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Rapid Communication

Nitric Oxide Mediates Capsaicin-Induced Cytotoxicity in Cultured Dorsal Root Ganglion Neurons

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Capsaicin exerts excitatory, desensitizing and toxic actions on a subset of sensory neurons by inducing influx of ions including calcium (Wood *et al.*, 1988). Recently, capsaicin receptor was cloned using an expression cloning strategy based on calcium influx (Caterina *et al.*, 1997). Capsaicin-induced neurotoxicity has been proposed to be caused by diverse cellular events such as mitochondrial damage, abnormal osmotic pressure, block of axonal transport, nerve growth factor (NGF) deprivation and activation of calcium-activated proteases (Szallasi, 1994). In addition to these cellular events, nitric oxide (NO) has been suggested to be involved in the capsaicin-induced neurotoxicity. Chemical injury elicited in small diameter afferent neurons by systemic administration of capsaicin can induce a change in nitric oxide synthase (NOS) expression similar to that elicited by peripheral nerve injury (Vizzard *et al.*, 1995). Subpopulations of NADPH diaphorase-positive rat dorsal root ganglion (DRG) neurons that express NOS mRNA are susceptible to the neurotoxic effects of capsaicin (Ren and Ruda, 1995). In line with the possible role of NO in neurotoxic cellular events, the present work was directed to determine whether NO is involved in capsaicin-induced neurotoxicity or neuroprotection of capsaicin-sensitive DRG neurons.

DRG neurons were prepared from neonatal Sprague-Dawley rats as previously described (Wood *et al.*, 1988) with modification. Dissociated cells were plated onto 96 well plates at a density of 12,000-15,000 neurons/well. Neuronal injury was quantitatively assessed by measuring the activity of lactate dehydrogenase (LDH) released from damaged or destroyed cells (Koh and Choi, 1987) and was expressed in conventional units per liter. In order to determine the capsaicin-specific responsiveness of DRG neurons undergoing neurotoxic challenge, ^{45}Ca uptake experiments were performed according to the procedure described previously (Park *et al.*, 1999).

Although the LDH efflux assay has not, to our knowledge, been used previously to assess viability of DRG neurons, the fact that LDH is an ubiquitous enzyme and is also plentiful in central neurons and glia (Tholey *et al.*, 1981) suggests that this assay method would be employed as an appropriate biochemical index of DRG neuronal injury. Overnight treatment of DRG neurons in culture with micromolar concentrations of capsaicin resulted in preferential loss of a subpopulation of capsaicin-sensitive small, dark sensory neurons (Wood *et al.*, 1988). Thus, cultured DRG neurons exposed for 16 hr to 2 μM capsaicin showed considerable neuronal death as revealed by the increased release of LDH into the culture medium (Table I). L^G-Nitro-L-arginine methyl ester (L-NAME, 0.1 to 1 mM) and sodium nitroprusside (SNP, 10 μM) did not affect capsaicin-induced neuronal cell injury. In contrast, a higher concentration of SNP (100 μM), either alone or in combination with capsaicin, enhanced the LDH relese (Table I). Considering the massive accumulation of calcium and other divalent cations in the cultured sensory neurons (Wood *et al.*, 1988), it is conceivable that DRG neuronal cell death by long-term treatment with capsaicin is mainly caused by pathological alterations in osmotic equilibrium and $[Ca^{2+}]_i$.

However, under such a harsh experimental condition of overnight treatment of capsaicin, determination of the possible involvement of the NOS-NO pathway in capsaicin-induced cytotoxicity may not be conclusive. Therefore, we adopted a milder exposure protocol, which measured LDH efflux 16 hr after 30 min treatment of capsaicin (10 μM). This short-term treatment of capsaicin was as potent as the 16 hr treatment in terms of inducing LDH release (Table II). In a subsequent experiment, we were able to clearly demonstrate that capsaicin-mediated cell death could be reduced by the specific NOS inhibitor L-NAME in a dose dependent manner. However, the NO-releasing agent SNP did not aggravate or ameliorate cell injury.

