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Research paper

Pattern of Mas expression in acute and post-acute stage of nerve injury in mice

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ABSTRACT

Angiotensin-(1–7) (Ang [1–7]) and its receptor Mas are involved in a number of physiological processes, including control of arterial pressure and modulation of nervous system actions. However, the involvement of the Ang-(1–7)/Mas axis in peripheral nerve injury has not been investigated. Using a model of sciatic nerve injury in mice, we demonstrated opposing changes in Mas receptor expression at days 2 and 14 post-injury. Mas receptor expression was more intense 2 days after the nerve lesion, compared with the intensity of the intact nerve. At this time point, the sciatic nerve functional index was −20. At day 14 after the lesion, the intensity of the immunostaining labeling in longitudinal sections of the nerve was reduced (~30%) and the functional index increased +36 (gait improvement). In the axotomized group treated with A779 (a Mas receptor antagonist), the functional recovery index decreased in relation to the untreated axotomized group. The Mas receptor inhibitor also altered the intensity of labeling of S-100, GAP43, and IBA-1 (morphological features compatible with delayed axon growth). This study demonstrated that Ang-(1–7)/Mas axis activity was differentially modulated in the acute and post-acute stages of nerve injury.

1. Introduction

Ang 1–7 is a metabolite of the renin-angiotensin system that plays a similar role to angiotensin II (Ang II) in the release of vasopressin [1]. Ang-(1–7) and its endogenous receptor, named “Mas” [2], have been identified in neurons from the bulbar areas involved in the tonic reflexes of arterial pressure control and in astrocytes in hypertensive rat models [3,4]. The Mas receptor has also been detected in the peripheral nervous system (PNS), particularly in the sensory neurons of the dorsal root ganglia. At that location, it appears to be a contributing regulatory factor for sensory information involved with the anti-nociceptive mechanism, regardless of opiates [5]. The presence of the Mas receptor in the PNS inspired the hypothesis about its involvement in response to nerve injury.

Sciatic nerve crush is one of the most common models of peripheral nerve injury in rodents [6–8]. Crushing interrupts all axons (axotomy), but preserves the Schwann cells (SCs) and basal laminae. This allows for a precise study of the ability of a growing axon. After axotomy in the distal segment, the disconnected axons, along with the myelin sheath, initiate a process called “Wallerian degeneration.” The products of this degeneration are eliminated through the cooperative action of SCs and macrophages [9]. Furthermore, SCs form the Büngner bands that function as channels into which axonal growth is guided. Cell adhesion molecules, along with the neurotrophic factors produced by SCs, have an essential role in the elongation of the axon [10,11].

The innate and acquired immune systems are activated during Wallerian degeneration [12]. In the first few days post-injury, tissue clearance is improved by macrophages, and phagocytic capacity is potentiated by pro-inflammatory cytokines released by infiltrating CD4 T cells and opsonins [13] as well as antibodies produced by B lymphocytes. Tissue clearance is an important step for the regenerative process, since axon growth inhibitory proteins, such as the myelin-associated glycoprotein, are present in the fragmented myelin [11]. When debris is removed, the inflammatory response is suppressed by M2 macrophages and T helper 2 lymphocytes, creating a microenvironment appropriate to the regenerative process [14].

Most available evidence supports a counter-regulatory role for Ang-(1–7) in which it opposes many actions of angiotensin II on AT_1 receptors, especially vasoconstriction and proliferation. Many studies have now shown that Ang-(1–7) is associated with acute and chronic

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inflammation, including leukocyte influx, fibrogenesis, and the proliferation of certain cell types [for review see 15], by acting via the Mas receptor. Macrophages are particularly affected by Mas expression. Mas signaling affects macrophage polarization and migration as well as macrophage-mediated T-cell activation [16]. Considering that the Mas axis is involved in regulating the physiological functions of the PNS, we theorized that it might also be involved in the nerve injury process. To date, no study has characterized Mas expression in acute and post-acute sciatic nerve injury.

2. Methods

2.1. Animals

Forty-five adult male wild-type C57BL6, 6–8 weeks old mice, weighting around 25 g were used. The animals were maintained in a light/dark cycle of 12 h with food and water *ad libitum*. All experiments were approved by the Ethics Committee for Animal Experimentation of the Federal University of Uberlandia, Uberlandia, MG, Brazil (CEUA/UFU Protocol 09/12).

2.2. Reagents and procedures

The animals were divided into two groups. One group, called AXOTOMY, consisted of 25 animals that suffered peripheral nerve injury and another group, called SHAM, consisted of 20 animals that underwent the same surgical stress of animals excepted for peripheral nerve injured (skin and muscle incisions and exposure of the sciatic nerve;

SHAM group was used only in gait analysis for the study of the functional return of the nerve after injury). In the surgical procedure, the animals were anesthetized with ketamine hydrochloride and xylazine hydrochloride (1:1; 0.10 ml/25 g, via intraperitoneal) and then subjected to a crush lesion on the left sciatic nerve, near the obturator foramen. Using a number 4 jeweler's forceps, constant pressure was maintained for 10 s, and then the procedure was repeated by the same period of time [17]. The paw contralateral to the injured side was used as control.

