Br. J. Pharmac. (1986), **89**, 199–205

Impairment of protein synthesis in the rat uterus following intrauterine delivery of indomethacin

P.R. Hurst & P.V. Peplow[1]

Department of Anatomy, University of Otago, Dunedin, New Zealand.

1 When indomethacin was incorporated into a slow-release preparation (initial content 1.6 mg drug) and placed in one horn of the rat uterus, a significant decrease in protein synthesis occurred for this horn in comparison with control animals (as determined by the incorporation of radioactive leucine) at three different times after insertion. Decreases of 20, 21% at the two times of dioestrus and 28% at the time of oestrus selected were determined. No significant reduction in protein synthesis was found for the contralateral horn, although there was a tendency for it to be lowered at the earliest time of examination when two complete oestrous cycles had passed following insertion.

2 Measurement of the uptake of radioactive leucine by the uterine horns showed no change in response to indomethacin delivery compared to the control animals with silastic implants, and suggested that the transport system for this amino acid in cells of the uterine horns was not affected by the drug.

3 It was apparent that in instances when the protein synthesis of the uterine horn was impaired by indomethacin that a decrease in RNA/DNA ratio existed. At the latest time examined, no alteration in DNA content occurred in the indomethacin-influenced horn but there was a significant reduction in RNA content.

4 For a small proportion of the animals with indomethacin-releasing preparations there was a tendency to show a lengthening of the oestrous cycle over the first three cycles following insertion. Whether this was due to a direct effect of indomethacin on the ovaries or an effect caused by decreased concentrations of prostaglandins in the uterus was unknown.

5 These results provide further evidence that non-steroidal anti-inflammatory drugs can interfere with the synthesis of macromolecular substances, and that such changes need to be taken into account when considering the overall effect of these drugs on tissues and organs.

Introduction

Many of the non-steroidal anti-inflammatory drugs cause a suppression of prostaglandin synthesis in tissues through an inhibition of cyclo-oxygenase activity (Kuehl & Egan, 1980). However, while having powerful anti-inflammatory action, some of these drugs possess only weak prostaglandin synthetase inhibiting properties e.g. salicylate (Vane, 1971; Flower, 1974) and benoxaprofen (Cashin *et al.*, 1977). The anti-inflammatory action of these compounds is thought to be due to interference with several components of the inflammatory response (Smith, 1975). Related to this, salicylates and other non-steroidal anti-inflammatory drugs have been shown to inhibit the biosynthesis of various macromolecular substances such as protein (Dawkins *et al.*, 1966; Reunanen *et al.*, 1967), glycoprotein (Rainsford, 1978), mucopolysaccharides (Kalbhen *et al.*, 1967), RNA and DNA (Janakidevi & Smith, 1970a,b). It was considered relevant to determine whether indomethacin, a potent anti-inflammatory drug, could influence uterine macromolecular synthesis *in vivo*. In this paper we describe the effect of local delivery of indomethacin on protein synthesis in the uterus, which could have important implications with regard to reproductive physiology and fertility control. The experiments were performed on rats in which indomethacin was administered by an intrauterine implant placed in one horn. This results in sustained release of indomethacin at low concentration within the uterus and would probably have minimal effect on other tissues and organs.

[1]Author for correspondence.

Methods

Preparation and insertion of intrauterine implants

Adult female Wistar rats (approx. 250 g body weight) having regular oestrous cycles were used. Groups of animals (matched for age) were fitted with unilateral intrauterine silastic implants in the shape of rods of 1 cm length × 0.09 cm diameter containing indomethacin (28% w/w). Other groups of control animals were fitted with silastic implants alone. The preparation and sterilization of the implants were performed as previously described (Peplow & Hurst, 1981) with the use of recrystallized indomethacin and silastic 382 M elastomer (Dow Corning) which is considered to be relatively non-toxic to tissues. The vinyl sleeve was removed from around the implants in order to maximize the amount of drug released from the indomethacin implants. For insertion of the implants, the animals were anaesthetized with sodium pentobarbitone and a small incision made in the left horn at approximately half-way along its length to allow the implant to be placed in the lumen of the horn. The implant was secured to the uterine wall and the incision closed by a short nylon suture. Vaginal smears were taken daily from the animals immediately following insertion of the implants and successive cycle lengths recorded up to the time of measurement of protein synthesis.

