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EFFECTS OF INFLAMMATORY AGENTS ON ENDOTHELIAL LYSOSOMAL FRAGILITY AND THEIR INHIBITION BY ANTI-INFLAMMATORY DRUGS

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1 Endothelial cells from human unbilical veins were maintained in tissue culture. The fragility of lysosomal membranes were studied by microdensitometry.

2 Histamine (50 μM to 10 mM), 4-methylhistamine (100 nM to 10 mM) and dimaprit (100 nM to 10 mM) increased lysosomal fragility. 2-Thiazolyethylamine and 2-pyridylethylamine (100 nM to 10 mM) had no effect.

3 Prostaglandins E_1 and E_2 (3 nM to 30 μM) and prostaglandin $F_{2\alpha}$ (2 nM to 20 μM) had no direct effect. Low concentrations of prostaglandins E_1 and E_2 inhibited the fragility induced by histamine 100 μM.

4 Bradykinin (100 nM to 100 μM) decreased fragility.

5 The increase in fragility induced by histamine 100 μM or dimaprit 100 μM was inhibited by cimetidine (100 μM and 1 mM) but not by mepyramine (1 μM to 1 mM).

6 Pretreatment with indomethacin, hydrocortisone, ibuprofen and sodium salicylate caused a dose-dependent inhibition of histamine-induced fragility. Threshold concentrations were 1 pM, 100 pM, 10 nM and 10 μM, respectively.

7 Lignocaine (1 μM to 1 mM) had no direct effect and did not decrease histamine-induced fragility.

Introduction

The inflammatory response is characterized by a series of vascular reactions. Vascular endothelium may modulate this response by the synthesis of mediators (Rosen, Hollis & Sharma, 1974; Gimbrone & Alexander, 1976; Mason, Sharp, Chuang & Mohammed, 1977), or by the removal of active substances (Erdös, 1975). It may also be actively involved in permeability changes (Hammersen, 1976) and leucocyte adhesion (Danon & Skutelsky, 1976).

Endothelial cells from lung (Heath, Gandy & Jacobsen, 1976), bone marrow sinusoids (de Bruyn, Michelson & Becker, 1975), lymphatic vessels (van Deurs, 1978) and endothelioid cells (Reed & Wenzel, 1975) have been shown to contain acid phosphatase positive granules, believed to be lysosomes. Lysosomal enzymes are latent under normal conditions due to the impermeability of the lysosomal membrane. Abnormal loss of latency may have a central role in maintaining the inflammatory response (Hirschorn, 1973). The aim of this study was to investigate the effects of some proposed inflammatory mediators on lysosomal membrane permeability in cultured endothelial cells and to see whether this could be inhibited by pharmacological antagonists and anti-inflammatory drugs.

Methods

Cell culture

Endothelial cells were removed from human umbilical veins by digestion with collagenase 1 mg/ml for 15 min, then plated on plastic coverslips in 35 mm tissue culture dishes and maintained in culture for 3 days (Jaffe, Nachman, Becker & Minick, 1973). The growth medium used was T.C.199 supplemented by foetal calf serum 20%, penicillin 200 u/ml and streptomycin 200 μg/ml. It was buffered with 15 mM HEPES and the pH adjusted to 7.4 with 0.1 M NaOH.

Fragility test

Changes in the permeability of the lysosomal membrane were assessed by means of a fragility test described by Bitensky, Butcher & Chayen (1973). In this test, cells are incubated at pH 5, which increases

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lysosomal permeability so that the substrate, sodium β-glycerophosphate, penetrates the lysosomes where it is hydrolyzed by acid phosphatase. The phosphate produced is trapped by precipitation as lead phosphate and stained by conversion to lead sulphide. The length of the lag phase before penetration depends on the permeability or 'fragility' of the lysosomal membrane. Labilising substances will decrease the lag phase and accelerate the rate of staining.

