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Inhibitory effect of quercetin on tryptase and interleukin-6 release, and histidine decarboxylase mRNA transcription by human mast cell-1 cell line

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Abstract Mast cells are involved in inflammatory processes and in allergic reactions where immunologic stimulation leads to degranulation and generation of numerous cytokines and inflammatory mediators. Mast cells have been proposed as an immune gate to the brain, as well as sensors of environmental and emotional stress, and are likely involved in neuropathologic processes such as multiple sclerosis. Among mast cell products, the protease tryptase could be associated with neurodegenerative processes through the activation of specific receptors (PARs) expressed in the brain, while interleukin (IL)-6 likely causes neurodegeneration and exacerbates dysfunction induced by other cytokines; or it could have a protective effect against demyelinisation. In this report we show that quercetin, a natural compound able to act as an inhibitor of mast cell secretion, causes a decrease in the release of tryptase and IL-6 and the down-regulation of histidine decarboxylase (HDC) mRNA from human mast cell (HMC)-1 cells. As quercetin dramatically inhibits mast cell tryptase and IL-6 release and HDC mRNA transcription by HMC-1 cell line, these results nominate quercetin as a therapeutical compound in association with other therapeutical molecules for neurological diseases mediated by mast cell degranulation.

Key words Quercetin • Mast cells • Cytokines • Histidine decarboxylase

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Introduction

Brain inflammation is an underlying factor in the pathogenesis of Alzheimer's disease (AD) and multiple sclerosis (MS), where mast cells are also likely involved [1, 2]. Pharmacological treatments of neurodegenerative diseases cause modulation of pro-inflammatory and anti-inflammatory cytokines and chemokines [3, 4]. Neurohormones [5] and cytokines secreted under stress [6] can further activate mast cells. Cytokines can also induce the transcription of the novel human tumour suppressor gene GERP, physiologically expressed in the brain [7] and presumably involved in the development of glioblastoma. Mast cells have been proposed as an immune gate to the brain, as well as sensors of environmental and emotional stress, and have been linked to many neuropathologic processes [6–8].

Human mast cells contain and release several inflammatory compounds and have been used to study mast cell mediators and their role in inflammatory mechanisms [1, 9]. Mast cells are essential not only for allergies [10, 11] but also for innate and acquired immunity, autoimmunity

and inflammation [12–16]. They mediate all these processes through the release of various mediators: histamine, proteases (tryptase, chymase), proteoglycans and prostaglandin D2. They also produce leukotrienes, whose receptors are expressed on rat microglia [17], and several multifunctional cytokines [18–21] and chemokines [22, 23]. Cytokines and chemokines are implicated in inflammation of the central nervous system [24]. Mast cells can accumulate at inflammatory sites in response to the specific C-C chemokine RANTES (acronym for Regulated upon Activation, Normal T Cell Expressed and Secreted) [25–27].

Tryptases are expressed by most human mast cells and are divided into two groups: alpha or beta. Beta tryptase appear to be the major type stored in secretory granules of mast cells, while alpha is the main type expressed in basophils. Protease-activated receptors (PARs) are G-protein-coupled receptors activated by proteases, including tryptase. Several studies suggest a role for tryptase in allergic diseases and inflammatory reactions mediated by human mast cells by stimulating PARs [28]. All four classes of PARs are expressed in neurons of the central and peripheral nervous systems; it has been proposed that tryptase could be associated with neurodegenerative processes through the activation of specific receptors (PARs) expressed in the brain, as also suggested by the finding that mast cells and tryptases are elevated in the CNS of MS patients.

Interleukin-6 (IL-6) is a pleiotropic cytokine that is produced upon activation of Th2 cells or mast cells [4], which mediate physiological processes, such as immune responses, inflammation, acute phase responses, haematopoiesis, various diseases [29–31], and acute stress and brain serotonin metabolism through the activation of the hypothalamic-pituitary-adrenal axis. IL-6 is a crucial cytokine for mast cell maturation and mast cell production [32]. IL-6 has been shown to exacerbate neuronal dysfunction induced by other cytokines, but also to have a protective effect against demyelinisation.

