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Assessment of topical non-steroidal anti-inflammatory drugs

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Abstract—A new non-invasive technique for assessing the efficacy of topical non-steroidal anti-inflammatory drugs (NSAID) in man is proposed. The NSAID are initially applied to the skin under occlusion before inflammation is induced by a methyl nicotinate solution. The inflammatory response is quantified in terms of cutaneous blood flow by a laser Doppler velocimeter (LDV). The efficacy of NSAID preparations is calculated by comparing the responses of the LDV to the methyl nicotinate challenge on the pretreated and the non-treated skin sites. This protocol has been used to investigate the effect of three different NSAID preparations (indomethacin, niflumic acid, palmitoyl collagenic acid) and the influence of the vehicle on the efficacy of indomethacin. The three preparations tested gave positive results but with different amplitudes in response. The efficacy of indomethacin varied with the vehicle used.

There are several methods for assessing the efficacy of anti-inflammatory drugs in man. They generally consist of treating the skin by either physical or chemical means to produce an inflammatory reaction on which the efficacy of the preparation is tested. This is evaluated by allocating a score to the clinical reaction observed (as described by Gomez & Trancik 1981), or by measurement of other parameters of inflammation, e.g. redness (Farr & Diffey 1986) or blood flow (Guy et al 1983). The inflammatory reaction can be induced by various means, including the application of croton oil (Ortega et al 1972), kerosene (Kaidbey & Kligman 1974), or by irradiation with ultraviolet B, A, or C light as previously described by Synder (1975) and Kaidbey & Kurban (1976). To date, the only non-invasive technique available for the measurement of the efficacy of dermocorticosteroids is the assessment of blanching, as proposed by MacKenzie & Stoughton (1962). The other techniques cause durable, sometimes painful lesions, and the inflammatory mechanism is poorly defined.

The aim of the present work was to define a new non-invasive technique enabling the efficacy of non-steroidal anti-inflammatory drugs (NSAID) to be quantified in terms of their effect on a cutaneous challenge by a nicotinic ester. It is well known that the application to the skin of methyl nicotinate solution rapidly produces a visible vasodilation, the duration of which varies from subject to subject and depends on the concentration used. According to Wilkin et al (1985), this reaction is due to the liberation of prostaglandin. The interest of this method is that the extent of the inflammatory reaction can be quantified in terms of cutaneous blood flow by means of a Doppler laser. Also, the reaction is painless, short-term and reproducible in a given subject using the same dose applied to the same site. Since the bioavailability of methyl nicotinate in solution is approximately 10 to 100 times more rapid than that of NSAID's such as indomethacin (Nowack et al 1985), we applied the anti-inflammatory drug before the vasodilator. A similar protocol has been used, without success, by Amantea et al (1983) to measure the effect of corticosteroids. The present study was divided into two parts. Firstly, to validate the method, three different types of NSAID were tested on 10 volunteers. Then, the same protocol was used to investigate the influence of the formulation on the percutaneous absorption of indomethacin.

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Materials and methods

Subjects. Ten adult volunteers of either sex, aged from 22–39 years, took part. They had healthy skin and were asked not to ingest or apply to the skin any anti-inflammatory drug one week before the study. Subjects rested for at least 15 min before the assay to acclimatize to the environment (21°C ± 2) (RH 60% ± 10). Clinical approval was obtained for the experiments.

Protocol. The different preparations (4 mg cm^{-2}) were applied to a delineated area (25 cm^2) of the skin of the ventral surface of the forearm. The drug or its vehicle alone were randomly applied to the right forearm. The preparations were spread uniformly with a gloved finger and allowed to dry. The site was then occluded by a plastic film for 1, 2 or 4 h according to the experiments (see later). After the occlusion period each zone was gently washed and allowed to dry for 15 min. Only one drug was tested on a given subject at a minimum of three days interval to avoid systemic reactions.

The vascular response to methyl nicotinate on the pretreated sites was evaluated by laser Doppler velocimetry (LDV), using a Periflux PF 2B laser Doppler flowmeter (Perimed KB, Stockholm, Sweden). First, the physiological base line was determined for each subject on an area of untreated skin. The recordings for the treated and untreated areas were then made over 30 min immediately following the application of methyl nicotinate solution (0·5%, 2 mg cm^{-2}). The areas between the response curves for each zone and the base line were calculated (see Fig. 1). Results were expressed as the percentage inhibition of the inflammatory reaction induced by methyl nicotinate, calculated from the following equation:

$$\text{Inhibition } (\%) = \frac{\text{AUC (V)} - \text{AUC(D)}}{\text{AUC(V)}} \times 100$$

AUC(V) is the area under the response-time curve on the vehicle-treated site, AUC(D) is the area under the response-time curve on the drug-treated site.

