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Research report

Sciatic nerve transection increases gluthatione antioxidant system activity and neuronal nitric oxide synthase expression in the spinal cord

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ABSTRACT

Glutathione (GSH) is a major non-enzymatic antioxidant which is present in all tissues. Its protective actions occur through different pathways such its role as a substrate of antioxidant enzymes, such as glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST). Nitric oxide (NO) is involved in many physiological processes in the central nervous system, including nociception. In spite of much evidence concerning oxidative and nitrosative stress and neuropathic pain, the exact role of these molecules in pain processing is still unknown. Sciatic nerve transection (SNT) was employed to induce neuropathic pain in rats. Glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST) activities, glutathione (GSH) content, GSH/GSSG ratio, nitric oxide metabolites (NOx) and neuronal nitric oxide synthase (nNOS) protein expression in the lumbosacral spinal cord were determined. All of these analyses were performed in the SNT and sham groups 1, 3, 7 and 15 days after surgery. There was an increase in GPx activity and in GSH content 3 days after surgery in both sham and SNT groups, but the GSH/GSSG ratio increased only in the SNT group in this time point. nNOS expression was upregulated 7 days post SNT. NOx was detected 1 day after surgery in sham and SNT groups, but at 7 and 15 days, the increase occurred only in SNT animals. These results support the role of the gluthatione system in pain physiology and highlight the involvement of NO as an important molecule related to nociception.

1. Introduction

Pain transmission is determined by neurochemical changes in the central nervous system (CNS), mainly in the spinal cord, including alterations in neurotransmitters, receptors, inflammatory mediators and transcriptional factors [59,64,20,39,24]. Over the last years, studies regarding reactive oxygen species (ROS) and antioxidant systems in nociceptive processing have emerged [22,17,14,17]. Evidence indicates that higher levels of ROS and changes in antioxidant activity in the CNS after peripheral nerve injury or tissue inflammation may be important factors in persistent pain [58,25].

Cellular metabolic reactions generate small amounts of ROS, including hydroxyl radicals (•OH), superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Under normal physiological conditions, ROS production is balanced by several cellular antioxidant mechanisms in order to avoid the harmful effects of these species [34,33,55]. Antioxidant systems are crucial in providing defenses against oxidative cell damage. Glutathione (GSH) is one of the most abundant non-enzymatic antioxidants in all tissues. GSH can scavenge free radicals, reduce peroxides, and be conjugated with electrophilic compounds through enzymatic or non-enzymatic reactions. The oxidation of GSH leads to the production of glutathione disulfide (GSSG). Since GSSG/GSH pair forms the major redox couple in cells [47,21], this ratio can be used to establish the oxidative status of the tissue [15,3,36]. Hence, the cell protective actions of the GSH occur through different pathways including its role as a substrate of antioxidant enzymes, such as glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST) [54]. GST plays a major part in the detoxification of xenobiotics and products of lipoperoxidation [30]. In turn, GPx catalyzes the reduction of hydrogen peroxide and organic hydroperoxides at the expense of GSH. GPx also functions as a defense line against peroxynitrite-mediated oxidations [33,48]. Using a model of neuropathic pain, a previous study has reported a decrease in GSH content in sciatic nerve after chronic constriction injury [38]. In addition, Tan et al. [51] showed an increase in GSH content in skeletal muscle and Costa et al. [10] observed an activation of the GSH system in rat paw tissue after peripheral nerve injury. However, there are no studies establishing a relationship between GSH content in the spinal cord and neuropathic pain.