Histidine decarboxylase (HDC) is an important mast cell biochemical and functional marker for the generation of histamine from histidine [33]. Histamine is also expressed by neurons involved in many brain functions and in the maintenance of wakefulness. The activity of the HDC enzyme is altered in various neurological diseases, treated and non-treated with therapeutical compounds. HDC is useful in studies in identification of histaminergic neurons and their function in the brain [34, 35].

Natural compounds have long been recognised to possess anti-inflammatory, anti-oxidant, anti-allergic, hepatoprotective, anti-thrombotic, anti-viral and anti-carcinogenic activities [32, 36–38]. Also the flavonoid quercetin has a variety of functions including anti-allergic activities, and it is known to inhibit histamine release from human basophils and murine mast cells [39]. Therefore, it is pertinent to evaluate the effect of quercetin on tryptase, HDC mRNA and IL-6 release by the human mast cell line HMC-1.

Materials and methods

Cells

Reagents for cell culture were purchased from Sigma-Aldrich (Milan, Italy). HMC-1 cells [40], a human mast cell leukaemia cell line, were kindly provided by Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN). HMC-1 were grown in Iscove's modified Dulbecco's medium supplemented with 10% bovine calf serum, 1.2 mM monothioglycerol, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin either in 25-cm^2 tissue culture plates or in six-well tissue culture plates (Costar). Cells were plated at a density of 0.2×10^6/ml taken from the 3-day-old culture grown under the same conditions. Cells were grown in an incubator in 5% CO_2 and 95% O_2 at 37°C.

Calcium ionophore A23187 (Sigma, St. Louis, MO, USA) was dissolved in dimethylsulphoxide (DMSO) (Sigma) at 0.5 µg/ml. Dilutions of the ionophore were made directly in Locke's medium with various final concentrations. In separate tubes, in each experiment, cells were exposed to the vehicle alone (i.e., DMSO or ethanol) at identical concentrations, to determine non-specific formation. Quercetin, a supplement-derived polyphenol contained in fruit and vegetables, was used at 10^{-5} M.

Determination of cell viability

HMC-1 cells were harvested, washed in PBS and centrifuged for 5 min at 400xg at room temperature. Cells were then resuspended in culture medium, 0.1% trypan blue solution was added for 5 min at room temperature and the cells were counted using a haemocytometer. Viability was 99%, expressed as percentage of cells that do not take up trypan blue.

Stimulation with anti-IgE or calcium ionophore

Calcium ionophore A23187 (Sigma, Milan, Italy) or anti-IgE at different dilutions were made directly in HMC-1 culture medium. In separate tubes, in each experiment, cells were exposed to the vehicle alone, to determine non-specific release.

HMC-1 cells (1×10^6) in a six-well tissue culture dish were washed with culture medium containing 1 mg/ml bovine serum albumin (BSA) and without Ca^{2+} to reduce spontaneous secretion. They were then sensitised in the same medium for 30 min at 37°C with 2 ml of mouse monoclonal anti-DNP IgE (500 ng/ml). After sensitisation the cells were washed again and treated for 30 min at 37°C with 2 ml of DNP-BSA (10 ng/ml) in the same medium, but now supplemented with 0.5 mM calcium to permit secretion. Control samples without IgE were run simultaneously in the presence of 0.5 mM Ca^{2+} and these values represented non-specific release.

