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MINOCYCLINE TREATMENT ATTENUATES MICROGLIA ACTIVATION AND NON-ANGIOTENSIN II [^{125}I] CGP42112 BINDING IN BRAINSTEM FOLLOWING NODOSE GANGLIONECTOMY

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Abstract—We have previously shown that following unilateral nodose ganglionectomy, [^{125}I] CGP42112 binds to a non-angiotensin II (Ang II) related binding site in rat dorsal motor nucleus of the vagus nerve, ambiguus nucleus and nucleus of the solitary tract. Furthermore, this up-regulated binding site localizes with activated microglia. Given that some tetracyclines may inhibit microglia activation in brain, we examined the effect of minocycline treatment on the binding of [^{125}I] CGP42112 and [^{3}H] PK11195 (an established radioligand for microglia), as well as OX-42 immunoreactivity (an immunomarker for activated microglia), following nodose ganglionectomy. Male Wistar Kyoto rats underwent unilateral nodose ganglionectomy or sham operation and were treated with saline or minocycline (50 mg/kg i.p.) 12 h before surgery and twice daily after surgery (each 50 mg/kg i.p.) for 3 days. Subsequent to nodose ganglionectomy, [^{125}I] CGP42112 binding (insensitive to PD123319 or Ang II) was increased approximately two-fold in the ipsilateral nucleus of the solitary tract and was also induced in the ipsilateral dorsal motor nucleus of the vagus nerve and ambiguus nucleus of saline-treated rats. Treatment with minocycline reduced this non-angiotensin II [^{125}I] CGP42112 binding (40–50% reduction) in the nucleus of the solitary tract, dorsal motor nucleus of the vagus nerve and ambiguus nucleus. Analogous experiments using [^{3}H] PK11195 also revealed up-regulated binding in the ipsilateral nucleus of the solitary tract (~205%), dorsal motor nucleus of the vagus nerve (~80%) and ambiguus nucleus (~210%) of saline-treated rats following nodose ganglionectomy, which was reduced by 40–100% with minocycline treatment. Immunoreactivity to OX-42 confirmed an increase in microglia activation and accumulation of macrophages in these brain stem nuclei following nodose ganglionectomy, which was also attenuated following treatment with minocycline.

These data demonstrate that non-Ang II [^{125}I] CGP42112 binding following nodose ganglionectomy is attenuated by minocycline treatment. This minocycline-induced effect was associated with reduced activation of microglia and an apparent reduction in the number of macrophages in the abovementioned nuclei. This evidence suggests that a non-Ang II [^{125}I] CGP42112 binding site is located on, or associated with, activated microglia and macrophages, providing a useful tool with which to quantitate the neuroprotective effects of centrally acting anti-inflammatory compounds. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: inflammation, neuronal degeneration, autoradiography, immunohistochemistry, reactive glia.

Injury to the CNS that results in degeneration of neurons, their axons or terminals, is accompanied by an acute inflammatory response which involves an increase in the number of non-neuronal cells, hypertrophy of glial cells and other cellular changes (Minghetti and Levi, 1998; Streit et al., 1999). In the brain there is little pathology that does not involve reactivity of glial cells, which kill invading microorganisms, remove debris, and facilitate tissue repair after injury (Kreutzberg, 1996; Streit et al., 1999). In particular, microglial cells undergo characteristic changes that, although they may vary somewhat from one lesion paradigm to another, are generally stereotypical and reproducible across a variety of models, including ischemic stroke (Streit et al., 1999).

A striking feature of microglia reactivity is their ability to synthesize and secrete a large number of substances including growth factors, cytokines, coagulation and complement factors, lipid mediators, extracellular matrix components, enzymes, free radicals, neurotoxins, nitric oxide and prostaglandins (Minghetti and Levi, 1998; Streit et al., 1999). Although at low levels these inflammatory mediators can play a vital role in the regeneration and repair of damaged tissue, at high levels these products have also been reported to contribute to the degeneration of neurons (Minghetti and Levi, 1998). Therefore, the usefulness of inflammatory markers in the delineation and treatment of neuropathological events is increasingly recognized. In the past, investigation of potential neuroprotective agents that target inflammatory events that occur following CNS injury has employed numerous staining techniques, including immunohistochemistry, to visualize and count the number of surviving neurones and invading inflammatory cells (Beech et al., 2001; Tikka and Koistinaho, 2001; Stirling et al., 2004). However, these techniques can be laborious and costly, and development of new techniques that enable quick and easy quantification of inflammatory events that occur following injury to the CNS will aid in the development of neuroprotective treatments.

A novel non-angiotensin II (Ang II) CGP42112 binding site has been reported in healing brain wounds (Viswanathan et al., 1994a,b, 1996) and macrophages derived from rat spleen (de Oliveira et al., 1994). Further-

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Abbreviations: Ang II, angiotensin II; ANOVA, analysis of variance; AT_2, angiotensin II receptor subtype 2; DMX, dorsal motor nucleus of the vagus nerve; MCID, microcomputer imaging device; n.amb, nucleus ambiguus; NTS, nucleus of the solitary tract; PBR, peripheral benzodiazepine receptor; WKY, Wistar Kyoto.

