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EFEK GANDA FLAVONOID QUERCETIN PADA KULTUR SEL *RAT BASOPHILIC LEUKEMIA (RBL-2H3)*: MENGHAMBAT PELEPASAN HISTAMIN DAN MENGURANGI PERTUMBUHAN SEL

DUAL EFFECTS OF FLAVONOID QUERCETIN ON RAT BASOPHILIC LEUKEMIA (RBL-2H3) CELLS : INHIBITS HISTAMINE RELEASE AND REDUCES CELL GROWTH

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ABSTRACT

The dual effects of the flavonoid quercetin on rat basophilic leukemia cells (RBL-2H3), tumor analog mast cells, was studied. Quercetin is known as an anti-inflammatory drug that inhibits mast cell secretion of histamine. This study aims to investigate the consequences of varying incubation times with quercetin on 2H3 cells to histamine release inhibitory action and the effects of long time incubation to the morphology of the cells.

The effect of quercetin on histamine synthesis was also observed. The histamine release from the cells was inhibited by quercetin as expected, but the ability of secretion was rapidly recovered when quercetin was removed before challenging. Incubation up to 6 hours decreased the inhibitory action, but longer than 6 hours increased the inhibitory activity.

In long time incubation, the cells exhibited cell damage, decreased cell growth, morphological changes, and detachment from the underlying surface in proportion with the concentration of quercetin. The instant and reversible inhibitory effects of quercetin appear to represent a first phase of actions, while reduced cell growth, elevated cell damage and morphological changes seem to be connected to a second phase.

Consequently, quercetin could be considered as a compound that acts dually on RBL-2H3 cells.

Key words : quercetin, histamine, RBL-2H3 cells

ABSTRAK

Telah diteliti efek ganda flavonoid quercetin terhadap kultur sel Rat Basophilic Leukemia (RBL-2H3), suatu sel yang menyerupai sel mast. Quercetin merupakan senyawa anti inflamasi yang dapat menghambat pelepasan histamin dari sel mast. Penelitian ini bertujuan mempelajari efek variasi waktu inkubasi sel dengan quercetin terhadap aktivitas pelepasan histamin dan mengetahui efek inkubasi terhadap morfologi sel.

Dari penelitian diperoleh hasil bahwa pelepasan histamin dihambat oleh quercetin, tetapi kemampuan pelepasan histamin segera diperoleh kembali secara cepat jika quercetin dihilangkan dari medium. Waktu inkubasi yang meningkat sampai dengan 6 jam menurunkan aktivitas penghambatan terhadap pelepasan histamin, tetapi setelah 6 jam aktivitas penghambatan kembali meningkat. Di samping itu, sel menunjukkan kerusakan, perubahan morfologi, penghambatan pertumbuhan sel, dan sel terlepas dari dasar wadah.

Efek quercetin yang cepat dan reversibel terhadap penghambatan pelepasan histamin nampaknya menunjukkan efek pada fase pertama aksi, sedangkan efek pada perubahan morfologi dan kerusakan sel berkaitan dengan fase kedua aksi.

Dengan demikian, quercetin dapat dipandang sebagai senyawa yang beraksi ganda terhadap sel RBL-2H3.

Kata kunci : quercetin, histamin, kultur sel RBL-2H3

INTRODUCTION

Flavonoids are benzo-γ-pyrone derivatives with low molecular weight that are found in plants and in various food products. The flavonoids display numerous pharmacological activities that affect the immune system, platelet aggregation and the relaxation of cardiovascular smooth muscle cells (Formica and Regelson, 1995). Quercetin is a biflavonoid that inhibits histamine release from basophils and mast cells (Fewtrell and Gomperts, 1977). In brain cells from rat, quercetin inhibits protein kinase C (PKC) (Ferriola *et al*, 1989) and in chloroplasts, Ca^{2+}-ATPase is inhibited (Shoshan, *et al.*, 1980). Quercetin is also known to be carcinostatic and to inhibit cell growth (Middleton, *et al*., 2000), which makes quercetin interesting as a possible cytotoxic agent in addition to the anti-allergic properties of the drug.

