ELSEVIER

European Journal of Pharmacology 361 (1998) 311–319

ejp

Novel mechanism by which hemoglobin induces constriction of cerebral arteries

Bozena Vollrath [a,*], David Cook [a], Joseph Megyesi [b], J. Max Findlay [b], Hiroki Ohkuma [b]

[a] *Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7*
[b] *Department of Surgery, University of Alberta, Edmonton, Alberta, Canada T6G 2H7*

Received 11 May 1998; revised 3 August 1998; accepted 13 October 1998

Abstract

Since oxyhemoglobin (OxyHb) is implicated in the pathogenesis of cerebral vasospasm, we have investigated the role of protein tyrosine phosphorylation in OxyHb-mediated signalling in canine cerebral arteries and cultured canine cerebrovascular smooth muscle cells. OxyHb produced a contraction of basilar artery preparations, which was reversed by genistein, an inhibitor of tyrosine kinases, and PD098059, an inhibitor of mitogen-activated protein kinase. In cerebrovascular smooth muscle cells, OxyHb induced tyrosine phosphorylation of 42, 46, 54–60 and 80–100 kDa proteins with a time-course which paralleled the contractile action of OxyHb, suggesting that these events might be functionally linked. The 42 and 60 kDa proteins were immunologically related to the mitogen-activated protein kinase, extracellular signal regulated protein kinase (ERK2), and to $p60^{c\text{-}Src}$ (c-Src), respectively. The increase in protein tyrosine phosphorylation was attenuated by genistein, and the phosphorylation of the 42 kDa protein (ERK2) was inhibited by PD098059. These results suggest that OxyHb-mediated signalling utilizes a protein tyrosine kinase-based mechanism.

Keywords: Tyrosine phosphorylation; Hemoglobin; Cerebral vasospasm; Genistein

1. Introduction

Oxyhemoglobin (OxyHb) produces a sustained constriction of cerebral arteries, and there is substantial evidence that the release of this spasmogen from lysed erythrocytes in the subarachnoid clot triggers the cerebral vasospasm that occurs after subarachnoid hemorrhage (Macdonald et al., 1991; Cook and Vollrath, 1995). The delayed ischemia which arises from the spasm of cerebral arteries can cause significant neurological deficit and sometimes death. Although OxyHb is likely to be the causative agent in cerebral vasospasm, the molecular mechanisms responsible for the prolonged vasoconstriction and the proliferative, hyperplastic changes observed in cerebral vessels after vasospasm, are not well understood. It is thought that free radicals generated by OxyHb may play a role (Steele et al., 1991); the autoxidation of OxyHb generates superoxide ion and hydroxyl radical which is formed by the reaction between iron present in this hemoprotein, and hydrogen peroxide (Macdonald and Weir, 1994). Superoxide can also react with nitric oxide to form the peroxynitrite radical, a powerful oxidant which decomposes rapidly to the hydroxyl radical (Kooy et al., 1994). These active oxygen species have been shown to initiate lipid peroxidation, a reaction which stimulates a variety of cellular processes including activation of phospholipases, protein kinases, protooncogene expression and cell growth (Wiseman and Halliwell, 1996; Lander, 1997). Free radicals have also been shown to promote a direct activation of critical components of signalling pathways mediated by receptor and non-receptor tyrosine kinases, such as $p21^{ras}$ and mitogen-activated protein kinases (Baas and Berk, 1995; Lander et al., 1995, 1996). Furthermore, superoxide ion has been shown to activate mitogen-activated protein kinase in rat aortic smooth muscle cells (Baas and Berk, 1995), an observation which suggests that the activation of this enzyme by free radicals may be an important process in the action of OxyHb on smooth muscle cells.

The significance of these actions became appreciated only recently, because of the intense interest in the $p21^{ras}$-regulated mitogenic cascade, involving the protein kinases

* Corresponding author. Tel.: +1-403-492-4747; Fax: +1-403-492-4325; E-mail: bozena.vollrath@ualberta.ca

0014-2999/98/$ - see front matter
PII: S0014-2999(98)00745-6

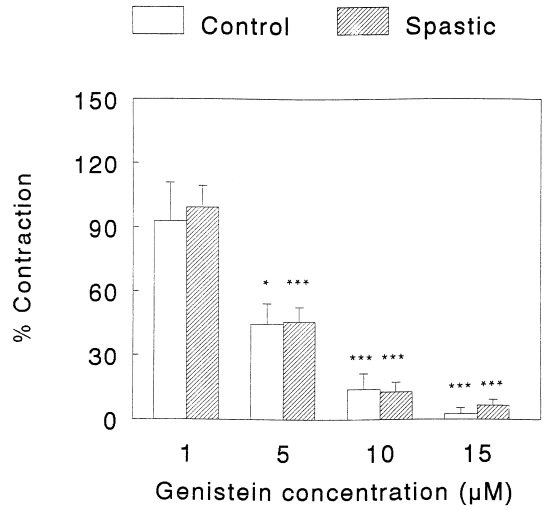

Fig. 1. Effects of genistein on the contraction produced by OxyHb in rings of control arteries (open bars) and in rings of spastic arteries from the canine double hemorrhage model of cerebral vasospasm (hatched bars). Ordinate: contraction expressed as the percentage of the maximum tension induced by 10 μM OxyHb. Bars represent the means ± standard error, for experiments conducted with tissue preparations ($n = 8$) from five separate animals. Asterisks indicate a significant difference ($* p < 0.05$, $* * * p < 0.001$) between contraction in the absence and in the presence of genistein.

Raf, MEK and mitogen-activated protein kinase, that ultimately alters gene expression. Critical enzymes in this cascade are the 44- and 42-kDa mitogen activated kinases, also called extracellular signal regulated protein kinases (ERK1 and ERK2, respectively) (Robinson and Cobb, 1997). A dual-specificity protein kinase called mitogen-activated protein kinase kinase or MEK (Crews et al., 1992), catalyzes the phosphorylation of mitogen-activated protein kinases on Thr and Tyr residues, and is itself regulated by serine phosphorylation by MEK kinase (Raf-1) (Seger et al., 1991). Activation of Raf-1, a downstream target of $p21^{ras}$, is mediated by $p21^{ras}$ in its active, GTP-bound state (Seger and Krebs, 1995). Among target proteins phosphorylated by mitogen-activated protein kinase, there is an actin binding protein, caldesmon, implicated in smooth muscle contraction (Adam et al., 1992; Childs et al., 1992). It has been suggested that phosphorylation of this protein by mitogen-activated protein kinase removes the inhibitory constraint imposed by caldesmon on the interaction of actin with myosin in smooth muscle cells, thus leading to a sustained smooth muscle contraction (Childs et al., 1992). Thus mitogen-activated protein kinase may be as important for the control of smooth muscle contraction as it is for the control of cell proliferation and differentiation. Since cerebrovascular spasm involves both processes, it is reasonable to suggest that vasospasm and the actions of OxyHb may be mediated via activation of this cascade.

