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Perfusion of the isolated trout heart coronary circulation with red blood cells: effects of oxygen supply and nitrite on coronary flow and myocardial oxygen consumption

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Summary

A method for perfusion of the isolated trout heart coronary circulation with red blood cells (RBCs) was developed. The method was used to analyse the influence of RBC perfusion on myocardial O_2 supply and O_2 consumption and to test the hypothesis that nitrite is converted to vasoactive nitric oxide in the RBC-perfused coronary circulation. Perfusion with RBCs significantly increased myocardial O_2 supply and O_2 consumption by increasing the incoming O_2 concentration and the O_2 extraction. Coronary flow did not differ between RBC perfusion and saline perfusion, but RBC perfusion established a strong linear increase in myocardial O_2 consumption with coronary flow. Nitric oxide was measured in the atrial effluent of the preparation. Perfusion with saline under hypoxic conditions was associated with NO production. The nitric oxide synthase inhibitor L-NA obliterated this NO production and significantly decreased coronary flow, showing that the NO was vasoactive and probably of endothelial origin. RBC perfusion at low P_{O_2} similarly caused an L-NA-inhibitable NO production. The change in NO production upon subsequent nitrite addition, by contrast, was not inhibited by L-NA. Nitrite entered trout erythrocytes independent of degree of oxygenation, but the O_2 saturation of RBCs showed a major decrease in the coronary circulation, and $[NO_2^-]$ decreased while methaemoglobin rose, suggesting that deoxyHb-mediated reduction of nitrite to NO may have occurred. However, other possibilities (e.g. $NO_2^- \rightarrow NO$ conversion in myocardial cells) cannot be excluded. The NO formation associated with nitrite had no effect on coronary flow, possibly because NO was produced after the resistance vessels.

Key words: erythrocyte, haemoglobin, microcirculation, nitrite, nitric oxide, vasodilation.

Introduction

The isolated trout heart provides a useful model for studying mechanical aspects of heart contraction and humoral control mechanisms involved in adjusting coronary blood flow to myocardial metabolic requirements. So far, this model has been used with a physiological saline perfusing the heart and/or coronary arteries. A further development of the technique to allow perfusion with red blood cells (RBCs) would constitute a significant advance. Perfusion of the coronary tree with RBCs represents a more '*in vivo*-like' model and would limit potential problems with insufficient O_2 supply to the myocardium or the use of very high O_2 tensions (which may create problems with reactive oxygen species). Perfusion with RBCs is also of interest in relation to recent hypotheses that suggest the RBCs to be involved in the regulation of local blood flow *via* an oxygenation-dependent release of vasodilatory compounds (Ellsworth, 2000; Pawloski et al., 2001; Gladwin et al., 2004). One timely idea is that nitrite, an oxidative metabolite of nitric oxide, serves as a vascular storage pool of NO activity (Gladwin et al., 2004). Nitrite that enters RBCs reacts with oxygenated haemoglobin (Hb) to form nitrate and methaemoglobin (metHb) (Kosaka and Tyuma 1987), whereas the reaction with deoxygenated Hb forms NO and metHb (Cosby et al., 2003; Nagababu et al., 2003). Nitrite is accordingly converted to NO to an extent that depends on the degree of deoxygenation, and a subsequent escape of NO from the RBCs may therefore produce vasodilation and increase blood flow to an extent that matches O_2 requirements (Cosby et al., 2003; Nagababu et al., 2003). This idea is supported by positive arterial–venous $[NO_2^-]$ differences (Gladwin et al., 2000) and nitrite infusion-caused vasodilation (Cosby et al., 2003) in the human forearm. Rainbow trout exposed to nitrite similarly show cardiovascular changes that support the generation of vasoactive NO from nitrite (Jensen, 2003). Reduction of nitrite to NO may also play a role during ischemia in the myocardium (Webb et al., 2004) and kidney (Okamoto et al., 2005).

The purposes of the present study were to develop a method for RBC perfusion of the trout heart coronary circulation and to evaluate the impact of RBC perfusion on coronary flow and myocardial O_2 consumption (as compared with saline perfusion). Furthermore, we tested the hypothesis that nitrite is converted to NO in the RBC-perfused trout heart coronary tree and that this has an influence on coronary blood flow. NO of endothelial origin has previously been implicated in vasodilatory mechanisms in trout coronaries (Mustafa et al., 1997; Mustafa and Agnisola, 1998).

Materials and methods

Animals and tissue sampling

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 303±21 g, *N*=23) were obtained from a freshwater fish farm in Jutland, Denmark. The fish were transported to Odense two weeks prior to the experiment and were housed in 400-litre aquaria in 15°C flowing, aerated Odense tapwater under a 12 h:12 h light:dark cycle.

The fish were euthanized with a rapid stunning blow to the head, and a blood sample was taken from the caudal vessels in the tail with a heparinized syringe. Fish to be dissected were subsequently injected with 0.5 ml 100 IU ml^{-1} heparin into the caudal vessel.