Preparation of the HDC probe

We used a probe made from a reverse transcribed rat brain polyA+ RNA. Total cellular RNA extracted from rat brains was purchased from New England Deconess Hospital. PolyA mRNA was purified according to Conti et al. [39]. A sample of 2 µg polyA+ mRNA was reverse transcribed at 42°C for 40 min in a 20-µl mixture containing 4 µl of 5x reverse transcriptase buffer (250 mM Tris HCl pH 8.3 at 42°C), 50 mM $MgCl_2$, 250 mM KCl, 15 mM DTT, 10 U placental RNase inhibitor, 0.5 mM each dNTP, 50 pmol oligo-dT primer and 20 U AMV (avian myeloblastosis virus) reverse transcriptase. After reverse transcription the HDC cDNA was amplified by polymerase chain reaction using two specific primers synthesised on a gene assembler plus (Amersham Pharmacia Biotech): 5' primer, 5'-ATGATGGAGCCCAGTGAATACC; 3' primer, 5'-CCAGAATTCGCATGTCTGAGGTAG. Single-stranded cDNA mixture (4 µl) was supplemented with 50 pmol each of sense and antisense primers in a volume of 50 µl denatured for 2 min in a boiling bath and added to a 50-µl mix prewarmed at 72°C containing 0.25 mM each dNTP, 10 µl 10x Taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3 at 25°C, 15 mM $MgCl_2$ and 0.1% gelatin) and 1.5 U of Taq polymerase (Perkin-Elmer Cetus). The polymerase chain reaction programme consisted of one cycle of 1 min at 94°C and 15 min at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 4 min at 72°C, and was completed by an additional annealing at 55°C for 30 s and a final elongation at 72°C for 15 min. Polymerase chain reaction was performed in a Techne PHC-2 programmable heating block. Amplified products of the expected Base-Pair size were purified by a glass-milk procedure (Geneclean BIO 101), blunt-ended with the Klenow fragment, and cut by EcoRI. The resulting blunt-end-EcoRI fragments were cloned in the p-MAL vector (New England Biolabs) cut by both StuI and EcoRI restriction enzymes. The resulting recombinant construct was transformed in the TB1 *Escherichia coli* strain. Plasmid DNA was sequenced according to the manufacturer's protocol on the double-stranded DNA sequencing using the sequenase kit V2.0 (USB Cleveland). Plasmid containing amplified HDC cDNA was prepared according to the alkaline lysis method and purified on a CL4B column.

Northern blot analysis

Total RNA was isolated from HMC-1 cells by lysing the cells in TRIzol solution (Life Technologies) according to the manufacturer's instructions, and 30 µg was treated with DNase I (Amersham Pharmacia Biotech). Total RNA (10 µg/lane) was fractionated by electrophoresis on a formaldehyde denaturing agarose gel, transferred to nylon membranes (Hybond N, Amersham) and ^{32}P labelled HDC cDNA probes were hybridised ($2x10^8$ cpm/mg). It was then washed four times at room temperature for 15 min in 2x solution of sodium citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS), heated to 48°C for 30 min and then washed twice in 0.1xSSC and 0.1% SDS. Membranes were finally exposed to Kodak XAR5 for 3 days at –70°C. Signals were compared with ribosomal RNA to evaluate an equal quantity of RNA for each lane. A densitometric analysis was performed using a computerised image analyser (Quantimed software, Leica, Heidelberg, Germany) for normalisation of the relative mRNA levels (as reported in Results).

Immunoassays for tryptase

Tryptase was measured in the supernatants and cell pellets by the UniCAPTryptase Fluoroenzyme-immunoassay System (Pharmacia Diagnostics AB, Uppsala, Sweden) as reported previously (Schwartz LB 1994). Briefly, anti-tryptase covalently coupled to ImmunoCAP reacts with the tryptase in the specimen. After washing, enzyme-labelled antibodies against tryptase are added to form a complex. After incubation, unbound enzyme-anti-tryptase is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured in the FluoroCount™ 96 microplate reader. The fluorescence is directly proportional to the concentration of tryptase in the sample. To evaluate the test results, the response for the samples is compared directly to the response of the standards.

IL-6 release determination

IL-6 was determined in cell-free supernatants with a commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions (sensitivity of the assay, 3 pg/ml).