In this study, we have explored whether protein tyrosine phosphorylation plays a role in the responses to OxyHb by measuring both the contractility of canine cerebral arteries in vasospasm and protein tyrosine phosphorylation in cerebrovascular smooth muscle cells.

2. Materials and methods

2.1. Materials

OxyHb was obtained from Sigma and purified by the technique of Martin et al. (1985) before use. Agents used in this study were the protein tyrosine kinase inhibitor genistein, and its inactive analog daidzein (Calbiochem), PD098059, a MEK inhibitor (Parke-Davis), monoclonal anti-phospho-tyrosine (4G10), and $p60^{src}$ antibodies (Upstate Biotechnology UPI), and polyclonal anti-mitogen-

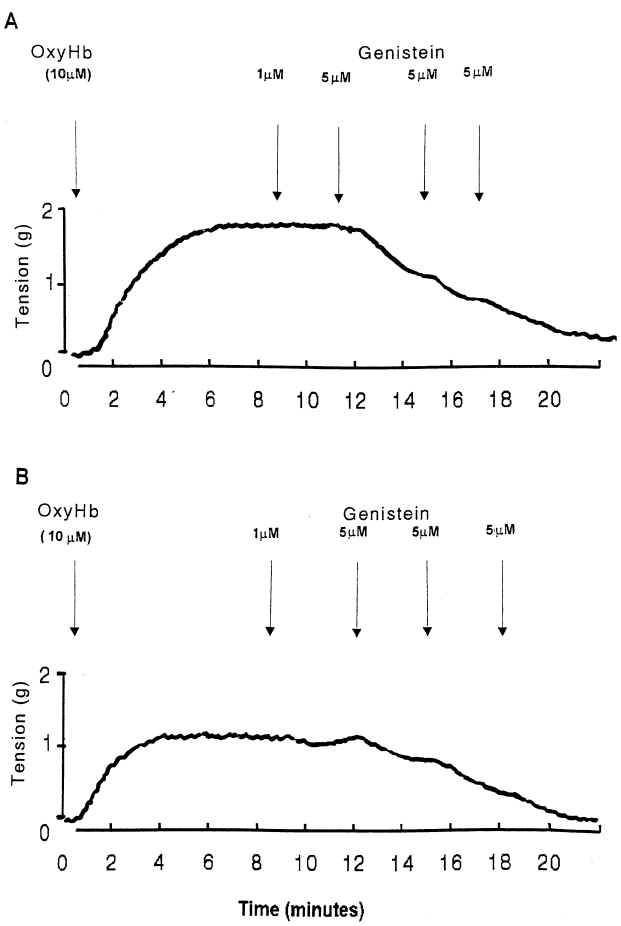

Fig. 2. Representative traces of the responses of rings of control arteries (panel A) and spastic arteries (panel B) contracted with OxyHb. Ring preparations were suspended under a resting tension of 1 g in tissue bath containing Krebs–Henseleit solution at 37°C, gassed with O_2 (95%) and CO_2 (5%) and equilibrated for 1 h. The experiments were performed as described in Section 2. Arrows indicate time of administration of OxyHb and genistein. Each tracing illustrating the response of an individual tissue preparation, is representative of five independently conducted experiments.

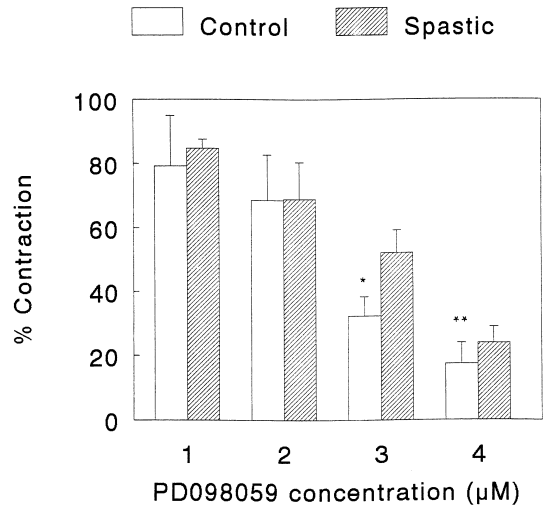

Fig. 3. Effects of PD098059 on the contraction produced by OxyHb in rings of control arteries (open bars) and in spastic preparations from the canine double hemorrhage model of cerebral vasospasm (hatched bars). Ordinate: contraction expressed as the percentage of the maximum tension induced by 10 μM OxyHb. Bars represent the mean ± standard error, for experiments conducted with tissue preparations ($n = 3–5$) from five separate animals. Asterisks indicate a significant difference ($* p < 0.05$, $** p < 0.01$) between contraction in the absence and in the presence of PD098059.

activated protein kinase ERK1 and ERK2 antibodies, horseradish peroxidase-coupled goat anti-mouse and goat anti-rabbit antibodies and the Enhanced Chemiluminescence Detection System (ECL) detection kit (all from Pierce). Dulbecco's modified Eagle's medium was obtained from GIBCO Canada. Chemicals for electrophoresis were purchased from Bio-Rad and a monoclonal antibody against α-actin was purchased from Sigma. All other reagents were of the highest available quality and were obtained from Sigma, Fischer Scientific or BDH.

2.2. Experimental subarachnoid hemorrhage

Ten mongrel dogs of either sex, weighing about 20 kg, were used in these studies. The protocol was evaluated and approved by the University of Alberta Animal and Ethics Review Committee, and experiments were conducted with strict adherence to the standards of the Canadian Council on Animal Care. Five animals underwent intracisternal administration of blood to mimic subarachnoid hemorrhage and to induce vasospasm. The control group consisted of five dogs without intracisternal administration of blood, but otherwise treated as the subarachnoid hemorrhage group. The animals were anesthetized with sodium pentobarbital (25 mg/kg, i.v.) and intubated for all procedures on the day of first administration of blood, as well as on the 3rd and 7th day after the first administration of blood. Intermittent boluses of pentobarbital were administered to maintain adequate anesthesia. Experimental subarachnoid hemorrhage was induced according to the method of Varsos et al. (1983). Briefly, 7 ml of autologous blood was injected into the cisterna magna, and the animal was placed in a special animal facilities for post-operative recovery and administration of analgesics. On day 3, a second injection of arterial blood was given. On day 7 vertebral angiograms, indicative of severe spasm, were obtained and the animals were killed with an overdose of sodium pentobarbital. The brain with the cerebral arteries attached was rapidly removed and placed in oxygenated Krebs–Henseleit solution of the following composition (mM): Na^+ 130, K^+ 5, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 12.2, HCO^{3-} 25, SO_4^{2-} 1.2, $H_2PO_4^{2-}$ 1.2 and dextrose 11. Each artery was cut into sections about 3 mm in length and these preparations were suspended under a resting tension of 1 g in tissue baths containing Krebs–Henseleit buffer at 37°C, gassed with 95% O_2 and 5% CO_2. After an equilibration period of 1 h, during which time the Krebs–Henseleit buffer was changed every 15 min, the response

