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INDOMETHACIN AND THE ROLE OF PROSTAGLANDINS IN ADIPOSE TISSUE

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Abstract—Indomethacin (2 or 10 μg/ml) or meclofenamic acid (4 μg/ml), two potent inhibitors of prostaglandin biosynthesis, did not affect basal or noradrenaline (10^{-6} M) stimulated lipolysis when added to isolated rat fat cells. Indomethacin (5 μg/ml blood) similarly was without effect on blood flow, on lipolysis or on ^{3}H-noradrenaline overflow before, during and after nerve stimulation (4 Hz) in perfused canine subcutaneous adipose tissue *in situ*. Indomethacin given to rats 5 × 5 mg/kg at 10–14 hr intervals p.o. had no effect on arterial glycerol concentration, but caused a significant hypoglycemia. Fat cells or fat pads extracted from such rats had an unchanged basal lipolytic rate but a lowered responsiveness to high concentrations of noradrenaline (4×10^{-7}–2×10^{-6} M), compared with controls. However, 10^{-7} M noradrenaline caused a higher lipolytic response in fat cells from indomethacin-treated rats than from controls, presumably because the former caused a smaller degradation of noradrenaline during the incubation period. Indomethacin (0·2–20 μg/ml) had no effect on cAMP binding to protein kinase, but apparently caused membrane stabilization, since erythrocytes from indomethacin-treated rats were more resistant to hypotonic lysis than red cells from control animals. Our results suggest that indomethacin has a multitude of biological effects, some of which may be unrelated to inhibition of prostaglandin biosynthesis. When considered together with previous results the findings also suggest that endogenous prostaglandins are of minor importance as feed back inhibitors of lipolysis in adipose tissue.

Prostaglandins (PGs) of the E-series are potent inhibitors of lipolysis stimulated by catecholamines *in vitro* [1] and of lipolysis induced by nerve stimulation in canine subcutaneous adipose tissue *in situ* [2, 3]. PGs are released from adipose tissue during incubation with lipolytic hormones *in vitro* [4], as well as by sympathetic nerve stimulation in canine subcutaneous adipose tissue *in situ* [5].

The anti-inflammatory drug indomethacin is a potent inhibitor of PG synthesis in a wide variety of tissues [6–9]. For that reason indomethacin has been used as a tool to evaluate the physiological role of PGs. In particular, a report by Illiano and Cuatrecasas [10] that indomethacin increased glycerol release and cyclic AMP formation in rat adipocytes following noradrenaline (NA) stimulation was taken as evidence for a physiological role of PGs as feed back inhibitors of lipolysis. Fain *et al.* [11], who failed to confirm these observations under a variety of *in vitro* conditions, conversely concluded that they had obtained no evidence that PG play a significant role in the regulation of basal or activated lipolysis. This conclusion is similar to the one drawn previously based on results in perfused canine subcutaneous adipose tissue [2, 12].

In the present study we have used indomethacin as a possible tool to determine the role of PG in adipose tissue by examining its effects on blood perfused canine subcutaneous adipose tissue as well as on blood substrate levels, erythrocyte membrane stability, adipose tissue and adipocytes of the rat.

METHODS

Dog experiments

The experimental technique has been described earlier [12, 13]. The experiments were conducted on fed female mongrel dogs weighing 11–19 kg anaesthetized with sodium pentobarbital (30 mg/kg i.v.). The animals were tracheotomized and artificially ventilated to maintain an essentially normal acid base balance: pH 7·33–7·39, pCO_2 30–33 mm Hg, pO_2 81–97 mm Hg in arterial blood as determined by an acid–base analyzer (BHM 71, BMS 3Mk2, Radiometer Copenhagen). Arterial blood pressure was measured from the carotid artery by means of a Statham P23 AC transducer. The right inguinal subcutaneous fat pad (22–51 g) was isolated from all surrounding tissues including skin under scrupulous hemostasis. Exteriorized loops of polyethylene tubing were inserted between the artery and vein to the preparation and the femoral artery and vein. A drop-counter to monitor blood flow was included in the arterial loop. A three way stop-cock was included in the venous loop to permit intermittent sampling of venous blood from the tissue. The nerve to the tissue was cut between ligatures and placed on a bipolar silver electrode, protected from drying by plastibase (Squibb). Pulses of supramaximal intensity and duration (10–12 V, 2 msec) were delivered at the rate of 4 Hz from a Grass S4D stimulator. Blood pressure and adipose tissue blood flow were recorded on a Grass 7B polygraph. Heparin (1200–1500 U/kg, Vitrum,

Sweden) was given i.v. to prevent clotting. ^{3}H-NA was slowly infused (4 μCi/min for 30–45 min) via a side branch in the arterial loop.

