PITUITARY-ADRENOCORTICAL STIMULATION AND ANTI-INFLAMMATORY ACTIONS OF 4-ETHOXY-2-METHYL-5-MORPHOLINO-3(2H)-PYRIDAZINONE (M73101)

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We investigated the mechanism of the elevation of serum corticosterone level by 4-ethoxy-2-methyl-5-morpholino-3(2H)-pyridazinone (M73101) and its participation in the anti-inflammatory action. M73101 when given to rats intraperitoneally at doses of 50–200 mg/kg, caused an increase in serum and adrenal corticosterone in a dose-dependent manner. Oral administration of 200 mg/kg of M73101 also elevated the serum corticosterone level. Such action of M73101 was completely abolished by adrenalectomy, hypophysectomy, or the pretreatment with pentobarbital and morphine. In vitro experiment demonstrated that M73101 had no direct effect on adrenocortical function. These results indicate that the response to M73101 must be mediated through the release of ACTH from the adenohypophysis, which is probably due to the secretion of corticotropin releasing factor from the hypothalamus. M73101 at an oral dose of 200 mg/kg significantly decreased the volume of exudative fluid and the number of leucocytes in carrageenin-induced pleurisy of intact rats. However, the inhibitory action of this drug on cell mobilization decreased by adrenalectomy but not on exudative fluid, indicating that anti-inflammatory actions of M73101 may be due in part to pituitary-adrenocortical stimulation.

Keywords—non-steroidal anti-inflammatory drug; 4-ethoxy-2-methyl-5-morpholino-3(2H)-pyridazinone; anti-inflammatory activity; pituitary-adrenocortical activity; plasma and adrenal corticosterone; hypophysectomy; adrenalectomy

INTRODUCTION

4-Ethoxy-2-methyl-5-morpholino-3 (2H)-pyridazinone (M73101), structurally related to aminopyrine and phenylbutazone with a pyrazolone ring, is a newly synthetized non-steroidal anti-inflammatory drug. The pharmacological profile of M73101 was found to be similar to that of aminopyrine in many aspects. However, details of mechanisms of anti-inflammatory action of M73101 remain unclarified.

During the course of investigations on the mechanism of action of M73101, we found that this drug elevated the serum corticosterone level in rats, indicating that M73101 has a possibility to stimulate the adrenocortical activity. It is well known that non-steroidal anti-inflammatory drugs stimulate the adrenocortical activity with a consequent production of steroids possessing anti-inflammatory properties. Therefore, it is of importance to investigate whether or not anti-inflammatory action of M73101 involve the serum corticosterone elevating action.

In the present paper, we describe the mechanism of the elevation of serum corticosterone level by M73101 and its participation in the anti-inflammatory action.

MATERIALS AND METHODS

Drugs and Chemicals — M73101 used in this experiments was synthetized in our laboratories. As a reference drug, aminopyrine (Marushi Seiyaku Co.) was used. Corticosterone and adrenocorticotropic hormone (ACTH) were purchased from Sigma Chemicals Co. Other chemicals were obtained from commercial sources.
Animals — Male Wistar rats weighing 160–200 g were used. The animals were fed on laboratory chow (Nihon CLEA Co., CE-2) and water ad libitum in an air-conditioned room (25±5°C, 60±5% humidity) that was illuminated from 07:00 to 19:00 o’clock. They were acclimatized to both handling and intraperitoneal or oral administration of 0.2 ml/kg of physiological saline solution between 09:00 and 10:00 o’clock for at least 7 consecutive days before the experiments. To avoid influences of daily fluctuations in corticosterone levels, the experiments were carried out between 09:00 and 11:00 o’clock with the exception of the time course study which was performed between 09:00 and 15:00 o’clock.

Studies on Normal Rats — Rats were given the test drugs intraperitoneally or orally and were immediately returned to their home cage. At various times after the injection, the concentration of corticosterone in the serum and adrenals was determined.

Studies on Hypophysectomized Rats — To determine whether the elevation of serum corticosterone level by M73101 is due to a central action mediated via the pituitary, or a peripheral action at the level of adrenal cortex, rats were hypophysectomized by the external auditory method of Koyama. Seven days after the operation, the rats were injected with the test drugs intraperitoneally and 30 min later decapitated to collect the blood samples. Completeness of hypophysectomy was confirmed by visual inspection at autopsy.

