Cerebrospinal Fluid from Patients with Subarachnoid Haemorrhage and Vasospasm Enhances Endothelin Contraction in Rat Cerebral Arteries

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Abstract

Introduction

Previous studies have suggested that cerebrospinal fluid from patients with subarachnoid hemorrhage (SAH) leads to pronounced vasoconstriction in isolated arteries. We hypothesized that only cerebrospinal fluid from SAH patients with vasospasm would produce an enhanced contractile response to endothelin-1 in rat cerebral arteries, involving both endothelin ETₐ and ETₐ receptors.

Methods

Intact rat basilar arteries were incubated for 24 hours with cerebrospinal fluid from 1) SAH patients with vasospasm, 2) SAH patients without vasospasm, and 3) control patients. Arterial segments with and without endothelium were mounted in myographs and concentration-response curves for endothelin-1 were constructed in the absence and presence of selective and combined ETₐ and ETₐ receptor antagonists. Endothelin concentrations in culture medium and receptor expression were measured.

Results

Compared to the other groups, the following was observed in arteries exposed to cerebrospinal fluid from patients with vasospasm: 1) larger contractions at lower endothelin concentrations (p<0.05); 2) the increased endothelin contraction was absent in arteries without endothelium; 3) higher levels of endothelin secretion in the culture medium (p<0.05); 4) there was expression of ETₐ receptors and new expression of ETₐ receptors was apparent;
5) reduction in the enhanced response to endothelin after ETB blockade in the low range and after ETA blockade in the high range of endothelin concentrations; 6) after combined ETA and ETB blockade a complete inhibition of endothelin contraction was observed.

Conclusions

Our experimental findings showed that in intact rat basilar arteries exposed to cerebrospinal fluid from patients with vasospasm endothelin contraction was enhanced in an endothelium-dependent manner and was blocked by combined ETA and ETB receptor antagonism. Therefore we suggest that combined blockade of both receptors may play a role in countering vasospasm in patients with SAH.

Introduction

Cerebral vasospasm is one of the most serious complications following aneurysmal subarachnoid hemorrhage (SAH) and is independently associated with poor outcome [1]. Endothelin-1 (ET-1) is thought to be of major importance for cerebral vasospasm [2,3].

In experimental models of SAH, increased sensitivity of cerebral arteries to ET-1 have been invariably reported [4]; increased levels of ETA receptor mRNA have been inconsistently demonstrated [5] [6,7] while increased levels of ETB mRNA have been unequivocally reported [8–10] [11]. So far studies on endothelin-1 receptor regulation did not differentiate between the two conditions of SAH with and without vasospasm [6,9,10,12]. Moreover, and in vitro studies on the cerebral microvasculature have often been used to explain events observed in conductive vessels [13]. Furthermore, although the cerebral endothelium is unique in terms of growth and responsiveness to various vasoactive agonists, results obtained in non-cerebral endothelial cells have been extrapolated to the brain vasculature [14,15].

The efficacy of selective receptor antagonists have been tested in experimental models of cerebral vasospasm [5,16,17]. Several randomized clinical trials tested the efficacy of ETA selective blockade and found a reduction in angiographic vasospasm but no improvement in measurable functional outcomes [18–22]. These results pose several questions: 1) the possibility that early brain injury occurring just after the hemorrhage may contribute to the poor outcome; 2) a functional interaction between ETA and ETB receptors named “cross talk” may play a role in the pathogenesis of SAH-induced vasospasm.

To overcome the above-mentioned methodological criticisms and to elucidate the functional interaction between the two receptors, we evaluated the upregulation of the endothelin system of the targeted vessels by vasospasm, incubating intact and denuded conductive cerebral vessels with CSF from SAH patients with a conclusive diagnosis of vasospasm obtained by angiography.

We hypothesized that CSF from SAH patients who developed vasospasm would produce an enhanced contractile response in intact rat cerebral arteries involving both ETA and ETB receptors.

Materials and Methods

Patient Selection

Ethical approval

The present study was conducted using CSF from patients with aneurysmal SAH confirmed by CT scan and angiography admitted to ICU. CSF had been collected for an observational study.
in SAH patients at risk of developing vasospasm (NCT01686763). The institutional review board (Comitato Etico Interaziendale A.O.U. San Giovanni Battista di Torino A.O. C.T.O./Maria Adelaide di Torino) approved the study. If the patient was unable to give consent at study entry, consent was delayed, and the family was informed of the study. Written permission for using collected data was thus obtained from the patient (if competent) or from the family (in case of death or if the patient remained incompetent).

