SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Trigeminovascular dysfunctions in the relationship between obesity and migraine: the role of meningeal chemosensitive nociceptors

by Balázs Marics, MSc

Supervisors:

Mária Dux, MD, PhD

Barna Peitl, MD, PhD

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Supervisors:

Mária Dux, MD, PhD

Barna Peitl, MD, PhD

Doctoral School of Pharmaceutical Sciences (Programme of Pharmacology),
University of Debrecen

Head of the Examination Committee:	Árpád Tósaki, PD, PhD, DSc
Members of the Examination Committee:	Csaba Csonka, MD, PhD
	Gábor Mogyorósy, MD, PhD

The Examination takes place at the Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, on 3rd May, 2017, at 11 AM

Head of the Defense Committee:	Árpád Tósaki, PD, PhD, DSc
Reviewers:	Viktor Horváth, MD, PhD
	Judit Zsuga, MD, PhD
Members of the Defense Committee:	Csaba Csonka, MD, PhD
	Gábor Mogyorósy, MD, PhD

The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen on 3rd May, 2017, at 1 PM

INTRODUCTION

Obesity is associated with the development of chronic diseases and represents a major public health concern. Clinical and population-based studies suggest that obesity may be comorbid with the common and disabling primary headache disorder migraine leading to enhanced frequency and severity of headache attacks. However, the mechanisms underlying the obesity-headache relationship remain largely elusive.

Peripheral events associated with the activation and sensitization of trigeminal primary afferents that densely innervate the main pain-sensitive intracranial structure dura mater encephali are considered as important pathophysiological correlates of primary headaches, in particular migraine. Earlier studies confirmed the role of chemosensitive afferent nerves expressing the transient receptor potential vanilloid type 1 (TRPV1) and the transient receptor potential ankyrin 1 (TRPA1) receptors in the processes of meningeal nociception. The activation of chemosensitive afferents of the dura mater promotes pain transmission to higher brain areas and elicits neurogenic inflammation in the meninges through the peripheral release of proinflammatory substances, such as calcitonin gene-related peptide (CGRP). The neuropeptide CGRP is regarded as one of the key mediators in migraine pathophysiology and has been hypothesized to be involved in the relationship of obesity and migraine. It is postulated that obesity may sensitize the trigeminovascular system and enhance the susceptibility to headache triggers, although direct experimental evidence to underpin these assumptions is lacking.

Recent findings revealed that many of the factors that have long been considered as potential headache triggers are able to modulate TRPV1 and TRPA1 channels. Additionally, obesity may lead to alterations in the function of sensory nerves, including their TRPV1 and TRPA1 receptors. Pathophysiological changes in trigeminal primary afferents and in peripheral events related to the activation of meningeal chemosensitive nociceptors may contribute to the increased prevalence and disability of headaches in obese individuals.

AIMS

This work was initiated in an attempt to reveal potential mechanisms underlying the relationship between obesity and the primary headache disorder migraine. Therefore, a western-type diet consisting of high-fat, high-sucrose (HFHS) intake was administered chronically to create a metabolic and immunological environment in rats that may also

characterize migraine patients with obesity. *In vivo* and *ex vivo* experiments were performed to determine whether obesity alters neuropeptide release and vascular responses in the dura mater encephali that may have a role in the obesity-related enhancement in meningeal nociception.

The main goal of the present study was to investigate the effects of diet-induced obesity on the:

1. vasomotor function of meningeal blood vessels along with the TRPV1- and TRPA1-mediated neurogenic sensory vasodilation.
2. function of CGRP-containing trigeminal afferents innervating the meninges with special interest to the basal and potassium-chloride (KCl)-induced CGRP release from their peripheral terminals.
3. responsiveness of meningeal chemosensitive nociceptors by the measurement of dural CGRP release after stimulation with the selective TRPV1 agonist capsaicin and the selective TRPA1 agonist acrolein.
4. appearance and distribution of CGRP- and TRPV1-immunoreactive nerve fibers of the dura mater.
5. expression of TRPA1 protein in the trigeminal ganglia.

