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Nitric oxide influences red blood cell velocity independently of changes in the vascular tone

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Abstract
Nitric oxide (NO) plays a key role in regulation of vascular tone and blood flow. In the microcirculation blood flow is strongly dependent on red blood cells (RBC) deformability. *In vitro* NO increases RBC deformability. This study hypothesized that NO increases RBC velocity *in vivo* not only by regulating vascular tone, but also by modifying RBC deformability. The effects of NO on RBC velocity were analysed by intra-vital microscopy in the microcirculation of the chorioallantoic membrane (CAM) of the avian embryo at day 7 post-fertilization, when all vessels lack smooth muscle cells and vascular tone is not affected by NO. It was found that inhibition of enzymatic NO synthesis and NO scavenging decreased intracellular NO levels and avian RBC deformability *in vitro*. Injection of a NO synthase-inhibitor or a NO scavenger into the microcirculation of the CAM decreased capillary RBC velocity and deformation, while the diameter of the vessels remained constant. The results indicate that scavenging of NO and inhibition of NO synthesis decrease RBC velocity not only by regulating vascular tone but also by decreasing RBC deformability.

Keywords: *Intra-vital microscopy, microcirculation, chorioallantoic membrane, NO, erythrocyte deformability.*

Introduction

Capillary blood flow supplies organs with oxygen and nutrients and is regulated by both vascular tone changes [1] and rheological properties of blood cells [2]. In the capillaries, RBC must deform to enter and transit vessels narrower than their own diameter. Therefore, RBC velocity and capillary blood flow strongly depend on RBC deformability [3–6], defined as the ability of RBC to deform under a given force.

Nitric oxide (NO) plays a key role in blood flow control [7,8]. In the cardiovascular system NO is continuously produced in endothelial cells from L-arginine in a reaction catalysed by the type III isoform of NO synthase (NOS3, EC 1.14.13.39), commonly defined as endothelial NOS (eNOS) [9]. Endothelium-derived NO relaxes the smooth muscle cells in the vascular wall and controls vascular resistance. In the vascular lumen, NO can be scavenged by oxyhaemoglobin, the most abundant protein expressed in RBC. In addition, RBC are known to accumulate and transport NO metabolites, like nitrite [10,11] and under hypoxic conditions induce aortic ring relaxation [12,13]. Moreover, RBC express an active NOS [14,15] and under normoxic conditions release NO metabolites *in vitro* in a NOS-dependent fashion [15].

A role of NO in the control of RBC deformability has been proposed. NO donors affect RBC deformability as measured *in vitro* [16–21] and *ex vivo* [20]. Treatment of freshly isolated human RBC with a

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NOS inhibitor decreased RBC deformability as measured *in vitro* [17,22]. It is not known whether NO-dependent changes of RBC deformability affect RBC velocity and blood flow in the microcirculation. *In vivo*, application of NOS inhibitors reduced RBC velocity and blood flow in the microcirculation of skeletal muscle [23] or the liver [24] and affected vascular tone [23,24]. In these models it was not possible to distinguish whether the NOS inhibitors decreased RBC velocity not only by inducing vasoconstriction, but also by decreasing RBC deformability.

Here we tested whether scavenging of NO or NOS inhibition decrease RBC velocity *in vivo* also by decreasing RBC deformability. We have taken advantage of using the microcirculation of the avian chorioallantoic membrane (CAM), which is an extra-embryonic vessel system arising from differentiated mesodermal endothelial cells. This model has previously been applied to study angiogenesis [25] and vasoreactivity in response to epoxyeicosatrienoic acids [26]. RBC velocity and deformation of single RBC passing through the capillaries of the CAM were quantified by intra-vital microscopy using a high-speed camera. The experiments were performed on day 7 post-fertilization, when all vessels lack smooth muscle cells [25,27]. Thus, in this model vascular diameter cannot be modified by NO, as shown previously [26]. This allowed us to focus on the effect of NO on RBC velocity and cell deformation *in vivo*, independently of changes on vascular tone.