All the patients fulfilled the following inclusion criteria: 1) angiographic proof of aneurysm; 2) admission within 24 hours of the SAH; and 3) presence of an intraventricular catheter placed either after admission or at the time of surgery. Patients were graded clinically according to the World Federation of Neurological Surgeons (WFNS) scale and classified according to the CT distribution of blood as described by the Fisher scale.

All patients underwent neurosurgical intervention or endovascular procedure to secure the aneurysm within 2 days of admission. Neurological status was evaluated daily using the Glasgow Coma Scale (GCS), transcranial Doppler of the middle cerebral arteries was performed daily until day 14 and patients were treated according to the guidelines [1].

On day 7 post-SAH, a second angiogram was routinely performed and the patients were then classified as having: 1) angiographic vasospasm (AV) if the angiogram showed 25% or more reduction from baseline caliber without any clinical deterioration; 2) clinical vasospasm (CV) if a reduction in the angiographic caliber >50% of the middle cerebral artery (MCA) was accompanied by controlateral weakness to or global neurological deterioration (two points reduction in GCS) occurring later than day 3 for anterior or diffuse vasospasm; 3) patients without vasospasm (NV) if there was reduction lower than 25% in the angiographic caliber [23] [24].

To test the hypothesis of the present study samples of CSF from patients with the most severe vasospasm (clinical vasospasm CV) and from those without vasospasm (no vasospasm NV) were included. As negative control a group of patients with normal pressure hydrocephalus (NPI) and normal GCS undergoing lumbar drainage for the diagnosis of NPI was included [25]. In SAH patients CSF was collected daily until day 7, with the samples collected on days 4–5 used for the present study. CSF was drawn into tubes containing EDTA from the intraventricular catheter and collected by ventricular drainage from hydrocephalus patients. Tubes were kept and transported in ice. All CSF samples were centrifuged at 1700g for 15 min at 4°C to remove intact cells and cellular debris, following which the supernatant was frozen at −80°C.

**Functional study of the Ex Vivo Model of SAH-induced Vasospasm**

Isolated rat basilar arteries were obtained from male Wistar rats (350–400g) (Charles River Laboratories, Calco, Italy). Rats were group-housed, had free access to standard chow and water in a controlled facility providing a 12-hour light/dark cycle, and were kept according to institutional animal welfare guidelines and legislation, submitted and approved by the local Animal Ethics Committee (Comitato di Bioetica dell’Ateneo dell’Università degli Studi di Torino) and all efforts were made to minimize suffering. Rats were sacrificed by carbon dioxide overdose followed by cervical dislocation and the cerebral basilar artery was isolated using a stereomicroscope and immersed in ice-cold physiological salt solution (PSS) (CaCl2 2H2O 2.5 mmol/L, NaCl 119 mmol/L, KCl 4.69 mmol/L, glucose 5.5 mmol/L, MgSO4 7H2O 1.17nmol/L, NaHCO3 25 mmol/L, KH2PO4 1.18 mmol/L and ethylenediaminetetraacetic (EDTA) 0.03 mmol/L). Segments of approximately 2 mm in length were incubated with 5% human CSF (v/v) obtained from ventricular drainage of the patients included in the study. Incubation lasted 24 hours at 37°C with 5% CO2 in O2 in Dulbecco’s modified Eagle’s medium (DMEM). The culture medium was then collected and frozen at −80°C for analysis of ET-1 concentrations.
Following incubation with human CSF, isolated rat basilar arteries were mounted on 40 μm tungsten wires in a small vessel myograph (D.M.T., Aarhus, Denmark) for isometric force recording [26]. Measurements were recorded using the ICU-Lab software program (KleisTEK Advanced Electronic Systems; Bari, Italy). The vessels were allowed to equilibrate for about 30 min in PSS, which was continuously aerated with oxygen enriched with 5% CO₂ to maintain a pH of 7.4. The relation between resting wall tension and internal circumference was determined, and the internal circumference, L₁₀₀, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel in situ was calculated. The vessels were set to the internal circumference L₁, given by \( L₁ = 0.9L₁₀₀ \). The effective internal lumen diameter was determined as \( l₁ = L₁/π \), and was 306±41 mm (mean±SD, N = 106) and 303±38 μm (mean±SD, N = 20) in vessels with and without endothelium respectively.

