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THE INFLAMMATORY EFFECTS OF HYDROPEROXY AND HYDROXY ACID PRODUCTS OF ARACHIDONATE LIPOXYGENASE IN RABBIT SKIN

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1 The inflammatory effects of hydroperoxy (HPETE) and hydroxy (HETE) acids, synthesized by arachidonic acid lipoxygenases, have been investigated in rabbit skin.

2 High doses (10–20 μg) of 5-, 12- or 15-HPETE or the 5,12-di-hydroxy acid, leukotriene B_4 (0.1–1 μg), caused small but significant increases in plasma exudation following intra-dermal injection.

3 Leukotriene B_4 was equipotent with prostaglandin E_2 and prostacyclin in potentiating bradykinin-induced plasma exudation, and was 100 times more active in this property than any other lipoxygenase product tested.

4 Leukotriene B_4-induced plasma exudation was enhanced by prostaglandin E_2.

5 The mono-HETEs were relatively inactive in causing or enhancing plasma exudation.

6 Leukotriene B_4 (0.1 μg) or prostaglandin E_1 (1.0 μg) significantly elevated leukocyte accumulation in rabbit skin, whereas PGE_2, 5-HPETE, 5-HETE, 12-HPETE or 12-HETE were inactive at doses up to 1 μg.

Introduction

Peroxidation of arachidonic acid at C-11 by cyclo-oxygenase leads to the formation of cyclic endoperoxides which are the precursors of prostaglandins, thromboxanes and prostacyclin. Lipoxygenases control peroxidation at several other positions in the arachidonic acid molecule. These lipoxygenase reactions, which are not followed by cyclization, lead to the formation of hydroperoxy acids which may be subsequently reduced enzymatically or non-enzymatically to the corresponding hydroxy acids. Lipoxygenase activity was first detected in platelets which were shown to convert arachidonic acid to 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE; Hamberg & Samuelsson, 1974; Nugteren, 1975). Peroxidation of arachidonic acid at C-5 is of particular interest since the 5-HPETE produced is an intermediate in the formation of leukotrienes (Murphy, Hammarstrom & Samuelsson, 1979). Initially, 5-HPETE is converted to a 5,6-epoxide (leukotriene A_4; LTA_4) which is then enzymatically hydrolyzed to a dihydroxy acid, 5,12-di-HETE (leukotriene B_4; LTB_4). The reaction of LTA with glutathione leads to the formation of a series of leukotrienes with a novel lipid-peptide structure (Samuelsson & Hammarstrom, 1980; for review see Samuelsson, Hammarstrom, Murphy & Borgeat, 1980).

Cyclo-oxygenase products such as prostaglandin E_2 or prostacyclin (PGI_2) contribute to inflammatory erythema, oedema and hyperalgesia (for review see Higgs, Moncada & Vane, 1980). In 1977, Williams & Peck proposed that oedema formation results from the interaction of mediators which separately cause vasodilatation and increased vascular permeability. PGE_2 and prostacyclin increase blood flow in rabbit skin without causing plasma exudation, whereas bradykinin and histamine have a direct effect on vascular permeability. When PGE_2 or prostacyclin is given in combination with bradykinin there is an augmentation of plasma exudation (Peck & Williams, 1978; Williams, 1979).

Although prostaglandins are clearly involved in the vascular responses in inflammation, the accummulation of leukocytes in inflamed tissues is independent of cyclo-oxygenase activity (Walker, Smith & Ford-Hutchinson, 1976; Higgs, Eakins, Mugridge, Moncada & Vane, 1980). However, recent reports indicate that lipoxygenase products of arachidonic acid metabolism could be involved in leukocyte migration. Some mono-hydroxy acids are chemotactic for polymorphonuclear leukocytes (PMNs) *in vitro* (Turner, Tainer & Lynn, 1975; Goetzl & Sun, 1979) and it has recently been shown that LTB_4 is one of the most potent endogenous chemotactic factors known

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(Ford-Hutchinson, Bray, Doig, Shipley & Smith, 1980; Palmer, Stepney, Higgs & Eakins, 1980).