Another important purpose of the study was to complement the findings on the trigeminovascular nociceptive system with data on the metabolic and immunological background. Accordingly, food and fluid intake, body weight (BW), white adipose tissue (WAT) and liver mass in addition to glucose and insulin homeostasis as well as the plasma levels of the proinflammatory cytokines tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were determined.

MATERIALS AND METHODS

Characterization of the diet-induced obese animal model

Animals and diet

Male Sprague-Dawley rats (6 weeks old) were housed in an environmentally controlled room and randomly divided into two groups. The control group was maintained on a regular diet consisting of standard laboratory chow (3.20 kcal/g, 59 % carbohydrate, 32 %

protein, 9 % fat) and tap water. The experimental group received a HFHS diet composed of high-fat chow (4.56 kcal/g, 35 % carbohydrate, 20 % protein, 45 % fat) and tap water containing 5 % sucrose. Diets were provided *ad libitum* for 20 weeks. BW, food and fluid intake of the animals were measured regularly and daily calorie intake was calculated. All experiments were carried out after the completion of the dietary treatment period in 26 weeks old animals.

Assessment of insulin sensitivity

Hyperinsulinemic euglycemic glucose clamp (HEGC)

After an overnight fast, rats were anesthetized with intraperitoneally (i.p.) administered thiopental sodium (100 mg/kg) and the trachea was cannulated to allow free breathing. The left and right jugular veins were cannulated for insulin and glucose (20%) infusion. Blood sampling was performed through a cannula inserted into the right carotid artery. After a short stabilization period, a continuous insulin infusion was commenced at a rate of 3 mU/kg/min over 120 min during which the blood glucose was maintained at euglycemic level (5.5 ± 0.5 mmol/l) by adjusting the rate of glucose infusion based on the blood glucose concentration determined at 10-min intervals. The average glucose infusion rate (GIR, mg/kg/min) needed to maintain euglycaemia during the steady state condition was used to characterize whole body insulin sensitivity. In order to determine plasma insulin concentration, arterial blood samples were taken before the start of insulin infusion and during the steady-state period. Blood samples were centrifuged for 2 min at 4 °C and 10,000 g, the plasma was aliquoted, frozen and stored at −70 °C for later analysis.

Calculation of simple estimates of insulin sensitivity

Homeostasis model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) were calculated by using the data on fasting blood glucose (FBG, mmol/l) and fasting plasma insulin (FPI, μU/ml) levels obtained from the HEGC study and from a separate set of experiments aimed to determine circulating levels of plasma biomarkers. HOMA-IR was established as the product of FBG and FPI divided by a constant of 22.5, whereas QUICKI score was determined as the inverse log sum of FPI and FBG.

Analysis of plasma biomarkers

Following an overnight fast, rats were anesthetized with thiopental sodium (100 mg/kg, i.p.). The carotid artery was cannulated for blood sampling. After a short stabilization period, blood glucose was determined and additional samples were taken to Eppendorf cups. Samples were processed and stored as described above. Plasma concentration of insulin was measured by immunoradiometric assay (IRMA), whereas TNFα, IL-1β and IL-6 were assessed by enzyme-linked immunoassay (EIA) according to the manufacturer's instructions.

Measurement of adiposity and liver mass

After completing the experiments, intra-abdominal and epididymal WAT fat pads as well as the liver were removed and weighed. Adiposity was evaluated by expressing the sum of the WAT fat pads as a percentage of the total BW. The total liver mass of the animals was also compared.