Methods

Materials

Glutaraldehyde was purchased from Merck (Darmstadt, Germany), phosphate buffered saline (PBS) from Serag Wiessner (Naila, Germany), Parafilm®M from Brand (Wertheim, Germany), haemoglobin and L-arginine from Sigma-Aldrich (Deisenhofen, Germany) and L-N^5-(iminoethyl)ornithine (LNIO) from Alexis Biochemicals (Enzo Lifescience GmbH, Lörach, Germany). 4–amino–5-methylamino-2, 7- difluorofluorescein (DAF-FM) diacetate was from Invitrogen (Darmstadt, Germany). S-nitrosocysteine (SNOC) was synthesized as described [28].

Experimental design

We analysed and compared the effects of NO scavenger and NOS inhibitors on RBC deformability and NO levels *in vitro*, as well as on RBC velocity and cell deformation *in vivo* in the microcirculation of the CAM of the avian embryo.

In vitro experiments

Avian blood samples. Blood samples were collected from the brachial vein of adult chicken, anti-coagulated with heparin and stored for max 2 h at 4°C. Probes were kindly provided by Professor Dr Schaub (Zoology/Parasitology Group, Ruhr-University of Bochum, Germany). All animal procedures were authorized by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany.

Intracellular staining with DAF-FM and flow cytometry

The effects of NOS inhibitors on intracellular NO levels in avian RBC were assessed by staining RBC with DAF-FM diacetate, which in the presence of NO and O_2 is converted to a highly fluorescence triazole derivate by an unstable nitrosating species mainly derived from oxidation of NO [29,30]. The best staining conditions (cell and dye concentrations, temperature, incubation time and pH) as well as the effects of NO donors and intracellular NO scavenging were carefully optimized by using human RBC. Moreover, the reactivity of DAF-FM for anion superoxide and hydrogen peroxide has been also tested (not shown).

Avian blood was diluted 1:500 (4×10^5 cells/µl) in PBS, divided in aliquots and treated for 30 min at 37°C with the NOS inhibitor L-NIO (3 mM) or with L-arginine (3 mM). As a positive control RBC were treated with the NO donor and nitrosating species SNOC (25 µM) [31]. Intracellular NO was s cavenged by incubation of RBC for 30 min at 37°C with 250 µM of iron diethyldithiocarbamate ($Fe(DETC)_2$) prepared as previously described [28]. Cells were stained for 30 min at 37°C with 10 µM DAF-FM diacetate, washed in cold PBS and measured within 15 min in a flow cytometer (FACS CANTO II) within the FITC channel (ex 488 nm, em 530 ± 30 nm). Number of counted cells: 30 000, $n = 3$. Median fluorescence intensity (MFI) was determined in the fluorescence distribution plot by using the FlowJo V7.5.5 (TreeStar, Ashland, OR) software package. For each experiment unstained cells served as autofluorescence control. Reliability of fluorescence acquisition was assured adjusting fluorescence acquisition voltage with fluorescent latex beads (Rainbow beads, BD Bioscience, Heidelberg, Germany).

Measurement of avian RBC deformability by ektacytometry

Blood samples were treated with glutaraldehyde (0.01%) to increase the stiffness of RBC, with the NO scavenger oxyhaemoglobin (0.1 mM), with the NOS inhibitor L-NIO (0.1 mM), with the NOS substrate L-arginine (3 mM) or with PBS as a control for 30 min at 37°C. These concentrations chosen have previously been shown to affect the filterability of human RBC *in vitro* [22]. Avian RBC deformability was determined at various fluid shear stresses by laser

diffraction analysis using an ektacytometer (laser-assisted optical rotational cell analyser (LORCA), RR Mechatronics, Hoorn, Netherlands). This system has been described elsewhere [32]. Briefly, an elongation index (EI) was calculated on the basis of the geometry of the ellipitical diffraction pattern: EI = (L - W)/(L + W), where L and W are the length and width of the diffraction pattern, respectively. EI values were calculated for shear rates between 0.5–15 Pa. An increased EI at a given shear stress indicates greater cell deformation and hence greater RBC deformability. All measurements were carried out at 37°C.

In vivo experiments

Preparation of the avian chorioallantoic membrane. Fertile eggs (Deindl, Rietberg, Germany) were incubated for 7 days at 37°C and 55% relative humidity in a custom-built glass bowl. The eggs were rotated twice per day until day 5. At this time point 2 ml of egg white were drawn using a syringe with a diameter of 0.9 mm, the upper half of the eggshell was removed and a 1.0 × 1.5 cm window was opened above the CAM. This 'window' was covered with Parafilm®M to prevent the drying of the egg and the incubation was continued without rotation until day 7.

