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CAFFEINE AND URIC ACID MEDIATE GLUTATHIONE SYNTHESIS FOR NEUROPROTECTION

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Abstract—Several lines of epidemiological studies have indicated that caffeine consumption and plasma uric acid (UA) level were negatively correlated with the incidence of some neurodegenerative diseases. We report here a novel mechanism by which these purine derivatives increase neuronal glutathione (GSH) synthesis. Intraperitoneal injection of caffeine or UA into male C57BL/6 mice significantly increased total GSH levels in the hippocampus. Neither SCH58261, an adenosine A2A receptor antagonist, nor rolipram, a phosphodiesterase-4 inhibitor, increased GSH levels. Pretreatment with allopurinol, a drug to inhibit UA production, did not change the GSH level in the caffeine-treated mice. Hippocampal CA1 pyramidal neurons treated with caffeine or UA were resistant to oxidant exposure in the slice culture experiments. In experiments with the SH-SY5Y cell line, cysteine uptake was sodium-dependent and pretreatment with caffeine or UA increased cysteine uptake significantly as compared with the control conditions. Slice culture experiments using the hippocampus also showed increased cysteine and GSH contents after the treatment with caffeine or UA. Immunohistochemical analysis showed increased GSH levels in the hippocampal excitatory amino acid carrier-1 (EAAC1)-positive neurons of mice treated with caffeine or UA. These findings suggest that purine derivatives caffeine and UA induce neuronal GSH synthesis by promoting cysteine uptake, leading to neuroprotection.

Key words: caffeine, uric acid, glutathione, neuroprotection, cysteine uptake, EAAC1.

Caffeine, 1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione, is the most favorable psychostimulant in beverages or foods for motor activation, mood changes, information processing and cognitive/motor performances (Fredholm et al., 1999). Several lines of epidemiological studies have indicated that caffeine consumption was negatively correlated with the incidence of some neurodegenerative diseases (Ascherio et al., 2001; Lindsay et al., 2002; Maia and de Mendonca, 2002). Experimental data have also demonstrated caffeine's neuroprotective effects on a variety of neurotoxins (Chen et al., 2001; Dall'Igna et al., 2003, 2007; Arendash et al., 2006). Caffeine is structurally similar to adenosine, an endogenous inhibitory neuromodulator, and binds to adenosine receptors to act as a nonselective antagonist (Fredholm et al., 1999). Most studies have suggested that caffeine has neuroprotective effects as an adenosine receptor antagonist (Chen et al., 2001; Dall'Igna et al., 2003, 2007; Arendash et al., 2006; Canas et al., 2009). However, caffeine-mediated neuroprotection is not exclusively attributable to the blockade of adenosine receptors (Alvira et al., 2007; Bateup et al., 2008; Nakaso et al., 2008). Caffeine might have other pharmacological effects against neurodegeneration in the CNS, although the precise entity is still elusive.

As another purine derivative, uric acid [UA; 7,9-dihydro-1H-purine-2,6,8(3H)-trion] is the final product of purine metabolism in human. Epidemiological studies have linked a reduced UA level in plasma to some neurodegenerative diseases (Kutzing and Firestein, 2008). Although UA has been widely known as an antioxidant in blood (Ames et al., 1981), the precise mechanism in the CNS is still unclear.

The brain is one of the major organs that generate large amounts of reactive oxygen species (ROS). Compared with other organs, the brain is especially vulnerable to oxidative stress because of its lower antioxidant enzyme activities, while it contains high quantities of lipids with unsaturated fatty acids, which are targets of lipid peroxidation (Dringen, 2000). Under normal conditions, the brain can equilibrate the generated ROS with its own antioxidant defense. Glutathione (GSH) is the most abundant thiol-reducing agent that plays a critical role as a major antioxidant in the CNS (Dringen, 2000). However, excessive production of ROS or a reduced antioxidant system induces oxidative stress to cause neurodegeneration. Some neurodegenerative diseases showed brain GSH depletion (Sian et al., 1994; Ramassamy et al., 2000), which is considered as an early event toward progression of the diseases (Jenner, 1994).

