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ORIGINAL PAPER

Structural basis of the anti-inflammatory activity of quercetin: inhibition of the 5-Hydroxytryptamine Type 2 Receptor

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Abstract The anti-inflammatory activity of quercetin was evaluated through serotonin-induced rat-paw edema. The experiments showed that quercetin had an important effect on acute inflammatory processes. Docking of serotonin and quercetin into the homology model of the 5-Hydroxytryptamine Type 2 Receptor allowed to analyze the structural basis of the anti-inflammatory activity. Results showed that serotonin and quercetin bind in the same region of the active site with a similar binding energy but quercetin has a much bigger inhibition constant. Therefore, it seems possible that quercetin may act as a natural inhibitor of the receptor blocking the acute inflammation generated by serotonin.

Keywords Anti-inflammatory activity · Serotonin · Receptor $5HT_2$ · Docking · Quercetin

Introduction

The symptoms of acute inflammation (redness, edema, heat, pain, and disturbed tissue function) are the results of complex pathophysiological processes. They include increased blood flow and vascular permeability, activation of humoral and cellular defense mechanisms, and sensibilization and activation of nociceptors. These processes are mediated by a variety of signaling molecules. The increased vascular permeability which is mediated mainly for early phase mediators produced by mast cells and platelets is especially important in acute inflammation. They are histamine and serotonin. Other substances (bradykinin, complements component, prostaglandin E_2, and LTC_4) have also been implicated.

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) mediates a wide range of physiological and pathological process by interacting with multiple receptors. In the past 16 years, seven distinct families of 5-HT receptors have been identified ($5\text{-}HT_1$–$5\text{-}HT_7$), and subpopulations have been described for several of these families. To date, there are no agents which display absolute specificity for one subpopulation of $5\text{-}HT_{2(A,B,C)}$ receptors over the others. Several semi-selective agents show different binding profiles for the three subpopulations, but with preference for one or two subpopulations over the other(s) (Glennon et al. 2000).

In the skin the local effects of serotonin e.g., pro edema, vasodilatory, proinflammatory, and pruritogenic is mediated by receptors (Fitzpatrick et al. 1993; Goldsmith 1991; Belmonte and Cervero 1996; Theoharides 1996; Askenase 1977; Tachibana et al. 1990). The vascular permeability in skin rodent is mediated by the activation of 5-Hydroxytryptamine Type (HT_1) and (HT_2) receptors (Fujii et al. 1994). These authors observed that subcutaneous injection of 5-HT induced a dose-related increase of vascular permeability at the injection site. This effect was inhibited by pre-treatment with intraperitoneal injection of ketanserin ($5\text{-}HT_{2A}$ antagonist) and methysergide ($5\text{-}HT_{1/2A}$ antagonist), less efficiently by NAN-190 ($5\text{-}HT_{1A}$ antagonist), but not by granisetron ($5\text{-}HT_3$ antagonist), suggesting that 5-HT

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increases vascular permeability by activating the 5-HT_2 receptors and that endogenous nitric oxide will be involved in this effect. Besides, anatomical evidence of the 5-HT_{2A} receptor present on sensory axons at dermal–epidermal junction in rat glabrous skin from the plantar surface was demonstrated by Carlton and Coggeshall (1997). The pruritogenic effect of serotonin may be mediated through direct activation of 5-HT_3 receptors or indirectly through mast cells (Belmonte and Cervero 1996; Bos 1997; Plotnikoff et al. 1999; Weisshaar et al. 1997).

