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NO-induced migraine attack: strong increase in plasma calcitonin gene-related peptide (CGRP) concentration and negative correlation with platelet serotonin release

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Abstract

The aim of the present study was to investigate changes in the plasma calcitonin gene-related peptide (CGRP) concentration and platelet serotonin (5-hydroxytriptamine, 5-HT) content during the immediate headache and the delayed genuine migraine attack provoked by nitroglycerin. Fifteen female migraineurs (without aura) and eight controls participated in the study. Sublingual nitroglycerin (0.5 mg) was administered. Blood was collected from the antecubital vein four times: 60 min before and after the nitroglycerin application, and 60 and 120 min after the beginning of the migraine attack (mean 344 and 404 min; 12 subjects). In those subjects who had no migraine attack (11 subjects) a similar time schedule was used. Plasma CGRP concentration increased significantly ($P < 0.01$) during the migraine attack and returned to baseline after the cessation of the migraine. In addition, both change and peak, showed significant positive correlations with migraine headache intensity ($P < 0.001$). However, plasma CGRP concentrations failed to change during immediate headache and in the subjects with no migraine attack. Basal CGRP concentration was significantly higher and platelet 5-HT content tended to be lower in subjects who experienced a migraine attack. Platelet serotonin content decreased significantly ($P < 0.01$) after nitroglycerin in subjects with no migraine attack but no consistent change was observed in patients with migraine attack. In conclusion, the fact that plasma CGRP concentration correlates with the timing and severity of a migraine headache suggests a direct relationship between CGRP and migraine. In contrast, serotonin release from platelets does not provoke migraine, it may even counteract the headache and the concomitant CGRP release in this model.

Keywords: Headache; Migraine; Nitroglycerin; Nitric oxide; Calcitonin gene-related peptide; Platelet serotonin

1. Introduction

Nitroglycerin is an organic nitrate ester that has vasodilatory properties. The direct formation of nitric oxide (NO) from organic nitrates has been shown in several tissues (Tassorelli and Joseph, 1996). Furthermore, endogenous NO is synthesized by various isoforms of nitric oxide synthase (NOS) (Ohkuma and Katsura, 2001), which are encoded by distinct genes (Griffiths et al., 1997). NO is an important mediator of vasodilatation in intra- and extracranial blood vessels, and is also an algogenic substance (Iversen, 1995). This small and almost ubiquitous messenger molecule does not interact with specific receptors, but diffuses freely across membranes (Ohkuma and Katsura, 2001). NO diffuses into vascular smooth

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; cGMP, cyclic guanylate monophosphate; SP, substance P; CGRP, calcitonin gene-related peptide; 5-HT, 5-hydroxytriptamine; ANOVA, analysis of variance.

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muscle where it activates soluble guanylate cyclase with the formation of cyclic guanylate monophosphate (cGMP), which in turn, relaxes the muscles and dilates the blood vessels (Zicari et al., 2001). Furthermore, NO modulates the neuronal release of neurotransmitters in both in vitro and in vivo conditions (Prast and Philippu, 2001).

Previous studies have shown that migraineurs experience a delayed migraine-like headache in association with nitroglycerin administration more often than non-migraineurs (Olesen et al., 1993). It appears that NO initiates a slow pathological reaction that, eventually, leads to a headache attack; it is also seen that migraineurs are supersensitive to both exogenous and endogenous NO (Olesen et al., 1994). Artificially increased concentrations of NO are able to increase NO synthase availability in nociceptive trigeminal neurons (Knyihar-Csillik and Vecsei, 1999; Pardutz et al., 2000). At the level of the trigeminal system, neuronal NOS appears to coordinate NO production to activate calcitonin gene-related peptide (CGRP) release from trigeminal fibres which in turn triggers vasodilatation. At the level of the blood vessels themselves, CGRP appears to activate endothelial NO synthase to cause further NO production and thus relaxation of blood vessel smooth muscle with attendant dilation (Akerman et al., 2002; Goadsby et al., 1990; Lassen et al., 2002; Sarchielli et al., 2000). However, it is not yet clearly understood why migraine sufferers respond more severely to NO than the controls.

The aim of the present study was to evaluate how plasma CGRP concentration and platelet serotonin content change in peripheral blood circulation during a reproducible and accurate human migraine model such as nitroglycerin-induced headache.

2. Materials and methods

2.1. Study subjects

Fifteen unrelated migraine patients without aura (migraineurs; mean age: 41.9 ± 2.3 years) and eight unrelated healthy controls (mean age: 38.5 ± 4.4 years) were included in the study. All individuals were females. A detailed medical history was taken from each subject, and they underwent complete physical, neurological and psychological examinations and laboratory studies before participating in the project. Control subjects have rare (less than 1/year) and mild headaches only. Patients using frequent analgesic medications (corresponding to more than 2 g of aspirin/day) were excluded from the study. The diagnoses for headache were made according to the criteria of the International Headache Society (IHS, 1988). None of the subjects were suffering from psychiatric disorders of any kind according to the criteria of the DSM-IV (DSM-IV, 1994). At the time of the study, all participants were medication-free (including oral contraceptives) and were not following any particular diet. All subjects were studied during the mid follicular phase (between days 5 and 10 of the menstrual cycle).

