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Oxaliplatin evokes P2X7-dependent glutamate release in the cerebral cortex: A pain mechanism mediated by Pannexin 1

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ABSTRACT

Anticancer therapy based on the repeated administration of oxaliplatin is limited by the development of a neuropathic syndrome difficult to treat. Oxaliplatin neurotoxicity is based on complex nervous mechanisms, the comprehension of the role of single neurotransmitters and the knowledge of the signal flow among cells is matter of importance to improve therapeutic chances.

In a rat model of oxaliplatin-induced neuropathy, we report increased P2X7-evoked glutamate release from cerebrocortical synaptosomes. The release was abolished by the P2X7 receptor (P2X7R) antagonists Brilliant-Blue-G (BBG) and A-438079, and significantly reduced by Carbenoxolone and the Pannexin 1 (Panx1) selective inhibitors Erioglaucine and ^{10}Panx suggesting the recruitment of Panx1. Aimed to evaluate the significance of P2X7R-Panx1 system activation in pain generated by oxaliplatin, pharmacological modulators were spinally infused by intrathecal catheter in oxaliplatin-treated animals. BBG, Erioglaucine and ^{10}Panx reverted oxaliplatin-dependent pain. Finally, the influence of the P2X7R-Panx1 system blockade on oxaliplatin anticancer activity was evaluated on the human colon cancer cell line HT-29. Prevention of HT-29 apoptosis and mortality was dependent by kind and concentration of P2X7R antagonists. On the contrary, the inhibition of Panx1 did not alter oxaliplatin lethality in tumor cells.

It is concluded that glutamate release dependent on P2X7R is increased in cerebrocortical nerve terminals from oxaliplatin-treated rats; the increase is mediated by functional recruitment of Panx1; P2X7R antagonists and Panx1 inhibitors revert oxaliplatin-induced neuropathic pain; Panx1 inhibitors do not alter the oxaliplatin-induced mortality of cancer cells HT-29. The inhibition of Panx1 channel is suggested as a new and safe pharmacological target.

1. Introduction

Neuropathic pain evolves from the physiological role of nociceptive pain to pathological aspects depending on the complex response of the nervous system to a lesion or disease of its somatosensory component. Electrical, molecular and cellular activity participate in sensitizing nervous circuits of periphery, dorsal horn as well as anterior cingulate gyrus, prefrontal cortex, amygdala, and periaqueductal gray leading to pain-mediating signals (von Hehn et al., 2012). The maladaptive plasticity of the central nervous system (CNS) assumes increasing evidence in the pathophysiology of chemotherapy-induced neuropathies (Di Cesare Mannelli et al., 2013a; Renn et al., 2011), a peculiar iatrogenic damage of the nervous tissue that results in therapy dose reduction or discontinuation and negatively influences quality of life on cancer survivors (Hershman et al., 2014).

ATP is recognized as one of the keys for the relay of sensory information from the periphery to the CNS (Burnstock, 2007). Both sensory neurons and glial cells inside and outside of the CNS release ATP to affect surrounding cells (Skaper et al., 2010). Accumulated

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evidence indicates that ATP and its purine receptors are involved in the regulation of neuropathic pain (Burnstock, 2015; Tsuda et al., 2010). Among the ATP-gated ionotropic P2X receptors the P2X7 subtype (P2X7R) plays a central role in glial/neuron crosstalk (Bennett et al., 2009; Sperlagh et al., 2006). Functional P2X7Rs are found on different nerve terminals (Marcoli et al., 2008; Sperlagh et al., 2006). In CNS glutamatergic neurons (Atkinson et al., 2004; Sperlagh et al., 2006), P2X7R triggers the release of glutamate, a neurotransmitter crucially involved in the central sensitization and in the related changes in the properties of central neurons (Latremoliere and Woolf, 2009).

P2X7R exhibits peculiar physiological and pharmacological characteristics (Skaper et al., 2010). Differently from the micromolar affinity for ATP shown by the other family members, P2X7R is activated by high concentrations of ATP (>100 μM), further its prolonged exposure to ATP causes the formation of a reversible plasma membrane pore permeable to hydrophilic solutes up to 900 Da (Skaper et al., 2010). The large pore is either formed by P2X7 itself in a process termed pore dilation (Browne et al., 2013) or as a result of recruitment and activation of Pannexin 1 (Panx1) (Di Virgilio, 2007; Pelegrin and Surprenant, 2006).

Aimed to study the role of P2X7R-Panx1 system in chemotherapy-induced neuropathic pain, the pharmacological analysis of glutamate release from nerve terminals was performed in the cerebral cortex of oxaliplatin-treated rats. P2X7R-Panx1 modulation was evaluated as pain reliever focusing on molecules that not interfere with oxaliplatin antitumor mechanisms.

2. Materials and methods

2.1. Animals

For all the experiments described below, male Sprague–Dawley rats (Harlan, Varese, Italy) weighing approximately 200–250 g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (N°54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guideline (Kilkenny et al., 2010). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Intrathecal catheterization

Rats were anesthetized with 2% isoflurane and intrathecal catheter was surgically implanted according to (Yaksh and Rudy, 1976). Rats were shaved on the back of the neck and placed in the stereotaxic frame with the head securely held between ear bars. The skin over the nap of the neck was cleaned with ethyl alcohol and incised for 1 cm. The muscle on either side of the external occipital crest was detached and retracted to expose about 3–4 mm^2 of the atlanto-occipital membrane. The membrane was incised by a needle, which led to the escape of cerebrospinal fluid. The caudal edge of the cut was lifted and about 7.0 cm of 28G polyurethane catheter (0.36 mm outer diameter; 0.18 mm inner diameter; Alzet, USA) was gently inserted into the intrathecal space in the midline, dorsal to the spinal cord until the lumbar enlargement. The exit end of the catheter was connected to 4.0 cm polyurethane (0.84 mm outer diameter; 0.36 mm inner diameter) and was taken out through the skin, flushed with saline solution, sealed and securely fixed on the back of the head with a silk wire. All animals used during behavioral tests did not shown motor impairment induced by surgical operation for the catheter implantation. The evaluation of potential motor dysfunctions were investigated using Rota rod test. The animals who represented any kind of motor disability were excluded from the behavioral measurements.

