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STRESS- AND NON-STRESS-MEDIATED MECHANISMS ARE INVOLVED IN PAIN-INDUCED APOPTOSIS IN HIPPOCAMPUS AND DORSAL LUMBAR SPINAL CORD IN RATS

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Abstract—Chronic pain has been reported to induce apoptosis. Both chronic excitation of neural pathways involved in pain transmission and control and the stress of pain may be potentially involved in apoptosis induced by pain. Here, we have investigated their possible role in pain-induced apoptosis. Inflammatory pain was induced by injection of formalin in intact and adrenalectomized (ADX) rats. Following exposure to repeated injections of 5% formalin, we detected Bax, Bcl-2, pro-caspase and activated caspase-3 proteins using immunoblotting. The results were compared with those obtained from animals suffered from chronic immobilization stress (IMO). These results showed an increased ratio of Bax/Bcl-2 and activated caspase-3 in hippocampus and dorsal lumbar spinal cord of animals treated with pain and IMO stress; these effects were reduced in ADX animals. On the other hand, the remaining apoptotic effect of pain in adrenalectomized rats was also significant. We surmise that both chronic neural activation and the stress induced by pain are involved in pain-induced apoptosis.

Key words: inflammatory pain, corticosterone, apoptosis, caspase-3, Bax, Bcl-2.

After injury or inflammation, plastic changes can take place in the periphery, spinal cord and higher brain centers. This neuroplasticity, as well as processes of cell death, contribute to pain perception and to the development and maintenance of chronic pain syndromes (Corasaniti et al., 2006). Inflammatory and neuropathic pain have been reported to induce cell death in CNS (Sugimoto et al., 1990; Scholz et al., 2005; Hassanzadeh and Ahmadiani, 2006; Pourahmad et al., 2006a, b). Morphological studies suggest the occurrence of apoptosis in the spinal cord following peripheral nerve insult (Azkue et al., 1998; Whiteside and Munglani, 2001). Neuropathic pain induced by 2 weeks of sciatic nerve ligature increases mRNA expression levels of the pro-apoptotic genes of Bcl-2 family in the lumbar dorsal horn of the spinal cord of rats (Maione et al., 2002).

Previously we reported a significant increase in the number of dark neurons in the superficial laminae I–II of the lumbar spinal cord following chronic intra-plantar injection of 5% formalin (Hassanzadeh and Ahmadiani, 2006). Intra-plantar injection of dilute formalin activates peripheral neural fibers, leading to activation of dorsal horn neurons (Vaccarino and Couret, 1995; Okuda et al., 2001). On the other hand, the hypothalamic–pituitary–adrenal (HPA) axis is also shown to respond to stressful stimuli, including pain (Vaccarino and Couret, 1995). Stress has been known to impair brain function and increase the vulnerability of neurons to injury, especially in the hippocampus (Uno et al., 1989; McEwen, 2000; Sapolsky, 1996). Stress induces significant dendritic atrophy and neuronal loss in the hippocampus, in parallel with impaired spatial memory performance (De Wied and Croiset, 1991; Mizoguchi et al., 1992; Watanabe et al., 1992).

The steroid glucocorticoid hormones (GCs) released from adrenal cortex are principal effectors of the stress response (Magarinos and McEwen, 1995; Sapolsky et al., 2000). While short-term elevation of GCs can protect against stress, chronic elevation of GCs due to sustained stress exerts deleterious effects on the brain (Sapolsky et al., 2000). The hippocampus, which has a high density of GC receptors, is a principal target of increased levels of GCs (McEwen et al., 1986). In spinal cord, glucocorticoid receptors are located in dorsal horn neurons, the region involved in pain transmission and its control (Cintra et al., 1993). In experimental models, administration of exogenous GCs induces significant dendritic atrophy and neuronal death in the hippocampus (Woolley et al., 1990; Hassan et al., 1996). Treatment with the synthetic GC dexamethasone stimulates apoptosis in the hippocampus, via increasing the expression of Bcl-2 family genes (Almeida et al., 2000). The proteins encoded by these genes are major regulatory components of the apoptotic pathway. The relative abundance of pro-apoptotic and anti-apoptotic protein members determines the cell susceptibility to programmed cell death. Bcl-2 family members regulate the release of cytochrome C from the mitochondrial intermembrane compartments into the cytoplasm (Hengartner, 2000), and subsequent formation of the cytosolic 'apoptosome' complex, which ultimately actives caspase-3 for execution of cells (Li et al., 1997; Zou et al., 1997).

Stress and pain may provoke increased stimulation of glutamatergic pathways, leading to excess intracellular Na^+ and Ca^+ concentrations, resulting in initial cytotoxic

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Abbreviations: ADX, adrenalectomized; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; GCs, glucocorticoid hormones; HPA, hypothalamic–pituitary–adrenal; IMO, immobilization stress; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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osmotic swelling of neurons and glial cells (Emerson et al., 1999). Stress exposure or glucocorticoid treatment has been shown to induce changes in glutamate receptor expression in hippocampus (Krugers et al., 1993; Bartanusz et al., 1995; Schwendt and Jezova, 2000). Selective blockade of glutamate receptors prevents the early over-expression of pro-apoptotic genes and morphological changes in dorsal horn lamina II neurons following sciatic nerve chronic constrictive injury (De Novellis et al., 2004). Several types of stress, including forced swim stress, cold stress and immobilization stress (IMO) can induce cell death (Hatanaka et al., 2001; Ohsaka et al., 2002; Tuder et al., 2003; Yun et al., 2003; Tseilikman et al., 2005). Chronic unpredictable stress induces dendric atrophy in CA_3 pyramidal neurons in rats (Karst and Joels, 2003). Other studies showed changes in the ratio of Bcl-2 to Bax, which was altered in favor of apoptosis in rat hippocampus following repeated IMO (Yun et al., 2003).

