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Research Report

Involvement of caspase cascade in capsaicin-induced apoptosis of dorsal root ganglion neurons

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Abstract

Capsaicin induces apoptosis in some types of neurons, but the exact molecular mechanism remains unclear. In this study, capsaicin was systemically administrated in newborn rats and the dorsal root ganglion (DRG) neurons were examined for caspase-immunoreactivity. Capsaicin-induced neuronal apoptosis was revealed by TUNEL. TUNEL-positive neurons rapidly increased, reaching the peak at 24 h post-injection when 10.6% of DRG neurons were apoptotic. Neurons expressing immunoreactivity for activated caspases-9 and -3 concomitantly increased. At 24 h, 15.9% and 17.7% of DRG neurons exhibited immunoreactivity for caspase-9 and caspase-3, respectively. DNA fragmentation signal and caspase-immunoreactivity were detected in less than 0.5% of DRG neurons of vehicle control rats. The immunoreactivity and TUNEL-positivity returned to the vehicle control level by 120 h. Double label immunohistochemistry revealed co-expression of caspase-9 and DNA fragmentation or caspase-3 and DNA fragmentation. These results suggest that the caspase cascade is involved in the primary neuronal apoptosis induced by neurotoxin capsaicin.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: Caspase-3; Caspase-9; Apoptosis; Dorsal root ganglion; Capsaicin; Newborn rat; TUNEL

1. Introduction

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a major pungent ingredient of red peppers. Its pharmacological and physiological effects have been widely investigated for many years. Recently, capsaicin was reported to efficiently induce apoptosis in some types of cells including glioma cells, hepatocarcinoma cells, rat thymocytes, and human B-cells [1,8,15,25]. In addition, our previous study also demonstrated that neonatal capsaicin treatment induced apoptotic cell death in rat trigeminal primary neurons [17,19]. However, the exact molecular mechanism underlying the capsaicin-induced apoptotic cell death remains unclear.

The caspase family of cysteine protease is involved in apoptotic mechanisms in a wide variety of cells in vertebrate species. In the nervous system, caspase activity has been demonstrated in neuronal apoptosis following peripheral nerve injury [20], ischemia [4], and in normal development [9]. Recent investigations have established that cytochrome *c*, Apaf-1, caspase-9 (ICE-LAP6, Mch6) and caspase-3 (CPP32, apopain, YAMA) form an evolutionary conserved cell death pathway in the mammalian nervous system [9].

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When cells receive apoptotic stimuli, cytochrome *c* is released from the intermembrane space of the mitochondria, and binds to Apaf-1 together with ATP. The resultant complex enhances autoactivation of procaspase-9 (47 kDa) into its active forms (35 kDa or 17 kDa). Thus activated caspase-9 cleaves downstream procaspase-3 (P32) into active caspase-3 fragments (P20/17 and P12) [3,4,9]. The activated caspase-3 affects a number of targets including the DNA repair enzyme poly-ADP-ribose polymerase (PARP). Inactivation of PARP, in turn, accelerates DNA fragmentation characterizing apoptotic cell death [14].

In this study, we investigated the potential involvement of caspases-9 and -3 activation in apoptosis of rat dorsal root ganglion (DRG) neurons induced by neonatal capsaicin. The active forms of caspases-9 and -3 were detected by immunohistochemistry, and apoptosis by the terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method.