Studies on Rats Pretreated with Pentobarbital and Morphine — To study whether the elevation of serum corticosterone by M73101 is due to the secretion of corticotropin releasing factor (CRF) by the hypothalamic stimulation, rats were given intraperitoneally pentobarbital sodium (40 mg/kg) and 10 min later morphine hydrochloride (10 mg/kg). It has been shown that the combination of two drugs blocks the secretion of CRF from the hypothalamus. The test drugs were given to rats 10 min after morphine administration, and 30 min later the concentration of corticosterone in the serum was determined.

Studies on in Vitro Experiment — According to the method of Saffran and Schally, adrenal quaters of rats were incubated with the test drugs in Krebs-Ringer bicarbonate buffer solution containing 0.2% glucose (pH 7.4) for 2 h at 38°C in a gas phase of 95% O2-5% CO2. Incubation of the tissue with the test drugs and ACTH was also carried out under the same conditions. After centrifugation at 3000 rpm for 10 min at 4°C, the concentration of corticosterone in the supernatants was determined.

Determination of Corticosterone — The animals were killed by decapitation immediately after removal from the home cage, and the blood was collected in tubes and centrifuged at 3000 rpm for 20 min at 4°C. The serum aliquots were stored at −20°C for subsequent assay. Adrenals, trimmed of adherent fat tissue, were weighed and frozed at −20°C. The serum or adrenal corticosterone concentration was fluoro metrically determined by the method of Guillenin et al.

Anti-inflammatory Effects on Carrageenin-induced Pleurisy in Adrenalectomized Rats

![Graph]

FIG. 1. Dose-Response Relationship of Effects of M73101 and Aminopyrine on Serum Corticosterone Levels in Rats. The results are the mean ± S.E. of 5–10 animals. White column, 30 min after injection; black column, 60 min after injection. Significant difference from control, a) p < 0.01.
Bilateral adrenalectomy was performed surgically under ether anesthesia. The rats were maintained on physiological saline solution. At 7th day after the operation, rats were slightly anesthetized with ether and 0.1 ml of 2% α-carrageenin (PASCO) suspended in 0.9% sterile physiological saline was injected into the right pleural cavity through the chest skin sterilized by 70% ethanol. After 3 h, the rats were exsanguinated and the exudate was collected. The total leucocyte count was determined with microcell-counter (TOA MEDICAL ELECTRONICS Co., Model CC-108). The test drugs were given orally immediately before the carrageenin injection. After the experiments, the animals were autopsied to confirm completeness of adrenalectomy.

All values obtained were statistically analyzed using Student's t-test.

RESULTS

Effect on Serum and Adrenal Corticosterone Levels in Normal Rats

When M73101 was given to intact rats intraperitoneally at doses of 50—200 mg/kg, the serum corticosterone levels were elevated in a dose-dependent fashion and significant differences were observed at 60 min after injection of 100 mg/kg, and at 30 and 60 min after 200 mg/kg. Aminopyrine elevated the serum levels significantly only at the dose of 200 mg/kg (Fig. 1). The corticosterone concentration in the adrenal was also elevated by these drugs in almost parallel with serum levels (Fig. 2).

Time course of the effects on serum corticosterone levels of these anti-inflammatory drugs when given orally at a dose of 200 mg/kg were shown in Fig. 3. The elevation of serum corticosterone by M73101 observed from 60 min after the administration and the response reached a maximum at 120 min, then the concentration of corticosterone decreased gradually. The duration of this drug was found to be relatively long, and the concentration at 360 min after dosing was still significantly higher than in control rats. Oral administration of aminopyrine also elevated the serum corticosterone level, but its potency and duration were weaker and shorter than those of M73101.

![Fig. 2. Dose-Response Relationship of Effects of M73101 and Aminopyrine on Adrenal Corticosterone Levels in Rats](image)

Significant difference from control, a) \( p < 0.05 \), b) \( p < 0.01 \). Further explanations are the same as in Fig. 1.

![Fig. 3. Time Course of Effects of M73101 and Aminopyrine on Serum Corticosterone Levels in Rats](image)

White column, control; black column, M73101 (200 mg/kg, p.o.); hatched column, aminopyrine (200 mg/kg, p.o.). The results are the mean ± S.E. of 5—10 rats. Significant difference from control, a) \( p < 0.05 \), b) \( p < 0.01 \).
Effect on Serum Corticosterone Levels in Hypophysectomized Rats

As shown in Fig. 4, the adrenocortical response found in intact rats by M73101 and aminopyrine (Fig. 1) completely disappeared in hypophysectomized rats whose adrenal cortices retained the normal responsiveness to exogenous ACTH.

