μg).

buffer solution as solvent was added to a 25-ml flask containing 2·0 ml of the above lysosome suspension. After the incubation mixture was kept for 5 min at 25°, 0·5-ml aliquots were removed to determine the initial activity of free enzymes in the supernatant. The residual mixtures were incubated at 37° for 30, 90 or 120 min with shaking at an agitation cycle of 75/min (8 cm stroke). After the incubation, the supernatant obtained by high speed centrifugation at 27,000 *g* for 15 min at 4° was used for the measurement of the marker enzymes released from lysosomes. The total activity of these enzymes was assayed using the supernatant obtained by centrifuging the lysosome suspension incubated at 37° for 30 min in 0·2% Triton X-100–0·04 M Tris–acetate buffer (pH 7·4).

Acid phosphatase (EC 3.1.3.2., orthophosphoric monoester phosphohydrolase) and aryl sulfatase (EC 3.1.6.1., aryl sulfate sulfohydrolase) activities were assayed using sodium *p*-nitrophenyl phosphate or dipotassium *p*-nitrocatechol sulfate as substrate by the methods described previously [4].

Table 1 shows the stability of lysosomes prepared by the above methods and the stabilizing activity of test drugs on lysosomes. The lysosomes prepared from PMN leukocytes in 0·25 M sucrose buffer were found to be more stable than those prepared in 0·34 M sucrose buffer. Both phenylbutazone and prednisolone inhibited the release of enzyme from the three lysosome fractions, as shown in Table 1. Therefore, in the following experiments, the supernatant fraction obtained by centrifuging at 600 *g* was used as the suspension of lysosomes.

The data in Table 2 indicate that the release of acid phosphatase from PMN leukocyte lysosomes is time dependent for periods up to 120 min. The inhibitory activity of phenylbutazone and tinoridine hydrochloride (Y-3642 HCl) [5], agents with anti-inflammatory activity, on the enzyme release decreased in proportion to the incubation times. On the other hand, both prednisolone and hydrocortisone were found to have inhibitory activity throughout the period of incubation. This suggests that the mode of action of predni-

Table 2. Time course of acid phosphatase release from PMN leukocyte lysosomes (600 *g* Sup.)*

Drug	Concn (μM)	Per cent of total acid phosphatase			
		0	30	90	120 min†
Control		5·2 ± 0·4	15·7 ± 0·5	33·0 ± 0·7	41·9 ± 0·9
Phenylbutazone	100	5·1 ± 0·4	10·4 ± 0·4‡ (50·6)	28·6 ± 0·5‡ (15·7)	42·0 ± 1·0 (−0·7)
Tinoridine HCl	100	5·2 ± 0·3	11·8 ± 0·4‡ (37·5)	26·8 ± 0·6‡ (22·2)	48·1 ± 1·0‡ (−17·0)
Prednisolone	100	5·2 ± 0·4	12·4 ± 0·4‡ (32·5)	23·7 ± 0·7‡ (33·3)	25·9 ± 0·8‡ (43·6)
Hydrocortisone	100	5·2 ± 0·4	12·9 ± 0·4‡ (26·9)	24·4 ± 0·7‡ (30·8)	27·6 ± 0·8‡ (38·7)

* Results are shown as the means ± S.E. ($N = 4$) of two separate experiments; the numbers in parentheses show the percent inhibition. Total activity of enzyme: 74·2 ± 4·0 μg *p*-nitrophenol formed.

† Incubation time at 37°.

‡ $P < 0.01$ (significant) vs control.

Table 3. Effects of anti-inflammatory drugs on release of enzymes from PMN leukocyte lysosomes*

Drug	Marker enzyme†	Per cent inhibition (means ± S.E., $N = 4$) 1 μM	10 μM	100 μM	300 μM
Acetylsalicylic acid	AP		12·2 ± 1·5‡	24·9 ± 3·2§	47·2 ± 6·2§
Ibuprofen	AP	9·2 ± 3·7	17·4 ± 4·8§	30·6 ± 4·0§	
Phenylbutazone	AP	15·8 ± 3·8§	32·6 ± 2·7§	50·6 ± 3·1§	
	AS	17·7 ± 2·5§	38·3 ± 2·7§	54·0 ± 2·0§	
Indomethacin	AS	10·1 ± 3·1	20·1 ± 3·6§	36·8 ± 2·1§	
Tinoridine HCl	AP	3·5 ± 2·5	20·7 ± 2·1§	35·4 ± 1·6§	
Prednisolone	AP	7·8 ± 1·9	13·3 ± 3·5‡	29·7 ± 6·3§	
Hydrocortisone	AP	8·4 ± 4·0	12·3 ± 4·1‡	26·3 ± 5·2§	

* The lysosome suspension (600 *g* Sup.) was incubated with each test solution in 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4) at 37° for 30 min.

