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Effect of permanent middle cerebral artery occlusion on Cytoglobin expression in the mouse brain

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ABSTRACT

Cytoglobin, a new member of the mammalian heme-globin family has been shown to bind oxygen and to have cell protective properties *in vitro*. Cytoglobin is specifically expressed in a subpopulation of brain neurons. Based on hypoxia-induced up regulation and proposed scavenging of reactive oxygen species Cytoglobin was suggested as a candidate for pharmaceutical stroke treatment. Since production of reactive oxygen species is a hallmark of ischemia, we hypothesized that Cytoglobin expression would be increased and that Cytoglobin expressing neurons would be spared after ischemic injury. Twenty male C57BL/6J mice were used in the experimental design. Ten were sham operated and ten were given permanent middle cerebral artery occlusion (pMCAo). All animals were euthanized after 24 h. From each group, three animals were used for histology and seven for QRT-PCR and western blotting. Immunohistochemical examination of the ischemic penumbra revealed neither changes in Cytoglobin immunoreactivity nor any changes in expression in the necrotic infarct area. The lack of expression change was confirmed by western blotting and QRT-PCR showing no significant difference between sham and pMCAo operated mice. This suggests that Cytoglobin is likely not important for global neuronal protection following ischemia and the role of Cytoglobin in relation to endogenous neuroprotection remains unresolved.

1. Introduction

Stroke is a tremendous burden on society being one of the most common causes of mortality and morbidity and leading to a vast socioeconomic cost [1,2]. Despite an enormous effort, there are no effective pharmaceutical stroke treatments and new therapeutic avenues should therefore be considered [3]. Recently an increasing interest in using up regulation of endogenously expressed proteins as potential targets for stroke treatment has emerged. Of these potential proteins are Neuroglobin (Ngb) and Cytoglobin (Cygb), two recently discovered neuronal expressed heme-globins, which both have been proposed to serve as therapeutic targets in stroke and neurodegenerative disorders [4–7]. Ngb and Cygb have, in spite of low sequence similarity with hemoglobin and myoglobin, retained the classic globin fold and can reversibly bind oxygen with an affinity in the range of myoglobin [8–11]. While many studies have focused on Ngb and its possible function in neuroprotection (for a review see [12]), the neuroprotective effects of Cygb have been less well studied. Cygb was reported to be up regulated by hypoxia both *in vitro* and *in vivo* [13–18] where its been shown to scavenge reactive oxygen species (ROS) and nitric oxide [13,14,19–21] and can protect cells from ischemic death when over-expressed *in vitro* [22–24]. Because Cygb is expressed in a subpopulation of brain neurons [16,18,25,26] and given it is suggested cell protective properties, examining Cygb expression and Cygb expressing neurons after *in vivo* ischemic stroke will give valuable insight into Cygb's potential as an endogenous neuroprotective target. No studies have to our knowledge investigated Cygb expression and endogenous neuroprotective properties in an *in vivo* model of brain ischemia. We have therefore used a mouse model of permanent middle artery occlusion (pMCAo) to test the hypothesis that Cygb expression is up regulated after ischemic injury and therefore might serve a neuroprotective role.

2. Material and methods

Animal care and all experimental procedures were conducted in accordance with Danish Ministry of Justice. The Danish National

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Committee for Ethics in Animal Research approved the experimental protocol in accordance with the European Community Councils Directive of November 24th, 1986 (86/609/EEC).

Animals were housed at the animal facility center, in the Panum Institute, University of Copenhagen with a 12:12 h light:dark cycle (lights on at 6 a.m.; lights off at 6 p.m.). Daily routines were carried out between 7 a.m. and 4 p.m. by authorized personnel. Standard laboratory chow and water were provided ad libitum, as well as soaked standard laboratory chow and nutritional gel postoperative.

2.1. Study design

Male C57BL/6 (8 weeks old) were randomized to one of the following two groups: I. pMCAo ($n = 10$) and II. Uninjured (sham) ($n = 10$). All animals were euthanized after 24 h. pMCAo ($n = 3$) and sham ($n = 3$) were used for immunohistochemistry (IHC). For QRT-PCR and western blotting, pMCAo ($n = 7$) and sham ($n = 7$) were used.