Under this protocol three types of experiments were conducted:

(i) *Time of occlusion.* A preliminary experiment was made to determine the prefered time of occlusion. In this experiment an indomethacin aqueous gel (pH 7·5) (concentration 1·5% w/w) was used and the results obtained with the methyl nicotinate challenge were compared for 1, 2 and 4 h after occlusion.

(ii) *Comparison of the effect of three topical NSAIDs.* For a given occlusion time (2 h) the effect of three different NSAIDs was compared: indomethacin at 1·5% w/w in an aqueous gel, niflumic acid (Nifluril) at 3% w/w in an o/w cream containing mineral oil, fatty acids and alcohols in the lipophilic phase and collagenic palmitoyl acid (Lenidermyl) at 10% w/w in an o/w cream containing fatty acids and alcohols and isopropyl myristate in the lipophilic phase.

(iii) *Influence of the vehicle on the efficacy of a given NSAID.* The effect of the pretreatment of the skin by different formulations of indomethacin was recorded. The vehicle was always an aqueous gel. In gel 1, 2 and 3 indomethacin was solubilized by sodium bicarbonate while in gel 4 the drug was dispersed (particle size 18 μm). In addition, supposed penetration enhancers were added, into gel 2 (urea 2%) and into gel 3 (ethanol 5%) (Table 1). The stability of indomethacin in the formulations was checked by HPLC.

Table 1. Composition and pH values of the four indomethacin gels.

Components	Gel 1	Gel 2	Gel 3	Gel 4
Indomethacin	1·5	1·5	1·5	1·5
Hydroxyethylcellulose	2	2	2	2
Polysorbate 80	2·3	2·3	2·3	2·3
Sodium bicarbonate	0·8	0·8	0·8	0
Urea	0	2	0	0
Ethanol	0	0	5	0
Preservative	0·15	0·15	0·15	0·15
Purified water	93·25	91·25	88·25	94·05
pH value	7·5	7·5	7·5	6

Statistics. The significance of the results was tested by ANOVA test with each volunteer as a factor.

Results

For every subject and for every preparation tested, the vasodilation curve recorded by LDV showed a decrease in amplitude, which varied from subject to subject (see Fig. 1 for example). The variation in the "area under the curve" for indomethacin and its vehicle alone versus the time of occlusion is shown in Fig. 2. The effect of indomethacin is highly significant for each occlusion time ($F = 54{\cdot}3$, $P < 10^{-4}$). The percentages of inhibition corresponding to the occlusion times of 1, 2 and 4 h are 22, 43 and 58%, respectively. It thus appears that a 2 h occlusion was a suitable time for further experiments.

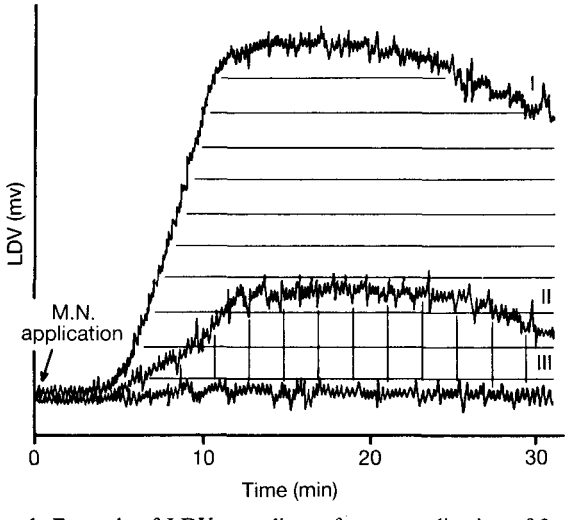

FIG. 1. Example of LDV recordings after an application of 2 mg cm^{-2} of a 0·5% solution of methyl nicotinate, on the control and pretreated sites. A typical recording of the physiological line is also shown.

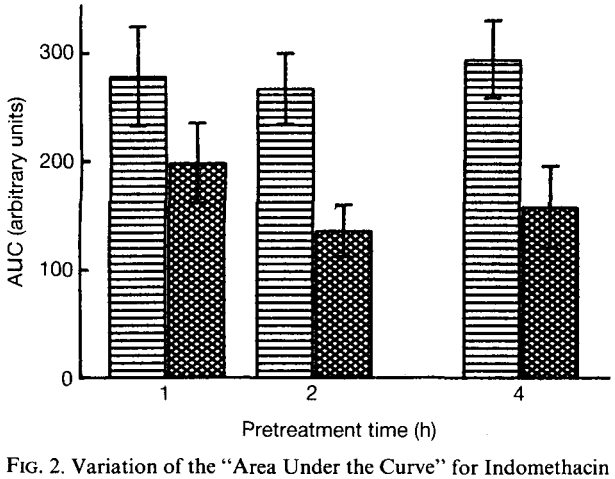

FIG. 2. Variation of the "Area Under the Curve" for Indomethacin and the vehicle alone vs the time of pretreatment (see text for statistics).