Preparation of red blood cell suspensions for heart perfusion

Freshly drawn trout blood was centrifuged (4600 ***g***, 5 min) and the plasma was discharged. The RBCs were subsequently washed three times in approximately seven times their volume of physiological saline. The saline had the following composition (g l^{-1}): 7.89 NaCl, 0.23 KCl, 0.23 $MgSO_4.7H_2O$, 0.016 $NaH_2PO_4.H_2O$, 0.28 $Na_2HPO_4.2H_2O$, 0.37 $CaCl_2.2H_2O$, 1 glucose, 10 polyvinylpyrrolidone. This saline was constructed to match the osmolality of plasma and has an increased [NaCl] compared with earlier versions (Farrell et al., 1986). The measured osmolality of samples taken randomly throughout the experiments was 293.6±0.5 mOsmol kg^{-1} for plasma (*N*=11) and 292.3±0.5 mOsmol kg^{-1} (*N*=8) for the saline (means ± S.E.M.). Following the final wash, the RBCs were suspended to the required haematocrit (Hct), and a minor amount of heparin was added (1.3 IU ml^{-1} final concentration). The RBC suspension was placed in two rotating glass tonometers, each containing 9–10 ml RBC suspension, and equilibrated for 45 min at 15°C to a humidified gas mixture of O_2 (3 or 4%) and CO_2 (0.5%), with N_2 as the balance gas. Gas mixtures were delivered from a Wösthoff (Bochum, Germany) Digamix 5KM432X gas-mixing pump.

Preparation of hearts and the isolated heart perfusion set-up

The coronary circulation of a non-working, electrically paced isolated heart was perfused under constant pressure. The heart was isolated and cannulated as previously described (Mustafa and Agnisola, 1994) with some modifications. The heart was dissected out, and the coronary artery was cannulated with a 5 mm-long, 0.30 mm outer diameter nylon tube. The atrium was cannulated with a 2.5 mm-diameter, 14 mm-long cannula, which, outside the atrium, continued into a 4.5 mm-diameter, 6 mm-long cannula. The ventral aorta was cannulated with an occluded cannula. The coronaries were pre-perfused with saline at a constant pressure (1 kPa) to wash out blood, and the heart was mounted into a jacketed chamber maintained at 15°C (Fig. 1). Next, the perfusion with saline from a saline head pressure reservoir was started. The saline was equilibrated to the same gas mixture as the RBCs (Fig. 1). The mounted heart was paced at a rate of 30 beats min^{-1} with two platinum electrodes connected with a Grass S6 Stimulator (stimulus 10 V, 20 ms). Under these conditions, the coronary perfusate drained into the atrium and then flowed out from the atrium *via* the atrial cannula. An ISO-NOP electrode (World Precision Instruments, Sarasota, FL, USA) was inserted through the atrial cannula to continuously detect the NO levels in the effluent from the atrium. The saline level in the chamber was kept constant by an overflow. Perfusate outflow from the atrium was helped by the regular atrial beating induced by electrical pacing. Because of the occluded aortic cannula, only the compact layer of the ventricle wall was perfused in this preparation, leaving out any possible contribution to NO release from the spongy myocardium.

Samples of input perfusate were collected *via* a sidearm of the coronary input tube, while samples from the atrium were collected *via* a secondary cannula (0.5 mm diameter) inserted into the atrium together with the main atrial cannula (Fig. 1).

For RBC perfusions, the equilibrated RBC suspension was transferred from the tonometer to an RBC head pressure reservoir (Fig. 1) in a gas-tight Hamilton syringe. The atmosphere of the reservoir received gas from the gas-mixing pump to guarantee that the RBCs continued their gas equilibration. A three-way tap allowed the switch between saline and RBC perfusion (Fig. 1) and to draw the erythrocyte suspension from the reservoir directly to the coronary input upon initiation of RBC perfusion. To avoid RBC sedimentation in the reservoir, the RBC suspension was intermittently mixed by gently sucking it into the gas-tight syringe and re-injecting it into the reservoir.

Experimental protocols

In initial experiments, we tested perfusion of the trout heart coronary circulation with RBC suspensions having Hct values of ~15%. In some of these experiments, the coronary resistance increased sharply after a while, and the hearts developed a few visual dark spots, which was ascribed to problems with precipitation or sedimentation of RBCs inside the vasculature. These problems were not encountered at lower Hct, which accordingly was chosen for the experimental protocols.

Protocol 1

Animal mass, 294±29 g; ventricle mass, 0.28±0.03 g (means ± S.E.M., *N*=6). In this protocol, the coronary circulation was first perfused with saline at a specific input oxygen tension