† AP, acid phosphatase. Activity in controls: before incubation, 3·4 ± 0·7; after incubation, 11·7 ± 0·9; total activity, 75·8 ± 10·2 μg *p*-nitrophenol formed. AS, aryl sulfatase. Activity in controls: before incubation, 0·3 ± 0·1; after incubation, 2·1 ± 0·2; total activity, 13·0 ± 1·9 μg *p*-nitrocatechol formed.

‡ $P < 0·05$.

§ $P < 0·01$ (significant).

solone and hydrocortisone on the lysosome membrane may differ from that of phenylbutazone or tinoridine hydrochloride.

Table 3 shows the per cent inhibition of enzyme release from PMN leukocyte lysosomes after 30 min of incubation with drugs. Nonsteroidal acidic anti-inflammatory drugs such as phenylbutazone and tinoridine hydrochloride, and steroidal anti-inflammatory drugs such as hydrocortisone inhibited the release of enzymes from lysosomes to a degree that was proportional to the drug concentration.

Recently, Ignarro and Colombo [6] have reported that both steroidal and nonsteroidal anti-inflammatory agents such as hydrocortisone and phenylbutazone had a stabilizing effect on lysosomes prepared from guinea-pig PMN leukocytes. Ignarro [3] also reported that glucocorticoids and chloroquine stabilized lysosomes prepared from rabbit peritoneal PMN leukocytes and that nonsteroidal acidic anti-inflammatory agents labilized them. Similarly, Brown and Schwartz [7] reported that anti-inflammatory drugs (i.e. phenylbutazone and indomethacin) had a labilizing activity on rat liver lysosomes *in vitro*.

Table 4. Effects of anti-inflammatory drugs on release of acid phosphatase from unstable lysosomes*

Drug	Concn (μM)	Per cent inhibition (means ± S.E., N = 4)
Phenylbutazone	100	−9·8 ± 3·8
Tinoridine HCl	100	−4·8 ± 5·9
Prednisolone	100	44·0 ± 1·7†
Hydrocortisone	100	44·1 ± 4·0†

* The lysosome suspension (600 *g* Sup.) in 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4) was preincubated at 37° for 15 min. After the addition of each test solution, incubations were carried out at 37° for 30 min. Enzyme activity: before preincubation, 3·7 ± 0·3 (4·5%); after preincubation, 7·4 ± 0·3 (9·1%); after incubation, 18·3 ± 3·2 (22·3%); total activity, 81·6 ± 4·3 (100%) μg *p*-nitrophenol formed.

† $P < 0·01$ (significant).

The discrepancy between these results may not arise from differences between the lysosomes from different species or tissues. However, the lack of agreement may result from differences in the stability of various lysosome preparations, as well as from the conditions of incubation employed in the different experiments. The findings that both steroidal and nonsteroidal anti-inflammatory drugs stabilize the lysosomes from rabbit liver [3], and guinea pig PMN leukocytes [4, 6] seem to agree with the results in Table 3. The data in Table 2 of this report support Ignarro's conclusion [8], which indicates that the anti-inflammatory drugs may labilize the lysosomes because they become extremely fragile during the incubation.

Since the initial activity of free acid phosphatase in this experiment is only less than 6 per cent of the total activity in the lysosome suspension, the lysosomes prepared from leukocytes in 0·25 M sucrose buffer seem to be more stable than those described by Ignarro [3]. The initial activity in his experiments was more than 11 per cent of the total activity, which is a value higher than that found in our experiments. The differences in the results of these two studies with anti-inflammatory drugs seem to arise from the stability of the lysosomes prepared in each case.

Table 4 summarizes the data obtained with the unstable lysosomes caused by the preincubation at 37° for 15 min. The results are in accord with the above hypothesis, namely, only steroidal anti-inflammatory drugs do stabilize the unstable lysosomes.