After a 30 min period to stabilize vessel tone, the contractile capacity was determined by exposing the vessels to an isotonic solution containing 123 mmol/l of K⁺ (KCl 123.70 mmol/L, MgSO₄ 7H₂O 1.17 mmol/L, KH₂PO₄ 1.18 mmol/L, CaCl₂ 2H₂O 2.5 mmol/L, NaHCO₃ 25 mmol/L, EDTA 0.03 mmol/L, glucose 5.5 mmol/L), obtained by equimolar change of NaCl for KCl in PSS. Only vessels responding by contraction corresponding to a pressure above 13.3 kPa to potassium were included in the study. The presence of an intact endothelium was checked by contracting the vessel using serotonin (5-HT, 10⁻⁵ mol/L, Sigma-Aldrich, Milan, Italy) and subsequently exposing the segments to histamine (10⁻⁵ mol/L, Sigma-Aldrich, Milan, Italy) to stimulate endothelium-dependent relaxation. Confirmation of the presence of endothelium was determined by a relaxation >50% (E⁺). In a separate set of experiments the endothelial cell layer was mechanically removed by gentle rubbing the intimal surface with a human hair (E⁻). After endothelial cells removal the contractility of the vessels was tested by adding serotonin.

We did not find any difference in serotonin response between groups with or without endothelial cells. The response to 5-HT was 1.59±0.56 (n = 6) and 1.43±0.48 mN/mm (n = 8), respectively, in vessels with and without endothelium exposed to CSF from the Control group, and 1.59±0.56 (n = 6) and 1.34±0.44 mN/mm (n = 6) in vessels with and without endothelium exposed to CSF from the Non-Vasospasm group, and 1.79±0.50 (n = 6) and 1.74±0.43 mN/mm (n = 6) in vessels with and without endothelium exposed to CSF from the Vasospasm group.

In a separate set of vessels, following exposure of the isolated basilar arteries to 5% human CSF, vessels were incubated with specific antagonists to 1) ETA (BQ-123, 1×10⁻⁵ mol/L, Sigma-Aldrich, Milan, Italy), 2) ETB (BQ-788, 1×10⁻⁶ mol/L, Sigma-Aldrich S.r.l, Milan, Italy) 3) both ETA and ETB (BQ-123 and BQ-788) 4) vehicle control for 30 min prior to performing concentration-response curves by cumulative application of ET-1.

**Enzyme-linked Immunosorbent Assay (ELISA)**

ET-1 concentrations in CSF and in culture medium obtained before and after the incubation of the arteries were determined by ELISA (Human ET-1 (1–21), with sensitivity <0.05 pg/ml; Biomedica Diagnostics Inc, Vienna, Austria).

**Immunofluorescence**

After the 24-hour incubation in CSF, basilar arteries were embedded in Tissue-Tek (Sakura, Zoeterwoude, NL) and frozen in liquid nitrogen. Cross-sections of the embedded vessels were then cut into 20 μm sections. The first antibodies used were rabbit anti-rat ETA (AB3260, Chemicon International, Vimodrone, Italy), diluted 1:200, and rabbit anti-rat ETB (AB3284,
Chemicon International, Vimodrone, Italy), diluted 1:200 or irrelevant isotype control antibody. All dilutions were performed in PBS with 5% bovine serum albumin (Sigma-Aldrich, Milan, Italy). The second antibodies used were goat anti-rabbit Alexafluor 488 conjugated (A11070, Invitrogen, San Giuliano Milanese, Italy), diluted 1:500 in PBS with 5% bovine serum albumin. The antibodies were detected at the appropriate wavelength using confocal microscopy (Leica TCS SP2, Mannheim, Germany). As control, only secondary antibodies were used. All samples were scanned in the same parameter setting throughout each series of immunoincubation, including same size of pin hole, gain level, black level, and laser power. The quantification analysis of immunoreactivity was carried out on single confocal image using Leica confocal software. As first step, background fluorescence was estimated by analysing the distribution of the pixel intensities in the image areas that did not contain any immunoreactive objects (the background threshold). The background was subtracted by setting the baseline of pixel intensities to the background value; autofluorescence was then subtracted. In the next step, an arbitrarily outlined polygon, which covered the vessel-occupied and imaged area, was chosen. In the polygon area, the relative area of immunolabelled pixels with an intensity value above the background was calculated. The fluorescence in five different areas on each artery was measured and the mean value for each vessel was used.