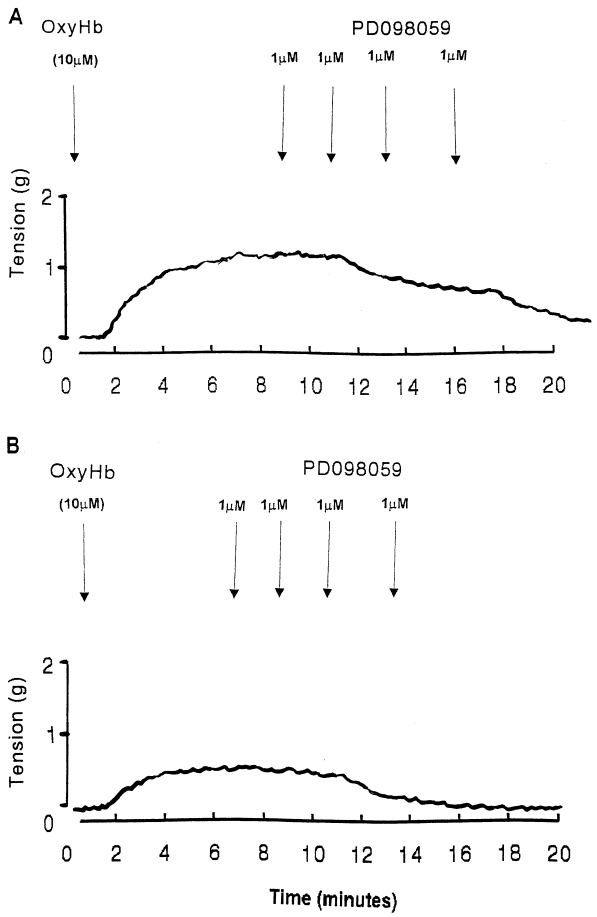

Fig. 4. Representative traces of the responses of rings of control arteries (panel A) and spastic arteries (panel B) contracted with OxyHb. The experiments were performed as described in Fig. 2 and in Section 2. Arrows indicate time of administration of OxyHb and PD098059. Each tracing illustrating the response of an individual tissue is representative of three to five independently conducted experiments.

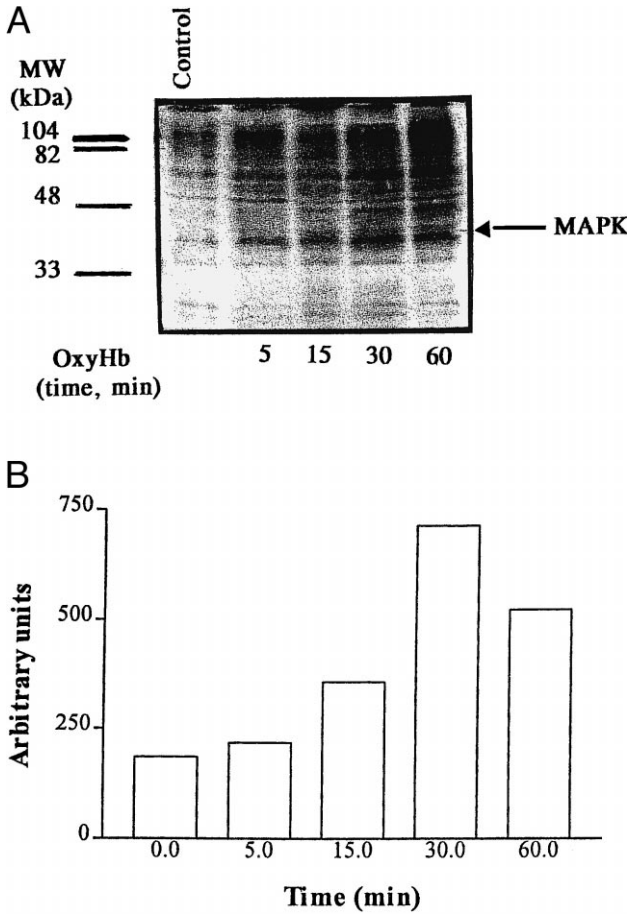

Fig. 5. Time-course of protein tyrosine phosphorylation stimulated by OxyHb in cerebrovascular smooth muscle cells. Serum starved cerebrovascular smooth muscle cells (lane 1) were stimulated with OxyHb (10 μM) for the indicated times (lanes 2–5). The cell lysates were resolved by SDS-PAGE and immunoblotted with monoclonal anti-Tyr (4G10) antibody as described in Section 2. Proteins were visualized using a goat anti-mouse antibody conjugated to horse radish peroxidaze and a chemiluminescence detection system. MAPK standard (42 kDa) electrophoresed on the same gel was used as a positive control for immunoblotting with anti-MAPK antibody. Results presented are representative of three independent experiments. The position of MAPK is indicated on the right and that of molecular weight (MW, kDa) markers on the left of the figure (upper panel). The density of each band was measured by densitometry and the analysis of the MAPK phosphorylation was performed using Sigma Gel software. The bars represent the time-course of MAPK tyrosine phosphorylation expressed in arbitrary units (lower panel).

to KCl (60 mM) was recorded and preparations were washed until resting tension was again obtained. Contractions to 10 μM OxyHb, which is an approximate minimal concentration of OxyHb in the subarachnoid blood clot (Pluta et al., 1998), were recorded isometrically using force-displacement transducers and a Grass 7D polygraph. Cumulative concentrations of genistein or PD098059 were added to the organ baths after sustained, tonic response to OxyHb had developed. In the end of the experiment 60 mM KCl was added to assure that the viability of the preparations was maintained.