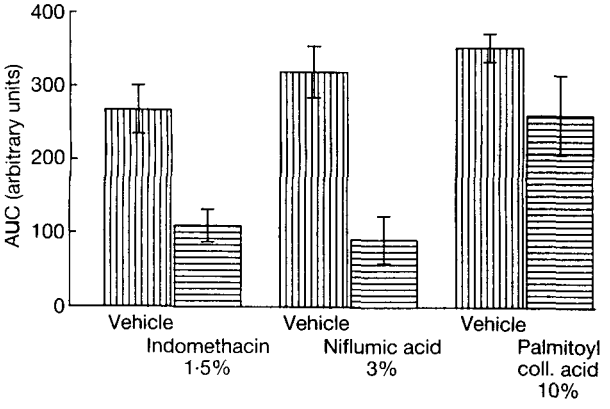

FIG. 3. Area under the curve of response of the LDV for NSAIDs compared with their vehicles.

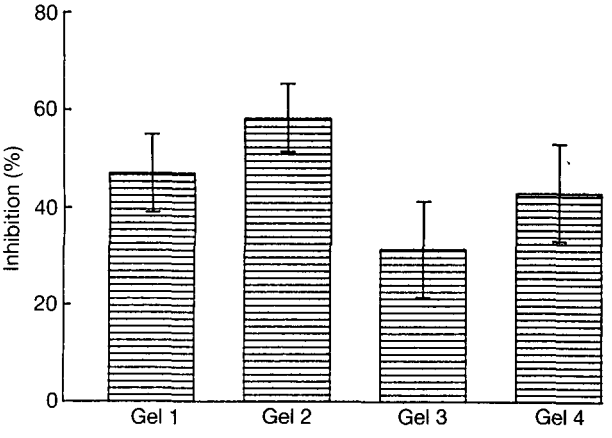

FIG. 4. Influence of the vehicle of indomethacin on its percentage of inhibition. All the percentages of inhibition are significantly positive. Only gel 2 and gel 3 present statistically different results (see text).

The areas under the response curve for each drug were calculated and compared with the control (placebo). A significant inhibitory effect was observed for each of the three NSAIDs ($F = 38$, $P < 10^{-4}$) versus the corresponding placebos (Fig. 3). Niflumic acid reduced blood flow by up to 70% (s.d. 28), indomethacin by up to 58% (s.d. 21), and palmitoyl collagenic acid by up to 26% (s.d. 10). There was no significant difference between niflumic acid and indomethacin, but their effect was statistically greater ($P < 0{\cdot}01$) than that of palmitoyl collagenic acid.

In the second part of this study, regardless of the type of formulation, indomethacin significantly limited the increase in blood flow induced by the application of methyl nicotinate. No significant difference was observed between formulations 1, 2 and 4, although urea (gel 2) did appear to have a greater enhancing effect than ethanol (gel 3 $P < 0{\cdot}05$) (Fig. 4).

Discussion

The kinetics of the vasodilation of cutaneous microcirculation induced by the topical application of methyl nicotinate under our experimental conditions are in agreement with the work of

Guy et al (1983, 1984) and Kholi et al (1987). The vasodilation appears following a lag period of 4 to 6 min, reaches a plateau, and begins to decrease slowly thereafter. The growth phase has a sigmoidal aspect. In healthy subjects, a 0·5% concentration of methyl nicotinate applied at 2 mg cm^{-2} induces a significant response which is easily detectable using LDV and shows inter-subject variations. These variations, of a physiological nature, require the drug to be tested simultaneously against a placebo. In effect, there is either a decrease in the duration of the plateau or in the amplitude of the peak. This polymorphism shows that it is preferable to calculate the area under the curve rather than to measure a single parameter such as the lag time, the height of the peak, or the length of the plateau, as is done for the bioavailability of nicotinic esters, since they do not fully reflect the phenomenon observed (as previously noted by Kohli et al 1987).