Protease inhibitors, such as E 64 and leupeptin, are known to markedly reduce the capsaicin-dependent cell death determined using a fluorescein diacetate/propidium iodide double staining procedure (Chard *et al.*, 1995). In line with the above findings, the capsaicin-induced LDH efflux level was significantly lowered by E

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Table I. Effect of NO on the loss of DRG neurons viability following exposure to capsaicin and inhibitors or inducers of NO generation for 16 hr

Exposure protocol	net LDH (U/L) efflux
DME/F12 medium only	0
CAP 0.3 μM	51.2±6.8
CAP 2 μM	67.3±8.1
L-NAME 100 μM	5.42±0.43
L-NAME 1 mM	4.32±0.61
SNP 10 μM	2.11±0.24
SNP 100 μM	35.2±5.0
CAP 2 μM+L-NAME 100 μM	68.4±5.7
CAP 2 μM+L-NAME 1 mM	72.9±9.8
CAP 2 μM+SNP 10 μM	70.1±6.2
CAP 2 μM+SNP 100 μM	95.7±12.8

In the capsaicin (CAP) and L-NAME co-treatment group, L-NAME was preincubated for 1 hr. Values are expressed as mean ± SEM from 4 separate experiments.

64 and leupeptin (Table II). However, co-application of L-NAME and E 64 failed to exert any noticeable synergistic effect under our experimental conditions. We then proceeded to examine whether the cell injury produced by exposure to 10 μM capsaicin for 30 min could be prevented by incubation with calcium channel blockers. As anticipated, the excitotoxic injury could be blocked by the specific capsaicin antagonist capsazepine in a dose dependent manner. Voltage-sensitive calcium channel blockers, nifedipine and verapamil, did not attenuate capsaicin-induced cell injury (Table II).

Table II. Prevention of capsaicin-induced neurotoxicity in DRG neurons by NOS inhibitor L-NAME and Ca^{2+}-activated protease inhibitors E 64 and leupeptin

Exposure protocol	net LDH (U/L) efflux
DME/F12 medium only	0
CAP 10 μM	55.3±5.3
CAP 10 μM+nifedipine/verapamil 20 μM	57.8±7.2
CAP 10 μM+capsazepine 20 μM	34.9±3.6
CAP 10 μM+capsazepine 100 μM	2.45±0.31
CAP 10 μM+L-NAME 100 μM	31.5±2.8
CAP 10 μM+L-NAME 1 mM	16.2±3.0
CAP 10 μM+SNP 1 μM	54.4±6.3
CAP 10 μM+SNP 10 μM	51.0±8.8
E 64 10 μM	2.85±0.43
leupeptin 10 μM	1.98±0.35
CAP 10 μM+E 64 10 μM	18.7±2.7
CAP 10 μM+leupeptin 10 μM	11.9±1.5
CAP 10 μM+L-NAME 100 μM+E 64 10 μM	17.9±3.2
CAP 10 μM+L-NAME 1 mM+E 64 10 μM	13.6±2.5

Cells were preincubated with L-NAME, E 64, and leupeptin for 30 min prior to and during exposure to 10 μM capsaicin. These agents and SNP were contained in the incubation medium (DME/F12) at indicated doses. Values are expressed as mean ± SEM from 4 separate experiments.

The present study strongly proposes a role of NO as a mediator of toxic effects exerted by capsaicin. There are several possible mechanisms by which NO exerts its cytotoxic action on DRG sensory neurons. NO can damage cells by attacking the iron-sulfur centers in various key proteins such as aconitase, which plays a role in the tricarboxylic acid cycle, and complex I in the electron transport system (Nathan, 1992). These non-specific target destruction by NO leads to inactivation of mitochondrial function, and, at high concentrations, inhibition of DNA replication and the induction of apoptosis (Sarih *et al.*, 1993). Recently, we suggested the modulatory role of constitutively synthesized NO on the vanilloid receptor function (Park *et al.*, 1999). Thus, it is conceivable that a robust production of NO by induced NOS could possibly alter properties of various channels to cause neurotoxic actions. Capsaicin has been demonstrated previously to increase cGMP production via NO in DRG neuronal cultures, especially in amphicytes (Bauer *et al.*, 1995). However, the possible cytotoxic potential of the elevated cGMP is not known.

Treatment of cultured rat sensory neurons with capsaicin causes the establishment of a reversible or irreversible desensitization depending on the experimental conditions (Cholewinski *et al.*, 1993). In this context, we have examined whether the cell cultures which exhibit lower cell death rate in capsaicin-induced neurotoxicity by virtue of L-NAME or protease inhibitors retain the capacity of capsaicin induced calcium uptake characteristic of the DRG neuronal subpopulation. Capsaicin-stimulated ^{45}Ca uptake was conducted to quantitatively assess the responsiveness to capsaicin. Regardless of the differences in the cell death rate, 30 min pretreatment of 10 μM capsaicin caused a irreversible desensitization, where subsequent capsaicin-induced ^{45}Ca accumulation was inhibited (data not shown).

The data presented here are indicative of the significant role of NO in capsaicin-induced cytotoxicity. A further mechanistic study will be necessary to precisely define the roles of various causative phenomena in capsaicin-induced neurotoxicity.

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