We used an open label case control design. The study protocol was approved by the local ethics committee for experimentation on humans and every subject gave written informed consent before participating in the research.

2.2. Procedure

The subjects arrived at the laboratory at 6.30 a.m. and were observed until 5.00 p.m. Headache intensity and characteristics were measured at baseline (7.00 a.m.) and every 20 min thereafter for the duration of the observation period. Headache intensity was scored on a verbal scale that measured from 0 to 10: 1, a very mild headache including pre-pain (feeling of pressure); 5, a headache of medium severity; and 10, a very severe headache. The subjects were asked after the standardized procedure about pain localization and quality, and side effects were recorded.

Sublingual administration of 0.5 mg nitroglycerin was used as a test at 8.00 a.m.

Venous blood was drawn from an antecubital vein four times during the test day using the Vacutainer® system. Baseline blood samples were collected at 7.00 a.m. after a 30 min rest period. At 8.00 a.m. subjects received a 0.5 mg dose of sublingual nitroglycerin. The second blood sample was taken after sublingual nitroglycerin at 9.00 a.m. The next two blood samples were taken 60 and 120 min after the beginning of the migraine attack (mean 344 and 404 min after nitroglycerin). In subjects with no migraine attack, similar time schedules were used based on our preliminary data (third blood sample 5 h and fourth blood sample 6 h after nitroglycerin).

Subjects were free to withdraw at any time during the study and treatment was offered if needed. None of the subjects whose data are presented here received any drug treatment before the last blood sampling.

2.3. Determination of plasma CGRP level

2.3.1. Drugs and chemicals

Rat Tyr-α-CGRP (23–37) (Bachem, Budendorf, Switzerland), EDTA, Na_2HPO_4, NaH_2PO_4 (Reanal, Budapest, Hungary), Aprotinin (Richter, Budapest, Hungary), 30% bovine albumin solution (Sigma, St. Louis, USA) were used. ^{125}I-labelled Tyr-α-CGRP (23–37) was prepared in our laboratory (Nemeth et al., 2002). CGRP antiserum was provided by Dr T. Gorcs, Semmelweis Medical University, Budapest.

For the determination of plasma CGRP immunoreactivity, venous blood samples (3 ml/patient) were taken into ice-cold tubes containing EDTA (7.5% 0.072 ml/3 ml blood) and aprotinin (2700 KIU/3 ml blood). Following centrifugation (3000 rpm for 15 min at 4 °C) the plasma was

aspirated and stored at −80 °C until plasma CGRP determination. Plasma CGRP concentrations were measured by means of a specific and sensitive radioimmunoassay (RIA) method (Nemeth et al., 1998). Plasma CGRP concentrations were measured by examiners who were not familiar with the subjects participating in the study.

2.4. Determination of platelet serotonin content

The 5-hydroxytriptamine (5-HT) concentration was determined from platelet-rich-plasma by high-pressure liquid chromatography (HPLC) coupled with electrochemical detection as described earlier (Guicheney, 1988; Kantor et al., 2001). Blood from the cubital vein was obtained in tubes containing EDTA. Platelet-rich plasma was routinely prepared by centrifugation and platelets were counted by phase contrast microscopy. Platelet lyses was induced by the addition of perchloric acid and methanol. The supernatant was kept at −80 °C until the assay was performed. The 5-HT concentration was measured by HPLC that included an LKB-Pharmacia 2248-010 pump, a 5 C-18 Nucleosil RP column and an electrochemical detector. Results were calculated from standard and sample peak height ratios. Serotonin content was expressed in ng/10^8 platelets. Platelet 5-HT concentrations were measured by examiners who were not familiar with the subjects participating in the study.

2.5. Statistical analysis

One-way, two-way and repeated measure analyses of variance (ANOVA) with post-hoc comparisons (Newman-Keuls) were applied for testing the statistical differences between the means of CGRP and 5-HT with regard to diagnoses and time. Friedman's non-parametric ANOVA was used to test changes in headache scores over time. Statistical differences in headache scores among groups were compared using the Mann–Whitney U test. Significance levels were adjusted for multiple comparisons using a Bonferroni correction. The χ^2 test was applied for statistical analysis to search for any differences between the ratios of patients who experienced headache in the different groups. Correlation for headache scores versus 5-HT or CGRP concentration was quantified using the Spearman rank correlation coefficient. Correlation for basal CGRP concentrations versus peak CGRP changes was done with Pearson product moment correlation test. All procedures were carried out using Statistica 5 for Windows (StatSoft, 1997). Results were presented as the mean ± standard error of mean; $P < 0.05$ was considered significantly different.

3. Results

All data were analyzed in two separate ways, primarily according to the development of migraine attack (migraine attack versus no migraine attack, see Figs. 1–4), and secondly according to the clinical diagnosis (migraineurs versus controls, see Tables 1–3).