Therefore, it is concluded that both steroidal and nonsteroidal anti-inflammatory drugs have a membrane-stabilizing activity on intact lysosomes, and the former also stabilizes the unstable lysosomes prepared from rabbit peritoneal PMN leukocytes *in vitro*.

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The effect of pretreating rats with 3-methylcholanthrene upon the enhancement of microsomal aniline hydroxylation by acetone and other agents

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Various compounds have been found to enhance the hydroxylation of aromatic amines by the hepatic microsomal fraction *in vitro*, following their addition to the reaction mixture [1, 2]. Anders [3] has suggested that the enhancing effect of acetone might be attributable to the presence of two aromatic amine hydroxylases in the microsomal fraction, one with a high substrate affinity and a low V_{max} which is inhibited by acetone, and the other with a low substrate affinity and a high V_{max} which is not inhibited by acetone and whose properties only become apparent when the former hydroxylase is inhibited. Vainio and Hänninen [4] found that acetone added to the microsomal fraction decreased the type II difference spectrum and increased the type I difference spectrum produced by aniline. They suggested a correlation between the increased type I spectral change and the enhancement of aniline hydroxylation by acetone. They also found that phospholipase C digestion of the microsomal fraction, which destroys the type I binding site [5], abolishes acetone enhancement of aniline hydroxylation [4].

The pretreatment of rats with polycyclic hydrocarbons such as 3-methylcholanthrene has been shown to diminish the type I difference spectrum produced by drugs and to decrease metabolism of type I substrates [6]. If acetone is producing its effects upon aniline hydroxylation by promoting the type I binding of aniline, then it might be expected that pretreating rats with 3-methylcholanthrene would result in a decrease in the ability of acetone to enhance aniline hydroxylation. Anders [7] has reported however that the aromatic hydroxylase from rats pretreated with 3-methylcholanthrene still responds to acetone. We have reinvestigated the effects of pretreating rats with 3-methylcholanthrene upon the ability of the microsomal fraction to respond to acetone and other enhancing agents.

Methods

Male Wistar rats weighing 250 g, were pretreated with four, daily intraperitoneal injections of 3-methylcholanthrene 80 mg/kg, dissolved in corn oil. Control animals were treated with an equivalent volume of corn oil. Hepatic microsomes were prepared by the method of Ernster *et al.* [8] and suspended in 0·25 M sucrose, 0·05 M Tris pH 7·4. The drug metabolising activity of the freshly prepared microsomal fraction was determined over 30 min at 37° using a supporting system utilizing glucose-6-phosphate dehydrogenase as described by Mazel [9]. The formation of *p*-aminophenol from aniline was measured by the method of Schenkman *et al.* [10] and formaldehyde from aminopyrine by the method of Nash [11]. The concentration of aniline in the reaction mixture was 2·5 mM and aminopyrine 5·0 mM. In studies to determine the kinetic constants of aniline hydroxylation aniline concentrations of 0·1, 0·2, 0·3, 0·5 and 1·0 mM were employed. The protein content of the microsomal fraction was determined by the method of Lowry *et al.* [12].

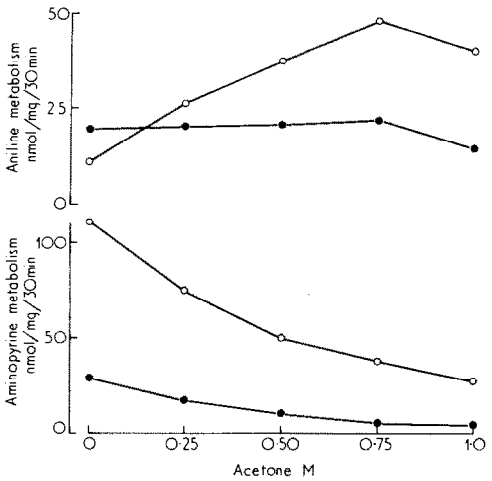

Fig. 1. The effect of acetone upon the metabolism of aniline and aminopyrine by the hepatic microsomal fraction from control (○) and 3-methylcholanthrene pretreated rats (●). Incubation conditions are described in the text. Acetone was added directly to the reaction mixture to give the concentrations shown.