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Cell and brain tissue imaging of the flavonoid fisetin using label-free two-photon microscopy

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ABSTRACT

Over the last few years, we have identified an orally active, novel neuroprotective and cognition–enhancing molecule, the flavonoid fisetin. Fisetin not only has direct antioxidant activity but it can also increase the intracellular levels of glutathione, the major intracellular antioxidant. Fisetin can also activate key neurotrophic factor signaling pathways. In addition, it has anti-inflammatory activity against microglia and astrocytes and inhibits the activity of lipoxygenases, thereby reducing the production of pro-inflammatory eicosanoids and their by-products. However, key questions about its targets and brain penetration remain. In this study, we used label-free two-photon microscopy of intrinsic fisetin fluorescence to examine the localization of fisetin in living nerve cells and the brains of living mice. In cells, fisetin but not structurally related flavonols with different numbers of hydroxyl groups, localized to the nucleoli suggesting that key targets of fisetin may reside in this organelle. In the mouse brain, following intraperitoneal injection and oral administration, fisetin rapidly distributed to the blood vessels of the brain followed by a slower dispersion into the brain parenchyma. Thus, these results provide further support for the effects of fisetin on brain function. In addition, they suggest that label-free two-photon microscopy may prove useful for studying the intracellular and tissue distribution of other intrinsically-fluorescent flavonoids.

1. Introduction

The flavonoid fisetin (3,7,3′,4′-tetrahydroxyflavone) is an orally active, novel neuroprotective and cognition–enhancing molecule (Maher, 2009). Fisetin protects nerve cells from multiple toxic insults including amyloid toxicity, ischemia and hyperglycemia. It has both direct antioxidant activity and maintains glutathione, the major intracellular antioxidant, under conditions of stress by inducing the transcription factors, Nrf2 and ATF4 (Ehren and Maher, 2013). Fisetin is also able to maintain ATP levels under ischemic conditions (Maher et al., 2007). In addition, fisetin possesses neurotrophic activity and can induce neurite outgrowth (Sagara et al., 2004). Further studies have shown that fisetin facilitates long-term potentiation (LTP) in hippocampal slices via modulation of ERK and CREB phosphorylation and that oral administration promotes learning and memory in mice using the object recognition test (Maher et al., 2006). Using three different models of Huntington's disease (mutant huntingtin-expressing PC12 cells, mutant huntingtin-expressing Drosophila and the R6/2 mouse) we found that fisetin was able to reduce the impact of mutant huntingtin in each of these disease models (Maher et al., 2011a). Fisetin is also effective in two different models of stroke (Gelderblom et al., 2012; Maher et al., 2007). Moreover, fisetin reduces markers of CNS inflammation and prevents learning and memory deficits in AD transgenic mice (Currais et al., 2014).

Despite these clear effects of fisetin on the CNS, there is an ongoing debate about whether flavonoids such as fisetin actually get into the brain (Faria et al., 2012; Schaffer and Halliwell, 2012). Although pharmacokinetic studies have provided some evidence for brain penetration, more direct evidence is needed especially as it is possible that many of the effects of fisetin could be mediated by its actions on the microvasculature. A promising approach to address this question is *in vivo* two-photon excited fluorescence (TPEF) imaging.

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Fisetin is an efficient fluorophore due to its conjugated ring structure and its fluorescence emission spectrum can be distinguished from endogenous tissue fluorophores such as NADH and FAD+ based on both intensity and spectral features. In this study, we report on the detection of fisetin both in nerve cells in culture and in the brain following both intraperitoneal and oral administration using label-free TPEF microscopy. To the best of our knowledge, this is the first study with any flavonoid that provides direct evidence for the entrance of the molecule into the brain parenchyma.

2. Materials and methods

2.1. Cell culture experiments

Mouse hippocampal HT22 nerve cells were plated at a density of 7.5×10^3 to 1×10^4 cells per 35 mm glass culture dish coated with 50 μg/mL polyornithine. The cells were grown for ~48 h in phenol red-free DMEM (Gibco 31053) containing 4 mM L-glutamine, 25 mM HEPES, 1 mM pyruvate and 10% FCS. The cells were treated with 10 μM of the compounds dissolved in DMSO (for a total addition of 10 μL) or 10 μL of DMSO as a control.