In this study, we used RBL-2H3 cell line (rat basophilic leukemia cells), which have similar properties to the mucosal mast cells (Maeyama *et al*., 1992). The 2H3 cells contain several hundred thousand IgE receptors on the membrane surface, and after sensitization with mouse monoclonal IgE, the cells respond to antigen and release histamine. Although the mucosal mast cells response to secretagogues and their sensitivity to inhibitors are different from those of cutaneous-type mast cells, the mechanism of histamine secretion is supposed to be general (Maeyama, *et al.,* 1992). Beside its similarity to mast cells, RBL-2H3 cell is a kind of cancer cell that is derived from rat basophil. That makes the cells are also possible to be used to study the cytotoxic effect of a compound.

Mast cell is one of inflammatory cells playing an important role in allergic disease, anaphylaxis and inflammation. When activated by antigen-IgE stimulation or other secretagogues, mast cells secrete inflammatory mediators, such as histamine, proteases, proteoglycans, cytokines, arachidonic acid and chemotactic factors. Histamine is the main preformed mediator released after mast cell activation (Dale and Foreman, 1994), formed by decarboxylation of histidine which is catalized by the intracellular enzyme histidine decarboxylase, and is stored in granules within the cell (Watanabe, *et al.,* 1991). For exocytosis, activation of protein kinase C, PKC, and/or elevation of $[Ca^{2+}]_i$ is necessary.

There is a wide phenotypic heterogeneity among mast cells, both in humans and animals. In humans, it is complicated to define the different types of mast cells exactly, but there are several types observed. In rats, at least two types of mast cells can be distinguished depending on their site of differentiation: connective tissue mast cells (CTMC) and mucosal mast cells (MMC). CTMC have predominantly the proteoglycan heparin in their granules, can be stained with safranin and contain the neutral protease rat mast cell protease I, RMCP I (Foreman, 1994). Mucosal mast cells, MMC, are smaller, have less histamine content, contain chondroitin instead of heparin and can not be stained with safranin. MMC contain the neutral protease RMCP II, which is similar to the actions of RMCP I (Foreman, 1994). For obscure reasons, polybasic peptides, such as compound 48/80 and substance P, exclusively release histamine from some types of mast cells, including rat peritoneal mast cells (CTMC-like cells), but not 2H3 cells.

The aim of the present study is to investigate the effects of quercetin on histamine release and synthesis, and to study whether the inhibitory effect is reversible or irreversible after various incubation times with the drug. Histamine release was assayed using HPLC-fluorometry, and the HDC-activity was examined to assay histamine synthesis. The side effects of quercetin, such as the reduction of cell growth, decreased cell attachment and morphological changes of the cells were investigated by trypan blue exclusion.

METHODOLOGY

Chemicals and drugs

Fetal calf serum (FCS) was purchased from JRH Bioscience (USA). Dinitrophenylated bovine serum albumine (DNP-BSA_{24}-BSA, which consists of 24 moles dinitrophenol bound per 1 mol BSA) was given by Dr. H. Metzger, NIH (Bethesda, MD, USA), and monoclonal IgE against DNP-BSA_{24}-BSA was purified from the supernatant in IgE-producing hybridoma which was obtained in our laboratory. MEM was purchased from Gibco BRL (USA). Phorbol myristyl acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). Histamine diphosphate, thapsigargin and quercetin dihydrate were obtained from Wako (Osaka, Japan). PIPES was purchased from Dojindo Lab (Kumamoto, Japan). Other chemicals were of the highest grade commercially available.

Methods

Culture of RBL-2H3 cells and treatment with various agents

RBL-2H3 cells were maintained in a flask with Eagle's minimum essential medium (MEM) supplemented with 15% fetal calf serum and antibiotics in a humidified atmosphere of 5% CO_2 in air according to Barsumian *et al*. (1981). For histamine assay, 2H3 cells were incubated overnight in 24-well culture plates (0.2×10^6 cells/well) and sensitized with monoclonal IgE against DNP-BSA (0.5 μg/ml).