Measurement of protein synthesis

To determine protein synthesis in the horn with the implant and also in the contralateral horn (no implant) at the selected stage of the cycle (either oestrus or dioestrus) the animals were anaesthetized and injected i.m. at 10 h 00 min with L-[4,5-^{3}H]-leucine (50 μCi in 0.1 ml saline, Amersham). After 2 h, the uterine horns of the animal were removed. The horns were trimmed of fat and mesometrium and cleaned on filter paper. Each was slit longitudinally to allow the escape of luminal fluid and to permit the removal of the implant. The luminal surface of the uterus was blotted on filter paper. The separate horns were weighed and homogenized in 5 ml ice-cold distilled water containing unlabelled L-leucine (50 μg ml^{-1}) in a glass homogenizer with an electrically-driven teflon plunger (10 strokes over 1 min). The homogenizer was washed in 1 ml ice-cold water with carrier leucine. The homogenate and washings were combined and subjected to a perchloric acid-based procedure (Miller, 1979) which firstly involved the immediate addition of 2 ml 1.2 N perchloric acid to precipitate RNA, DNA and protein. The sample was centrifuged (1,000 *g*, 20 min, 4°C) and the supernatant recovered. The perchloric acid-insoluble pellet was washed twice with 5 ml 0.3 N perchloric acid, and after centrifuging the washings were removed. All precipitations and washings were carried out with the tubes placed in an ice-bath and the acid supernatant and pooled washings were stored at − 20°C until required for counting of radioactivity. RNA and DNA were extracted from the perchloric acid-insoluble pellet by hydrolysis at 90°C for 20 min in 10 ml 0.5 N perchloric acid and were recovered in the supernatant by chilling and centrifuging the sample (1,000 *g*, 20 min, 4°C). The supernatant was stored at − 20°C until required for RNA and DNA assay. The perchloric acid-insoluble pellet remaining after extraction of nucleic acids was solubilized by heating at 90°C for 1 h in 10 ml 0.25 N sodium hydroxide and cooled. The solutions were stored frozen.

Radioactivity in the acid supernatant and washings was determined by combining aliquots of the supernatant (0.1 ml) and corresponding pooled washings (0.125 ml) and counted in 14 ml toluene: Triton X-100 (2:1) scintillation fluid with 0.5% PPO and 0.01% POPOP. Radioactivity in the sodium hydroxide solubilized precipitate was measured by treating aliquots (2 ml) with 13 ml toluene:Triton X-100 scintillation fluid and counted after 3 days to allow for decay of chemiluminescence.

Biochemical assays

Assay of RNA in the 0.5 N perchloric acid extract was performed by treating suitably diluted samples (1 ml) with 0.02% ferric chloride hexahydrate in concentrated HCl (1 ml) and 6% orcinol in ethanol (0.1 ml), with the period of heating at 100°C for colour development extended to 30 min (Kerr & Seraidarian, 1945). D-Ribose or solubilized RNA (Sigma type IV, calf liver) in 0.5 N perchloric acid was used as a standard.

DNA in the 0.5 N perchloric acid extracts was assayed by reacting suitably diluted samples (0.5 ml) with 1.5% diphenylamine: 8 mg% acetaldehyde in acetic acid:sulphuric acid (100:1.5) (1 ml) for 18 h in the dark at room temperature for colour development (Burton, 1956). Solubilized DNA (Sigma, calf thymus) in 0.5 N perchloric acid was used as a standard.

Estimation of indomethacin in silastic implants

Indomethacin in the implants removed from the animals was extracted into ethanol and measured fluorimetrically (Peplow & Hurst, 1981; Hurst & Peplow, 1985). A uniform distribution of the drug was shown for a small number of the prepared indomethacin implants which were not fitted into animals.

