Br. J. clin. Pharmac. (1987), **24**, 747–752

Effects of silybin on histamine release from human basophil leucocytes

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1 Effects of a naturally occurring flavonoid, silybin, on histamine release from human basophils were examined, in order to assess the potential utility in the treatment of allergic disorders.

2 The f-met peptide and anti-IgE-induced histamine release was significantly ($P < 0.05$) inhibited in a concentration-dependent fashion. Conversely, no significant ($P > 0.05$) effect on calcium ionophore A23187-induced histamine secretion was documented. The inhibitory activity was significantly ($P < 0.05$) reversed by elevating extracellular calcium concentrations.

3 The anti-allergic properties of silybin can be reasonably ascribed to a membrane-stabilizing activity, possibly related to an interference in calcium influx. These results indicate that an *in vivo* evaluation of the anti-allergic activity of silybin would be worthwhile.

Keywords silybin flavonoids histamine release basophil leucocytes

Introduction

Naturally occurring flavonoids have been shown to possess many biological activities; among these the inhibition of several enzymes (Middleton & Drzewiecki, 1982), such as transport ATPases, cyclic nucleotide phosphodiesterases, phospholipase A_2 (Lee *et al.*, 1982), phospholipase C (Cockcroft, 1982) and protein kinase C (Gschwendt *et al.*, 1983). Moreover certain flavonoids have anti-allergic properties, since they can inhibit histamine release from basophils and mast-cells and arachidonate 5-lipoxygenase activity (Yoshimoto *et al.*, 1983). Fewtrell & Gomperts (1977a) observed that some flavonoids could inhibit histamine release from rat mast-cells stimulated with calcium ionophore A23187, antigen and concanavalin A. Subsequently Middleton and coworkers (Middleton *et al.*, 1981; Middleton & Drzewiecki, 1985) investigated the effects of different flavonoids on histamine release from human basophils. Quercetin, apigenin and fisetin were shown to exert a potent inhibition on antigen-induced histamine release. This inhibitory effect was concentration-dependent, slightly influenced by extracellular calcium concentration and not related to intracellular cyclic AMP content.

In the present study we have investigated the effect of silybin, one of the main constituents of the naturally occurring flavonoid silymarin, on the *in vitro* histamine release from human basophils, in view of its potential use in the treatment of allergic disorders.

Methods

Subjects

Fifteen normal subjects (eight males and seven females), with ages ranging from 23 to 34 years (mean ± s.e. mean 28 ± 2.1) were included in our study.

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Preparation of leucocyte suspensions

Venous blood (60 ml) was collected from each subject and was anticoagulated with 0.1 M EDTA. Leucocytes were separated by dextran sedimentation, according to the method described by Lichtenstein & Osler (1964). The supernatant was aspirated and centrifuged and the cell pellet was washed twice in Tyrode's buffer, pH 7.4, containing 135 mM NaCl, 5.5 mM dextrose, 2.7 mM KCl, 0.36 mM NaH_2PO_4 and 12 mM $NaHCO_3$. Finally, the cells were suspended in Tyrode's buffer with 1.8 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Basophils were counted in duplicate in a Fuchs Rosenthal chamber, using the Alcian blue staining (Gilbert & Ornstein, 1975); a final concentration of 4×10^4 basophils/ml was obtained adjusting the volume of Tyrode's buffer.

Histamine release from washed leucocytes

Basophil leucocytes were stimulated with calcium ionophore A23187 (Sigma Chemical, St. Louis, MO), f-met peptide (Sigma Chemical) and anti-IgE (Behringwerke AG, Marburg, FRG).

2×10^4 basophils, suspended in 0.5 ml Tyrode's buffer with $CaCl_2$ and $MgCl_2$ were added to 12 × 75 mm polyethylene tubes, containing 0.5 ml of the different stimuli. Calcium ionophore A23187 was used at final concentrations of 0.5, 1, 2, 10 and 20 μM, f-met peptide at concentrations of 1 and 10 μM and anti-IgE at concentrations of 8,000 and 80,000 i.u. ml^{-1}.

Histamine concentration in the cell supernatants was evaluated by an automated fluorometric method, modified from Siraganian (1974). The percentage of histamine release was calculated as follows:

$$\% \text{ release} = \frac{e - b}{t - b} \times 100$$

where e = fluorometric reading for experimental supernatant, b = mean of blanks, t = total histamine content.

Spontaneous histamine release (mean of blanks ± s.e. mean) was 1.2 ± 0.3 ng ml^{-1}, and did not exceed 3% of total histamine content.