Effects on Serum Corticosterone Levels in Rats pretreated with Pentobarbital and Morphine

Serum corticosterone levels (μg/100 ml, mean±S.E., N=5) observed in this experiment were as follows; normal group (11.5±2.0) control group (10.5±1.5), M73101-treated group (9.0 ± 2.8) and aminopyrine-treated group (10.5 ± 1.2). Consequently, it was found that the combination of pentobarbital and morphine did not modify and resting serum corticosterone levels and that completely blocked the serum corticosterone elevation after the administration of M73101 or aminopyrine.

Effects on Spontaneous and ACTH-induced Release of Corticosterone from Adrenal Gland

As shown in Fig. 5, addition of M73101 at the concentrations of 10^{-6} and 10^{-4} g/ml to the incubation system of rat adrenal quarters did not show any influence on both the spontaneous and ACTH-induced release of corticosterone in the incubation medium. Same results were obtained with 10^{-4} g/ml of aminopyrine.

Anti-inflammatory Effects on Carrageenin-induced Pleurisy in Adrenalectomized Rats

The results obtained after oral administration of M73101 and aminopyrine to normal and bilaterally adrenalectomized rats are shown in Table I. Adrenalectomy enhanced two important aspects of inflammatory responses; volume of exudates was increased approximately triple, and leucocyte accumulation double. M73101 reduced

![Graph showing effects of M73101, Aminopyrine and ACTH on Serum Corticosterone Levels in Hypophysectomized Rats](image1)

**FIG. 4. Effects of M73101, Aminopyrine and ACTH on Serum Corticosterone Levels in Hypophysectomized Rats**

Drugs were given intraperitoneally at 7th day after the operation. Corticosterone concentration in serum was determined 30 min after dosing. The results are the mean ± S.E. of 5 animals. White column, normal rats; black column, hypophysectomized rats. a) p < 0.05, b) p < 0.01.

![Graph showing in vitro effects of M73101 and Aminopyrine on Corticosterone Release from the Rat Adrenal Gland](image2)

**FIG. 5. In Vitro Effects of M73101 and Aminopyrine on Corticosterone Release from the Rat Adrenal Gland**

Adrenal quarters were incubated with drugs only, and drug and ACTH (5 mU/flask) for 2 h at 38°C. The results are the mean ± S.E. of 4–6 incubations. White column, spontaneous release; black column, ACTH-induced release.
exudate formation to the same degree both in the sham-operated and the adrenalectomized rats. However, the action of this drug on cell mobilization was decreased by adrenalectomy. On the other hand, adrenalectomy did not essentially influence the anti-inflammatory action of the same dose of aminopyrine.

DISCUSSION
The present study revealed that the administration of M73101 could stimulate the pituitary-adrenocortical function in rats. M73101 when given to rats intraperitoneally at doses of 50—200 mg/kg, caused an increase in serum and adrenal corticosterone in a dose-dependent manner. Oral administration of M73101 in the dose of 200 mg/kg also elevated the serum corticosterone level and the action lasted for over 6 h after administration. Similar results were obtained with the same dose levels of aminopyrine used as a reference drug, but its potency and the duration were much weaker and shorter than those of M73101, respectively.

In vitro study with the adrenal quarters of intact rats, M73101 (10^-6 and 10^-4 g/ml) and aminopyrine (10^-4 g/ml) affected neither the spontaneous nor the ACTH-induced release of corticosterone from the adrenal gland, which indicates that these drugs have no direct effect on adrenocortical function and do not affect the response to the corticosterone release from the adrenal induced by ACTH.

### TABLE I. Effects of M73101 and Aminopyrine on Carrageenin-induced Pleurisy in Sham-operated and Adrenalectomized Rats