Initially it was thought that hydroxy acid lipoxygenase products were not involved in vascular permeability, as LTB_4 did not cause or enhance oedema in the rat paw (Smith, Ford-Hutchinson & Bray, 1980). Furthermore, intra-dermal injections of 5-HETE or LTB_4 did not cause plasma exudation in guinea-pig skin although leukotriene D_4 was active (Williams & Piper, 1980). We have recently reported to the Physiological Society however, that LTB_4 is equi-active with PGE_2 and prostacyclin in potentiating bradykinin-induced plasma exudation in rabbit skin (Eakins, Higgs, Moncada, Salmon & Spayne, 1980). These observations have now been confirmed (Bray, Cunningham, Ford-Hutchinson & Smith, 1981) and Wedmore & Williams (1981) have also described synergy between LTB_4 and PGE_2.

In this paper we have compared the effects of some cyclo-oxygenase and lipoxygenase products on plasma exudation and leukocyte infiltration in rabbit skin. Some of these results have been described to the Physiological Society and the British Pharmacological Society (Eakins *et al.*, 1980; Carr, Higgs, Salmon & Spayne, 1981).

Methods

Plasma exudation

Plasma exudation in rabbit skin was measured according to the method described by Williams (1979). The backs of New Zealand White rabbits (2–3.5 kg) were closely shaved and injection sites were marked out in blocks of six according to a fixed pattern. Each animal received an intravenous injection of 50 μCi [^{125}I]-human serum albumin (Radiochemical Centre, Amersham) in a 2.5% solution of Evans blue dye dissolved in sterile 0.9% w/v NaCl solution (saline), prior to administration of a short-acting anaesthetic (1% methohexitone sodium; 10 mg/kg). Test substances were dissolved in 50 mM Tris buffer (pH 7.5) in 0.9% chloride solution and 100 μl volumes were injected intradermally into 6 separate sites with 12 × 0.4 mm disposable needles; each animal received 8–12 different treatments. After 30 min, three blood samples (2.5 ml) were taken from an ear vein and each animal was killed by an overdose of anaesthetic, the skin removed and injection sites separated with a 5/8 inch steel punch. Radioactivity in plasma and skin samples was measured and the plasma volume at each injection site was calculated.

Leukocyte infiltration

For the estimation of leukocyte infiltration, skin samples were taken 1–4 h after intradermal injection. Following histological processing, sections of skin (4–5 μm thick) were cut at four different points of each site. The sections were stained with haematoxylin and eosin before microscopic examination at low power (× 160) to locate the injection site. Total leukocyte numbers were counted in five high power fields (× 1000; hpf) arranged vertically through the dermis; the fields were chosen to avoid hair follicles.

Preparation of lipoxygenase products

Hydroperoxy acids were synthesized by the oxygenation of arachidonic acid (Porter, Wolf, Yarbro & Weenan, 1979) and the corresponding hydroxy acids were prepared by reduction with triphenyl phosphine. The HETEs were purified by high performance liquid chromatography (h.p.l.c.) and identified using gas liquid chromatography and mass spectrometry (g.l.c./m.s.).

LTB_4 was generated biologically by incubating 2×10^9 glycogen-stimulated rabbit peritoneal leukocytes with 1 mg arachidonic acid and 50 μg of the calcium ionophore A23187. The acidic lipid products of this reaction mixture were extracted into ether and subjected to silicic acid column chromatography according to Borgeat & Samuelsson (1979). The ethyl acetate fraction was evaporated to dryness and further purified by reversed phase h.p.l.c. The sample was

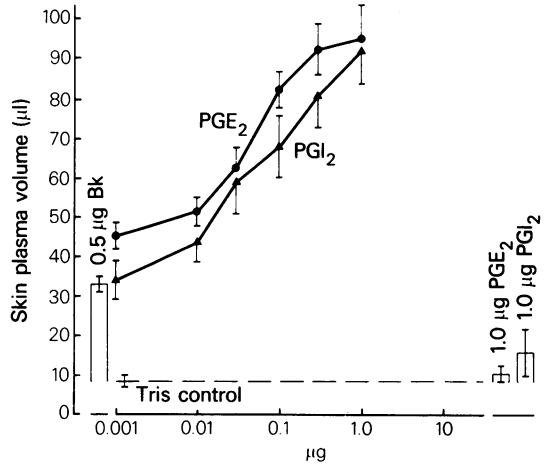

Figure 1 The effects of arachidonate cyclo-oxygenase products on bradykinin-induced plasma exudation in rabbit skin. The dose-response curves show the effects of increasing concentrations of prostaglandin E_2 (PGE_2) or prostacyclin (PGI_2) on plasma exudation when given in combination with 0.5 μg bradykinin (Bk). The dashed line indicates the effect of intra-dermal injections of vehicle alone and the histograms show the effects of the highest doses of each substance when given alone. Each point is the mean of at least six values and the bars represent s.e.mean.