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more, increased non-Ang II [^{125}I] CGP42112 binding associated with retrograde damage leading to neuronal degeneration has been reported following kainic acid lesions (Jöhren et al., 1995). These authors suggested that the presence of a non-Ang II CGP42112 binding site correlated with the accumulation of macrophages that accompany inflammation following brain injury (Jöhren et al., 1995; Viswanathan et al., 1994a,b) and balloon catheter injury in the rat carotid artery (Viswanathan et al., 1996). Recently we have used known markers for activated microglia to study the inflammatory response that occurs following retrograde degeneration of neuronal cell bodies in brainstem nuclei following unilateral nodose ganglionectomy (Roulston et al., 2004). We demonstrated for the first time that non-Ang II [^{125}I] CGP42112 binding is associated with activated microglia, as well as macrophage accumulation, three and 14 days following unilateral nodose ganglionectomy. Furthermore we demonstrated the potential use of non-Ang II [^{125}I] CGP42112 binding as a marker for quantitating inflammatory events which occur as a result of damage to the CNS.

Members of the tetracycline family of broad-spectrum antibiotics have been found to exhibit anti-inflammatory effects independent of their antimicrobial activity. Minocycline, a semisynthetic second-generation tetracycline, has been reported to inhibit mammalian collagenases and several other matrix metalloproteinases responsible for the degradation of connective tissue matrices in inflammatory arthritis (Ryan et al., 2001), and is currently in clinical trial for the treatment of rheumatoid arthritis (Stone et al., 2003), Huntington's disease and amyotrophic lateral sclerosis (Blum et al., 2004). Recently, minocycline has also been shown to be an effective neuroprotective agent in animal models of spinal cord injury (Stirling et al., 2004), Parkinson's disease (Du et al., 2001), multiple sclerosis (Brundula et al., 2002) and against focal ischemia, with a wide therapeutic window (Yrjänheikki et al., 1998, 1999). Minocycline is rapidly and completely absorbed, and has adequate tissue penetration into the brain and cerebrospinal fluid (Du et al., 2001; Colovic and Caccia, 2003). Beneficial effects of minocycline are associated with reduced inducible nitric oxide synthase and cyclooxygenase-2 expression, decreased cytokine and prostaglandin release and decreased induction of IL-1β-converting enzyme in microglia (Yrjänheikki et al., 1998, 1999; Du et al., 2001). However of particular interest to the present study are the reports that treatment with minocycline prevents the activation and proliferation of microglia, but not astrocytes, at the site of injury (Yrjänheikki et al., 1998, 1999; Stirling et al., 2004).

In light of the above reports that demonstrate minocycline prevents the activation of microglia following CNS injury, the initial aim of the present study was to extend these reports by investigating the effect of minocycline treatment on [^{3}H] PK11195 binding and OX-42 immunoreactivity following nodose ganglionectomy (Roulston et al., 2004). Given that a non-Ang II [^{125}I] CGP42112 binding site is also up-regulated in brain stem nuclei where increases in [^{3}H] PK11195 binding and OX-42 immunoreactivity occur following nodose ganglionectomy (Roulston et al., 2004), the second aim of the present study was to investigate the effect of minocycline treatment on non-Ang II [^{125}I] CGP42112 binding. These data will provide a technique for investigating the ability of anti-inflammatory agents such as minocycline to prevent microglia activation and macrophage accumulation in response to a nerve injury of this kind.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar Kyoto (WKY) rats, 16–20 weeks, weighing approximately 300–350 g, were obtained from the Austin Hospital Biological Research Laboratories (Heidelberg, Victoria, Australia) and housed in the Monash University Pharmacology Animal House. Animals were maintained on a 12-h light/dark cycle at a temperature of 18–22 °C and were allowed to feed/drink *ad libitum*. All efforts were made to minimize animal suffering and the number of animals used. All experiments described here were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the NH&MRC code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Surgical procedures

WKY rats were divided randomly into four groups and then underwent the procedure of right nodose ganglionectomy, or sham operation as described previously (Roulston et al., 2003). Briefly, rats were anesthetized with sodium methohexitone (60 mg/kg, i.p. Eli Lilly, USA) and a midline incision in the neck was made so that the right nodose ganglion could be cleared of connective tissue. In the case of unilateral nodose ganglionectomy, the right nodose ganglion was then excised (n=8). Sham control operations were also performed in a separate group of animals whereby the right nodose ganglion was exposed but not removed (n=8).

Drug treatment

Based on previously published work describing the use of minocycline (Yrjänheikki et al., 1998, 1999), rats from all groups were injected i.p. with either 50 mg/kg of minocycline hydrochloride (equivalent to 45 mg/kg of base) (Sigma, USA), or saline (1 ml/kg, i.p.) 12 hours before surgical procedure. Thereafter, the animals were injected twice daily with either minocycline (50 mg/kg) or saline, for 3 days.

In vitro autoradiography

Autoradiographic experiments were carried out using standard techniques as previously described (Roulston et al., 2003, 2004). Following a 3-day recovery period and treatment with minocycline or saline, animals were killed by decapitation, brainstems dissected out and immediately frozen over liquid nitrogen and stored at −80 °C. Coronal brainstem sections (14 μm) were prepared as described previously (Roulston et al., 2004). Adjacent slide mounted brainstem sections from all surgical groups were exposed to either [^{3}H] PK11195 (a marker for activated microglia) (Gehlert et al., 1997; Stephenson et al., 1995) or [^{125}I] CGP42112 (Roulston et al., 2003).

[^{3}H] PK11195

Sections were pre-incubated for 15 min at room temperature in buffer containing 50 mM Tris (pH 7.4), 120 mM NaCl and 5 mM KCl. Consecutive sections were then incubated at room temperature in fresh buffer containing 50 mM Tris (pH 7.4), 300 mM NaCl