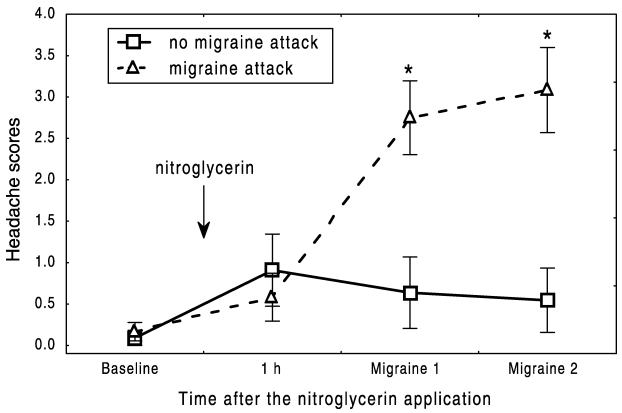

Fig. 1. Effects of nitroglycerin (0.5 mg sublingual) on headache scores (0–10 verbal scale) in subjects with migraine attack ($n = 12$) and subjects with no migraine attack ($n = 11$). Values are mean ± SEM headache scores. Baseline blood samples were collected at 7.00 a.m. A secondary blood sample was taken 1 h after sublingual application of nitroglycerin, at 9.00 a.m. The next two blood samples were taken 60 (migraine 1) and 120 min (migraine 2) after the beginning of the migraine attack (mean 344 and 404 min after nitroglycerin). In subjects with no migraine attack, similar time schedules were used based on our preliminary data (migraine 1: 5 h and migraine 2: 6 h after nitroglycerin, respectively). *Significant changes after sublingual nitroglycerin compared to baseline, $P < 0.01$.

3.1. Headaches

Two out of the eight control subjects and 10 out of the 15 migraineurs developed an immediate headache (mean

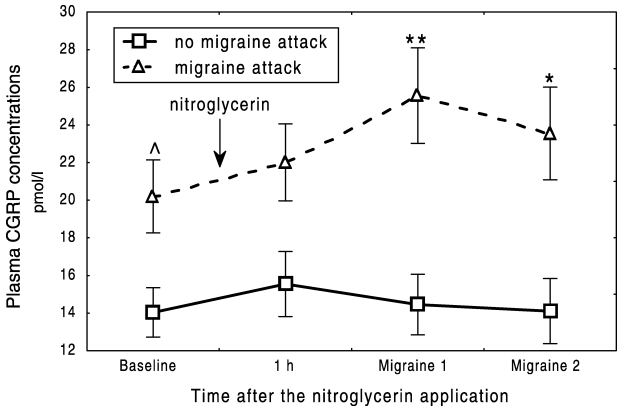

Fig. 2. Effects of nitroglycerin (0.5 mg sublingual) on plasma CGRP concentrations in subjects with migraine attack ($n = 12$) and subjects with no migraine attack ($n = 11$). Values are mean ± SEM CGRP concentrations. Baseline blood samples were collected at 7.00 a.m. A secondary blood sample was taken 1 h after sublingual application of nitroglycerin, at 9.00 a.m. The next two blood samples were taken 60 (migraine 1) and 120 min (migraine 2) after the beginning of the migraine attack (mean 344 and 404 min after nitroglycerin). In subjects with no migraine attack, similar time schedules were used based on our preliminary data (migraine 1: 5 h and migraine 2: 6 h after nitroglycerin, respectively). ^Significant difference in baseline values, $P < 0.05$. Significant changes after sublingual nitroglycerin compared to baseline, *$P < 0.05$; **$P < 0.001$.