2.3. Oxaliplatin model

Oxaliplatin treatment started 4 days after the surgical procedure for the spinal catheter implantation. Rats were treated with 2.4 mg kg^{-1} oxaliplatin (Sequoia Research Products, Pangbourne, UK), administered intraperitoneally (i.p.) for 5 consecutive days every week for 2 weeks (10 i.p. injections) (Cavaletti et al., 2001; Di Cesare Mannelli et al., 2013a). Oxaliplatin was dissolved in a 5% glucose-water solution. The model used for the present research is consistent with the clinical practice (Cavaletti et al., 2001; Zanardelli et al., 2014).

2.4. Preparation of purified nerve terminals

Purified nerve terminals (synaptosomes) were prepared from the cerebral cortex of oxaliplatin-treated (and vehicle-treated) rats on day 15 of treatment, as previously reported (Marcoli et al., 2008). Briefly, the cerebral cortex was rapidly removed and placed in ice-cold medium, then homogenized in 0.32 mM sucrose with Tris–HCl, pH 7.4, using a glass-Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 4 °C, 1000 g) to remove nuclei and debris; the supernatant was stratified on a discontinuous Percoll gradient (2%, 6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 g (5 min). The layer containing synaptosomes (between 10% and 20% Percoll) was collected and washed by centrifugation; synaptosomes were then suspended in HEPES medium (mM: NaCl 128, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.0, and HEPES 10 with glucose 10, pH 7.4).

2.5. Glutamate release from superfused synaptosomes

Glutamate release was studied by measuring tritium efflux from synaptosomes pre-labeled with [^{3}H]D-aspartate (Amersham Radiochemical Centre, Buckinghamshire, UK (see (Alloisio et al., 2008; Cervetto et al., 2012; Marcoli et al., 2008)). Briefly, synaptosomes were incubated (15 min, 37 °C) with [^{3}H]D-aspartate (0.03 μM), transferred to parallel superfusion chambers at 37 °C and superfused (0.5 ml/min) with standard medium. After 33 min superfusion, superfusate fractions were collected (3-min samples) till the end of the experiment; after 38 min superfusion, synaptosomes were exposed (120 s) to 2′-3′-O-(benzoylbenzoyl) ATP (BzATP; Sigma–Aldrich, Milan, Italy). The effect of Brilliant Blue G (BBG; Sigma–Aldrich, Milan, Italy), A-438079 (Tocris Bioscience, Bristol, UK), Carbenoxolone (Sigma–Aldrich, Milan, Italy), Brilliant Blue FCF (Erioglaucine; Sigma–Aldrich, Milan, Italy) or ^{10}Panx (Trp-Arg-Gln-Ala-Ala-Phe-Val-Asp-Ser-Tyr; WRQAAFVDSY; Proteogenix, Schiltigheim, France) was evaluated by adding the drug 8 min before BzATP. The radioactivity in synaptosomes and superfusate fractions was determined by liquid scintillation counting at the end of superfusion. The radioactivity released in each fraction was calculated as a percentage of the synaptosomal tritium content at the start of the respective collection period (fractional efflux). The BzATP-evoked release (overflow) in the presence or absence of antagonists was calculated by subtracting the basal efflux from the total tritium released in the fractions collected during and after stimulation. In each experiment at least one chamber was used as a control for each condition and was superfused with standard medium or with medium appropriately modified.

When possible, drugs were dissolved in distilled water or in physiological medium. ^{10}Panx was dissolved in 1% DMSO to obtain 1 mM solution and then diluted in physiological medium at final concentration.

2.6. In vivo pharmacological treatments

BBG and Erioglaucine were dissolved in sterile saline solution. ^{10}Panx was dissolved in 1% DMSO to obtain 1 mM solution and then diluted in sterile saline solution to obtain the final concentrations. Behavioral measurements were performed on day 15 of oxaliplatin treatment after the intrathecal (i.t.) infusion of 0.01–1 nmol BBG, 0.01–1 nmol Erioglaucine and 0.01–10 nmol ^{10}Panx. All compounds were infused in a final volume of 10 μl. Control animals received equivalent volumes of vehicles. The i.t. route of administration was used for all compounds in order to compare the effects induced by BBG and Erioglaucine with those of 10Panx (unable to cross the blood brain barrier) and to specifically evaluate their central properties.

2.7. Paw-pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to a previously published method (Leighton et al., 1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (25%). For analgesia measures, mechanical pressure application was stopped at 120 g.

2.8. Von Frey test

The animals were placed in 20 cm × 20 cm Plexiglas boxes equipped with a metallic mesh floor, 20 cm above the bench. Animals were allowed to habituate themselves to their environment for 15 min before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: the withdrawal threshold was evaluated by applying forces ranging from 0 to 50 g with a 0.2 g accuracy. Punctuate stimulus was delivered to the mid-plantar area of each anterior paw from below the mesh floor through a plastic tip and the withdrawal threshold was automatically