Assay of histamine release

Quercetin (working concentration 20 μM, unless otherwise stated) was added to specified wells, and the cells were pre-incubated for 10 min., 30 min., 2, 4, 6, 12, 24 or 48 hours. The quercetin was prepared as a stock solution of 60 mM in DMSO. After incubation, the cells were washed with 0.5 ml PIPES buffer (25 mM PIPES, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 40 mM NaOH, 0.1 % BSA, pH 7.2). The subsequent incubation for 30 minutes was done in PIPES buffer alone or with the following challengers: thapsigargin (0.5 μM) or DNP-BSA (20 ng/ml). Selected wells also contained quercetin (20 μM). Thapsigargin and DNP-BSA were prepared as stock solutions in PIPES buffer, 5 μM and 200 ng/ml, respectively.

To assay the floating cells, supernatants from cell suspensions that had been incubated for 48 hours with quercetin (20 μM), were carefully collected and centrifuged at 300 x g for 5 min. The pellet was washed and suspended in MEM, and put in a small flask in 37°C. The suspension was later transferred to a tube, centrifuged at 300 x g for 5 min and re-suspended in PIPES buffer. 200 μl each were transferred to Eppendorf tubes, which were incubated for 30 min with PIPES buffer containing thapsigargin (0.5 μM), DNP-BSA (20 ng/ml), or PIPES buffer only.

Assay of histamine content

Histamine released in the supernatants was assayed by HPLC-fluorometry according to Yamatodani, *et al*. (1985). Briefly, one hundred microliters of cell medium from each well and from the 24 tubes with the floating cells were centrifuged at 300 x g for 5 min. To 50 μl of supernatant, 250 μl perchloric acid (3%) and 30 μl KOH/KH_2PO_4 (2 M and 1 M, respectively) was added. The mixture was centrifuged at 10 000 x g for 20 min, and each of the supernatants was directly injected onto a column packed with TSKgel SP-2SW Cation Exchanger (Tosoh, Tokyo, Japan). At a flow rate of 0.6 ml/min, histamine was eluted with potassium phosphate (0.25 M). The histamine was post-labeled with o-phthalaldehyde in alkaline conditions, and detected fluorometrically (F1080 Fluorometer, Hitachi, Tokyo, Japan), using excitation and emission wavelengths of 360 and 450 nm, respectively.

To obtain the total content of histamine in the cells, cell suspensions were diluted 5 times and sonicated. Fifty microliters of each suspension was then mixed with perchloric acid and KOH/KH_2PO_4 and proceeded with as described above.

To calculate the percentage of net histamine release, the spontaneous histamine release and percentage of inhibition of histamine release, the following equations were used (HA=histamine):

$$\text{net HA release (\%)} = \frac{\text{challenged HA-release (pmoles) - spontaneous HA-release (pmoles)}}{\text{total HA-release (pmoles)-spontaneous release (pmoles)}} \times 100$$

$$\text{spontaneous HA release (\%)} = \frac{\text{HA-release without drugs (pmoles)}}{\text{total HA-content (pmoles)}} \times 100$$

$$\text{uinhibition (\%)} = \frac{\text{HA-release without drugs (pmoles) - HA-release with drugs}}{\text{HA-release without drugs}} \times 100$$

(Watanabe *et al.*, 1991).

Extraction of HDC and assay of its activity

Cells were pre-incubated with quercetin (30 uM) on a 24-well plate at 37°C, and then incubated with MEM containing, phorbol myristate acetate, (10 nM) for 4 hours. Then, the media were discarded, and the attached cells were suspended in 0.75 ml ice cold HDC buffer (0.1 M potassium phosphate buffer pH 6.8, 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 1 % (w/v) polyethylene glycol (average molecular weight: 300 g/mole) and 100 μg/ml phenyl-methylsulfonyl flouride) and sonicated for 20 sec. The homogenate was centrifuged at 10 000 x g for 20 min, and the supernatant was dialyzed three times against 50 volumes of HDC buffer. The enzyme was incubated for 2 hours at 37°C in 1.0 ml HDC-buffer containing 0.25 mM L-histidine, and the reaction was stopped by adding 0.04 ml perchloric acid (6.2 M). It was then added with 20 μl PCA (60%) and 42μl KOH/KH_2PO_4 (2 M and 1 M, respectively) and centrifuged at 10 000 x g for 20 min, the histamine in the supernatant was assayed using HPLC as described above. The protein amount was analized using a Bio-Rad protein assay kit. HDC activity is expressed as formation of histamine (pmoles) per min per mg protein.