Statistical analysis of measurements of radioactivity and biochemical assays

Values for the two uterine horns in animals with

Table 1 Effect of intrauterine implants of indomethacin on oestrous cycle length in rats

With silastic implant (controls)

	Cycle length			
	3-day	*4-day*	*5-day*	*6-day*
1st cycle	1	12	5	1
2nd cycle	2	16	0	1
3rd cycle	0	11	1	0
4th cycle	0	12	0	0
5th cycle	0	9	2	0

With indomethacin implant

	Cycle length			
	3-day	*4-day*	*5-day*	*6-day*
1st cycle	0	10	11	0
2nd cycle	0	16	4	1
3rd cycle	0	12	2	0
4th cycle	0	13	1	0
5th cycle	0	12	0	0

Oestrous cycle records were combined for the several groups of animals with silastic implants, as well as for the several groups with indomethacin implants. All the animals showed regular 4-day cycles before insertion of the implants.

indomethacin implants have been compared with the corresponding horns in animals with silastic implants at the selected times following insertion. This allowed the effect of local delivery of indomethacin on parameters measured in the horn with the implant and also in the contralateral horn to be evaluated. It was considered that indomethacin might affect the contralateral horn either by diffusion through the intervening tissues or transport by the vascular system. All statistical tests were made using an unpaired Student's *t* test.

Results

Effect of intrauterine delivery of indomethacin on oestrous cycle lengths

The data relating to the lengths of cycles observed for animals with silastic implants and those with indomethacin implants immediately following insertion is presented in Table 1. The most frequently encounted cycle lengths were 4 and 5 days; cycles of 3 or 6 days were relatively few. Accordingly the data collected on the 4-day and 5-day cycles has been tested for statistical significance between the two groups of animals using a χ^2 (2 × 2)-test. Regarding the 1st cycle, no significant difference was found in the proportion of animals having a 5-day cycle in the two groups ($\chi^2 = 2.03$, $P > 0.05$), but for the 2nd cycle there was a tendency for a higher proportion of animals with an indomethacin implant (4/20) to have a 5-day cycle compared to those with silastic implants (0/16) ($\chi^2 = 3.60$, $P \sim 0.05$). Summing the data obtained over the 1st and 2nd cycles and also over the 1st to 3rd cycle showed a significantly higher occurrence of 5-day cycles in animals with indomethacin implants compared to animals with silastic implants ($\chi^2 = 4.26$ and 4.32 respectively, $P < 0.05$ in both cases).

Protein synthesis and uptake of labelled leucine by rat uterine horns

The radioactivities measured on a wet weight basis in the acid-soluble and acid-insoluble components of the uterine horns are given in Table 2. The values for the acid-soluble components reflected the uptake of labelled leucine into the precursor pools, while those for the acid-insoluble components represented incorporation into synthesized protein.

At dioestrus after the completion of 2 cycles, the radioactivity in the acid-insoluble component of the horn with an indomethacin implant was significantly lowered when compared to the horn with a silastic implant. No difference was found in the radioactivity of the acid-soluble components of these two horns. The radioactivity of the acid-insoluble component in the contralateral horn of animals with indomethacin implants tended to be reduced in comparison with the corresponding horn in animals with silastic implants ($0.05 < P < 0.10$), and no difference in radioactivity for the soluble components of these two horns was found.

At both oestrus and dioestrus upon completing or following 5 cycles, respectively, the radioactivity in the acid-insoluble component was significantly reduced for the horn with an indomethacin implant compared to the horn with a silastic implant. Again no marked alteration occurred in the radioactivity of the acid-soluble components for these two horns. By contrast, no significant change was found in the radioactivity of the acid-insoluble component of the contralateral horns in the two different groups, and the radioactivity for the acid-soluble components of these two horns were similar.

Biochemical composition of rat uterine horns

The measurements from the biochemical assays at the three times are summarized in Table 3A and B.

