Meningeal trigeminovascular reactions mediated by TRPV1 and TRPA1 receptors: effect of adriamycin treatment

PhD Thesis

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Szeged

2018

Publications related to the thesis:

I. Dux M, **Deák É**, Tassi N, Sántha P, Jancsó G.: Endovanilloids are potential activators of the trigeminovascular nocisensor complex. J Headache Pain. 2016;17:53. doi: 10.1186/s10194-016-0644-7. Impact factor: 3.58

II. **Deák É**, Rosta J, Boros K, Kis G, Sántha P, Messlinger K, Jancsó G, Dux M.: Chronic adriamycin treatment impairs CGRP-mediated functions of meningeal sensory nerves. Neuropeptides 2018; Epub: 10 April 2018. Impact factor: 2.486

INTRODUCTION

Intracranial nociceptive processes play significant role in the pathophysiology of headaches. A large body of evidence supports the hypothesis that primary headaches, such as migraine, are of trigeminovascular origin. They are induced or influenced by nociceptors innervating the cranial meninges, particularly the dura mater encephali and large intracerebral blood vessels. Sensory and autonomic nerve fibers innervate the dura mater, they form dense plexuses around dural arterial blood vessels. Sensory innervation of the dura mater is served by all three branches of the trigeminal nerve. Ultrastructural analyses of trigeminal afferents revealed that the majority of meningeal Aδ and C fibers terminate as free nerve endings.

Chemosensitive primary sensory neurons represent a unique population of trigeminal ganglion neurons. They express different members of the transient receptor potential (TRP) receptor family; the transient receptor potential vanilloid 1 (TRPV1) and the transient receptor potential ankyrin 1 (TRPA1) ion channels. TRPV1 receptor can be activated by high temperature (>43°C), acidic pH, and a wide range of exogenous (e.g. capsaicin) and endogenous compounds. Different metabolites of membrane lipids have been recently characterized as endogenous activators of the TRPV1 receptor. Arachidonylethanolamide (anandamide) and N-arachidonoyl-dopamine (NADA) have been previously identified in dorsal root ganglion neurons as potential endogenous activators of TRPV1 and cannabinoid (CB) receptors under physiological or pathophysiological conditions.

The TRPA1 channel is expressed in a subset of TRPV1 receptor-expressing chemosensitive primary sensory neurons. Recent studies found evidence that TRPA1 is involved in sensory neural responses to mustard oil (allyl isothiocyanate), allicin, cinnamaldehyde and gingerol. Also acrolein and other environmental irritants that are known triggers of migraine headache attacks in susceptible individuals, activate TRPA1 receptors.

A significant population of TRPV1 and TRPA1 receptor-expressing trigeminal nociceptors are peptidergic. Retrograde tracing experiments indicated that nearly 80 % of dural afferents expressing TRPV1 receptors also exhibit immunoreactivity for calcitonin gene-related peptide (CGRP). Meningeal nerve fibers immunoreactive for CGRP, substance P (SP) or neurokinin A are considered to be afferents of the trigeminal

sensory system. The majority of the CGRP-immunoreactive fibers are distributed to branches of the anterior- and middle meningeal arteries and to the superior sagittal and transverse sinuses. Chemosensitive meningeal afferents containing neuropeptides have a dual function. Peptides contained in primary sensory neurons serve not only as transmitters at the central synapses of nociceptive afferents but they also play a role in the mechanisms of the neurogenic inflammatory response of the innervated tissue. Release of neuropeptides induces neurovascular reactions; neurogenic vasodilatation induced by CGRP release, and neurogenic plasma extravasation mediated by SP. A large variety of transmitters and mediators has been shown to influence the neuropeptide release from peripheral endings of primary sensory neurons. Stimulation of cannabinoid (CB) receptor 1 of chemosensitive neurons decreasing intracellular cyclic adenosine monophosphate (cAMP) production reduces the transmitter release from nerve terminals. In the trigeminal system cannabinoid CB1 receptor immunoreactive neurons were found mainly in the maxillary and mandibular divisions of the trigeminal nerve. Activation of CB1 receptors may have a particular role in the regulation of CGRP release from TRPV1 expressing neurons, since both receptors can be activated by the same endogenous lipid metabolites as anandamide and NADA acting on both TRPV1 and CB1 receptors with different efficacies.

