

# SAH-Induced MMP Activation and $K_v$ Current Suppression is Mediated Via Both ROS-Dependent and ROS-Independent Mechanisms

Masayo Koide and George C. Wellman

**Abstract** Voltage-gated potassium ( $K_v$ ) channels regulate cerebral artery tone and have been implicated in subarachnoid hemorrhage (SAH)-induced pathologies. Here, we examined whether matrix metalloprotease (MMP) activation contributes to SAH-induced  $K_v$  current suppression and cerebral artery constriction via activation of epidermal growth factor receptors (EGFRs). Using patch clamp electrophysiology, we observed that  $K_v$  currents were selectively decreased in cerebral artery myocytes isolated from SAH model rabbits. Consistent with involvement of enhanced MMP and EGFR activity in SAH-induced  $K_v$  current suppression, we found that: (1) oxyhemoglobin (OxyHb) and/or the exogenous EGFR ligand, heparin-binding EGF-like growth factor (HB-EGF), failed to induce further  $K_v$  current suppression after SAH and (2) gelatin zymography detected significantly higher MMP-2 activity after SAH. The removal of reactive oxygen species (ROS) by combined treatment with superoxide dismutase (SOD) and catalase partially inhibited OxyHb-induced  $K_v$  current suppression. However, these agents had little effect on OxyHb-induced MMP-2 activation. Interestingly, in the presence of a broad-spectrum MMP inhibitor (GM6001), OxyHb failed to cause  $K_v$  current suppression. These data suggest that OxyHb suppresses  $K_v$  currents through both ROS-dependent and ROS-independent pathways involving MMP activation. The ROS-independent pathway involves activation of MMP-2, whereas the ROS-dependent pathway involves activation of a second unidentified MMP or ADAM (a disintegrin and metalloprotease domain).

**Keywords**  $K^+$  channels • Heparin-binding EGF-like growth factor (HB-EGF) • Parenchymal arteriole • Patch clamp • Vascular smooth muscle • Vasospasm

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## Introduction

Subarachnoid hemorrhage (SAH) following cerebral aneurysm rupture is associated with substantial morbidity and mortality and existing therapeutic options have limited efficacy. A major contributor to poor outcome is delayed cerebral ischemia (DCI) manifesting 4–10 days after aneurysm rupture. Despite decades of study, mechanisms contributing to SAH-induced DCI remain controversial. Factors contributing to the development of DCI after SAH may include early brain injury, cortical spreading depression, disruption of the blood-brain barrier, activation of inflammatory pathways, and enhanced constriction of brain surface arteries/arterioles and intracerebral arterioles [5, 8, 16–18].

The membrane potential of cerebral artery myocytes is a key regulator of vascular diameter, with membrane potential depolarization leading to an increase in the open-state probability of voltage-dependent  $Ca^{2+}$  channels, enhanced  $Ca^{2+}$  entry, and vasoconstriction [15]. Studies using intracellular microelectrodes to measure smooth muscle membrane potential in intact cerebral arteries have found enhanced membrane potential depolarization concomitant with enhanced constriction in tissue from SAH model animals [6, 16, 22]. Voltage-gated potassium ( $K_v$ ) channels play an important role in the regulation of smooth muscle membrane potential and arterial diameter with decreased  $K_v$  channel activity leading to membrane potential depolarization [1, 3, 4]. Evidence indicates that  $K_v$  current suppression contributes to enhanced membrane potential depolarization and constriction of cerebral arteries isolated from SAH model animals [7, 11, 20, 22]. Further, we have previously demonstrated that acute application of the blood component oxyhemoglobin (OxyHb) leads to matrix metalloprotease (MMP) activation, shedding of heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), epidermal growth factor receptor (EGFR) activation, and  $K_v$  channel suppression via internalization [11]. However, the mechanism underlying OxyHb-induced MMP activation and HB-EGF shedding is unclear. The objec-

tive of this study was to examine the contribution of reactive oxygen species (ROS) on enhanced MMP activity and  $K_v$  current suppression in cerebral artery myocytes after SAH.

## Materials and Methods

### Rabbit Double-Hemorrhage SAH Model

As previously described, two injections of unheparinized autologous arterial blood (3 mL) were delivered via the cistern magna onto the brain surface of anesthetized rabbits at an interval of 48 h [7, 8, 10]. Five days after the initial surgery, rabbits were euthanized and posterior cerebral and cerebellar arteries (100–200- $\mu$ m diameter) were isolated from the brain surface for in vitro studies. All experiments were conducted in accordance with The Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996) and followed protocols approved by the Institutional Animal Care and Use Committee of the University of Vermont.

