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Acute anti-inflammatory effects of aspirin and dexamethasone in rats deprived of endogenous prostaglandin precursors

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Paw oedema, induced by carrageenan, was potentiated in normal rats by arachidonic acid and bishomo-γ-linoleic acid, but not by 5,8,11-eicosatrienoic acid. The latter is not an endogenous prostaglandin precursor, but replaces the other two in essential fatty acid deficient (EFAD) rats. Carrageenan oedema was partially suppressed in these EFAD rats. Aspirin exhibited equal suppression of carrageenan oedema in both normal and EFAD rats, despite the fact that, in the latter, prostaglandins are of negligible importance. The anti-inflammatory effect of dexamethasone was also identical in both normal and EFAD rats. The view that interference with the prostaglandin-system explains the acute anti-inflammatory effects of the two drugs, is discussed, in relation to the present results.

In mammals, the endogenous precursors of prostaglandins (PGs) are essential fatty acids (Van Dorp, 1974). In rats kept on an essential fatty acid-deficient (EFAD) diet the tissues are markedly deficient in bishomo-γ-linoleic acid and arachidonic acid, which in such animals are to a large extent replaced by 5,8,11-eicosatrienoic acid (Ziboh & Hsia, 1972). The latter is not a substrate (Van Dorp, 1971) but rather a competitive inhibitor of the cyclo-oxygenase (Ziboh, Vanderhoek & Lands, 1974), an indispensable enzyme in the biosynthesis of PGs. In EFAD rats, which are deprived of endogenous PG-precursors, the carrageenan- or kaolin-induced inflammatory hind paw oedema is partially suppressed when compared with rats on a normal diet (Bonta, Chrispijn & others, 1974). A similar finding was reported when acute hind paw inflammation was induced by implantation of urate crystals (Denko 1974). Acute supplementation of EFAD rats with PG precursor by administration of arachidonic acid to the hind paw, restores the diminished carrageenan oedema to the level of normal rats (Bonta, Bult & others, 1976). This suggests that shortage of adequate substrate for the cyclo-oxygenase results in suppression of the development of the inflammatory response. It is not known whether eicosatrienoic acid can potentiate the response of normal rats to carrageenan, However, the bradykinin-potentiating peptide, BPP_{9a}, enhances carrageenan oedema in EFAD rats to a similar extent as it does in normal animals (Bonta & others, 1976), thus providing circumstantial evidence for maintenance of the bradykinin-mediated component of tissue injury under conditions of PG-precursor deprivation. The apparently selective abolition of the inflammatory role of substances (thromboxane A_2, PG-endoperoxides, PGs) derived from PG-precursors (Hamberg, Svensson & Samuelsson, 1975), indicates that EFAD rats are a useful tool in studying pharmacological aspects of inflammation outside the PG-system.

Particular interest in this area has arisen for three reasons: firstly, because the original demonstration of inhibition of PG biosynthesis by aspirin (Vane, 1971) has led to the seminal concept that this effect sufficiently explains the acute anti-inflammatory effect of aspirin-like drugs (Vane, 1973); secondly, because this attractive key theory, while being accepted by several researchers as beyond the range of challenge, is now becoming the subject of more critical appraisal (Smith, 1975); thirdly, because the recent finding that corticosteroids, though not inhibiting the biosynthesis of PGs, do prevent their release (Lewis & Piper, 1975) has led to the proposal that reduction of PG output accounts for at least the acute effects of all anti-inflammatory agents, whether non-steroidal or steroidal (Lewis, 1976). In order to investigate the importance of an active PG system in the acute anti-inflammatory effects of non-steroidal and steroidal agents, we have examined the effects of aspirin and dexamethasone on carrageenan-induced paw oedema in normal and EFAD rats.

