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HOMOCYSTEINE-INDUCED ATTENUATION OF VASCULAR ENDOTHELIUM-DEPENDENT HYPERALGESIA IN THE RAT

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Abstract—We have recently demonstrated a role of the vascular endothelium in peripheral pain mechanism by disrupting endothelial cell function using intravascular administration of octoxynol-9, a non-selective membrane active agent. As an independent test of the role of endothelial cells in pain mechanisms, we evaluated the effect of homocysteine, an agent that damages endothelial cell function. Mechanical stimulus-induced enhancement of endothelin-1 hyperalgesia in the gastrocnemius muscle of the rat was first prevented then enhanced by intravenous administration of homocysteine, but was only inhibited by its precursor, methionine. Both homocysteine and methionine significantly attenuated mechanical hyperalgesia in two models of ergonomic muscle pain, induced by exposure to vibration, and by eccentric exercise, and cutaneous mechanical hyperalgesia in an ischemia–reperfusion injury model of Complex Regional Pain Syndrome type I, all previously shown responsive to octoxynol-9. This study provides independent support for a role of the endothelial cell in pain syndromes thought to have a vascular basis, and suggests that substances that are endothelial cell toxins can enhance vascular pain.

Key words: vascular pain, homocysteine, methionine endothelium, muscle pain.

INTRODUCTION

We recently discovered a novel phenomenon that drives an active contribution of vascular endothelial cells to peripheral pain mechanisms (Joseph et al., 2011). This phenomenon, referred to as stimulus-dependent hyperalgesia, is elicited by two potent vasoactive compounds: endothelin-1 (ET-1) and epinephrine. ET-1 and epinephrine act at their cognate receptors on the endothelial cell to produce a state in which mechanical stimulation produces enhanced release of ATP, which acts on the P2X3 purinergic receptor on sensory neurons to produce stimulus-dependent hyperalgesia (Joseph et al., 2013). Stimulus-dependent hyperalgesia is distinct from the direct hyperalgesic effect of ET-1 and epinephrine mediated by their cognate receptors on the peripheral terminals of the nociceptor (Joseph et al., 2011; Joseph and Levine, 2012a). The discovery of the role of vascular endothelial cells in stimulus-dependent hyperalgesia was made possible by adaptation of a method from the cardiovascular and renal vascular literature using the intravenous administration of octoxynol-9 to attenuate endothelial cell function (Connor and Feniuk, 1989; Jamal et al., 1992; Sun et al., 1997). We found that administration of octoxynol-9 eliminated ET-1-induced stimulus-dependent hyperalgesia it being dependent on the function of the endothelium; the direct hyperalgesic effect of ET-1 acting on the nociceptor was unaffected by octoxynol-9 administration. Since octoxynol-9 is a non-selective membrane active agent, in the present study we employed an alternative method to impair endothelial cell function. It has been demonstrated in rats as well as in humans that hyperhomocysteinemia produces endothelial dysfunction (Kanani et al., 1999; Edirimanne et al., 2007). Since hyperhomocysteinemia affects several functions of endothelial cells (Abahji et al., 2007; Pushpakumar et al., 2014), we tested whether homocysteine or its precursor, L-methionine (Kanani et al., 1999; Edirimanne et al., 2007) affects ET-1-induced stimulus-dependent hyperalgesia. We found that homocysteine, in a time-dependent manner, both inhibited and enhanced stimulus-dependent hyperalgesia, while methionine only produced inhibition. Both substances also inhibited preclinical models of vascular pain syndromes previously shown to be attenuated by octoxynol-9 (Joseph and Levine, 2012a; Joseph et al., 2013). Our data provide independent support for a role of the endothelial cell in mechanical stimulus-dependent hyperalgesia and vascular pain syndromes.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on adult male Sprague–Dawley rats, (200–250 g; Charles River, Hollister, CA, USA). Animals were housed three per cage, under a 12-h light/dark cycle, in a temperature- and

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Abbreviations: CRPS, Complex Regional Pain Syndrome; ET-1, endothelin-1; i.d., intradermal.

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humidity-controlled environment. Food and water were available *ad libitum*. All behavioral nociceptive testing was performed between 10:00 AM and 4:00 PM. Rats were acclimatized to the testing environment, by bringing them to the experimental area, in their home cages, left in the home cage for 15–30 min, after which they were placed in restrainers, cylindrical acrylic tubes that have side openings that allow extension of the hind limbs from the restrainer, for nociceptive testing. Rats were left undisturbed in the restrainer for another 15–30 min before nociceptive testing was started.