Trypan blue staining

Cells were incubated at 37°C for 12, 24 or 48 hours in 400 μl RPMI media (without phenol red), with or without quercetin (20 uM). To study the floating cells, media from selected wells were transferred to new wells after incubation, and 400 μl RPMI media without quercetin was added to the old wells. Cells were stained with 10 μl trypan blue (0.4 %), and pictures were taken in an inverse light microscope for observation. Also, assay of histamine content of the cells was performed using HPLC as previously described.

RESULT AND DISCUSION

Effect of quercetin on histamine release

Quercetin showed inhibitory effect on histamine release induced by DNP-BSA from IgE-sensitized 2H3 cells in a concentration dependent manner (Fig. 1a). Induction of histamine release by thapsigargin was also inhibited by quercetin (Fig. 1a).

Effect of quercetin on HDC-activity

When the cells were pre-incubated with quercetin, the HDC-activity induced by PMA was inhibited (Fig. 1b), indicating that histamine synthesis was inhibited by quercetin.

Effect of quercetin after various incubation timesIgE-sensitized cells were challenged with DNP-BSA and thapsigargin after different incubation times with quercetin, both experiments showing similar results. When quercetin was removed by washing before challenging, the property of releasing histamine was recovered independently of incubation time (Fig. 2a and b, white bars). If the quercetin was not removed before challenging, histamine release was inhibited, but after 6 hours incubation with quercetin the inhibition was decreased (Fig. 2a-d). Further incubation gave rise to increased inhibition (Fig. 2a-d).

Recovery of histamine releasing ability

If quercetin is removed from the cell medium before challenging, the histamine release will no longer be inhibited. Fig. 3 shows the time course of histamine release from IgE-sensitized cells challenged with DNP-BSA or thapsigargin, with or without pre-incubation with quercetin.

Damaged cells and morphological changes

After 48 hours incubation with several concentrations of quercetin, some of the cells tended to detach from the underlying surface with increasing concentration of the drug. Histamine content of the attached and detached cells respectively, was assayed and the results was established at Fig. 4. The attached cells were stained with trypan blue, and the number of damaged cells increased with higher concentrations of quercetin while the total number of cells was decreased. Furthermore, the morphology of the cells changed from the characteristic appearance of 2H3 cells to more spherically shaped cells that lacked processes extending from the cell body.

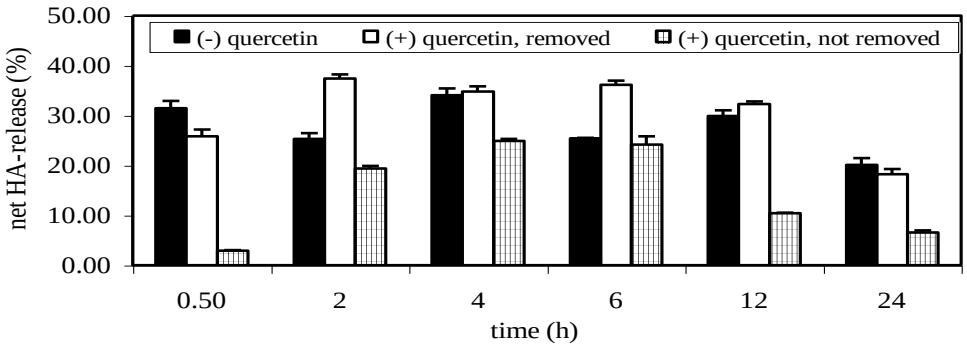

Fig. 2c. Histamin release after incubation with quercetin and challene with thapsigargin. Positve SEM of uplicateso values are shown if eceeding 0,01

Fig.1a.Concentration-response curve of quercetin effect on histamine release from RBL-2H3 cells. Histamine release was induced by DNP-BSA or thapsigargin. Positive SEM of duplicates of values are shown if exceeding 0.01.