applied to a C18-μ-Bondapak column (Waters Associates) and eluted with methanol-water-acetic acid (70:30:0.01, v/v/v) at a flow rate of 0.7 ml/min. The eluant was monitored for ultra-violet absorbance at 254 and 280 nm. Two small peaks of absorption at 280 nm were detected at 13.0 and 13.75 min and these were believed to be the C-12 epimers of the all *trans*-triene of 5,12-di-HETE (isomers 1 and 2; see results). The major absorption peak at 280 nm eluted at 14.5 min and corresponded to LTB_4 (isomer 3). Purified LTB_4 was quantified by uv spectrophotometry at 273 nm and its identity confirmed by g.l.c./m.s.

Results

Plasma exudation

Intradermal injections of bradykinin (0.01–10 μg) caused a dose-dependent increase in plasma exudation. In contrast, PGE_2 (0.01–30 μg) or PGI_2 (0.01–30 μg) did not significantly increase plasma volume above that seen in skin samples receiving injections of vehicle alone. 5-, 12-, and 15-HPETE (10–20 μg) and LTB_4 (0.1–1.0 μg) caused a small but significant plasma leakage of up to 30 μl. 11-HPETE and the mono-hydroxy acids were inactive at doses up to 20 μg.

When bradykinin (0.5 μg) was injected in combination with increasing concentrations of PGE_2 or PGI_2 (0.001–1.0 μg), there was a dose-dependent potentiation of plasma exudation (Figure 1); 0.5 μg bradykinin plus 1 μg 6-oxo-$PGF_{1\alpha}$ caused a plasma leakage of 41.4 μl ± 2.7 ($n = 5$) which was not significantly greater than 0.5 μg bradykinin alone in the same experiment. Combinations of 0.5 μg bradykinin with any of the following, LTB_4 (0.001–0.2 μg), 5-HPETE (1–30 μg), 12-HPETE (0.01–10 μg) or 15-HPETE (0.01–30 μg) also caused a dose-dependent enhancement of plasma exudation (Figure 2). LTB_4 was equi-potent with PGE_2 and PGI_2 in enhancing bradykinin-induced exudation and was at least 100 times more active than any of the mono-hydroperoxy acids tested (Figure 2). The diasteromers of 5,12-di-HETE were less active than LTB_4. In preliminary experiments, a combination of isomers 1 and 2 (0.003–0.03 μg) did not enhance bradykinin-induced plasma exudation. 11-HPETE (1–10 μg) also potentiated plasma leakage but the mono-