Meningeal blood vessels, nociceptors and dural mast cells are both anatomically and functionally interconnected and may be regarded as an important entity in pathophysiological processes of meningeal nociception. Neurogenic plasma extravasation seems to be of minor significance in migraine pathogenesis, while neurogenic sensory vasodilatation mediated by CGRP appears to play an important role in it. Release of CGRP into the jugular blood has been demonstrated during migraine attacks in humans.

Adriamycin, an anthracycline-type antitumor agent is used in the treatment of various malignancies. Besides its therapeutic effect adriamycin may also have serious side effects altering cardiac functions. Clinical observations and experimental results indicate that adriamycin exerts also a neurotoxic effect. Recent studies demonstrated marked structural, neurochemical and functional impairments of primary sensory neurons in animal models of adriamycin toxicity. Observations in our laboratory indicated profound changes in the density of intraepidermal chemosensitive afferents

while the distribution and density of subepidermal nerve fibers were apparently unaltered. Since somatosensory and visceral chemosensitive nerves share common characteristics, it can be assumed that systemic adriamycin treatment may produce alterations in meningeal chemosensitive afferent nerves resulting in impairments of dural vascular functions.

AIMS OF THE STUDY

Chemosensitive peptidergic sensory neurons expressing the TRPV1 and TRPA1 receptors play a significant role in nociceptive reactions. Functional changes in trigeminal chemosensitive nociceptors innervating the dura mater encephali contribute to pathophysiological mechanisms of headaches. In our study we aimed to reveal the possible contribution of endovanilloids in meningeal TRPV1 receptor-mediated vascular reactions and CGRP release. We studied also the role of CB1 receptor activation in reactions induced by topically applied endogenous vanilloid/cannabinoid compounds, anandamide and NADA, which have been previously identified in primary sensory neurons.

Systemic treatment with the antitumor agent adriamycin has been shown to result in alterations of cutaneous chemosensitive afferent nerves and vascular reactions of the skin. Since somatosensory and visceral chemosensitive nerves share many common functional traits, in the present study we aimed to reveal the effect of systemic adriamycin treatment on changes in meningeal blood flow and CGRP release measured upon dural applications of specific agonists of TRPV1 and TRPA1 receptors. We also examined changes in TRPV1 protein content of trigeminal ganglia, expression and distribution of TRPV1 receptor immunoreactivity, CGRP immunoreactivity and immunoreactivity of vascular CGRP receptor components in the rat dura mater after systemic adriamycin treatment.

MATERIALS AND METHODS

In vivo measurement of meningeal blood flow

Control, capsaicin-desensitized and adriamycin-treated adult male Wistar rats weighing 270-350 g were used. All experiments were performed 2-7 days after the termination of the treatment of the animals. A cranial window for the measurement of

dural blood flow was prepared in anesthetized rats. Dural blood flow was measured with needle-type probes of a laser Doppler flowmeter in a cranial window preparation. The probes were placed over branches of the middle meningeal artery lying distant from visible cortical blood vessels. The mean blood flow measured during a 3-5-min period prior to drug application was regarded as the basal flow in different experiments. Changes induced in blood flow by the application of drugs were expressed as percentage changes relative to the basal flow (mean ± SEM) calculated for the 3-min application period. The blood flow increasing effect of capsaicin (100 nM) placed onto the exposed surface of the dura mater was measured, then anandamide (100 nM - 10 μM) or NADA (10 nM - 1 μM) were applied. The effects of TRPV1- (capsazepine 10 μM), CGRP- ($CGRP_{8-37}$ 100 μM) and CB1 (AM 251 100 μM) receptor antagonists on the endovanilloid-induced blood flow changes were determined by comparing the changes in blood flow in response to stimulation before and after the application of the respective antagonist(s).