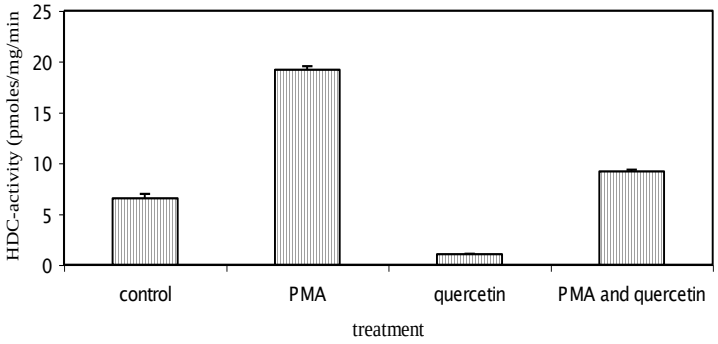

Fig.1b. HDC-activity measured after pre-incubation with vehicle, quercetin, PMA or both PMA and quercetin. Positive SEM of duplicates of values are shown if exceeding 0.01.

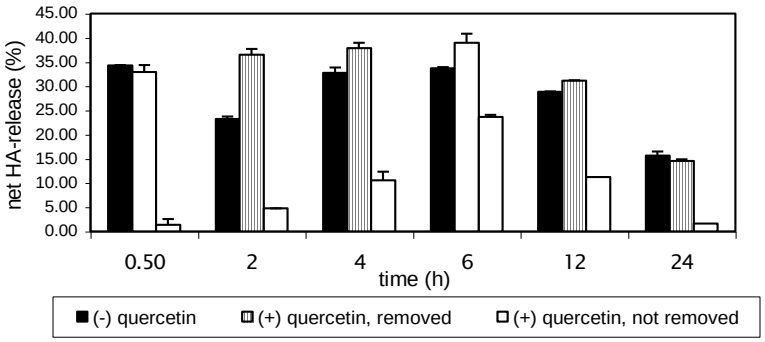

Fig. 2a. Histamine release from IgE-sensitized cells after incubation with quercetin and challenge with DNP-BSA. Positive SEM of duplicates of values are shown if exceeding 0.01.

After incubation with 20 uM quercetin, cells were only slightly affected by the damaging effect, cell growth inhibition and the morphologically changing effects of the drug, while the inhibitory effect on histamine release was significant. This is the concentration of quercetin used in most experiments in this study.

It seems that the effects of quercetin on 2H3 cells can be divided into at least two phases; the first occurring if the cells were incubated for a shorter time than 6 hours, and the second one was after 6 hours incubation or longer. This study can not determine the reasons for the biphasic behavior of quercetin, but the different effects are discussed. The points represents inhibition of challenge after removal of quercetin. Positive

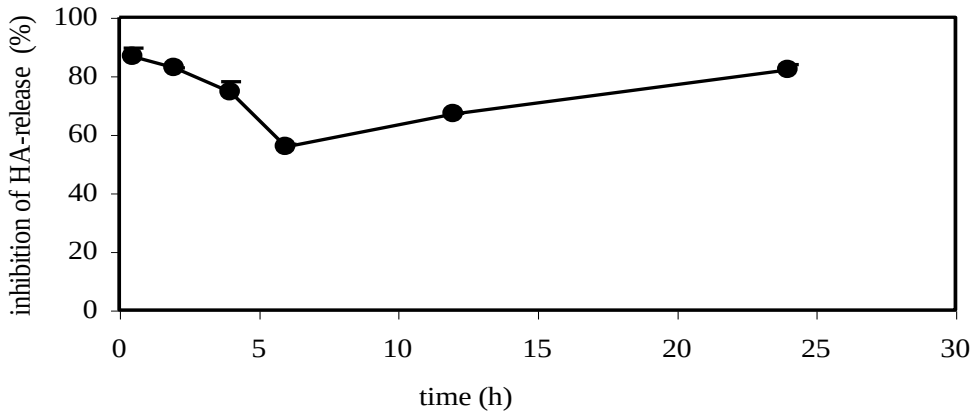

Fig. 2b. Inhibition of histamin release induced by DNP-BSA after various incubation time with quercetin