At dioestrus after completing 2 cycles

While no significant alteration in % RNA was found for the horn with an indomethacin implant compared

Table 2 Effect of intrauterine implants of indomethacin on uptake and incorporation of radioactive leucine by rat uterine horns

	Radioactivity (c.p.m. $\times 10^{-3}$ per 100 mg wet wt)			
	Implant horn		*Contralateral horn*	
Group of animals	*Soluble*	*Insoluble*	*Soluble*	*Insoluble*
At dioestrus after 2 cycles				
with silastic implant ($n = 6$)	4.7 ± 0.3	19.0 ± 1.1	4.2 ± 0.2	17.0 ± 1.0
with indomethacin implant ($n = 6$)	4.8 ± 0.3	15.1 ± 0.1	4.7 ± 0.2	14.1 ± 1.0
At oestrus on completing 5 cycles				
with silastic implant ($n = 5$)	3.7 ± 0.1	15.8 ± 1.1	3.4 ± 0.1	12.2 ± 2.3
with indomethacin implant ($n = 6$)	3.5 ± 0.2	11.3 ± 0.7	3.3 ± 0.2	8.7 ± 0.4
At dioestrus after 5 cycles				
with silastic implant ($n = 6$)	4.0 ± 0.3	27.9 ± 1.3	3.5 ± 0.3	23.7 ± 1.3
with indomethacin implant ($n = 6$)	4.2 ± 0.2	22.2 ± 1.5	3.8 ± 0.2	22.6 ± 1.4

Means ± s.e.
Values underlined for animals with indomethacin implants were significantly different from corresponding ones in controls ($P < 0.05$).

to the horn with a silastic implant, a significant increase in % DNA together with a significant reduction in RNA/DNA ratio was shown for the indomethacin implant horn. Similar observations were made for the contralateral horns of the two groups of animals.

At oestrus upon completing 5 cycles

No significant changes occurred in % RNA or % DNA for the horn with an indomethacin implant compared to the horn with a silastic implant. However, a significant reduction in RNA/DNA ratio was found for the indomethacin implant horn when compared to the silastic implant horn. No significant alterations in % RNA, % DNA and RNA/DNA ratio occurred for the contralateral horns in the two groups of animals.

At dioestrus after completing 5 cycles

A significant decrease in % RNA occurred, with no change in % DNA, for the horn with an indomethacin implant compared to the horn with a silastic implant. These changes led to a significant reduction in RNA/DNA ratio for the indomethacin implant horn relative to the silastic implant horn. No significant alteration in any of these parameters was found for the contralateral horns of the two groups.

Discussion

The present study has shown that the intrauterine release of indomethacin from a silastic implant in the rat tends to cause a lengthening of the oestrous cycle in a small proportion of animals and is most marked for the second cycle following insertion of the indomethacin implant. Previous studies in rats having unilateral implants with an initial loading of 28% indomethacin showed that approximately 1 mg indomethacin was released *in utero* over 21 days, and corresponds to an average daily release of 48 μg. The release rate in the earlier part of this period would be much higher than this (Hurst & Peplow, 1985). While it is possible that indomethacin may reach the ovary from an intraluminal site of release, it is not known whether sufficiently high levels accumulate there for the drug to have a direct effect on luteal function. It is also unknown as to what extent uterine prostaglandins are responsible for regulating ovarian physiology in the rat. If these were involved then the decreased prostaglandin levels in the indomethacin-influenced uterus could be an important factor. In this regard it has been found that the intraluminal release of indomethacin in the guinea-pig caused a pronounced elongation of the oestrous cycle (Horton & Poyser, 1973) and in this species uterine prostaglandin $F_{2\alpha}$ was markedly luteolytic (Blatchley & Donovan, 1969).