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MATERIALS AND METHODS

Male albino rats of an inbred Wistar strain (Animal Farm TNO, Zeist) 220–280 g were used. New born animals were taken from females which were put on an EFAD diet (Hope Farms, Woerden) five days before the expected day of delivery. The EFAD diet contained 4% hydrogenated cocos fat and had a fatty acid composition as described previously (Vincent, Zijlstra & Bonta, 1975). After weaning the litters were kept on the same diet until required for the experiment (13–14 weeks old). Control rats were from mothers fed on a diet containing 3·5% of its calories as linoleic acid. The litters were kept on this diet. The animals receiving the EFAD diet exhibited the well-known symptom of growth retardation, reaching 25–35% at the time of the experiments. As the EFAD and normal rats used in the rat paw experiments were selected to match according to body weight, the EFAD rats were older. The effect of this age difference, however, was ruled out, since the carrageenan oedema proved identical in rats on a normal diet at 8 and 20 weeks of age (Bonta & others, 1976). Besides retarded growth, several rats showed hair loss and scaly tails, which are recognized symptoms of EFAD condition (Ziboh & Hsia, 1972). Fatty acid analysis of erythrocytes showed that, whereas in normal rats the ratio eicosatrienoic acid/arachidonic acid was 0·013, in the EFAD rats a ratio of 1·97 was found (Bult & Bonta, 1976). Hence the criterion of essential fatty acid deficiency, being a ratio greater than 0·4 (Holman, 1960), was amply exceeded.

Hind paw oedema was induced by injecting 0·1 ml of 1% carrageenan (Viscarine Marine Colloids) into the subplantar pad. Development of the oedema was evaluated in some of the experiments by assessing the thickness of the paws, using a measuring device as published earlier (Bonta & Noordhoek, 1973). The first measurement was made immediately before the subplantar injection and thereafter measurements were made at hourly intervals. In these experiments, carrageenan was injected into both hind paws of each rat; each of the hourly values were expressed as percentages of the pre-carrageenan values. In two series of experiments groups of rats were killed with chloroform at given time intervals (specified in legend of figures) after the subplantar injection. The paws were severed at the tibio-tarsal joint with a small guillotine and the paw weight was assessed to the nearest mg. In these experiments one paw from each rat was injected with carrageenan and the other paw received an equal volume of saline. The percent difference between the wet weights of the two paws was calculated and this value served as a measure of the development of the oedema. Unless otherwise stated groups of five rats were used.

In the experiments devised to examine the effect of fatty acids on the development of paw oedema, the fatty acids were injected into the foot pad at the same time as the carrageenan, The fatty acids were made up in an aqueous solution of 5% ethanol, 0·1% Na_2CO_3 and 1% carrageenan. The injection volume was 0·1 ml and a concomitant control group received an equal volume of the vehicle without fatty acid. The paw thickness in each fatty acid-treated group was compared with the carrageenan group receiving vehicle only and the results were expressed as percent potentiation. The dose of each fatty acid was 50 μg. The following fatty acids were used: arachidonic acid (Grade 1, Sigma), bishomo-γ-linoleic acid (Unilever), 5,8,11-eicosatrieonic acid (Unilever) and eicosatetraynoic acid (TYA, Roche). Fatty acid solutions were prepared freshly before the experiment. Aspirin (acetylsalicylate calcium, Amsterdamsche Chinine Fabriek ACF) and dexamethasone (Organon) were made up in saline. As paw measurements were made up to 8 h after the carrageenan injection, it seemed important to ensure that blood concentrations of the drugs should not decline during the experiment. Therefore, the drugs were given in three divided doses; 30 min before, 3 h and 6 h after the carrageenan injection. The doses are specified in the legends to the figures and the subcutaneously injected volume was kept at 0·2 ml/100 g body weight. In the corresponding control groups each rat received an equal volume of saline subcutaneously. For statistical evaluation the two-tailed Student's t-test was used.

RESULTS

The results of administering fatty acids together with carrageenan into the foot pad of normal rats are shown in Fig. 1. The carrageenan-induced oedema was strongly potentiated by arachidonic acid, the effect being most pronounced at 1 h, and showing a rapid decay thereafter. However, a small effect was still demonstrable for up to 6 h. A qualitatively similar, but somewhat less pronounced, effect was obtained with bishomo-γ-linoleic acid. 5,8,11-eicosatrienoic acid which is not a PG-precursor, produced a negligible effect. Only a slight, transient depression of the oedema was apparent with eicosatetraynoic acid.