Chronic pain is a stressor, but also activates neurons involved in pain transmission and control. Both chronic excitation of neurons involved in pain transmission and control, and the stress caused by pain could be involved in pain-induced apoptosis. Here we investigated their possible role in pain-induced apoptosis, comparing the apoptotic effect of chronic inflammatory pain and chronic IMO in hippocampus and dorsal spinal cord of both intact and adrenalectomized (ADX) rats. Hippocampal neurons are strongly affected by stress, and dorsal spinal cord cells play a major role in pain transmission. Changes in the protein level of Bax, Bcl-2, pro-caspase-3 and activated caspase-3 were measured using immunoblotting technique to evaluate apoptosis level.

EXPERIMENTAL PROCEDURES

Animals

All experiments were carried out on male Wistar rats, weighting 200–250 g. Animals were housed four per cage under a 12-h light/dark cycle in a room with controlled temperature (23±2 °C). Food and water were available *ad libitum*. ADX rats received saline instead of tap water. Prior to the experiments onset, animals were handled daily (between 9:00 and 10:00 a.m.) for 5 days to minimize nonspecific stress responses. Rats were placed randomly into experimental groups, each comprising six to eight animals. All experiments followed the guidelines on ethical standards for investigation of experimental pain in animals (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their suffering.

General procedure

Inflammatory pain was induced by single or repeated 50 μl injections of 5% formalin solution. In chronic pain groups, formalin was injected into the plantar surface of the left or right hind paws for 4 days. Injections into the hind paws were carried out as follows: day 1, plantar surface of both paws; day 2, dorsal surface of both paws; on days 3 and 4, the injections were same as those of days 1 and 2, respectively. To produce IMO, rats were daily immobilized for 2 h in tightly fitted restrainers for seven or nine consecutive days.

To adrenalectomize the animals, rats were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). Both adrenal glands were removed through two dorsal incisions using a pair of sterile fine forceps. The sham operation consisted of bilateral dorsal incision, plus locating and exposing the adrenals. All ADX rats were maintained on 0.9% NaCl drinking solution; whereas the sham-operated rats were kept on tap water. The animals began the experimental procedures 5 days after the adrenalectomy or sham operations.

Animals were killed by decapitation between 9:00 and 10:00 a.m., immediately after the 7th or 9th IMO, or on day 5 for pain-treated groups. The trunk blood was collected into tubes containing 5% EDTA. Plasma was obtained by centrifuging the blood at 2500 rpm (10 min). Samples were frozen immediately and stored at −20 °C until the time of corticosterone assay. The hippocampus and spinal cord were rapidly removed and the dorsal half of the lumbar cord was dissected. Tissue samples were frozen in liquid nitrogen then stored at −80 °C until the time of protein extraction for Western blot analysis.

Corticosterone assay

Plasma level of corticosterone was measured by radioimmunoassay (RIA) using a commercial kit for rats corticosterone ([^{125}I], DRG International, Inc., USA). The sensitivity of assay was 0.25 ng/ml with 100% antibody cross-reaction with corticosterone, 0.34% with desoxycorticosterone, and less than 0.10% with other steroids.

Immunoblotting analysis

The hippocampus and the dissected spinal tissues were homogenized on ice in cold lysis buffer containing 50 mM Tris–HCl (pH = 8.0), 150 mM NaCl, 0.1% Triton X-100, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After centrifugation, the supernatant was collected and assayed for protein concentration using the Bradford method. Lysates containing equal amounts of protein were resolved on SDS–12.5% polyacrylamide gel electrophoresis, and transferred to PVDF membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), blocked in 2% ECL advanced kit blocking reagent (Amersham Biosciences) and probed with primary mouse monoclonal antibodies to Bax (1:100,000) or Bcl-2 (1:50,000) (Sigma-Aldrich, St. Louis, MO, USA), or rabbit monoclonal antibody to caspase-3 (1:1000 Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After washing, membranes were incubated for 60 min at room temperature with horseradish peroxidase–conjugated secondary antibody (1:100,000 Santa Cruz Biotechnology, CA, USA). Blots were revealed by ECL advanced kit (Amersham Biosciences). To normalize for protein content, blots were stripped in stripping buffer containing 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris–HCl (pH=6.7) and then probed with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

Band densities on the blots were measured using Laboratory Work software. The densities obtained for Bax, Bcl-2 and activated caspase-3 were normalized to actin band density. The results are expressed as mean±S.E.M. Data from all the experiments were analyzed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc. $P<0.05$ was considered significant.

RESULTS

Corticosterone assay

Chronic inflammatory pain and repeated IMO for 7 or 9 days significantly increased the level of plasma corticosterone compared with intact groups ($P<0.001$). The level of corticosterone in the pain-treated group was significantly