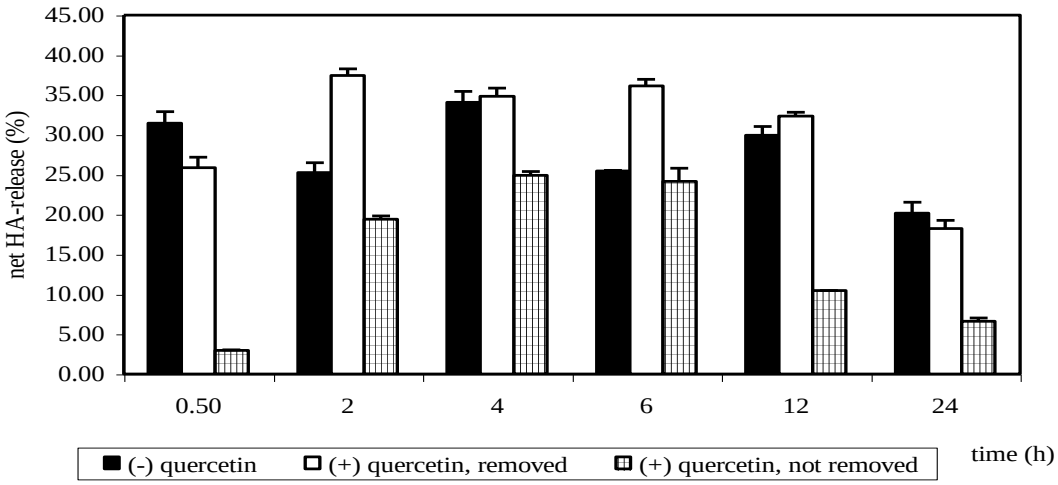

Fig. 2c.Histamin release after incubation with quercetin and challenge wiyh thapsigargin. Positive SEM of duplicates of value are shown if exceeding 0,01

Inhibition of histamine release and synthesis

As expected, quercetin inhibited histamine release induced by DNP-BSA in a concentration dependent manner, supporting previously published data on the inhibitory effect of quercetin on tyrosine kinases ((Ferriola *et al*, 1989), since the lyn- and syk tyrosine kinases associated with the $Fc_{\varepsilon}RI$ most probably are inhibited.

The histamine release triggered by thapsigargin was also inhibited (Fig. 1a), which might indicate an inhibitory effect of quercetin on Ca^{2+}-ATPases in the cell membrane that regulates Ca^{2+}-influx, which in this case prevents the cell from releasing histamine by preventing sufficient elevation of $[Ca^{2+}]_i$.

Fig. 1b shows that quercetin to some extent inhibits PMA-induced HDC-activity, which most likely contributes to the decreased content of the cells. The results also implies that quercetin inhibits PKC since PMA is a specific protein kinase activator. However, the inhibition of the HDC activity was not complete, which stresses the importance of other sites of inhibition.

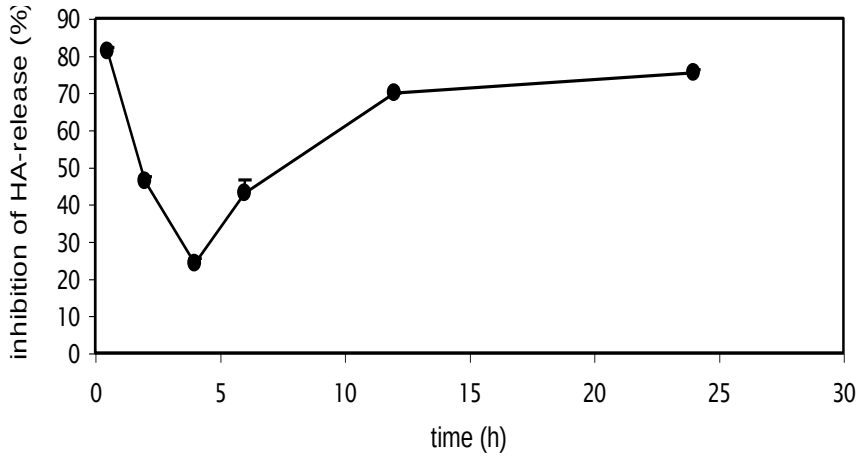

Fig.2d. Inhibition of histamine release induced by thapsigargin after various incubation times with quercetin

In conclusion, there are several sites of action for the inhibitory effect of quercetin on histamine release and synthesis. The points represents inhibition of challenge after removal of quercetin. Positive SEM of duplicates of values are shown if exceeding 0.01.