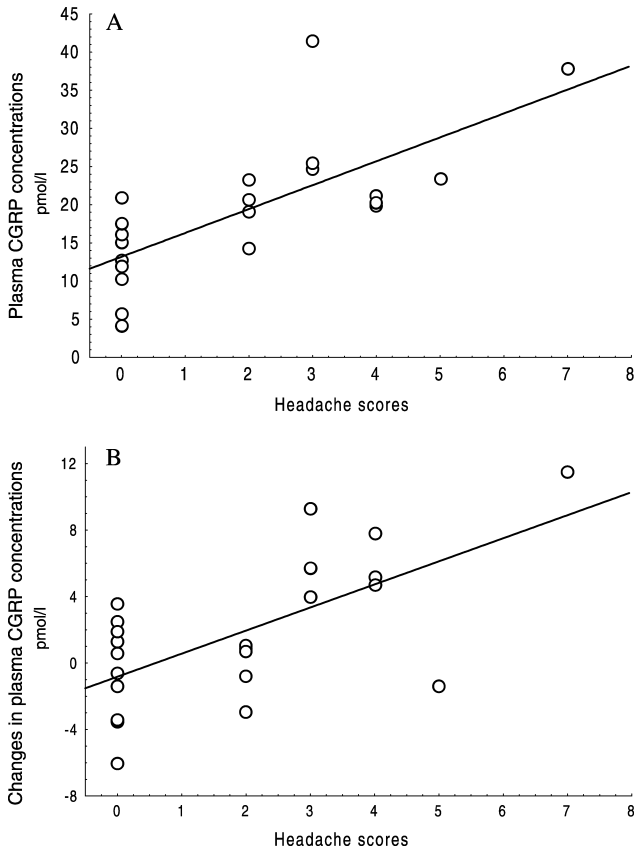

Fig. 3. Significant correlations were observed between headache scores and plasma CGRP concentrations at the time of the fourth blood sampling (Spearman $R = 0.78$, $P < 0.0001$; A). Significant correlation was also observed between headache scores and changes in plasma CGRP concentration, when compared to the baseline, at the time of fourth blood sampling (Spearman $R = 0.57$, $P = 0.005$; B).

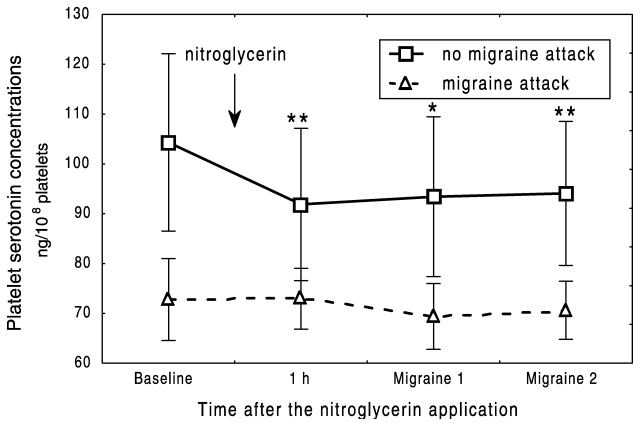

Fig. 4. Effects of nitroglycerin (0.5 mg sublingual) on platelet serotonin concentrations in subjects with migraine attack ($n = 12$) and subjects with no migraine attack ($n = 11$). Values are mean ± SEM serotonin concentrations. Baseline blood samples were collected at 7.00 a.m. A secondary blood sample was taken 1 h after sublingual application of nitroglycerin at 9.00 a.m. The next two blood samples were taken 60 (migraine 1) and 120 min (migraine 2) after the beginning of the migraine attack (mean 344 and 404 min after nitroglycerin). In subjects with no migraine attack, similar time schedules were used based on our preliminary data (migraine 1: 5 h and migraine 2: 6 h after nitroglycerin, respectively). Significant changes after sublingual nitroglycerin compared to baseline, $^{*}P < 0.05$; $^{**}P < 0.01$.

latency: 12.5 ± 4.7 min) after the nitroglycerin test (sublingual 0.5 mg), see Tables 1 and 2. This headache did not fulfill the IHS criteria for migraine without aura (median headache score 1, range 0–4), lasting only a few minutes (mean 31.7 ± 5.2 min), and disappearing spontaneously. Typical migraine attacks that fulfilled the IHS criteria for migraine without aura, developed in two of the control subjects and 10 of the migraineurs (median headache score 3.5, range 2–7). Characteristics and accompanying symptoms of usual migraine headache and migraine-type headache after nitroglycerin are summarized in Table 2. Out of the 12 subjects with migraine attack, only seven had immediate headache, see Tables 1 and 2. The mean latency for the migraine attacks was 284.2 ± 90.3 min.

Immediate headache and migraine headache scores in subjects with no migraine attack and in subjects with migraine attack are shown in Fig. 1. There was no significant difference in immediate headache scores between the two groups (Mann–Whitney U test: $U = 61.0$, $P = 0.76$).

There was a statistically significant increase in headache scores (Friedman ANOVA: $\chi^2 = 20.7$, $N = 15$, df = 3, $P < 0.00012$) only in migraineurs, see Table 3. As expected, migraine headache developed more frequently among migraineurs (χ^2 test: $\chi^2 = 3.63$, df = 1, $P = 0.057$) and the severity of the attacks was also higher. For further information about the headache characteristics of migraine attacks and accompanying symptoms, see Table 2.

3.2. Basal neurochemical measures

3.2.1. According to the development of headache

Basal CGRP concentrations were significantly higher (one-way ANOVA: $F = 6.65$, df = 1, 21, $P = 0.018$) in those subjects who developed migraine attack (20.2 ± 1.9 pmol/l) when compared to the subjects with no migraine attack (14.0 ± 1.3 pmol/l). In contrast, basal CGRP concentration was not significantly different in subjects who developed immediate headache

Table 1
Immediate headache and migraine attack frequency in the study population

Diagnosis (number)	Immediate headache		Migraine attack		Both types of headache
	Yes	No	Yes	No	
Migraineurs (15)	10	5	10	5	6
Controls (8)	2	6	2	6	1
Totals	12	11	12	11	7