Assessment of the functional condition of the trigeminal nociceptive system

In vivo recordings of meningeal blood flow

Control and obese rats were anesthetized with thiopental sodium (150 mg/kg, i.p.) and the trachea was cannulated to allow spontaneous breathing. Systemic blood pressure was recorded with a pressure transducer connected to a cannula inserted into the femoral artery. The head of the rats was stabilized in a stereotaxic frame and a cranial window was drilled into the parietal bone to expose the dura mater. Blood flow was recorded with a needle-type probe of a laser Doppler flowmeter positioned over a branch of the middle meningeal artery lying distant from visible cortical blood vessels. Stimulation of the dura mater was performed by topical application of capsaicin (100 nM and 10 μM), acrolein (50, 100 and 300 μM), CGRP and histamine (both 100 μM) at a volume of 40 μl for 5 min followed by repeated washouts with pH 7.4 synthetic interstitial fluid (SIF). In control rats, the effects of pretreatments with the TRPA1 receptor antagonist HC-030031 (50 μM) and the CGRP receptor antagonist CGRP8-37 (100 μM) on acrolein-induced (300 μM) blood flow increase were also tested. Both antagonists were topically applied for 5 min prior to stimulation with acrolein (300 μM). Basal blood flow was determined as the mean flow during 3 min prior to drug application. Percentage changes in meningeal blood flow in response to drug application were determined by comparing basal flow with the mean value of 1 min where change in blood flow was maximal within the 5 min application period or as mean flow values within

the 5 min application period calculated separately at one-minute intervals relative to the basal flow.

Ex vivo measurement of CGRP release

Control and obese rats were decapitated following deep anesthesia with thiopental sodium (150 mg/kg i.p.). The skull was divided into halves along the midline after the removal of the skin and the muscles. The cerebral hemispheres were removed, skull halves were washed at room temperature for 30 min in carbogen-gassed SIF, then the cranial fossae were filled with 300 µl SIF. Samples of the superfusate were collected with a micropipette at periods of 5 min for CGRP measurement. A control sample was taken to determine basal CGRP release. Then the dura was stimulated with capsaicin (10 and 100 nM), acrolein (10, 50, 100 or 300 µM) or KCl (60 mM). In some control experiments the effect of TRPA1 receptor antagonist HC-030031 (50 µM) pretreatment on acrolein-induced (300 µM) CGRP release was also measured. Samples diluted with EIA buffer were placed into Eppendorf cups and immediately frozen at -70 °C for subsequent analysis. EIA method was used for CGRP measurement.

Immunohistochemistry

The distribution of TRPV1- and CGRP-immunoreactive nerve fibers was studied in dural whole mount preparations of rats. Control and obese animals were anesthetized deeply with thiopental sodium (150 mg/kg, i.p.) and perfused transcardially with physiological saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The skin and muscles of the skull 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 hours 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 DL488 were used as secondary antibodies. Whole mount preparations of the dura mater were examined under a confocal fluorescence microscope.

Western blot analysis

Overnight fasted rats were anesthetized with thiopental sodium (150 mg/kg, i.p.) and decapitated. Trigeminal ganglia were removed and homogenized in ice-cold NP40 buffer supplemented with 1mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail.

Homogenates were sonicated twice for 10 seconds each and agitated constantly for two hours at 4 °C. Lysates were centrifuged for 20 min at 13,680 g at 4 °C. The protein concentration of the supernatant was determined by BCA Protein Assay. Whole-cell lysates (40-50 mg protein) were analysed on 10% sodium dodecyl sulphate (SDS) polyacrylamide gel under reducing conditions. Immunoblotting was carried out on a nitrocellulose membrane using a rabbit anti-TRPA1 antibody (1:2500) together with β-actin (1:1000) overnight at 4 °C followed by incubation with donkey anti-rabbit immunoglobulin (Ig)G-horseradish peroxidase (HRP) (1:5000) for an hour at room temperature. Immunoreactive bands were visualised by the SuperSignal West Pico Chemiluminescent Substrate using a Gel Logic 1500 Imaging System.