Measurement of rheological parameters by intra-vital microscopy

The microcirculation of the CAM was analysed by using an intra-vital microscope (DML Leica, Wetzlar, Germany) equipped with a 10-fold (10 ×) magnification ocular. The focal plane was set as shown in Figure 1. To analyse rheological parameters of RBC before and after intervention, images obtained by differential interference microscopy (DIC) were recorded at a rate of 50 fields/s with a long working distance 50 × objective by using a charge-coupled device camera (AVT B-C71, Horn, Germany), obtaining videos showing single RBC passing through the microcirculation of the CAM (Figure 1). Videos were taken before intervention as well as 10 and 60 min after intervention and analysed by using Cap-Image 1.1 (Dr. Zeintl GmbH, Heidelberg, Germany).

Vessel diameters and RBC velocity were measured in the pre-capillary and capillary over a period of 2 min. Diameter of pre-capillary, capillary and the large arteries upstream were measured before and after intervention by using the appropriate software tool. RBC velocity was analysed according to the line-shift-diagram method, a semi-automatic computer analysis method, following the manufacturer's instruction. Briefly, a line is drawn along the main axis of the vessel. Each RBC that passed the measurement line causes a shift in grey level pattern, measured in μm, over time, measured in seconds (s). The velocity (μm/s) of all RBC passing through the vessel during 2 mins was averaged.

RBC length (L) and width (W) were measured during the entrance of the RBC from pre-capillary into a following capillary (Figure 1) before and after intervention. The passage of a single RBC from the pre-capillary to capillary was tracked by direct visualization. L and W were measured at two defined measurement points in the pre-capillary and capillary. Entering a capillary the process of RBC deformation led to an increase in L and a decrease in W. Values obtained from the analysis of 30 RBC per time-point per intervention per egg were averaged (the total number of cells analysed per treatment was 90 RBC). RBC deformation was assessed by the deformation index (DI) [33], defined as in (1):

$$DI = L/W \tag{1}$$

A decrease in *DI* indicates a decrease in RBC deformation.

RBC flow rate (Q_{RBC}) along the pre-capillary–capillary flow was estimated using the following equation:

$$\text{RBC flow rate} = \tfrac{1}{4}\,\pi\, Dc^2\, V_{RBC} \tag{2}$$

where *Dc* is the interior diameter of the pre-capillary or capillary and V_{RBC} is the RBC velocity measured in the pre-capillary or capillary.

Shear rate was calculated from RBC velocity and capillary diameter according to the Poiseuille parabolic intra-arterial velocity distribution:

$$\text{Shear rate} = 8\, Vi/Di \tag{3}$$

where *Vi* is the velocity and *D* is the vessel diameter at point *i*.

Heart rate was assessed by directly observing the heart contraction and counting the number of beats over a period of 60 s before intervention, as well as 5 and 10 min after intervention. Data are expressed as beats per minute (bpm). During all experimental procedures eggs were placed in a custom-made glass bowl perfused with water at 37°C.

Intervention procedure

Experiments were performed on day 7 of embryonic development. All compounds were injected into a conduit artery of the CAM using a smoothed borosilicate glass capillary with an exterior diameter of 0.5 mm. Glutaraldehyde (0.01%) was injected to assess the effect of changes in the RBC deformability on RBC velocity. To influence vascular NO bioavailability we applied the NO scavenger oxyhaemoglobin (0.1 mM). To modify endogenous NO synthesis the NO synthase inhibitor LNIO (0.1 mM) or the NOS substrate L-arginine (3 mM) were injected.