The curve of the percentage inhibition of vasodilation against the time of pretreatment shows that after a 1 h occlusion a slight anti-inflammatory effect develops. Nowack et al (1985) carried out a study in man of the percutaneous bioavailability of a 1% indomethacin gel applied under occlusion. The drug was detected in the blood after 1 h, but the maximal concentration was reached at between 2 and 4 h, decreasing thereafter. These data should be viewed in the light of the present study (Fig. 2) since the pharmacological response was detected after 2 h occlusion. In our experiment, the effect of indomethacin is clearly observed, as previously reported by Wilkin et al (1985) who showed that the absorption of indomethacin from the tract suppressed the vascular response to methyl nicotinate in man.

Under our experimental conditions, niflumic acid induced the strongest anti-inflammatory response, while that of palmitoyl collagenic acid's action was significant but much less intense. However, to our knowledge there are no data in the literature concerning the efficacy of palmitoyl collagenic acid. The indomethacin gel containing urea had an intermediate effect.

Thus, this objective method for the evaluation of the pharmacological effect of NSAID applied percutaneously allows the measurement of the activity of various molecules in dermatological preparations. All three molecules tested showed an anti-inflammatory effect but with significantly different amplitudes in strictly similar experimental conditions. It should be noted that the time of pre-application of the NSAIDs was only optimized for indomethacin, although an occlusion time of 2 h would appear to be generally suitable.

For indomethacin, a significant reduction in the inflammatory reaction was observed with all the formulations used versus placebo ($P < 0{\cdot}01$). From these findings it may be inferred that indomethacin is able to penetrate the skin barrier and that the formulation used affected drug release.

These results are in agreement with those of Naito & Tsai (1981) who also found an enhancing effect of urea in indomethacin preparations given to rabbits. However, the addition of ethanol to gel 3 had little effect which confirms the work of Inagi et al (1981) who reported that even high ethanol concentrations (up to 30%) did not increase the percutaneous absorption of indomethacin in the guinea-pig. Ionized drugs are generally supposed to show slight skin penetration; however, the absorption of indomethacin observed under our experimental conditions was the same for gels 1 and 2 (partly ionized) as for gel 4 where the drug was simply dispersed in the vehicle and required solubilization before being absorbed.

This result partly confirms those of Naito & Tsai (1981) who showed in rabbits the percutaneous penetration of crystallized indomethacin dispersed in a w/o emulsion. However, those authors did not observe the release of the active drug from an aqueous gel. This disagreement with our results may stem from the use by those authors of an NSAID with a particle size of approximately 150 μm, compared to that of 18 μm for the indomethacin in the gel we used.

Any objective experimental test designed to measure in man the efficacy of topical anti-inflammatory drugs must be able to discriminate significantly between agents known to differ in their potencies and to assess the effects of the formulation on drug bioavailability. The procedure we propose fulfils both these conditions. In addition, it can be directly applied in man for routine testing since it employs non-invasive techniques.

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Amino acids dilate resistance blood vessels of the perfused rat mesentery

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Abstract—The vasodilator effect of several L-amino acids in the perfused, noradrenaline-preconstricted rat mesentery preparation has been investigated. *N*-α-Benzoyl-L-arginine ethyl ester (BAEE) (ED50, 1·4±0·09 μmol) and L-alanine methylester (ED50, 0·9±0·007 μmol) were the most potent although L-arginine methylester, hydroxamate and hydrochloride, *N*-α-benzoyl-L-arginine methyl ester (BAME), L-methionine methylester, L-lysine hydroxamate and L-glutamic acid methylester exhibited similar potency with ED50 values in the range 2·4–3·7 μmol. L-Homoarginine chloride was inactive at doses up to 20 μmol. D-Arginine hydrochloride and D-lysine hydroxamate were inactive at doses up to 50 μmol whilst D-methionine methylester (50 μmol) produced small falls in perfusion pressure in only 3 out of 7 preparations studied. Responses to BAEE, BAME, L-arginine hydrochloride, L-alanine methylester, L-methionine methylester, L-lysine hydroxamate and acetylcholine (but not nitroprusside) were significantly inhibited by CHAPS (4·7 mg mL^{-1}, 30 s) de-endothelialization as well as pretreatment of mesentery preparations with gossypol (3 μM). Responses to BAEE, BAME, L-arginine hydrochloride, L-alanine methylester and acetylcholine were similarly selectively reduced by NDGA (10 μM) pretreatment. We propose that these L-amino acids exhibit vasodilator activity in the perfused rat mesentery by virtue of releasing endothelium-derived nitric oxide (EDNO).