Statistics

All values were expressed as means ± SEM. Statistical analysis of the data was performed using Statistica 12. For the statistical comparisons of CGRP and cytokine concentrations, meningeal blood flow changes, TRPA1 protein expression as well as food and fluid intake of the animals the Student's t-test was used for group sizes of $n \geq 10$ and the Mann–Whitney U-test for independent measurements of group sizes $n<10$. One-way ANOVA followed by the Bonferroni test was used to compare metabolic parameters of the animals. A probability level of $p < 0.05$ was regarded as statistically significant.

RESULTS

Characterization of the diet-induced obese animal model

Food-, fluid- and energy intake

On average, the HFHS group of animals consumed smaller quantities of food (28,5 ± 0,4 vs. 18,4 ± 0,2 g/day, $p < 0,001$, n = 17-17) and greater amount of fluid (39,7 ± 0,5 vs. 125,9 ± 1,8 ml/day, $p < 0,001$, n = 17-17) than animals fed with a regular diet. The mean daily caloric intake of HFHS rats significantly exceeded the control group (90,7 ± 1,7 vs. 110,1 ± 1,2 kcal/day, $p < 0,001$, n = 17-17).

Body weight, adiposity and liver mass

Rats maintained on a HFHS diet had significantly higher body weight at the end of the dietary period (616 ± 11 vs. 740 ± 15 g, $p < 0,001$). The HFHS group also exhibited greater intra-abdominal (10,09 ± 0,64 vs. 27,09 ± 1,84 g, $p < 0,001$, n = 22-23) and epididymal (12,59

± 0,70 vs. 23,73 ± 1,15 g, $p < 0,001$, n = 22-23) fat pads along with higher ratio of WAT to BW (3,93 ± 0,16 vs. 7,35 ± 0,3 %, $p < 0,001$, n = 22-23) as well as increased total liver mass (13 ± 0,36 vs. 17,53 ± 0,59 g, $p < 0,001$, n = 22-23) at the end of the 20-week dietary treatment period.

Glucose and insulin homeostasis

Diet-induced obesity led to elevations in fasting blood glucose (5,61 ± 0,13 vs. 6,31 ± 0,19 mmol/l, $p < 0,01$, n = 22-23) and plasma insulin concentrations (18,05 ± 1,68 vs. 43,08 ± 4,36 μU/ml, $p < 0,001$, n = 22-23) accompanied by a significant increase in HOMA-IR (4,49 ± 0,44 vs. 12,53 ± 1,59, $p < 0,001$, n = 22-23) and a decrease in QUICKI score (0,31 ± 0,004 vs. 0,28 ± 0,004, $p < 0,001$, n = 22-23) and glucose infusion rate (9,66 ± 1,35 vs. 4,60 ± 1,34, $p < 0,05$, n = 5-5).

Plasma concentrations of proinflammatory cytokines

In obese animals significantly higher concentrations of IL-1β (61,63 ± 3,67 vs. 195,87 ± 29,57 pg/ml, $p < 0,001$, n = 17-18) and IL-6 (53,21 ± 1,33 vs. 126,27 ± 9,74, $p < 0,001$, n = 17-18) were measured, whereas TNFα levels were similar in control and obese rats (19,03 ± 1,22 vs. 16,87 ± 1,43 pg/ml, $p = 0,26$, n = 9-9).

The functional condition of the trigeminovascular nociceptive system

Effect of diet-induced obesity on meningeal vasoreactivity and neurogenic vascular responses

The basal flow values were in the same range in control and obese rats (226,1 ± 20,29 vs. 214,4 ± 20,1 PU, $p = 0,96$). No significant difference was observed in systemic blood pressure between groups (128 ± 17 vs. 134 ± 13 mmHg, $p = 0,96$), although it was slightly above the values measured earlier in non-obese animals under the same experimental conditions. Drugs administered topically to the dura mater failed to influence systemic blood pressure.