Statistical analysis

All assays were performed in triplicate. The results were expressed as mean±SD. Data from three different experiments were combined and reported as the mean±SD. Student's *t*-test for independent means was used to provide statistical analyses ($P>0.05$ was considered as not significant).

Results

Inhibition of histidine decarboxylase (HDC) mRNA by quercetin in HMC-1 cell line

HDC cDNA probe was cloned into the p-Mal plasmid and used for Northern blot hybridisation. As quercetin is an inhibitor of mast cells, in these studies we cultured HMC-1 cells in the presence or absence of quercetin 10^{-5} M and mRNA expression modulation was determined for HDC. When quercetin was added, HDC mRNA was significantly inhibited compared to the controls. Figure 1 shows the steady-state levels of HDC mRNA after treatment of HMC-1 with quercetin 10^{-5} M and with or without a classic secretagogue anti-IgE (10 µg/ml) or a non-specific acti-

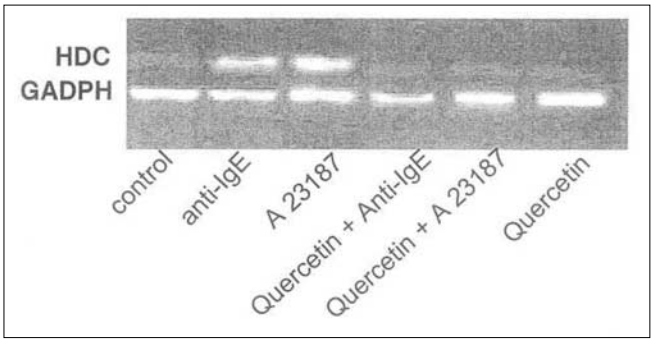

Fig. 1 Effect of quercetin on HCD mRNA expression in HMC-1 cells cultured for overnight incubation. The cells were activated by A23187 (0.5 μg/ml) or anti-IgE (10 μg/ml)

vator, calcium ionophore A23187 (0.5 μg/ml). The transcription of IL-6 mRNA was also inhibited by quercetin (data not shown).

Tryptase release from HMC-1 treated or not with quercetin

HMC-1 are cells that have tryptase-containing granules and receptors for IgE. In this report we studied HMC-1 under the influence of quercetin (10^{-5} M), a natural inhibitor of mast cells, with and without a classic specific secretagogue anti-IgE, or a non-specific compound calcium ionophore A23187. Table 1 shows in three representative

Table 1 Tryptase (ng/ml) release from HMC-1 (10^5 or 10^6 cells/ml) following the addition or not of quercetin (10^{-5} M) and the corresponding samples treated with calcium ionophore (A23187) plus the addition of quercetin (10^{-5} M) or not

	Tryptase release (ng/ml)			
	10^5 cells	*P* values	10^6 cells	*P* values
Spontaneous	14.5±1.0	–	24.0±6.0	–
A23187 (0.5 μg/ml)	129.0±11.5	–	210.0±32.0	–
Quercetin (10^{-5} M)	4.5±1.0	0.05	10.0±2.0	0.05
Quercetin (10^{-5} M)+A23187 (0.5 μg/ml)	86.7±12.1	0.05	140.0±21.0	0.05

The cells were cultured overnight at 37°C, 5% CO_2. *P* values (Student's *t*-test) are calculated by comparing quercetin untreated HMC-1 with quercetin-treated cells. The values±SD are representative of three experiments in triplicate

Table 2 Tryptase (ng/ml) release from HMC-1 (10^6 cells/ml) following the addition or not of quercetin (10^{-5} M) and the corresponding samples treated with anti-IgE (10 μg/ml) plus the addition of quercetin (10^{-5} M) or not