### Artery Diameter Measurements

Freshly isolated arteries were cannulated and pressurized to 60 mmHg, superfused with artificial cerebrospinal fluid (aCSF) and diameter measurements obtained using video edge-detection equipment [8, 16]. Constriction (tone) is expressed as percent decrease from maximum diameter obtained using  $Ca^{2+}$ -free aCSF with 100  $\mu$ M diltiazem and 1  $\mu$ M forskolin.

### Patch Clamp Electrophysiology

Whole-cell  $K^+$  currents were measured using the conventional whole-cell configuration of the patch-clamp technique [7, 10, 11]. Outward currents were elicited by 800-ms depolarizing voltage steps from a holding potential of  $-70$  to  $+50$  mV [7, 11]. The bath solution contained (in mM): 134 NaCl, 6 KCl, 1  $MgCl_2$ , 0.1  $CaCl_2$ , 10 glucose, and 10 HEPES (pH 7.4). Patch pipettes (3–5  $M\Omega$ ) were filled with an internal solution that contained (in mM): 87 potassium aspartate, 20 KCl, 1  $CaCl_2$ , 1  $MgCl_2$ , 10 HEPES, 10 EGTA, and 25 KOH (pH 7.2). Inwardly rectifying  $K^+$  ( $K_{IR}$ ) channel currents were measured as 100- $\mu$ M barium-sensitive currents using voltage ramps from  $-100$  to  $+40$  mV [23]. For  $K_{IR}$  recordings, the bath solution contained (in mM): 140 KCl, 1  $MgCl_2$ , 0.1  $CaCl_2$ , 10 glucose, and 10 HEPES (pH 7.4) and the patch pipette contained (in mM): 87 potassium aspartate, 20 KCl, 1  $CaCl_2$ , 1  $MgCl_2$ , 10 HEPES, 10 EGTA,

and 25 KOH (pH 7.2). For all recordings, cell capacitance was not different between groups (control:  $10.9 \pm 0.4$  pF,  $n=37$ ; SAH:  $11.3 \pm 0.3$  pF,  $n=30$ ). Current density was calculated by dividing the  $K^+$  current by the cell capacitance.

### Zymography

MMP activity was measured using gelatin zymography [11]. Cerebral arteries were homogenized in gel loading buffer (100 mM Tris, 2 % SDS and 20 % glycerol). Lysate (15  $\mu$ g protein, quantified by Bradford assay) was applied to a 10 % polyacrylamide gel copolymerized with the MMP2/9 substrate, gelatin (1 mg/mL). After electrophoresis, the gel was rinsed overnight then incubated at 37 °C for 20 h to allow gelatinolytic activity. The gel was stained with Coomassie Brilliant Blue and MMP activity was detected as unstained bands against the background of the blue-stained gelatin [11].

### PCR

Expression of mRNA was examined by semi-quantitative RT-PCR [9]. Total RNA was extracted from intact cerebral arteries, and converted into cDNA. Amplification of cDNA was performed using Taq DNA polymerase (GenScript) and primers for MMP-2 (forward: 5'-CCG TGT GAA GTA TGG CAA TG-3', reverse: 5'-CGT AGA GCT CTT GAA TGC CC-3'). Band intensity in the linear range of amplification was normalized to band intensity for 18S ribosomal RNA.

