Hemolysed blood elicits - calcium antagonist and high CO_2 reversible - constrictions via elevation of Ca^{2+} in isolated cerebral arteries

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Abstract

During acute subarachnoid hemorrhage blood is hemolysed, which is followed by a significant cerebrovascular spasm resulting in serious clinical condition. Interestingly, however the direct vasomotor effect of perivascular hemolysed blood (HB) has not yet been characterized preventing the assessment of contribution of vasoconstrictor mechanisms deriving from brain tissue and/or blood and development of possible treatments. We hypothesized that perivascular HB reduces the diameter of the cerebral arteries (BA: basilar artery; MCA: middle cerebral artery) via elevating vascular tissue $[Ca^{2+}]_i$ level. Vasomotor responses were measured by videomicroscopy and intracellular Ca^{2+} by the fura2-AM ratiometric method. Adding HB to the vessel chamber reduced the diameter significantly (BA: from 264±7 μm to 164±11μm; -24±3 % of PD; MCA: from 187±14 μm to 155±14μm), which was reversed to control level by wash-out of HB. Potassium chloride (KCl), HB, serum, hemolysed red blood cell (RBC), plasma and platelet suspension (PLTs), elicited significant constrictions of isolated basilar arteries. There was a significant increase in K^+ concentration in hemolysed HB (7.02±0.22 mmol/L) compared to whole blood (6.20±0.01 mmol/L). Before HB, acetylcholine (ACh), sodium-nitroprussid (SNP), nifedipin, and CO_2 elicited substantial dilations in cerebral arteries. In contrast, in the presence of HB dilations to ACh, SNP, decreased, but not to nifedipine and CO_2. After washout of HB, NO-mediated dilations remained significantly reduced compared to control. HB significantly increased the ratiometric Ca-signal, which returned to control level after washout.

In conclusion, perivascular hemolysed blood elicits significant - nifedipine and high CO_2 reversible - constrictions of isolated basilar and middle cerebral arteries, primarily via increasing intracellular Ca^{2+}, findings, which can contribute to the refinement of local treatment of subarachnoid hemorrhage.

Introduction

Clinical and experimental studies showed that acute subarachnoid hemorrhage due to traumatic brain injury or stroke[1] is followed by serious local vasospasm[2], which can severely reduce regional cerebral blood flow, with the consequent loss of brain function. Proper resistance (i.e. diameter) of cerebral arteries plays an important role in maintaining continuous blood supply of the brain to preserve its functions.[1, 3, 4] Disturbances of cerebrovascular autoregulation[3] may occur as a result of intracranial hemorrhage and brain injury, as well. The hemolysed blood (HB) then can affect local tissues (neurons, glia cells, vascular cells, etc.), but primarily impairs the regulation of cerebrovascular tone endangering maintenance of normal flow to brain and thus functions.[5, 6 7, 8] Increased vascular contractility to hemolysed blood may be attributed to endothelial dysfunction and/or increased contractility of vascular smooth muscle.[9] However, in such conditions several cell types can be involved in the pathological regulation of vascular resistance in addition to HB. Earlier experiments showed that purified hemoglobin induces vasoconstriction[10] of cerebral vessels, which was explained by its nitric oxide scavenging effect.[11] However, the vasoconstrictor effects of perivascular hemolysed blood (HB), which is present in vivo during hemorrhage and traumatic brain injury have not yet been fully characterized.[12] We hypothesized that perivascular HB reduces the diameter of the cerebral vessels via elevating $[Ca^{2+}]_i$ levels.

In order to test this hypothesis we have utilized isolated basilar and middle cerebral arteries of rat, known to be importantly involved in the blood supply of brain and allowing us to single out the vasomotor effects of hemolysed blood without the interference of other mechanisms associated with tissue hemorrhage and traumatic brain injury.

Materials and Methods

Animals

For these experiments ~2 months-old (250±50 g) male Wistar rats (Crl:WI, Charles River Hungary Kft; n=6-12 in each group) were used. Animals were housed on a 12h light/dark cycle and were ad-libitum fad on standard rat chow and free access to tap water. All experiments and interventions were undertaken according to the general rules and special approval of the University of Pecs Ethical Committee for the Protection of Animals in Research (BA 02/2000-8/2008), in accordance with the directives of the National Ethical Council for Animal Research and those of the EU Directive (2010/63/EU), in accordance with the ARRIVE guidelines.

Isolation of cerebral arteries and measurements of diameter

Cerebral vessels were isolated as previously described.[13-15] In brief, animals were anesthetized by ether and decapitated according to Institutional Animal Care and Use Committee of University of Pecs, Medical School, Pecs, Hungary. The brains were immediately removed and placed in Krebs' buffer. Basilar arteries (BA) and middle cerebral arteries (MCA) were isolated from the brain of each animal. Segments of the BAs and MCAs were isolated using microsurgery instruments. Both ends of the arteries were mounted onto two glass micropipettes in a vessel chamber and pressurized to 80 mmHg with zero flow. The hydrodynamic resistances of the micropipettes were matched. Inflow and outflow pressures were controlled and measured by a pressure servo-control system (Living Systems Instrumentation, Burlington, VT, USA). Inner vascular diameter was measured with a video-micrometer system and continuously recorded using a computerized data acquisition system (LabChart 7 pro by PowerLab, ADInstruments, Australia). All vessels were allowed to stabilize for 60 min in oxygenated (21% O_2; 5% CO_2;

74% N_2) Krebs' buffer (at 37°C). After the equilibration period, during which spontaneous myogenic tone developed (measured as a basal diameter; BD), and the vascular responses were assessed, as reported previously.[15-17] At the end of each experiment the passive diameters (PD) of the vessels were measured at 80 mmHg intraluminal pressure in the presence of Ca^{2+}-free Krebs' buffer containing the L-type Ca^{2+} channel inhibitor nifedipine (10^{-4} mol/L) to achieve maximal vasodilatation.

