PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 187, 273–277 (1988)

Temporal Relationships of the Anti-inflammatory Effect of Etodolac in the Adjuvant Arthritic Rat (42663)

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Abstract. Using the curative model of adjuvant arthritis, adult male Sprague–Dawley rats were treated with vehicle or etodolac (1, 3, and 8 mg/kg/day, po) for 9 days. Rats were sacrificed after 1, 2, 4, or 9 daily doses, and paw volume, PGE_2 concentrations, and *N*-acetyl-β-D-glucosaminidase (NAG) activity were determined in the left adjuvant-injected hindpaws. All three doses of etodolac caused a significant decrease in PGE_2 concentrations after the first dose, and the decreases persisted for 2, 4, and 9 days of treatment, respectively. In rats given four daily doses of 3 and 8 mg/kg/day of etodolac, the paw volume was significantly decreased by about 50%, compared with that of the arthritic controls. A significant decrease in NAG activity was observed only after nine daily doses of 8 mg/kg/day etodolac. The sequence of anti-inflammatory events manifested following etodolac treatment would appear to be an initial inhibition of PGE_2 synthesis, followed by resorption of fluid, and then by a reduction in macrophage infiltration.

Inhibition of prostaglandin (PG) production by nonsteroidal anti-inflammatory drugs (NSAIDs) at the site of inflammation is believed to account for much of their efficacy in the treatment of arthritis (1). The various manifestations of arthritis include edema, increase in PG concentrations at the inflamed site, and infiltration of inflammatory cells into the inflamed site (2). It has been shown that PG may be involved in all of these manifestations (3, 4).

Etodolac (Ultradol, Lodine, Ramodar; Fig. 1) is a unique anti-inflammatory agent that exhibits, both in man (5–7) and in rats (8–11), a large degree of anti-inflammatory efficacy coupled with a profile of gastrointestinal and renal safety. In the curative model of adjuvant arthritis in the rat, etodolac, but not naproxen, ibuprofen, or aspirin, caused a dose-related retardation, and even reversal, of joint damage (12–14). In the present study, using a similar curative adjuvant arthritis model, we have determined the effects of etodolac on inflamed hindpaw edema volume, PGE_2 concentrations, and macrophage infiltration (measured by the marker enzyme *N*-acetyl-β-D-glucosaminidase (NAG)). The results provide insight to the primary mechanism of the anti-inflammatory activity of etodolac.

Materials and Methods. *Animals.* Adult male Sprague–Dawley rats weighing about 200 g were used (Charles River, Wilmington, MA). Food and water were given *ad libitum.* The animals were maintained in a 12-hr light:12-hr dark cycle. Etodolac (Ayerst) was dissolved in 0.2% Tween 80 in saline and administered by gastric intubation. Freund's complete adjuvant (FCA) was prepared by suspending 5 mg killed and dried *Mycobacterium butyricum* (Difco) in 1 ml liquid paraffin.

On Day 0, all animals were weighed, and the paw volume of both hindpaws was measured using a plethysmograph (Buxco Electronics, Sharon, CT). Animals in the arthritic groups were injected in the left hindpaws with 0.05 ml FCA. On Day 21, the body weight and the hindpaw volumes were measured. Rats showing an injected paw volume of 5.5 ml or more were selected and divided into 16 groups of eight rats each, such that the mean paw volume of each group was comparable. All rats were dosed once daily with either vehicle (Tween 80/saline) or etodolac (1, 3, and 8 mg/kg/day) by gavage for the following 9 days. On Days 21, 22, 24, and 29, body weights and paw volumes of the appropriate groups of rats were measured. The rats were then euthanized 2 hr after dos-

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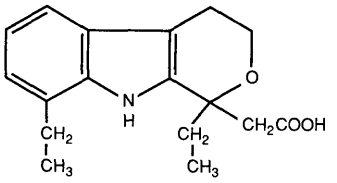

FIG. 1. Etodolac: 1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4,-β]-indole-1-acetic acid.

ing. Both hindpaws were severed above the ankle joint, immediately frozen by submersion in liquid nitrogen, and stored frozen until processing. Parallel groups of normal rats were also included for comparison.

Tissue preparation. For the preparation of the paw homogenate, the frozen hindpaw was taken from liquid nitrogen storage and placed in a stainless steel dish with a stainless steel top, which had been precooled in dry ice. The paw was quickly pulverized in the dish by use of a mallet, and the resulting fragments were transferred to a 50-ml polypropylene conical tube (Falcon) kept on ice. Twenty milliliters of 50 m*M* Tris buffer, pH 7.4, containing 11.5 m*M* aspirin (to arrest further PG biosynthesis) was then added, and the mixture was homogenized with a Tissumizer (Tekmar) fitted with a 50-ml probe until a uniform homogenate was achieved. The homogenate was filtered through a double layer of cheesecloth. Aliquots of 1 ml homogenates were then pipetted into 50-ml polypropylene conical tubes containing trace amounts (about 3000 dpm) of [^{3}H]PGE_2 to monitor extraction recovery. Another 1-ml aliquot of the paw homogenates was pipetted into another tube for the determination of NAG activity. All tubes were kept on ice at all times. All tubes were briefly vortexed and quickly frozen in methanol–dry ice for storage until assay.