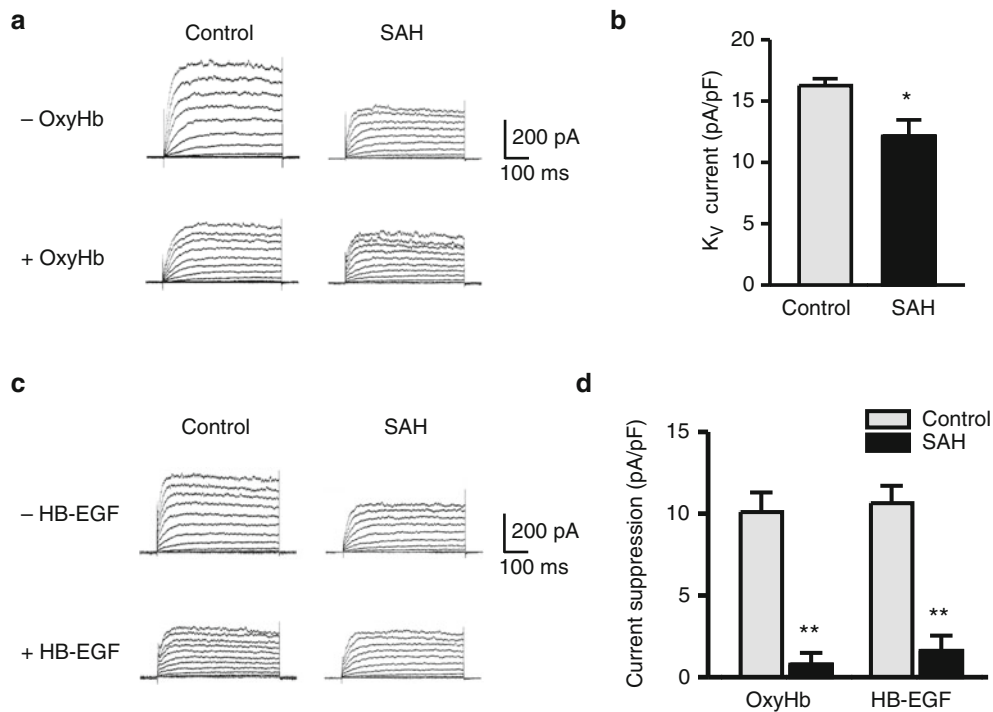
### Statistical Analysis

Data are expressed as mean  $\pm$  SEM with n representing the number of cells or samples per group. Student's paired or unpaired *t*-test were used to determine statistical significance at the level of  $P < 0.05$  (\*) or  $P < 0.01$  (\*\*).

## Results

### Selective Suppression of $K_v$ Channel Function After SAH Involves EGF Receptor Activation

Whole-cell voltage-dependent and inwardly rectifying  $K^+$  currents were examined in cerebral artery myocytes freshly isolated from control and SAH model rabbits



**Fig. 1** OxyHb and HB-EGF suppressed  $K_V$  currents of cerebral artery myocytes from control animals but not from SAH model animals. (a) Example of whole-cell  $K^+$  currents before and after 10 min application of OxyHb (10  $\mu$ M) to cerebral artery myocytes isolated from control and SAH model animals. (b) Summary of 4-AP-sensitive  $K_V$  currents obtained from control ( $n=6$ ) and SAH model ( $n=4$ ) animals. (c) Examples of whole-cell  $K^+$  currents before and after 10 min applica-

tion of HB-EGF (30 ng/ml) to cerebral artery myocytes isolated from control and SAH model animals. (d) Summary data demonstrating that OxyHb and HB-EGF significantly suppressed  $K_V$  currents in cerebral artery myocytes from control, but not SAH model animals. OxyHb treatment: control  $n=7$ , SAH  $n=4$ ; HB-EGF treatment: control  $n=6$ , SAH  $n=5$ . \*\* $P < 0.01$  vs control, unpaired Student's  $t$ -test

using the conventional whole-cell configuration of the patch-clamp technique. Whole-cell voltage-dependent  $K^+$  currents were measured during 10-mV voltage steps from a holding potential of  $-70$  mV and represent the combined activity of large-conductance  $Ca^{2+}$ -activated (BK) and voltage-gated ( $K_V$ )  $K^+$  channels. As illustrated in Fig. 1a, the current amplitude of whole-cell composite  $K^+$  currents was reduced in cerebral artery myocytes from SAH model animals. To separate BK and  $K_V$  currents, selective blockers of BK channels (paxilline, 1  $\mu$ M) and  $K_V$  channels (4-aminopyridine, 4-AP, 10 mM) were applied to myocytes isolated from control and SAH model animals. The current densities of paxilline-sensitive BK currents were similar in myocytes isolated from both groups; controls:  $6.5 \pm 0.7$  pA/pF at +50 mV ( $n=6$ ) and SAH:  $5.6 \pm 0.6$  pA/pF at +50 mV ( $n=4$ ). In marked contrast, 4-AP-sensitive  $K_V$  currents were significantly decreased in myocytes from SAH animals ( $12.1 \pm 1.4$  pA/pF at +50 mV,  $n=4$ ) compared with myocytes from control animals ( $16.3 \pm 0.6$  pA/pF at +50 mV,  $n=6$ ) (Fig. 1b). Inwardly rectifying  $K^+$  ( $K_{IR}$ ) channel currents, determined as inward currents sensitive to 100  $\mu$ M  $Ba^{2+}$  using voltage ramps from  $-100$  to +40 mV, were not significantly different between