Effect of in vitro *preincubation with silybin on histamine release from basophil leucocytes*

Dextran-sedimented leucocytes were incubated for 1, 5 and 30 min at 37° C with different concentrations (1, 10 and 100 μM) of silybin (kindly provided by Istituto Biochimico Italiano, Milan, Italy). Histamine release was subsequently induced with the different stimuli, according to the previously described method.

In five cases basophil leucocytes were incubated for 30 min with silybin, washed twice in Tyrode's buffer without Ca^{++} and Mg^{++}, resuspended in Tyrode's buffer with Ca^{++} and Mg^{++} and finally exposed to the degranulating stimuli. These experiments were performed in order to evaluate if the presence of the drug in the extracellular medium was essential for its effect on histamine release.

The activity of silybin on the basophil cells from five subjects was also evaluated in the presence of different extracellular calcium concentrations (1.8, 3.6 and 5.4 mM).

The results (mean ± s.e. mean) were expressed in terms of percentage inhibition of the control release in the presence of silybin, using the following formula:
(HR in absence of silybin − HR in presence of silybin)/(HR in absence of silybin) × 100.
where HR = histamine release.

Statistical analysis

Student's *t*-test for paired data was used to compare the histamine release from basophil leucocytes in the absence or in the presence of silybin.

Results

Inhibitory effect of silybin on histamine release

The dose-response curves of the histamine release induced by anti-IgE, f-met peptide and calcium ionophore A23187 are reported in Figure 1.

The *in vitro* preincubation of basophils with silybin at concentrations of 1, 10 and 100 μM exerted a significant concentration-dependent inhibition on the histamine release induced by anti-IgE (Figure 2) and f-met peptide (Figure 3). Conversely, no significant protection ($P > 0.05$) was afforded by the drug on the calcium ionophore-induced histamine secretion, even if a slight inhibition was observed when the cells were preincubated with 100 μM silybin and stimulated with the lowest concentration (0.5 μM) of calcium ionophore (Figure 4). The drug effect was more marked at low secretagogue concentrations. In addition, the incubation time did not affect the inhibitory activity of silybin, and the percentage of inhibition did not change appreciably when the cells were incubated with silybin for 1, 5 and 30 min. Large variations were observed from subject to subject. The data concerning IC_{50} s, with 95% confidence limits, are summarized in Table 1. We failed to calculate

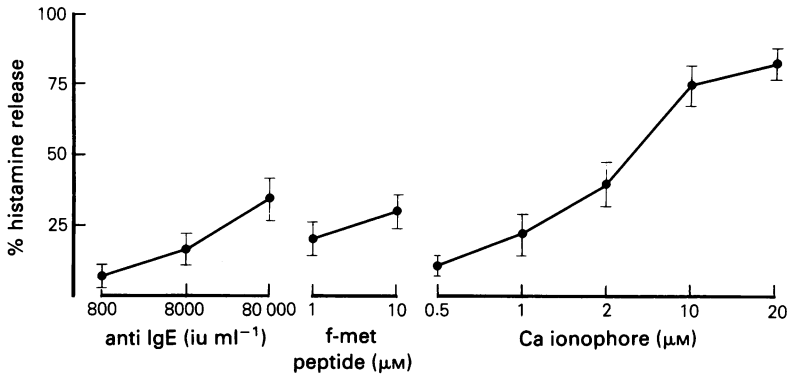

Figure 1 Histamine release as a function of anti-IgE, f-met peptide and calcium ionophore A23187 concentrations in 15 normal donors.

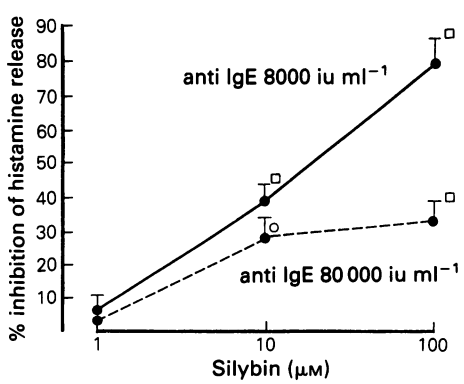

Figure 2 Concentration-effect (inhibition) curves of silybin on anti-IgE (8000 iu ml^{-1} ●——● and 80,000 iu ml^{-1} ●— — —●)-induced histamine release from basophil leucocytes of 15 normal donors. Significantly lower than control release in the absence of silybin (□ $P < 0.02$; ○ $P < 0.05$).

