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Doxycycline exerted neuroprotective activity by enhancing the activation of neuropeptide GPCR PAC1

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ABSTRACT

Doxycycline has significant neuroprotective effect with anti-inflammatory and anti-apoptotic activity. We found for the first time that doxycycline specially promoted the proliferation of Chinese hamster ovary (CHO) cells with high expression of neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) preferring G protein-coupled receptor (GPCR), PACAP receptor 1(PAC1) and induced the internalization of PAC1 tagged with yellow fluorescent protein (YFP) indicating doxycycline interacted with PAC1. The homology modeling of PAC1 and molecular docking of doxycycline with PAC1 showed the theoretical binding of doxycycline to PAC1 at the site where PACAP(30–37) recognized. The competition binding assay and PAC1 site-specific mutation of Asp116, which formed two hydrogen bonds with Dox, confirmed the binding of doxycycline to PAC1 imitating PACAP(30–37). Doxycycline (100 ng/mL) significantly promoted the proliferative activities of vasoactive intestinal polypeptide (VIP) and oligopeptide HSDGIF responsible for the activation of PAC1 in PAC1-CHO cells, indicating that doxycycline facilitated the binding and the activation of PAC1 imitating PACAP(28–38). In Neuro2a cells with endogenous expression of PAC1 and its ligands, doxycycline not only promoted the proliferation of Neuro2a cells but also protected the cells from scopolamine induced apoptosis, which was inhibited by cAMP-PKA signal pathway inhibitor H-89, PAC1 shRNA or PACAP antagonist PACAP(6–38). The *in vivo* study showed long-term treatment with doxycycline (100ug/kg) had significant effect against scopolamine induced amnesia, and the synergetic anti-apoptotic, anti-oxidative and neuroprotective effect of doxycycline with VIP was more efficient than doxycycline alone or VIP alone, indicating doxycycline enhanced the activation of PAC1 *in vivo* effectively. Furthermore, doxycycline analogue minocycline also had similar theoretically binding site on PAC1 to doxycycline and displayed corresponding similar activity on PAC1 to doxycycline. All these results confirmed for the first time that doxycycline specially targeted PAC1 imitating PACAP(30–37) and acted as an enhancer by facilitating the subsequent ligand binding and the activation of PAC1. The confirmation of PAC1 as a novel molecular target of doxycycline and the novel mechanism by which doxycycline enhances the activity of PAC1 will help further clinical development of doxycycline as novel therapy for nervous system diseases such as neurodegenerative diseases targeting PAC1.

1. Introduction

Synthetic antibiotic doxycycline is a tetracycline derivative, and it has been widely reported and accepted that doxycycline has significant anti-inflammatory and anti-oxidative activity (Krakauer and Buckley, 2003; Tilakaratne and Soory, 2014). And the significant inhibitory effect of doxycycline against systemic inflammation (Payne et al., 2011) has made the subantimicrobial-dose-doxycycline (SDD) be clinically used as novel therapy for periodontitis (Bretz, 2011), cystic fibrosis (Beringer et al., 2012), preventing acute coronary syndromes (Brown et al., 2004) and reducing the risk of cardiovascular disease (Salminen et al., 2013) and so on. The neuroprotective effect of doxycycline due to its anti-inflammatory and anti-apoptotic activity is also dominant. For example, doxycycline inhibits the neuron-inflammation after

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hypoxia-ischemia in neonatal rats (Jantzie and Todd, 2010) and exerts neuroprotective effect in brain and cochlea in experimental pneumococcal meningitis (Meli et al., 2006 Jul). And the *in vivo* test has proven that doxycycline increases neurogenesis and reduces microglia in the adult hippocampus (Sultan et al., 2013). Although doxycycline displays significant anti-inflammatory and neuroprotective activity by regulating cytokines levels and inhibiting matrix metalloproteinases activity (Nukarinen et al., 2015), the target protein or receptor of doxycycline is still not explicit till now.

PACAP (pituitary adenylate cyclase-activating polypeptide) was first isolated from ovine hypothalamic extract on the basis of its ability to stimulate cAMP formation in anterior pituitary cells (Miyata et al., 1989), which belongs to the vasoactive intestinal polypeptide (VIP)/secretin/growth hormone releasing hormone/glucagon superfamily (Harmar et al., 1998). PACAP has two forms: a 38-amino-acid form PACAP38 and its C-terminal truncated form PACAP27, which has 68% amino acid homology with VIP (Harmar et al., 1998). PACAP elicits various biological actions via three types of G protein-coupled receptor (GPCR), a PACAP-preferring receptor 1 (PAC1) and two VIP-shared receptors (VPAC1 and VPAC2) termed by the International Union of Pharmacology according to their relative affinity for PACAP and VIP (Harmar et al., 1998). PAC1 has affinity for PACAP almost 1000 folds higher than for VIP, whereas VPAC receptors recognize two peptides with similar affinity (Vaudry et al., 2009). PACAP preferring receptor PAC1 is abundantly located in the central nervous system and peripheral nervous system, mediates the significant anti-apoptotic (Seaborn et al., 2011), anti-inflammatory (Banki et al., 2014; Martínez et al., 2006), neurogenetic (Nakajima et al., 2013) and neuroprotective (Bourgault et al., 2009a) effect of PACAP. So PAC1 is considered as a drug target for nervous system disease, especially for the neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Yang et al., 2015).