The measurements of incorporation of radioactive leucine in the uterine horns show a decrease in protein synthesis in the horn with an indomethacin implant. The two experiments performed at dioestrus at different times following insertion of the implant gave very similar results with protein synthesis for the indomethacin implant horn being decreased by 21 and 20% following the completion of 2 and 5 cycles, respectively. A further series of measurements was performed at oestrus corresponding to the later time

Table 3 Effect of intrauterine implants of indomethacin on wet weight, RNA and DNA levels in rat uterine horns

A *Implant horn*

Group of animals	*Wet wt* (g)	*Biochemical composition* *% RNA*	*% DNA*	*RNA/DNA*
At dioestrus after 2 cycles				
with silastic implant ($n = 6$)	0.257 ± 0.012	0.55 ± 0.03	0.23 ± 0.01	2.35 ± 0.12
with indomethacin implant ($n = 6$)	0.187 ± 0.005	0.52 ± 0.01	0.27 ± 0.01	1.92 ± 0.06
At oestrus on completing 5 cycles				
with silastic implant ($n = 5$)	0.296 ± 0.013	0.60 ± 0.02	0.23 ± 0.01	2.60 ± 0.06
with indomethacin implant ($n = 6$)	0.245 ± 0.008	0.57 ± 0.03	0.25 ± 0.01	2.29 ± 0.06
At dioestrus after 5 cycles				
with silastic implant ($n = 6$)	0.229 ± 0.010	0.58 ± 0.01	0.25 ± 0.01	2.41 ± 0.06
with indomethacin implant ($n = 6$)	0.217 ± 0.010	0.54 ± 0.01	0.25 ± 0.01	2.17 ± 0.06

B *Contralateral horn*

Group of animals	*Wet wt* (g)	*% RNA*	*% DNA*	*RNA/DNA*
At dioestrus after 2 cycles				
with silastic implant ($n = 6$)	0.188 ± 0.007	0.53 ± 0.03	0.26 ± 0.01	2.04 ± 0.06
with indomethacin implant ($n = 6$)	0.157 ± 0.005	0.57 ± 0.03	0.33 ± 0.01	1.73 ± 0.06
At oestrus on completing 5 cycles				
with silastic implant ($n = 5$)	0.224 ± 0.017	0.59 ± 0.03	0.25 ± 0.02	2.48 ± 0.12
with indomethacin implant ($n = 6$)	0.215 ± 0.007	0.60 ± 0.03	0.26 ± 0.02	2.29 ± 0.06
At dioestrus after 5 cycles				
with silastic implant ($n = 6$)	0.168 ± 0.010	0.60 ± 0.01	0.29 ± 0.02	2.10 ± 0.12
with indomethacin implant ($n = 6$)	0.174 ± 0.006	0.56 ± 0.02	0.29 ± 0.01	2.04 ± 0.06

Means ± s.e. are given
Values underlined for animals with indomethacin implants were significantly different from corresponding ones in controls ($P < 0.05$).

and showed that protein synthesis was reduced by 28% for the indomethacin implant horn compared to the silastic implant horn. The value at oestrus for the silastic implant horn, as well as for the indomethacin implant horn, was lower than that for the same horn at dioestrus after 5 cycles ($P < 0.05$), and is in agreement with the cyclical change in protein synthesis described for the normal rat uterus (Reid & Heald, 1971). The decreases in protein synthesis observed in the present study were not caused by reduction in the leucine amino acid pools in the uterine tissue since the measurements of radioactivity for the perchloric acid-soluble components were unaltered. Moreover, while in the two experiments performed at the later times no significant changes in protein synthesis were detected for the contralateral horns, at the earlier time the protein synthesis in the contralateral horn of animals with indomethacin implants tended to be reduced suggesting that the drug may be reaching this horn in sufficient quantity at this time.

In all three experiments the changes in protein synthesis shown for the indomethacin implant horn were found to be related to a lowering in the RNA/DNA ratio for that horn. While the % DNA in the horn with the indomethacin implant at dioestrus at the earlier time was significantly higher than in the horn with a silastic implant, no significant alteration in % DNA occurred between these horns at the two stages examined in the later time. It was also observed that the % DNA in the silastic implant horn was similar at all three times examined. It is suggested that the increase in % DNA that occurred for the indomethacin implant horn after 2 cycles may reflect a decreased extracellular fluid content caused by indomethacin, as the wet weight was also significantly reduced. After 5 cycles this effect is apparently over, as no significant alteration in wet weight occurred and DNA content was unchanged compared to the horn with a silastic implant. At this time, however, the RNA content was significantly reduced for the horn with an indometh-

acin implant. It is possible that reductions had occurred in % RNA at the two earlier times, but that this was not disclosed on account of slight rises in % DNA which were accompanied by small increases in % RNA.