Administration of Vasoactive Agents and Inhibitors

The vasomotor effect of perivascular blood was investigated by adding autologous hemolysed whole blood (HB) directly into the vessel chamber. Hemolysed whole blood (200 µL) was prepared by osmolysis from 40 µL whole blood (B) and 160 µL bidestillated water (DW) at ratio B:DW=1:4. In other series of experiments vasomotor function of cerebral arteries were studied in response to blood components, such as blood serum, blood plasma, hemolysed red blood cell (RBC), platelet concentrate (PLTc), platelet suspension (PLTs) and purified hemoglobin (Hgb). For testing the receptor-independent vasoconstriction 60 mmol/L KCl was use

endothelial function, was tested by vascular responses to acetylcholine (ACh, 10^{-4} mol/L), whereas that of smooth muscle by sodium nitroprusside (SNP; 10^{-4} mol/L) and the L-type Ca^{2+} channel inhibitor nifedipine (10^{-6} mol/L), which was also used to assess the passive diameter (PD) of arteries (10^{-4} mol/L).

To assess the vasodilator effect of carbon dioxide (CO_2), normal (5% CO_2; 21% O_2; 74% N_2) and elevated CO_2 (15% CO_2; 21% O_2; 64% N_2) gas mixture were used to bubble Krebs' buffer (for 5 minutes; at 37°C; n=10) in vessel chamber. All drugs were purchased from Sigma Aldrich (Budapest, Hungary).

Potassium concentration was measured by Nova Biomedical pHOx plus blood gas analyzer (Massachusetts, USA).

Assessment of intravascular calcium ion level

As described previously[19] changes in intracellular Ca^{2+}-ion concentration were assessed with ratiometric (R) calcium-measurement at the wavelength of 340 nm and 380 nm using 5-Oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-, (acetyloxy)methyl ester (Fura2-AM; Invitrogen, Life Technologies, Budapest, Hungary) fluorescent dyes.[20, 21] The physiological Krebs solution was supplemented with Fura2-AM (5 µmol/L) fluorescent Ca^{2+} indicator dye and BSA (bovine serum albumin; 1%) for 60 min during which spontaneous myogenic tone developed. We have used fluorescent microscope to measure ar Ca^{2+} concentrations by an IncyteIm2 instrument (Intracellular Imaging Inc, Cincinnati, OH, USA) by recording images (cut off >510 nm) excited alternatively by 340 and 380 nm wavelengths. Images were recorded every 4 s and evaluated offline. Arterial Ca^{2+} concentrations were detected by calculating ratios (R) between averaged signal intensity at 340 and 380 nm excitation in the whole arterial segment.

Statistical Analysis

Experimental results are presented as mean ± S.E.M. Data are expressed as either micrometer or percentage of basal [BD%] and passive diameter [PD%]. The changes in ratiometric intracellular calcium measurements are indicated either as ratio (R) or as a delta ratio (ΔR). Statistical analysis was performed by one-way ANOVA (Holm-Sidak method) or Student's t-test as appropriate by SPSS 11.0 for Windows software. P-values <0.05 were considered to be statistically significant. Figures were made by SigmaPlot 11.0 for Windows software..

Results

Effect of perivascular hemolysed blood and its components on the diameter of cerebral arteries

The basal diameter of BA was 264±7 µm and MCA was 185±15 µm in the presence of 80 mmHg intraluminal pressure, whereas the passive diameter of BA was 392±8 µm and MCA was 282±10 µm. Summary data (Fig. 1) shows that HB elicited significant constrictions of BA (top, 164±11µm, -23.9±3 of PD%) and also in MCA (bottom; 155±14µm, -11.4±0.8 of PD%). Importantly, after wash-out of HB the basal diameters of cerebral arteries reached level (BA: 288±12 µm; MCA: 195±12 µm).

Figure 2 shows that KCl (control: 255±18 µm, KCl: 170±20 µm; -21±2 of PD%), and HB elicited constrictions of cerebral arteries and at the same time there was a significant increase in K^+ concentration in hemolysed blood (HB 7.02±0.22 mmol/L) compared to whole blood (6.20±0.01 mmol/L).

Figure 3 shows summary data of diameter changes [PD%] of BA in response to hemolysed blood (HB), blood serum, hemolysed red blood cell (RBC), blood plasma, platelet suspension (PLTs), platelet concentration (PLTc) and hemoglobin (Hgb). HB (control: 264±7 µm, HB: 164±11µm, -23.9±3 of PD%), Blood serum (control: 246±8 µm, serum: 170±6 µm; -19±0.9 of PD%), the hemolysed red blood cell (RBC), (control: 217±9 µm, RBC: 166±6 µm; -14±1of PD%), blood plasma (control: 258±7 µm, plasma: 226±7 µm; -7,7±0,5 of PD%), platelet suspension (PLTs), (control: 191±15 µm, PLTs: 165±16 µm; -7,5±2 of PD%). Whereas, hemoglobin (Hgb) (control: 263±16 µm; Hgb 10^{-12} M: 263±15 µm; -0.27±2.11 of PD%; Hgb 10^{-6} M: 274±21 µm; -0.12±1.91 of PD%) and platelet concentrate (PLTc) (control: 188±11 µm, PLTc: 185±12 µm; -0.8±0.9 of PD%) did not affect the diameter in the present experimental conditions.

