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Clonidine reduces hypersensitivity and alters the balance of pro- and anti-inflammatory leukocytes after local injection at the site of inflammatory neuritis

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Abstract

Perineural α2-adrenoceptor activation relieves hypersensitivity induced by peripheral nerve injury or sciatic inflammatory neuritis. This effect is associated with a reduction in pro-inflammatory cytokines, as well as a reduction in local leukocyte number and their capacity to produce pro-inflammatory cytokines. Curiously, clonidine's antinociceptive effect appears with a 2–3-day delay after injection. Previous observations have shown that α-adrenoceptor activation induces apoptosis in leukocytes, which would reduce leukocyte number. Additionally, macrophage scavenging of apoptotic cells results in a shift to an anti-inflammatory phenotype, with expression of transforming growth factor (TGF)-β1. We therefore examined the effects of perineural clonidine 24 h and 3 days after its injection on apoptosis, TGF-β1 expression and lymphocyte and macrophage phenotype in acute sciatic inflammatory neuritis. Perineural clonidine reduced ipsilateral neuritis-induced hypersensitivity in a delayed manner (3 days after treatment), along with a reduction at this time in lymphocyte number and an increase in caspase-3 and TGF-β1 expressing cells and macrophages co-expressing TGF-β1 in the sciatic nerve. One day after injection clonidine treatment was associated with a reduction in lymphocytes and pro-inflammatory Th-1 cells as well as increased numbers of caspase-3 and TGF-β1 expressing cells and macrophages co-expressing TGF-β1 in sciatic nerve. Clonidine's effects were prevented by co-administration of an α2-adrenoceptor antagonist. These data suggest that α2-adrenoceptor activation in sciatic inflammatory neuritis increases local apoptosis and anti-inflammatory products early after treatment. This early effect likely underlies the delayed anti-inflammatory and anti-hypersensitivity effects of perineural clonidine in this setting.

Keywords: Immune response; α2-Adrenoceptor; Leukocytes; Neuropathic pain; Caspase-3; TGF-β1; IL-1β; TNFα; Hypersensitivity; Sciatic nerve

1. Introduction

Peripheral nerve injury induces chronic pain in part due to immune activation orchestrated by stimulation and migration of leukocytes and by their expression of pro-inflammatory products. Immune activation near healthy peripheral nerves is sufficient to produce pain states (Watkins et al., 1995) and peripheral immune modulation reduces hypersensitivity to somatic stimuli in several neuropathic pain models (Twining et al., 2004; Romero-Sandoval and Eisenach, 2006).

Sympathetic nervous system activity or exogenously applied adrenoceptor (AR) agonists can modulate activity and responses of lymphocytes and macrophages (Moynihan et al., 2004), due to actions on multiple AR subtypes, including α2-ARs (Titnchi and Clark, 1984; Spengler et al., 1990; Josefsson et al., 1996). Sympathetic and α-AR activation have been suggested to contribute to peripheral nerve injury-induced pain (Sato and Perl, 1991; Koltzenburg and McMahon, 1991; McLachlan et al., 1993). However, we have also shown that perineural α2-AR activation relieves neuropathic pain by modulating immune responses

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(Romero-Sandoval et al., 2005; Romero-Sandoval and Eisenach, 2006).

Because of the potential relevance of understanding how immune response modulation in neuropathic pain conditions alters pain processing, we sought to further probe the mechanisms by which α2-ARs affect hypersensitivity and immune responses in the sciatic inflammatory neuropathy (SIN) model. This model, consisting of local application of zymosan on the sciatic nerve through an implanted catheter (Gazda et al., 2001; Chacur et al., 2001), results in intense and bilateral hypersensitivity to mechanical stimuli, dependent upon interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, reactive oxygen species and complement expression and generation (Twining et al., 2004).

Perineural administration of the α2-AR agonist, clonidine, reverses hypersensitivity in the SIN model (Romero-Sandoval et al., 2005). Antinociception from clonidine is associated with a reduction in pro-inflammatory cytokines, macrophage and lymphocyte number in the inflammatory exudates, and capacity of the leukocytes present to produce pro-inflammatory cytokines in response to further challenge. Since clonidine's antinociceptive effects occur in a delayed manner several days after transient drug exposure, we hypothesize that clonidine acutely alters immune responses, leading over time to reduced migration and leukocyte function.

We based our study on five previous observations: (1) when macrophages recognize and engulf apoptotic cells, they alter their phenotype to a primarily anti-inflammatory one, expressing TGF-β1 (Fadok et al., 2000; Huynh et al., 2002), (2) α-AR agonists change the proportion and increase the number of apoptotic leukocytes (Stevenson et al., 2001), (3) clonidine produces apoptosis in the developing brainstem (Dygalo et al., 2004) and in skin melanocytes (Uchida-Oka and Sugimoto, 2001), (4) clonidine increases TGF-β1 expression in sciatic nerve tissue in a mechanical nerve injury model of neuropathic pain (Lavand'homme and Eisenach, 2003) and (5) perineural clonidine treatment reduces pro-inflammatory but increases anti-inflammatory responses in immune cells in the inflammatory exudates in the SIN model (Romero-Sandoval et al., 2005). These observations suggest that α2-AR mediated acute apoptosis could underlie the delayed beneficial effects of perineural clonidine in neuritis. Here we tested whether perineural clonidine induced apoptosis and resulted in altered immune cell phenotype in the inflamed nerve of the SIN model, and examined its time course and pharmacologic mechanism of action.