In normal rats, aspirin inhibited the carageenan-induced oedema after 2 h. In EFAD animals the

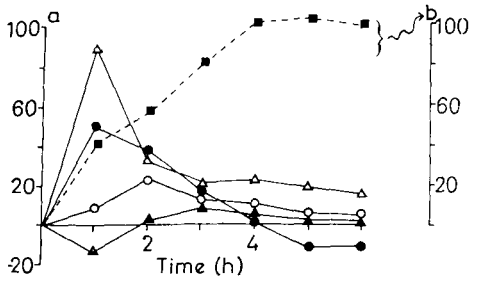

FIG. 1. a—Potentiation (%) of the carrageenan-induced paw oedema by simultaneous administration of fatty acids in the foot pad. b—% paw increase due to injecting only carrageenan (■----■) is shown as averages of 4 groups of 5 rats each. The percent potentiation is calculated as a percentage of that particular carrageenan only group which concomitantly ran with each of the fatty acid groups. Potentiation by arachidonic acid (△——△) was significant at each measuring point (1 h $P<0{\cdot}001$, 2 h $P<0{\cdot}01$, other points $P<0{\cdot}05$). Bishomo-γ-linoleic acid (●——●) caused significant potentiation at 1 h ($P<0{\cdot}001$) and 2 h ($P<0{\cdot}01$). The slight effect of 5,8,11-eicosatrienoic acid (○——○) was significant only at 2 h ($P<0{\cdot}05$). Depression by eicosatetraynoic acid (▲——▲) was significant ($P<0{\cdot}05$) at 1 h. The dose of each fatty acid was 50 μg.

response to carrageenan was partially suppressed, when compared with the oedema in normal rats and a further significant decrease (at 2 and 7 h, $P<0{\cdot}025$, at other times, $P<0{\cdot}01$) was caused by aspirin, as shown in Fig. 2. Similar results were observed with dexamethasone (Fig. 3), which also produced a significant reduction of the carrageenan effect

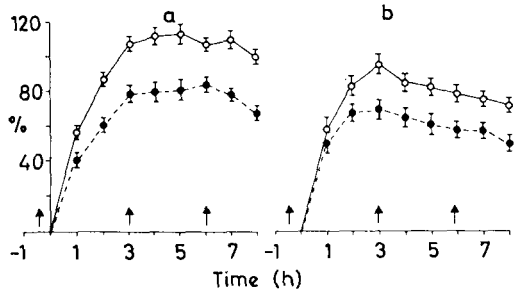

FIG. 2. Effects of aspirin on the carrageenan-induced paw oedema in a—normal and b—EFAD rats. Results show paw diameter as % increase in comparison to the values at 0 h. Each point is the mean ± s.e.m. of a group of 5 rats. Aspirin (●----●) was s.c. administered (125 mg kg^{-1}) at time points indicated with arrows. The corresponding control groups (○——○) received at the same times 0·2 ml/100 g saline subcutaneously. At 1 h the effect of aspirin was not significant either on normal or on EFAD rats. At other measuring points the effect of aspirin was significant. at the level of $P<0{\cdot}01$, except that on EFAD rats at 2 and 7 h the effect of aspirin was significant at $P<0{\cdot}025$.

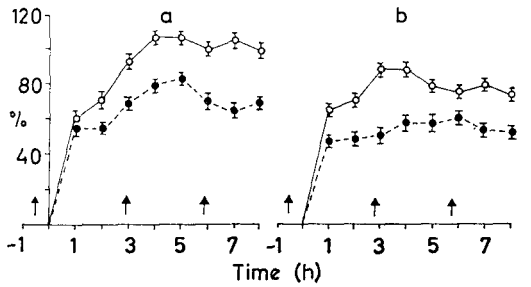

FIG. 3. Effects of dexamethasone on the carrageenan-induced paw oedema in a—normal and b—EFAD rats. Results show paw diameter as % increase in comparison to the values at 0 h. Each point is the mean ± s.e.m. of a group of 5 rats. Dexamethasone (●----●) was subcutaneously administered (0·125 mg kg^{-1}) at time points indicated with arrows. The corresponding control groups (○——○) received 0·2 ml/100 g saline s.c. at the same times. At 1 h the effect of dexamethasone was not significant in normal rats. At other points dexamethasone caused significant inhibition at the level of $P<0{\cdot}01$, except at 2 h in normal rats and 1 and 6 h in EFAD rats when significance was $P<0{\cdot}05$.

irrespective of whether tested in normal or EFAD rats. In the latter, the carrageenan-oedema was already reduced and a further suppression was caused by the steroid.