GSH is a tripeptide composed of glutamate, cysteine, and glycine. Neuronal GSH synthesis is exclusively regulated by cysteine, but not glutamate or glycine, uptake (Dringen et al., 1999; Dringen, 2000). In mature neurons, approximately 90% of total cysteine uptake is mediated by sodium-dependent systems, mainly by excitatory amino acid transporter (EAAT) (Shanker et al., 2001). Excitatory amino acid carrier-1 (EAAC1) is a neuronal EAAT widely expressed in the brain (Zerangue and Kavanaugh, 1996). EAAC1-deficient mice showed an approximately 40% de-

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Abbreviations: aCSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; BSO, buthionine sulphoximine; CMFDA, chloromethylfluorescein diacetate; DHK, dihydrokainic acid; DTT, dithiothreitol; EAAC1, excitatory amino acid carrier-1; EAAT, excitatory amino acid transporter; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; GSSG, glutathione disulfide; PBS, phosphate-buffered saline; PD, Parkinson's disease; PDE, phosphodiesterase; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine; UA, uric acid.

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creased GSH content, increased oxidant levels and increased susceptibility to oxidative stress in the brain (Aoyama et al., 2006). EAAC1 dysfunction could impair neuronal cysteine uptake to reduce GSH synthesis, leading to neurodegeneration (Aoyama et al., 2006; Li et al., 2010).

Here we describe a novel function of two purine derivatives, caffeine and UA, to protect neurons by inducing GSH synthesis via increased cysteine uptake.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 male mice aged 4 weeks or 8 weeks were used for slice culture experiments or drug-injection experiments, respectively. All mice were kept in a temperature-controlled room at 23 °C under a 12 h light/dark cycle with food and water available *ad libitum*. The protocols were approved by the Animal Ethics Committee of Teikyo University School of Medicine.

Drug manipulations *in vivo*

Mice were fasted overnight before the experiments and were administered 10 or 40 mg/kg caffeine (Sigma, Tokyo, Japan) or 10 mg/kg UA (Sigma) by i.p. injection 1–4 h before their brains were harvested. Different doses of SCH58261 (Tocris, Ellisville, MO, USA), an adenosine A_{2A} receptor antagonist, were injected i.p. 1–2 h before the harvest or 1.25 mg/kg rolipram (Sigma), a specific phosphodiesterase (PDE)-4 inhibitor, 2 h before the harvest. Allopurinol (Sigma) was pre-injected at a dose of 4 mg/kg i.p. 30 min before injection with 10 mg/kg caffeine. Control mice were injected with the same volume of saline i.p. in each experiment.

Total GSH assay

Total GSH [reduced GSH plus GSH disulfide (GSSG)] was measured by the NADPH-dependent GSH reductase method as previously described (Tietze, 1969; Watabe et al., 2008). Hippocampus samples were homogenized with ice-cold 5% sulfosalicylic acid to precipitate cellular macromolecules and extract GSH from both cells and tissues. After centrifugation at 14,400 rpm for 15 min, the supernatant solution was used for measurements. Brain solutions were diluted with phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and adjusted to pH 7.0. A reaction mixture containing 1 mM EDTA/0.3 mM dithionitrobenzoic acid/0.4 mM NADPH/2 IU/ml GSH reductase was added to the same volume of diluted brain solution and GSH standard solutions. Total absorbance at 405 nm was measured and calibrated to GSH standards.