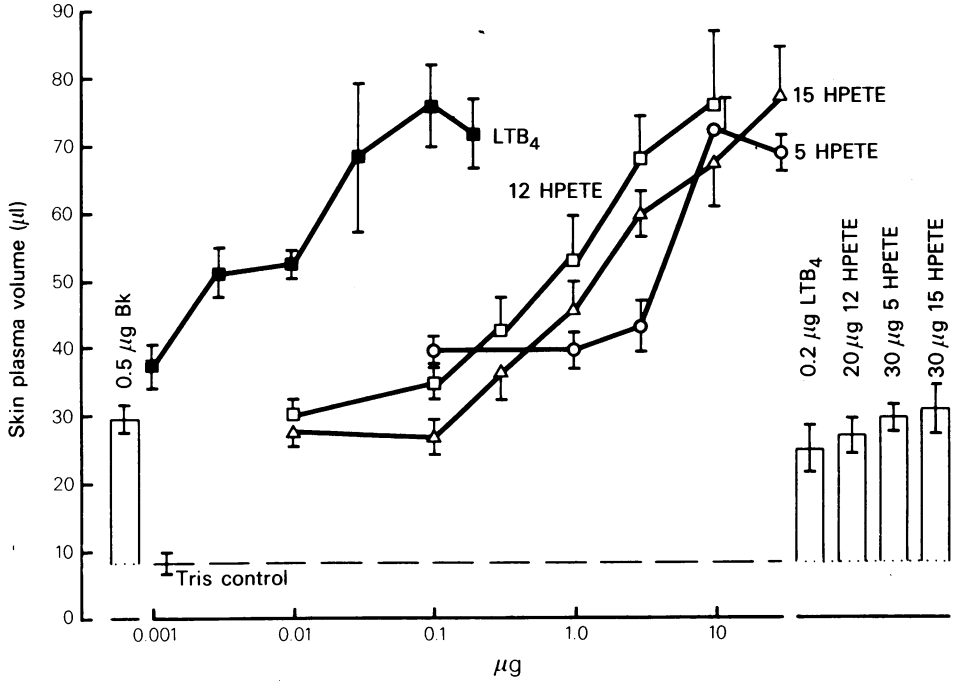

Figure 2 The effects of arachidonate lipoxygenase products on bradykinin-induced plasma exudation in rabbit skin. The dose-response curves show the effects of increasing concentrations of leukotriene B_4 (LTB_4), 12-hydroxyperoxy (HPETE), 15-HPETE or 5-HPETE acids on plasma exudation, when given in combination with 0.5 μg bradykinin (Bk). The dashed line indicates the effect of intra-dermal injections of vehicle alone and the histograms show the effects of the highest doses of each substance when given alone. Each point is the mean of at least six values and the bars represent s.e.mean.

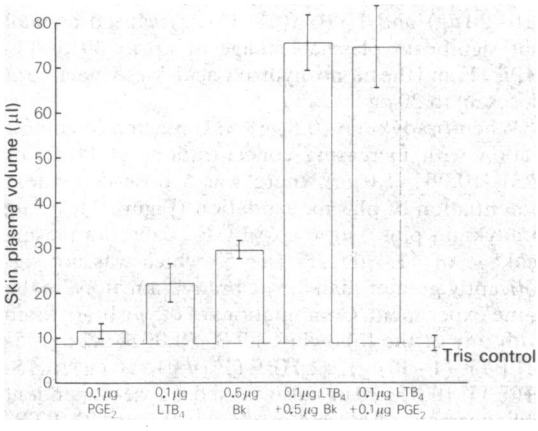

Figure 3 The effects of prostaglandin E_2 (PGE_2) and bradykinin (Bk) on leukotriene B_4 (LTB_4)-induced plasma exudation in rabbit skin. The dashed line indicates the plasma volume in samples of skin following intra-dermal injection of 100 μl Tris buffer. Each histogram is the mean of at least 6 values and the bars represent s.e.mean.

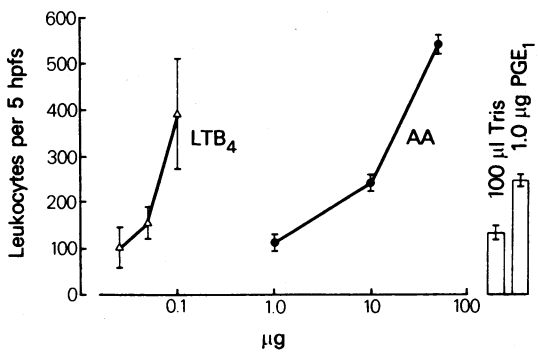

Figure 4 The total number of leukocytes seen in 5 high power fields (hpfs) in the dermis of rabbit skin 4 h after the intra-dermal injection of leukotriene B_4 (LTB_4 0.025–0.1 μg) or arachidonic acid (AA, 10–50 μg). Each point represents the mean of counts in skin samples taken from at least three separate animals and the bars are s.e.mean. The histograms indicate the leukocyte accumulation following injection of 100 μl Tris buffer or 1 μg prostaglandin E_1 (PGE_1).

HETEs (20 μg) were inactive. Increased plasma exudation was observed when LTB_4 was given in combination with either bradykinin or PGE_2 (Figure 3).