Partial suppression of the carrageenan-induced paw inflammation in EFAD rats has been demonstrated previously by assessing the increase in paw diameter (Bonta & others, 1976). In an attempt to confirm this, the change in wet weight of the paws was determined. In agreement with the paw diameter-results, the rats deprived of endogenous PG-precursors developed a smaller oedema after carrageenan than normal rats (Fig. 4). The difference

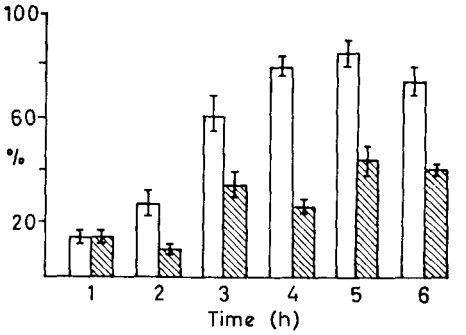

FIG. 4. Carrageenan-induced paw oedema in normal (open columns) and EFAD rats (hatched columns). Results show the % increase in wet weight of the carrageenan treated paws in comparison to the paws receiving 0·1 ml of saline. Each bar represents the mean ± s.e.m. of a group of 6 rats. Except for the measurement of 1 h the difference between normal and EFAD rats was significant (at 2 and 3 h $P<0{\cdot}05$, at 5 h $P<0{\cdot}01$, at 4 and 6 h $P<0{\cdot}001$).

was more pronounced after 3 h than at earlier measuring times.

Finally the effect of aspirin was re-examined in normal and EFAD rats, using the increase in the wet weight of the paws as the inflammatory parameter 6 h after subplantar injection of carrageenan. The results (Fig. 5) show that aspirin proved to be very effective irrespective of whether it was administered to normal animals or to those deprived of endogenous PG-precursors. In the latter group of rats the dose of 125 mg kg^{-1} aspirin was in fact slightly more effective than in non-deprived animals (48% inhibition in EFAD versus 31% in normal rats).

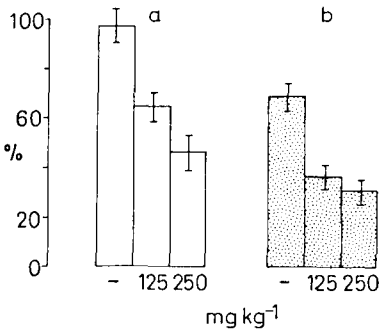

FIG. 5. Effect of aspirin (mg kg^{-1}) on the carrageenan-induced wet weight increase of paws in a—normal and b—EFAD rats. Results are expressed as in Fig. 4. Each bar is the mean ± s.e.m. of a group of 5 rats. The first column in each set shows paw weight increase of rats without aspirin. Such animals received a subcutaneous injection of 0·2 ml/100 g saline 30 min before and 3 h after carrageenan. The second columns represent groups receiving 125 mg kg^{-1} aspirin administered in two divided doses at the time points when the corresponding controls had saline. The third columns represent the groups receiving 250 mg kg^{-1} aspirin in two divided doses. The effect of each aspirin dose, both in normal and EFAD rats, differed significantly ($P<0·001$) from the corresponding control group. The difference between the saline-treated normal and EFAD rats was significant at the level of $P<0·01$.