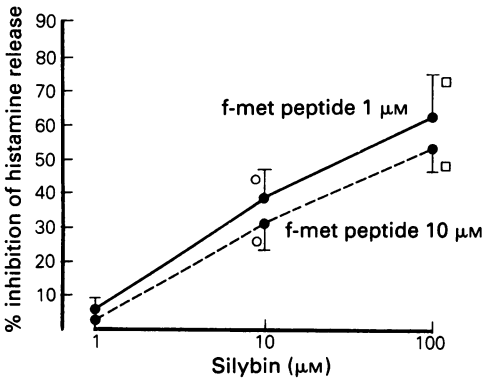

Figure 3 Concentration-effect (inhibition) curves of silybin on f-met peptide (1 μM ●——● and 10 μM ●— — —●)-induced histamine release from basophil leucocytes of 15 normal subjects. Significantly lower than control release in the absence of silybin (□ $P < 0.01$; ○ $P < 0.05$).

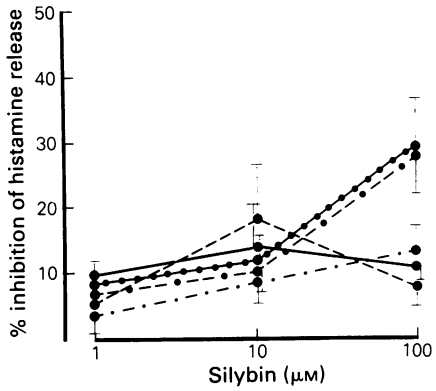

Figure 4 Concentration-effect (inhibition) curves of silybin on calcium ionophore A23187 (0.5 μM ●—●—●—●—●—●, 1 μM ●--●--●, 2 μM ●———●, 10 μM ●———● and 20 μM ●—•—●)-induced histamine release from basophil leucocytes of 15 normal subjects.

the IC_{50} s for calcium ionophore A23187 and for the higher concentration (80,000 i.u. ml^{-1}) of anti-IgE. Therefore in four experiments the cells were incubated with higher doses of silybin (200 and 500 μM) and then stimulated with 80,000 iu ml^{-1} anti-IgE and 0.5, 1, 2, 10 and 20 μM calcium ionophore A23187. In these conditions the mean IC_{50} for anti-IgE was 200 μM; conversely, the effect on the calcium ionophore A23187-induced histamine release did not change appreciably by elevating silybin concentrations.

In five cases dextran-sedimented leucocytes were preincubated with silybin (100 μM) for 30 min and thereafter they were washed twice in Tyrode's buffer without Ca^{++} and Mg^{++}. This procedure did not influence significantly ($P > 0.05$) the inhibitory effect of silybin.

Effect of extracellular calcium concentrations on the inhibitory activity of silybin

In the absence of silybin the different buffer calcium concentrations (1.8, 3.6 and 5.4 mM) did not significantly ($P > 0.05$) modify anti-IgE and f-met peptide-induced histamine release. The drug activity on the anti-IgE (8,000 iu ml^{-1}) and f-met peptide-induced histamine release was significantly ($P < 0.05$) reversed by increasing extracellular calcium concentrations from 1.8 to 5.4 mM (Figure 5). The inhibition of the histamine secretion induced by the highest dose of anti-IgE (80,000 iu ml^{-1}) was partially, but not significantly ($P > 0.05$) reversed by increasing buffer calcium concentrations.

Discussion

Our results suggest that silybin can reduce histamine release from human blood basophils. This inhibitory effect is highly variable depending on the stimulus and the subject. No significant influence was exerted on the calcium ionophore A23187-induced histamine release; conversely, the f-met peptide and anti-IgE-induced histamine secretion was significantly inhibited. The drug effect was concentration-dependent and more marked at lower secretagogue concentrations; moreover, the degree of inhibition was not affected by the time of incubation. Also Pearce and coworkers (1984) observed that the inhibitory effect of the flavonoid quercetin did not change significantly whether the cells were preincubated with the drug for up to 10 min or not.