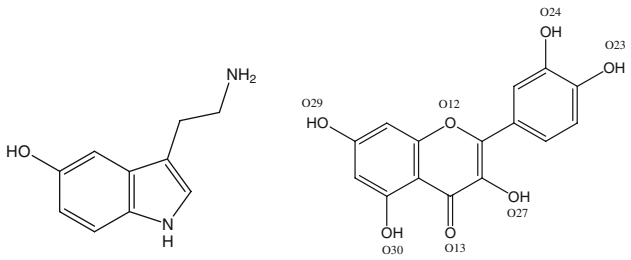
On the other hand, flavonoids are low-molecular weight compounds found in citrus fruits, olive oil, tea, red wine, seeds, and several medicinal plants. Anti-inflammatory properties of these compounds have been broadly studied both in vitro and in vivo (Harborne and Williams 2000; Middleton and Kandaswami 1994; Miksicek 1993). Besides, Berg and Daniel (1988) have reported that flavonoids are also known for their anti-allergic effects. Other authors have demonstrated that flavonoids possess anti-bradykinin activity (Agarwal 1982). Also, they cause the inhibition of prostaglandin synthesis (Ferrandiz and Alcaraz 1991). Recently, Gafner et al. (2003) demonstrated that flavonoids isolated from *Scutellaria lateriflora* (baicalein, baicalin, scutellarin, wogonin, 5,6,7,trihydroxy 2, methoxyflavonas, three flavone glycoside, and a flavone glycoside) showed a high affinity for the $5HT_7$ receptor. Besides, Lee et al. (2005) have shown the interaction between quercetin and $5HT_3$ receptor. In competition experiments, they exposed the possibility that quercetin may inhibit the binding of 5-HT to the binding site in the 5-HT_{3A} receptor. In previous studies, we have demonstrated the anti-inflammatory activities of numerous flavonoids on acute inflammation in rat-paw edema on carrageenan (Pelzer et al. 1998); therefore, the aim of this work was to investigate if quercetin (a) inhibits the paw edema induced by serotonin, an early mediator of inflammation and (b) its possible interaction with the 5-Hydroxytryptamine Type 2 Receptor.

Materials and methods

Pharmacological assay

Animals

Wistar albino rats weighing 160–180 g fed on standard chow and tap water ad libitum were used. The animals were housed at a room temperature of 24 ± 1°C with 12 h light/dark cycle. The animals were randomly assigned to different groups and a period of 4 days was allowed to adapt to each experiment. All experiments were in compliance with the ANMAT Nº 6344/96, for animal care guidelines.



Serotonin Quercetin

Scheme 1 Serotonin and quercetin scheme

Drugs

Serotonin and quercetin were purchased from Sigma, USA, see, Scheme 1.

Serotonin-induced paw edema in rat

Wistar rats were divided into groups of 6 animals each. One group received normal saline and served as a control. The other groups were injected intraperitoneally with a suspension of quercetin in normal saline: (80 mg kg^{-1}). One hour after drug administration, all groups received an intradermic injection (0.1 ml) of a serotonin 0.1% solution in normal saline (Kalbhem and Smalla 1977).

Paw volume was measured using a plesthymometer, pretreatment, and post-treatment of serotonin at 30, 60, and 120 min. Percent inhibition of inflammation induced by each was calculated with respect to its vehicle treated control group.

Statistical analysis

Data obtained from pharmacological experiment are expressed as mean values ± S.D. Differences between the control and the treatments in these experiments were tested for significance using analysis of variance followed by Dunnet's test. A probability of $P < 0.05$ was considered significant; a probability of $P < 0.01$ was considered very significant.

Homology modeling

The three-dimensional model of the serotonin receptor was constructed with MODELLER 9.4 (Sali and Blundell 1993), using as template the high resolution crystallographic structure of the human β_2-adrenergic G protein-coupled receptor (HAGPCR) (PDB dataset 2RH1) which has 31.79% sequence identity and 49.29% similarity. BLAST (Altschul et al. 1997) was used to search for the

Table 1 Effect of intraperitoneal doses of quercetin on serotonin-induced paw edema in rats

Drug	Doses (mg kg^{-1})	30 min		60 min		120 min	
		Edema volume (ml)	Inhibition (%)	Edema volume (ml)	Inhibition (%)	Edema volume (ml)	Inhibition (%)
Control		1.05 ± 0.10	–	0.86 ± 0.13	–	0.62 ± 0.14	–
Quercetin	80	0.68 ± 0.15	35**	0.53 ± 0.13	38**	0.35 ± 0.11	44*

The increase in volume caused by serotonin was measured by subtracting the volume of the untreated right paw from that of the treated left paw. Inhibition percent represent percent reduction paw volume compared with the controls. Values are the mean ± SD for 6 rats. Dunnet's test: * $P < 0.05$, ** $P < 0.01$

templates and to do the alignment. The quality of the model was assessed by WHAT_CHECK (Hooft et al. 1996).