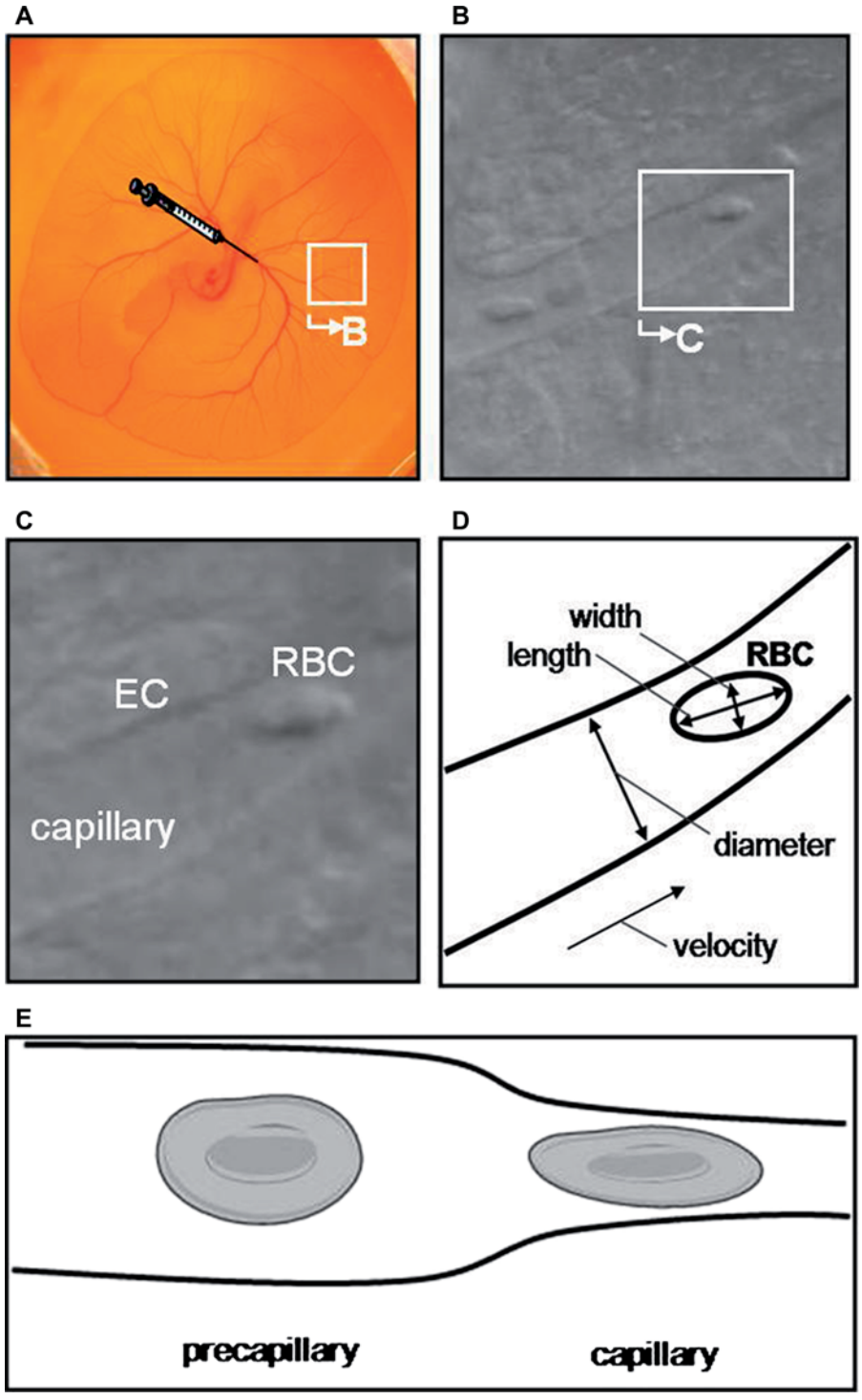

Figure 1. The avian chorioallantoic membrane model: a functional microcirculatory system. Representative picture of a chicken embryo at day 7 of embryonic development showing the chorioallantoic membrane and the site of injection (A), the microvascular bed (B) with a magnified (C) and a schematic (D) illustration of the measured parameters using an intra-vital microscope with a 10 × ocular and 50 × objective at long working distance. Schematic illustration of RBC deformation (increase in cell length and a decrease in cell width) within the capillary (E). EC = endothelial cell.

Application of PBS served as control. Final concentrations have been calculated considering a total blood volume of 750 µl at day 7 of embryonic development. All compounds were injected as a 10 µl bolus over 10 s and at a temperature of 37°C to exclude influence of volume changes or temperature on blood flow. Each treatment was repeated in five different eggs (total number of eggs: 30).