Changes in agonist-induced vasomotor responses to presence of HB

Responses were measured before (control), in the presence of HB and after wash-out of HB. Summary data shows in Figure 4 that in control, the ACh-induced dilations were 19.9±4.6 (% of basal diameter, BD%), presence of HB significantly decreased the dilation to 7.4±1.4 of BD% and after wash-out HB it remained at 5.7±1.7 of BD%). As Figure 4 shows, dilations to SNP in control were 26±2.6 of BD%, which was reduced to 11.8±1.7 of BD% by HB and remained at 13.9±2.2 of BD%. In contrast nifedipine-induced dilations were not significantly affected by HB: [illegible]e 32.6±5.1 of BD%, during HB 28.7±3.5 of BD%, after wash-out the HB 30±2.3 of BD%.

Reversal of HB-induced cerebrovascular constrictions in the presence of high CO_2 and nifedipine

Dilations to increased level of CO_2 were measured before (control), during (HB) and after (wash-out) of hemolysed blood (Fig. 4). High CO_2 elicited significant dilations in control (25.7±2.7 of BD%), which did not change significantly in the presence of HB (29.5± 1.7 of BD%), or after wash-out HB (27±3 of BD%). Similarly, nifedipine-induced dilations (control: 32.6±5.1 of BD%) were not affected by the presence of HB (28.7±3.5 of BD%) or after wash-out of HB (30.1±2.3 of BD%, respectively).

Changes in vascular $[Ca^{2+}]_i$ in response to HB

Summary data (Fig. 5) shows that perivascular HB elicited increases in the ratiometric (R) Ca signal in a concentration-dependent manner (by 20 µL steps from 0 µL up to 200 µL), indicating increase in intravascular $[Ca^{2+}]_i$ concentrations. In control conditions, before administration of HB the ratio was 1.118±0.043; 100 µL HB it increased to 1.352±0.019 (Δratio=0.154±0.013) and 200 µL HB it significantly increased to 1.397±0.016 (Δratio=0.211±0.022), respectively. The

HB-induced constrictions of basilar arteries paralleled with the increases in intracellular Ca^{2+} concentration. After wash-out the ratio significantly decreased (1.076±0.069; Δratio= -0.293±0.079) resulting in dilation.

Discussion

The salient findings of the present study are: 1) perivascular hemolysed blood elicited substantial constrictions of isolated basilar and middle cerebral arteries, 2) which corresponded with increases in vascular wall Ca^{2+}, and could be reversed by the calcium-channel antagonist nifedipine and increased level of CO_2. In addition it reduced agonists-induced dilations

HB elicits vasoconstriction both in basilar and middle cerebral arteries

In all experiments vessels developed myogenic tone [3, 13, 16, 17] (passive diameters vs. basal diameters), thus vasomotor capacity of both basilar (BA) and middle cerebral arteries (MCA) could be observed in the presence of optimal tone, without the use of pre-constrictor, which could interfere with cellular vasomotor mechanisms. The data show that addition of HB to the chamber caused significant constrictions in basilar arteries. Interestingly, after washout of HB, basal diameter returned to the control level (Fig. 1). Importantly smaller intracerebral arteries (middle cerebral artery) are also responded with constriction to HB. It is likely that even smaller arterial vessels are affected by HB as previous studies showed that myogenic tone of pial vessels were impaired even after washout of blood[22]. Nevertheless, HB may elicit vasomotor responses, which are region specific.

Potential mechanisms of reversal of HB-induced constrictions by high pCO_2

Interestingly, data reported in the literature regarding the effect and mediation of pCO_2-induced dilations of cerebral vessels are not unequivocal. For example, the nature of response (dilation or constriction) varied depending on the experimental conditions. The potential effect of changes in pH was supported by some, but refuted by other studies.[23-25] There were studies suggesting endothelial[26, 27] and nitric oxide mediations[28], and role for arachidonic acid metabolites[27, 29], SK_{Ca}/IK_{Ca} channels[27, 30] and also changes in vascular cell membrane polarization. [31, 32] Because of the above mentioned we felt it is important to establish the effects of pCO_2 on the vasomotor tone of isolated cerebral arteries, especially in the presence of HB; a condition in which the presence of in vivo confounding factors can be excluded. The finding that vasoconstrictor effect of HB can be reversed by wash-out of blood or decreasing intracellular Ca^{2+} concentration using locally applied Ca-channel antagonists, or increase locally perivascular pCO_2 suggest a key role for intracellular Ca^{2+} level rather than to calcium sensitivity. Also, it seems that high pCO_2 is powerful enough to overcome any constrictor mechanisms or factors operating during hemolysis of blood. We believe that extrapolating these experimental findings to clinical conditions may open up novel therapeutic avenues for subarachnoid hemorrhage especially the powerful effect of perivascular application of high pCO_2 should be explored and documented.

