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Article ID: WMC003708

Article Type: Original Articles

Submitted on: 18-Sep-2012, 10:38:59 AM GMT **Published on:** 18-Sep-2012, 12:58:10 PM GMT

Article URL: http://www.webmedcentral.com/article_view/3708

Subject Categories: TOXICOLOGY

Keywords: Capsaicin, Apoptosis, DNA ladder, Okadaic acid

How to cite the article: Hiura A, Nakagawa H. Analysis of DNA Extracted From the Trigeminal Ganglion Cells After Neonatal Capsaicin Treatment by Agarose Gel Electrophoresis . WebmedCentral TOXICOLOGY 2012;3(9):WMC003708

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Source(s) of Funding:

No source of funding

Competing Interests:

No competing interests

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Abstract

The aim of this study was to examine whether the neonatal injection of capsaicin induces DNA ladder formation, as an indicator of apoptosis, in the rat trigeminal ganglion (TG). The DNA extracted from the TGs after treatment with capsaicin (50 mg/kg) at 2 days of age exhibited diffuse DNA fragmentation instead of "ladder formation", as determined by agarose gel electrophoresis. In contrast, clear DNA ladder formation was induced in human osteosarcoma cells treated with okadaic acid (OA) at concentrations of 10 and 20 nM. These results suggest that capsaicin cannot induce apoptosis of TG neurons sufficient for detectable DNA ladder formation. Thus, it is conceivable that capsaicin mainly causes necrosis rather than apoptosis in rodent primary sensory neurons.

Introduction

Previously, we reported that neonatal treatment with capsaicin (50 mg/kg), a component of the pungent red pepper, selectively destroys small-type sensory neurons in the mouse dorsal root ganglion (DRG) [1], and results in the permanent loss of approximately 50% of lumbar DRG cells (70% in smaller cells) [2]. In the case of mouse DRGs, the first sign of degeneration induced by capsaicin is cytolysis but not nuclear morphological changes [1]. The features of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) –positive apoptotic cell death are scarcely seen (at most 1-2 cells/section 24 h after treatment) [3] within 3 days after treatment with capsaicin [1]. From the above results, it was concluded that the majority of cell deaths by capsaicin are due to necrosis rather than apoptosis, at least in mice [3].

There are a few reports demonstrating that neonatal capsaicin injection (50 mg/kg) induces apoptotic cell death in approximately 10% of trigeminal ganglion (TG) cells [4,5] 24 h after treatment. Recently, it was reported that TUNEL-positive DRG cells reached a

peak at 24 h after neonatal capsaicin treatment, with capsaicin coinciding with the apoptosis of 10.6% of DRG neurons of rats [6]. Moreover, about 17% of DRG neurons exhibited immunoreactivity for caspase (cysteine protease)-3 and -9, indicating their involvement in the capsaicin-induced apoptotic pathway of the primary sensory neurons [6].

Apoptotic cells undergo DNA fragmentation: multiple double-strand cleavage of nuclear DNA at the intra-nucleosomal DNA (linker regions) into fragments of 200-1400 bp [7], resulting in a characteristic "ladder" pattern on agarose gel electrophoresis [8]. The purpose of the present study was to reveal whether the DNA ladder as a signal for apoptosis is visible or not after neonatal capsaicin treatment using the rat trigeminal ganglion.

Materials and Methods

Animals:

Neonatal Wistar rats born in the Experimental Animal Institute of the School of Dentistry, University of Tokushima, were used in these studies. The animals were housed at a temperature of 25°C under a 12:12-h light/dark cycle. The present protocol was approved by the Committee on Experimental Animals and the Ethical Treatment of Animals of the University of Tokushima. The parental animals had free access to food pellets, and water was available ad libitum.