2.2. Animal studies

The fisetin solution for intraperitoneal injection was prepared in PEG200:DMSO (Sigma) (7:3), filtered through a 0.45 μm filter and then sonicated in a water bath sonicator. For administration by gavage, the fisetin solution was prepared in 10% ethanol: 40% Solutol HS15:50% PBS and administered at 25 mg/kg as described (Maher et al., 2006). The preparation of the mice was as described (Lin et al., 2014) except that the mice were anesthetized using ketamine (10 mg/ml)-xylazine (1.49 mg/ml). Briefly, C57Bl6 mice were placed on a heating pad in a stereotactic frame. The scalp was removed to create a field of view and the skull was thinned. Vaseline was used to create a saline well and covered by a glass cover slip to aid in two-photon imaging. All procedures were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.3. In vitro two-photon excited fluorescence imaging

Label-free two-photon excited fluorescence (TPEF) emission spectra were obtained using the 32-channel Meta detector of the Zeiss LSM 510 Meta NLO microscopy system equipped with a Zeiss EC Plan-Neofluar (100×, Ph3, n.a.1.3) oil immersion objective. Detector (Hamamatsu PMTs) settings were chosen to keep the signal intensity on a linear part of the response curve. TPEF excitation at 780 nm was generated using a Chameleon-Ultra femtosecond pulsed tunable laser (Coherent Inc.). This particular wavelength was chosen to be close to the maximum according to the known one-photon excitation spectrum of fisetin (Sengupta et al., 2005). Two channels were acquired in imaging mode: the blue channel (390–465 nm) detected free fisetin emission in an aqueous environment ($\lambda_{max} = 460$ nm) while the green channel (500–550 nm range) detected the emission of protein-bound fisetin ($\lambda_{max} = 540$ nm) (Sengupta et al., 2005). The detector settings of both channels were chosen to minimize the fluorescence signal of cellular NAD(P)H and FAD so detector sensitivity was set by imaging control cells. These settings were established and used in all experiments. Laser scanning did not induce any visible damage to the cells or noticeable bleaching of the sample.

2.4. In vivo two-photon excited fluorescence imaging

Microscopy imaging and measurements of fisetin fluorescence emission spectra in mouse brain *in vivo* were performed on a Zeiss LSM NL META microscope equipped with an objective inverter (LSM Tech. Inc, Stewartstown, Pennsylvania). The inverter converts any inverted microscope into an upright configuration by simultaneously changing the orientation of the objective and moving the sample off of the stage. This allows maximum flexibility in sample positioning and handling while still retaining the ability to automatically acquire z-stacks. A mouse was positioned by a stereotactic device, which was placed on a small platform next to the microscope stage; a round coverslip with a ring glued to its surface was placed on the skull opening and a drop of water added on top of it to provide a matching refractive index media for a chosen objective (Fig. 1). The objective lens for the *in vivo* experiments was a long working distance Zeiss 40x 0.8 na water immersion objective. The maximum depth for imaging was up to 80 μm from the brain surface using an excitation wavelength of 780 nm (Chameleon-Ultra Titanium:Sapphire laser, Coherent, Inc.). Spectral acquisition was obtained using a META polychromatic 32-channel detector.

3. Results

Emission spectra for fisetin in DMSO were obtained using two-photon excitation at 780 nm (Fig. 2). This wavelength was chosen based on the reported one-photon excitation spectrum of fisetin (Sengupta et al., 2005). A distinct emission peak at ~550 nm as well as an expected blue-shifted peak at 460 nm were observed. These results were similar to those reported using conventional one-photon excitation (Sengupta et al., 2005).

Fisetin was solubilized in DMSO and added to the medium of HT22 mouse hippocampal nerve cells (10 μM) in order to determine its detectability in cultured cells. The cells were examined by TPEF microscopy both before the addition of fisetin and at increasing times after the addition of the flavonoid. To distinguish the fluorescence emission of fisetin from endogenous NADH (blue channel) and FAD+ (green channel) signals, cells were treated with an equal amount of DMSO and imaged 30 min after treatment (Fig. 3a). The exact same parameters (scanner, detector, laser power) were used in the imaging of fisetin-treated cells. As shown in Fig. 3, after

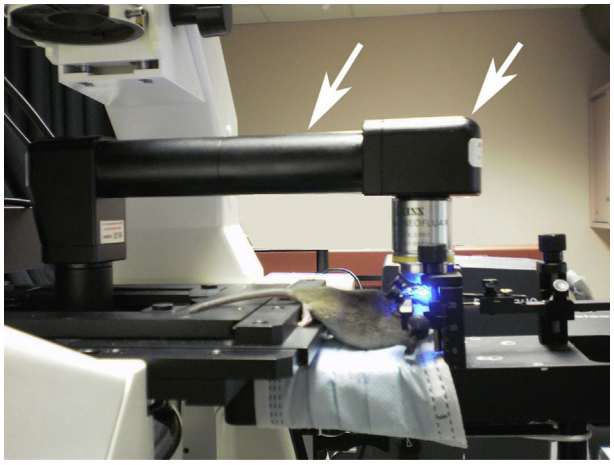

Fig. 1. Zeiss LSM 510 microscopy system equipped with objective inverter for the *in vivo* study. Shown is the general layout of the experimental set-up and, in particular, the one unusual optical element in it, an objective inverter, which is indicated by the white arrows.