Proposed mechanisms of action of hemolysed blood (HB) and blood component-induced constrictions of cerebral arteries

Blood contains myriad of vasoactive components[9, 11, 12, 33] thus future studies need to single out the mechanisms finally leading to constriction. For example, blood serum via activating coagulation cascade may contain eicosanoids[34]/prostanoids[35], low molecule weight peptides (endothelin-1[36]) and thrombin.[37, 38] Blood plasma circulating with inactive coagulation factors has

less vasoactive properties than serum, but containing fibrinogen[39-41] or plasma protein[42] may result in vasoconstriction. Interestingly, while others[10, 11] demonstrated that hemoglobin causes vasoconstriction, we could not confirm it in isolated basilar arteries. Hemolysed red blood cell suspension induces vasoconstriction, which could be explained -in part- by released hemoglobin and bilirubin oxidation products[11] and potassium[12] ions (see Fig. 2) derived from de-compartmentalized RBC. Interestingly, while platelet concentration had no vasoconstrictor effect, platelet suspension elicited vasoconstriction (also see Fig. 3), likely due to release of thromboxane-A_2 from platelets.[43] In addition to these mechanisms, we propose a po

for high K^+ in HB-induced constrictions. During hemolysis high amount of K^+ is released from red blood cells, which can reach a constrictor level. Perivascular application of KCl shows (Fig. 2) that it can elicit substantial constrictions, similar to that of HB.

The finding that HB impaired the endothelium and smooth muscle nitric oxide pathways (Ach, SNP; Fig. 4) suggest in the presence of HB high level of K^+ effects directly the smooth muscle eliciting depolarization[22] and thus increases Ca^{2+} level and similar level of constriction as exogenous KCl. Nevertheless, it is a clinically relevant finding that washing out of blood reversed the constrictions (Fig. 1).

Effect of HB on agonists-induced dilations

Many previous studies[3, 44, 45] established that endothelium-derived factors are important in the modulation of vasomotor tone of cerebral arteries. Previous data showed that oxyhemoglobin induces significant constriction of cerebral arteries which was explained – in part - by binding nitric oxide (NO).[10, 11] On the other hand hemoglobin may act directly on smooth muscle cell by activating tyrosin-kinase thus inactivating voltage dependent potassium channels (K_V1,5).[22] In the presence of HB (Fig. 4), ACh- and SNP-induced dilations mechanisms were significantly reduced which, after washout of HB remained impair, suggesting that HB affects both endothelial

an smooth muscle NO-related mediations, which remain impaired even after washout of HB. These findings suggest that although HB-induced constrictions can be reversed, some of the important vasomotor mechanisms remain impaired, which may have clinical significance.

HB increases the level of vascular wall $[Ca^{2+}]_i$

Summary data (Fig. 5) shows that HB, in a concentration-dependent manner (by 20 µL steps from 0 µL up to 200 µL) increased the ratiometric (R) Ca signal indicating increase in ...ar $[Ca^{2+}]_i$ concentration[19-21]. Since we have found that HB elicited constrictions of basilar arteries, we hypothesized that regardless of proximal signaling pathways, HB by increasing the intravascular Ca^{2+} level, results in constrictions. Interestingly, wash-out of HB, significantly decreased the $[Ca^{2+}]_i$ reaching the control level. The findings regarding the parallel changes in the vascular $[Ca^{2+}]_i$ and the diameter suggests that the final signaling mechanism by which HB elicits constriction of cerebral arteries is an elevation of smooth muscle intracellular Ca^{2+} concentration.

Clinical importance

Searching for effective pharmaceutical treatments to improve cerebral blood flow in diseased conditions, such as hemorrhagic stroke [46] or traumatic brain injury (TBI)[47-49] is an ongoing clinical effort. In these conditions the resistance of cerebral vessel greatly increases reducing the regional blood supply of brain. Our findings that direct perivascular application of HB (without traumatic brain injury, and in the absence of neural or other tissue factors) elicited substantial constrictions, which however can be reversed by local application of calcium channel antagonist or high pCO2 suggest that they could be utilized in clinical area and may open up novel therapeutic possibilities for subarachnoid hemorrhage.

In conclusion, extravascular hemolysed blood elicits substantial constriction of cerebral arteries of different sizes by increasing the level of smooth muscle Ca^{2+}, which however could be reversed by perivascular administration of calcium antagonist and increasing CO_2 level. These findings could advance the development of novel therapies during hemorrhagic stroke, traumatic brain injury and surgery to overcome cerebrovascular spasm and thereby providing appropriate blood flow to the affected brain regions.

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Disclosure/conflict of Interest

No competing financial interests exist.

