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In vitro evidence of the role of hemoglobin during vasospasm on the modifications of the expression of PKCα and ζ

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Received May 30, 2007; Accepted July 2, 2007

Abstract. The cellular events leading to cerebral vasospasm after subarachnoid hemorrhage (SAH) are poorly understood, although the family of protein kinase C (PKC) is already known to play crucial roles in this pathology. Hemoglobin (Hb) is one of the major causes of the cerebral vasospasm that follows SAH. In the present study we investigated whether Hb can *in vitro* regulate PKC expression in endothelial as opposed to smooth-muscle cells. The levels of expression of PKCα and PKCζ were quantitatively determined by means of computer-assisted fluorescence microscopy in the A7r5 smooth-muscle rat cells and human umbilical endothelial cells (HUVECs). Hb significantly modified both calcium-dependent PKCα and calcium-independent PKCζ expression in HUVECs and A7r5 smooth-muscle rat cells. Our data showed that, *in vitro*, Hb promptly and markedly modified the levels of expression of both calcium-dependent PKCα and calcium-independent PKCζ. We are currently investigating the effects of specific PKC antagonists associated or not with calcium channel blockers on the expression of PKC and the *in vivo* severity of SAH-induced vasospasm. Our results encourage the prophylactic use of specific PKC isoform antagonists associated with calcium channel blockers early after SAH to prevent cerebral vasospasm.

Introduction

Subarachnoid hemorrhage (SAH) is a unique disorder and a major clinical problem that most commonly occurs when an aneurysm in a cerebral artery ruptures and causes bleeding and clot formation (1). Despite advances in the treatment of aneurysmal SAH, cerebral vasospasm remains a common complication and has been correlated with a 1.5- to 3-fold increase in death during the first 2 weeks after hemorrhage (2). A number of medical, pharmacological, and surgical therapies are currently in use or being investigated in an attempt to reverse cerebral vasospasm, but only a few have proven to be useful (2).

Two major mechanisms concur to cause cerebral vasospasm after SAH, i.e. first, activation of calcium-calmodulin-dependent protein kinases, and second, the increase in protein kinase C (PKC) (3-11). Whereas the first mechanism leads to a transient contraction, most of the experimental results *in vitro* or in animal models support the view that activation of PKC in cerebral vasospasm results in a delayed but prolonged contraction of major arteries after SAH (9-11). We have recently shown that SAH-induced vasospasm in rat basilar artery markedly modifies the levels of expression of PKCα and PKCζ, and, to a lesser extent, of PKCß and PKCη in endothelial and smooth-muscle cells (12). While PKCα and PKCß are Ca^{2+}-dependent, PKCη and PKCζ are calcium-independent (13,14).

The PKC family consists of at least 12 isoforms with clear differences in structure, substrate requirements, expression and locus (13,14). Differences in their structures and substrate requirements have enabled them to be divided into three groups, namely i) the conventional PKCs (cPKC)α ßI, ßII and γ, which are Ca^{2+}-dependent and activated by both phosphatidylserine (PS) and the second messenger diacylglycerol (DAG); ii) the novel PKCs (nPKC)δ, ε, η and θ, which are Ca^{2+}-independent and regulated by PS and DAG; and iii) the atypical PKCs (aPKC)ζ and ι/λ, which are Ca^{2+}-independent and do not require DAG for activation even though PS does regulate their activity (13,14).

Oxyhemoglobin (oHb) is also suspected of being one of the causes of the cerebral vasospasm that follows SAH (15-18). This indicates that endothelin-1 (ET-1), which is a potent vasoconstrictor, can be induced with Hb in both vascular endothelial and smooth-muscle cells through PKC activation (19). In cerebrovascular smooth muscle, oHb induced Rho and both PKCα and PKCε translocation (15), and a threshold concentration of ET-1 potentiated the contraction induced by oHb via RhoA/Rho kinase- and PKCε-dependent mechanisms (20). Moreover, in a canine two-hemorrhage model, PKC and Rho kinase proteins

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Key words: in vitro vasospasm, human umbilical endothelial cells, A7r5 smooth-muscle cells, protein kinase C, computer-assisted fluorescence microscopy, hemoglobin

interacted in the development of cerebral vasospasm (21). Therefore, we investigated *in vitro* the influence of hemoglobin on the calcium-dependent PKCα and the calcium-independent PKCζ levels of expression in A7r5 smooth-muscle rat cells and human umbilical endothelial cells (HUVECs).