Leukocyte infiltration

Sections of normal skin did not show any evidence of leukocyte infiltration whereas intra-dermal injection of 100 μl Tris buffer resulted in the appearance of PMNs in the dermis. The numbers of cells in the dermis were significantly increased by LTB_4 (0.1 μg) or arachidonic acid (10–50 μg) (Figure 4). The numbers of leukocytes in skin samples taken 4 h after injection of LTB_4 were not significantly higher than in samples taken at 1 h. PGE_2, 5-HPETE, 5-HETE, 12-HPETE or 12-HETE at doses up to 1 μg did not significantly elevate the numbers of leukocytes above those seen with vehicle alone. PGE_1 (1 μg), however, did enhance leukocyte accumulation but was approximately twenty times less active than LTB_4 (Figure 4). With every treatment, the leukocytes seen were predominantly (> 98%) polymorphonuclear and large numbers were eosinophilic in appearance.

Discussion

The results presented in this paper confirm the observations that PGE_2 and PGI_2 enhance bradykinin-induced plasma exudation in rabbit skin (Williams, 1979) and they indicate that a lipoxygenase product, LTB_4, is equi-active in this property. The HPETEs tested also potentiate bradykinin effects but are at least two orders of magnitude less potent while the hydroxy acid derivatives are relatively inactive.

Williams & Peck (1977) have suggested that plasma exudation is the result of the action of two mediators which separately cause increased vascular permeability and vasodilatation. PGE_2 and PGI_2 are powerful vasodilators but by themselves they do not cause significant plasma leakage. Bradykinin, however, appears to be a mixed mediator, increasing permeability and also having intrinsic vasodilator activity. The observation that LTB_4-induced exudation is enhanced by both bradykinin and PGE_2 indicates that LTB_4 has a direct effect on permeability.

Previous studies have shown that LTB_4 is a potent chemotactic agent *in vitro* (Ford-Hutchinson *et al.*, 1980; Palmer *et al.*, 1980) and we have now demonstrated that LTB_4 enhances leukocyte accumulation *in vivo* and is more active than any other lipoxygenase or cyclo-oxygenase product tested. LTB_4 has also been reported to enhance leukocyte migration following intra-peritoneal injection (Smith *et al.*, 1980). Furthermore, LTB_4 is equi-active with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) in inducing leukocyte accumulation in the aqueous humour of rabbit eyes (Bhattacherjee, Hammond, Salmon, Stepney & Eakins, 1981).

There is evidence that leukocytes may be involved in vascular permeability changes and, therefore, in the oedema formation in acute inflammation. Chemotactic factors such as LTB_4, FMLP and the complement fraction C5a, when in combination with PGE_2, induce plasma exudation in rabbit skin and this activity is reduced in neutropaenic animals (Wedmore & Williams, 1981). The results presented

in this paper support the idea that there is a link between leukocyte accumulation and vascular permeability. This hypothesis may also explain why reduction of oedema corresponds more closely to the inhibition of leukocyte accumulation than the inhibition of prostaglandin synthesis (Higgs *et al.*, 1980).

Clearly, arachidonic acid lipoxygenase products have potent inflammatory properties *in vivo*. It is possible that LTB_4, produced by stimulated leukocytes (Borgeat & Samuelsson, 1979), acts as a central mediator in the control of vascular permeability and leukocyte migration. These observations endorse the proposal that dual inhibitors of lipoxygenase and cyclo-oxygenase such as BW755C have a more comprehensive anti-inflammatory activity than selective cyclo-oxygenase inhibitors such as aspirin or indomethacin (Higgs, Flower & Vane, 1979).

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References

BHATTACHERJEE, P., HAMMOND, B., SALMON, J.A., STEPNEY, R. & EAKINS, K.E. (1981). Chemotactic response to some arachidonic acid lipoxygenase products in the rabbit eye. *Eur. J. Pharmac.* (in press).

BORGEAT, P. & SAMUELSSON, B. (1979). Arachidonic acid metabolism in polymorphonuclear leukocytes. Effects of ionophore A23187. *Proc. natn. Acad. Sci., U.S.A.*, **76,** 2148–2152.

BRAY, M.A., CUNNINGHAM, F.M., FORD-HUTCHINSON, A.W. & SMITH, M.J.H. (1981). Leukotriene B_4: a mediator of vascular permeability. *Br. J. Pharmac.*, **72,** 483–486.

CARR, S.C., HIGGS, G.A., SALMON, J.A. & SPAYNE, J.A. (1981). The effects of arachidonate lipoxygenase products on leukocyte migration in rabbit skin. *Br. J. Pharmac.*, **73,** 253–254P.