Table 2
(a) Clinical characteristics of participants' usual headache (control subjects have rare—less than 1/year—and mild headaches only). (b) Presence of immediate, non-migraine type headache within the first hour and clinical characteristics of maximal headache scores from 1 to 12 h after the nitroglycerin test

Subject	Immediate headache		Headache characteristic of usual and NO-induced late headache					Accompanying symptoms			
			Time	Localization	Intensity	Quality	Aggravate	Nausea	Photo	Phono	IHS
1	a			Left	Mod/sev	Puls	Yes	Yes	Yes	Yes	M
	b	No	5	Left	3	Puls	Yes	No	Yes	No	Yes
2	a			Right/left	Severe	Puls	Yes	Yes	Yes	Yes	M
	b	Yes	1	Bilat	4	Cons	No	No	No	No	No
3	a			Left	Mod/sev	Puls	Yes	Yes	No	Yes	M
	b	Yes	6	Bilat	5	Puls	Yes	Yes	No	Yes	Yes
4	a			Left	Severe	Puls	Yes	Yes	Yes	Yes	M
	b	No	7	Bilat	7	Puls	Yes	Yes	Yes	Yes	Yes
5	a			Bilat	Mod/sev	Puls	Yes	No	Yes	Yes	M
	b	Yes	6	Right/bilat	6	Puls	Yes	No	Yes	No	Yes
6	a			Right	Severe	Puls	Yes	Yes	Yes	No	M
	b	Yes	7	Right	6	Puls	Yes	Yes	Yes	Yes	Yes
7	a			Right	Mod	Puls	Yes	No	Yes	No	M
	b	No			0		No	No	No	No	No
8	a			Left	Mod/sev	Puls	Yes	Yes	Yes	No	M
	b	No	6	Left	4	Puls	Yes	Yes	Yes	No	Yes
9	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	No			0		No	No	No	No	No
10	a			Right/left	Severe	Puls	Yes	Yes	Yes	Yes	M
	b	Yes	5	Left	3	Puls	Yes	Yes	Yes	Yes	Yes
11*	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	Yes	5	Left	2	Puls	Yes	No	Yes	Yes	Yes
12	a			Right	Severe	Puls	Yes	Yes	Yes	Yes	M
	b	Yes	5	Right	3	Puls	Yes	Yes	Yes	Yes	Yes
13	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	Yes	1	Bilat	2	Cons	No	No	No	No	No
14	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	No			0		No	No	No	No	No
15	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	No			0		No	No	No	No	No
16	a			Right	Severe	Puls	Yes	Yes	Yes	Yes	M
	b	No	7	Right	2	Puls	Yes	No	Yes	Yes	Yes
17	a			Right	Mod/sev	Puls	Yes	Yes	Yes	No	M
	b	Yes	1	Bilat	3	Cons	No	No	No	No	No
18	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	No			0		No	No	No	No	No
19	a			Right	Mod/sev	Puls	Yes	Yes	Yes	No	M
	b	Yes	8	Bilat	5	Puls	Yes	Yes	Yes	No	Yes
20	a			Bilat	Mod/sev	Puls	Yes	Yes	Yes	Yes	M
	b	Yes	1	Bilat	1	Cons	No	No	No	No	No
21**	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	No	5	Bilat	2	Puls	Yes	Yes	Yes	No	Yes
22	a			Left	Severe	Puls	Yes	Yes	No	No	M
	b	Yes			0		No	Yes	No	No	No
23	a			Bilat	Mild	Cons	No	No	No	No	CO
	b	No			0		No	No	No	No	No

Immediate headache, atypical headache (not fulfilling the IHS criteria for migraine without aura) within the first hour; time, time to peak headache after the sublingual application of nitroglycerin; localization, bilateral or right/left hemisphere; intensity, intensity of headache scored on a scale from 0 to 10; quality, constant or pulsating; aggravate, intensity of headache aggravated by physical activity; photo, photophobia; phono, phonophobia; IHS, yes, M, fulfilling the IHS criteria for migraine without aura; IHS, no, CO, not fulfilling the IHS criteria for migraine without aura. *One bilateral, pulsating headache with vomiting several years ago. **Rare headaches after sleep deprivation with intensity of headache aggravated by physical activity.

(18.8 ± 1.9 pmol/l) when compared to those without immediate headache (15.6 ± 1.8 pmol/l), see Fig. 2.