Dual behavior of quercetin

Quercetin inhibited histamine release after incubation for 0.5-4 hours (Fig. 2), but notably, if quercetin was removed before challenging, the cells recovered their ability to release histamine (Fig. 2a and c). This effect was observed after challenge with either DNP-BSA or thapsigargin. Fig. 3 shows that IgE-sensitized cells have almost the same responsiveness to DNP-BSA independently of the time course after removal of quercetin (recovery time) compared to control. These results suggest that the inhibitory effect on histamine release of quercetin could be quickly reversed. The chemical structure of quercetin (Fig. 5) reveals a rather hydrophobic compound, which makes it suitable for interactions within the plasma membrane. If a part of the inhibitory effect on histamine release is due to interactions of quercetin and enzymes in, or in the proximity of, the plasma membrane, this could be one of the explanations why the inhibitory effect is so quickly eliminated by washing.

However, if a portion of the quercetin is trapped in the cytosol, or more likely in organelles, is not known. Probably a small amount of quercetin remains in the cells since it has been reported that the drug has long term effects, such as growth inhibition (Middleton, *et al*., 2000) and cell maturation (Senyshyn, *et al*., 1998).

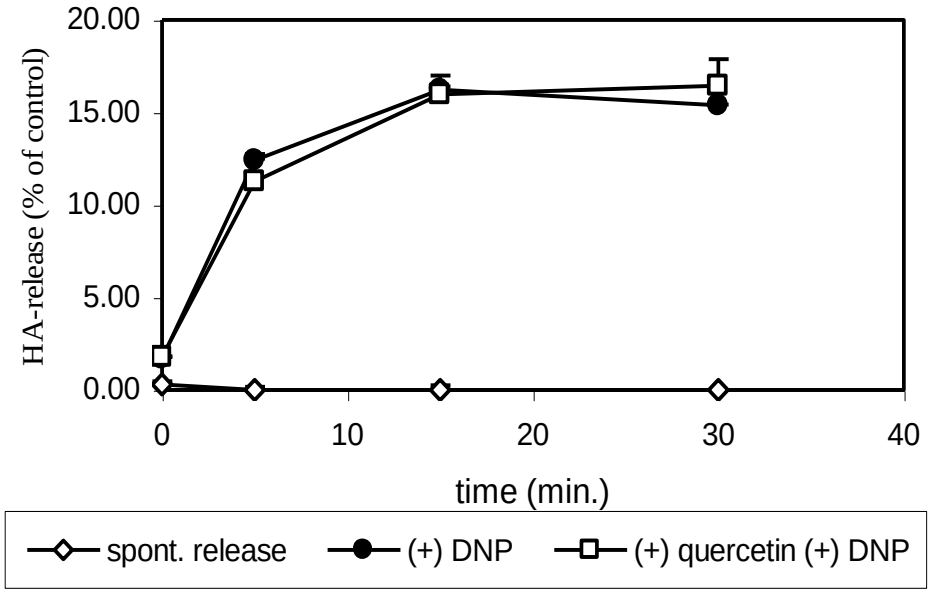

Fig. 3. Histamine release from 2H3 cells after various recovery times, with or without pre-incubation with quercetin. Positive SEM of duplicates of values are shown if exceeding 0.01.