2. Methods

2.1. Animals and surgery

After approval by the Animal Care and Use Committee (Wake Forest University School of Medicine, Winston-Salem, North Carolina) male Wistar rats (200–250 g at the day of surgery), underwent implantation of a gelfoam-attached silastic catheter near the sciatic nerve as previously described (Gazda et al., 2001; Chacur et al., 2001; Romero-Sandoval et al., 2005). Briefly, the left sciatic nerve was exposed and wrapped with gelfoam attached to the end of the catheter. The catheter was anchored to the muscles using 3–0 silk suture and passed subcutaneously, exiting at the base of the tail. The external end was held in a plastic structure protected by an aluminum frame and sealed with rubber and silicone glue.

At the end of the experiments, the animals were anesthetized with halothane and were sacrificed by decapitation. All catheters were dissected, and only animals with correctly placed catheters were included in the analysis.

2.2. Drugs and in vivo treatments

Drugs were administered in awake and freely moving animals through the catheter 4–5 days after surgery. The drugs used were: incomplete Freund's adjuvant (IFA) as vehicle, zymosan (in vehicle, both from Sigma, St. Louis, MO), clonidine (in saline, from Roxane, Columbus, OH) and BRL44408, an α2-AR antagonist (Tocris, Ellisville, MO).

The animals received a single dose of zymosan (40 μg, 50 μl) to induce acute inflammatory neuritis and bilateral hypersensitivity as previously described (Milligan et al., 2003; Romero-Sandoval et al., 2005). Clonidine, which has a terminal elimination half life of 8–25 h (Anavekar et al., 1982; Conway et al., 1992) was administered 3 h after zymosan (when hypersensitivity was already evident) in a single dose (30 μg, 60 μl, a dose previously shown to be effective to relieve hypersensitivity in this model; Romero-Sandoval et al., 2005) or together with BRL44408 (45 μg, 10 μl in saline, a dose previously shown to block clonidine's anti-hypersensitive effects; Lavand'homme et al., 2002). Control groups received vehicle instead of zymosan, and saline instead clonidine. The groups studied were: vehicle plus saline (v + s, $n = 4$), zymosan plus saline (z + s, $n = 4$), zymosan plus clonidine (z + c, $n = 5$) and zymosan plus BRL44408 plus clonidine (z + BR + c, $n = 5$).

2.3. Behavioral testing

Withdrawal threshold to von Frey filament (Stoelting, Wood Dale, IL) probing was determined using an up-down statistical method (Chaplan et al., 1994). Thresholds were measured ipsi- and contralaterally to neuritis before catheter implantation, 4–5 days after catheter implantation (immediately before zymosan or vehicle injection), 3 h after zymosan injection and 1 or 3 days thereafter. Withdrawal thresholds were measured twice at each site, and the average used for data analysis.

2.4. Tissue preparation and immunostaining

Animals ($n = 4$ for all groups) on post-treatment day 1 or 3 were deeply anesthetized with pentobarbital and perfused transcardially with buffer (0.01 M phosphate buffered saline + 1% sodium nitrite, 100 ml) followed by 4% paraformaldehyde (400 ml) at room temperature. The sciatic nerve (~1 cm) ipsilateral to neuritis was removed and postfixed in the same fixative for 2–3 h followed by cryoprotection in 30% sucrose for 48–72 h at 4 °C. Tissue was cut at a 16 μm thickness using a cryostat and sections immunolabeled on slides. After four washes with 0.01 M phosphate buffered saline + 0.15% Triton 100-X (PBS + T), the slides were incubated in 50% alcohol for 45 min, washed four times with PBS + T and blocked with 1.5% normal goat serum. Slides were then incubated overnight at 4 °C in primary antibodies, washed two times with PBS + T, then incubated for 1 h with fluorescein (1:200, West Grove, PA) or biotin (1:200, Vector, Burlingame, CA) plus Alexa 568 (1:400, Molecular Probes, Eugene, OR) secondary antibodies.

Apoptotic cells were identified using an antibody to cleaved caspase-3 (rabbit polyclonal antibody, 1:1000, Wako Pure Chemical Industries, Richmond, VA). Lymphocytes and monocytes/macrophages were identified using CD2 and ED1 antibodies, respectively (both mouse monoclonal antibodies from Serotec, Raleigh, NC; CD2 at 1:500 and ED1 at 1:200). We also co-localized caspase-3 with CD2 or ED1. We identified TGF-β1 expressing cells using a rabbit polyclonal antibody (1:1000, Biovision, Mountain View, CA). We chose TGF-β1, since others have shown that