Basal platelet serotonin content was somewhat lower (one-way ANOVA: $F = 2.74$, df $= 1, 21$, $P = 0.11$) in subjects with typical migraine attack (72.8 ± 8.2 ng/10^8 pts) when compared to the subjects with no migraine attack (104.3 ± 17.8 ng/10^8 pts). Basal platelet serotonin content was not significantly different (one-way ANOVA:

Table 3
Headache scores, plasma CGRP concentrations and platelet serotonin concentrations (mean ± SEM) in controls and migraineurs during the nitroglycerin test

Time	Controls (mean ± SEM)				Migraineurs (mean ± SEM)			
	B	1	2	3	B	1	2	3
Headache scores (1–10)	0.0 ± 0.0	0.25 ± 0.25	0.5 ± 0.33	0.5 ± 0.33	0.2 ± 0.10	1.0 ± 0.35	2.4 ± 0.48**	2.6 ± 0.53**
Plasma CGRP (pmol/l)	15.1 ± 2.0	17.5 ± 2.3	16.5 ± 2.3	15.3 ± 2.0	18.4 ± 1.7	19.7 ± 1.9	22.2 ± 2.6**	21.0 ± 2.4*
Platelet serotonin (ng/10^8 plts)	111.1 ± 23.3	97.7 ± 20.0*	101.0 ± 21.3*	98.1 ± 18.9*	75.5 ± 7.7	73.6 ± 5.8	70.2 ± 5.9	73.2 ± 5.9

Baseline blood samples were collected at 7.00 a.m. A secondary blood sample was taken 1 h after the sublingual application of nitroglycerin, at 9.00 a.m. The next two blood samples (2 and 3) were taken 60 and 120 min, respectively, after the beginning of a migraine attack (mean 344 and 404 min after nitroglycerin). In subjects with no migraine attack, similar time schedules were used based on our preliminary data (2: 5 h and 3: 6 h after nitroglycerin, respectively). Significant changes after sublingual nitroglycerin compared to baseline, *$P < 0.05$; **$P < 0.01$.

$F = 1.49$, df $= 1, 21$, $P = 0.24$) in patients with immediate headache (74.2 ± 9.3 ng/10^8 pts) when compared to those without immediate headache (102.7 ± 17.4 ng/10^8 pts), see Fig. 4.

3.2.2. According to the clinical diagnosis

Basal CGRP concentrations were not significantly higher in migraineurs when compared to controls (M, 18.4 ± 1.7 pmol/l; CO, 15.1 ± 2.0 pmol/l; one-way ANOVA: $F = 1.44$, df $= 1, 21$, $P = 0.24$), see Table 3.

Basal platelet serotonin content was somewhat lower (one-way ANOVA: $F = 3.24$, df $= 1, 21$, $P = 0.086$) in migraineurs (75.5 ± 7.7 ng/10^8 pts) when compared to the control subjects (111.1 ± 23.3 ng/10^8 pts), see Table 3.

3.3. Changes in neurochemical measures over time

3.3.1. According to the development of headache

Nitroglycerin highly significantly increased the plasma CGRP concentration over time during the migraine attack (two-way ANOVA, no migraine attack vs. migraine attack subjects: $F = 10.17$, df $= 1, 21$, $P = 0.004$; group × time interaction: $F = 3.58$, df $= 3, 63$, $P = 0.019$; Fig. 2). When separate ANOVA tests were run, significant increase (one-way ANOVA: $F = 6.92$, df $= 3, 33$, $P = 0.001$) in plasma CGRP concentrations were found in subjects with migraine attack but not in the subjects with no migraine attack (one-way ANOVA: $F = 0.57$, df $= 3, 30$, $P = 0.63$), see Fig. 2.

Our results demonstrate that there is strong correlation between plasma CGRP concentrations and migraine headache scores (Spearman $R = 0.70$, $P < 0.001$ and Spearman $R = 0.78$, $P < 0.0001$ in the 60th and 120th min after the beginning of migraine, respectively; Fig. 3). In addition, the changes in plasma CGRP content when compared to the basal values, showed significant correlation with migraine headache intensity (Spearman $R = 0.55$, $P = 0.006$ and Spearman $R = 0.57$, $P = 0.005$ in the 60th and 120th min after the beginning of migraine, respectively; Fig. 3). Furthermore, the peak changes of plasma CGRP concentrations when compared to basal values, showed significant correlation with basal CGRP concentration (Pearson $R = 0.45$, $P = 0.034$).

There were, however, no significant changes (one-way ANOVA: $F = 1.79$, df $= 1, 11$, $P = 0.21$) in plasma CGRP concentrations during the immediate headache, and the immediate headache scores did not correlate with the plasma CGRP concentration (Spearman $R = 0.1$, $P = 0.63$).

An early and long-lasting decrease in platelet serotonin content (one-way ANOVA: $F = 4.85$, df $= 3, 30$, $P = 0.007$) was found during the nitroglycerin test in those subjects who did not develop a migraine attack but not in the subjects who experienced a migraine attack (one-way ANOVA: $F = 0.17$, df $= 3, 33$, $P = 0.92$; Fig. 4). The decrease in platelet serotonin content was prolonged, and had not yet returned to the starting level at the time of the last blood sampling.

Furthermore, in subjects without immediate headache, a significant decrease in platelet serotonin content was observed 60 min after the nitroglycerin test (one-way ANOVA: $F = 5.2$, df $= 1, 10$, $P = 0.046$). There were no significant changes in platelet serotonin content in those subjects who developed an immediate headache (one-way ANOVA: $F = 0.05$, df $= 1, 11$, $P = 0.83$). A significant negative correlation was observed between immediate headache scores and platelet serotonin content 60 min after nitroglycerin, when data of all subjects were included in the analysis (Spearman $R = -0.45$, $P = 0.03$).