groups. For example,  $Ba^{2+}$ -sensitive current densities at  $-100$  mV were  $-5.4 \pm 0.9$  pA/pF and  $-5.2 \pm 0.6$  pA/pF in myocytes from control ( $n=24$ ) and SAH ( $n=23$ ) animals, respectively. This data demonstrates that  $K_V$  currents are selectively suppressed in cerebral artery myocytes from SAH model rabbits. Consistent with SAH-induced  $K_V$  current suppression, constrictions to 4-AP (10 mM) were significantly reduced in arteries isolated from SAH ( $17.4 \pm 3.7$  % decrease in diameter,  $n=4$ ) model animals compared with control ( $38.9 \pm 6.2$  % decrease in diameter,  $n=4$ ) animals.

Our previous work has demonstrated that acute application of the blood component OxyHb decreased  $K_V$  currents in cerebral artery myocytes through a mechanism involving HB-EGF shedding and EGFR activation [11]. As with this previous work, OxyHb decreased  $K_V$  currents in myocytes isolated from control animals. However, OxyHb failed to reduce  $K_V$  currents obtained from SAH animals (Fig. 1a, d); indicating that acute application of OxyHb and 4-day exposure of subarachnoid blood in vivo may work through a common mechanism to decrease  $K_V$  channel activity. Further, exogenous application of HB-EGF mimicked the actions of acute application of OxyHb, causing a reduction in  $K_V$

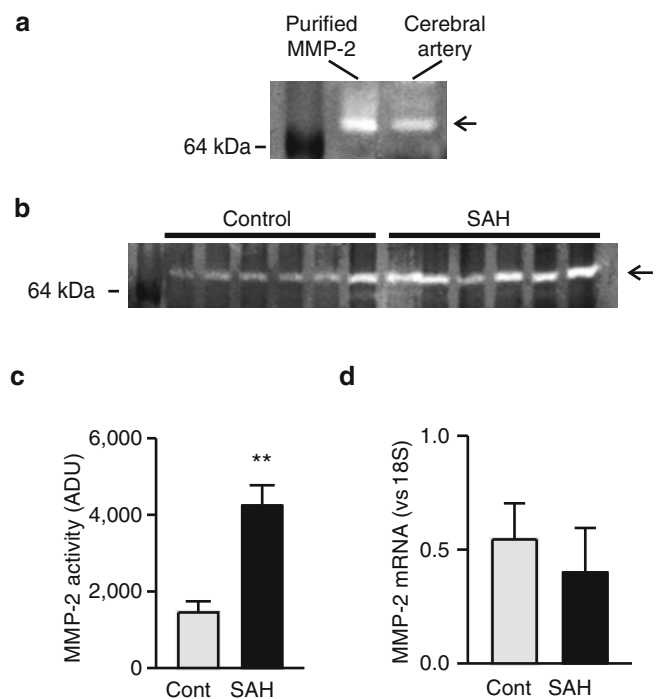
currents in myocytes obtained from control, but not SAH model animals (Fig. 1c, d). This data suggests SAH-induced  $K_V$  suppression involves HB-EGF shedding and EGFR activation in a manner similar to that caused by OxyHb.

### MMP-2 Activity Is Enhanced in Cerebral Arteries from SAH Model Animals

The matrix metalloprotease subtype, MMP-2, has been implicated in HB-EGF shedding and mesenteric artery constriction [12]. In addition, our previous work has demonstrated that OxyHb increases MMP-2 activity in cerebral artery homogenates from control animals [11]. To examine if increased MMP-2 activity may be involved in SAH-induced HB-EGF shedding and  $K_V$  current suppression, zymography using the MMP-2 substrate gelatin was performed using cerebral artery homogenates from control and SAH model animals. Gelatin zymography from cerebral artery homogenates produced a single 65-kDa band similar to commercially purified MMP-2 (Fig. 2a). Interestingly, MMP-2 band intensity was significantly greater in homogenates from SAH model animals (Fig. 2b, c). Although MMP-2 activity was increased after SAH, mRNA levels of MMP-2 were similar in cerebral arteries from control and SAH model animals (Fig. 2d). This data demonstrates that MMP-2 activity, but not expression, is increased in cerebral arteries after SAH in a manner similar to that observed with acute application of OxyHb.