PG extraction and determination. The extraction and radioimmunoassay for PGE_2 were carried out, as described previously (15), with kits purchased from Seragen (Boston, MA). The limit of detection was 25 pg.

NAG activity determination. The NAG activity of the samples was assayed using a modification of the method as described by Schorlemmer *et al.* (16). In the test, 50 μl of tissue extract in double dilution, 50 μl of 2.24 m*M* *p*-nitrophenyl-2-acetamide-2-β-D-glucopyrandoside, and 50 μl of 0.1 *M* phosphate buffer, pH 7.4, was introduced into a single well of a 96-well microtiter plate. After mixing, the plate was incubated in a 37°C water bath for 15 min, followed by the addition of 50 μl of 0.2 *M* glycerol buffer, pH 10.4, to stop the enzymatic reaction. The color formation was read with an automatic Dynatech micro-ELISA MR580 reader. An enzyme unit of NAG activity was defined as the amount of enzyme which produces a change of the optical density of 0.001 under the described experimental conditions as measured as 405 nm.

Statistical analysis. The differences in all parameters were tested for statistical significance by analysis of variance (ANOVA). Pairwise multiple-comparison follow-ups were performed using the Dunnett multiple-comparison *t*-test. Descriptively, results were expressed as means ± SEM.

Results. *Paw volume.* The results are presented in Table I. In the left adjuvant-in-

TABLE I. EFFECT OF ETODOLAC ON THE LEFT PAW VOLUME OF THE ADJUVANT ARTHRITIC RAT

Treatment	Number of daily doses			
	1	2	4	9
	Paw volume (ml/paw ± SEM)			
Arthritic control	7.3 ± 0.3	7.5 ± 0.4	7.7 ± 0.4	8.4 ± 0.4
Arthritic + etodolac 1[a]	7.1 ± 0.5	7.4 ± 0.4	7.0 ± 0.4	6.3 ± 0.2**
Arthritic + etodolac 3	7.0 ± 0.3	6.8 ± 0.3	5.7 ± 0.2**	5.5 ± 0.3**
Arthritic + etodolac 8	7.2 ± 0.4	6.8 ± 0.3	5.5 ± 0.3**	5.0 ± 0.3**
Normal control	2.7 ± 0.1**	2.7 ± 0.1**	3.1 ± 0.1**	2.8 ± 0.1**

Note. $N = 8$.
[a] Dose in mg/kg/day, po.
** $P < 0.01$ vs arthritic control group.

TABLE II. EFFECT OF ETODOLAC ON THE AMOUNT OF PGE_2 IN THE LEFT PAWS OF THE ADJUVANT ARTHRITIC RAT

Treatment	Number of daily doses			
	1	2	4	9
	Amount of PGE_2 (ng/paw ± SEM)			
Arthritic control	383 ± 65	373 ± 82[a]	335 ± 38	294 ± 60
Arthritic + etodolac 1[b]	195 ± 41*	209 ± 27*	260 ± 33	190 ± 26
Arthritic + etodolac 3	183 ± 24*	180 ± 36*	131 ± 14*	173 ± 47
Arthritic + etodolac 8	247 ± 50*	236 ± 35*	170 ± 45*	111 ± 15*
Normal control	55 ± 10*	51 ± 7*	67 ± 10*	74 ± 11*

Note. $N = 8$.
[a] $N = 7$.
[b] Dose in mg/kg/day, po.
* $P < 0.05$ vs arthritic control group.

jected paw, the arthritic control paw volume was 2.7 times greater than that of the normal control group. A gradual increase in paw size of the arthritic control rats over the 9-day period was observed. Administration of 1 mg/kg/day of etodolac caused a significant decrease in the paw volume after nine daily doses only, whereas doses of 3 and 8 mg/kg/day produced significant decreases after four doses.

PGE_2 concentrations. Data are expressed on a per paw basis, which reflects the total levels in the paw. As shown in Table II, the amounts of PGE_2 in the left paws of the untreated arthritic rats were about four to seven times higher than those of the normal control group. Etodolac, at the 1 mg/kg/day dose, significantly lowered the amount of PGE_2 by about 50%; however, the effect was not maintained throughout the 9 days of treatment. The decreases observed with the higher doses of 3 and 8 mg/kg/day etodolac remained significant after 4 and 9 days of treatment, respectively.

NAG activity. The results shown in Table III indicate that the NAG acitvity was much greater in the adjuvant-injected paws than in the normal paws. Etodolac did not affect NAG activity at the 1 and 3 mg/kg/day doses; only after nine daily doses of 8 mg/kg/day did etodolac significantly lower NAG activity.