In all the experiments performed, the impairment of protein synthesis (calculated on a wet weight basis) occurred in the absence of any apparent decrease in cell population in the uterine tissue (as judged from % DNA values) and may have even occurred in the presence of increased cell numbers at dioestrus after 2 cycles. Relevant to these findings is that the two stages of the oestrous cycle chosen for this study at midday correspond to ones having low rates of mitotic activity in the normal rat (Marcus, 1974). These two stages differ in respect of there being slightly higher levels of mitotic division in the luminal/glandular epithelium and stroma at dioestrus, and while low levels of mitotic activity occurred in the myometrium at oestrus no such activity was found at dioestrus. It is likely that the tissues most influenced by the intrauterine delivery of indomethacin are those contained within the endometrium, lying closest to the site of release of the drug and, therefore, exposed to the highest concentration of the drug.

The present study, which is the first on the biochemical effects of sustained delivery of indomethacin on uterine macromolecular synthesis, has confirmed that non-steroidal anti-inflammatory drugs can interfere with protein synthesis. Previous studies with salicylate had shown that at low concentrations (0.1–0.3 mM and above) it could inhibit the incorporation of radioactive leucine into the protein of cell-free systems prepared from rat liver (Dawkins *et al.*, 1966; Reunanen *et al.*, 1967). It was also found that at higher concentrations the synthesis of various aminoacyl-*t*RNA species was affected by salicylate (at 0.6 mM and above) (Burleigh & Smith, 1970), and at even greater concentrations of salicylate (3 mM and above) a lowering in the concentrations of RNA and DNA polymerases (Janakidevi & Smith, 1969) and inhibition of RNA and DNA synthesis occurred (Janakidevi & Smith, 1970a, b). No such previous studies have been performed on the detailed biochemical effects of indomethacin. However, working with isolated cells in culture, Bayer *et al.* (1980) showed that the primary effect of indomethacin was an inhibition of protein synthesis (found after 1 h of exposure to the drug) and that a secondary effect of decreased cell proliferation occurred some considerable time afterwards (~24 h) (Bayer *et al.*, 1979). The inhibition of cell proliferation was not overcome by the addition of exogenous prostaglandins and the inhibition of protein synthesis was considered to be due to an interference with the uptake of various amino acids. That such an effect has not been shown in the present study with radioactive leucine may possibly be due to the occurrence of different transport systems for the various amino acids within the cells (Christensen, 1977; Kilberg *et al.*, 1980), and that these are selectively affected by indomethacin.

The effects of intrauterine delivery of indomethacin on uterine protein synthesis and RNA/DNA ratio shown in this study support the contention that non-steroidal anti-inflammatory drugs are able to affect more than one molecular or cellular event contributing to the inflammatory response (Whitehouse, 1974). It has been indicated that the simple correlation of anti-prostaglandin synthetase activity with anti-inflammatory action for these drugs was not justified and that the overall anti-inflammatory activity was due to multiple interactions with several components of inflammatory reactions (Smith, 1975). To provide a better understanding of the changes brought about by indomethacin, further studies are needed to determine the nature of the proteins whose synthesis is affected by the drug, and also to examine the effects of other potent non-steroidal anti-inflammatory substances (such as meclofenamic acid, ibuprofen) when delivered in a similar way. Such changes in protein synthesis could have importance with regard to improving the effectiveness of the intrauterine device as a method of fertility control, since if similar findings occurred in pregnant animals they could interfere with the development of the embryo within the uterus.

This work was supported by a grant from the Medical Research Council of New Zealand.

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(Received February 22, 1986.
Accepted May 12, 1986.)