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg, p.o.)</th>
<th>No. of rats</th>
<th>Exudate volume (ml)</th>
<th>Mobilized cells (× 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean±S.E.</td>
<td>ED₉₀ (95% C.L.)</td>
</tr>
<tr>
<td>Sham-operated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>7</td>
<td>0.47±0.04 (20.3)</td>
<td>165 (94—289)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10</td>
<td>0.41±0.07 (30.5)</td>
<td>7.6±1.5 (75.8)</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>12</td>
<td>0.25±0.04 (57.6)</td>
<td>22.7±3.5 (27.7)</td>
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<tr>
<td></td>
<td>400</td>
<td>11</td>
<td>0.16±0.03 (72.9)</td>
<td>22.7±3.5 (27.7)</td>
</tr>
<tr>
<td>Aminopyrine</td>
<td>50</td>
<td>8</td>
<td>0.45±0.12 (23.7)</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8</td>
<td>0.21±0.04 (64.4)</td>
<td>9.0±1.9 (71.3)</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10</td>
<td>0.15±0.03 (74.6)</td>
<td>9.0±1.9 (71.3)</td>
</tr>
<tr>
<td>Adrenalectomized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>6</td>
<td>1.81±0.27</td>
<td>75.8±7.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10</td>
<td>1.98±0.12</td>
<td>79.0±6.8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>11</td>
<td>1.26±0.20 (30.4)</td>
<td>68.8±5.8 (9.2)</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>12</td>
<td>0.69±0.14 (61.9)</td>
<td>63.5±6.6 (16.2)</td>
</tr>
<tr>
<td>Aminopyrine</td>
<td>50</td>
<td>8</td>
<td>1.33±0.17 (26.5)</td>
<td>43.8±7.0 (42.2)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10</td>
<td>0.63±0.10 (65.2)</td>
<td>48.0±5.0 (36.7)</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10</td>
<td>0.43±0.06 (76.2)</td>
<td>38.2±4.2 (49.6)</td>
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</table>

Adrenalectomy was performed 7 days before the experiment. Rats were given the test drugs orally immediately before carrageenin injection, and 3 h later killed to collect pleural exudates. Figures in parenthesis represent % inhibition. ED₉₀ values and 95% confidence limits were calculated by Litchfield-Wilcoxon's method. a) p < 0.05, b) p < 0.01, c) p < 0.001 (control group versus drug-treated group). d) p < 0.01 (sham-operated group versus adrenalectomized group at the same dose).
The fact that the stimulation of the adrenal cortex with these two anti-inflammatory drugs is completely abolished by hypophysectomy indicates that the drugs stimulate ACTH secretion from the pituitary in rats via adrenocortical function. Furthermore, the corticosterone elevation induced by M73101 or aminopyrine was completely abolished by the pretreatment of pentobarbital and morphine HCl which are known to block the secretion of CRF from the hypothalamus. These results suggest that both drugs may act on the hypothalamus rather than on the pituitary gland. It is well known that a stress stimulates the midbrain reticular formation to release CRF, which is transported via the hypophyseal-portal system to the anterior pituitary and induces secretion of ACTH. In a previous paper, we reported that on the electroencephalographic study in rabbits, M73101 as well as aminopyrine produced an arousal pattern in spontaneous EEG and stimulated the brainstem reticular formation. Therefore, such stimulatory actions on the central nervous system were thought to involve the release of CRF from the hypothalamus.

M73101 has been shown to inhibit the edema provoked with various phlogistic agents and the leucocyte accumulation into the inflamed site in CMC pouch. This was confirmed also by the present study using carrageenin-induced pleurisy in rats, in which M73101 as well as aminopyrine decreased the volume of exudative fluid and the number of leucocyte in a dose-dependent fashion at the oral doses of 50–400 mg/kg. To determine whether the elevation of serum corticosterone by these drugs plays an important role in their anti-inflammatory action, adrenalectomy was performed. As a result, it was found that only the inhibitory effect of M73101 on cell mobilization but not the exudative fluid decreased by adrenalectomy. On the other hand, the inhibitory effect of aminopyrine on these two aspects was not essentially affected. The difference in the effects of adrenalectomy on the action of M73101 and aminopyrine may be due to differences in the degree and the duration of serum corticosterone levels after dosing as seen in Fig. 1 and 3.

We have already reported that since the anti-edematous activity of M73101 in carrageenin-induced rat paw edema was not affected by adrenalectomy, the anti-inflammatory action of this drug was independent of the pituitary-adrenal axis. Concerning anti-edematous activity, the present results confirm the previous observation. However, in the present study using carrageenin-induced pleurisy rats, the inhibitory action of M73101 on cell mobilization, which might be one of the important mechanism of anti-inflammatory action of this drug, was explained, at least in part, by the release of endogenous corticosteroids. According to Vinegar et al., the inhibition on leucocyte migration is the main action of corticosteroid in carrageenin-induced inflammation.

It is worth noting that adrenalectomy enhanced the inflammatory reaction in carrageenin-induced pleurisy, which supports the concept that endogenous steroids control inflammatory responses. On the contrary, in carrageenin-induced rat paw edema, such enhancement has never been observed. These findings seem to indicate that carrageenin-induced rat pleurisy may be the more suitable model than carrageenin-induced rat paw edema in a study on the participation of adrenocortical stimulation in the anti-inflammatory action.

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