The experiment was started 60 min after the end of the ^{3}H-NA infusion by the withdrawal of an arterial and a venous blood sample. Subsequently the nerve was stimulated before and after the close arterial infusion of indomethacin. Blood samples were drawn before, during and after each nerve stimulation period (5 min). An aliquot of blood was taken for the determination of hematocrit (31–38%). After centrifugation aliquots which were shaken with 10 ml Instagel® (Pack- [14]. Radioactivity was determined in 0·2 ml plasma aliquots which were shaken with 10 ml Instagel (Packard) and counted in a liquid scintillation spectrometer (Packard). The net release rate of glycerol and radioactivity was calculated from the arteriovenous difference and the plasma flow. The net release caused by nerve stimulation was calculated by adding the total amount released from the tissue during and 20 min after the stimulation and subtracting the basal release for the same overall time period.

Rat experiments

The experiments were conducted on male Sprague–Dawley rats (180–230 g) given food and water *ad lib.* The animals were kept either at room temperature or at 4°. To half of the animals indomethacin (Indomée®, Merck) was given p.o. in a dose of 5–25 mg/kg five times at 10–14 hr intervals. Two or three hr after the last administration the animals were sacrificed.

Blood substrate levels. Seventy rats, 11–23 in each of the above four groups, were lightly anaesthetized with ether and blood was collected from the carotids. The blood was analyzed for lactate [15], pyruvate [16], glucose (glucose oxidase reagent, Glox®, KABI, Stockholm, Sweden) and glycerol [14].

Isolated adipose tissue in vitro. From four animals in each of the four groups inguinal subcutaneous and epididymal fat pads were extracted, cut in small pieces and distributed among plastic vials in Krebs–Ringer bicarbonate buffer pH 7·4 [18] containing 5·5 mM glucose and 2% bovine serum albumin and gassed with 5% CO_2 in O_2. The lipolytic rate was determined by analyzing samples of the medium taken at the start and after 60 min incubation for glycerol content [14].

Isolated fat cells. Adipocytes were prepared according to Rodbell [18] from rat epididymal adipose tissue taken either from control rats or from rats treated with indomethacin as described above. After 15 min preincubation in the above mentioned medium at 37°, aliquots were taken for glycerol determination [14] and NA was added to the final concentrations given in the text. Another aliquot was taken at 60 min. The lipolytic rate is expressed as μmoles glycerol released per hr per 10^6 cells. In some experiments ^{3}H-NA was added together with cold NA at a final concentration of 10^{-7} M. After 60 min incubation at 37° an aliquot of the medium was taken for alumina chromatography [19] which separates catechol from noncatechol NA metabolites. In the same experiments ascorbic acid was added to some of the vials to a final concentration of 10 μg/ml.

Binding to cAMP-dependent protein kinase. Protein kinase was prepared according to Walsh *et al.* [20] through the first DEAE-chromatography step. The binding of cAMP and indomethacin to protein kinase was determined according to the method of Brown *et al.* [21]. Incubations were performed with 1 and 5 pmoles ^{3}H-cAMP in a total volume of 0·3 ml containing 3 μg protein kinase at 0° for 90 min.

Erythrocyte membrane stability. Erythrocytes from control and indomethacin treated rats were prepared and their stability towards hypotonic lysis was tested according to Seeman and Weinstein [22]. 100% hemolysis was produced by distilled water, while the hemolysis produced in 154 mM NaCl was considered as zero.