Docking

The docking of serotonin and quercetin were done using AUTODOCK 4 (Scripps Research Institute. La Jolla, Cal.) (Morris et al. 1998; Huey et al. 2007; Sanner 1999). All water molecules were removed and non-polar hydrogens were added using UCSF CHIMERA (Pettersen et al. 2004). Atomic partial charges for the receptor and the ligands were computed by the Gasteiger method (Gasteiger and Marsili 1980). The atomic interaction energy grids were calculated using probes corresponding to each atom type found in the substrate. They were tested every 0.375 Å in a 60 Å cubic box centered in approximately the active site of the template (human β_2-adrenergic G protein-coupled receptor, 2RH1). The docking of each compound consisted of two stages or runs. In the first run the compound was placed in the position occupied by the substrate in the template. For the second stage, the global minimum structure, the low-energy structures of significant clusters were subjected to redocking under the same conditions and to cluster analysis using a 2.0 Å RMSD. Genetic algorithm with Local Search or Lamarckian with 250,000 evaluations was used for the dockings.

Results

Pharmacology assay

Effect on serotonin-induced paw edema in rat

The results are shown in Table 1. At 30 min quercetin showed significant anti-inflammatory activity, this is maintained for 120 min.

5-Hydroxytryptamine Type 2 Receptor (5HT$_2$)

The homology model of the serotonin 5HT$_2$ receptor included 265 amino acids. The N-terminus (residues 1–77) and the C-terminus (residues 390–465) were not modeled as they were disordered in the template. The fold is composed of seven transmembrane helices forming a helical bundle (Fig. 1).

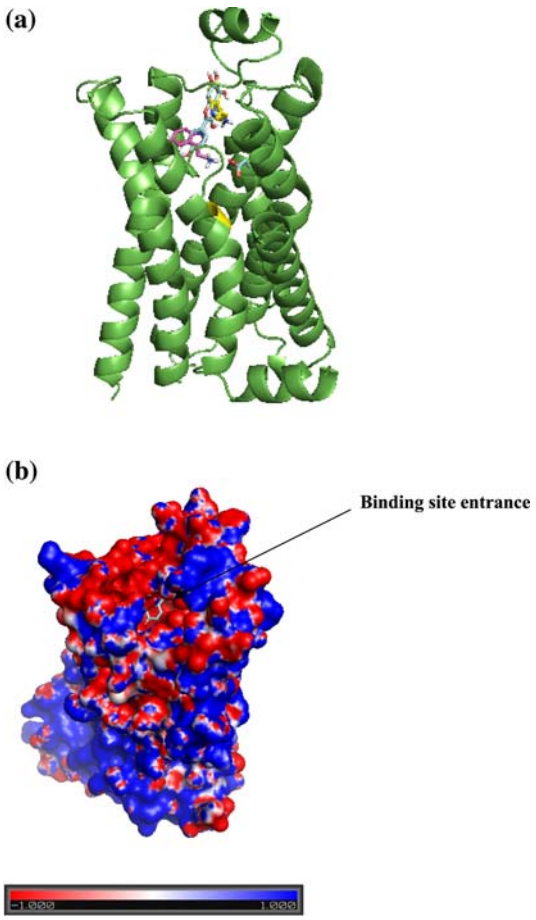


Fig. 1 **a** Overall fold of the 5-Hydroxytryptamine Type 2 Receptor, with quercetin and serotonin bound in subsites I and II. PYMOL (DeLano 2002) was used in the preparation of all figures. **b** Surface representation of 5-Hydroxytryptamine Type 2 Receptor colored by calculated charge from red −1 to blue +1, using a dielectric constant of 78. Poisson–Boltzman electrostatics was calculated using APBS (Baker et al. 2001) as implemented in AUTODOCK