2.3. Canine cerebrovascular smooth muscle cell culture

Smooth muscle cells were isolated from cerebral vessels from untreated dogs (Takanashi et al., 1992). Basilar or middle cerebral arteries were isolated under sterile conditions and placed in a Petri dish containing Dulbecco's modified Eagle medium (DMEM). The adventitia was removed mechanically, the vessels were cut into segments approximately 5 mm in length along the longitudinal axis, and the endothelium was removed by gently scraping the inner surface of the segments. The tissue was then chopped into 1 to 2 mm pieces and the explants were transferred to

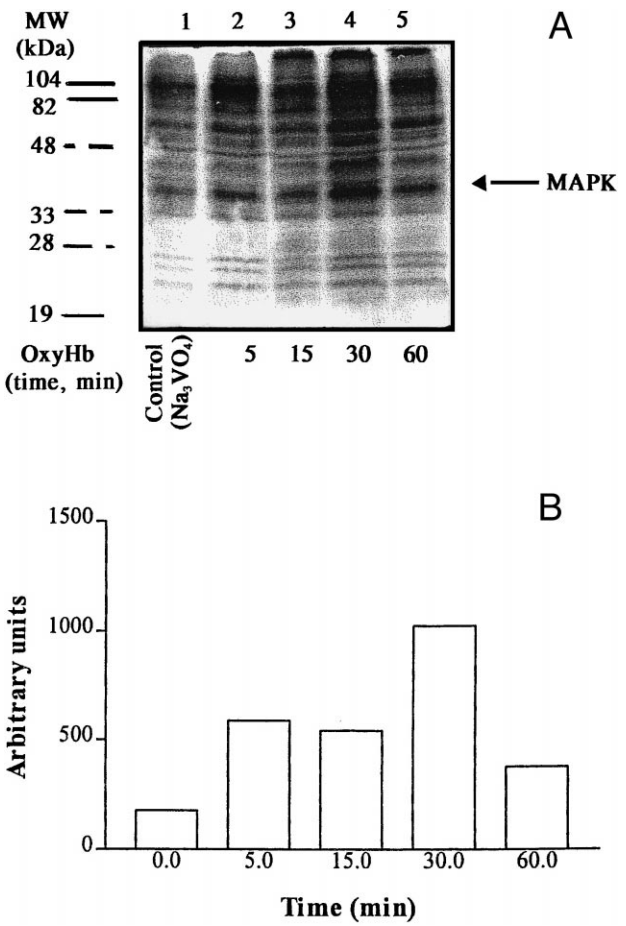

Fig. 6. Time-course of protein tyrosine phosphorylation stimulated by OxyHb in cerebrovascular smooth muscle cells in the presence of sodium orthovanadate (Na_3VO_4). Serum starved cerebrovascular smooth muscle cells were treated with Na_3VO_4 (100 μM, 30 min) alone (lane 1) or were stimulated with OxyHb (10 μM), in the presence of Na_3VO_4 for the indicated times (lanes 2–5). The cell lysates were resolved by SDS-PAGE and immunoblotted with anti-Tyr antibody as described in Fig. 5 and in Section 2. Results presented are representative of three independent experiments. The position of MAPK is indicated on the right and that of molecular weight (MW, kDa) markers on the left of the figure (upper panel). The density of each band was measured by densitometry and the analysis of the MAPK tyrosine phosphorylation was performed as described in Fig. 5. Bars represent the time-course of MAPK tyrosine phosphorylation, stimulated by OxyHb in the presence of Na_3VO_4, expressed in arbitrary units (lower panel).

25 cm^2 culture flasks containing 1 ml of DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). After the explants had adhered, the volume was made up gradually over the next 4 days to 4 ml per flask, and thereafter the culture medium was changed weekly. When the primary cultures were almost confluent the cerebrovascular smooth muscle cells were transferred to 75 cm^2 flasks and then routinely subcultured at a split ratio of 1:3. The cells were test positive for smooth muscle α-actin and at confluence, the cells demonstrated the typical 'hill and valley' pattern. The studies were carried out on cells in passages 6–10.

2.4. Western blot analysis

Cerebrovascular smooth muscle cells were serum-starved for 48 h, treated where indicated with Na_3VO_4, genistein and PD098059 for 30 min, and stimulated with OxyHb. After stimulation, the cells were harvested, washed twice with saline and lysed at 4°C with 100 μl of lysis buffer containing 25 mM Tris/HCl (pH 7.4), 25 mM sodium chloride, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 20 μM leupeptin. The lysates were incubated on ice for 20 min and centrifuged for 20 min at 4°C. Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. In some blots, Ponceau S (Sigma) staining was performed to confirm that an equal amount of protein was loaded in each lane. Laemmli's sodium dodecyl sulphate-polyacylamide gel electrophoresis (SDS-PAGE) sample buffer (5×) was added, and the lysates were boiled for 5 min, matched for protein content and subjected to SDS-PAGE in 10% gels. After gel electrophoresis (100 V applied for approximately 90 min), the proteins were transferred to nitrocellulose. The membranes were blocked in PBS (20 mM Tris, 120 mM sodium chloride) containing 3–5% non-fat milk, for 20 min at 20°C with constant agitation.

For phosphotyrosine detection, the nitrocellulose was incubated at 4°C overnight with 1 μg/ml of monoclonal phosphotyrosine antibody (4G10). The blots were washed twice with water and incubated with a goat anti-mouse secondary antibody linked to horseradish peroxidase (1:3000), in PBS containing non-fat milk for 1.5 h at 20°C. Proteins were visualized using an enhanced chemiluminescence detection system. For mitogen-activated protein kinase detection, the membranes were incubated with the anti-mitogen-activated protein kinase polyclonal antibody using a 1:1000 dilution to identify the mitogen-activated protein kinase in the cell lysate. Purified mitogen-activated protein kinase (40 ng/10 μl) was used as a positive control. Proteins were visualized using a secondary antibody conjugated to horseradish peroxidase, and a chemiluminescence detection system. To quantify and compare levels of mitogen-activated protein kinase, phosphorylation films were subjected to densitometric analysis using a Scan Jet 6100C imaging densitometer. The density of bands corresponding to mitogen-activated protein kinase was measured using Sigma Gel software (Jandel, San Rafael, CA). For $p60^{c\text{-}Src}$ detection, the membranes were probed with monoclonal anti-Src antibody (3 μg/ml); proteins were visualized using a horseradish peroxidase-coupled goat anti-mouse antibody and a chemiluminescence detection system. Molecular weights of the proteins were estimated by using pre-stained markers (Bio-Rad).

2.5. Statistical methods

Data are expressed as the mean ± the standard error of the mean, with the number of preparations used in parentheses. Statistical significance was assessed using one way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test when significant probability was reached. Values of $p < 0.05$ were considered to be significant.

3. Results

To examine the involvement of protein tyrosine phosphorylation in cerebral vasoconstriction, we have measured both contraction of normal and spastic cerebral arteries and protein tyrosine phosphorylation in cerebrovascular smooth muscle cells stimulated with OxyHb.