Large variations were observed from subject to subject, concerning the inhibitory effect of silybin; individual differences in basophil releasability may partially account for the variable drug effect on the cells from different donors. Furthermore large variations in basophil response, depending upon the stimulus and the subject were observed by Findlay & Lichtenstein (1980). Anti-IgE, f-met peptide and calcium ionophore A23187 induce histamine release through different mechanisms. Anti-IgE interacts with membrane bound IgE (Ishizaka *et al.*, 1969), while f-met peptide interacts with a separate surface receptor, which is different

Table 1 Inhibitory effect of silybin on the histamine release from human basophil leucocytes, elicited by anti-IgE and f-met peptide: the calcium ionophore A23187-induced histamine secretion was not significantly ($P > 0.05$) reduced by silybin

Stimulus	*Concentration*	*IC_{50} (μM)*	*95% confidence limits*
Anti-IgE	8,000 i.u. ml^{-1}	47	25.4 – 68.6
	80,000 i.u. ml^{-1}	61	39.0 – 83.2
F-met peptide	1 μM	32	16.1 – 48.0
	10 μM	42	23.2 – 62.4

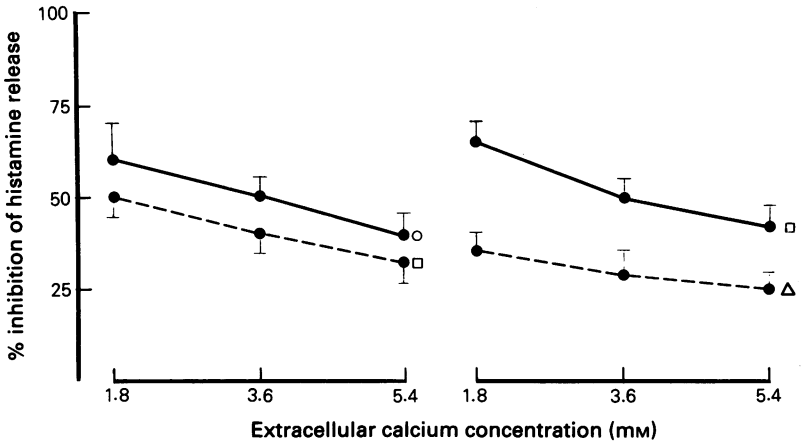

Figure 5 Influence of different extracellular calcium concentrations on the inhibitory effect of 100 μM silybin on f-met peptide (1 μM ●———● and 10 μM ●— — —●) and anti-IgE (8,000 iu ml^{-1} ●———● and 80,000 iu ml^{-1} ●— — ●)-induced histamine release in five different donors. Significantly lower than the inhibitory activity in the presence of 1.8 mM $CaCl_2$ (□ $P < 0.01$; ○ $P < 0.05$). Not significantly different from the inhibitory-activity in the presence of 1.8 mM $CaCl_2$ (△ $P > 0.05$).

from IgE and C5a receptors (Siraganian & Hook, 1977). The histamine releasing activity of calcium ionophore A23187 is related to its property of calcium carrier (Lichtenstein, 1975). In order to elucidate if the effect of silybin was due to an interference with calcium dependent steps, leading to histamine release, the cells were suspended in Tyrode's buffer, containing different calcium concentrations (from 1.8 to 5.4 mM). These experiments showed that the inhibitory activity of silybin on anti-IgE and f-met peptide-induced histamine release was reversed by increasing buffer calcium concentrations. Thus it is reasonable to suppose that silybin exerts a membrane-stabilizing activity, possibly influencing calcium influx. Also Middleton *et al.* (1981) suggested a similar mechanism of action for quercetin, another naturally occurring flavonoid with a potent inhibitory activity on histamine release. The lack of activity on calcium ionophore A23187-induced histamine secretion may be explained with a selective action of silybin on receptor-operated calcium channels. In fact cell activation induced by calcium ionophore A23187 does not involve the opening of receptor operated calcium channels and is due to a calcium carrier activity (Lichtenstein, 1975). However, the mechanism of action of flavonoids is controversial and deserves further studies. Some authors (Fewtrell & Gomperts, 1977a,b; Middleton *et al.*, 1981) supposed that these compounds can interfere with the receptor-mediated activation of calcium channels, while others hypothesized a more complex activity, since quercetin can reduce histamine release from rat peritoneal mast cells in the absence of extracellular calcium and inhibits the histamine secretion caused by ionophores (Ennis *et al.*, 1981) and surface-active agents (Pearce & Clements, 1982). Cell washing after incubation with silybin did not affect appreciably the inhibitory activity on histamine release. This finding is in contrast with the observation by Middleton & Drzewiecki (1985): these authors found that cell washing after preincubation with quercetin was followed by the loss of inhibitory activity. Thus it is conceivable that silybin and quercetin present some different characteristics. On the basis of the currently available data, it is possible to suggest an *in vivo* evaluation of anti-allergic activity of flavonoids.

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(Received 20 May 1987, accepted 24 July 1987)