The residues that form the helices are as follow: Helix I, Leu 1 to Leu 23, helix II, Asn 29 to Tyr 59, helix III, Ser 67

to Gln 100, helix IV, Arg 111 to Val 134, helix V, Asp 153 to Glu 186, helix VI, Glu 188 to ILe 218, helix VII, and Asn 226 to Leu 252. The residues making up the intracellular loops (ICLs) and the extracellular loops (ECLs) are: ICL1, Glu 24 to Gln 28, ECL1, Gly 60 to Pro 66, ICL2, Asn 101 to Ser110, ECL2, Pro 133 to Ala 152, ICL3, missing in the template, and ECL3, Cys 219 to Glu 225. The disulfide bond, Cyst70–Cys 149, that links helix III with ECL2, is conserved. The main topology differences between the template and $5HT_2$ are the position of ECL2. This is due, perhaps, to an intraloop disulfide bond which is present in the template but missing in $5HT_2$.

Binding site

The extracellular loops and the N termini of GPCRs (G protein coupled receptors) together with the extracellular halves of the transmembrane helices are thought to define the ligand binding site of each receptor (Angers et al. 2000). Small molecule ligands, like serotonin or quercetin, are believed to bind much deeper within the space generated by the transmembrane helices, whereas larger ligands, such as peptides bind closer to the membrane surface near the ECLs (Ji et al. 1998; Gether 2000). In the human β_2-Adrenergic G Protein-coupled receptor the deep binding site, defined by the binding of carozolol (Cherezov et al. 2007), is formed by the following residues making hydrophobic and aromatic interactions within a 4 Å radius of the substrate: Trp 109 (73), Val 114 (78), Val 117 (Ser 81), Thr 118 (82), Phe 193 (Leu 151), Tyr 199 (Asn155), Ser 207 (Val 165), Trp 286 (206), Phe 289 (209), Phe 290 (210), and Tyr 308 (Gly 229). The salt bridge and hydrogen bond contacts are held by Asp 113 (77), Asn 312 (233), and Tyr 316 (240). The numbers within brackets are referred to the corresponding residue number in $5HT_2$. Although the overall sequence identity between HAGPCR and $5HT_2$ is about 31% the binding site is highly conserved. The most important differences in the binding site are caused by the mutations of Phe 193 into Leu 151, Tyr 199 into Asn 155, and Tyr 308 into Gly 224 which produced a widening of the binding site.

Docking of serotonin

The key results in a docking run are the docked structures found at the end of each run, the energies of these docked structures, and their similarities to each other. The similarity of docked structures is measured by computing the root-mean-square deviation, rmsd, between the coordinates of the atoms. A docking experiment usually has several solutions. The reliability of a docking result depends on the similarity of its final docked conformations. One way to measure the reliability of a result is to compare the rmsd of the lowest energy conformations and their rmsd to one another, to group them into families of similar conformations or "clusters".