Materials. L-7-^{3}H-NA (11·3 Ci/m-mole) was obtained from the Radiochemical Centre (Amersham, U.K.) and 2-^{14}C-indomethacin (10 μCi/mg) was a kind gift from Merck, Sharpe and Dohme, as was unlabelled indomethacin. Sodium meclofenamate was a gift from Parke, Davis and Co. (Pontypool, U.K.). Indomethacin was dissolved in a minimal amount of ethanol and diluted in Krebs–Ringer bicarbonate buffer before use. Crude bacterial collagenase was obtained from Worthington and bovine serum albumin from Pentex. Enzymes and coenzymes were obtained from Boehringer & Sohn (Mannheim, Germany). Other chemicals used were reagent grades from ordinary commercial sources.

RESULTS

Dog experiments. The normal responses to sympathetic nerve stimulation in canine subcutaneous adipose tissue *in situ* have been described extensively [3, 12]. In brief, stimulation causes a marked vasoconstriction but little or no increase in lipolytic rate during a 5-min period. The release of ^{3}H-radioactivity actually decreases somewhat during the stimulation, although the proportion of intact NA increases [3, 23]. After the termination of the stimulation there is a period of hyperemia, increased glycerol release and increased transmitter overflow, which returns to normal within 20–30 min.

The intraarterial infusion of indomethacin (5 μg/ml blood) had no significant effect on the vascular resistance before, during or after nerve stimulation. Similarly, indomethacin did not modify basal or stimulated lipolysis or transmitter overflow (Table 1).

Rat experiments in vivo. Following the repeated administration of indomethacin (5 × 5 mg/kg during 2·5 days) rats suffered a 15 per cent weight loss ($P < 0·01$) relative to the controls receiving vehicle (1% ethanol in saline). This was true irrespective of the temperature at which the rats were kept, even though cold storage by itself caused a weight loss of the same magnitude. Treatment with indomethacin induced a

Table 1. The effect of indomethacin (5 μg/ml blood) on vascular resistance (PRU_{100}), glycerol release and on ^{3}H-outflow during and after stimulation of the nervous supply to canine subcutaneous adipose tissue (22–51 g) prelabelled with ^{3}H-NA

	Control (n = 5)	Indomethacin (n = 3)	Paired difference
Vascular resistance (PRU_{100})			
Basal	29·8 ± 7·7	40·2 ± 14·5	5·7 ± 10·6
During stim.	104·8 ± 21·7	129·5 ± 91·5	11·3 ± 63·5
Min following stim.	17·7 ± 2·3	23·1 ± 5·6	4·6 ± 3·7
Glycerol release			
Basal (μmoles/min/100 g^{-1})	0·53 ± 0·09	0·49 ± 0·21	−0·01 ± 0·06
Stimulated* (μmoles/100 g)	39·2 ± 6·1	40·9 ± 8·8	4·7 ± 4·9
^{3}H-overflow			
Stimulated* (10^3 counts/min 100 g^{-1})	224 ± 31	198 ± 14	−40 ± 30

* Calculated by adding the amount released during and following nerve stimulation and subtracting the basal release for the same overall time period.

The results are expressed as mean ± S.E.M. from experiments in three dogs.

Table 2. Effects of indomethacin (5 × 5 mg/kg twice daily for 2½ days) and of cold storage on the concentration (mM) of lactate and pyruvate in arterial blood and of glycerol and glucose in plasma

	Room temperature		Cold-storage		Change due to indomethacin	
	Untreated	Indomethacin	Untreated	Indomethacin	Room	Cold
Glycerol	0·102 ± 0·013 (13)	0·085 ± 0·013 (13)	0·096 ± 0·011 (22)	0·083 ± 0·006 (23)	−0·017	−0·013
Glucose	7·74 ± 0·43 (13)	6·24 ± 0·55 (13)	9·34 ± 0·57 (21)	6·69 ± 0·47 (23)	−1·50	−2·65
			P < 0·01		P < 0·05	P < 0·01
Lactate	1·90 ± 0·18 (11)	1·53 ± 0·13 (13)	3·38 ± 0·51 (19)	4·04 ± 0·50 (23)	−0·37	+0·66
			P < 0·05			
Pyruvate	0·120 ± 0·013 (9)	0·105 ± 0·010 (11)	0·220 ± 0·038 (15)	0·234 ± 0·043 (20)	−0·015	+0·014
			P < 0·05			

The results are expressed as mean ± S.E.M.(N) where N is the number of animals.