Materials and methods

Cell lines and reagents. The A7r5 smooth-muscle cells from thoracic rat aorta from the American Type Culture Collection (ATCC CRL-1444; Manassas, VA) were grown as monolayers cultured at 37˚C in closed Falcon plastic dishes (Nunc, Life Technologies, Merelbeke, Belgium) containing Eagle's minimum essential medium (MEM, Gibco, Life Technologies), to which 5% fetal calf serum (FCS; Gibco) was added. The FCS was heat-inactivated for 1 h at 56˚C. The HUVECs were grown as *in vitro* monolayers. The monolayers were obtained by means of a method adapted from the procedure described by Gimbrone *et al* (22) and cultured at 37˚C in Endothelial Cell Growth Medium 2 (ECGM2, ImTec Diagnostics, Antwerp, Belgium). The same batch of HUVEC primo-culture was used in the present study. All the media were supplemented with a mixture of 0.6 mg/ml glutamine, 200 IU/ml penicillin, 200 g/ml streptomycin and 0.1 mg/ml gentamycin (all from Gibco).

The two anti-PKC antibodies were purchased from Santa Cruz Biotechnology (Boechout, Belgium) and used at a dilution 1/200.

Fluorescent detection of protein expression

Experimental conditions. The levels of PKCα and PKCζ expression in the A7r5 and HUVECs were analyzed in the absence (control) or the presence of hemoglobin (with concentrations ranging from 10^{-11} M to 10 μM) for 30 min and 3 h.

Immunocytochemical procedures. A7r5 and HUVEC suspensions were seeded onto glass coverslips 48 h prior to the immunofluorescence staining, after which the cells were fixed with 4% formaldehyde in PBS, pH 7.4, for 20 min. Five coverslips were available for each experimental condition and for each cell line. In order to detect the PKC isoforms, the cells were permeabilized with 0.2% Triton X-100 for 5 min, and unspecific bounds were blocked for 20 min with 1% horse serum (Vector Laboratories; Burlingame, CA). The binding of the primary antibodies was detected by Alexa Fluor-594-conjugated secondary antibody (10 μg/ml; Molecular Probes, Invitrogen, Merelbeke, Belgium).

Computer-assisted microscopy. The level of expression of each PKC isotype (relating to the intensity of the fluorescence staining) was quantitatively determined by means of computer-assisted fluorescence microscopy using a Provis Olympus Microscope (Omnilabo S.A., Antwerp, Belgium) coupled to a Megaview 2 camera (Omnilabo S.A.) feeding digitized information into a computer using an AnalySIS® software (Soft Imaging System, GmbH, Munster, Germany). Five hundred cells were analyzed per cell line for PKCα and PKCζ in each of the experimental conditions under study.

Data analysis. All the values shown are the means ± standard error (SEM). All the statistical analyses were carried out using Statistica (Statsoft, Tulsa, Oklaoma). The statistical significance of the comparisons between the different experimental conditions was tested by the non-parametric Mann-Whitney U test and is indicated by $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

Results

In vitro characterization of hemoglobin (Hb)-induced modifications to the expression of the calcium-dependent cPKCα and the calcium-independent aPKCζ in smooth-muscle and endothelial cells. The influence of the Hb-induced effects on PKC*α* and PKCζ expression was determined 30 min and 3 h after its addition to the culture medium. Fig. 1A illustrates the influence of Hb-induced effects on PKC*α* expression in HUVECs 30 min after its addition to the culture medium. The data show that 30 min after addition of Hb, the levels of PKC*α* were markedly decreased for Hb concentrations ranging from 10^{-11} to 10^{-9} M (Fig. 1A). These marked effects vanished almost completely 3 h after Hb addition to the HUVEC culture medium (data not shown). In sharp contrast to what was observed concerning the negative influence of Hb on the expression of PKC*α* in the HUVECs (Fig. 1A), Hb stimulated the expression of PKCζ in these cells in doses ranging from 10^{-10} to 10^{-8} M, 3 h after its addition to the culture medium (Fig. 1B). No statistically significant influence was observed 30 min after its addition to the culture medium (data not shown).