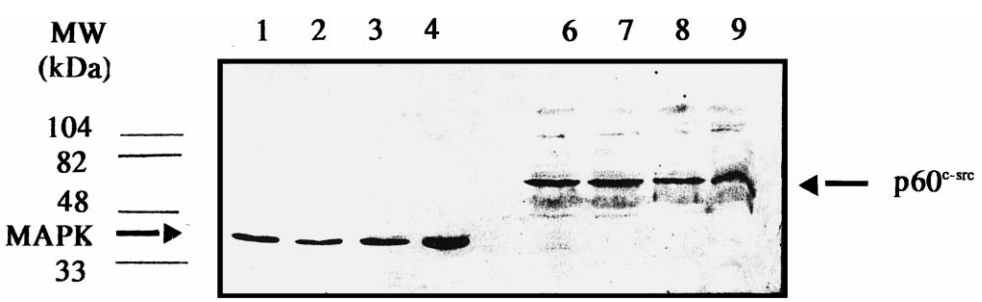

Fig. 7. Expression of $p60^{c\text{-}Src}$ and MAPK in canine cerebrovascular smooth muscle cells. Cell lysates were resolved by SDS-PAGE, transferred onto nitrocellulose and probed with anti-MAPK (lane 1–4) and anti-Src (lanes 6–9) antibodies. The position of $p60^{c\text{-}Src}$ is indicated on the right and that of MAPK and molecular weight markers on the left of the figure.

The minimal concentration of OxyHb in the blood clot after subarachnoid hemorrhage is in the micromolar range (Pluta et al., 1998) thus a concentration of 10 μM OxyHb was used in the present contractility experiments. This treatment induced a slowly developing sustained arterial constriction of isolated canine basilar arteries, which reached a plateau at about 10–20 min and was maintained for at least 60 min (Figs. 1–4). The protocol was designed to approximate to the effects of OxyHb released slowly from lysed erythrocytes, and permits the analysis of the action of inhibitors administered after the sustained response had developed. The tension developed in spastic arterial ring preparations in response to 10 μM OxyHb (0.78 ± 0.08 g) was less than that observed in control preparations (0.99 ± 0.17), in agreement with other reports (Onoue et al., 1995). To mimic the situation in vasospasm in which therapeutic agents must be administered after vasospasm has developed, genistein, an inhibitor of tyrosine kinases, and PD098059 which selectively blocks the activity of MEK (Akiyama et al., 1987; Alessi et al., 1995), were administered to preparations in which a tonic contraction to OxyHb had developed (Figs. 2 and 4). Genistein and PD098059 elicited concentration-dependent relaxation of both control, and spastic cerebral arteries from an experimental model of subarachnoid hemorrhage, thus demonstrating that vasoconstriction could be reversed by inhibition of a tyrosine kinase and MEK (Figs. 1 and 3). The IC_{50} values against OxyHb for genistein and PD098059 were 3.8 and 3.2 μM, respectively. Daidzein, which is an inactive analog of genistein, had no effect on the vascular contraction produced by OxyHb.

In the experiments in which the time course of protein tyrosine phosphorylation was examined, cultured canine cerebrovascular smooth muscle cells were exposed to OxyHb (10 μM) for 5, 15, 30 or 60 min, in the absence (Fig. 5) or in the presence (Fig. 6) of 100 μM of sodium orthovanadate (Na_3VO_4), and then washed, scraped, lysed and subjected to SDS-PAGE followed by Western blotting using the 4G10, anti-phosphotyrosine monoclonal antibody. After application of OxyHb, an increase in tyrosine phosphorylation of several proteins with molecular masses of 42, 46, 54–60 and 80–100 kDa was observed (Figs. 5 and 6). The increase in protein tyrosine phosphorylation occurred within first 5 min, peaked between 15 and 30 min and declined after 60 min. The time course of the changes in intensity of the tyrosine phosphorylation therefore closely corresponds to the development of contraction induced by OxyHb in cerebral arteries. Among several tyrosine-phosphorylated proteins, a band corresponding to 42 kDa protein may represent ERK2, a mitogen-activated protein kinase involved in contraction and in mitogenic processes. Comparison of phosphotyrosine and mitogen-activated protein kinase blots of the same cell lysates electrophoresed on the same SDS-PAGE gel showed that the protein recognized by anti-mitogen-activated protein kinase antibody co-migrates with tyrosine phosphorylated p42 (ERK2) (Figs. 5 and 6). The presence of a 60 kDa tyrosine phosphorylated protein after OxyHb treatment suggests identity with $p60^{c\text{-}Src}$. The application of anti-Src-PTK monoclonal antibody revealed that this enzyme is expressed in dog cerebrovascular smooth muscle cells (Fig. 7).

The presence of a 54 kDa tyrosine-phosphorylated protein after treatment with OxyHb suggests identity with the recently-described p54 stress activated protein kinase (SAPKs) known to be activated by oxidative stress (Kyriakis and Avruch, 1996). However, the identity and functional role of this protein remains to be established. The band at 80–100 kDa is broad and may consist of

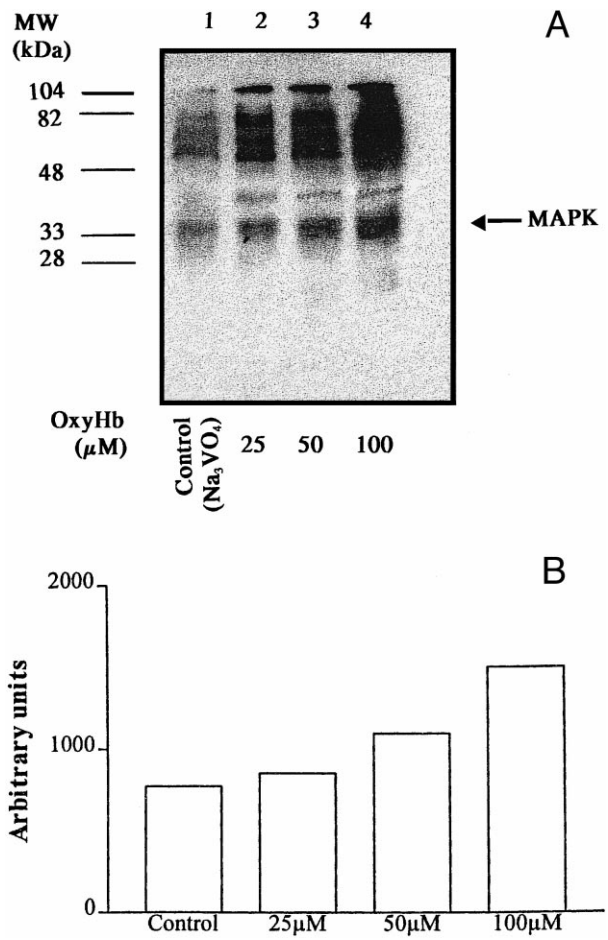

Fig. 8. Concentration-responses of OxyHb-mediated stimulation of protein tyrosine phosphorylation. Serum starved cerebrovascular smooth muscle cells were treated with Na_3VO_4 (100 μM, 30 min) alone (lane 1), or were stimulated for 30 min with the indicated concentrations of OxyHb (lanes 2–4) in the presence of Na_3VO_4 (100 μM, 30 min). The cell lysates (70 μg/lane) were resolved by SDS-PAGE and immunoblotted with anti-Tyr (4G10) antibody as described in Section 2. The position of molecular weight markers is shown on the left and that of MAPK on the right of the figure (upper panel). The density of each band was measured by densitometry and the analysis of the MAPK tyrosine phosphorylation was performed as described in Fig. 5Fig. 6. Bars represent the concentration dependence of MAPK tyrosine phosphorylation stimulated by OxyHb in the presence of Na_3VO_4, expressed in arbitrary units (lower panel).

multiple proteins phosphorylated on tyrosine after stimulation with OxyHb.