Table 3. The effect of indomethacin and meclofenamic acid on basal and NA (10^{-6} M) induced lipolysis in rat epididymal fat cells

	Glycerol release (μmoles hr^{-1} 10^{-6} cells)	
	Basal	Noradrenaline
Control	0·29 ± 0·04	3·30 ± 0·42
Indomethacin 2 μg/ml	0·29 ± 0·03	3·26 ± 0·26
Indomethacin 10 μg/ml	0·24 ± 0·06	3·22 ± 0·34
Meclofenamic acid 4 μg/ml	0·23 ± 0·05	3·24 ± 0·32

The results are expressed as mean ± S.E.M. for four determinations.

hypoglycemia, but no significant changes in arterial concentration of glycerol, lactate and pyruvate were noted (Table 2).

Rat experiments in vitro. Indomethacin 2 and 10 μg/ml was without effect on basal or NA (10^{-6} M) stimulated lipolysis in isolated rat fat cells. Sodium meclofenamate, another potent inhibitor of PG-biosynthesis, was similarly ineffective in a concentration of 4 μg/ml (Table 3).

While the addition of indomethacin *in vitro* thus had no effect on lipolysis we found that fat cells extracted from indomethacin treated rats responded differently from cells prepared from control rats (Fig. 1). There was no difference in basal lipolytic rate between the

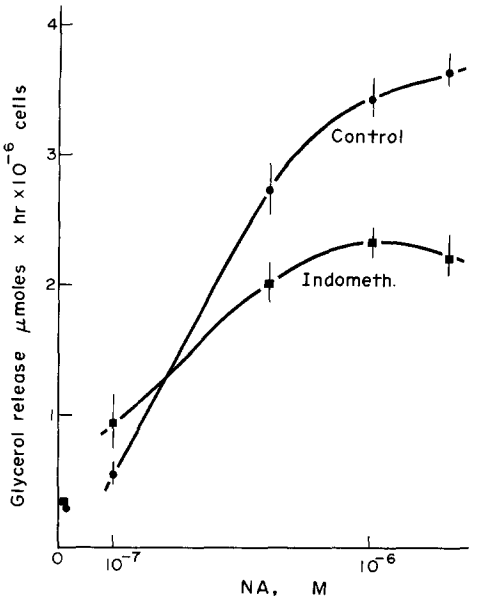

Fig. 1. Glycerol release by fat cells isolated from animals receiving vehicle (1% ethanol in saline) ●——● and animals receiving indomethacin (5 mg/kg × 5) ■——■. Cells were incubated in Krebs–Ringer bicarbonate buffer pH 7·4 containing 2% albumin and 5·5 mM glucose gassed with 95% O_2 and 5% CO_2. Control cells (3·88–4·60 × 10^5 cells/ml). Indomethacin cells (1·87–7·8 × 10^5 cells/ml). Control animals 218 ± 9 g, indomethacin treated animals 194 ± 5 g. Fat pad weights: control 0·50 g, indomethacin 0·48 g. Incubation with the indicated addition for 1 hr following a 20 min preincubation.

two types of cells, but with 10^{-7} M NA in the incubation medium more glycerol was released from the indomethacin cells than from control cells. On the other hand, with higher concentrations of NA (4×10^{-7}–2×10^{-6} M) the opposite was seen. Thus the shape of the dose–response curve was markedly different between the two types of cells. A similar type of shift in the dose–response curve was seen by Fassina [17] when ascorbic acid, which protects NA from degradation, was included in the incubation medium. We therefore studied the effect of ascorbic acid (10 μg/ml) on glycerol release induced by 10^{-7} M NA in control and indomethacin cells. Ascorbic acid significantly augmented lipolysis in control cells (0·56 ± 0·10 and 0·80 ± 0·07 μmoles/hr/10^6 cells), but was without effect in indomethacin cells (0·94 ± 0·20 to 0·98 ± 0·07). As a result the difference between control and indomethacin cells seen without ascorbic acid in the medium vanished. When incubated with control cells for 1 hr under the same conditions only 17% of 10^{-7} M ^{3}H-NA cochromatographed with NA on alumina columns. In contrast, 52 per cent remained "intact" when incubated with cells from indomethacin cells. When ascorbic acid was included in the incubation medium the corresponding values were 80 and 78 per cent from control and indomethacin cells respectively.