Statistical Analysis

Data analysis
All recorded data were analyzed and expressed as tension (mN/mm) developed by the artery, defined as the force induced divided by two times the vessel length because we did not find any differences on potassium induced contraction among groups. In a given experiment, E\textsubscript{max} denotes the maximal contractile response, and pEC\textsubscript{50} denotes the negative logarithm of the concentration that elicits one-half of the maximal response. For biphasic responses, E\textsubscript{max1} and pEC\textsubscript{50(1)} were used to describe the high-affinity phase and E\textsubscript{max2} and pEC\textsubscript{50(2)} were used to describe the low-affinity phase of the concentration-response curves.

Continuous data are presented as mean ± standard error of the mean. Data normally distributed were analyzed by one-way or two-way ANOVA, followed by a Student’s t-test or Tukey-Kramer post-hoc test where appropriate, and data not normally distributed were analyzed by a non-parametric Kruskal-Wallis with Dunn’s post-hoc test. Differences were considered significant when the probability value was <0.05. Statistical analysis was performed using SPSS-statistical software, version 17.0 (SPSS Inc. Chicago, IL).

Results

Inclusion criteria, demographic and clinical data of patients with subarachnoid hemorrhage included in the present study are reported in Table 1. The control group consisted of six patients (4 females, mean age 54±10) with diagnosed communicating hydrocephalus and normal GCS undergoing lumbar drainage.

Functional Analysis

Rat basilar arteries incubated with CSF from Vasospasm patients developed greater contractile forces at lower ET-1 concentrations compared to vessels incubated with Non-Vasospasm CSF, as shown in the representative myograph trace (Fig. 1).
In E⁺ vessels an enhanced concentration-dependent contractile response to ET-1 was present in the Vasospasm group when compared with the Non-Vasospasm and Control groups at ET-1 concentrations between $1 \times 10^{-12.5}$ and $1 \times 10^{-7}$ mol/L ($p < 0.05$) (Fig. 2 upper panel). Interpolation of the concentration-response curves showed a biphasic response in all three groups. Analysis of the $E_{\text{max}}$ and $pEC_{50}$ for each phase showed that the $E_{\text{max}}$, $E_{\text{max}}$, and $pEC_{50}$ were significantly higher in the Vasospasm group compared to the Non-Vasospasm or Control groups (Table 2).

In E⁻ vessels all the three groups exhibited a similar biphasic response (Table 2). The enhanced contractile response to ET-1 observed in arteries with endothelium and exposed to CSF from patients with vasospasm was absent in arteries without endothelium (Fig. 2 lower panel). ET-1 concentrations in CSF used for vessels incubation were $2.45 \pm 0.81$ pg/ml in Non-Vasospasm group and $2.63 \pm 1.31$ pg/ml in Vasospasm group. After dilution, ET-1 concentrations in the culture medium at the beginning of the incubation were undetectable in all groups (data not shown), but ET-1 concentration after 24 hours incubation was significantly higher in the Vasospasm (1.71 ± 0.4 pg/ml) compared to the Non-Vasospasm (0.82 ± 0.2 pg/ml) and Control groups (0.91 ± 0.04 pg/ml) ($p < 0.05$).

Endothelin Receptor Expression in the Ex Vivo Model of SAH-induced Vasospasm

There was a faint autofluorescence in the arterial segments examined by confocal microscopy. When autofluorescence was subtracted ETₐ receptors were found to be constitutively expressed in the smooth muscle cell layer of arteries exposed to CSF from all three patient groups (Fig. 3A). Semiquantitative immunofluorescence for ETₐ receptors revealed that the expression apparently was markedly enhanced in the smooth muscle cell layer comparing arteries...
incubated with CSF from patients with Vasospasm than in those incubated with CSF from Non-Vasospasm and Control patients (Fig. 3B) (260±9%; p < 0.001, results not shown).