The coverslips with confluent monolayers attached were rinsed in isotonic saline and immersed in the drug solution for 5 min at 37°C. They were rinsed again, placed in substrate medium for 120 min at 37°C, then in a saturated solution of hydrogen sulphide in isotonic sucrose for 1 min, then blotted dry and mounted in Farrant's medium. The substrate medium was prepared by dissolving 265 mg of lead sulphide in 200 ml of 10 mM acetate buffer, pH 5, and mixing it with 600 mg sodium β-glycerophosphate dissolved in 20 ml of distilled water. The mixture was incubated at 37°C overnight, then cooled and filtered immediately before use (Gomori, 1950; Chayen, Bitensky & Butcher, 1973). In this way, released phosphate was trapped as lead phosphate which in turn was converted to lead sulphide. The amount of lead sulphide was determined with a Vickers M85 microdensitometer which scanned cells at 550 nm (light beam <0.25 μm diameter) so that the chromophore was optically homogeneous; 50,000 readings of each cell were electronically integrated. In spite of the variability of cytochemical staining, a linear relationship has been demonstrated between the optical density of end product measured in this way and enzyme activity measured biochemically (Clark & Picton, 1975). For all experiments, except 4-methylhistamine, nine slides were assessed, in duplicate, reading 20 individual cells. In this study only optical densities were compared but these may be converted to absolute units (mg PbS/cell) by calibration. The amount of lead sulphide after drug treatment was expressed as a percentage relative to that in untreated cells. The variance ratios confirmed that a two tailed Student's *t* test, with significance at the 95% fiducial limit, could be used to indicate whether the difference between two sample means was significant.

Materials

Materials were obtained from the following sources; T.C.199 and foetal calf serum (Gibco-Biocult), collagenase (Worthington Biochem. Grade IV), benzylpenicillin (BDH), streptomycin sulphate (Sigma), HEPES (Fisons), sodium β-glycerophosphate (Sigma) and lead nitrate (Fisons). The drugs used were bradykinin triacetate (Sigma), cimetidine (S.K.F. Ltd.), histamine acid phosphate (BDH), hydrocortisone sodium succinate (Organon), indomethacin (Merck, Sharp and Dohme), lignocaine hydrochloride (Astra Chemicals), mepyramine maleate (May and Baker), and sodium salicylate (Fisons). The following gifts are gratefully acknowledged; 2 pyridylethylamine, 2-thiazolylethylamine, 4-pyridylethylamine, 4-methylhistamine and dimaprit (Dr C.R. Ganellin, S.K.F. Ltd.), prostaglandins E_1, E_2, and $F_{2\alpha}$ (Dr. J. Pike, Upjohn Co.) and ibuprofen sodium dihydrate (Dr P. Bressloff, Boots Co.).

Table 1 Effect of histamine and H_1-agonists

Conc.	*Histamine*	*Dimaprit*	*4-Methylhistamine* (n=3)
10 nM	–	97 ± 6	–
100 nM	103 ± 3	119 ± 7*	137 ± 5*
1 μM	115 ± 15	116 ± 5*	136 ± 18*
10 μM	113 ± 19	118 ± 5*	133 ± 17*
50 μM	133 ± 7*	–	–
100 μM	143 ± 10*	123 ± 4*	146 ± 8*
500 μM	163 ± 9*	–	–
1 mM	160 ± 15*	126 ± 6*	122 ± 9*
10 mM	157 ± 14*	128 ± 4*	128 ± 27*

Staining intensity given as percentage of intensity measured in untreated cells.
* Value statistically greater than untreated cells (P<0.05)

Results

Histamine 100 μM had a rapid effect, increasing the staining intensity to 128% ± 6 within 20 s. Continued incubation for periods up to 10 min did not cause further change. Histamine and its analogues were therefore applied for 5 min.