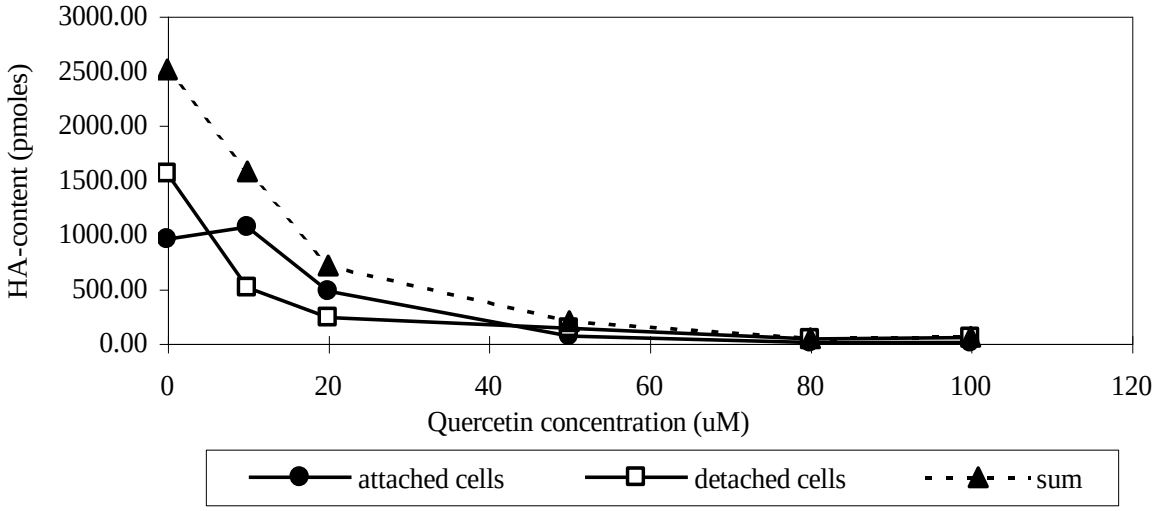

Fig. 4. Histamine content of attached and detached cells. Positive SEM of duplicates of values are shown if exceeding 0.01.

When the cells were incubated for about 6 hours, the inhibitory effect of quercetin seemed to decrease, while it would increased again when was incubated longer (Fig. 2b and d). These data propose that quercetin behaves dually, which needs to be further evaluated.

Cell changes

After 48 hours incubation, the cells became round, lacked extending processes and the number of attached cells drastically decreased when the concentration of quercetin was increased (Fig. 5). Also, the cells exhibited a high degree of cell damage with increasing concentration of the drug as visualized by trypan blue exclusion. The fraction of detached cells increased while their histamine content was reduced, which partly could be explained by the reduction of HDC-activity. However, the results prompted for a phenotypic change of the cells, which means that quercetin most likely exerts effects that was not instantly reversible by removing the drug from the medium. One of the effects might involve rearrangement of the actin cytoskeleton, which can lead to changes in adhesive properties and morphology of the cells. Polymerizing and depolymerizing of actin determines the cytoskeleton, and these reactions are, in addition to the motor protein myosin, regulated by $[Ca^{2+}]_i$ and PKC, which in turn are affected by quercetin. Thus both morphological changes and detachment of the cells could be influenced by quercetin-induced cytoskeletal rearrangement, and this hypothesis must be further investigated.

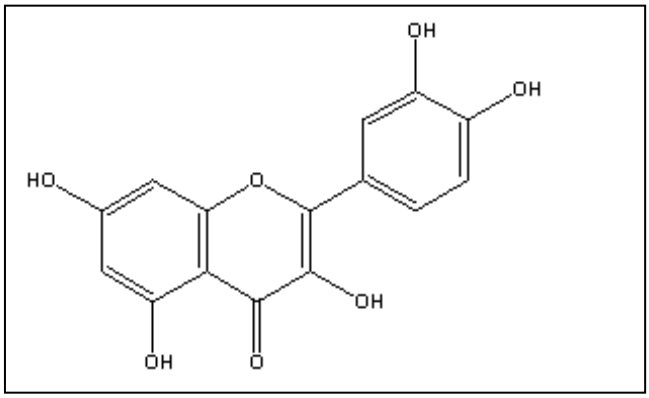

Fig. 5. Chemical structure of quercetin

It has been reported that quercetin induces apoptosis in tumor cells, such as cultured colorectal tumor cells (Richter *et al.*, 1999). In the cell lines HeLa and COLO320 DM, the induction of cytoprotective heat shock proteins (*hsps*) is supressed by quercetin, leading to apoptosis (Yoshida, *et al.*, 1990). Since 2H3 cells origin form basophilic leukemia cells, the observed cytotoxicity and cell number decrease in this study might be influenced by quercetin-dependent supression of *hsps* leading to apoptosis, along with the growth inhibitory effect of the drug.

In conclusion, it seems like quercetin has many sites of action of inhibiting the histamine release and synthesis, the most important being inhibition of tyrosine kinases, PKC, Ca^{2+}-ATPases and HDC. These results also suggest that quercetin behaves dually, with a first phase of anti-allergic properties and a latter phase dominated by growth inhibition and cytotoxicity. The mechanisms of the two phases are most likely different, possibly the previous is mainly sited at/within the plasma membrane, and the latter one located in the cytosol, organelles or in the nucleus.

This dual behavior of quercetin needs to be further analyzed, since it represents both anti-allergic and cytotoxic effects.

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