Statistical analysis

Data are reported as mean ± SEM. Paired *t*-test was used to assess significant differences in the mean values for data from the same egg before and after intervention. Mann-Whitney rank sum test and analysis of variance with Bonferroni correction for multiple *t*-tests were used to compare different eggs. *p*-values < 0.05 were considered as statistically significant.

Results

NOS inhibition and NO scavenging decreased deformability and intracellular NO levels of avian RBC in vitro

Addition of L-NIO (0.1 mM) as well as treatment with the extracellular NO scavenger oxyhaemoglobin (0.1 mM) significantly decreased RBC deformability as compared to treatment with PBS (Figure 2A), while treatment with the NOS substrate L-arginine (3 mM) did not affect RBC deformability. As a positive control, avian RBC were treated with glutaraldehyde (0.01%), which has been previously applied to cross-link RBC membrane proteins and thus decrease their deformability [34,35]. Treatment with glutaraldehyde decreased RBC deformability as compared to PBS-treated control ($p < 0.05$).

The effects of L-NIO and L-arginine on the intracellular NO levels of avian RBC were assessed by staining with DAF-FM and flow cytometry. Avian RBC stained with DAF-FM diacetate were strongly fluorescent, as compared to the unlabelled control. Addition of the NOS inhibitor L-NIO (0.1 mM) decreased intracellular fluorescence activity (Figure 2B), while treatment with the NOS substrate L-arginine (3 mM) did not affect intracellular DAF-FM fluorescence. To control for reactivity and specificity of intracellular DAF-FM for NO we added SNOC (25 μM) or the cell-permeable NO scavenger $Fe(DETC)_2$ (250 μM). We found that SNOC increases intracellular DAF-FM fluorescence activity (Figure 2B), while $Fe(DETC)_2$ decreased it. Thus, intracellular DAF-FM fluorescence in the RBC depends on NO levels.

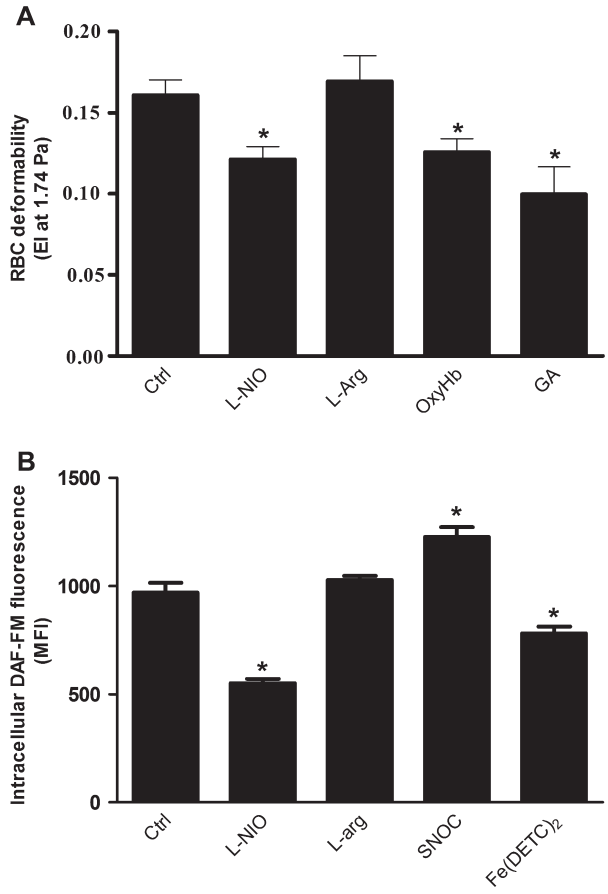

Figure 2. NOS inhibition and NO scavenging decrease deformability and intracellular NO levels of avian RBC *in vitro*. (A) RBC were treated with L-NIO (0.01 mM), oxyhaemoglobin (OxyHb; 0.01 mM), L-arginine (L-Arg; 3 mM) and glutaraldehyde (GA; 0.01%) or left untreated (Ctrl). RBC deformability was assessed by ektacytometry (*indicates significant difference as compared to Ctrl). (B) RBC were treated with L-NIO (3 mM) or with L-Arginine (L-Arg; 3 mM) or left untreated (Ctrl). As control, RBC were treated with SNOC (25 μM) or with the NO scavenger $Fe(DETC)_2$ (250 μM). Cells were washed, stained with 10 μM DAF-FM and analysed by flow cytometry. Median fluorescence intensity (MFI) within the RBC gate was calculated by analysing the fluorescence distribution plot of the green channel (Ex 488 Em 530 ± 30 nm). Data represent the difference between MFI of stained cells minus the autofluorescence. *indicates significant difference as compared to Ctrl ($p < 0.05$).