### Oxyhemoglobin Activates MMPs and Suppresses $K_V$ Currents Via ROS-Dependent and ROS-Independent Pathways

To examine whether reactive oxygen species (ROS) such as superoxide anions ( $O_2^-$ ) contribute to increased MMP activity caused by OxyHb, studies were performed in cerebral artery myocytes from control animals using a combination of superoxide dismutase and catalase. Superoxide dismutase catalyzes the conversion of superoxide anions ( $O_2^-$ ) into oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ), whereas catalase converts  $H_2O_2$  to  $H_2O$  and  $O_2$ . The combination of superoxide dismutase (150 U/mL) and catalase (500 U/mL) decreased OxyHb-induced  $K_V$  suppression by approximately 40% (Fig. 3a, b). In comparison, the broad-spectrum MMP inhibitor, GM6001 (10  $\mu$ M), caused a substantially greater decrease in OxyHb-induced  $K_V$  suppression of nearly 80%. These findings suggest that ROS partially mediates OxyHb-induced  $K_V$  suppression; however, ROS-independent MMP activation also contributes to suppression of  $K_V$  currents by

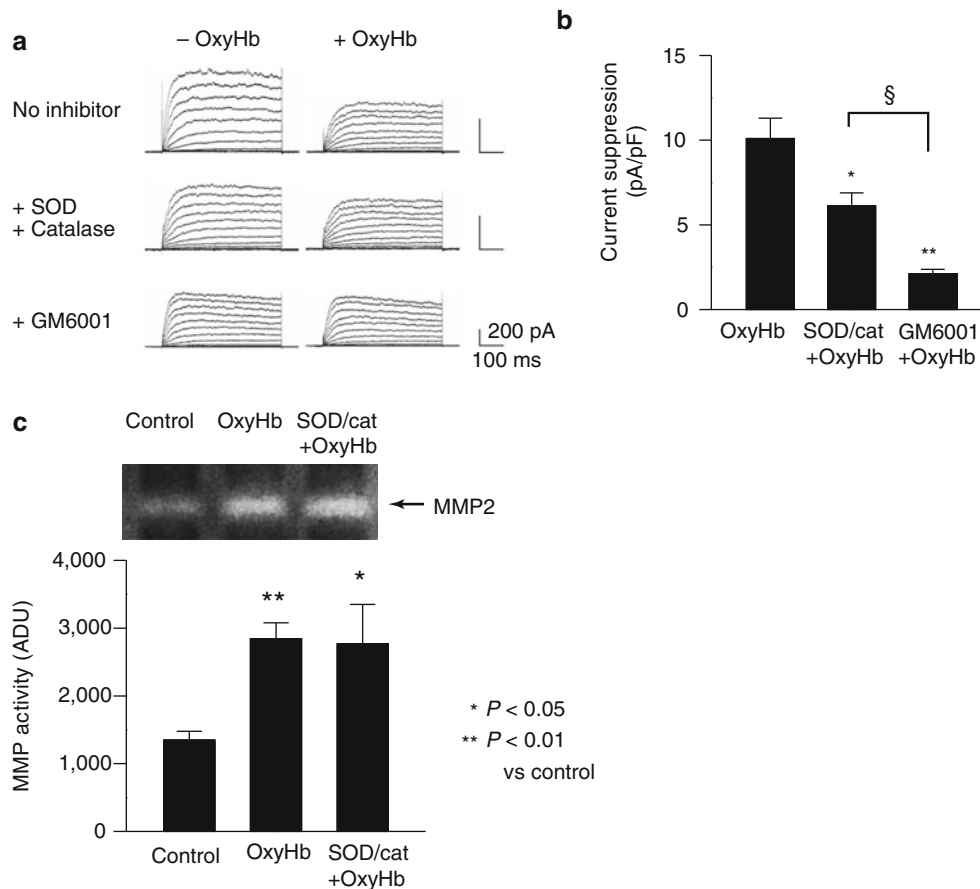


**Fig. 2** MMP-2 activity is enhanced in cerebral arteries from SAH model animals. (a) Gelatin zymography demonstrating activity of commercially purified MMP-2 and cerebral artery homogenate from a control animal. (b) Gelatin zymography demonstrating enhanced MMP-2 activity in homogenates obtained from SAH model animals. (c) Summary data showing significantly greater MMP-2 activity in cerebral artery homogenates from SAH model animals compared with cerebral artery homogenates from unoperated control animals ( $n=8$  for each). (d) Summary data demonstrating that MMP-2 mRNA levels are similar in cerebral artery homogenates obtained from control and SAH model animals ( $n=7$  for each). \*\* $P<0.01$  vs control, unpaired Student's  $t$ -test

OxyHb. Further, as illustrated in Fig. 3c, OxyHb-induced MMP-2 activity was not altered by superoxide dismutase and catalase. These findings indicate that both ROS-dependent and ROS-independent MMP activation contribute to OxyHb-induced  $K_V$  current suppression and that OxyHb increases MMP-2 activity independently of ROS generation.