2. Materials and methods

A total of 32 Sprague–Dawley rat pups of either sex were obtained from 6 litters. Twenty-eight pups were given a single dose of 50 mg/kg of capsaicin on postnatal day 1 (12–24 h after birth) by subcutaneous injection into the nape. Under hypothermic anesthesia, capsaicin was given as a 1.5% (W/V) solution in a vehicle consisting of 10% (V/V) alcohol, 10% Tween-80 and 80% saline. At post-injection intervals of 12, 16, 20, 24, 48, 72, and 120 h, the rats were anesthetized by ether inhalation to the level at which respiration was markedly suppressed, and perfused through the left ventricle with saline for 20–40 s for exsanguination followed by 0.1 M sodium phosphate buffer containing 4% formaldehyde (prepared fresh from paraformaldehyde, pH 7.3). For vehicle control, 4 newborn rats were given an equivalent volume of vehicle, and killed at 24 h post-injection. In addition to the above, one adult rat (b.w. = 200 g) received capsaicin injection under anesthesia by i.p. injection with a mixture of pentobarbital sodium (20 mg/kg) and ethyl carbamate (650 mg/kg), and was killed 24 h later. The 3rd, 4th, and 5th lumbar (L3, L4, L5) DRGs were dissected and further fixed in a fresh volume of fixative overnight at 4 °C. They were cryoprotected by immersing in 0.02 M phosphate-buffered saline (PBS, pH 7.3) containing 20% sucrose. Horizontal cryosections of 10 μm in thickness were mounted on silan-coated glass slides. The complete series of sections were divided into 10 subseries so that every tenth section was mounted on the same slides.

The sections were thawed, incubated for 1 h with 0.3% H_2O_2 in 80% methanol for quenching endogenous peroxidase activity, and further processed for TUNEL or immunohistochemistry for caspase-9 or -3. Immunohistochemistry was performed using the avidin–biotin–horseradish peroxidase complex (ABC) method. The sections were thoroughly rinsed with PBS and incubated with rabbit antibodies that recognize cleaved caspase-3 (17–20 kDa) (1:3000, Cell Signaling Technology, USA) or cleaved caspase-9 (17 kDa or 38 kDa with prodomain) (1:3000, Cell Signaling Technology, USA) for 40 h at 4 °C. For negative control, the primary antibody was substituted with an equivalent volume of PBS. The sections were sequentially incubated with biotinylated goat anti-rabbit IgG and ABC complex according to the manufacturer's instruction (Vectastain ABC kit, Vector Laboratories, USA). The label was visualized by nickel ammonium sulfate-intensified diaminobenzidine (DAB) histochemistry. The sections were counter-stained with methyl green. TUNEL was performed for the detection of fragmented DNA. After thorough rinsing with Tris–HCl-buffered saline (TBS, pH 7.4), the sections were incubated with a tailing buffer (25 mM Tris–HCl buffer, 1 mM $COCl_2$, 200 mM sodium cacodylate, 0.025% bovine serum albumin, pH 6.6) containing 0.1 mM dATP, 0.01 mM biotin-16-dUTP and 250 U/ml terminal deoxyribonucleotidyl transferase from calf thymus (Boehringer-Mannheim, FRG) for 70 min at 37 °C. The enzymatic reaction was stopped by rinsing with PBS. For negative control, either the enzyme or biotin-16-dUTP was omitted from the system. The sections were further reacted with ABC complex, and the label was visualized by nickel ammonium sulfate-intensified DAB histochemistry. The sections were counter-stained with cresyl violet. DAB reaction was not detected in negative controls for either immunohistochemistry or TUNEL.

For numerical analysis, every tenth section of the L4 DRG on one side was used for each rat. Unambiguously identified immunoreactive (ir) or TUNEL-positive neuron profiles and apparently healthy neurons were counted and the proportion of positively stained neurons to the total number was recorded for each rat. The data from capsaicin-treated rats ($n = 4$ for each survival period) were compared with those obtained from vehicle-treated rats at 24 h ($n = 4$) by the use of one-way analysis of variance (ANOVA).

Sections obtained from two rats, which survived for 24 h after the capsaicin treatment, were processed for a sequential double staining procedure for detecting caspase-9-immunoreactivity (ir) or caspase-3-ir and DNA fragmentation signal in the same sections. The sections were first processed for indirect immunofluorescence. They were incubated with the primary antibody for caspase-9-ir or caspase-3-ir (1:200) for 24 h at room temperature followed by lissamine rhodamine B chloride-conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch Laboratories, USA). The sections were coverslipped with glycerin and photomicrographs were taken. Thereafter, the coverslip was removed and the sections were processed for TUNEL as described above.

The experiments were carried out under the control of the Animal Research Control Committee in accordance with The Guidelines for Animal Experiments of Okayama University Medical School, Government Animal Protection and Management Law (No. 105), and Japanese Government Notification on Feeding and Safekeeping of Animals (No.