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In addition, nitrosative stress, which is characterized by an overproduction of reactive nitrogen species (RNS), such as peroxynitrite, does also interfere in pain modulation [50,12,56,4]. Because it is a diffusible molecule that contains one unpaired electron, nitric oxide (NO) can also be regarded as a RNS. NO acts as an inter- and intracellular messenger in many tissues and is involved in many physiological processes in the CNS. It promotes survival and differentiation of neural cells, and it is also involved in synaptic activity, neural plasticity and memory formation. These effects occur through the regulation of transcription factors and the modulation of gene expression [9]. In nociceptive transmission, NO is a mediator usually referred to as pronociceptive [26,61]. This effect possibly occurs via release of glutamate in the spinal cord dorsal horn which activates N-methyl-D-aspartate (NMDA) receptors and NO production. NO produced in response to nociceptive stimulation acts as a retrograde messenger that enhances presynaptic activity, intensifying pain signaling [35,63]. However, analgesic effects of nitric oxide synthase (NOS) inhibitors are controversial. While some studies show that NOS inhibitors decrease hyperalgesia [45,40], others demonstrate that these compounds have no effect on pain behaviors after injury [29].

Thus, due to the existence of a correlation among antioxidants, ROS, RNS and pain, the aim of the present study was to establish the involvement of the GSH antioxidant system and NO in the lumbosacral spinal cord after neuropathic pain induction using sciatic nerve transection (SNT) as an experimental model.

2. Materials and methods

2.1. Animals, surgical procedure and preparation of samples

Under intraperitoneal anaesthesia (ketamine 80 mg/kg and xylazine 2 mg/kg) and sterile conditions, the right sciatic nerve of male Wistar rats weighing 200–250 g was exposed and transected at mid thigh level. In sham-operated animals, the right sciatic nerve was exposed but not transected. For further comparisons, a naïve group was included in which the animals did not undergo surgical manipulation. Groups of five animals were sacrificed 1, 3, 7 and 15 days after surgery. All animal procedures were approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul.

Rats were killed by decapitation and their whole lumbosacral spinal cords were promptly dissected out. In order to determine GSH and GSSG contents, the samples were immediately cooled in liquid nitrogen and processed. In order to determinate GPx and GST activities and NO metabolites (NOx), the tissues were immediately cooled in ice and homogenized in 1.15% KCl diluted 1:5 (w/v) containing 1 mmol/L PMSF. The homogenates were centrifuged at 1000 × *g* for 20 min to discard nuclei and cell debris. The supernatant fraction obtained was frozen at −70 °C for further measurements. For Western blot analyses, samples were homogenized (Ultra-Turrax) with lysis buffer (NP40), pH 7.4, proportionally to weight (20 mL/g of tissue) with leupeptin and aprotinin.

2.2. Determination of oxidized and reduced glutathione concentration

To determine oxidized and reduced GSH concentration, tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 × *g* and supernatant was neutralized with 2 mol/L potassium hydroxide. The reaction medium contained 100 mmol/L phosphate buffer (pH 7.2), 2 mmol/L nicotinamide dinucleotide phosphate acid, 0.2 U/mL glutathione reductase, 70 μmol/L 5,5′ dithiobis (2-nitrobenzoic acid). To determine reduced glutathione, the supernatant was neutralized with 2 mol/L potassium hydroxide, to react with 70 μmol/L 5,5′ dithiobis (2-nitro benzoic acid), and read at 420 nm [2].

2.3. Determination of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities

GPx activity was measured by following NADPH oxidation at 340 nm as described by Flohé and Gunzler [13]. GPx results were expressed as nmol of peroxide/hydroperoxide reduced/min/mg protein. GST activity, expressed as nmol/mg protein, was measured by the rate of formation of dinitrophenyl-*S*-glutathione at 340 nm. The reaction medium consisted of 19 mmol/L sodium phosphate buffer (pH 6.5), 1 mmol/L GSH, and 1 mmol/L chloride dinitrobenzene [31].

2.4. Determination of NO metabolites (NOx)

For NOx measurement, nitrites (NO_2^-) were determined using the Griess reagent, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthyl-ethylenediamine (0.1%) and sulfanilamide (1%). Nitrates (NO_3^-) were determined as total nitrites (initial nitrite plus nitrite reduced from nitrate) after their reduction using nitrate reductase from the *Aspergillus* species, in the presence of NADPH. A standard curve was established with a set of serial dilutions (10^{-8} to 10^{-3} mol/L) of sodium nitrite. Results were expressed as mmol/L of nitrates plus nitrites [16].