To study the effect of adriamycin treatment on meningeal vascular responses, the dura mater was stimulated with repeated applications of a TRPV1 receptor agonist capsaicin (100 nM), a TRPA1 receptor agonist acrolein (300 μM) and CGRP (10 μM) for 3 min. The effects of single histamine (10 μM), acetylcholine (100 μM) and forskolin (10 μM) applications were also tested in control and adriamycin-treated animals.

***Ex vivo* measurement of CGRP release**

Control and adriamycin-treated rats were deeply anesthetized and decapitated. After removal of the skin and muscles, the skull was divided into halves along the midline and the cerebral hemispheres were removed. The skull preparations were washed with carbogen-gassed synthetic interstitial fluid (SIF) at room temperature for 30 min and then mounted in a humid chamber at 37°C. The cranial fossae were filled with 300 μl of SIF. Samples of the superfusate were collected at periods of 5 min by carefully removing the content of the skull halves with a pipette.

Control samples were taken to determine basal CGRP release. In some experiments the second sample was collected after incubation in the presence of NADA at 100 nM and the third sample after capsaicin at 100 nM. In some series of experiments

the effect of CB1 receptor antagonist pretreatment was studied on the anandamide-induced CGRP release. In these preparations after measuring the basal CGRP release, anandamide (10 μM) was applied twice for 5 min. CB1 receptor antagonist AM 251 was applied at 100 μM prior to the second anandamide application.

In other experiments CGRP releasing effect of repeated applications of capsaicin (100 nM) or acrolein (300 μM) was studied in control and adriamycin-treated animals. Capsaicin and acrolein were applied three times separated by washout periods with SIF. CGRP releasing effect of KCl (60 mM) application was also measured and compared to basal CGRP release. 100 μl of samples diluted with 25 μl of enzyme-linked immunoassay (EIA) buffer were placed into Eppendorf cups and immediately frozen at -70^{o}C for later analysis. The CGRP contents of the samples were measured with an EIA kit. The absorbance of the reaction product representing the CGRP content of the sample was determined photometrically, using a microplate reader. The CGRP concentrations of the superfusates were expressed in pg/ml. Changes induced in CGRP release were expressed as percentage changes relative to the basal release.

Measurement of TRPV1 protein content in the trigeminal ganglion

Control and adriamycin-treated animals were deeply anesthetized and decapitated. The skin and muscles were removed and the skull was divided into halves along the midline. Trigeminal ganglia were cut out and homogenized in phosphate buffered saline. The samples were stored overnight at -20°C. Two freeze-thaw cycles were performed before centrifuging the homogenates at 4°C for 5 min at 5 *g*. The supernatants were removed and frozen at -70°C for subsequent analysis. Concentration of TRPV1 protein in tissue samples was determined by EIA method and expressed in pg/mg tissue.

Immunohistochemistry

Control and adriamycin-treated rats were perfused transcardially with physiological saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The skin and muscles of the skull of decapitated animals were removed and the skull was divided into halves along the sagittal suture. After removing the brain, samples of the dura mater containing branches of the middle meningeal artery were cut out,

postfixed for 2 h in the same fixative and processed for staining with the indirect immunofluorescence technique using a rabbit polyclonal antiserum raised against the TRPV1 receptor in combination with a monoclonal mouse anti-CGRP antibody. IgGs labeled with Cy3 and DyLight 488 were used as secondary antibodies. CGRP receptor components receptor component protein (RCP) and receptor activity-modifying protein 1 (RAMP1) were visualized using mouse antiserum against RCP in combination with goat anti-RAMP1 primary and corresponding secondary antibodies labeled with Alexa 555 and Alexa 488. Whole mount preparations of the dura mater were examined under a confocal fluorescence microscope.

Statistical analysis of the data

All values were expressed as means ± SEM. Statistical analysis of the data was performed using Statistica 12 or 13. In all groups normality was tested by the Shapiro-Wilk test. According to the distribution of data the Student's t-test or the Wilcoxon test was used. ANOVA with repeated measurements and Fisher's least significant difference test were used to analyze the consecutive measurements of CGRP levels and blood flow increases induced by repeated applications of capsaicin, acrolein and CGRP. One-way ANOVA followed by the Bonferroni test was used for the statistical analysis of blood flow increasing effect of endovanilloids. A probability level of $p < 0.05$ was regarded as statistically significant.