EAKINS, K.E., HIGGS, G.A., MONCADA, S., SALMON, J.A. & SPAYNE, J.A. (1980). The effects of arachidonate lipoxygenase products on plasma exudation in rabbit skin. *J. Physiol.*, **307,** 71P.

FORD-HUTCHINSON, A.W., BRAY, M.A., DOIG, M.V., SHIPLEY, M.E. & SMITH, M.J.H. (1980). Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature*, **286,** 264–265.

GOETZL, E.J. & SUN, F.F. (1979). Generation of unique mono-hydroxy eicosatetraenoic acids from arachidonic acid by human neutrophils. *J. exp. Med.*, **150,** 406–411.

HAMBERG, M. & SAMUELSSON, B. (1974). Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. natn. Acad. Sci., U.S.A.*, **71,** 3400–3404.

HIGGS, G.A., EAKINS, K.E., MUGRIDGE, K.G., MONCADA, S. & VANE, J.R. (1980). The effects of non-steroid anti-inflammatory drugs on leukocyte migration in carrageenin-induced inflammation. *Eur. J. Pharmac.*, **66,** 81–86.

HIGGS, G.A., FLOWER, R.J. & VANE, J.R. (1979). A new approach to anti-inflammatory drugs. *Biochem. Pharmac.*, **28,** 1959–1961.

HIGGS, G.A., MONCADA, S. & VANE, J.R. (1980). The mode of action of anti-inflammatory drugs which prevent the peroxidation of arachidonic acid. *Clin. Rheum. Dis.*, **6,** 675–693.

MURPHY, R.C., HAMMARSTROM, S. & SAMUELSSON, B. (1979). Leukotriene C: a slow-reacting substance from murine mastocytoma cells. *Proc. natn. Acad. Sci., U.S.A.*, **76,** 4275–4279.

NUGTEREN, D.A. (1975). Arachidonate lipoxygenase in blood platelets. *Biochim. biophys. Acta.*, **380,** 299–307.

PALMER, R.J., STEPNEY, R., HIGGS, G.A. & EAKINS, K.E. (1980). Chemokinetic activity of arachidonic acid lipoxygenase products on leukocytes from different species. *Prostaglandins*, **20,** 411–418.

PECK, M.J. & WILLIAMS, T.J. (1978). Prostacyclin (PGI_2) potentiates bradykinin-induced plasma exudation in rabbit skin. *Br. J. Pharmac.*, **62,** 464–465P.

PORTER, W.A., WOLF, R.A., YARBRO, E.M. & WEENAN, H. (1979). The autoxidation of arachidonic acid: formation of a proposed SRS-A intermediate. *Biochem. biophys. Res. Commun.*, **89,** 1058–1064.

SAMUELSSON, B. & HAMMARSTROM, S. (1980). Nomenclature for leukotrienes. *Prostaglandins*, **19,** 645–649.

SAMUELSSON, B., HAMMARSTROM, S., MURPHY, R.C. & BORGEAT, P. (1980). Leukotrienes and slow reacting substance of anaphylaxis (SRS-A). *Allergy*, **35,** 375–384.

SMITH, M.J.H., FORD-HUTCHINSON, A.W. & BRAY, M.A. (1980). Leukotriene B: a potential mediator of inflammation. *J. Pharm. Pharmac.*, **32,** 517–518.

TURNER, S.R., TAINER, J.A. & LYNN, W.S. (1975). Biogenesis of chemotactic molecules by the arachidonate lipoxygenase system of platelets. *Nature*, **257,** 680–681.

WALKER, J.R., SMITH, M.J.H. & FORD-HUTCHINSON, A.W. (1976). Anti-inflammatory drugs. Prostaglandins and leukocyte migration. *Agents & Actions*, **6,** 602–606.

WEDMORE, C.V. & WILLIAMS, T.J. (1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature*, **289,** 646–650.

WILLIAMS, T.J. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *Br. J. Pharmac.*, **65,** 517–524.

WILLIAMS, T.J. & PECK, M.J. (1977). Role of prostaglandin-mediated vasodilation in inflammation. *Nature*, **270,** 530–532.

WILLIAMS, T.J. & PIPER, P.J. (1980). The action of chemically pure SRS-A on the microcirculation *in vivo*. *Prostaglandins*, **19,** 779–789.

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