2.2. Anesthesia and surgical procedure

Anesthesia was induced by inhalation of 8% sevoflurane (Abbott Laboratories, Inc.). Loss of the pedal reflex was used as an index of onset of surgical anesthesia. The animals were connected to a mouse ventilator (Minivent type 845 Hugo-Sachs Elektronik Harvard Apparatus GmbH, Germany) and a Capnograph (Type 340 Hugo-Sachs Elektronik Harvard Apparatus GmbH, Germany). Anesthesia was maintained with a mixture of 5.5% sevoflurane and medical air (delivered by AGA, Denmark) at a rate of 120–130 breaths/min and an inspiratory volume of 200 μL. By making a standard curve that correlates arterial partial CO_2 pressure with the $ETCO_2$. The ventilator was adjusted according to a standard curve where p_{aCO2} correlated to $ETCO_2$. The core body temperature was kept at around 37 °C using a feed-back system with a temperature-controlled heating pad coupled to a rectal probe (Small homeothermic Blanket Control Unit, Hugo-Sachs Elektronik Harvard Apparatus GmbH, Germany).

Before surgery all animals received an intramuscular injection of 5 μg/100 g bodyweight of atropine (Atropinsulfat 1 mg/ml, Denmark) to reduce mucus production. Bupivacain (Bupivacain SAD 5 mg/ml, Denmark) and Lidocaine (SAD 5 mg/ml) mixed 1:1 was injected subcutaneously at the incision sites to ease postoperative pain.

A skin incision between the lateral part of the orbit and the external auditory meatus was made. A burr-hole was drilled directly over the distal part of the MCA, the dura mater was removed, and the MCA was coagulated by applying bipolar forceps coupled to an electrosurgical unit (ERBE VIO 100 C Mediplast NCN-ielsen A/S, Denmark) [27]. The animals received a subcutaneous injection of 1.5 ml saline 37 °C to prevent postoperative dehydration, and recovered in heated cages. Sham operated animals were subjected to the exact same procedure except coagulation of the MCA.

2.3. Immunohistochemistry

Sham ($n = 3$) and pMCAo injured ($n = 3$) mice were perfusion fixed with 4% paraformaldehyde and brains were removed and postfixed in the same fixative overnight. Following fixation the brains were cryoprotected in 30% sucrose and cut in 40 μm sections in series of four.

For free floating IHC, sections were incubated with polyclonal rabbit anti-Cygb (in house, code# 5092/7, diluted 1:30.000) overnight at 4 °C. The primary antibody was detected by a donkey anti-rabbit $F(ab)_2$ secondary antibody (code# 711–066-152 Jackson Immunoresearch Laboratories, Baltimore, PA, USA, diluted 1:2000) in combination with Avidin–Biotin-peroxidase Complex (ABC) (VWR international, Roedovre Denmark) and visualized with 0.05% diaminobenzidine. Sections were counterstained with Mayer's haematoxylin (Mayer from Th. Geyer Denmark ApS) for 30 s. The expression pattern of the rabbit anti-Cygb antibody was validated using in situ hybridization and a guinea pig anti-Cygb polyclonal antibody (in house, code# 12168/7) showing the identical staining pattern (data not shown).

2.4. Western blotting

Brains from sham ($n = 7$) and pMCAo ($n = 7$) C57BL6/J male mice were removed 24 h after sham or pMCAo operation. The brains were divided in the two hemispheres and snap frozen on dry ice and stored at −80. Protein and RNA from the pMCAo injured or sham hemisphere were extracted using the PARIS kit (code# MA1921, Invitrogen, Carlsbad, CA, USA) supplemented with 1% Halt Phosphatase Inhibitor Cocktail (Pierce, Rockford, IL, USA) and protease inhibitors (code# P8340, Sigma Aldrich, Brondby, Denmark) according to the manufacturer's instructions.