The results of serotonin's docking showed that the lowest energy conformations were grouped into three clusters of similar binding energy (See, Table 2). The structures in cluster 1 and 2 docked occupying a common zone in the binding site defining a subsite (I). Structures in cluster 3 occupied a nearby zone that defined a second subsite (II). Subsite I was located at the entrance of the binding site, with serotonin amino group making an electrostatic interaction with Ile 132 backbone oxygen and the hydroxyl making a strong hydrogen bond to Asp 139. Serotonin double ring was making hydrophobic and aromatic interactions within a 4 Å radius with Leu 151, Asn 155, Phe 156, and Ile 159. Subsite II was located at the bottom of the binding site with the serotonin amino group making a very strong salt bridge with Asp 77 and hydrogen bonds with Asn 233 and Tyr 240. The following residues made hydrophobic and aromatic interactions within a 4 Å radius: Phe 209, Phe 210, Trp 206, Asn 213, Val 78, and Ser81. Subsite II is equivalent to the binding site of carozolol in the beta adrenergic receptor. The analysis of the interactions was based in the lowest energy conformations of clusters 1 and 3. The chosen structures also fulfil the selection criteria used in docking studies like: a good fit in the binding pocket, a chemical match between atoms in the ligand versus receptor, for example, carbon atoms in the ligand are near hydrophobic atoms in the receptor, nitrogen and oxygen in the ligand are near complementary hydrogen bonding atoms and charge complementarity. Figure 2 shows serotonin in subsites I and II.

Docking of quercetin

The lowest energy conformation structure was chosen as the most significant solution despite being outside the most populated cluster. Visual analysis showed that binds very deep in the site (Fig. 2), satisfying all the previously

Table 2 Results of the docking experiments

Compound	Cluster	Binding energy	Inhibition constant	Subsite
Serotonin	1_1	−6.43	19.25	I
	1_2	−6.36	21.76	
	2_1	−6.38	20.89	
	2_2	−6.36	21.69	
	3_1	−6.00	39.85	II
	3_2	−5.64	73.42	
Quercetin	1	−8.39	712.71	I–II
	3_1[a]	−7.76	2.05	

The table shows the binding energies and the inhibition constant after the second stage of docking as calculated for AUTODOCK

[a] Most populated cluster

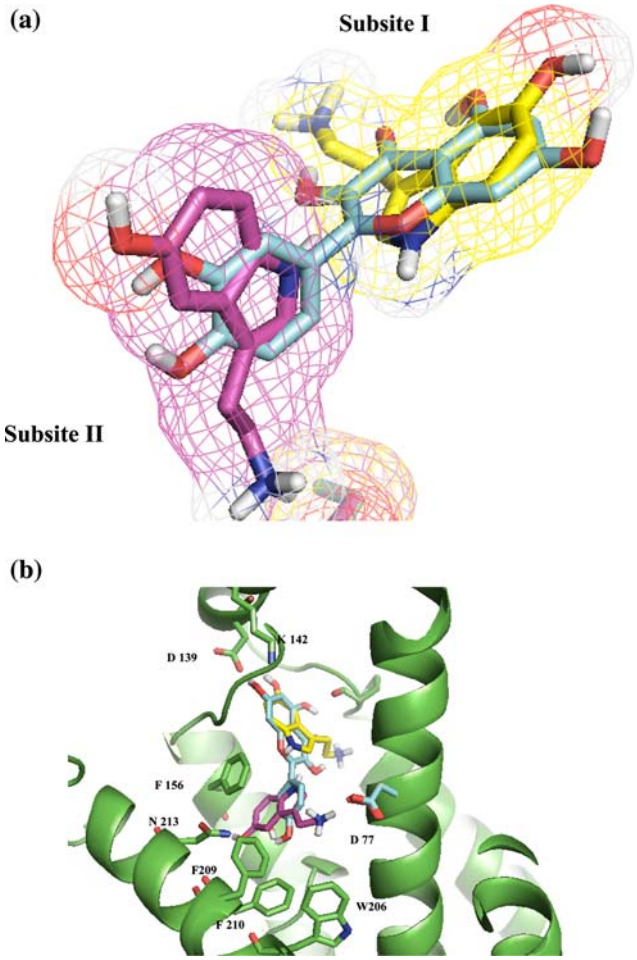


Fig. 2 **a** Stick representation of Quercetin (*cyan*) and serotonin bound in subsites I (*yellow*) and II (*magenta*). **b** Main interactions of quercetin and serotonin in the binding site of 5-Hydroxytryptamine Type 2 Receptor. Aminoacids are labeled using the one letter code