Effects of Selective and combined Endothelin Receptor Blockade

In a separate set of experiments, the ET-1 concentration-response curve was obtained with and without blockade of the individual and combined ET_A and ET_B receptors (Table 3). In cerebral arteries incubated with CSF from Non-Vasospasm group pretreatment with ET_A antagonist (BQ-123) reduced the contractile response for ET-1 concentrations between $1 \times 10^{-11}$ to $1 \times 10^{-8}$ mol/L; pretreatment with ET_B antagonist (BQ-788) did not alter ET-1 induced contraction, while pretreatment with both ET_A and ET_B antagonists abolished the contractile response for ET-1 concentrations between $1 \times 10^{-11.5}$ to $1 \times 10^{-8}$ mol/L (Fig. 4, upper panel). In cerebral arteries incubated with CSF from the Vasospasm group pretreatment with ET_A antagonist (BQ-123) significantly reduced the contractile response in the high range of ET-1 concentrations.
 Pretreatment with ETB antagonist reduced the contractile response in the low range of ET-1 concentrations (between $1 \times 10^{-11.5}$ to $1 \times 10^{-7.5}$ mol/L), while pretreatment with both ETA and ETB antagonists completely abolished the ET-1 induced contraction (Fig. 4, lower panel).

![Figure 2. Concentration-response curves elicited by ET-1 in isolated rat basilar arteries with endothelium (E⁺) and without endothelium (E⁻) following incubation with 5% human CSF from Control (n = 6), Non-Vasospasm (n = 6) and Vasospasm (n = 6) patients (upper panel) and from Control (n = 8), Non-Vasospasm (n = 6) and Vasospasm (n = 6) patients (lower panel). The response to ET-1 was significantly increased in the Vasospasm group compared to Control and Non-Vasospasm groups only in intact vessels. Significance by Kruskal Wallis with Dunn’s post-hoc test where *$p$ < 0.05 = Vasospasm versus Control and Non-Vasospasm.

Table 2. Contractile response to ET-1 in rat basilar arteries with (E⁺) and without (E⁻) endothelium following incubation with human CSF.

|                | E⁺ | E⁻ |
|----------------|----|----|
|                | Ctrl (n = 8) | Non-Vasospasm (n = 6) | Vasospasm (n = 6) | Ctrl (n = 8) | Non-Vasospasm (n = 6) | Vasospasm (n = 6) |
| $E_{\text{max}1}$ ± SEM (mN/mm) | 1.04±0.13 | 0.86±0.11 | 2.10±0.28* | 0.81±0.08 | 0.83±0.15 | 0.92±0.07 |
| $E_{\text{max}2}$ ± SEM (mN/mm) | 2.27±0.15 | 2.09±0.15 | 3.38±0.12* | 1.82±0.11 | 1.83±0.17 | 2.03±0.13 |
| pEC$_{50(1)}$ ± SEM | 12.13±0.50 | 12.33±0.44 | 13.01±0.36 | 12.3±0.51 | 12.0±0.49 | 12.01±0.28 |
| pEC$_{50(2)}$ ± SEM | 9.19±0.34 | 8.50±0.27 | 10.26±0.37* | 8.69±0.22 | 8.47±0.34 | 8.23±0.24 |

Values are expressed as mean ± SEM. Significance was determined by one-way ANOVA with Tukey-Kramer post-hoc where; ctrl = control

* $p$ < 0.05 = Vasospasm versus Control and Non-Vasospasm.

doi:10.1371/journal.pone.0116456.t002
Discussion

This study for the first time clearly describes the functional response, protein expression, and effects of selective and combined blockade of both ETA and ETB receptors in two well-defined conditions: intact conductive cerebral vessels exposed to the biological effects of the hemorrhagic event (SAH without vasospasm) and vessels exposed to the combined biological effects of SAH and vasospasm. When CSF from patients with SAH and vasospasm was used both receptors were present and mediated the vasoconstriction; therefore combined blockade of both receptors led to the maximal inhibition of the ET-1 induced contraction. The enhanced endothelin contraction was absent in vessels without endothelium suggesting that an endothelium-dependent mechanism contribute to the enhanced contractile endothelin-1 response observed in arteries conditioned by CSF from SAH patients with vasospasm.