All reagents and equipment used for electrophoresis and transfer of proteins were used according to manufacturers' instructions regarding the NuPAGE® system (Invitrogen). Frozen brain lysate was briefly thawed on ice, followed by the addition of equal amounts of 2 × SDS sample buffer (100 mM Tris (pH 6,8), 8% SDS, 24% glycerol, 80 mM HCl and 25% Coomasie brilliant blue) freshly supplemented with 1 × NuPAGE Reducing agent (Invitrogen, Carlsbad, CA, USA) and incubated for 10 min at 70 °C. Equal amounts (20 μL) of each sample (sham, pMCAo) were loaded on a 4–20% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and immunoblotted for Cygb as described below. Immunoblotting was conducted over night at 4 °C with polyclonal rabbit anti-Cygb (code# 5092/7, in house diluted 1:5000) and rabbit anti-beta-actin (code# 4790, Cell Signaling, Danvers, MA, USA diluted 1:5000) as the loading control. Immunoreactivity was detected with swine anti-rabbit IgG (code# P0399, Dako, Glostrup, Denmark, diluted 1:2500) horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized with enhanced chemiluminescence according to the manufacturer's protocol (Western Lightning Plus-ECL, PerkinElmer, Waltham, MA, USA). The experiments were performed in duplicates. Validation of the rabbit anti-Cygb polyclonal antibody was conducted using a polyclonal guinea pig anti-Cygb antibody (in house code# 12168/7) both detecting Cygb at an identical molecular weight of approximately 22 kDa (data not shown).

2.5. Quantitative real time PCR

500 ng of total RNA was reverse transcribed using Superscript III Reverse Transcriptase kit according to the manufacture's guidelines (Invitrogen, Carlsbad, CA, USA). cDNA was diluted 2-fold and expression of target transcripts were quantified using the following Taqman (Applied Biosystems) assays: Cygb (Mn00446071_m1) and actin beta (Mn01205647_g1). Cygb expression was normalized to actin beta using the delta-delta CT method [28]. The experiments were performed in replicas of three.

2.6. Statistics

All data were analyzed using GraphPad Prism software. Infarct effects on mRNA and protein levels were tested with a Mann–Whitney two-tailed test and $p < 0.05$ was considered statistically significant.

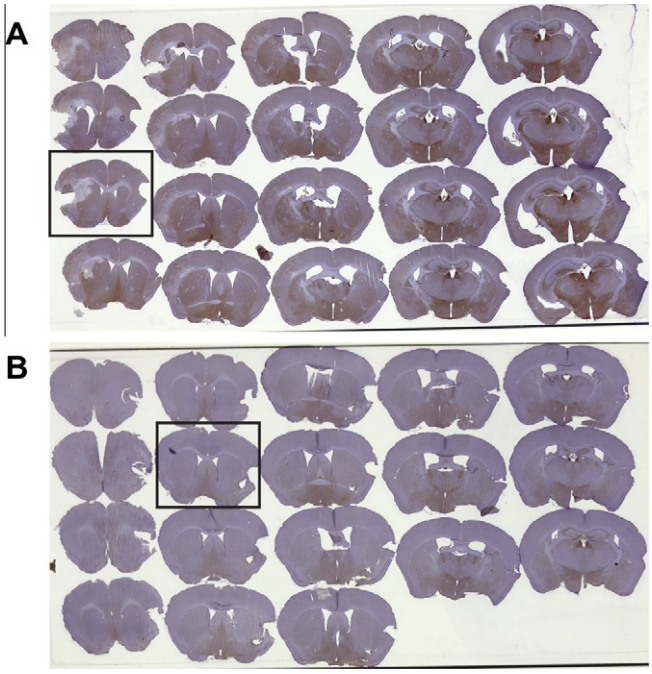

Fig. 1. Overview of Cygb-immunoreactivity (IR) (brown) following pMCAo ischemia. (A) showing a brain from a pMCAo and (B) a sham operated mice counterstained with Mayer's haematoxylin. The sections within the black squares are shown in higher magnification in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Histology

To answer if Cygb is differentially expressed after pMCAo, we used IHC to investigate Cygb-immunoreactivity (IR) in the penumbra and necrotic infarct areas. Cygb-IR in the cortex and striatum was observed in small and large sized neurons (Figs. 1 and 2). Following pMCAo no visible changes in Cygb-IR could be detected in the penumbra and no viable Cygb-IR neurons were detected in the damaged necrotic area (Fig. 2).

3.2. Quantitative measurements of Cygb mRNA and protein expression

QRT-PCR and western blotting was used to determine the effect of pMCAo on Cygb mRNA and protein expression levels. There were no significant difference in either Cygb mRNA or protein levels between sham and pMCAo operated mice (Fig. 3A and B).