It has been known for several decades that L-amino acids have important roles in the intermediary metabolism of carbohydrates and proteins and as neutrotransmitters in the mammalian central nervous system (see reviews by Bender 1985; Davidson 1976). The recent demonstration that L-arginine (Palmer et al 1988) or an L-arginine-containing peptide (Thomas & Ramwell 1988) is the natural precursor for endothelium-derived nitric oxide (EDNO) has raised the possibility that this amino acid may be at the centre of a local haemostatic mechanism controlling blood vessel calibre. In this context we have recently made a preliminary report that L-benzoyl arginine methyl and ethyl esters (BAME, BAEE) exhibit endothelium-dependent vasodilatation in the perfused rat mesenteric vascular bed (Al-Swayeh et al 1989). In this report we show that endothelium-dependent vasodilator activity in the perfused rat mesentery is not restricted to L-arginine but is a more general phenomenon shared by a number of chemically unrelated L-amino acid analogues.

Materials and methods

The experimental procedures used were those of Bhardwaj & Moore (1988). Briefly, male Sprague-Dawley rats (250–350 g) were killed by a blow to the head and exsanguinated. The mesenteric vascular bed was perfused with warmed (37°C), oxygenated (95% O_2:5% CO_2) Krebs solution via a cannula inserted into the superior mesenteric artery. Perfusion pressure was monitored continuously by means of a Bell & Howell pressure transducer connected to a Devices pen recorder. Indomethacin (7 μM) was routinely added to the perfusing Krebs solution to inhibit vascular cyclo-oxygenase activity and prostaglandin $(PG)I_2$ biosynthesis.

The vasodilator effect of drugs was assessed in so-called 'high tone' preparations in which noradrenaline was added to the Krebs reservoir at a concentration (0·06–2·5 mM) sufficient to increase perfusion pressure by 70–90 mm Hg representing approximately 65–75% of the maximum response. No significant loss of vasoconstrictor tone or change in mesentery weight was detected over a 4 h perfusion which was thus the maximum length of each experiment. Acetylcholine (ACh, 0·01–10 nmol), sodium nitroprusside (NP, 0·1–20 nmol), L-arginine methylester, L-arginine hydroxamate, D- and L-arginine hydrochloride, L-benzoyl arginine ethyl and methylesters (BAEE and BAME), L-homoarginine chloride, L- and D-methionine methylester, L-alanine methylester, L-glutamic acid methylester and L- and D-lysine hydroxamate (all 0·3 μmol–60 μmol) were injected in small volumes (< 10 μL) to prevent changes in perfusion pressure due to an injection artefact. In some experiments, mesentery preparations were perfused (30 s) with 4·7 mg mL^{-1} 3-(3-cholamidopropyl) 1-propanesulphonate (CHAPS) to remove endothelial cells lining resistance blood vessels. Alternatively, gossypol (3 μM) or nordihydroguaiaretic acid (NDGA, 10 μM) was added to the Krebs reservoir and allowed to perfuse preparations for 1 h in order to prevent the biosynthesis or promote the inactivation of EDNO, respectively.

All drugs were purchased from Sigma Ltd. Stock solutions of indomethacin were prepared in 0·5% Na_2CO_3. All other drugs were dissolved on the day of the experiment in saline (0·9% w/v NaCl). Results show mean ± s.e.m with the number of observations indicated in parentheses. Statistical analysis of differences between groups was determined using Student's unpaired *t*-test. A probability value of 0·05 or less was taken to indicate statistical significance.

Results

The mean resting perfusing pressure of rat mesentery preparations used in this study was 15·4±4·9 mm Hg (n=26). 'High tone' rat mesentery preparations perfused for 4 h gained an average 4% in weight indicating the absence of significant oedema formation over this period. Bolus injection of ACh and NP produced transient and dose related falls in perfusion pressure. The dose of each drug required to produce 50% of the maximum response (ED50) was 6·1±0·2 nmol (n=5) and 8·2±0·9 nmol (n=5), respectively. The maximum vasodilatation which could be achieved with each drug was approximately 45 mm Hg. CHAPS-induced de-endothelialization reduced the vasodilator effect of a dose of ACh required to produce 80% of the maximum response (ED80) by approximately 85% without influencing the response to an appropriate ED80 of NP. Similarly, both gossypol and NDGA pretreatment of perfused rat mesentery preparations significantly inhibited vasodilatation due to ACh without an effect on the response to NP. These results are summarized in Table 1.

All of the L-amino acids tested produced dose related vasodilatation of rat mesenteric resistance blood vessels with the exception of L-homoarginine chloride which was inactive at doses up to 20 μmol. In contrast, D-arginine hydrochloride and D-lysine methylester were without vasodilator activity at doses up to 50 μmol (n=6). D-Methionine methylester (50 μmol)

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