As shown in Fig. 8, there was a concentration-dependent enhancement in protein tyrosine phosphorylation induced in cerebrovascular smooth muscle cells after stimulation with OxyHb for 30 min. The several bands changed in intensity including p42 and a broad band at approximately 54 to 100 kDa which may consist of multiple proteins (Fig. 8).

To examine the effects of tyrosine kinase inhibitors, cerebrovascular smooth muscle cells were pretreated with PD098059 (1 μM), genistein (10 μM) and daidzein (50 μM) for 30 min in the presence of 100 μM of Na_3VO_4 and then stimulated with OxyHb (10 μM) for another 30 min (Fig. 9). Inclusion of Na_3VO_4, an inhibitor of tyrosine phosphatase enhanced tyrosine phosphorylation induced by OxyHb (Fig. 9), suggesting that the increase in phosphorylation was caused by the activation of a tyrosine kinase and not by the inhibition of tyrosine phosphatase. PD098059 (1 μM), a MEK inhibitor administered to cerebrovascular smooth muscle cells 30 min before application of OxyHb markedly inhibited phosphorylation of the proteins which co-migrated with the mitogen activated protein kinase standard detected by the anti-mitogen-activated protein kinase antibody (Fig. 9). A 30 min pretreatment of cerebrovascular smooth muscle cells with genistein suppressed the OxyHb-induced increase in tyrosine phosphorylation of multiple proteins including proteins corresponding to mitogen-activated protein kinase (Fig. 9). Daidzein, an inactive analog of genistein, did not have any effect on tyrosine phosphorylation induced by OxyHb (Fig. 9).

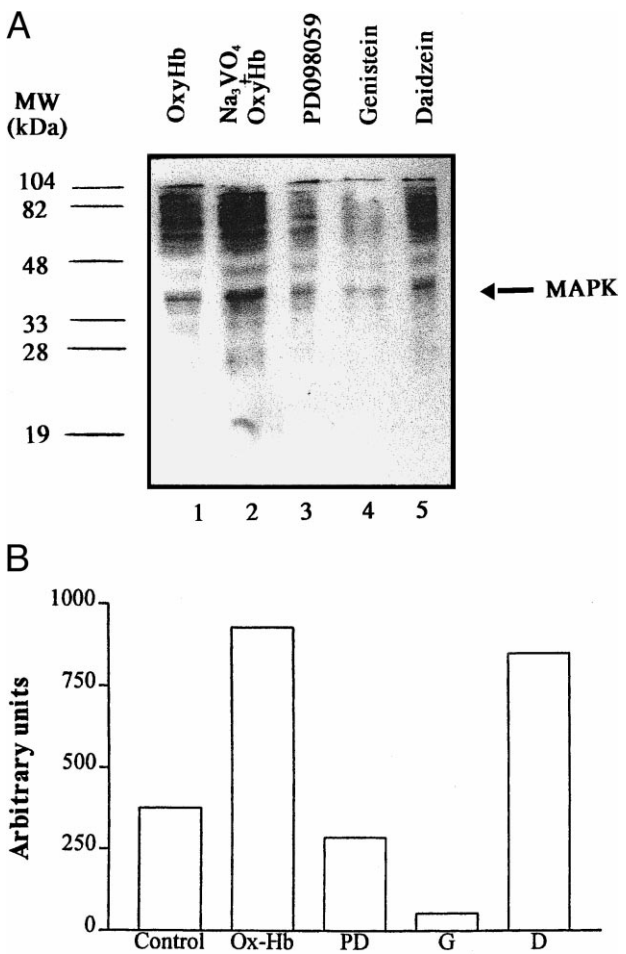

Fig. 9. Effects of PD098059 and genistein on protein tyrosine phosphorylation induced by OxyHb. Quiescent cerebrovascular smooth muscle cells were pretreated with indicated agents for 30 min in the presence of 100 μM of Na_3VO_4 and then stimulated with OxyHb (10 μM) for another 30 min. Total cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-Tyr antibody as detailed in Section 2. Results presented are representative of three experiments. The position of MAPK is indicated on the right and that of molecular weight markers on the left of the figure (upper panel). The density of each band was measured by densitometry and the analysis of the MAPK tyrosine phosphorylation was performed as described in Fig. 5Fig. 6Fig. 8. Bars represent the effects of OxyHb, administered in the presence of Na_3VO_4, PD098059 and genistein on MAPK tyrosine phosphorylation expressed in arbitrary units (lower panel).

4. Discussion

In these studies we show that vasoconstriction induced by OxyHb can be reversed by genistein, a tyrosine kinase inhibitor, and by PD098059, an inhibitor of MEK, which operates upstream of mitogen-activated protein kinase (Alessi et al., 1995; Seger and Krebs, 1995). The time-course of contraction to OxyHb corresponds to an increase in the tyrosine phosphorylation of multiple proteins, including a 42 kDa protein which may represent the mitogen-activated protein kinase implicated in cell proliferation and differentiation, as well as in smooth muscle contraction (Adam et al., 1992; Childs et al., 1992; Adam and Hathaway, 1993). These results suggest a novel mechanism for OxyHb-dependent signalling and provide evidence that links OxyHb to cell proliferation and differentiation, phenomena which occur in cerebral arteries after subarachnoid hemorrhage (Mayberg et al., 1990a,b).