4. Discussion

The present study was initiated to give the first evaluation of Cygb expression regulation following ischemic stroke using an *in vivo* mouse model. The main results from this study are the lack of *in vivo* regulation of Cygb expression and no apparent selective sparing of Cygb-IR neurons following pMCAo ischemia. If we accept the premise that there is causal relation between up regulation of Cygb and cellular protection [16], it can be inferred from the lack of Cygb regulation on the transcriptional and translational level reported here that it is questionable whether Cygb functions as a protective protein following ischemic stroke *in vivo*. This conclusion is further corroborated by the apparent lack of selective sparing of Cygb expressing neurons judged from our IHC observations. The scattered anatomical distribution of Cygb-IR with relative low levels in the cortex and striatum reported in this and others studies [18,25] and with the highest levels found in the hindbrain [25] is also not supportive for a major role in protection against ischemic injury unless the specific Cygb expressing neurons, for some unknown reason, demand more protection. *In vivo* studies have found hypoxia to up regulate Cygb expression. An explanation for the differences in Cygb regulation may relate to dissimilarities in the pathogenesis of hypoxia and ischemia in terms of oxygen availability and cell survival. In the case of hypoxia only oxygen levels are reduced while nutrient supply is unchanged whereas in ischemia

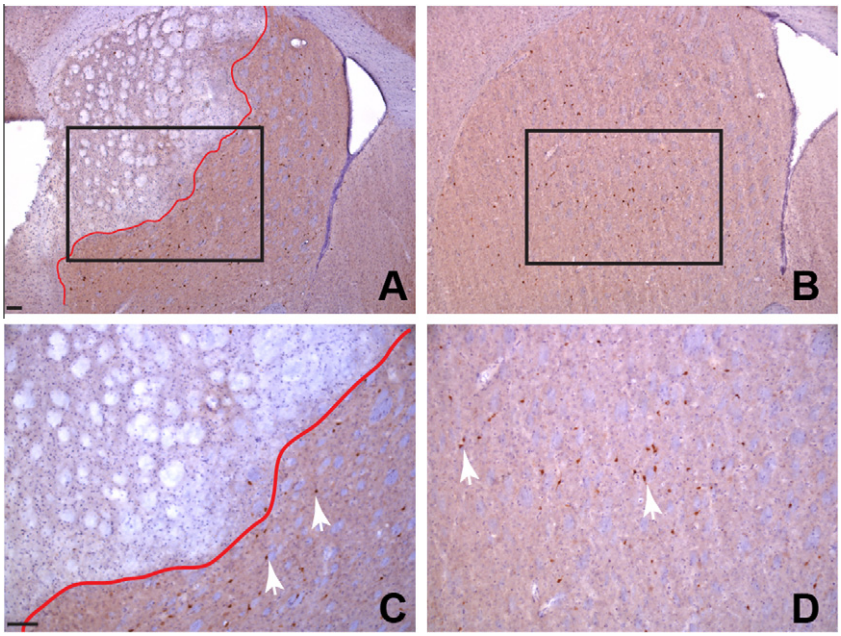

Fig. 2. Cygb-IR in striatum and cortical areas. In (A) pMCAo and (B) sham operated mouse brain is shown. The penumbra area (black square) of the pMCAo mouse and corresponding area of the sham mouse (black square) is shown in higher magnification in C and D, respectively. The red line denotes the penumbra area where to the left nuclei from necrotic cells can be seen. No visible up regulation of Cygb-IR can be seen in the penumbra and no viable Cygb-IR neurons can be seen in the necrotic left side of the red line. White arrows indicate representative Cygb-IR neurons. Scale bar 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

