RESEARCH PAPER

TRPV1, CGRP and SP in scalp arteries of patients suffering from chronic migraine

Marina Del Fiacco, Marina Quartu, Marianna Boi, M Pina Serra, Tiziana Melis, Riccardo Boccaletti, Elliot Shevel, Carlo Cianchetti

ABSTRACT

Objective The transient receptor potential vanilloid type-1 receptor (TRPV1) and the neuropeptides calcitonin gene related peptide (CGRP) and substance P (SP) appear to be differently involved in migraine pain. A role of neovascular scalp structures is also suggested by several data. We performed a quantitative study of TRPV1-like immunoreactive (LI), CGRP-LI and SP-LI innervation of scalp arterial samples from patients affected with chronic migraine (CM).

Methods Short segments of scalp arteries were collected from 17 participants undergoing vascular surgery for treatment-resistant CM and from 6 controls who underwent neurosurgery for various indications. The immunoreactivity of the arterial innervation to TRPV1, CGRP and SP were evaluated in the par-arteriolar marker protein gene product 9.5 (PGP9.5) examined. The immunoreactive nerve fibres in vessel cross-sections were quantified by computerised image analysis.

Results A significant increase of TRPV1-LI nerve fibres was found in the arterial wall from CM compared with control patients (p<0.05), while no significant difference was found for CGRP and SP.

Conclusions This study yields the first evidence for the existence of a TRPV1-LI innervation in human scalp arteries and provides the first quantitative assessment of the TRPV1-LI, CGRP-LI and SP-LI innervation of those vessels. The increase of TRPV1-LI perivascular nociceptive fibres of scalp arteries may represent, at least in some participants, a structural conditioning factor of CM (and possibly migraine), for example, by causing a higher sensitivity to algogenic agents.

INTRODUCTION

The transient receptor potential vanilloid type-1 receptor (TRPV1) and the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) appear to be differently involved in migraine pain. Migraine pathogenesis classically implicates central and neurovascular intracranial structures. However, a possible role of the neovascular scalp structures is also suggested by several data recently reviewed, and the literature of scalp arteries has been reported as beneficial in chronic migraine (CM). The availability of arterial specimen from such a surgical practice prompted us to study by immunohistochemistry the presence of TRPV1, CGRP and SP in them. Our results indicate a significantly increased number of TRPV1-like immunoreactive (LI) nerve fibres in the wall of scalp arteries from patients suffering from CM compared with controls.

MATERIALS AND METHODS

Sampling

Short segments (about 3 mm long) of the superficial temporal (STA) and occipital (OA) scalp arteries were collected from 17 patients with CM undergoing vascular surgery as described by Shevel. Inclusion criteria were diagnosis of CM according to the International Classification of Headache Disorders (ICHD-2) and absence of any concomitant disorder contraindicating the operation. Groups of 3–4 consecutive patients with CM undergoing surgery on the same day or on the following day were included, and the samples were sent by express courier from the site of collection, Johannesburg. This caused long intervals in collecting samples, requiring the possibility of having more patients to operate in close proximity. Recruitment began in January 2009 and ended in February 2013. Scalp arterial segments from non migraine patients were collected at surgery for intracranial pathologies in Sassari and used as controls. The collected segments belonged to the distal part of the artery, separated by the scalp incision to access the skull and destined to obliteration.

Table 1 shows the clinical characteristics of the patients. Improvement after surgery was evaluated by the patient according a 100 point numeric rating with a follow-up of at least 3 months.

Ethics approval

The sampling and handling of human specimens conformed to the programme approved by the Ethics Committee of the Azienda Ospedaliero-Universitaria (National Health System) of Sassari, in compliance with the principles enunciated in the Declaration of Helsinki, and the patients gave their informed consent to the surgery and to the use of specimens in the study.

Technical procedures

In Johannesburg and Sassari, specimens were collected at surgery and immediately fixed by immersion in 4% freshly prepared phosphate-buffered formaldehyde, pH 7.3, for 4–6 h at 4°C, and rinsed overnight in 0.1 M phosphate buffer (PB), pH 7.3, containing 10% sucrose. Soon on arrival in the laboratory of Cagliari, all samples were cryostat cut transversely at 12 μm. Sections were collected on chrome-alum-gelatin coated slides, stored at −80°C and processed within 3 months. The avidin–biotin–peroxidase complex (ABC) immunohistochemical technique was applied for the TRPV1 receptor, the neuropeptides CGRP
# Migraine

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F, female; M, male; OA, occipital artery; STA, superficial temporal artery.