### Discussion

The present study indicates that in vivo administration of subarachnoid blood or acute ex vivo application of OxyHb act via multiple pathways that converge to induce HB-EGF shedding and  $K_V$  current suppression in cerebral artery myocytes. We provide evidence that one of these pathways involves activation of MMP-2 via a mechanism independent of ROS generation and that a second pathway involves ROS-dependent activation of a matrix metalloprotease (MMP) or a disintegrin metalloprotease (ADAM) distinct from MMP-2. The combination of these ROS-dependent and ROS-independent mechanisms and the resultant shedding



**Fig. 3** ROS-dependent and ROS-independent MMP activation and  $K_V$  current suppression caused by exogenous OxyHb. **(a)** Representative  $K_V$  current recordings demonstrating the ability of the free radical scavengers superoxide dismutase (SOD) and catalase or the MMP inhibitor, GM6001 to reduce OxyHb-induced  $K_V$  current suppression. **(b)** Summary data demonstrating that GM6001 caused a greater inhibition of OxyHb-induced  $K_V$  current suppression than SOD/catalase. \* $P < 0.05$ ; \*\* $P < 0.01$  SOD/catalase and GM6001 on OxyHb-induced  $K_V$

suppression ( $n = 7$ ); § $P < 0.05$  GM6001 ( $n = 5$ ) vs. SOD/catalase treatment on OxyHb-induced  $K_V$  suppression ( $n = 5$ ). ANOVA followed by Tukey test. **(c)** Representative gel and summary of zymography data demonstrating that SOD/catalase treatment did not prevent OxyHb-induced MMP-2 activation. \* $P < 0.05$ ; \*\* $P < 0.01$  versus control. (Control:  $n = 8$ , OxyHb:  $n = 8$ , SOD/cat + OxyHb:  $n = 4$ ) ANOVA followed by Tukey test

of HB-EGF and EGFR activation account for the selective suppression of  $K_V$  channels in cerebral artery myocytes from SAH model animals.

Our observation that OxyHb does not cause additional suppression of  $K_V$  currents in myocytes isolated from SAH model animals indicates that OxyHb is the blood component largely responsible for reduced  $K_V$  currents leading to enhanced cerebral artery constriction after SAH. Consistent with this concept, both OxyHb and subarachnoid blood suppress  $K_V$  currents through a pathway involving MMP activation, HB-EGR shedding, and EGFR activation. Our present findings also indicate that OxyHb activates at least two distinct MMPs or ADAMs responsible for HB-EGF shedding—MMP-2 and an unidentified additional MMP/ADAM. It also appears that ROS are involved in the activation of the unidentified MMP/ADAM, but not MMP-2. The oxidation of OxyHb to methemoglobin releases  $O_2^-$  and secondarily leads to the production of hydroxyl radicals [14, 21]. Other studies

[2, 13, 19] have demonstrated that these ROS can increase activity and expression of MMPs, including MMP-2. However in the present study, super oxide dismutase and catalase, scavengers of  $O_2^-$  and  $H_2O_2$ , did not prevent OxyHb-induced stimulation of MMP-2 activity (Fig. 3). This finding indicates that OxyHb can also act independently of ROS to enhance MMP-2 activity. Future studies are needed to determine the mechanism of ROS-independent MMP-2 activation and to determine the identity of additional MMPs/ADAMs involved in OxyHb-induced  $K_V$  current suppression.

## Conclusions

Enhanced cerebral artery constriction represents one component of the multifactorial and interrelated series of pathological events leading to DCI in patients after aneurysmal SAH.

Our data indicate that OxyHb contributes to SAH-induced cerebral artery constriction via activation of multiple MMPs/ADAM, leading to HB-EGF shedding and  $K_v$  current suppression in cerebral artery myocytes. Evidence is also provided that ROS-dependent and ROS-independent pathways are involved in OxyHb-induced MMP activation. These findings suggest that SAH-induced MMP/ADAM activation may play an important role in the development of DCI after SAH and represent a new target for therapies to alleviate the detrimental consequences of cerebral aneurysm rupture.

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**Conflict of Interest Statement** We declare that we have no conflict of interest.

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