Capsaicin-sensitive neurogenic vasodilation

In control rats topical administration of capsaicin at 100 nM concentration induced a moderate increase in meningeal blood flow reaching significance in the last two minutes of the 5 minutes application period ($p = 0,048$ and $p = 0,035$, respectively, n = 8). Blood flow increasing effect of the same capsaicin concentration was more robust in obese animals

throughout the whole application period, it reached 20 ± 3,1 % increase in the last minute ($p \leq 0,023$, n = 9). This increase significantly exceeded the effect measured in control rats ($p \leq 0,049$).

Capsaicin-induced vasoconstriction

Administration of capsaicin at 10 μM reduced meningeal blood flow in both groups of animals. In control animals, decrease in meningeal blood flow varied in the range from 4,7 ± 2,7 and 2,7 ± 2,5 %, while this range was 14,5 ± 2,9 and 8 ± 2,8 % in obese rats. Blood flow reducing effect of 10 μM capsaicin was significantly stronger in obese animals from the 2nd to the 4th min compared to controls ($p \leq 0,037$).

Acrolein-evoked increase in meningeal blood flow

In control rats acrolein at 50 μM did not affect meningeal blood flow (0,35 ± 0,94 %, $p = 0,71$, n = 10), while a dose-dependent increase was observed at 100 μM (7,7 ± 1,4 %, $p < 0,001$, n = 10) and 300 μM (14,4 ± 2,3 %, $p < 0,001$, n = 12) concentrations. The blood flow-increasing effect of acrolein at 300 mM was abolished by the pretreatment of the dura mater with the TRPA1- (2,05 ± 1,2 %, $p = 0,001$, n = 8) and CGRP receptor antagonists (1,9 ± 1,2 %, $p = 0,001$, n = 8). Dural administration of acrolein (100 μM) also led to elevation in meningeal blood flow (13,1 ± 2,2 %, $p < 0,001$, n = 9) in obese animals that was significantly higher compared to the controls ($p = 0,049$).

Endothelial- and smooth muscle cell-dependent vasorelaxation

Histamine and CGRP acting directly on endothelial- or smooth muscle cells of blood vessels induced significant increases in meningeal blood flow in both control ($p \leq 0,015$, n = 8 both) and obese ($p \leq 0,035$, n = 8-10, respectively) rats. No difference regarding the vasodilation induced by histamine ($p \leq 0,98$) and CGRP ($p \leq 0,92$) administrations could be observed between control and HFHS diet-induced obese animals.

Effect of diet-induced obesity on basal and stimulated CGRP release from the dura mater

Basal release of CGRP

In the *ex vivo* dura mater preparation of obese rats the basal release of CGRP was significantly higher than that of the controls (16 ± 1,5 vs. 42,1 ± 6,6 pg/ml, $p = 0,005$, n = 13-22).

Capsaicin-induced CGRP release

Stimulation with capsaicin significantly enhanced the release of CGRP from meningeal afferents in skull preparations obtained from both control and obese animals. Capsaicin at a concentration of 10 nM elevated CGRP release to 34,7 ± 2,3 pg/ml (196,6 ± 21,2 % of basal release, $p < 0,001$, $n = 7$) and at 100 nM concentration to 75,6 ± 7 pg/ml (612,7 ± 58,9 % of basal release, $p < 0,001$, $n = 6$) in rats maintained on a regular diet. In obese animals capsaicin at 10 nM concentration increased the release of CGRP to 120 ± 27,6 pg/ml (300,1 ± 60,1 % of basal release, $p = 0,004$, $n = 11$) and at 100 nM to 358,1 ± 95,5 pg/ml (853,1 ± 76 % of basal release, $p = 0,004$, $n = 11$). HFHS diet-induced obesity resulted in a significantly greater CGRP release in response to both capsaicin concentrations as compared with the control ($p = 0,027$ and $p < 0,048$, respectively).