NOS inhibition and NO scavenging decreased RBC velocity and cell deformation in the microcirculation of the CAM

We aimed to determine whether the NOS inhibition and NO scavenging-mediated decrease in RBC deformability affect RBC velocity and deformation in the microcirculation of the CAM independently of changes in vascular tone. We found that the microcirculation of the CAM was suitable for the reliable and precise measurements of rheological parameters, e.g. vessel diameter, RBC velocity and cell deformation, by direct visualization of single cells (Figure 1). The basal rheological parameters are described in Table I.

Injection of oxyhaemoglobin to scavenge extracellular NO decreased RBC velocity and deformation index (DI) both in the pre-capillary and capillary as compared to basal values before injection ($p < 0.05$; Figure 3). The NOS inhibitor L-NIO decreased RBC velocity and DI in the capillary ($p < 0.05$). Injection of L-arginine did not influence RBC velocity or DI. Injection of glutaraldehyde decreased RBC velocity and DI both in pre-capillaries ($p < 0.05$) and in capillaries ($p < 0.05$) as compared to basal levels measured before intervention (Figure 3). Injection of PBS did not exert any effect on RBC velocity or DI as

Table I. Basal rheological parameters.

Rheological parameters	pre-capillary	capillary	*p*-value
Eggs (*n*)	30	30	
Vessel Diameter (μm)	13.5 ± 0.3	6.2 ± 0.2	<0.05
No. of measured RBC (*n*)	900	900	
Mean RBC length (μm)	14.8 ± 0.1	16.1 ± 0.2	<0.05
Mean RBC width (μm)	7.5 ± 0.2	5.8 ± 0.2	<0.05
DI	2.02 ± 0.03	2.94 ± 0.04	<0.05
RBC velocity (μm/s)	100 ± 15	304 ± 20	<0.05
Shear rate (s^{-1})	57 ± 3	340 ± 24	<0.05
RBC flow rate ($\mu m^3/s$)	12 500 ± 400	11 500 ± 700	n.s.
Heart rate (bpm)	216 ± 12		
Diameter of artery upstream (μm)	342 ± 8		

Values are means ± SE. RBC length increased up to 11% and RBC width decreased about 25% when RBC enter from pre-capillary into the capillary. RBC length-to-width ratio increased in capillaries up to 28% under basal conditions. RBC velocity in the artery upstream was measured with 720 ± 30 μm/s. The RBC flow rate did not change on passing from pre-capillary to capillary.

compared to basal conditions (Figure 3). The observed effects were stable over the duration of the experiments (60 min). Other physiological parameters such as diameter of pre-capillary, capillary and the arteries upstream, as well as heart rate did not change after any treatment (Table II—supplemental material).

To investigate the relationship between RBC velocity and RBC deformation, we calculated the shear rate, i.e. the force deforming the RBC in the microcirculation, from RBC velocity and vessel diameter (Figure 3C). We formed a linear relationship ($R^2 = 0.87$, $p < 0.01$) between shear rate and DI, suggesting that in our model RBC deformation is a function of shear rate.

Discussion

Our findings demonstrate that NOS inhibition and NO scavenging decrease RBC deformability *in vitro* and RBC velocity and deformation in the microcirculation of the CAM independently of changes in vascular tone.

The microcirculation of the CAM offers many advantages as a model for measuring NO-dependent changes on rheological parameters and blood flow. Study of rheological parameters by intra-vital microscopy in this model does not require application of anaesthetic and single circulating cells can be directly observed and measured. Moreover, until day 12 post-fertilization the vessels of the CAM lack smooth muscle cells [27]. Therefore, NO does not influence vascular diameter via regulation of smooth muscle cell relaxation, as previously shown by using NO donors [26]. Thus, we measured rheological parameters independently from changes in vascular diameter.