As shown above, the neuroprotective effect mediated by PAC1 in nervous system is similar to that of doxycycline, indicating some possible relationship between doxycycline and PAC1. And a recent report showed that doxycycline and PAC1 antagonist PACAP(6–38) exerted similar effects on kainic acid induced seizures also indicates the possible relationship between doxycycline and PAC1 (Bhandare et al., 2015). By accident, it was found by us for the first time that doxycycline promoted the proliferation of the Chinese hamster ovary (CHO) cells with stable high expression of PAC1 significantly, which was inhibited by shRNA for PAC1. Moreover, it was also observed for the first time that doxycycline induced the internalization of PAC1-YFP (YFP, yellow fluorescent protein), and the internalization of GPCRs is always involved in their activation. So we hypothesized that doxycycline might exert the neuroprotective effect by interacting with PAC1. The verification of this hypothesis may not only help explain the clinical neuroprotective effect of doxycycline and its analogues, but also push forward the further clinical development of doxycycline and its analogues in nervous system diseases targeting PAC1.

In order to verify the hypothesis that doxycycline interacts with PAC1, the computer molecular docking was firstly used to predict the theoretical binding of doxycycline to PAC1. The result of molecular docking after the homology modeling of PAC1 indicated that doxycycline bond PAC1 at the site PACAP(30–37) recognized. Then the competition binding assay and site-specific mutation of PAC1 were further used to confirm the targeting of doxycycline to PAC1 imitating PACAP(30–37). And the assays on the synergic effect of doxycycline with VIP or oligopeptide HSDGIF in PAC1-CHO cells were used to detect the role of doxycycline as an enhancer by facilitating the PAC1 binding by its ligands imitating PACAP(28–38). Moreover the proliferative and anti-apoptotic activities of doxycycline in Neuro2a cells were determined combined with cAMP-PKA inhibitor H-89, PAC1 shRNA and PACAP antagonist PACAP(6–38) to further verify doxycycline acted as an enhancer on the activity of PAC1. At last, the scopolamine induce amnesia combined with step-through test was used to test the *in vivo* effect of doxycycline on PAC1. Furthermore, the molecular docking of doxycycline analogue minocycline with the same neuroprotective effect as doxycycline onto PAC1 combined with some experiment data confirmed the binding of minocycline to PAC1 at the similar site to doxycycline.

2. Materials and methods

2.1. Homology modeling

In this work, only the extracellular N-terminus and seven-transmembrane domain of the human PAC1 were modeled, because the intracellular C-terminus is unrelated with the binding of small molecule agonists or antagonists to PAC1. The sequence of PAC1 was obtained from the Universal Protein Resource Knowledgebase (UniProtKB, http://www.uniprot.org/) with accession number P41586-3. Actually, the 3D structure of extracellular N-terminus has been resolved (PDB code: 2JOD and 3N94). The 3D structure 2JOD which is the complex structure of extracellular N-terminus and PACAP38 was chosen. The crystal structure of human glucagon class B G protein coupled receptor (GCGR) with PDB code 4L6R was selected as template to model the seven-transmembrane region of PAC1. Homology modeling was performed using the Modeler program in Discovery Studio 2.5 (DS2.5). The model with the best score was then minimized by 5000 steps of steepest descent and 2000 steps of conjugate gradient. Finally, the ProCheck procedure was used to verify the quality of the model.

2.2. Molecular docking

The optimized PAC1 3D structure acquired from homology modeling was selected as the initial conformation for the docking study. The binding sites were defined according to the complex structure of PAC1 N-terminus and PACAP38 (PDB code: 2JOD). The preprocessing of the PAC1 3D structure was implemented using DS2.5, such as hydrogenation and applying Charm Forcefield. Hydrazide, which has been identified as a small molecular antagonist of PAC1 (Beebe et al., 2008) was used as a control in molecular docking. The 3D structures of hydrazide, doxycycline and minocycline were sketched in DS2.5, and stored structure data files following energy minimization. The docking procedure was implemented using LibDock program of the DS simulation software package. LigScore1, LigScore2, PLP1, PLP2, Jain, PMF and PMF04 were used to scoring receptor-ligand binding affinity. PyMol 1.5 (Schrödinger LLC, Portland, OR, USA) was used for visual inspection of the results and the graphical representations.

2.3. Fluorescence confocal microscopy

Cellular trafficking of PAC1 was observed by visualizing fluorescence in CHO cells expressing YFP-tagged PAC1 which had been constructed previously (Yu et al., 2012). The cells expressing PAC1-YFP grown on Petri dish were mounted on microscopic slides. YFP fluorescence was acquired using appropriate spectral settings (excitation, 488 nm argon laser; emission, 545 nm filter; pinhole diameter 2.3 airy units) of the confocal microscope (LSM 510 META; Zeiss, Thornwood, NY) equipped with a Plan-Apochromat63 × /1.4 numerical aperture oil objective. Fluorescent images were collected at 0min, 5min and 10 min after the addition of doxycycline (100 ng/mL).