RESULTS

Effect of endovanilloids on the trigeminovascular system

Topical application of NADA significantly and dose-dependently increased meningeal blood flow. However, NADA at highest concentration (1 μM) slightly decreased meningeal blood flow. In accord with previous observations systemic capsaicin desensitization of experimental animals completely abolished the blood flow increasing effect of capsaicin and the increase in meningeal blood flow induced by NADA (10 nM) was also reduced. Pretreatment of the dura mater with a TRPV1- or CGRP receptor antagonist significantly inhibited the NADA-induced blood flow increases. Although anandamide at 10 μM decreased meningeal blood flow in control rats by 2.1 ± 0.8 %, following the administration of AM 251 this effect turned into an

increase by 4.1 ± 0.6 %. This vasodilatory effect of anandamide was abolished by additional blockage of CGRP receptors. Anandamide (10 μM) applied after simultaneous blockage of CB1 and CGRP receptors reduced meningeal blood flow.

In control dura mater preparations basal release of CGRP was 22.6 ± 5 pg/ml. NADA at 100 nM produced a marked increase in the release of CGRP amounting to 140.3 ± 16.2 % of the basal release. In this series of experiments the capsaicin-induced CGRP release measured following the challenge with NADA was 328.8 ± 63.6 % of the basal value. Anandamide (10 μM) increased release of CGRP to 122.2 ± 9.6 %. After blocking the CB1 receptors with AM 251 (100 μM) an increase of 170.4 ± 23.7 % was measured after anandamide application.

Effect of adriamycin treatment on TRPV1 and TRPA1 receptor function in the dura mater

In control animals capsaicin-induced increases in meningeal blood flow were reproducible, the three consecutive applications of capsaicin separated by washout periods increased blood flow by 28.6 ± 7.9 %, 30.3 ± 4 and 21.5 ± 3.4 %, respectively. In adriamycin-treated rats, the blood flow increasing effect of the same capsaicin concentration was attenuated during the first application (11 ± 3 %) and almost completely abolished during the further applications (2.6 ± 1.8 and 1 ± 0.6 %).

Topical application of acrolein (300 μM) had similar effects on meningeal blood flow. In control animals three consecutive applications increased meningeal blood flow by 14.4 ± 2.4, 13.3 ± 3.7 and 8.9 ± 2.5%. Acrolein-induced increases in blood flow were significantly reduced in adriamycin-treated animals amounting to 4.1 ± 1.1, 2.6 ± 1.3 and 1.4 ± 1.8%. Vasodilatory effect of repeated applications of CGRP was also reduced in adriamycin-treated animals, but in this case no tendency of further reduction could be observed upon repeated stimulations. No significant difference in meningeal blood flow increasing effects of single topical applications of histamine (21.3 ± 3.9 vs. 22.3 ± 4.6 %), acetylcholine (15.8 ± 3.7 vs. 16.9 ± 4.5 %) or forskolin (22 ± 8 vs. 22.9 ± 9.8 %) could be observed.

In *ex vivo* dura mater preparations of control rats three consecutive applications of capsaicin at 100 nM concentration induced significant increases in CGRP release (294.2 ± 51.6, 229.5 ± 56.7 and 251.4 ± 101.4 %). In contrast, in

adriamycin-treated animals first capsaicin application produced a significant increase in CGRP release that was even higher than the effect of the first capsaicin application in control rats. In adriamycin-treated animals; it was 564.1 ± 71.2 % of the basal release. Further capsaicin administrations failed to augment the basal release, it was 117.1 ± 19.9 and 80.1 ± 12.5 % after the second and third applications, respectively.

In control rats TRPA1 receptor activation by acrolein (300 μM) increased CGRP release to 277.2 ± 25.9, 361.9 ± 50.6 and 385.6 ± 83.3 % of the basal level at three consecutive applications. In adriamycin-treated animals the CGRP-releasing effect of the first acrolein application was comparable to that in control animals (273.9 ± 56.2 % of the basal release), but the second and the third administrations of acrolein induced only moderate increases in CGRP release (162.5 ± 40.3 and 189.1 ± 56.8 % of basal release, respectively). KCl at 60 mM depolarised meningeal afferents that increased CGRP release both in control and adriamycin-treated animals to 141.2 ± 24 % and 189.3 ± 29 %, respectively. The difference between control and adriamycin-treated groups was statistically not significant.