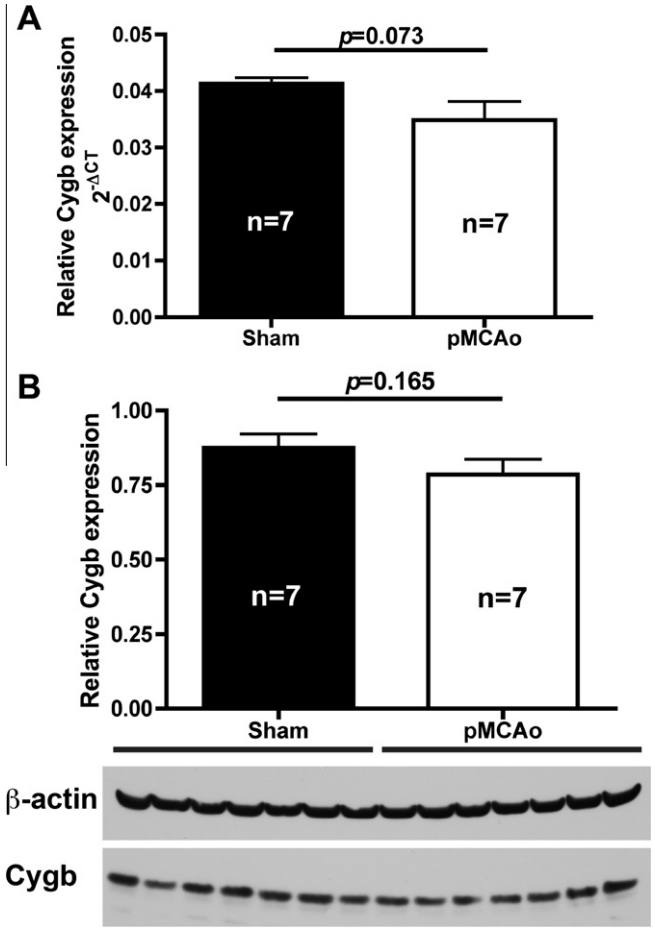

Fig. 3. QRT-PCR and Western blot analysis of Cygb expression in the mouse brain after ischemia. A shows the regulation of Cygb mRNA in sham (black) (SE ± 0.001) and pMCAo (white) (SE ± 0.003) operated mice. Cygb expression was normalized to ß-actin. No difference was observed between the groups. B top panel Cygb protein expression in sham (black) (SE ± 0.046) and pMCAo (white) (SE ± 0.051) operated mice. There was no significant difference between the two groups. Bottom panel the corresponding western blot showing ß-actin (loading control) and Cygb protein.

the situation is more complex. Here a reduction in both nutrient supply and oxygen levels leads to greater cell death in most areas except the penumbra where viable tissue is preserved. This difference in pathogenesis may confound a direct comparison between hypoxia and ischemic Cygb regulation. Since we investigated Cygb expression 24 h after the onset of ischemia, we cannot exclude that Cygb up regulation may have occurred at an earlier time point where more cells were still viable. However, in an *in vitro* ischemia study no change in Cygb mRNA or protein expression was found after oxygen and glucose deprivation (OGD) over a time course of 8–32 h [22]. The lack of Cygb regulation in the OGD model is in line with our observations and is further supported by lack of change in Cygb-IR observed in the penumbra. These results suggest that Cygb expression is not affected by ischemia over a broad time span. It therefore seems that the *in vivo* regulation of Cygb reported previously is related to low oxygen levels rather than cellular stress *per se*, which is in line with hypoxia response elements observed in the Cygb promotor region [29]. In contrast to the present results, showing no selective sparing of Cygb-IR neurons, ischemia *in vitro* studies showed that Cygb over expression conferred protection against anoxic and ROS mediated cell death and decreasing Cygb expression increased cell death [22–24]. Since we did not manipulate the levels of Cygb, it cannot be excluded that *in vivo* up- or down regulation of Cygb could have resulted in a decrease or increase in the ischemic infarct in line with the *in vitro* studies. However, there are many confounding factors, which are difficult to account for when comparing *in vitro* and *in vivo* studies and this challenges the conclusions drawn from an *in vitro* setting about the *in vivo* function of Cygb.

This study demonstrated that Cygb on the transcriptional and translational level is not regulated by pMCAo ischemia, and endogenous Cygb expression apparently confers neurons no protection against ischemic cell death. We argue that Cygb's *in vivo* role in neuronal protection is still contentious and more research is needed before Cygb conclusively can be linked to cellular protection *in vivo*.

5. Author contributions

Initiated and planed the study: ZR, AHS, CAH. Performed and planed the pMCAo experiments: ZR. Performed the IHC, WB and QRT-PCR: RR, CAH. Analyzed the data: ZR, CAH. Wrote the paper: ZR, CAH. Contributed reagents/material/equipment: ZR, AHS, CAH. Approved the final manuscript: ZR, RR, AHS, CAH.

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