Although avian RBC are nucleated and elliptical, they present many similarities with mammalian non-nucleated RBC. Like human [14,22] and mouse RBC [36], avian RBC produce NOS-derived NO metabolites, as shown here by staining with DAF-FM. Avian and mammalian RBC present similar membrane protein composition. Despite a lower deformability of nucleated avian RBC, the deformation process of nucleated and non nucleated RBC is qualitatively similar [27]. We here show that NOS inhibition by adding L-NIO and NO scavenging by treatment with oxyhaemoglobin significantly decreased RBC deformability *in vitro* as compared to treatment with PBS. Similar effects of NOS inhibition or NO scavenging on RBC deformability have previously been measured in non-nucleated mammalian RBC *in vitro* [17,22]. Thus, it has been proposed that NOS-derived NO in RBC plays a central role in maintaining deformability [17,21,22]. We have also found that treatment with the NOS substrate L-arginine (3 mM) did not affect avian RBC deformability as shown for human RBC elsewhere [17]. In contrast, we have observed previously that L-arginine increased filterability of human RBC [22]. While ektacytometry measures deformability, RBC filterability is influenced by both RBC deformability as well as aggregability [37]. Thus, in our previous setting L-arginine might have affected aggregability of RBC. As expected, increasing RBC stiffness by treatment with glutaraldehyde, which is known to cross-link RBC membrane proteins in mammals [34] and avians [35], decreased deformability of the cells *in vitro*. The mechanisms involved in NO-mediated modulation of RBC deformability are not well understood. NO may affect RBC deformability depending on guanylate cyclase activity and cyclic guanosine monophosphate concentrations [17,38], on $Ca2^+$-dependent K^+ channels [39], on K^+ transporters [17] or on cyclic adenosine monophosphate concentrations [40].

In the microcirculation of the CAM, we found that the NO-dependent changes in RBC deformability measured *in vitro* are reflected by changes in capillary blood flow *in vivo.* Inhibition of enzymatic NO synthesis by L-NIO not only reduced RBC deformability *in vitro*, but also decreased RBC velocity and cell deformation *in vivo*, while the diameters of pre-capillary, capillary and the arteries upstream remained