Nociceptive threshold was defined as the mean of three readings taken at 5-min intervals. All experimental protocols were approved by the University of California, San Francisco Committee on Animal Research and conformed to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. All efforts were made to minimize the number of animals used and their suffering.

Nociceptive testing

Cutaneous nociception. The nociceptive flexion reflex was quantified with an Ugo Basile Analgesymeter (Stoelting, Wood Dale, IL, USA), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw. Nociceptive threshold was defined as the force, in grams, at which the rat withdrew its hind paw from the stimulus. Hyperalgesia was defined as a decrease in mechanical nociceptive threshold, here presented as percentage change from baseline. Both paws of the same rat received the same treatment, and paw withdrawal thresholds were averaged for each rat. Each experiment was performed on separate groups of rats. These animals acted as their own controls, with a test agent injected either intradermally, into the dorsal surface of the hind paws, or intravenously before the intradermal administration of ET-1. Paw-withdrawal thresholds before and after drug treatment were compared.

Muscle nociception. Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a Chatillon digital force transducer (model DFI2; Ametek, Berwyn, PA, USA) (Dina et al., 2010). In lightly restrained rats (as described above), a 6-mm-diameter probe, attached to the force transducer, was applied to the skin overlying the gastrocnemius muscle, to deliver an increasing compression force. This probe width allows for evaluation of muscle nociceptive threshold without interference from nociceptive threshold of the overlying cutaneous nociceptive afferents (Murase et al., 2010). The nociceptive threshold was defined as the force (in milliNewtons) at which the rat withdrew its hind limb; results are presented as percentage change from baseline. Each hind limb (gastrocnemius muscle) is treated as an independent measure, and each experiment is performed on a separate group of rats.

Preclinical models

Vibration-induced hyperalgesia. It has been suggested that musculoskeletal pain induced by exposure to vibrating devices used in various occupations may have a vascular component (Ogasawara et al., 1997; Dowd et al., 1998). We demonstrated previously that exposure to vibration produces chronic muscle pain in the rat (Chen et al., 2010). The rat's hind limb was vibrated with a Digital Vortex Genie II laboratory Vortex mixer (Thermo Fisher Scientific, Waltham, MA, USA) that has a variable-speed motor with a real-time digital readout of the vibration speed. Rats were anesthetized with 3% isoflurane in oxygen and one hind limb affixed to the platform with Micropore surgical tape (3M, St. Paul, MN, USA) so that the knee and ankle joint angles were both 90°, and without rotational torque on the leg. The leg was vibrated at a frequency of 60–80 Hz, with a 5-mm peak-to-peak displacement amplitude. These vibration frequencies are within the ranges produced by hand-held power tools (35–150 Hz) (Radwin et al., 1990). In previous studies in the rat, more intense hind limb vibration at 80 Hz for 5 h daily for 2 d did not cause muscle necrosis (Lundborg et al., 1990). In the present experiments, hind limbs were vibrated once for 15 min.

Eccentric exercise-induced hyperalgesia. The method used to eccentrically exercise the rat hind limb (Alvarez et al., 2010) is similar to that described by Kano et al. (1994) and Taguchi et al. (2005). Briefly, isoflurane-anesthetized rats were placed in the supine position and the right hind paw was affixed to the foot bracket of the exercise apparatus (model RU-72; NEC Medical Systems, Tokyo, Japan) with Micropore surgical paper tape, such that the angle of the knee and ankle joints was at 90° (with the paw 30° from vertical). The gastrocnemius muscle was stimulated via subcutaneous needle-type electrodes attached to a model DPS-07 stimulator (Dia Medical System, Tokyo, Japan) that delivered trains of rectangular pulses (100 Hz, 700 ms, 3 V) every 3 s to give a total of 300 contractions. During these electrical stimulus-induced contractions of the gastrocnemius muscle, the electromotor system rotated the foot to produce extension of the gastrocnemius muscle.

Ischemia reperfusion-induced hyperalgesia. We used an ischemia–reperfusion injury model of Complex Regional Pain Syndrome (CRPS) type I (Coderre et al., 2004; Millecamps et al., 2010; Ragavendran et al., 2013). Rats were anesthetized with isoflurane and placed on a heating pad to maintain body temperature, and ophthalmic ointment used to prevent the corneas from drying out. A nitrile O-ring (5.5-mm internal diameter, durometer rating 70 Shore A, Grainer, Inc., San Francisco, CA) was placed around a hind limb, proximal to the ankle joint, to reduce arterial blood flow to the hind paw for 3 h, after which the O-ring was removed and the rats were allowed to recover from anesthesia. Nociceptive testing was performed 3 days after ischemia–reperfusion procedure.