3.3.2. According to the clinical diagnosis

Generally, nitroglycerin showed a strong tendency to increase the plasma CGRP concentration over time (two-way ANOVA: $F = 2.56$, df $= 3, 63$, $P = 0.06$) but there was no significant difference between migraineurs and the control subjects (two-way ANOVA, migraineurs vs. control subjects: $F = 1.77$, df $= 1, 21$, $P = 0.2$; group × time interaction: $F = 1.63$, df $= 3, 63$, $P = 0.19$). When separate ANOVA tests were run, a significant increase in plasma CGRP concentrations was found in migraineurs (one-way ANOVA: $F = 4.24$, df $= 3, 42$, $P = 0.01$) but not in the control subjects (one-way ANOVA: $F = 0.96$, df $= 3, 21$, $P = 0.43$), see Table 3.

Generally, nitroglycerin showed a tendency to decrease platelet serotonin concentration over time (two-way

ANOVA: $F = 2.07$, df $= 3, 63$, $P = 0.099$) and there was a slight difference between migraineurs and control subjects (two-way ANOVA, migraineurs vs. control subjects: $F = 2.97$, df $= 1, 21$, $P = 0.11$; group × time interaction: $F = 1.03$, df $= 3, 63$, $P = 0.39$). When separate ANOVA tests were run, a significant decrease in platelet serotonin concentration was found in the control subjects (one-way ANOVA: $F = 4.22$, df $= 3, 21$, $P = 0.017$) but not in migraineurs (one-way ANOVA: $F = 0.39$, df $= 3, 42$, $P = 0.76$), see Table 3.

3.3.3. Return of elevated CGRP concentration after the cessation of migraine attack

Further blood samples were available from two patients after the migraine attack was eased using sumatriptan. In these patients, plasma CGRP concentration had decreased to the basal value (*Headache*: basal, 0 and 0; maximal, 3 and 3; after treatment, 0 and 1. *CGRP*: basal, 20.7 and 22.4 pmol/l; at the time of maximal headache, 26.3 and 26.5 pmol/l; after treatment, 19.8 and 20.2 pmol/l) but no consistent changes were observed in platelet serotonin concentration (*Platelet 5-HT*: basal, 52.8 and 68.6 ng/10^8 pts; at the time of maximal headache, 70.4 and 66.9 ng/10^8 pts; after treatment, 72.2 and 49.3 ng/10^8 pts).

4. Discussion

The aim of the present study was to evaluate the neurochemical changes after sublingual nitroglycerin application that may cause headache in migraineurs and sensitive healthy subjects. Even low doses of sublingual nitroglycerin are able to evoke a migraine attack in sensitive patients. High doses even cause a migraine-type headache in non-migraineurs (Castellano et al., 1998; Christiansen et al., 2000; Iversen and Olesen, 1996). In our study, 10 out of the 15 migraineurs and two out of the eight controls developed a typical migraine attack during the nitroglycerin test. Previously all control subjects had only rare (less than 1/year) and mild headaches. However, one out of the two controls who experienced migraine attack, had one bilateral, pulsating headache with vomiting several years ago and the other had rare headaches after sleep deprivation with the intensity of the headache aggravated by physical activity. These signs may predict sensitivity to NO and the development of the provoked headache in these subjects. The relatively variable effectiveness of sublingual nitroglycerin on headache, allowed us to compare induced headache to that of the neurochemical responses in those subjects who developed typical migraine attack and those did not. Furthermore, correlation between the intensity of the migraine attack and the neurochemical measures could be evaluated.

Trigeminal activation may lead to the release of CGRP into the cranial circulation, both in humans and in animal models (Goadsby et al., 1990; Knyihar-Csillik et al., 1997; Lassen et al., 2002). Several primary headaches, such as migraine (Goadsby et al., 1990; Sarchielli et al., 2000), cluster (Fanciulacci et al., 1995; Edvinsson, 2000) and chronic paroxysmal hemicrania (Goadsby and Edvinsson, 1996) are associated with an increase in the CGRP level in the external and/or internal jugular vein during a pain attack. In addition, trigeminovascular activation has been shown to increase CGRP concentration in the jugular vein too. Similar, but less pronounced changes, were also seen in young migraineurs when CGRP was measured in the antecubital vein during the spontaneous migraine attacks (Gallai et al., 1995). Another study suggested that NO-induced immediate headache was not associated with the release of CGRP (Ashina et al., 2001).

Our results showed consistent, highly significant increases, in the peripheral plasma CGRP concentration during the nitroglycerin-induced migraine attack. These data support the hypothesis that migraine attacks are a result of trigeminovascular activation (Akerman et al., 2002). It seems that nitroglycerin triggers a cascade of events that subsequently lead to the release of CGRP in subjects who develop migraine attack. The mechanism of NO-induced pain is not clear, but animal studies suggest, that CGRP release does not require the production of cGMP, because guanilate cyclase inhibitor does not prevent NO-induced CGRP release (Garry et al., 2000).