2.5. Western blot

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (12%) was carried out using a miniprotein system (Bio-Rad). The molecular weights of the bands were determined by reference to a standard molecular weight marker (rainbow full range Amersham). Protein (60 μg) was loaded in each lane with a loading buffer containing 0.375 mol/L Tris (pH 6.8), 50% glycerol, 10% SDS, 0.5 mol/L mercaptoethanol, and 0.002% bromophenol blue. Samples were heated at 100 °C for 2 min prior to gel loading. After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham) using an electrophoretic transfer system at 110 V for 1 h. The membranes were then washed with TTBS (20 mmol/L Tris–HCl, pH 7.5; 150 mmol/L NaCl; 0.05% Tween-20, pH 7.4) and 8% non-fat dry milk for 1 h. The membranes were incubated overnight at 4 °C with the primary antibody diluted in TTBS plus 2.5% BSA. Rabbit anti-universal NOS (Sigma) was used as the primary antibody. After washing with TTBS, the membranes were incubated for 2 h at room temperature with secondary antibody (1:5000, peroxidase conjugated anti-rabbit IgG; Santa Cruz), washed with TBS (20 mmol/L Tris–HCl; 150 mM NaCl, pH 7.5) and revealed by chemiluminescence followed by apposition of the membranes to autoradiographic films. These films were analyzed with an image densitometer (Imagemaster VDS CI, Amersham). The results were expressed in % of pixels mean. The amount of protein transfer per lane was corrected by the Ponceau method [23].

2.6. Protein measurement

Protein was measured by the Bradford method [5], using bovine serum albumin as standard.

2.7. Statistical analyses

Enzyme activities, GSH content, NOx and Western blot results were compared by one-way ANOVA followed by the Student–Newman–Keuls post hoc multiple comparison test. The GSH/GSSG ratio was analyzed by the Kruskal–Wallis nonparametric test followed by post hoc Dunn's method. Differences were considered statistically significant when the *P*-value was <0.05. All statistical analysis was carried out with Sigma Stat 2.0 software.

3. Results

GSH content in the lumbosacral spinal cord was increased 3 days after surgery ($P < 0.001$) (Fig. 1). This increase was similar in both sham and SNT animals, showing that changes in GSH content were not specific to neuropathic pain. No differences in GSH content were found in the other periods analyzed. GPx activity (Fig. 2) also showed a marked increase at the same time point of GSH, at 3 days

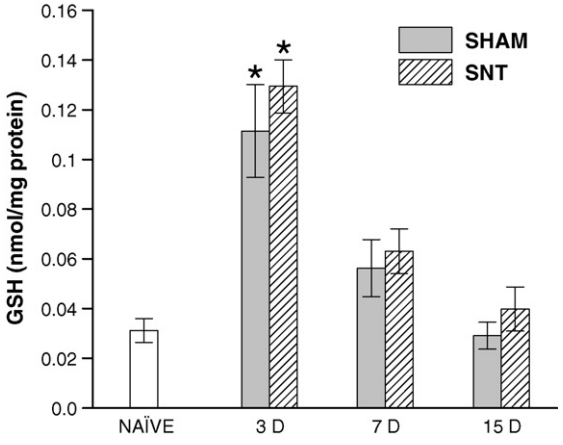

Fig. 1. Glutathione content in the lumbosacral spinal cord of naïve, sham and SNT groups 3, 7 and 15 days. $^*P < 0.001$ (one-way ANOVA followed by Student–Newman–Keuls post hoc test) for naïve vs. sham and naïve vs. SNT at 3 days. Glutathione content is expressed as nmol/mg of protein. Data represent mean ± SEM ($n = 5$ for each group). SNT, Sciatic nerve transection; 3, 7 and 15 days after surgery.