Histamine (50 μM to 500 μM) caused a dose-dependent increase in lysosomal fragility (Table 1). Concentrations less than 50 μM had no effect and

Table 2 Effect of bradykinin

Conc.	*Medium/5% FCS 10 min*	*0.9% Saline 10 min*	*0.9% Saline 60 min*
1 nM	91 ± 4	85 ± 4	91 ± 7
10 nM	93 ± 6	96 ± 4	96 ± 6
100 nM	101 ± 4	100 ± 8	81 ± 4*
1 μM	98 ± 7	90 ± 2	79 ± 4*
10 μM	83 ± 3	82 ± 2*	84 ± 5*
100 μM	84 ± 3	92 ± 5	80 ± 5*

Staining intensity given as percentage of intensity measured in untreated cells.
* Value significantly less than untreated cells (P<0.05).

Table 3 Effect of prostaglandins

Conc.	*Prostaglandin alone* PGE_1	PGE_2	$PGE_{2\alpha}$ [§]	*Prostaglandin + histamine 100 μM* PGE_1	PGE_2
Control	100 ± 5	100 ± 5	100 ± 5	130 ± 7*	124 ± 7*
3 nM	102 ± 8	106 ± 7	96 ± 9	123 ± 5*	114 ± 4
30 nM	97 ± 9	107 ± 5	98 ± 13	105 ± 8†	109 ± 7
300 nM	104 ± 9	103 ± 6	109 ± 6	120 ± 10	95 ± 6†
3 μM	105 ± 8	112 ± 9	106 ± 9	129 ± 9*	129 ± 7*
30 μM	105 ± 7	108 ± 4	107 ± 4	127 ± 9*	124 ± 10*

Staining intensity given as percentage of intensity measured in untreated cells.
[§] Prostaglandin $F_{2\alpha}$ was tested at concentrations of 2 nM to 20 μM
* Value significantly greater than untreated cells ($P < 0.05$)
† Value significantly less than histamine-treated cells ($P < 0.05$)

concentrations greater than 50 μM did not further increase fragility. The correlation coefficient of the rectilinear portion of the log dose curve was 0.999. The dose causing a half maximal response (ED_{50}) was 64 μM.

The selective H_1-agonists, 2-pyridylethylamine and 2-thiazolyethylamine (100 μM to 10 mM) had no effect, whereas the selective H_2-agonists, dimaprit and 4-methylhistamine were more potent than histamine although their maximal effect was smaller. 4-Methylhistamine produced its maximal effect at all the concentrations tested (100 nM to 10 mM). 4-Pyridylethylamine, which has negligible effects on H_1 and H_2-receptors (Durant, Ganellin, & Parsons, 1975) significantly decreased the fragility to 85 to 90% at concentrations of 1 μM to 10 mM.

When cells were treated for 10 min with bradykinin, dissolved in medium supplemented with 5% foetal calf serum, it had no significant effect. In the absence of serum, bradykinin 10 μM decreased lysosomal fragility (Table 2). After treatment for 1 h this stabilization was significant at concentrations of 10 nM to 100 μM.

Prostaglandins E_1 and E_2 (3nM to 30 μM) and prostaglandin $F_{2\alpha}$ (2 nM to 20 μM) had no effect on lysosomal fragility (Table 3). When given in combination with 100 μM histamine, low doses of prostaglandin E_1 (3 nM to 300 nM) reduced the staining intensity, although only at 30 nM was this significant. Higher concentrations (3 μM to 30 μM) had no such stabilizing effect. Prostaglandin E_2 influenced the effect of histamine in a similar manner, with significant stabilization at 300 nM.

Mepyramine, a selective H_1-blocker, increased lysosomal fragility at 1 μM and 1 mM but not at 100 nM, 10 μM and 100 μM (Table 4). It did not reduce the effect of 100 μM histamine or 100 μM dimaprit. Cimetidine, a selective H_2-blocker (Durant, Ganellin & Parsons, 1977), caused a nonsignificant and non dose-dependent increase in lysosomal fragility (100 nM to 1 mM). The increased fragility induced by 100 μM histamine or 100 μM dimaprit was inhibited by 100 μM and 1 mM cimetidine.