TRPA1-mediated CGRP release

Acrolein at a concentration of 10 and 50 μM failed to influence CGRP release in control animals ($p = 0,88$, $n = 7$ and $p = 0,203$, $n = 6$, respectively), whereas acrolein at 10 μM concentration was also ineffective in obese animals ($p = 0,51$, $n = 7$). Higher concentrations of acrolein (100 and 300 μM) significantly increased the release of CGRP (227,1 ± 29,9 % of basal release, $p < 0,001$, $n = 8$ and 232,7 ± 18,8 %, $p < 0,001$, $n = 8$, respectively), which was inhibited by pretreatment with the TRPA1 antagonist HC-030031 (50 μM) (132,2 ± 15,9 % of basal release, $p = 0,001$, $n = 8$). Acrolein at a concentration of 100 μM also increased the release of CGRP in obese rats (336 ± 33,1 % of basal release, $p = 0,001$, $n = 14$), however, the effect was significantly greater compared to the controls ($p = 0,039$).

KCl-induced CGRP release

KCl (60 mM) increased CGRP release from the dura mater in both control (307,4 ± 38,3 % of basal release, $p < 0,001$, $n = 8$) and obese (182,7 ± 18,2 % of basal release, $p < 0,001$, $n = 13$) animals. Although depolarisation induced enhancement in the release of CGRP was significantly different from basal values in both groups, the high potassium-induced release was markedly lower in the obese rats as compared with the controls ($p = 0,001$).

Effect of diet-induced obesity on TRPV1- and CGRP-immunoreactive nerve fibers of the dura mater

In the dura mater of control animals TRPV1- and CGRP-immunoreactive nerve fibres were distributed over the whole supratentorial dura mater. CGRP was colocalized with

TRPV1 in most of these nerve fibres. Although in whole mount dura preparations of obese rats, distribution of TRPV1- and CGRP-immunoreactive afferents was similar to that seen in control dura mater preparations, many single axons showed characteristic changes. While CGRP-immunoreactivity was distributed evenly in the afferents of control dura, in whole mounts of obese animals the immunoreactivity was observed in the form of a string of pearls.

Effect of diet-induced obesity on the expression of TRPA1 protein in the trigeminal ganglia

The TRPA1 antibody detected a band at 110 kDa from whole-cell lysates of rat trigeminal ganglia. Following incubation of anti-TRPA1 antibody with its cognate peptide provided by the manufacturer, the band could no longer be detected indicating the specificity of the antibody for the TRPA1 protein. There was a significant reduction in the expression of this 110 kDa protein in the trigeminal ganglia of obese rats compared with the control group (0,62 ± 0,1 vs. 0,52 ± 0,09 normalized to β-actin, $p = 0,048$, $n = 7-8$).

DISCUSSION

The animal model of diet-induced obesity

Our data indicate that HFHS dietary treatment influenced the processes involved in the regulation of food intake and energy balance. An increase in the energy density of the chow is often accompanied by a decrease in food intake due to homeostatic mechanisms to maintain energy balance. The consumption of sucrose solution is also known to increase fluid intake, which may be associated with its effects on the reward system of the brain. The greater energy intake of HFHS animals indicates that this particular combination of obesogenic dietary factors caused an imbalance in energy homeostasis leading to increased BW, WAT pads and adiposity index. These results show that our HFHS diet protocol is an effective method to correspond certain criteria of both experimental and human obesity. The elevations in fasting blood glucose and insulin levels, the subsequent increase in HOMA-IR index and the decrease in QUICKI score and glucose infusion rate clearly indicate the development of insulin resistance and impairments in glucose and insulin homeostasis in diet-induced obese rats. This metabolic disturbance was demonstrated in the presence of low-grade systemic inflammation as was indicated by the enhanced plasma concentrations of IL-1β and IL-6. Furthermore, the increased liver mass in obese animals suggests the development of hepatic steatosis. A major role of the proinflammatory cytokine TNFα is not very likely since its plasma level was not

affected in obese rats. This, however, does not exclude the involvement of local TNFα production in the sensitization of the trigeminovascular system.