After surgery, the two groups (axotomy and sham) were divided into subgroups as follows: Sham placebo (n = 10) and Axotomy placebo (n = 15) subgroups received subcutaneous injections of 100 µl saline (placebo treatment), Sham A779 (n = 10) and Axotomy A779 (n = 10). Potent antagonists of the Ang-(1–7) receptor have been generated by substituting the C-proline with a d-alanine, to form d-Ala^7-Ang-(1–7), called A779 (Asp1-Arg2-Val3-Tyr4-Ile5-His6-D-Ala7, MW 872.98KDa; code number H-2888; Bachem, Switzerland). Selective for the heptapeptide Ang-(1–7), the A779 peptide binds to Mas receptor with an IC50 of 0.3 nM, blocking the central and peripheral actions of Ang-(1–7) and exhibiting no significant affinity for AT_1 or AT_2 receptors at a concentration of 1 µM [18]. Utilization of these antagonists has been useful in unmasking specific effects of Ang-(1–7). The treatments took place daily with a dose of 72 µg/kg [19], always at the same time, during 14 days after surgery. Collection of footprints was carried out in all animals during gait for measurements and calculation of the Sciatic Functional Index (SFI) as described by Inserra et al. [20]. Analyzes were performed three days before the injury and two, five, nine, and thirteen days after surgery. SFI index is presented as percentage considering SFI before the surgery 100% (corresponding to healthy nerve).

Five axotomized animals of the placebo subgroup were euthanized after 48 h of nerve lesion to study the early Mas receptor expression. All other analyses of axotomized animals were performed only after 14 days of treatment. For this purpose, animals were anesthetized with ketamine hydrochloride and xylazine hydrochloride (1:1; 0.15 ml/25 g; via intraperitoneal) and subjected to euthanasia through thoracotomy and transcardiac perfusion with phosphate buffered saline (20 ml 0.1 M phosphate buffer plus 0.9% NaCl, pH 7.4; PBS) and fixative solution (20 ml 3.7% formaldehyde). Then, the sciatic nerves on both sides, contra and ipsilateral to the lesion, were drawn. The nerve was removed so as to leave the lesion site, identified by a surgical knot in the circumjacent muscles, at the median nerve segment removed. All the extracted material was frozen at −40 °C in isopentane cooled in liquid nitrogen. From the frozen blocks, longitudinal sections from the nerves were obtained using a cryostat (−25 °C, Microm, Heidelberg, Germany).

To perform the immunofluorescence, histological slides were acclimated at room temperature, immersed and washed in PBS, pH 7.4 and then incubated in a humidified chamber with 150 µl of 5% bovine albumin solution in PBS for 1 h. Next, the primary antibodies were added and incubated for 12–18 h at 4 °C in sections of the nerve (mouse anti-neurofilament, 1:200 [Sigma, N2912], rabbit anti-S100 beta, 1:200 [Abcam, AB868], mouse anti-GAP-43 1:100 [Sigma Aldrich, G9264], mouse anti-Mas, 1:50 [Alomone, AAR-013] and mouse anti-IBA-1, 1:100 [Imuny, IM0565]).

After the first incubation, slides were washed and incubated with secondary antibodies conjugated with fluorescent particle (Alexa Fluor 488 or 594, Jackson Laboratories, USA) for 1 h. The slides were rinsed and mounted with coverslip in glycerol/PBS (1:1) containing DAPI (4′-6′-diamino-2-phenyl-indole). The material was then examined and documented using a fluorescence microscope (TS-100, Nikon) coupled to a digital camera using the filters for fluorescein (488 nm) and rhodamine (594 nm).

For each animal, three representative images were captured from sections of the right (contralateral) and left (ipsilateral to the axotomy) nerves, with a 20 x objective, right distally the local of nerve crush. The images were quantitatively analyzed by pixel density using the ImageJ software (version 1.33u, National Institutes of Health, USA) by a blind researcher. For neurofilament immunolabeling, only qualitative evaluation was performed also by a blind researcher. There were ten measurements for each image (three images for each side of the animal). From the mean measurements of the ten measures, the average was calculated for each side of the animal. Finally, the mean for each group was calculated and comparison between these values was analyzed by using the Anova one way with Bonferroni post-test. All results were expressed as mean ± standard error of mean (SEM). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Mas receptor expression in the sciatic nerve after axotomy

Fig. 1 shows Mas immunostaining in nerves of axotomized animals. Mas receptor expression was more intense qualitatively and quantitatively (pixel density) at the site of the injury 2 days after axotomy (ipsilateral side; Fig. 1B) compared with the intensity of the intact nerve. At day 14 after the lesion, the immunostaining in longitudinal sections of the nerve was reduced (30%). Thus, in the acute phase of the lesion the intensity of Mas labeling was higher than in the chronic phase of the lesion.

3.2. Treatment with Mas antagonist A779 interfered with the functional recovery after peripheral nerve injury

To further examine this relationship, groups of animals were treated with Mas receptor antagonist A779 for 14 days. Functional recovery test was performed at days 2, 5, 9 and 13 post-injury. In the early days after axotomy, the animals were unable to sustain their bodies on the ipsilateral paw and the fingers were in adduction, reflecting sciatic nerve dysfunction. Within two weeks following the axotomy, the animals gradually recovered the ability to support themselves on the ipsilateral limb with the fingers in abduction. Fig. 2 shows that all animals started from a sciatic nerve function considered normal before the surgical procedure (score of 100%). Two days after nerve axotomy, a