The anti-inflammatory drugs tested (Table 5) had no direct effect, except for indomethacin 1 μM, ibuprofen 10 nM and 10 μM and sodium salicylate 1 μM which caused increased fragility. When applied for 5 min before exposure to 100 μM histamine all anti-inflammatory drugs caused a dose-dependent inhibition of the histamine-induced increase in fragility. Staining

Table 4 Effect of histamine antagonists

Conc.	*Antagonist alone* Mepyramine	Cimetidine	*Antagonist + histamine 100 μM* Mepyramine	Cimetidine	*Antagonist + dimaprit 100 μM* Mepyramine	Cimetidine
Control	100 ± 4	100 ± 5	120 ± 6*	123 ± 7*	119 ± 4*	121 ± 5*
100 nM	93 ± 4	112 ± 8	–	–	–	–
1 μM	120 ± 7*	123 ± 8	114 ± 7*	126 ± 6*	130 ± 3*	118 ± 6*
10 μM	89 ± 7	125 ± 11	119 ± 5*	115 ± 5*	122 ± 6*	118 ± 6*
100 μM	101 ± 8	126 ± 11	123 ± 6*	104 ± 6†	125 ± 2*	99 ± 8†
1 mM	133 ± 4*	127 ± 6*	130 ± 3*	102 ± 6†	120 ± 4*	107 ± 6†

Staining intensity given as percentage of intensity measured in untreated cells.
* Value significantly greater than untreated cells ($P < 0.05$)
† Value significantly less than histamine-treated cells ($P < 0.05$)

Table 5 Effect of anti-inflammatory drugs and histamine 100 μM

Conc.	*Indomethacin*	*Hydrocortisone*	*Ibuprofen*	*Salicylate*
Control	131 ± 6*	143 ± 7*	132 ± 10*	122 ± 5*
0.01 pM	128 ± 2*	–	–	–
0.1 pM	119 ± 7	–	–	–
1 pM	102 ± 5†	–	–	–
10 pM	102 ± 9†	–	127 ± 12	–
100 pM	103 ± 8†	113 ± 3*†	136 ± 9*	–
1 nM	97 ± 7†	89 ± 3†	127 ± 6	–
10 nM	99 ± 4†	97 ± 6†	109 ± 7†	–
100 nM	92 ± 5†	93 ± 4†	100 ± 8†	–
1 μM	98 ± 4†	99 ± 10†	83 ± 8†	120 ± 7*
10 μM	96 ± 7†	99 ± 6†	94 ± 13†	110 ± 7
100 μM	103 ± 5†	103 ± 10†	76 ± 4†	104 ± 9
1 mM	–	94 ± 2†	85 ± 8†	108 ± 5
10 mM	–	–	–	103 ± 2†

Staining intensity given as percentage of intensity measured in untreated cells.
* Value statistically greater than untreated cells ($P < 0.05$)
† Value statistically less than histamine-treated cells ($P < 0.05$)

intensity was significantly reduced below that of cells treated only with histamine by indomethacin 1 pM to 100 mM, hydrocortisone 100 pM to 1 mM, ibuprofen 10 nM to 1 mM and sodium salicylate 10 mM. Indomethacin 0.1 pM, ibuprofen 1 nM and sodium salicylate 10 μM to 1 mM produced staining intensities significantly greater than control values, but not significantly less than that of histamine-treated cells. Lignocaine (1 μM to 1 mM) had no direct effect on lysosomal fragility, and no effect on the histamine-induced increase in fragility.

Discussion

When a lipophilic phosphatase substrate which penetrates the lysosomal membrane freely, is used, staining appears without a lag phase and is not influenced by histamine (Yoffe, 1980). This suggests that histamine does not increase the specific activity of acid phosphatase in this system, but increases the fragility of the lysosomal membrane.