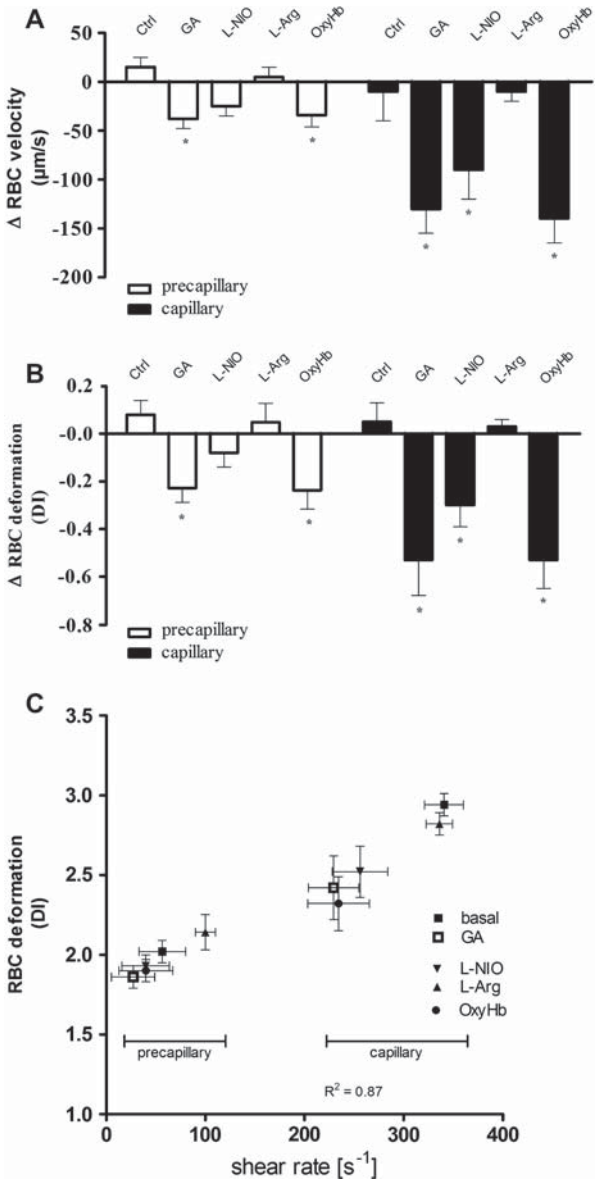

Figure 3. NOS inhibition and NO scavenging decrease RBC velocity and RBC deformation *in vivo*. A bolus volume (10 µl) of oxyhaemoglobin (OxyHb; 0.1 mM), LNIO (0.01 mM), Larginine (L-Arg; 3 mM), glutaraldehyde (GA; 0.01%) or PBS were injected into a conduit artery of the CAM. (A) RBC velocity and (B) RBC deformation index (DI) were measured off-line at a defined point of the pre-capillary and at a defined point of the capillary by using CapImage® software and were compared to basal values before intervention. *indicates significant differences ($p < 0.05$). (C) Shear rate and RBC DI significantly correlate in pre-capillary and capillary ($R^2 = 0.87$, $p < 0.01$).

constant. Injection of oxyhaemoglobin produced a more pronounced decrease in RBC velocity and deformation, as compared to L-NIO. While L-NIO inhibits enzymatic NO synthesis within the cells, oxyhaemoglobin is not cell permeable and scavenges NO present in the interstitium and in the vessel lumen, affecting the circulating NO pool [41]. For technical reasons (difficulties in blood drawing and small blood volume), it was not possible to assess NO metabolites in embryonic blood cells or rheological parameters of embryonic avian RBC *ex vivo*. Injection of L-arginine did not affect RBC velocity and cell deformation *in vivo*, as expected considering the lack of effect of L-arginine on RBC deformability *in vitro*.

RBC velocity depends on vessel diameter, pulse pressure or changes in rheological parameters, i.e. RBC deformability, RBC aggregation, RBC adhesion, hematocrit or blood viscosity [42]. Many of these parameters might be affected by NO. Changes of vascular diameter due to NO-mediated vasodilation can be excluded as smooth muscle cells are absent [25–27]. In fact, we did not measure any changes of the vessel diameter of pre-capillary, capillary or large arteries upstream after treatment with L-NIO or oxyhaemoglobin. However, minor changes in the internal diameter of the vessel lumen due to changes of the glycocalyx [43] cannot be excluded. Heart rate and the diameter of the arteria upstream did not change, which indicate that in our model pressure gradients or other haemodynamic parameters also did not change. The effects of blood dilution on hematocrit due to injection of the compounds were the same for all treatments as same volumina were injected. Effects of L-NIO or oxyhaemoglobin on RBC adhesion or aggregation *in vivo* cannot be fully excluded. However, we did not observe any formation of RBC aggregates *in vivo*. Taken together, our data indicate that the decrease in RBC velocity *in vivo* after injection of L-NIO and oxyhaemoglobin was strongly dependent on reduced RBC deformability.

The correlation between decreased RBC deformability and RBC velocity *in vivo* within the microcirculation has previously been demonstrated by others who injected glutaraldeyde-treated RBC in the microcirculation of rodents [3–6]. In our model, injection of glutaraldehyde also decreased RBC velocity and deformation. We cannot exclude that some of the effects of glutaraldeyde were due to cross-linking of vessel or plasma proteins. However, we did not observe any changes in heart rate or vessel diameter after glutaraldehyde injection.

To explain the correlation between RBC deformability and RBC velocity *in vivo*, it has been proposed that a decrease in RBC deformability causes RBC plugging into the capillary, followed by a more heterogeneous distribution of hematocrit and an increase of blood viscosity [5,44]. As a consequence of these, regional flow resistance increases and RBC velocity within the capillaries decreases.

Conclusions

To summarize, our data indicate that endogenous NO directly affects RBC velocity and cell deformation *in vivo* not only by regulating vascular diameters as previously shown [23,24], but also by affecting RBC deformability. These data point to new aspects of

NO-mediated regulation of blood flow, which are independent of changes of the vascular tone.

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Declaration of interest

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Supplementary material available online

Table 2. Supplemental Material.

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