Fig. 1C and D give morphological illustrations of PKC*α* expression in the control HUVECs as opposed to the HUVECs cultured for 30 min in the presence of 10^{-10} M Hb.

No statistically significant Hb-induced modifications to the PKC*α* expression were observed in the A7r5 cells 30 min after the addition of Hb to the culture medium (data not shown). Hb significantly decreased the PKCζ expression in the A7r5 cells 3 h after its addition to the culture medium (Fig. 2B). We observed no statistically significant effects of Hb on PKCζ expression in the A7r5 cells 30 min after its addition to the culture medium (data not shown). Fig. 2C and D illustrate the morphological patterns of PKCζ expression in the A7r5 control cells as opposed to the A7r5 cells cultured for 3 h in the presence of 10^{-9} M Hb.

Discussion

Over the past decade a variety of molecular mechanisms have been explored and a number of mediators including endothelium-derived mediators (nitric oxide, oxygen-free radicals, endothelin, lipooxygenases, and cyclooxygenases and their metabolites) (3,4); vascular smooth-muscle-derived mediators [potassium channel inhibition, calcium channel activation, reduction in second messengers (cAMP and cGMP), PKC activation] (3,4); pro-inflammatory mediators involved in blood-brain barrier disruption (serotonin, histamine and bradykinin), cytokines (IL-1, TNF-α and IL-6), and adhesion molecules (23); and stress-induced gene activation (heat shock proteins and hemeoxygenase-1) (3,4,24) have been implicated in SAH-induced vasospasm

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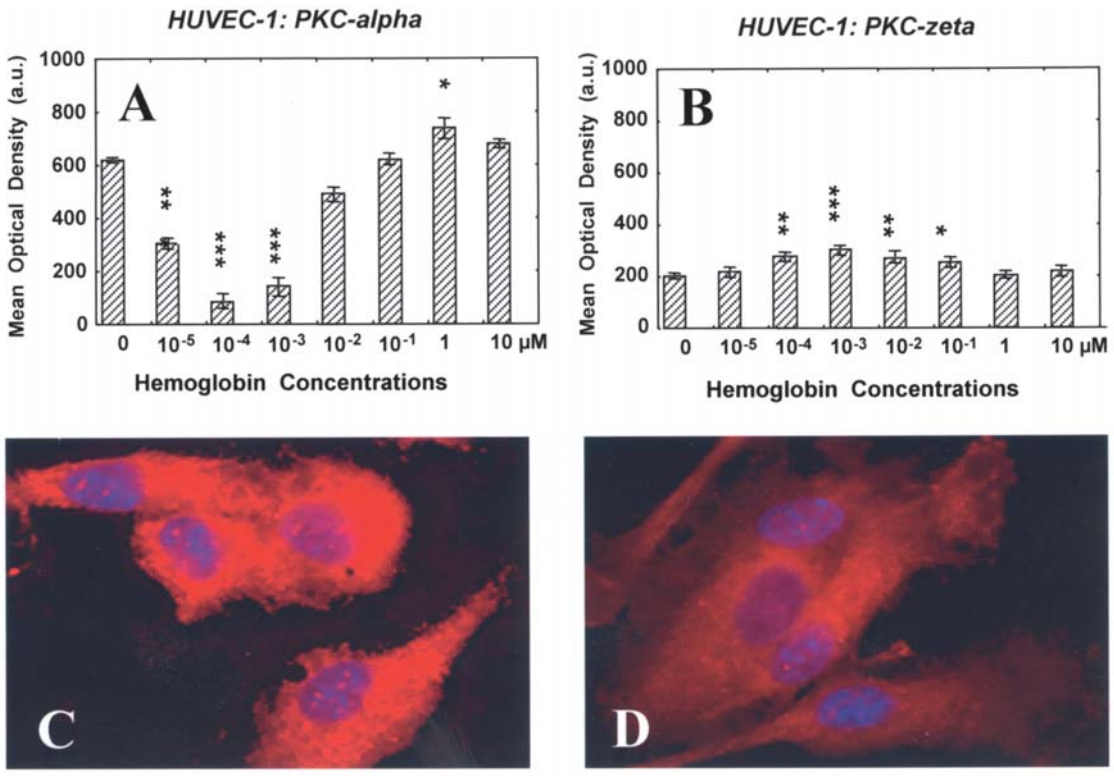