In another experiment epididymal and subcutaneous fat tissue from rats kept at room temperature or at 4° and given indomethacin or vehicle was incubated in the presence or absence of 10^{-6} M NA. NA increased lipolysis by 2 μmoles g^{-1} hr^{-1} in epididymal and by 1·2 in subcutaneous fat pads from rats kept at room temperature. Fat pads from rats at 4° was stimulated to a smaller degree (45–60%) by NA. NA induced lipolysis was significantly lower (30–50%) in fat tissue from indomethacin treated rats except in the case of subcutaneous adipose tissue from rats kept at 4°, where the difference was insignificant.

Binding to protein kinase. Acetylsalicylic acid and salicylic acid were recently found to inhibit binding of cAMP to protein kinase in a competitive manner [23]. We failed to note any similar inhibition of cAMP binding by indomethacin over the dose range 0·2–20 μg/ml. Similarly, at a concentration of 2 μg/ml less than 0·5 per cent of ^{14}C-indomethacin was bound to the protein kinase preparation.

Erythrocyte stability. Washed erythrocytes from control rats or rats given 5 or 25 mg/kg indomethacin 5 times p.o. at 10–14 hr intervals were incubated in hypotonic saline. One such experiment is illustrated in Fig. 2. Over a range of saline concentration cells from rats given indomethacin were more resistant to lysis than control cells. The "protection" offered was greater the higher the dose of indomethacin.

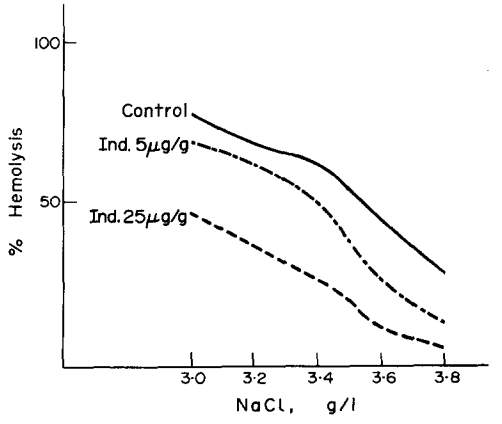

Fig. 2. Lysis of washed erythrocytes from 3 control rats (——), 3 rats given indomethacin 5 mg/kg 5 times at 10–14 hr intervals (– · – · –) and 3 rats given indomethacin 25 mg/kg at the same dosage scheme (·····), exposed to different hypotonic saline media. The hemolysis was expressed as % of the lysis due to distilled water. The blank, subtracted from all values was 9 g/l NaCl.

DISCUSSION

The acute administration of indomethacin had no discernible effect on basal lipolysis either in perfused canine adipose tissue *in situ* or in rat adipocytes *in vitro*. These observations are at variance with those of Illiano and Cuatrecasas [10] but agree with those of

Fain *et al.* [11]. Similarly there was no effect on evoked lipolysis whether this was induced by sympathetic nerve stimulation *in vivo* or by the addition of NA *in vitro*. Bowery and Lewis [27] similarly report that indomethacin (100 μg/ml) has no clearcut effect on lipolysis induced by ACTH in rabbit adipose tissue.

Therapeutic concentrations of another anti-inflammatory drug, acetylsalicylic acid are clearly antilipolytic [23–25]. This action seems to involve an inhibition of cAMP binding to cAMP-dependent protein kinase and a consequent inhibition of cAMP-stimulated protein kinase activity [23, 26]. The present findings that indomethacin had no such effect parallel our findings of no inhibitory effect on lipolysis. An interaction with cAMP-stimulated protein kinase might not be an obligatory part of the spectrum of actions of an anti-inflammatory drug.