The initial steps involved in the contraction and tyrosine phosphorylation which result from the action of OxyHb may involve generation of free radicals such as superoxide and hydroxyl radical implicated in the pathogenesis of cerebral vasospasm (Macdonald and Weir, 1994; Shishido et al., 1994). Reactive oxygen species have been shown to act as potent vasoconstrictors in blood vessel preparations (Auch-Schwelk et al., 1989) and to increase mitogen-activated protein kinase activity with the time-course corresponding to smooth muscle contraction (Baas and Berk, 1995). The molecular mechanism by which free radicals produce vasospasm is still far from clear but, in addition to direct activation of $p21^{ras}$ (Lander et al., 1995, 1996; Lander, 1997), an increase in intracellular calcium (Takanashi et al., 1992; Vollrath et al., 1994) and a prolonged activation of protein kinase C (Sako et al., 1993; Takuwa et al., 1993) may play a role. We have recently

shown that OxyHb and free radicals induce a sustained increase in intracellular calcium and that this action can be inhibited by agents which scavenge oxygen radicals, thus indicating that there is a link between free radical formation in the process of autoxidation of OxyHb and enhancement of the levels of intracellular calcium (Vollrath et al., 1995). How an increase in calcium is linked to the OxyHb -mediated tyrosine phosphorylation is unknown, but it is conceivable that the activation of a calcium-dependent proline rich tyrosine kinase 2 such as proline-rich tyrosine kinase 2 (PYK2) (Lev et al., 1995; Dikic et al., 1996) or cell-adhesion kinase-β (Sasaki et al., 1995) and a member of Src family protein tyrosine kinases may play a role in OxyHb-mediated signalling. It has been shown in neuronal tissue that an increase in intracellular calcium induces tyrosine phosphorylation of PYK2 and complex formation between this kinase and c-Src that leads to c-Src activation (Dikic et al., 1996). This interaction leads to complex formation with the adapter protein called growth-factor receptor binding protein (Grb2), and a guanidine exchange factor 'Son-of-sevenless' (Sos) and hence to activation of the mitogen-activated protein kinase signalling pathway.Whether these signalling events are activated in a similar manner by OxyHb in vascular smooth muscle is unknown. The c-Src tyrosine kinase is likely to be involved on the basis of the recent finding that c-Src activity is stimulated in smooth muscle and it is inhibited by the tyrosine kinase inhibitors geldanomycin, genistein and tyrphostin (Di Salvo et al., 1993). Furthermore, recent studies indicate that the exposure to oxidative stress enhances the catalytic activity of Src followed by the activation of Ras in HeLa cells (Devary et al., 1992). Although Src activity is known to be activated by dephosphorylation, there is evidence in favour of a positive regulatory role for tyrosine phosphorylation in control of Src activity; phosphorylation of a second conserved tyrosine residue, Tyr^{416} of c-Src enhances c-Src tyrosine kinase activity (Cooper and Howell, 1993). In the present studies we have found that c-Src is expressed in cerebrovascular smooth muscle cells and that tyrosine phosphorylation of a 60 kDa protein immunologically related to c-Src, is enhanced by OxyHb. However, the functional role of the Src kinases in OxyHb-related signalling remains to be established.

The ability of OxyHb to activate protein kinase C (Cook et al., 1993) may also play a role in stimulation of tyrosine phosphorylation. By activating protein kinase C, active oxygen species would be expected also to activate mitogen-activated protein kinase; in vascular smooth muscle cells, mitogen-activated protein kinase is activated by phorbol esters which owe their effects to activation of protein kinase C, and it is also known that mitogen-activated protein kinase translocation in the same cell type is dependent on protein kinase C activity (Khalil and Morgan, 1993). In fact, early activation of mitogen-activated protein kinase by superoxide ion is protein kinase C dependent, because when this enzyme is downregulated, the superoxide-mediated activation of mitogen-activated protein kinase is blocked. Activation of mitogen-activated protein kinase has been found to coincide with smooth muscle contraction, both of which are attenuated by protein kinase C inhibitors (Khalil and Morgan, 1993). Thus activation of protein kinase C may lead to the phosphorylation and activation of Raf-1 which in turn triggers a cascade of phosphorylation events with the sequential phosphorylation of MEK and mitogen-activated protein kinase. The involvement of MEK is suggested in our studies by the observation that PD098059, an inhibitor of MEK (Alessi et al., 1995), attenuated both contraction of cerebral arteries induced by OxyHb and the phosphorylation of mitogen-activated protein kinase. The mechanism by which activation of mitogen-activated protein kinase stimulates smooth muscle contraction may involve phosphorylation of caldesmon, an actin binding inhibitory protein. The expression of mitogen-activated protein kinase and the ability of this enzyme to phosphorylate caldesmon (Childs et al., 1992) has been demonstrated in vascular smooth muscle, and it is also known that the immunoreactivity of caldesmon in cerebral arteries after subarachnoid hemorrhage is significantly decreased (Takenaka et al., 1993). This suggests that the inhibitory effect of caldesmon on actin–myosin interaction is attenuated, an effect which may well be responsible for the vasoconstriction.

In conclusion, the data presented here suggest that protein tyrosine phosphorylation plays a role in sustained vasoconstriction initiated by OxyHb and free radicals derived from this agent. The observation that ERK2 is one of the tyrosine phosphorylated proteins would suggest that ERK2 and its upstream target, MEK, may play a role in the pathogenesis of cerebral vasospasm and the subsequent cerebral ischemia. An understanding of this signalling system and characterization of functional roles of the component involved will provide insight into the mechanism by which OxyHb induces cerebral vasospasm.

Acknowledgements

We thank the Alberta Heart and Stroke Foundation for financial support of this work.

References

Adam, L.P., Hathaway, D.R., 1993. Identification of mitogen-activated protein kinase phosphorylation sequences in mammalian h-caldesmon. FEBS Lett. 322, 56–60.

Adam, L.P., Gapinski, C.J., Hathaway, D.R., 1992. Phosphorylation sequences in h-caldesmon from phorbol ester-stimulated canine aortas. FEBS Lett. 303, 223–226.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y., 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. 262, 5592–5595.

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., Saltiel, A.R., 1995. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J. Biol. Chem. 270, 27489–27494.

Auch-Schwelk, W., Katusic, Z.S., Vanhoutte, P.M., 1989. Contractions to oxygen-derived free radicals are augmented in the aorta of the spontaneously hypertensive rat. Hypertension 13, 859–864.

Baas, A.S., Berk, B.C., 1995. Differential activation of mitogen-activated protein kinases by H_2O_2 O_2 in vascular smooth muscle cells. Circ. Res. 77, 29–36.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Childs, T.J., Watson, M.H., Sanghera, J.S., Campbell, D.L., Pelech, S.L., Mak, A.S., 1992. Phosphorylation of smooth muscle caldesmon by mitogen-activated protein (MAP) kinase and expression of MAP kinase in differentiated smooth muscle cells. J. Biol. Chem. 267, 22853–22859.

Cook, D.A., Vollrath, B., 1995. Free radicals and intracellular events associated with cerebrovascular spasm. Cardiovasc. Res. 30, 493–500.

Cook, D.A., Luong, T., Doi, M., Vollrath, B., 1993. Prolonged activation of protein kinase C in oxyhemoglobin-induced vasoconstriction in canine basilar artery. In: Findlay, J.M. (Ed.), Cerebral Vasospasm. Elsevier, Amsterdam, pp. 125–128.