described criteria is needed to assess a good docking. In addition, it had the largest inhibition constant, with a value several times larger than values found for the other conformations (Table 2). In this solution, quercetin is occupying subsites I and II. Quercetin double ring is superposed to serotonin double ring in subsite I, while the single ring is rotated at 90° and superposed to serotonin five-membered ring in subsite II. The observed hydrophobic and aromatic interactions were similar to the ones described previously for subsites I and II in the docking of serotonin. Strong hydrogen bonds were observed between Lys 142 and Asp 139 versus hydroxyl O29, Ile 132, and Pro 133 backbone oxygen versus hydroxyl O30.

Discussion

Rat paw edema induced by serotonin is a model of acute inflammation characterized by an increase of vascular permeability developed in an early phase (1–2 h).

Fujii et al. (1994) showed that the activation of 5-Hydroxytryptamine Type (HT_1) and (HT_2) receptors generate vascular permeability. Doak and Sawynok (1997) observed that subcutaneous injection of 5-HT induced edema and the co-injection of 5-HT/formalin results in significantly greater paw swelling than is seen with formalin alone but this is comparable to that seen with 5-HT alone. The co-injection of formalin/5-$HT_{1, 2, 3, 4}$ receptor subtype-selective agonists suggest that 5-HT_1, 5-HT_2, and possibly 5-HT_4 receptors may be involved in edema formation, while the activation of 5-HT_3 receptors is not involved in edema formation. The injection of DOI (5-HT_2 receptor agonist) augmented edema associated with formalin, although 5-HT_2 receptor activation does not appear to be directly involved in the nociceptive response.

On the other hand, it is known that serotonin is a potent vasodilator acting on healthy endothelial cells through nitric oxide release or inhibition of norepinephrine release (Rang et al. 2004). Besides, it is know that serotonin is able to activate nNOS, leading to the generation of reactive oxygen species (ROS) in addition to the NO(−) production (Breard et al. 2007).

Flavonoids have a wide range of biological activities (Harborne and Williams 2000; Middleton and Kandaswami 1994; Miksicek 1993). They are powerful antioxidants, they act as potent metal chelators and free radical scavengers (Hughes and Wilson 1977; Torel et al. 1986; Kandaswami and Middleton 1994). However, the flavonols, despite structural similarity, have different antioxidant and antiinflammatory effects (Wang et al. 2006). The capacity to act as antioxidants in vitro has been the subject of several studies and it is very important structure–activity relationships. The antioxidant efficacy of flavonoids in vivo is less documented, possibly due to the limited knowledge on their pharmacokinetics (Pietta 2000). The quercetin has an anti-inflammatory activity (Landolfi et al. 1984; Mascolo et al. 1988) and exhibited radical scavenging and antioxidative effect (Kim et al. 1999; Lu et al. 2009).

Cos et al. (2001) evaluated the lipid peroxidation—inhibiting activity of several flavonoids and interestingly many of the flavonoids was used as nutraceutical, e.g., quercetin shows a low antioxidant selectivity index.

Our experiment shows that quercetin has a potent antiserotonin effect on rat-paw edema and that it seems possible that quercetin may act as a natural inhibitor of the receptor $5HT_2$ blocking the acute inflammation generated by serotonin.

This hypothesis was confirmed by the docking studies of these compounds onto the homology model of the $5HT_2$ receptor. Although, homology models have shortcomings, usually generated in the available template, in our case, the target $5HT_2$ and the template, the human β_2-Adrenergic receptor belong to the same family of G protein-coupled

seven transmembrane receptors, which binds similar sort of substrates. This fact gives us confidence in the quality of the model. Besides, AUTODOCK is the most used docking program with lots of success in the field of drug design. Therefore, we trust that these results are very reliable and strongly confirm the hypothesis of quercetin as having a potent anti-serotonin effect through blocking the $5HT_2$ receptor, acting as a natural inhibitor, and neutralizing the acute inflammation generated by serotonin in this way.

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