Upregulation of the endothelin system after incubation with CSF from patients with vasospasm

In most functional studies on isolated vessels the two conditions of SAH, with and without vasospasm were not differentiated [6,9,10,12]. Only one study with human CSF clearly differentiated the two SAH conditions but in that study the role of endothelin-1 was not investigated [27]. The main novelty of the present study is the selection of human CSF with a definitive diagnosis of vasospasm established by angiography and integrated with TCD and clinical examination to investigate the endothelin-1 pathway in a model of isolated cerebral vessels. Interestingly, we found that intact vessels incubated with CSF from SAH patients with vasospasm resulted in a greater contractile response to ET-1 demonstrated by the left shift of the
concentration response curve with higher $E_{\text{max}}$ and $pE_{\text{C50}}$ (i.e. increased potency and sensitivity to ET-1) when compared to vessels incubated with CSF from non-vasospasm and control patients. The increased sensitivity to ET-1 induced by organ culture was presumably present in all our different experimental groups and therefore equally influenced all our results. In our study cerebral arteries were incubated with Dulbecco medium containing 5% human CSF. Although ET-1 was detectable in all CSF samples of the patients studied, we applied a dilution factor which brought ET-1 concentrations below the detectable range in both groups. Therefore, the amount of ET-1 measured in the culture medium was the result of the stimulation of the arterial segments by CSF from the patients. The choice of CSF from day 4–5 for the present study is explained by the fact that we verified that patients with severe angiographic vasospasm on day 7 (clinical vasospasm) had a significant increase in MCA velocity on TCD on day 4–5 suggesting that molecular mechanisms responsible for vasospasm were already activated at that time. Among the soluble factors derived from the blood, oxidative stress [28], increased coagulation, fibrinolysis cascade, arachidonic acid metabolites and inflammatory mediators [29] may be involved. Recently it has been suggested that SAH induces early activation of the MEK-ERK1/2 pathway in cerebral artery walls, which is associated with upregulation of proinflammatory cytokines and MMP-9 [30].

The vessels incubated with CSF from patients with SAH and vasospasm had higher secreted ET-1 levels suggesting that soluble factors present in the CSF of this group of patients were able to trigger greater ET-1 secretion compared to CSF from patients without vasospasm. It is a limitation that only CSF obtained at day 4–5 were examined, but on the other hand that was where the vasospasm was most pronounced. Moreover, it would be an advantage if secretion and “de novo” production of ET-1 could have been discriminated by measurements of mRNA levels.

### Table 3. Contractile response to ET-1 of intact rat basilar arteries following incubation with human CSF and ETA antagonist (BQ-123), ETB antagonist (BQ-788) and combined ETA and ETB antagonists (BQ-123 and BQ-788).

|                  | Biphasic Curves | Sigmoidal Curves |
|------------------|-----------------|------------------|
|                  | $E_{\text{max1}} \pm \text{SEM}$ (nN/mm) | $E_{\text{max2}} \pm \text{SEM}$ (nN/mm) | $pE_{\text{C50(1)}} \pm \text{SEM}$ | $pE_{\text{C50(2)}} \pm \text{SEM}$ | $E_{\text{max}} \pm \text{SEM}$ (nN/mm) | $pE_{\text{C50}} \pm \text{SEM}$ |
| Ctrl Vehicle (n = 7) | 0.79±0.26      | 1.52±1.18       | 13.13±0.71       | 8.67±0.54       | 0.65±0.07               | 14.57±3.40               |
| BQ-123 (n = 8)     | 0.65±0.07       | 14.57±3.40      | 9.74±0.19        | 7.67±0.52       |
| BQ-788 (n = 7)     | 2.17±0.13       | 9.74±0.19       | 7.67±0.52       |
| BQ-123 + BQ-788 (n = 5) | 0.26±0.08  | 7.67±0.52       |
| NVS Vehicle (n = 8) | 1.00±0.09       | 1.65±0.07       | 12.40±0.29       | 9.35±0.32       | 0.88±0.32               | 7.42±0.42               |
| BQ-123 (n = 8)     | 0.53±0.19†      | 1.56±0.51       | 12.49±0.62       | 7.39±0.53†      | 0.88±0.32               | 7.42±0.42               |
| BQ-788 (n = 8)     | 1.02±0.08       | 2.06±0.21       | 11.36±0.35       | 8.79±0.29       |
| BQ-123 + BQ-788 (n = 5) | 0.61±0.27 | 7.31±0.23       |
| VS Vehicle (n = 9) | 1.61±0.32*      | 2.29±0.16*      | 13.03±0.54       | 9.22±0.64       | 0.61±0.27               | 7.31±0.23               |
| BQ-123 (n = 9)     | 0.86±0.43†      | 1.91±0.82       | 12.81±0.54       | 7.29±0.78†      | 0.61±0.27               | 7.31±0.23               |
| BQ-788 (n = 9)     | 1.16±0.10†      | 2.17±0.10       | 11.83±0.33       | 9.16±0.31       |
| BQ-123 + BQ-788 (n = 5) | 0.61±0.27 | 7.31±0.23       |