DISCUSSION

In the light of the widely accepted view that PGs are involved either as mediators or modulators of acute inflammatory reactions (for review of the subject: Ferreira, Moncada & Vane, 1974) it was expected that animals which have a marked shortage of PGs would display a modified response to inflammatory stimuli. Indeed, it was shown that inflammatory hind paw oedemas caused by different irritants are partially suppressed in EFAD rats (Bonta & others, 1974); Denko 1974). Shortage of endogenous PG-precursors was proposed as the mechanism underlying this reduced inflammatory response in EFAD rats. This assumption obtained support by the finding that local acute supplementation with arachidonic acid, the precursor of PGE_2, restored the inflammatory responsiveness of EFAD rats to the level of normal animals (Bonta & others, 1976). Potentiation of carrageenan oedema by local administration of arachidonic acid is thought to be due to conversion to PGs, because this enhancement of the carrageenan response is reduced by a variety of PG-biosynthesis inhibitors. Paracetamol had no effect (Lewis, Nelson & Sugrue 1975), as it does not appear to influence the PG-synthetase system in tissues other than the brain (Flower & Vane, 1972). The arachidonic acid-induced potentiation of carrageenan-oedema also remained unaltered after sodium salicylate (Smith, Ford-Hutchinson & Elliott, 1975), which, in contrast to aspirin, has negligible inhibitory activity against PG-synthetase (Vane, 1971). The potentiating effect of arachidonic acid was confirmed in the present investigation and, in addition, it was shown that carrageenan oedema was enhanced, albeit to a slightly smaller extent, by the concurrent administration of bishomo-γ-linoleic acid. The latter is a precursor of PGE_1 and $F_{1\alpha}$ in rats (Danon, Heimberg & Oates, 1975). However, PGE_2 can also be indirectly synthesized from bishomo-γ-linoleic acid, because the latter can be converted into arachidonic acid by rat tissue (Bernert & Sprecher, 1975). Enhancement of carrageenan oedema by PGE_1 and PGE_2 has been reported (Lewis & others, 1975), thus forming a basis for understanding the potentiating effect of bishomo-γ-linoleic acid in the present experiments. It is not clear however, to what extent mutual interaction of the two precursors of PGs might have been involved in the potentiation by bishomo-γ-linoleic acid which was qualitatively similar, but less marked than that produced by arachidonic acid. Eicosatetraynoic acid, which is a competitive inhibitor of PG-synthetase (Downing, Ahern & Bachta, 1970) has been shown to have anti-inflammatory properties following intraperitoneal administration of high doses in rats (Willis, Kuhn & Weiss, 1974). However, in the present investigation this compound only caused a very slight depression of the oedema when given concomitantly with carrageenan. The dose might have been too low, but solubility problems precluded the local administration of larger amounts. A further interesting observation made during the present investigation was the lack of appreciable potentiation of carageenan oedema by 5,8,11-eicosatrienoic acid. To a large extent, this fatty acid replaces the PG-

precursors in EFAD rats (Ziboh & Hsia, 1972) and is not a substrate for PG-synthetase (Van Dorp, 1971). Its inability to produce appreciable enhancement of carrageenan-oedema in normal rats supports the suggestion that the poor development of the inflammatory response to carrageenan in EFAD rats is due to the replacement of the normal substrates for the cyclo-oxygenase. Markedly reduced PG release due to precursor shortage in EFAD-rats has been shown by directly assessing the output of thromboxane A_2, PG-endoperoxides and PGs during collagen-induced platelet aggregation (Bult & Bonta, 1976).

We have further confirmed earlier results showing partial reduction in inflammatory paw oedema in rats deprived of endogenous PG-precursor (Bonta & others, 1974; 1976). In agreement with previous findings (Bonta & others, 1974) the difference between carrageenan-induced oedema in normal and EFAD rats was most pronounced after 3 h (see Fig. 4). The time dependency of this difference is in agreement with the suggestion that the PG-component of inflammation is represented by the delayed, rather than by the early phase carrageenan-induced paw oedema (Di Rosa, Giroud & Willoughby, 1971). The time course of the anti-inflammatory effect of indomethacin in EFAD rats gave further support to this idea. This PG-synthesis inhibitor failed to suppress further the poorly developed carrageenan-oedema in EFAD rats after 4–5 h (Bonta & others, 1974). Nevertheless, even in EFAD rats indomethacin partially inhibited the early phase of carrageenan oedema which preceeds the so called PG-component (Bonta & others, 1976). This observation led to the suggestion that, besides inhibition of PG-biosynthesis an additional anti-inflammatory mechanism might be exerted by indomethacin. Obviously, the next step was to use EFAD rats to investigate whether the presence of a PG-component of carrageenan oedema was a necessary prerequisite for the demonstration of the anti-inflammatory effect of aspirin. The contrary appears to be the case as shown by two sets of experiments in the present work.