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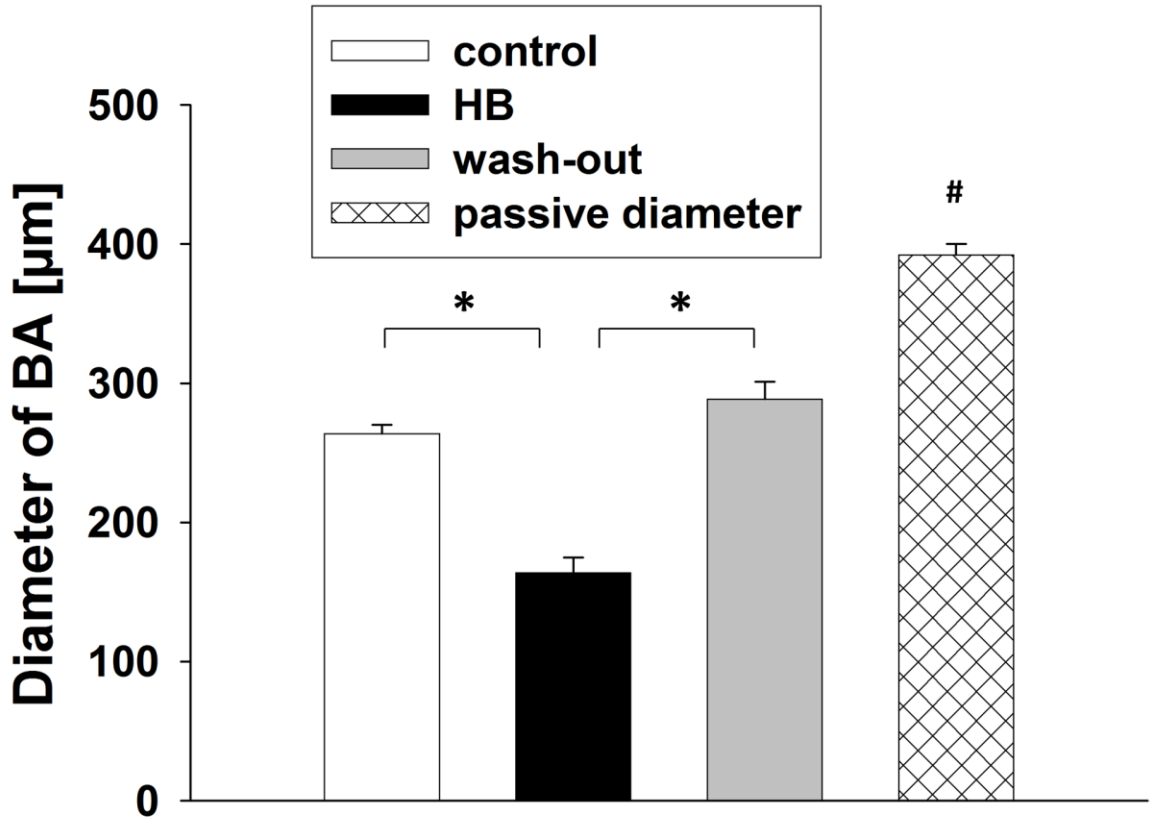
Figure Legends:



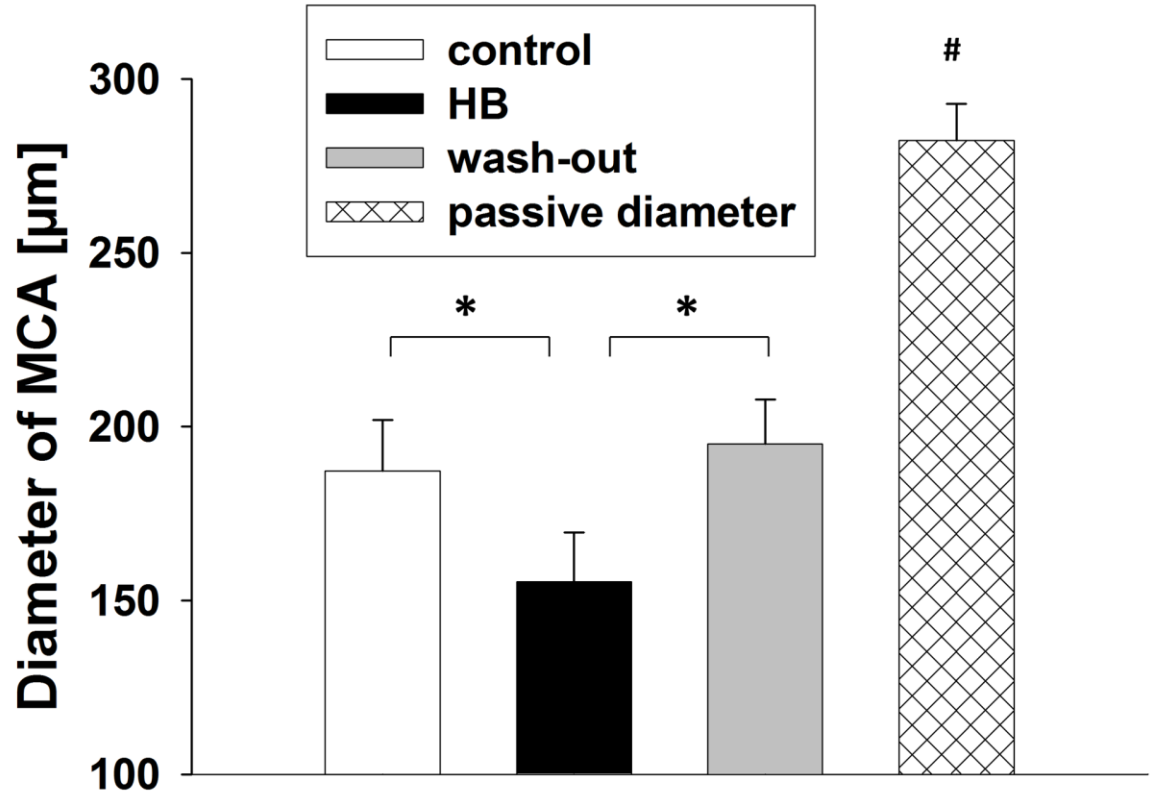


FIG. 1. Summary data of changes in diameter (µm) of basilar arteries BA (top, from 264±7 µm to 164±11µm, -23.9±3 [PD%]; n=12) and middle cerebral arteries MCA (bottom, from 185±15 µm to 155±14 µm, -11.4±0.8 [PD%]; n=6) in response to hemolysed blood (HB). Data are mean ± S.E.M. (* $p<0.05$ between either HB and control or HB and wash-out; # $p<0.05$ between control and passive diameter).