and SIP and the pan-neuronal marker protein gene product 9.5 (PGP9.5). The latter, considered the best immunohistochemical marker for neuronal processes at all levels of the central and peripheral nervous system, was used to label the whole vessel innervation so as to quantify the TRPV1-LI, CGRP-LI, and SP-LI nerve fibres in relation to the total innervation. Endogenous peroxidase activity was blocked with 0.1% phenylhydrazine in phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (PBS-T). The following antisera were used as primary antibodies: rabbit anti-TRPV1 (Thermo Scientific), raised against a 16 amino acid peptide near the carboxy terminus of human TRPV1, diluted 1:500; rabbit anti-CGRP (Chemicon), raised against the whole molecule of human origin, diluted 1:1000; guinea-pig anti-SIP (Abcam), raised against a synthetic peptide corresponding to amino acids 1–11 of rat SIP; diluted 1:2000; guinea-pig anti-PGP9.5 (Neuromics), raised against the amino acid sequence GASSFDTLKIDAAKVCRCR of the rat molecule, diluted 1:300. Biotin-conjugated goat anti-rabbit and anti-guinea-pig sera (Vector), both diluted 1:400, were used as the secondary antisera. The immunoreactivity was revealed with ABC (BioSpa Div.), diluted 1:250, followed by incubation with a solution of 0.1 M PB, pH 7.3, containing 0.05% 3,3'-diaminobenzidine (Sigma), 0.04% nickel ammonium sulfite and 0.01% hydrogen peroxide. Incubations with the primary antisera were carried out overnight at 4°C. Incubations with the secondary antisera and ABC lasted 60 and 30 min, respectively, and were performed at room temperature. All antisera and the ABC were diluted in PBS-T. Negative control preparations were obtained by incubating tissue sections in parallel with either PBS-T alone or with the primary antisera preabsorbed with an excess of the corresponding peptide antigen. Alternate vessel sections were stained with modified Mayer’s haematoxylin for morphological evaluation. Slides were dehydrated, cover-slipped and observed with an Olympus BX51 microscope. Digital images were captured with a ColorViewII Olympus camera.

Immunoreactive nerve fibres in the vessel sections were quantified by computerised image analysis (Olympus AnalySIS software) by an observer unaware of sample origin. The total length in μm of the immunostained fibre segments detectable in each entire cross-section of the vessel wall was captured using a ×20 objective magnification. The density of the positive innervation for each examined substance was calculated in three sections of each specimen as the ratio of such value to the relevant section area in μm². Density values obtained for PGP9.5 were further used to normalise those relevant to TRPV1, CGRP and SP in each section.

After the first collection of eight CM (2 different dispatches) and three control samples (3 different dispatches), a first blind evaluation of the results was performed by the laboratory staff (MQ, MB, MPS TM); at least three persons gave the evaluation of each sample and the mean of read values was used for the calculation. Later, the other two groups of CM (5 and 4 samples, respectively) and controls (1 and 2 samples) were studied with the same criteria.

**Statistics**

Non-parametric Mann-Whitney test (GraphPad Prism 5.00) was used for comparison between samples, and Pearson’s test for the correlation.
RESULTS

Histological analysis proved the good preservation of the structural tissue organisation of the collected specimens (figure 1). TRPV1-LI, CGRP-LI, SP-LI and PGP9.5-LI material was detected in varicose nerve fibres localised to the depth of the tunica adventitia, at the boundary with the tunica media of the vessel wall (figure 2). In addition, small nerve bundles displaying immunoreactivity for the examined molecules could be seen in the immediate surrounding area (e.g., see figure 2C for CGRP-LI). Density of stained nerve fibres differed for each marker and varied among specimens (figures 2 and 3).

Values of mean density normalised to PGP9.5 in superficial temporal artery (STA) samples (15 patients with CM vs 6 controls) were (figure 3): (a) for TRPV1, CM specimens 0.60±0.51 (range 0.18–1.83) versus controls 0.22±0.17 (range 0.09–0.49), Mann-Whitney U=18.0, p=0.039; (b) for CGRP, CM 0.60±0.80 (range 0.10–2.5) versus controls 0.69±0.86 (range 0.23–1.52), Mann-Whitney U=35.0, p=0.28; (c) for SP, CM 0.18
Migraine

Figure 3 Density of TRPV1-LI, CGRP-LI, SP-LI innervation normalised to that of PGP9.5 in specimens from superficial temporal arteries of 15 sufferers (black columns) and 6 controls (open columns). Each column represents the average of the innervation quantified in three cross sections of each specimen of the relevant group. Bars indicate the standard errors. Differences between the two groups, as analysed by Mann-Whitney test, are statistically significant only for TRPV1/LI(PGP9.5) (p<0.003), CGRP, calcitonin gene-related peptide, CM, chronic migraine; LI, like immunoreactive; PGP9.5, protein gene product 9.5; SP, substance P; TRPV1, transient receptor potential vanilloid type 1 receptor.

±0.02 (range 0.03-0.80) versus controls 0.16±0.10 (range 0.07-0.34), Mann-Whitney U=36.0, p=0.31. The 2 OAs had TRPV1/LI(PGP9.5) values in the range of the STAs (figure 4); with their inclusion in the STA CM group, the normalised density of TRPV1 positive innervation remained statistically different between the two groups (Mann-Whitney U=20.0, p=0.033).