Responses were characterized by $E_{\text{max}}$ and $pE_{\text{C50}}$ values (negative logarithm of the molar concentration that produced half-maximum contraction). NVS = non vasospasm, VS = vasospasm. Values are expressed as mean ± SEM. Significance was determined by a one-way ANOVA with a Tukey-Kramer post-hoc analysis between CSF, where $^* p<0.05$ = Vasospasm versus Control and Non-Vasospasm, and by a Student’s t-test between antagonists and vehicle, where $^†p<0.05$ = BQ-123 versus Vehicle.

doi:10.1371/journal.pone.0116456.t003
Involvement of ETA and ETB receptors after incubation with CSF from patients with vasospasm

In physiological conditions the biological effects of ET-1 are mediated through activation of the two ET receptors: ETA which induces vasoconstriction on smooth muscle cells, whereas the endothelial ETB mediates vasodilation [31]; consequently endothelium removal leads to the abolishment of the vasodilator modulation. After SAH a different endothelium dependent effect of ET-1 may be expected and the upregulation of vascular smooth muscle ETB receptor may contribute to vasoconstriction [32]. Indeed a functional interaction between ETA and ETB also named “cross talk” has been recently proposed to explain the limited efficacy of selective ET receptors antagonists [33].

Figure 4. Concentration-response curves for ET-1 after ETA (BQ-123), ETB (BQ-788) or both receptors blockade on isolated rat basilar arteries following incubation with 5% CSF from Non-Vasospasm (panel A) and Vasospasm (panel B) patients. In the Non-Vasospasm group, the response to ET-1 was significantly reduced by the ETA antagonist (n = 8), was not altered by the ETB (n = 8) antagonist and greatly reduced by both antagonists (n = 5) compared to Vehicle (n = 8), whereas in the Vasospasm group, the increased sensitivity to ET-1 was significantly reduced by the ETB (n = 9) antagonist in the low range, by the ETA antagonist (n = 9) in the high range of ET-1 concentrations and was completely abolished by combined receptors blockade (n = 5) compared to Vehicle (n = 9). Significance at each concentration by a two-way ANOVA followed by a one-way ANOVA with Dunnett’s post-hoc analysis between groups, where *p < 0.05 = ETA antagonist versus vehicle, †p < 0.05 = ETB antagonist versus vehicle, #p < 0.05 = ETA and ETB antagonists versus vehicle.

doi:10.1371/journal.pone.0116456.g004
ET_\text{A} receptors have previously been suggested as the main responsible for the pathogenesis of vasospasm. Thus, after SAH, ET_\text{A} receptor expression on smooth muscle cells was unchanged but blockade of ET_\text{A} receptor with clazosentan reduced the contraction to ET-1 [16]. Another two studies in SAH animal models showed upregulation of mRNA expression of the ET_\text{A} receptor after SAH [5,6]. In the present study, ET_\text{A} receptors were expressed in the smooth muscle layer of arteries exposed to CSF from all three groups of patients. These findings together with the increased functional endothelin-1 response in arteries exposed to CSF from patients with vasospasm suggest that antagonism of ET_\text{A} receptors would be attractive. However, ET_\text{B} receptors have also been suggested to play a role in the pathogenesis of vasospasm [10,34,35]. In different models of SAH increased levels of ET_\text{B} receptor mRNA with associated enhanced functional response to ET-1 have been shown [8,11]; in a rat model of SAH, upregulation of ET_\text{B} receptor, specific functional response, and mRNA and protein expression were reported [9]. Selective decrease in ET_\text{B} receptor-dependent relaxation [36] and reduced vasospasm after specific ET_\text{B} receptor antagonist [17] have also been reported. In the present study, we found that the ET_\text{B} receptor was not constitutively expressed in smooth muscle cells in control conditions and, more importantly, when using CSF from SAH patients without vasospasm but only CSF from SAH patients with vasospasm induced an apparently marked expression of ET_\text{B} receptors in the vascular smooth muscle cell layer.