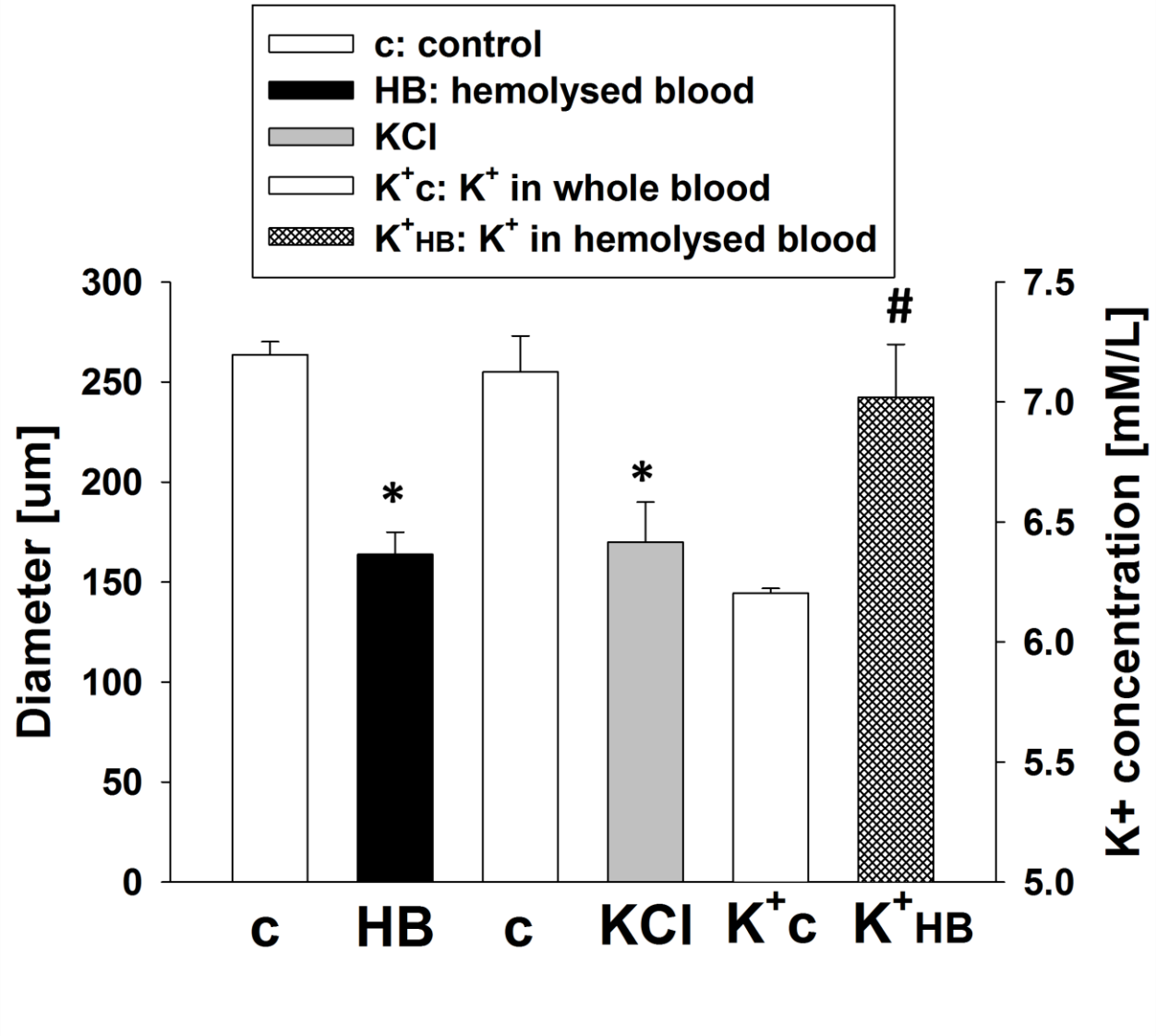


FIG. 2. Summary data of changes in diameter ([µm] on axis Y1) of basilar arteries (BA) in response to hemolysed blood (HB) or potassium chloride (KCl). Summary data of changes in K^+ concentration [mmol/L] in whole blood (K^+c; 6.20±0.01 mmol/L) and in hemolysed blood (K^+_{HB}; 7.02±0.22 mmol/L) on axis Y2. Data are mean ± S.E.M. (* $p<0.05$ between either control and HB or control and KCl, n=9-12 in each group; # $p<0.05$ between K^+c and K^+_{HB}; n=9).