Figure 4 shows the distribution of normalised TRPV1 mean density values in patients with CM and controls: two control samples show lower values than any other CM, and seven CM samples show values exceeding the highest values of controls.

Of the 17 patients with CM, 12 improved more than 50% after the operation, but 5 did not (table 1). The specimens of 2 of these 5 had TRPV1/LI(PGP9.5) values higher than the mean of the whole group, while the other 3 had lower.

Comparison of the normalised mean density in the 12 patients with CM under migraine attack at surgery (mean density values: 0.56 for TRPV1/LI(PGP9.5); 0.66 for CGRP/LI(PGP9.5); 0.20 for SP(LI(PGP9.5)) with those in the five patients with CM who were not under attack at surgery (mean density values: 0.63 for TRPV1/LI(PGP9.5); 0.40 for CGRP/LI(PGP9.5); 0.10 for SP(LI(PGP9.5)) resulted in no statistically significant difference between the two groups for any marker.

The increase of TRPV1/LI(PGP9.5) did not correlate with age (for CM r=-0.046; for controls r=0.230) nor with duration of migraine (r=0.268); TRPV1/LI(PGP9.5) values did not differ by gender (F=0.60±0.54; M 0.59±0.42). Moreover, the values obtained in the samples collected in different periods were randomly distributed.

DISCUSSION

To the best of our knowledge, this is the first study reporting evidence of the occurrence and evaluation of a TRPV1-LI innervation in human scalp arteries, providing a quantitative assessment of the TRPV1-LI, CGRP-LI and SP-LI innervation in those vessels, and examining the presence of TRPV1-LI, CGRP-LI and SP-LI in scalp arteries of patients with CM.

Previous studies have shown the presence of CGRP and SP in nerve fibres of scalp arteries of normal participants and that they elicit there a potent vasodilator response. In agreement with Jansen Olesen et al., we found a higher density of CGRP-LI than of SP-LI vessel innervation.

Our main result is that, compared with controls, the TRPV1-LI innervation of scalp arteries is frequently increased in CM sufferers. TRPV1-LI innervation does not show changes in relation to the presence or not of migraine at sampling and did not correlate with the duration of migraine. Of the CM STA samples, 8 of 15 showed TRPV1/LI(PGP9.5) values in the range of the controls (figure 4). Moreover, 5 of the 17 patients with CM did not improve after the operation, although of 2 of those had TRPV1/LI(PGP9.5) values higher than the mean of the CM group. These results could suggest a different pathophysiology in these CM cases, including a more relevant role of central mechanisms, and, peripherally, the possible participation of another receptor of the TRP family (eg, the ankyrin type TRPA1).

In front of the increase of the membrane receptor TRPV1-LI nerve fibres, we did not find a significant variation of CGRP-LI and SP-LI nerve fibres in arterial samples of patients with CM, indicating no significant variations of these peptides in the nerve fibres. CGRP has a definite role in migraine; besides the fact that it can trigger an attack and that the block of its receptor relieves migraine attacks, CGRP is reported to be increased in blood from patients with migraine and CM. The increase in blood suggests an increased release of CGRP, which is favoured by the activation of TRPV1 and could be higher in the presence of a higher number of vanilloid receptors in nerve fibres. The lack of CGRP (and SP) variations in nerves fibres could therefore be due to an equilibrium or compensation between a probable neuronal hyperproduction and an increased release. Although no experimental data are available in this respect, a reduction of CGRP in the STA nerve fibres in case of...
an inflammatory condition, where the vasodilatation suggests an increased release of CGRP has been reported.\(^{18}\)

The involvement of TRPV1 in migraine has been suggested by genetic\(^{19}\) and clinical\(^{20-22}\) reports, although contrasted by a study on rats.\(^{23}\) TRPV1 appears to be implicated in the antinociceptive mechanism of botulinum toxin A,\(^{14}\)\(^{15}\) which is effective in CM\(^{26}\) and its activation, as said above, elicits CGRP exocytosis.\(^{17}\)

The increase of TRPV1-LI innervation in scalp arteries may be a structural condition favouring migraine or CM. It may cause a greater sensitivity to nocuous agents. Although a role of central mechanisms in migraine is strongly supported by several data,\(^{6}\) the sharing of a peripheral mechanism mainly acting on scalp arteries is also reliable.\(^{6}\) Pressure painful scalp arteries are present in a large percentage of migrainous participants, during and in the interval between the migraine attacks.\(^{7}\) This suggests a susceptibility to pain of such structures in migraine. All these data support the hypothesis of a peripheral vulnerability that, in synergy with a central dysfunction, can predispose to and cause the attacks.

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Contributors All authors have made substantial contributions to this work. CC, MDP and MQ prevalent in study design, laboratory analysis, monograph preparation and revision. EJ, RB, MB, MPS and TM prevalent in sampling and laboratory data collection and analysis. All authors critically revised the manuscript and approved the submitted version.

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Patient consent Obtained.

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REFERENCES
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