First round experiment:

Five and seven rats 2 days after birth were subcutaneously (sc) injected with capsaicin (50 mg/kg, Nacalai Tesque Inc., Kyoto, Japan) or vehicle (containing 10% ethanol and 10% Tween 80 in saline), respectively. After 24 h, 10 bilateral trigeminal ganglions (TGs) and 14 bilateral TGs were extracted under ether anesthesia. Excised TGs were washed in 0.1 M phosphate-buffered saline (PBS), and then incubated in 0.1% collagenase in 0.1 M PBS (37°C, 40 min), followed by incubation in 0.05% trypsin in 0.1 M PBS (37°C, 40 min). After repeated pipetting, TG cells

were transferred to the vial for centrifugation. Floating TG cells were harvested by a few centrifugations (3,000 rps, 10 min). The pellet of TG cells was stored in a refrigerator (-80°C) until use for DNA analysis.

The handling of TG and cultured cells described below followed the previous report [9].

Osteosarcoma cell culture:

For the positive control of DNA analyses using TG cells, human osteosarcoma cell lines, MG63 cells (in 1st round experiment) and Saos-2 cells (in 2nd round experiment), were cultured in α -MEM containing 10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% O₂. For the positive control of DNA ladder formation, subconfluent cells (at 5 days in culture) were treated with an established apoptosis-inducing agent (protein phosphatase inhibitor), okadaic acid (OA, Wako Chemical, Osaka Japan) extracted from Japanese sponge (*Halicondria okadai*), dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany), by adding to the culture medium at concentrations of 0, 10, 20, and 100 nM and culturing for 24 h.

DNA isolation and agarose gel electrophoresis:

TG and cultured cells were washed twice with cold PBS and lysed in cold lysis buffer containing 10 nM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.5% Triton X-100. After lysing, debris was removed by centrifugation at 15,000 g for 20 min. RNase (Sigma) was added at a final concentration of 40 μ g/ml to the lysates, which were incubated for 1 h at 37°C with gentle agitation. Proteinase K (Merck, Darmstadt, Germany) was added to the RNase-treated lysates at a final concentration of 40 μ g/ml. The lysates were further incubated for 1 h at 37°C with gentle agitation. DNA in the supernatant was precipitated and dissolved in TE buffer (10 nM Tris, 1 mM EDTA, pH 8.0). The extracted DNA and molecular marker (100 bp DNA ladder, New England BioLabs Inc., Ipswich, USA) were subjected to 2.0% agarose gel (containing 0.5 μ g/ml ethidium bromide) electrophoresis with the Mupid electrophoretic apparatus (Advance, Tokyo, Japan). DNA ladder formation was visualized under a UV trans-illuminator (Vilber Lourmat, Cedex, France). Photographs were taken with a Polaroid DS-300 camera.

Second round experiment:

In the first round experiment, it seemed that the

amount of DNA was insufficient to detect the DNA ladder in the capsaicin-treated rats, and so we attempted to increase the number of animals treated with capsaicin. Fourteen neonatal rats aged 2 days were similarly injected with capsaicin (50 mg/kg). Twenty-eight bilateral TGs were extracted 24 h after treatment under anesthesia. The results were the same as in the first round experiment.

Results and Discussion

If a DNA ladder appears in capsaicin-treated TGs, it could be attributable to exclusive neuronal cells, because no apoptotic figures were observed without neuronal cells (Schwann and satellite cells) [1].

In the first round experiment, the amount of DNA extracted from capsaicin-treated rats was small compared with that of the control (Fig. 1a). Visible DNA bands could not be detected, but it showed dispersion. To eliminate the possibility that the DNA concentration is too low to detect DNA ladder formation, about 3 times as many as TGs were used for analyses in the second round experiment. However, consistent with the results of the first round experiment, DNA ladder formation was not detected in the second round experiment (Fig. 1b).

Since ladder formation in the DNA extracted from human promyelocytic leukemic HL-60 cells was derived from only 8% apoptotic cells [7], non-formation of DNA ladder firmly indicates an insufficient number of apoptotic TG neurons after treatment with capsaicin in the present study.