In our study, plasma CGRP concentration failed to increase during the immediate headache. These data support the conclusion of previous studies (Ashina et al., 2001; Eltrop et al., 2000). This suggests that the initial headache may be caused by a direct action of the NO-cGMP pathway, that causes vasodilatation by vascular smooth muscle relaxation, independently of the CGRP release (Akerman et al., 2002).

It has been suggested that plasma levels of CGRP in the peripheral circulation are increased in adult migraineurs interictally (Ashina et al., 2000). It is remarkable that our results demonstrate that the higher basal plasma CGRP concentrations predispose subjects to develop migraine attack during the nitroglycerin test. In our study, increase in the CGRP concentrations correlates markedly with the migraine headache intensity. Furthermore, CGRP concentration returned to baseline after the migraine headache had ceased. These data suggest that blocking CGRP synthesis, release or receptors, may successfully prevent or alleviate migraine pain (Akerman et al., 2002; Buzzi and Moskowitz, 1992; Doods et al., 2000; Durham and Russo, 1999; Edvinsson, 2001; Goadsby and Edvinsson, 1993; Lassen et al., 2002). Several drugs, that in various ways antagonize the effects of CGRP, are presently known to be in pre-clinical and clinical trials, but no results are available in the public domain (Doods, 2001).

Thus, it is likely that nitroglycerin-induced migraine attack can only occur when particular pathophysiological conditions are present (Lassen et al., 2002). Evidence from experimental models of pain suggests that NO plays

a crucial role in both central and peripheral sensitization (Knyihar-Csillik and Vecsei, 1999; Pardutz et al., 2000, 2002; Reuter et al., 2001). The long latency period, between nitroglycerin exposure and the fully developed migraine attack, indicates that the activation of NO—or steps in the NO-cGMP cascade—initiates a rather slow ongoing process which results in a migraine attack in sensitive patients. It is possible that this process takes place in the trigemino-vascular system, with the increased activity of neuronal NOS and the consequent activation of trigeminal fibres and nucleus (Akerman et al., 2002) or in the nociceptive modulation system (Tassorelli et al., 2002). Furthermore, increased NOS activity was demonstrated in patients with chronic daily headache and a previous history of migraine (Sarchielli et al., 2002), and in migraineurs (D'Amico et al., 2002; Sarchielli et al., 2000). This higher NOS activity may cause the higher basal CGRP level and, in addition, the correlation between the peak changes of plasma CGRP concentration compared to basal value supports this hypothesis.

Several investigations support that platelet serotonin content and its turnover is reduced in migraineurs between attacks, while the platelet serotonin is released during migraine crises (Anthony, 1986; Ferrari et al., 1989; Goadsby, 1997; Juhasz et al., 2003; Nakano et al., 1993; Sarchielli and Gallai, 2001; Waldenlind et al., 1985). Our recent data support that the platelet 5-HT concentrations are somewhat lower in subjects who developed migraine. The hyposerotonergic status facilitated the pial microvascular dilation in response to intravenous nitroglycerin infusion in an animal model (Srikiatkhachorn et al., 2001). Furthermore, increased NOS activity was observed in a model of serotonin depletion (Tagliaferro et al., 2001). These results suggest that a chronic hyposerotonergic condition is one of the main risk factors that may cause a more intense response to NO in migraineurs when compared to the responses of the controls.

According to our knowledge, only one study has previously investigated the platelet serotonin content during a nitroglycerin-induced headache attack and it failed to show any changes (Dalsgaard-Nielsen et al., 1975). In our studies, we could not detect any significant changes in platelet serotonin content in the subjects with nitroglycerin-induced migraine attack. On the other hand, an early and prolonged, highly significant decrease in platelet serotonin content was found in those subjects who did not develop migraine attack during the nitroglycerin test. These data provide evidence for a massive release of serotonin in subjects who did not develop migraine attack. This serotonin release, by acting on 5-$HT_{1B/1D}$ receptors such as tryptans, may cause vasoconstriction and inhibition of evoked trigeminal nucleus firing, release of CGRP and other inflammatory peptides from perivascular nerve terminals, and thus alleviates inflammation and pain (Goadsby and Hoskin, 1998; Johnson et al., 1998; Knyihar-Csillik et al., 1997, 2001). In this regard, changes in 5-HT content do not causally relate to migraine, but secondarily, 5-HT may influence the neurochemical changes during the migraine-induced pain attack.

In conclusion, our results support that lower platelet serotonin concentration and higher basal CGRP concentrations are risk factors that express greater susceptibility to develop both spontaneous and NO-induced migraine attack. Furthermore, plasma CGRP concentration is a dynamically changing trait marker for the migraine-induced pain that suggests a possible causative role of CGRP in migraine. On the other hand, hyposerotonergic conditions may prevent the early induced release of serotonin, which may counteract pain mechanisms by 5-$HT_{1B/1D}$ receptor activation.

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