Discussion. The term rheumatoid arthritis defines a syndrome that may consist of a group of related diseases that are metabolic-, endocrine-, and immune-mediated (2). The

TABLE III. EFFECT OF ETODOLAC ON THE NAG ACTIVITY IN THE LEFT PAWS OF THE ADJUVANT ARTHRITIC RAT

Treatment	Number of daily doses			
	1	2	4	9
	NAG activity (unit[a]/paw ± SEM)			
Arthritic control	161 ± 25+	166 ± 28	171 ± 26	168 ± 19[b]
Arthritic + etodolac 1[c]	150 ± 23[d]	207 ± 28	187 ± 19	166 ± 21
Arthritic + etodolac 3	153 ± 31	181 ± 26	140 ± 17	173 ± 23[b]
Arthritic + etodolac 8	164 ± 37	182 ± 25	144 ± 20	103 ± 12*
Normal control	35 ± 4*	28 ± 3*	28 ± 5*	26 ± 4*

Note. $N = 8$.
[a] Enzyme unit = Δ 0.001 OD/15 min.
[b] $N = 7$.
[c] Dose in mg/kg/day, po.
[d] $N = 6$.
* $P < 0.05$ vs arthritic control group.

various manifestations include edema (pharmacological manifestation), increase in PG concentrations at the inflamed site (biochemical change), and infiltration of inflammatory cells into the inflamed site (immunological response). It has been shown that PG may be involved in all of these manifestations (3, 4) and that reduction of PG concentrations may reverse these processes. The most common therapy for arthritis is the use of NSAIDs, whose main action is to inhibit PG biosynthesis (1). Recent studies have shown that these drugs may also affect the immune system by inhibiting the activation of the inflammatory cells (17). Our study examined the effect of etodolac on indicators of each of these processes, namely, PGE_2 concentrations, edema volume, and macrophage infiltration.

PGE_2 concentration, edema volume, and NAG activity (an indicator of macrophage population) in the adjuvant-injected hindpaw were greatly increased over those of the normal control group. The results obtained for the arthritic control group confirmed the observations reported previously (1–4, 17). Right paws (site of secondary inflammation) were also examined; however, the differences between arthritic and control animals in the parameters examined did not provide a sufficient window against which the effect of etodolac could be readily evaluated.

The data demonstrate that the initial change achieving statistical significance is a decrease in PGE_2 concentration. The effect is more persistent at higher doses of etodolac and precedes the changes in edema (paw volume) or NAG concentrations. The reductions in PGE_2 (Table II) are followed by reductions in paw volume, seen initially after four doses (Table I), and finally by a change in NAG concentration seen only after nine doses of 8 mg/kg/day etodolac (Table III). The values obtained for edema volume are in agreement with those of Martel *et al.* (10, 12).

The temporal onset of changes induced by etodolac in the inflamed paws of arthritic rats are best illustrated using the 8 mg/kg/day dosage regimen as seen in Fig. 2. For all parameters, the values obtained in the normal control rats were first subtracted from all values obtained in the arthritic rats, and the

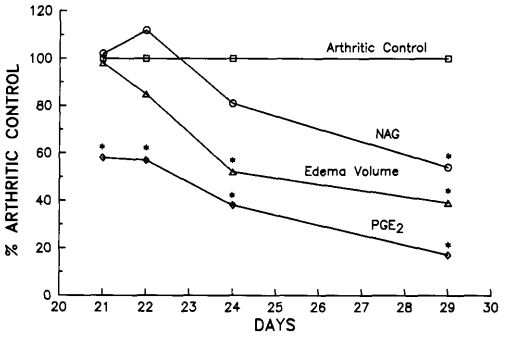

FIG. 2. Net effect of an 8 mg/kg/day dose of etodolac in the left paw of the adjuvant arthritic rat. Values are average means expressed as percentages of arthritic control (*$P < 0.05$ from arthritic control).

results were expressed as percentages of the arthritic controls. Etodolac treatment produced first a reduction in the amount of PGE_2, followed by reductions in edema volume and then in NAG activity.

That the decrease in PGE_2 was not secondary to a reduction in paw volume is demonstrated by the facts that first, it occurred prior to any paw volume changes, and second, when the PGE_2 reductions are expressed per paw volume, i.e., corrected for changes in paw volume, a statistically significant reduction is still demonstrable. This pattern of change contrasts with changes in paw NAG concentrations, which, after 8 mg/kg/day of etodolac, were not reduced in statistically significant measure when expressed per paw volume. These results suggest that the inhibitory effect of etodolac on PG synthesis at the inflamed site cannot be totally accounted for by changes in paw volume, whereas the decrease in macrophage infiltration appears to be due to a decrease in edema volume.

The results obtained in the present study lead us to hypothesize that one of etodolac's prime actions is biochemical: the inhibition of PG synthesis at the site of inflammation. This is followed by a decrease in edema volume, probably due to resorption of fluid from the intercellular space. Last, a decrease in macrophage population occurs, probably due to an inhibition of macrophage infiltration or to migration of macrophages from the inflamed site together with the resorption of fluid.

The authors acknowledge the excellent assistance of M. Osborne, T. Walter, R. Cohen, J. Gilliar, B. Mulder, B. Klein, M. B. Barkley, and J. Tamasi and his staff. Thanks are also due to P. Carabelli and M. Mendoker for typing this manuscript.

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Received September 9, 1987. P.S.E.B.M. 1988, Vol. 187.
Accepted November 2, 1987.