Lysosomal fragility was increased in endothelial cells by a histamine concentration (100 μM) similar to that required in cultured skin cells (Chayen, Bitensky & Ubhi, 1972) and cultured fibroblast-like cells (Tyas, 1978), but lower than that required to release enzymes from isolated lysosomes (Ignarro, personal communication). This suggests that the observed labilising effect requires an intact cell system. Changes in intracellular levels of calcium and cyclic nucleotides may be involved, as histamine's effects on endothelial cells are enhanced by calcium (Northover, 1975), and histamine stimulates adenyl cyclase (Allouche, Santais, Foussard, Ruff & Parrot, 1977) and activates cyclic AMP-dependent protein kinase (Mangeat, Marchis-Mouren, Cheret & Lewin, 1980) via the H_2-receptor. The increase in lysosomal fragility was clearly mediated through the H_2-receptor. The H_2-agonists, 4-methylhistamine and dimaprit were more effective than histamine, whilst the H_1-agonists, 2-pyridylethylamine and 2-thiazolylethylamine, had no significant effect. 4-Pyridylethylamine decreased lysosomal fragility but the mechanism and relevance of this is not clear.

Cimetidine antagonized histamine in equimolar concentration and itself slightly increased staining, which suggests that it acts as a competitive inhibitor and not as a general membrane stabilizer (Rooney, Gore & Lee, 1979).

Prostaglandins E_1, E_2, and $F_{2\alpha}$ had no direct effect and did not potentiate the histamine-induced fragility. This agrees with the view that the *in vivo* inflammatory effects of these prostaglandins are due to vasodilatation (Williams, 1977) The stabilization of endothelial lysosomes by prostaglandins E_1 and E_2 (Table 3) and of isolated liver lysosomes (Ignarro, 1975) may be related to their ability to interact with lecithin and alter membrane packing (Colaccico, Basu & Ray, 1977).

Bradykinin did not increase lysosomal fragility, even in the absence of serum kininases. It is possible that the endothelium may inactivate bradykinin (Erdös, 1975). Prolonged treatment with bradykinin decreased lysosomal fragility, possibly by stimulating endothelial prostaglandin production (Dumonde, Jose, Page & Williams, 1977).

As lysosomes contain many damaging substances, increased fragility may *in vivo* maintain inflammation. The role of H_2-receptors in vascular inflammatory changes is controversial (Brimblecombe, Farrington, Lavender & Owen, 1976; Little,

Savic & Stoner, 1978; Robertson & Greaves, 1978). Marks & Greaves (1977) have shown that treatment of vascular responses with a combination of H_1 and H_2-antagonists is more effective than with either alone. This investigation offers a possible explanation of the role of H_2-stimulation.

All the anti-inflammatory drugs tested protected the endothelium against histamine. The order of potency (Table 5) was in good agreement with their anti-inflammatory potency *in vitro* and *in vivo*. The protection may be due to stabilization of the lysosomal membrane and inhibition of enzyme release (Weissman, 1969; Mizushima, Ishii & Masumoto, 1975). However, no direct stabilizing effect was seen and membrane stabilizing drugs such as lignocaine and mepyramine did not antagonize histamine. There is now considerable evidence that inhibition of prostaglandin synthesis is the major mechanism by which anti-inflammatory drugs act (Vane, 1978; Appleton & Brown, 1979). However, the lack of prostaglandin-induced labilisation and the protective effects of sodium salicylate at concentrations which do not inhibit platelet prostaglandin synthesis (Vargaftig, 1978), support the view that other mechanisms may also be important (Famaey, Brooks & Dick, 1975; Smith, 1978). Non-steroidal anti-inflammatory drugs can act as calcium antagonists (Northover, 1977) and can interfere in the production (Ortman & Perkins, 1977) and effects of cyclic AMP (Dinnendahl, Peters and Schönhofer, 1973; Kantor & Hamptom, 1978; Catalan, Aragones, Martinez, Armijo & Pina, 1980). The observed prevention of the increase in lysosomal fragility induced by histamine suggests that this technique may be useful in screening steroidal and non-steroidal anti-inflammatory drugs.

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