Aspirin exerted a further suppression of the entire course of the paw oedema induced by carrageenan in EFAD rats (Fig. 2). In fact, when increased paw weight was used as the parameter of inflammation 6 h after carrageenan, the anti-inflammatory effect of aspirin was not smaller but even slightly greater in EFAD than in normal rats. On the basis of the arguments eleborated above, the role of an activated PG-system can be ruled out in EFAD rats. Even under these conditions the effect of aspirin is fully maintained and the conclusion seems to be justified that the anti-inflammatory effect of this drug involves at least one mechanism which is independent of PG-biosynthesis inhibition. The discovery that aspirin was a potent inhibitor of PG-biosynthesis (Vane, 1971), followed by allied studies by the same group (reviewed by Ferreira & others, 1974), was a breakthrough in understanding several puzzling effects of this particular drug and related anti-inflammatory agents. It also fertilized medical research far outside the field of the original finding. The correlation between inhibition of PG-synthesis by aspirin *in vivo* and suppression of signs and symptoms of inflammation appeared very strong (Vane, 1973). The concept was attractive enough to seduce some investigators into believing that the pharmacological control of a pathological condition as complicated as inflammation could be explained on the basis of a single, albeit extremely important, mechanism. This exaggerated view has been criticised recently (Smith, 1975). The finding that sodium salicylate and aspirin are equally effective as acute anti-inflammatory drugs, but largely incomparable as PG-biosynthesis inhibitors, led to the proposal that these two effects are not necessarily related (Smith & others, 1975). This proposal is now further supported by the demonstration that a potent inhibitor of PG-synthetase can suppress inflammation under circumstances whereby an appreciable participation of the PG-system is highly unlikely. The experimental situation used in this study may accommodate at least three mechanisms which were recently suggested as possibly underlying the anti-inflammatory action of aspirin. First, it was shown that migration of leucocytes to the inflamed tissue site in rats can be inhibited by aspirin (Vinegar, Truax & Selph, 1973). The effect of aspirin on cellular migration does not necessarily involve interference with the PG-system (Smith & others, 1975). Preliminary data indicate that marked depression of peripheral leucocyte count does not occur in EFAD rats (Vincent & others, 1975), but it is not known whether the leucocytes of such animals are still functionally intact. A second mechanism by which aspirin might influence the inflammatory reaction apart from the PG-system is through inhibition of phosphodiesterase (Stefanovich, 1974), increasing the intracellular concentration of cyclic AMP. Acute and subacute inflammatory models can indeed be suppressed through increasing c-AMP concentrations (Ichikawa, Nagasaki & others, 1972). Again, however, this needs to be confirmed under

circumstances of deprived PG-precursors. Aspirin also belongs to a group of compounds which may form copper chelates and the possible interference with copper has been studied (Sorenson, 1974). However, in view of the ambigous (inflammatory or anti-inflammatory) role of copper (Whitehouse, 1976), the interference of aspirin with this metal needs clarification. Nevertheless this is also a possible mechanism for the non-PG-mediated anti-inflammatory activity of aspirin.

Finally, the observation that dexamethasone was equally effective in suppressing carrageenan oedema in normal and EFAD rats indicates that interference with the PG system is not involved in the acute anti-flammatory action of corticosteroids. In the subcutaneous adipose tissue of rabbits the vasodilatation and PG-release occurring during lipolysis were prevented by corticosteroids (Lewis & Piper, 1975). Earlier, such an effect had been reported with aspirin (Bowery & Lewis, 1973). The two findings led recently to the speculation that all anti-inflammatory agents, whether steroids or non-steroids, may have a final common pathway, i. e. reduction of increased PG-amounts, irrespective of whether prevention of release or inhibition of synthesis are the underlying mechanisms (Lewis, 1976). A common final pathway in the acute anti-inflammatory effect of the two classes of drugs cannot be excluded on the basis of the present experiments, as representatives of each of the two drug classes were found to suppress paw inflammation in EFAD rats, However, this finding suggests that, in contrast to lipolysis in rabbits, carrageenan-induced rat paw oedema is not a situation where interference with increased PG-amounts is the common pathway of the two drug classes.

This work has not unravelled any, as yet, unknown anti-inflammatory mechanism. However, it does demonstrate a model situation which appears to be useful in investigating those mechanisms which, under normal conditions, might be masked by interference with the PG-system.

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Note added upon proof:

Recently it has been shown (Nijkamp, F. R., Flower, R. J., Moncada, S. and Vane, J. R. (1976) *Nature*, **263**, 479–482) that corticosteroids may act by inhibiting the activity of RCS-RF (rabbit aorta contracting substance-releasing factor) and thus the consequent release of PG-endoperoxides. It is possible that PG-endoperoxides and RCS might play a role in some inflammatory reactions. However in the present study dexamethasone retained its anti-inflammatory activity in EFAD rats. Thus, the activity of dexamethsone, at least in the carrageenan-induced inflammation, is not exerted by inhibiting the activity of RCS-RF alone, but an alternative mechanism is also involved.

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