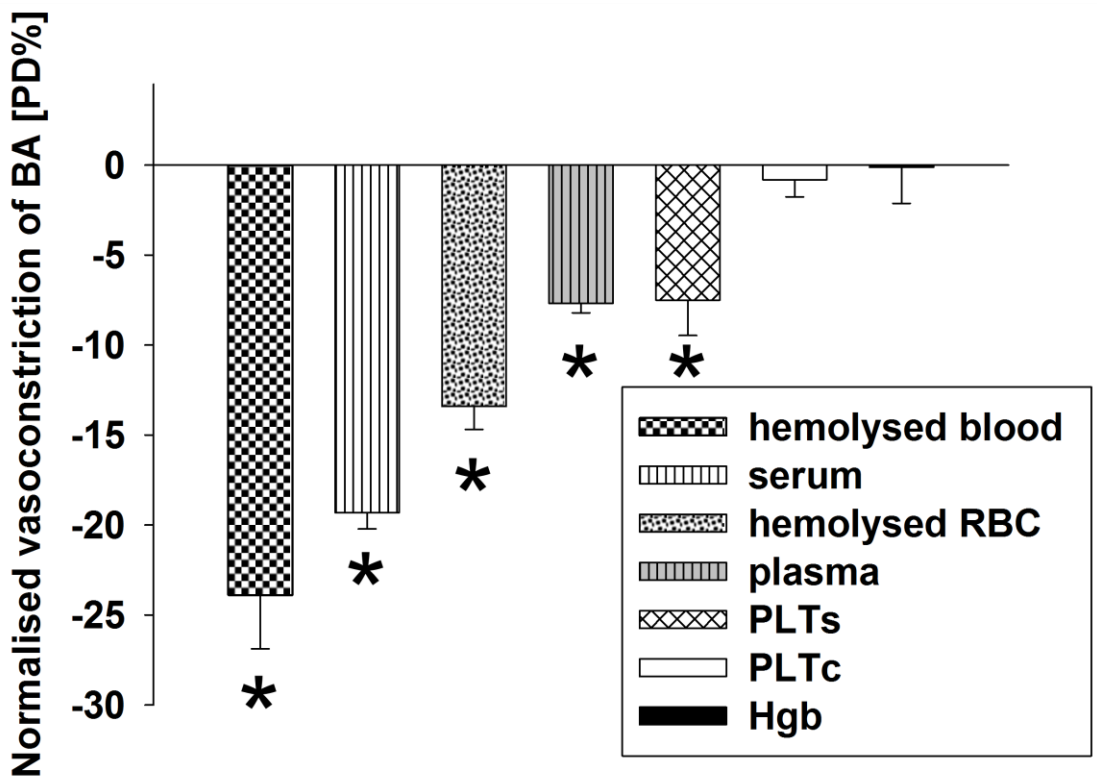


FIG. 3. Summary data of changes in diameter ([PD%]; % of passive diameter) of basilar arteries (BA) in response to hemolysed blood (HB), blood serum, hemolysed red blood cell (RBC), blood plasma, platelet suspension (PLTs), platelet concentration (PLTc) and hemoglobin (Hgb). Data are mean ± S.E.M. (* $p<0.05$, n=9-12 in each group). KCl (control: 255±18 µm, KCl: 170±20 µm; -21±2 [PD%]), HB (control: 264±7 µm, HB: 164±11µm, -23.9±3 [PD%]), Blood serum (control: 246±8 µm, serum: 170±6 µm; -19±0.9 [PD%]), the hemolysed red blood cell (RBC), (control: 217±9 µm, RBC: 166±6 µm; -14±1 [PD%]), blood plasma (control: 258±7 µm, plasma: 226±7 µm; -7,7±0,5 [PD%]), platelet suspension (PLTs), (control: 191±15 µm, PLTs: 165±16 µm; -7,5±2 [PD%]) caused significant vasoconstriction. However, hemoglobin (Hgb) (control: 263±16 µm; Hgb 10^{-12} M: 263±15 µm; -0.27±2.11 [PD%]; Hgb 10^{-6} M: 274±21 µm; -0.12±1.91

[PD%]) and platelet concentrate (PLTc) (control: 188±11 µm, PLTc: 185±12 µm; -0.8±0.9 [PD%]) did not elicit significant vasoconstriction.