While no effect of indomethacin either on basal or NA stimulated lipolysis could be seen when the drug was acutely administered *in vitro* or *in vivo*, there were differences in lipolysis induced by NA between adipocytes from control animals and from rats that had received 5 doses of 5 mg/kg indomethacin.

Thus the lipolytic activity of low doses of NA was higher in adipocytes from rats given repeated p.o. doses of indomethacin than from control rats. This difference in responsiveness was eliminated by the inclusion of ascorbic acid in the incubation medium. The destruction of NA during incubation was higher with cells from control animals than with cells from indomethacin treated animals. Ascorbic acid eliminated this difference as well. Potentiation of NA induced lipolysis in epididymal fat pads from rat was shown by Fassina [17]. On the other hand, ascorbic acid did not affect dibuturyl cyclic AMP induced lipolysis suggesting that the effect is localized early in the activation sequence. Our own results suggest that preservation of NA from destruction might well be a sufficient explanation for the effect of ascorbic acid. The shift in the NA dose response curve reported by Fassina [17], involving a marked potentiation of low doses of NA with no effect at high concentrations of the agonist, is also compatible with this view. Our results therefore suggest that indomethacin treatment inhibits degradation of NA. With higher doses of NA (4×10^{-7}–2×10^{-6} M) cells from indomethacin-treated rats responded with a smaller glycerol release than did control fat cells. Lipolysis induced by NA (10^{-6} M) was lower also in minced adipose tissue from such rats. It seem reasonable to conclude that indomethacin treatment induces changes in rat fat cells which lead to both a decreased lipolytic capacity and to a decreased destruction of NA. The mechanism behind these changes is unknown. It is interesting to note that hypotonic lysis of erythrocytes from indomethacin treated rats was less pronounced than in control red cells. This suggests a membrane stabilizing effect, that might have something to do with the observed changes in the fat cells.

In a recent paper, Deby and Bacq [31] report that indomethacin in high doses inhibits the rise in plasma FFA during fasting. Our finding that indomethacin-treated animals had an unchanged or a slightly lower plasma glycerol level is compatible with these findings. Thus indomethacin-treated rats lost weight relative to the controls and were also hypoglycemic, which is suggestive of starvation. Preliminary results from this laboratory also indicate an increased turnover of NA in adipose tissue in indomethacin treated rats. Starvation and increased sympathetic nervous activity both cause increased plasma glycerol levels. We would therefore have expected increased glycerol levels in the indomethacin animals. Yet none was seen, indicating that some kind of inhibition of lipolysis had taken place.

Following sympathetic nerve stimulation in canine subcutaneous adipose tissue there is a period of post-stimulatory hyperemia [28]. A similar phenomenon is seen in rabbit epigastric adipose tissue stimulated by ACTH [27, 29, 30], probably at least partly mediated by a PG. In rabbit tissue indomethacin inhibited the "functional hyperemia" following ACTH [27]. On the other hand, indomethacin had no effect on the post-stimulatory hyperemia in canine subcutaneous adipose tissue. This supports our earlier contention that PGs probably are of minor physiological significance in the determination of the vascular response to sympathetic nerve stimulation in this tissue [12].

We found recently that indomethacin increases NA overflow from the field stimulated guinea pig vas deferens [32]. This was taken as further evidence that endogenous PGs serve to negatively modulate the release of adrenergic transmitter in a number of organs [33]. On the other hand, PGE_2 had no definite effect on transmitter overflow in canine subcutaneous adipose tissue [3]. Our present finding that indomethacin had little or no effect on transmitter overflow further indicates that this tissue is not one of those where a PG mechanism is very important in the regulation of transmitter release.

Our conclusion is therefore the same as that of Fain *et al.* [11], namely that our results provide no evidence for an important role of PGs in feed back regulation of lipolysis. In fact the results obtained by administration of indomethacin to intact rats are the opposite to those one would expect if a blocked formation of a lipolysis inhibitor was the main effect of indomethacin. Similarly, our results do not favour the hypothesis that PGs are of major importance in the regulation of transmitter overflow or in the post-stimulatory hyperemia in canine subcutaneous adipose tissue.

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