TRPV1 protein content of trigeminal ganglia obtained from control rats was 6.25 ± 2.7 pg/mg. Adriamycin treatment reduced TRPV1 expression to 4 ± 0.5 pg/mg, although this difference did not reach statistical significance.

In whole mount preparations TRPV1- and CGRP-immunoreactive nerve fibers were distributed over the whole parietal dura mater. TRPV1- and CGRP-immunoreactive nerve fibers were observed in nerve bundles running parallel with branches of the middle meningeal artery and as single axons in regions at a distance from larger blood vessels. CGRP and TRPV1 were colocalized in most of these nerve fibers. No obvious difference in the density and distribution of TRPV1- and CGRP-immunoreactive afferents was seen in dura mater preparations of control and adriamycin-treated animals.

In dura mater preparations of control animals the CGRP receptor component proteins RCP and RAMP1 were present in the wall of meningeal arteries and veins. In dura samples of adriamycin-treated animals RAMP1-immunoreactivity was identifed, but no RCP-immunoreactive structures could be observed in the wall of meningeal blood vessels.

DISCUSSION

The present findings demonstrate that endovanilloids, through an action on the TRPV1 receptor, and acrolein, a TRPA1 agonist are potent activators of the trigeminovascular system. The findings also indicate that systemic adriamycin treatment results in marked alterations in the function of the trigeminovascular system.

Endovanilloids are endogenous membrane lipid metabolites that can be synthetised in sensory ganglion neurons or they can be taken up by neurons from the surrounding tissue. Although tissue content of anandamide measured under physiological conditions is moderate, its level may increase through inflammatory processes, such as neurogenic inflammation. The cellular concentration of endovanilloids may be elevated either by increased activity of the synthetizing enzymes and/or by increased endovanilloid transport across the cell membrane.

Anandamide and NADA act on both TRPV1 and CB1 receptors of nociceptors with different efficacies. In contrast to activation of the TRPV1 receptor, activation of the CB1 receptor inhibits the release of CGRP from trigeminal sensory nerves. The present findings indicate that the vascular effects of anandamide and NADA are different in the trigeminovascular system. While anandamide induced only slight, if any increase, NADA produced a marked increase in meningeal blood flow. This difference may be explained by the different activities of these agents on TRPV1 and CB1 receptors. Desensitization of the animals with capsaicin significantly inhibited NADA-induced increases in meningeal blood flow. In contrast, histamine-induced vasodilatation mediated by a direct action on endothelial and smooth muscle receptors was not affected by capsaicin-desensitization. Our results indicate, that the endogenous vanilloid substances may activate trigeminal afferents expressing the TRPV1 nociceptor ion channel, which, in turn, results in the release of CGRP from their terminals. NADA and also anandamide reduced meningeal blood flow at higher concentrations. The capsaicin/endovanilloid-induced vasoconstriction is brought about by a direct vascular action, although a TRPV1 receptor-mediated effect can not be excluded. In our *in vivo* experimental model anandamide evoked only moderate vasodilatation compared to NADA; the relatively strong effect of anandamide on CB1 receptors may counteract the effect of TRPV1 activation reducing the amount of CGRP released by the stimulated nerve terminals. Indeed, blockage of CB1 receptors with AM251 potentiated the

anandamide-induced vasodilatation.