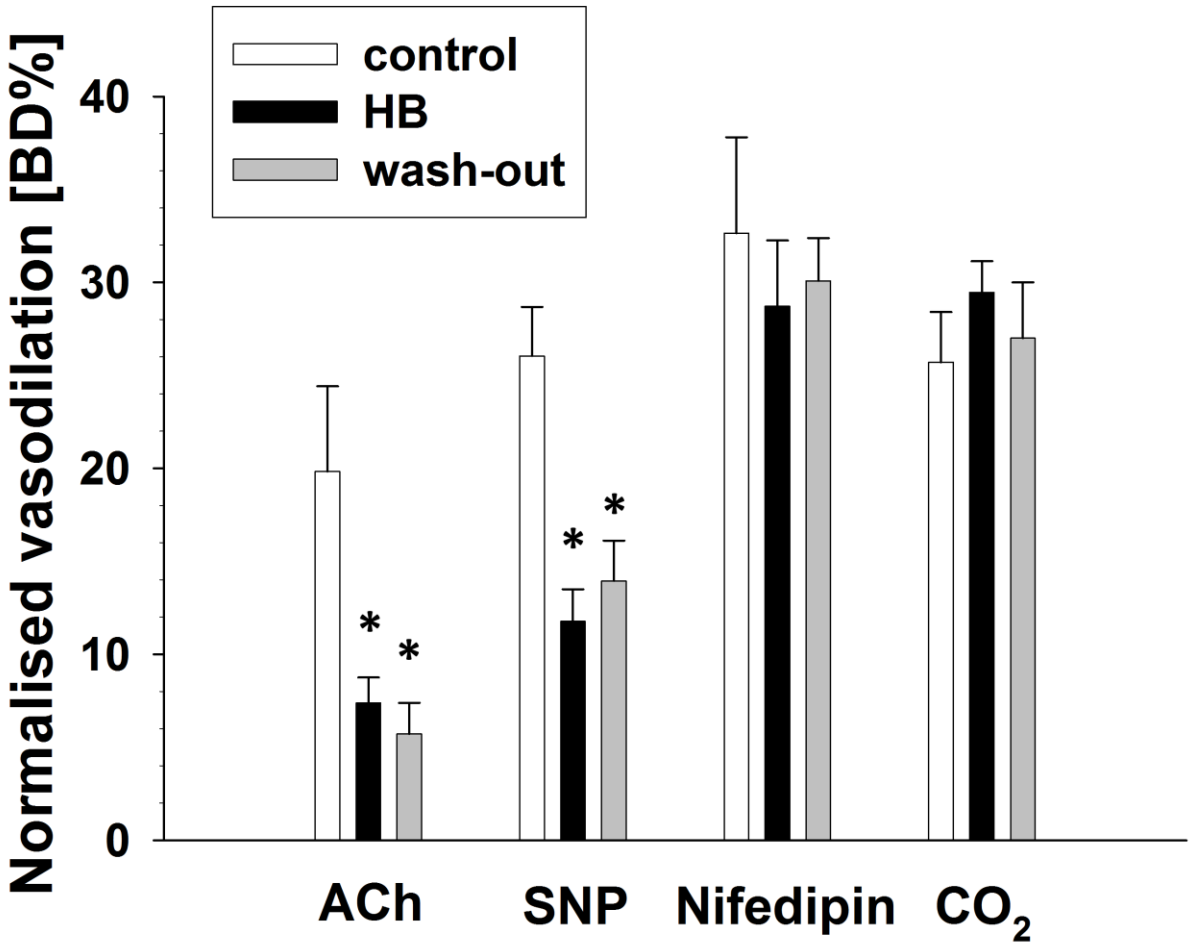


FIG. 4. Summary data of changes in diameter of isolated basilar arteries (BA). (% of basal diameter at 80 mmHg; [BD%]) in response to ACh, SNP, nifedipine and CO_2 before (control), during (HB) and after (wash-out) of hemolysed blood (HB). Data are mean ± S.E.M. (* $p<0.05$ between either control and HB or control and wash-out; n=10-12 in each group).

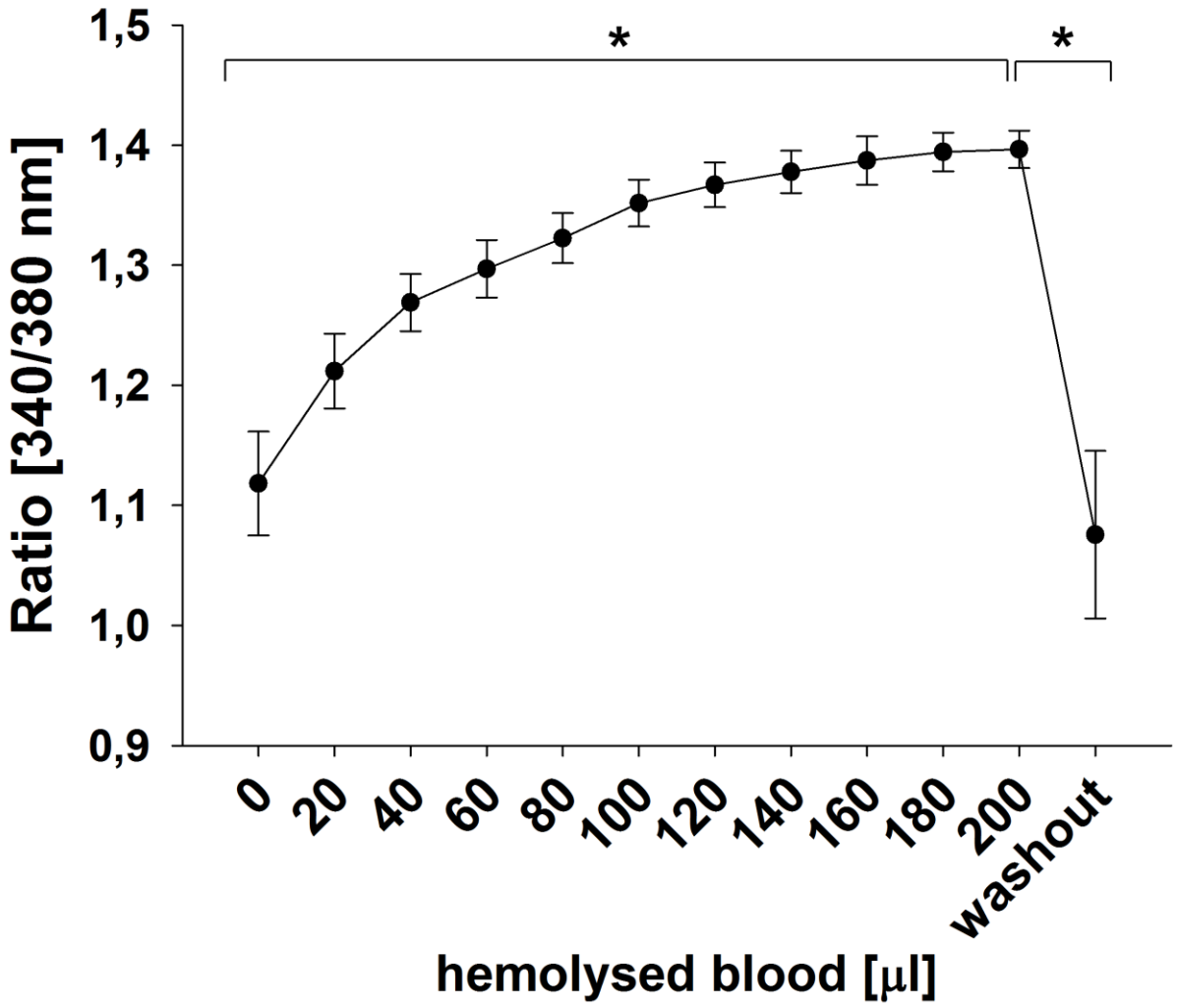


FIG. 5. Summary data of ratiometric (R) changes indicating the level of intravascular $[Ca^{2+}]_i$ of basilar arteries in response to increased concentrations of hemolysed blood (HB). Data are mean ± S.E.M. (* $p<0.05$; n=18).