Since typical apoptotic ultrastructural figures were demonstrated by OA using several mammalian cells [10] including human osteosarcoma cell lines [9], it has been frequently used as an inducer of apoptosis. In the first round experiment, OA at a concentration of 100 nM induced diffuse DNA fragmentation instead of DNA ladder formation (Fig. 1a). However, clear DNA ladder formation was observed in the cells treated with 10 and 20 nM OA in the second round experiment (Fig. 1b). These observations suggest that the higher concentration of OA (100 nM) induces necrosis, whereas the lower concentrations of OA (10 and 20 nM) induce apoptosis. Taken together, an application of capsaicin at a dose of 50 mg/kg is likely to be too high, so that the cell death of TG neurons occurred by the neurotoxic action of capsaicin, mainly leading to necrosis.

As it was recently reported that apoptosis was induced in cultured hepatoma HepG2 cells by capsaicin [11], the induction of apoptosis by the action of capsaicin has been well known to date. However, the action of capsaicin causing cell death may be mainly due to necrosis rather than apoptosis, at least in the primary sensory neurons. Probably, the induction of apoptosis depends on the dose of capsaicin, differences in animal species and many kinds of cells used, and the way of the experiment, i.e., in vivo or in vitro.

Acknowledgement

The harvesting of cells from TGs and cultured human osteosarcoma cells and analyses of their DNA were conducted by A. Ozaki (DDS) and H. Okamura (DDS).

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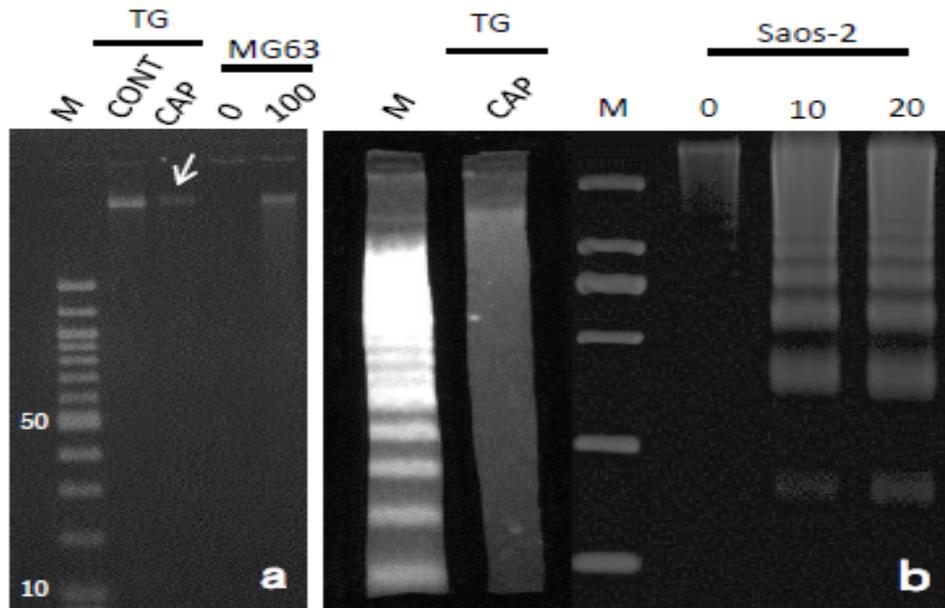
Illustrations

Illustration 1

Figure

Figure Legend

Fig 1. DNA analyses by agarose gel electrophoresis from the rat trigeminal ganglion (TG) neurons after neonatal treatment of vehicle (CONT) or capsaicin (CAP), and from the cultured cells of human osteosarcoma cell lines (MG63 and Saos-2) after treatment with okadaic acid (OA) at concentrations of 0, 10, 20, and 100 nM. DNA ladder formation was not seen in both TG neurons capsaicin-treated (arrow) and MG63 cells OA-treated at a concentration of 100 nM (a). They exhibit the indistinct dispersion of DNA. An increased amount of DNA from TG cells also did not exhibit DNA ladder formation, showing the indistinct dispersion of DNA (b). However, DNA collected from Saos-2 cells treated with OA (10 and 20 nM) exhibited a clear DNA ladder. From this, capsaicin does not seem to induce apoptosis of TG neurons sufficient to visualize DNA fragmentation. Numbers along the lane M of Fig. 1a indicate base pairs (X10). M: marker



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