Our results revealed a marked impairment of meningeal neurogenic sensory vasodilatation in adriamycin-treated rats. Diminished blood flow responses were even more obvious after repeated stimulations. In control rats, specific agonists of TRPV1 and TRPA1 receptors, capsaicin and acrolein, respectively, elicited reproducible releases of CGRP of largely similar magnitudes. In dura mater preparations obtained from rats treated with adriamycin, a strikingly different pattern of CGRP release appeared at repeated measurements, i.e. CGRP release was impaired only after the second and the third stimulation period with capsaicin or acrolein. In adriamycin-treated dural specimens there was even an increase in initial CGRP release after capsaicin application, probably reflecting a disturbed calcium homeostasis and consequent neuropeptide release. The discrepancy between the quantity of CGRP released by the first stimulation of chemosensitive nociceptors with capsaicin or acrolein and the intensity of the vasodilatory response elicited by the release of CGRP has drawn our attention to possible alterations in vascular functions that may lead to impaired vasodilatation in adriamycin-treated animals. Since earlier observations indicated apoptotic and necrotic changes in vascular smooth muscle after chronic exposure to adriamycin, we tested the effects of different vasodilator agents applied directly onto the dura mater. Blood flow increasing effects of histamine, acetylcholine and, in particular, exogenous CGRP were measured. Blood flow increases induced by these substances are independent of the functional condition of meningeal nerves, since they act directly on receptors localized on endothelial and/or smooth muscle cells of dural blood vessels. Histamine- and acetylcholine-induced vasodilatations were unaltered in adriamycin-treated animals suggesting that damage to vascular smooth muscle is unlikely to be responsible for the decreased vasodilatation in response to stimulation with capsaicin or acrolein. Conversely, in adriamycin-treated animals the blood flow increasing effect of CGRP was reduced in all three applications to about half of that measured in control. Immunohistochemistry revealed a loss of CGRP receptor component RCP-staining in arterial and venous dural blood vessels of adriamycin-treated animals. RCP component of the receptor complex is considered to enhance receptor coupling to the G-protein signaling machinery. Its expression seems to correlate with CGRP efficacy *in vivo*, suggesting its crucial role in the regulation of CGRP signaling. RCP protein staining

was undetectable in dura samples of adriamycin-treated animals indicating a significant change in protein structure resulting in a loss of immunohistochemical staining and an impairment of CGRP binding of the receptor complex.

Our functional studies indicate an altered TRPV1 and TRPA1 receptor function in meningeal afferents of adriamycin-treated animals. Decreased TRPV1 protein content of the trigeminal ganglia clearly signalised the impairment of chemosensitive nociceptors although decreased TRPV1 content of the trigeminal ganglia was not reflected by the peripheral axons of trigeminal neurons innervating the dura mater. Whole mount preparations of control and adriamycin-treated animals displayed similar density and distribution of TRPV1- and CGRP-immunoreactive nerves.

Taken together, our study demonstrated that endovanilloids similar to exogenous vanilloid compounds are effective in activation of the trigeminovascular nociceptive complex resulting in a release of CGRP and a consequent increase in meningeal blood flow. Chemosensitive afferents expressing the TRPV1 and TRPA1 receptors may contribute significantly not only to the vascular reactions but also to the nociceptive mechanisms of the dura mater possibly associated with the pathomechanisms of headaches. Increased production and/or uptake of endovanilloids may be implicated in the sustained activation of the trigeminal sensory system leading to the peripheral and/or central sensitization of the nociceptive pathway and, eventually head pain.

Chemotherapy with adriamycin impaires the nociceptive function of peptidergic meningeal afferents provoked by activation of TRPV1 and TRPA1 that results in diminished sensory neurogenic vasodilatation of meningeal blood vessels. Alterations in neuronal CGRP release and changes in RCP receptor complex protein expression may underlie the altered vascular responses. Adriamycin-induced impairment of vascular functions may affect sensory nerve-mediated local tissue reactions and protective mechanisms, such as neurogenic inflammation, operative in the meninges.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to Prof. Dr. Gábor Jancsó and Dr. Mária Dux for their scientific guidance and continuous support of my research activity, for their patience, motivation and immense knowledge. Their guidance helped me very much in my professional life. Futhermore, I would like to thank my dear colleagues in the laboratory for their professional and psychical support. I would like to thank Prof. Dr. Gábor Jancsó and Prof. Dr. Gyula Sáry, the previous and current Heads of the Department of Physiology in the Faculty of Medicine, for encouraging my research. Last but not least, I am grateful to Dr. Viktor Horváth for introducing me in the scientific activity.