Cooper, J.A., Howell, B., 1993. The when and how of Src regulation. Cell 73, 1051–1054.

Crews, C.M., Alessandrini, A., Erikson, R.L., 1992. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science 258, 478–480.

Devary, Y., Gottlieb, R.A., Smeal, T., Karin, M., 1992. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell 71, 1081–1091.

Dikic, I., Tokiwa, G., Lev, S., Courtneige, S.A., Schlessinger, J., 1996. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature (London) 383, 547–550.

Di Salvo, J., Steusloff, A., Semenchuk, L., Satoh, S., Kolquist, K., Pfitzer, G., 1993. Tyrosine kinase inhibitors suppress agonist-induced contraction in smooth muscle. Biochem. Biophys. Res. Commun. 190, 968–974.

Khalil, R.A., Morgan, K.G., 1993. PKC-mediated redistribution of mitogen-activated protein kinase during smooth muscle cell activation. Am. J. Physiol. 265, C406–C411.

Kooy, N.W., Royall, J.A., Ischiropoulos, H., Beckman, J.S., 1994. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic. Biol. Med. 16, 149–156.

Kyriakis, J.M., Avruch, J.S., 1996. Sounding the alarm: protein kinase cascades activated by stress and inflammation. J. Biol. Chem. 271, 24313–24316.

Lander, H.M., 1997. An essential role for free radicals and derived species in signal transduction. FASEB J. 11, 118–124.

Lander, H.M., Ogiste, J.S., Teng, K.K., Novogrodsky, A., 1995. $p21^{ras}$ as a common signalling target of reactive free radicals and cellular redox stress. J. Biol. Chem. 270, 21195–21198.

Lander, H.M., Milbank, A.J., Tauras, J.M., Hajjar, D.P., Hempstead, B.L., Schwartz, G.D., Kraemer, R.T., Mirza, U.A., Chait, B.T., Campbell-Burk, S., Quilliam, L.A., 1996. Redox regulation of cell signalling. Nature (London) 381, 380–381.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J.M., Plowman, G.D., Rudy, B., Schlessinger, J., 1995. Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. Nature (London) 376, 737.

Macdonald, R.L., Weir, B.K.A., 1994. Cerebral vasospasm and free radicals. Free Radic. Biol. Med. 16, 633–643.

Macdonald, R.L., Weir, B.K.A., Runzer, T.D., Grace, M.G.A., Findlay, J.M., Saito, K., Cook, D.A., Mielke, B.W., Kanamaru, K., 1991. Etiology of cerebral vasospasm in primates. J. Neurosurg. 75, 415–424.

Martin, W., Villani, G.M., Jothianandan, D., Furchgott, R.F., 1985. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation of rabbit aorta by certain ferrous hemoproteins. J. Pharmacol. Exp. Ther. 23, 679–685.

Mayberg, M.R., Okada, T., Bark, D.H., 1990a. The role of hemoglobin in arterial narrowing after SAH. J. Neurosurg. 72, 634–640.

Mayberg, M.R., Okada, T., Bark, D.H., 1990b. The significance of morphological changes in cerebral arteries after subarachnoid hemorrhage. J. Neurosurg. 72, 626–633.

Onoue, H., Kaito, N., Akiyama, M., Tomii, M., Tokudome, S., Abe, T., 1995. Altered reactivity of human cerebral arteries after subarachnoid hemorrhage. J. Neurosurg. 83, 510–515.

Pluta, M.R., Afshar, J.K.B., Boock, R.J., Oldfield, E.H., 1998. Temporal changes in perivascular concentrations of oxyhemoglobin after subarachnoid hemorrhage. J. Neurosurg. 88, 557–561.

Robinson, M.J., Cobb, M.H., 1997. Mitogen-activated protein kinase pathways. Curr. Opin. Cell Biol. 9, 180–186.

Sako, M., Nishikaro, J., Ohta, S., 1993. Role of protein kinase C in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage. J. Cereb. Blood Flow Metab. 13, 247–254.

Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K., Sasaki, T., 1995. Cloning and characterization of cell adhesion kinase β, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. J. Biol. Chem. 270, 21206–21219.

Seger, R., Krebs, E.G., 1995. The MAPK signalling cascade. FASEB J. 9, 726–735.

Seger, R., Ahn, N.G., Boulton, T.G., Yancopoulos, G.D., Panayotatos, N., Radziejewska, E., Ericksson, L., Bratlier, R.L., Cobb, M.H., Krebs, E.G., 1991. Microtubule-associated protein 2-kinases, ERK1 and ERK2 undergo autophosphorylation on both tyrosine and threonine residues: implication for their mechanism of activation. Proc. Natl. Acad. Sci. USA 88, 6142–6146.

Shishido, T., Suzuki, R., Qian, L., Hirokawa, K., 1994. The role of superoxide anions in the pathogenesis of cerebral vasospasm. Stroke 25, 864–868.

Steele, J.A., Stockbridge, N., Maljkovic, G., Weir, B., 1991. Free radicals mediate actions of oxyhemoglobin on cerebrovascular smooth muscle cells. Circ. Res. 68, 416–423.

Takanashi, Y., Weir, B.K.A., Vollrath, B., Kasuya, H., Macdonald, R.L., Cook, D., 1992. The time course of changes in the concentration of intracellular free calcium in cultured cerebrovascular smooth muscle cells after prolonged exposure to oxyhemoglobin. Neurosurgery 30, 346–350.

Takenaka, K., Goto, Y., Kassell, N.F., Lee, K.S., 1993. Modification of vascular smooth muscle proteins in rabbit basilar artery after subarachnoid hemorrhage. In: Findlay, J.M. (Ed.), Cerebral Vasospasm. Elsevier, Amsterdam, pp. 93–96.

Takuwa, Y., Matsui, T., Abe, Y., 1993. Alterations in protein kinase C activity and membrane lipid metabolism in cerebral vasospasm after subarachnoid hemorrhage. J. Cereb. Blood Flow Metab. 13, 409–415.

Varsos, V.G., Liszczak, T.M., Zervas, N.T., 1983. Delayed cerebral vasospasm is not reversible by aminophylline, nifedipine, or papaverine in a 'two-hemorrhage' canine model. J. Neurosurg. 58, 11–17.

Vollrath, B., Weir, B.K.A., Macdonald, R.L., Cook, D.A., 1994. Intracellular mechanisms involved in the responses of cerebrovascular smooth muscle cells to hemoglobin. J. Neurosurg. 58, 261–268.

Vollrath, B., Chan, P., Findlay, J.M., Cook, D.A., 1995. Lazaroids and deferoxamine attenuate the intracellular effects of oxyhaemoglobin in vascular smooth muscle. Cardiovasc. Res. 30, 619–626.

Wiseman, H., Halliwell, B., 1996. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem. J. 313, 17–29.