Figure 1. Quantitative determination (by means of computer-assisted fluorescence microscopy) of PKCα (A) and PKCζ (B) expression in HUVECs cultured with Hb concentrations ranging from 10^{-11} to 10^{-5} M (control, 0 M). (A) The influence of the Hb-induced effects on PKCα expression was determined 30 min after the addition of Hb to the culture medium. (B) The effects of Hb on PKCζ expression were determined 3 h after its addition to the culture medium. (C,D) Morphological illustrations of the PKCα expression in control HUVECs as opposed to HUVECs cultured for 30 min in the presence of 10^{-10} M Hb. The data are presented as the means ± SEM. The levels of statistical significance; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

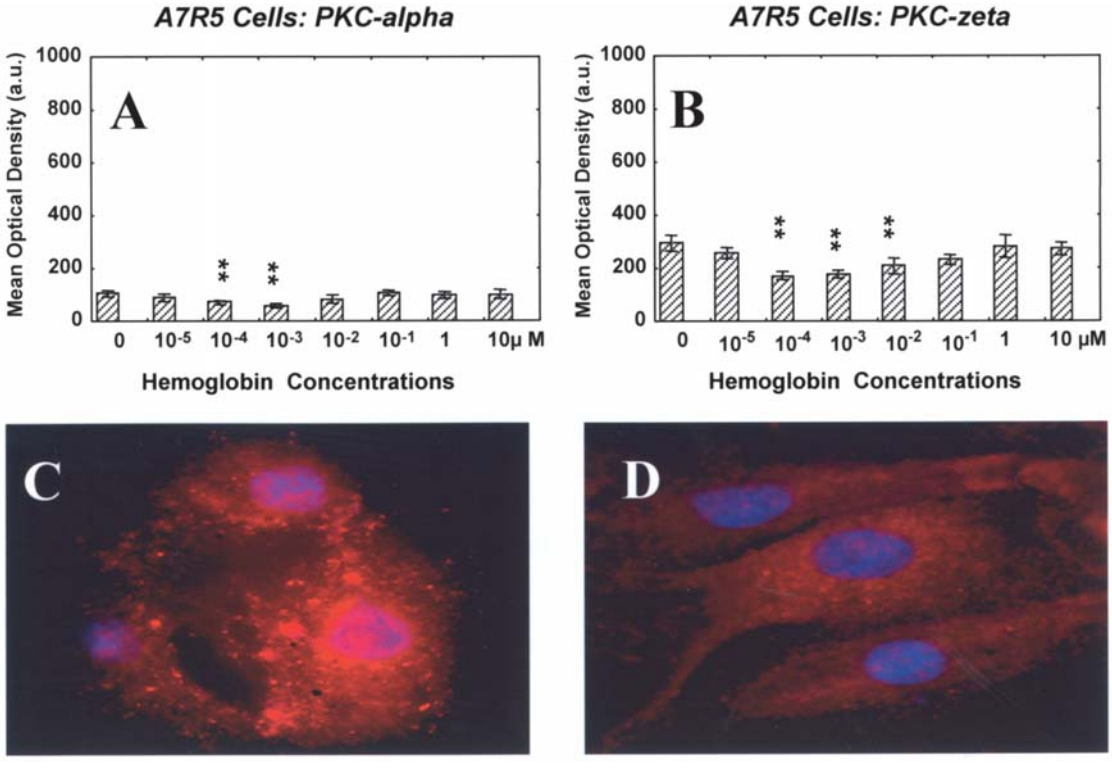

Figure 2. Quantitative determination (by means of computer-assisted fluorescence microscopy) of PKCα (A) and PKCζ (B) expression in A7r5 smooth-muscle rat cells cultured with Hb concentrations ranging from 10^{-11} to 10^{-5} M (control, 0 μM). (A,B) The Hb-induced effects on PKCα (2A) and PKCζ (B) expression in A7r5 smooth-muscle rat cells 3 h after its addition to the culture medium. (C,D) The morphological patterns of PKCζ expression in the A7r5 control cells as opposed to the A7r5 cells cultured for 3 h in the presence of 10^{-9} M Hb. The data are presented as the means ± SEM. The level of statistical significance, $^{**}p<0.01$.