Nitrosative stress in hippocampal slice culture

Caffeine or UA was administered i.p. at a dose of 10 mg/kg, and hippocampal slice culture experiments were started 2 h later. Control mice were injected with the same volume of saline i.p.. After decapitation, the brain was cut into 300-μm-thick slices in gassed (95% oxygen/5% CO_2) ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM) NaCl 130; KCl 3.5; NaH_2PO_4 1.25; $MgSO_4$ 2; $CaCl_2$ 2; $NaHCO_3$ 20; glucose 10 at pH 7.4. All of the experiments were initiated by transferring hippocampal slices to tubes at 30 °C, each of which contained aCSF that was continuously bubbled with 95% O_2/5% CO_2. Hippocampal slices were exposed to 1 mM 3-morpholinosydnonimine (SIN-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA), an NO donor, for 30 min and were fixed in 4% paraformaldehyde and then stored in 30% sucrose for nitrotyrosine staining. Fixed sections were incubated overnight at 4 °C with 5 μg/ml of mouse anti-nitrotyrosine antibody (Upstate, Lake Placid, NY, USA). After washing with PBS, the slices were incubated for 2 h with a 1:1000 dilution of Alexa Fluor 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). The sections were mounted using a ProLong Antifade Kit (Molecular Probes) and photographed with an FV1000 laser-scanning fluorescent microscope using FV10-ASW software (Olympus, Tokyo, Japan). Nitrotyrosine signal intensity was measured in the hippocampal cell body layer of each slice by an observer blinded to the experimental groups. The intensity was represented as the ratio of the mean fluorescence to that of a hippocampal slice from the same brain without SIN-1 treatment.

Cell culture experiment

SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum at 37 °C under 5% CO_2 in air. SH-SY5Y cells were treated with 100 μM caffeine or 3 μM UA for 3 h. After washing with PBS, cells were incubated with OPTI-MEM (Invitrogen, Tokyo, Japan) containing 10 μM cysteine and 100 μM dithiothreitol (DTT) for 5 min. For studies on the sodium-dependence of cysteine uptake, cells were placed in sodium-free medium containing 122 mM $C_5H_{14}NOCl$ (choline chloride)/3.3 mM KCl/0.4 mM $MgSO_4$/1.3 mM $CaCl_2$/1.2 mM KH_2PO_4 (pH was adjusted to 7.4 with KOH). After the medium was collected, cysteine amount in it was measured using an HPLC-fluorescence detection system.

Slice culture experiment for cysteine/GSH measurements

Methods for hippocampal slice culture were described as above. The slices were pre-incubated with different concentrations of caffeine or UA for 30 min and then co-incubated with 100 μM cysteine/DTT for 15 min. In the experiments using dihydrokainic acid (DHK, Tocris), the slices were pre-incubated for 30 min with 100 μM DHK plus 0.1 mM caffeine or 3 μM UA. In the experiments using L-buthionine sulfoximine (BSO, Sigma), the slices were pre-incubated for 30 min with or without 500 μM BSO plus 0.1 mM caffeine or 10 μM UA and then co-incubated with 100 μM cysteine/DTT for 30 min. After incubation, the slices were washed with aCSF containing 100 μM DTT on ice and frozen at −80 °C until HPLC analysis.

HPLC-fluorescence detection

Brain tissues were homogenized with a 10-fold volume of 5% trichloroacetic acid containing 5 mM Na_2EDTA and centrifuged at 14,400 rpm for 15 min. The supernatants were used for measurements. Tissue cysteine and GSH were detected with 4-fluoro-7-sulfamoylbenzofurazan (Dojindo, Kumamoto, Japan), a fluorogenic labeling reagent for thiols, according to the protocol. The LC-20AD liquid chromatography system (Shimadzu, Kyoto, Japan) was used for detection. An analytical column, Inertsil ODS-2 (150×4.6 mm ID 5 μm) (GL Sciences, Tokyo, Japan), was fixed at 40 °C and connected through a corresponding guard column (10×4.0 mm ID 5 μm) (GL Sciences). A stepwise gradient elution was programmed with solvents A (50 mM potassium biphthalate at pH 4.0) and B (8% acetonitrile in solvent A). The mobile phase was held at 80% solvent A and 20% B for 6 min followed by a 10 min program held at 100% solvent B. The flow rate of the eluate was 1.0 ml/min. All samples were injected into the column with an Auto Injector (Shimadzu). An RF-530 fluorescence spectrometer (Shimadzu) was used with excitation and emission at 380 nm and 510 nm, respectively. The signals from the detector were recorded on a Chromatopac C-R4A (Shimadzu). Tissue cysteine concentrations were calculated by the peak area standardized with known amounts of cysteine.