The present findings demonstrate that our animal model of diet-induced obesity possessed many of the metabolic alterations that have been previously suggested to be associated with obesity as well as with the pathophysiology of migraine.

Effect of diet-induced obesity on meningeal vascular reactions

Our results demonstrate that obesity is associated with enhanced capsaicin- and acrolein-induced sensory neurogenic vascular reactions in the dura mater, which were selective for the applied stimulus, since neither CGRP- nor histamine-induced vasodilation in obese rats were different from the control. The lack of changes in the vasodilatory actions of CGRP and histamine indicates that HFHS diet-induced obesity did not modify the processes involved in their vasodilatory effects in endothelial- or smooth muscle cells. Our measurements on capsaicin- and acrolein-induced CGRP release in *ex vivo* dura mater preparations indicate that the enhanced sensory neurogenic vasodilatory responses in obese rats may be accounted for the increased CGRP release from meningeal chemosensitive afferents upon the activation of TRPV1 and TRPA1 receptors. It is important to note that a large population of TRPV1 expressing neurons does not contain neuropeptides, but may also contribute to nociceptive transmission to higher brain areas. Obesity-related factors that alter the function of TRPV1 receptor in CGRP-containing dural afferents may also have the potential to affect TRPV1 in non-peptidergic trigeminal sensory neurons. Our observations that acrolein-induced vasodilation was inhibited by the antagonism of TRPA1- or CGRP receptors in the dura mater provide further support for acrolein, a common environmental pollutant, being an agonist on TRPA1 receptors of meningeal afferents and initiating CGRP-mediated increases in meningeal blood flow that are implicated in the generation of headaches. The obesity-related enhancement in capsaicin-induced vasoconstriction may also be of relevance to obesity-headache relationship. The increased vasoconstrictor activity of meningeal blood vessels may impair the effective removal of pain-producing tissue mediators involved in the induction or aggravation of headache attacks or may delay the restoration of tissue homeostasis.

Effect of diet-induced obesity on the CGRP release from the dura mater

The enhanced basal release of CGRP from the dura mater of obese rats suggests that obesity increases the resting activity of dural afferents or at least one of their subpopulations. The responsiveness of meningeal chemosensitive afferents to nociceptive stimuli was also potentiated by diet-induced obesity as was demonstrated by the augmented CGRP release in obese animals after dural application of capsaicin or acrolein. It is well established that capsaicin elicits CGRP release via the activation of TRPV1 channels in the trigeminal sensory neurons. However, our study provide further support for existing data that acrolein-induced CGRP release from meningeal afferents is mediated by the activation of TRPA1 channels. It should be mentioned that the concurrence of increased basal activity of dural afferents and the enhanced responsiveness of TRPV1- and TRPA1 expressing meningeal nociceptors may result in an abnormally high CGRP release in the dura mater that, in turn, may significantly contribute to the obesity-related enhancement of peripheral events involved in the initiation and maintenance of headache attacks. Our metabolic measurements draw attention to the possible pathophysiological role of IL-1β and IL-6 in the observed neurovascular alterations of obese animals. Accumulating experimental evidence suggests that these proinflammatory cytokines are able to influence sensory neurons as well as to modulate TRPV1 and TRPA1 functions via several direct and indirect mechanisms. Additionally, impairments in glucose and insulin homeostasis may also have a role in the sensitization of trigeminal nociceptors, including their TRPV1 and TRPA1 channels, for instance by the enhancement of oxidative and nitrosative stress.

The attenuation in high potassium-induced release of CGRP from the dura mater may be attributed to the altered resting membrane potential of meningeal afferents, which is generally considered as one of the main mechanisms underlying the sensitizing effects of inflammatory mediators in sensory neurons.