pathogenesis. Thus, PKC seems to be a key in cerebral vasospasm. PKC plays a role in the regulation of myogenic tone by sensitization of myofilaments to calcium (9). PKC phosphorylates various ion channels including augmenting voltage-dependent Ca^{2+} channels and inhibiting K^+ channels, which both lead to vessel contraction (9). Many signaling pathways interact with PKC activities during vasospasm (10). These factors include nitric oxide (NO) and cGMP, ET-1 and potassium channels (3,4,25) and Rho kinase (21,26). The rise in the intracellular diacylglycerol level causes PKC activation in spastic cerebral arteries, presumably because of the overexpression of ET-1 as well as the generation of free radicals; at the same time the cGMP level is inversely reduced owing to the inactivation of soluble guanylate cyclase by means of some as yet unknown mechanism (3,4,18). In fact, whereas NO induces vascular relaxation by inhibiting PKC activity (3,4,27), SAH impairs this inhibition causing PKC-dependent vascular contraction, such as vasospasm (27,28). Our previous results (12) have shown that, *in vivo*, SAH-induced vasospasm in rat cerebral arteries mainly modifies PKCα expression in smooth-muscle cells and PKCζ expression in endothelial cells. These modifications have been observed over an extensive period with respect to the potential role of Hb in PKC expression following SAH-induced vasospasm since the quantitative determination of PKC expression was carried out 24 h after the second cisternal injection of autologous blood. In the current series of *in vitro* experiments and with the goal of pinpointing early molecular events, we investigated the early effects of Hb on calcium-dependent PKCα and calcium-independent PKCζ expression in HUVECs and A7r5 smooth-muscle rat cells. Our results showed that the levels of both calcium-dependent and calcium-independent PKC expression were promptly modified when Hb was added to the culture medium of both endothelial and smooth-muscle cells, consistent with an early modification of PKC expression after SAH.

The treatment of patients with vasospasm remains a challenge in clinical practice (2). At present, the mainstay of treatment of cerebral vasospasm includes triple-H (hypervolemia, hemodilution, and hypertension) therapy and oral administration of nimodipine and intracranial angioplasty (2). Nimodipine, the most widely administered calcium channel blocker, was introduced as a therapeutic agent for the prophylaxis of vasospasm based on findings of a small randomized clinical trial in the US (29) and a large clinical trial in the UK (30). Both trials revealed modest improvements in neurological outcomes following nimodipine administration. In a metaanalysis of nimodipine treatment after SAH, Barker and Ogilvy (31) demonstrated that the prophylactic use of this drug is effective in increasing the odds of a good outcome after SAH. Although the drug is accepted as the standard of care, the mechanism by which it works remains controversial (32). Rinkel and colleagues (33) examined the efficacy of calcium antagonists from 12 different trials in a systematic review. The use of calcium antagonists was associated with improved outcomes, but, although calcium channel blockers significantly diminished the rate of secondary ischemia after aneurysmal SAH (from 40 to 27%), their use did not reduce the high mortality rate associated with SAH. Our study shows that, *in vitro*, Hb markedly and promptly modifies the levels of expression of calcium-dependent PKCα and also calcium-independent PKCζ. Our results encourage the prophylactic use of specific PKC isoform antagonists associated with nimodipine in cerebral vasospasm trials. Because cerebrospinal fluid (CSF) from SAH patients with cerebral vasospasm accumulates various components like NO, endothelin-1, transferin, bilirubin, bilirubin oxidation products, heme oxygenase and peroxidized lipids (34) which are directly involved in cerebral vasospasm following SAH, we are now investigating the effects of CSF on PKC levels of expression. We also intend to investigate the effects of specific PKC antagonists associated or not with calcium channel blockers on the expression of PKC and the *in vivo* severity of SAH-induced vasospasm.

Acknowledgements

We are deeply indebted to Dr J.B. Parys (Laboratory of Physiology, Katholieke Universiteit Leuven, Belgium) for providing us with vials of A7r5 smooth-muscle cells taken from thoracic rat aorta. F.L. is a Clinical Research Fellow with the Fonds National de la Recherche Scientifique (FNRS, Belgium).

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