	Tryptase release (ng/ml)	
	10^6 cells	*P* values
Spontaneous	14.5±1.9	–
Anti-IgE (10 μg/ml)	201.4±3.4	–
Quercetin (10^{-5} M)	4.9±1.1	0.05
Quercetin (10^{-5} M)+Anti-IgE (10 μg/ml)	112.6±12.8	0.05

The cells were cultured overnight at 37°C, 5% CO_2. *P* values (Student's *t*-test) are calculated by comparing quercetin untreated HMC-1 with quercetin-treated cells. The values±SD are representative of three experiments in triplicate

Table 3 IL-6 production from HMC-1 (10^5 or 10^6 cells/ml) following the addition or not of quercetin (10^{-5} M) and the corresponding samples treated with the calcium ionophore A23187 (0.5 μg/ml) plus the addition of quercetin (10^{-5} M) or not

	IL-6 release (ng/ml)			
	10^5 cells	*P* values	10^6 cells	*P* values
Spontaneous	15.0±9.0	–	28.0±14.0	–
A23187 (0.5 μg/ml)	150.0±4.0	–	182.0±21.0	–
Quercetin (10^{-5} M)	32±18	0.05	85±12	0.05
Quercetin (10^{-5} M)+A23187 (0.5 μg/ml)	92.0±6.0	0.05	104.0±18.0	0.05

The cells were cultured overnight at 37°C, 5% CO_2. *P* values (Student's *t*-test) are calculated by comparing quercetin untreated HMC-1 with quercetin-treated cells. The values±SD are representative of three experiments in triplicate

experiments the release of tryptase following HMC-1 treatment and incubated overnight with or without quercetin (10^{-5} M), with A23187 then used as a secretagogue at 0.5 μg/ml. Table 2 shows tryptase release following HMC-1 treatment and incubated overnight with or without quercetin (10^{-5} M) plus anti-IgE at 10 μg/ml.

IL-6 release from HMC-1 treated or not with quercetin stimulated through the IgE receptor (FcεRI) cross-linking

Stimulated HMC-1 were treated overnight with quercetin and then the physiological specific secretagogue anti-IgE was added (Table 3). After these treatments, the percent generation of IL-6 was calculated. The results indicate that quercetin (10^{-5} M) significantly affects ($P<0.05$) the release of IL-6.

Discussion

Quercetin has a negative effect on intracellular regulator signalling events initiated by FcεRI cross-linking and other activating receptors on mast cells [41]. Increased secretion of mast cell tryptase in certain disorders, such as bronchial asthma, may augment neurogenic inflammation [28]. In spite of this, it is not clear if quercetin is capable of directly inhibiting tryptase or IL-6 release from basophilic cells. In this report, we found that generation of the potent antioxidant, cytoprotective and anti-inflammatory flavonoid quercetin is an inhibitor of tryptase and IL-6 production. HMC-1 cells exposed to quercetin provoke a significant inhibition of tryptase and IL-6 release compared to the calcium ionophore A23187 or anti-IgE alone. Also, the addition of quercetin determines an inhibition of HDC mRNA expression in HMC-1 cells. We found that actinomycin D, a drug that blocks the transcription of new RNA, and cycloheximide, a protein synthesis inhibitor, abolished the reduction of HDC mRNA in cells treated overnight with quercetin. These results suggest that HDC mRNA down-regulation by quercetin is a complex process that could require the transcription and translation of one or several genes to decrease HDC mRNA expression [40, 42–45].

It has been proposed recently that mast cells can be modulated to secrete effector molecules at different kinetics and magnitudes depending on environmental factors [12]. Quercetin might have such modulating properties on mast cells: as mast cell secretion products have some effects that promote and others that suppress inflammation, quercetin may reduce the release of pro-inflammatory mediators or even favour the release of anti-inflammatory ones. The inhibition of HDC mRNA transcription and tryptase and IL-6 generation by quercetin on mast cells may be useful in many pathological conditions associated with a chronic inflammation status such as MS, AD, cancer and allergies [46–56].

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