Effects of diet-induced obesity on TRPV1- and CGRP-immunoreactive nerve fibers of the dura mater

In the dura mater whole mount preparations of lean and obese animals, in accord with previous data, the density and distribution of TRPV1- and CGRP-immunoreactive nerve fibers were similar. Our findings suggest that obesity-associated potentiation of capsaicin-induced neurogenic sensory vasodilation was brought about in the absence of obvious changes in TRPV1-immunoreactivity of dural afferents and the sensitization of TRPV1

receptor is more likely the consequence of altered gating properties of the receptor channel. A novel approach to modulate TRPV1 receptor function may be the development of drugs preventing the phosphorylation of the receptor, thereby counteracting the increased probability of channel opening. Therefore, kinase inhibitors may provide a promising strategy for migraine therapy, especially in obese headache sufferers, where altered TRPV1 receptor function seems to be an important pathophysiological factor. The pearl-like appearance of CGRP-immunoreactivity might be the morphological correlate of enhanced CGRP release, but the role of neurodegenerative processes also cannot be excluded.

Effect of diet-induced obesity on TRPA1 protein expression in the trigeminal ganglia

Considering the wide variety of factors that may influence sensory neurons and TRPA1 receptors in obesity, the reduced TRPA1 protein expression in the trigeminal ganglia of obese animals can be considered as part of compensatory mechanisms due to persistently increased neuronal activity.

NEW FINDINGS

High fat, high sucrose diet-induced obesity:

- potentiates both the capsaicin- and acrolein-induced neurogenic sensory vasodilation due to augmented release of CGRP from meningeal chemosensitive nociceptors upon the activation of TRPV1 and TRPA1 receptors.
- enhances the basal release of CGRP, but attenuates the high KCl-induced CGRP release from dural afferents.
- augments the vasoconstrictor effect of capsaicin in the dura mater, which may draw attention to the development of obesity-related dysfunctions in vasoconstrictor processes of the meningeal blood vessels.
- leads to characteristic changes in CGRP-immunoreactivity within meningeal afferents, but does not seem to alter the density and distribution of CGRP- and TRPV1 immunoreactivity in the dura mater.
- is associated with a small but significant decrease in the expression of TRPA1 protein in the trigeminal ganglia.

Our results suggest that obesity:

- sensitizes CGRP-containing trigeminal primary afferents innervating the dura mater.
- potentiates TRPV1- and TRPA1-mediated trigeminovascular responses, which may be the result of altered gating properties of these receptor channels.

The proinflammatory cytokines IL-1β and IL-6 along with the impairments in glucose and insulin homeostasis may have a role in the development of obesity-related trigeminovascular alterations.

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APPENDIX

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Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Marics, B.**, Peitl, B., Varga, A., Pázmándi, K. L., Bácsi, A., Németh, J., Szilvássy, Z., Jancsó, G., Dux, M.: Diet-induced obesity alters dural CGRP release and potentiates TRPA1-mediated trigeminovascular responses.
   Cephalalgia. [Epub ahead of Print], 2016.
   DOI: http://dx.doi.org/10.1177/0333102416654883
   IF: 6.052 (2015)
2. **Marics, B.**, Peitl, B., Pázmándi, K. L., Bácsi, A., Németh, J., Oszlács, O., Jancsó, G., Dux, M.: Diet-induced obesity enhances TRPV1-mediated neurovascular reactions in the dura mater.
   Headache. [Epub ahead of Print], [1-30], 2017.
   DOI: http://dx.doi.org/10.1111/head.13033
   IF: 2.961 (2015)

Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

List of other publications

3. Docsa, T., **Marics, B.**, Németh, J., Hüse, C., Somsák, L., Gergely, P., Peitl, B.: Insulin sensitivity is modified by a glycogen phosphorylase inhibitor: glucopyranosylidene-spiro-thiohydantoin in streptozotocin-induced diabetic rats.
Curr. Top. Med. Chem. *15* (23), 2390-2394, 2015.
IF: 2.9

Total IF of journals (all publications): 11,913
Total IF of journals (publications related to the dissertation): 9,013

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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