Finally, we evaluated a) the functional response of intact vessels after selective and combined blockade of ET_\text{A} and ET_\text{B} receptors and b) the functional response of intact and denuded isolated vessels to CSF from SAH patients with and without vasospasm. In the non vasospasm group, only selective ET_\text{A} blockade induced a dose-dependent right shift of the concentration-response curve. This is explained by the fact that ET_\text{A} is constitutively expressed in control conditions; the hemorrhagic event on its own does not cause upregulation of ET_\text{B} receptors so that ET_\text{B} blockade had no effect. In the vasospasm group, both receptor types were upregulated. ET_\text{B} expression on smooth muscle cells was associated with increased tone and consequently antagonism of both receptors induced a right shift of the ET-1 concentration-response curves: the effect of ET_\text{B} blockade was significant at low ET-1 concentrations (high affinity phase) whereas the effect of ET_\text{A} blockade was significant at high ET-1 concentrations (low affinity phase). After blocking both receptors the increased tone was abolished throughout the ET-1 concentration range suggesting the involvement of both receptors in the enhanced contractile response to ET-1. Furthermore the contractile response elicited by incubation with CSF from patients with vasospasm was abolished in vessels without endothelium suggesting that the condition of vasospasm after SAH induced an alteration in endothelial function contributing to the enhanced tone. These results may be explained by 1) ET-1 secreted by endothelial cells in the culture medium contributes to vasoconstriction [37]; 2) the existence of ET_\text{A} receptor in the spastic vessels [7]; 3) the dual "vasodilator-vasoconstrictor" role of ET_\text{B} receptors as described in pulmonary vessels [38,39]; 4) the heterodimerization of ET receptors described in pulmonary hypertension [38]. The latter hypothesis may explain why combination of selective ET_\text{A} and ET_\text{B} antagonists resulted in near maximal inhibition of ET-1 induced vasoconstriction.

Conclusion and Perspectives

What additional information does the present translational study provide after several clinical trials [18–20,22,40] with endothelin receptor antagonists which showed reduction in angiographic vasospasm but no improvement in measureable functional outcomes?

In a recent metanalysis Vergouwen et al [41] suggested that endothelin receptor antagonists might be effective in selective subgroups of patients. In this perspective our data indeed suggest
that only a well-defined subgroup of patients who develop severe vasospasm induced expression of both receptors mediating vasoconstriction and therefore specific targeting ETA receptors was not sufficient to maximally inhibit vasospasm. The limited efficacy of selective ET receptor antagonists may be due to the functional interaction between ETA and ETB receptors named “cross talk”.

The “cross talk” between the two receptors can be functionally interpreted as a cooperative inhibition by combined selective ET receptor blockade of the ET-1 induced contraction [33,42]. In line with this hypothesis in the present study, only blockade of both receptors induced an optimal reduction in the contractile response to ET-1.

In conclusion we studied the functional response, protein expression, and the effects of selective and combined blockade of both receptors in two well-defined conditions: intact conductive cerebral vessels exposed to the biological effects of the hemorrhagic event (SAH without vasospasm) and vessels exposed to the combined biological effects of SAH and vasospasm. When CSF from patients with SAH and vasospasm was used both receptors were upregulated and mediated vasoconstriction; therefore combined blockade of both receptors led to a maximal inhibition of ET-1-induced contraction. This enhanced ET-1 contraction was abolished in vessels without endothelium suggesting that the condition of vasospasm after SAH induced an alteration of endothelial function contributing to the enhanced tone. Further studies are required to demonstrate the exact nature of the functional interaction or “cross talk” between the two receptors [33].

Acknowledgments

The authors thank Prof. Alessandro Vercelli as animal facility’s supervisor, Dr Federico Sizzano as expert in confocal microscopy, Dr Davide Flamini for his help in interpolation analysis, Flavio Cristofani as and Valeria Puntorieri from Departement of Anatomy, University of Turin, Italy for technical assistance, animal care support and constructive discussion.

Author Contributions

Conceived and designed the experiments: BA MF AD US LM. Performed the experiments: BA ELM FC. Analyzed the data: BA ELM ES FC. Contributed reagents/materials/analysis tools: MF MB RB. Wrote the paper: BA ELM ES ATM US LM.

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