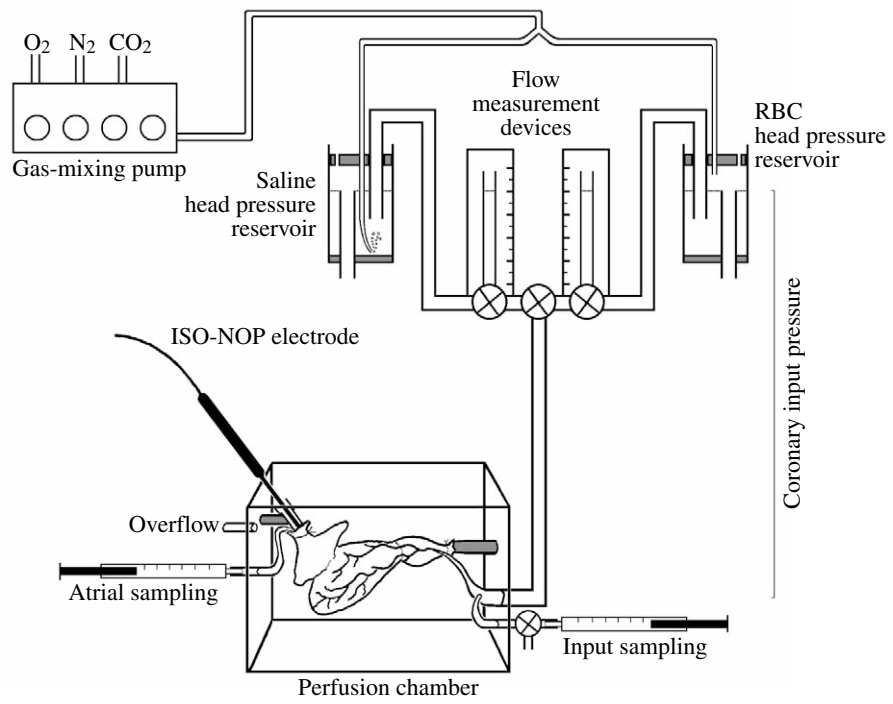

Fig. 1. Scheme of the perfusion set-up. The isolated heart was mounted into a saline-filled, temperature-controlled chamber. The coronary cannula was connected to two input reservoirs, one filled with saline (maintained at constant level by a re-circulating pump; not shown) and the other filled with the red blood cell (RBC) suspension. Perfusion could be shifted between the two reservoirs *via* a three-way tap. Each reservoir was associated with a coronary flow measurement device similar to that described by Agnisola et al. (1994), but computer controlled. The aortic cannula was occluded so the output from the preparation was into the chamber *via* the atrial cannula. An ISO-NOP electrode was inserted into the atrial cannula to measure relative changes in NO level in the effluent from the preparation. The saline level in the chamber was kept constant by an overflow. Samples could be taken from both input and atrium.

(P_{O_2}). The preparation was allowed to stabilize until the coronary resistance did not change between two successive measurements made 5 min apart (which usually occurred within 15–20 min). Samples were then taken from the input and the atrium for perfusate measurements (see below). The perfusion was subsequently shifted to RBC suspension (typically having an Hct of ~6%), and after a few minutes of stabilization, input and atrium samples were taken, and pressure, flow and resistance were determined. Finally, nitrite was added to the RBC suspension in the RBC reservoir to an extracellular concentration of 400 μmol l^{-1}. The concentration was chosen to be lower than the millimolar values in nitrite-exposed fish but higher than the low micromolar values in fish from clean water (Stormer et al., 1996) to promote nitrite effects. Nitrite was added from a 140 mmol l^{-1} $NaNO_2$ stock solution, and the amount added was calculated taking into account the volume of RBC suspension and Hct. The suspension was gently mixed (as above) and drawn into the input. After a few minutes of perfusion with the nitrite-containing RBC suspension, further samples were taken and measurements made.

Protocol 2

Animal mass, 315±51 g; ventricle mass, 0.31±0.04 g (N=5). This protocol was the same as protocol 1, apart from the presence of the nitric oxide synthase (NOS) inhibitor L-NA (N_ω-nitro-L-arginine; Sigma-Aldrich, Steinheim, Germany) at a concentration of 10^{-4} mol l^{-1} in both the saline and the RBC suspension.

Protocol 3

Animal mass, 301±7 g; ventricle mass, 0.29±0.03 g (N=3). This protocol involved saline perfusion in the absence and presence of nitrite, to test the effect of nitrite on the preparation in the absence of RBCs.

There were no significant differences in ventricle mass or animal mass between the three protocols. Samples from the input were typically taken and measured 2–3 min before samples from the atrium were taken.

Nitrite uptake and metHb formation in red blood cells

To study the uptake of nitrite into RBCs, washed RBCs were suspended in the physiological saline and equilibrated in the tonometers (cf. above). In one experiment (Hct=9.1%), RBCs were equilibrated to 3% O_2/0.5% CO_2/96.5% N_2 for 45 min, giving an intermediate O_2 saturation (close to 50%). Following withdrawal of a control sample, nitrite was added from a 140 mmol l^{-1} $NaNO_2$ stock solution to reach a concentration of 400 μmol l^{-1} (time zero). Further samples were taken at predetermined times for measurements of Hct, Hb, metHb and extracellular [NO_2^-]. After 107 min, the gas supply was shifted to 0.5% CO_2/99.5% N_2 to fully deoxygenate the cells, and at 150 min the gas supply was shifted to 30% O_2/0.5% CO_2/69.5% N_2 to oxygenate the cells. In another set of experiments (N=4), RBC suspensions (Hct ~20%) were equilibrated in parallel to 99.5% air/0.5% CO_2 (oxygenated RBCs) or 99.5% N_2/0.5% CO_2 (deoxygenated RBCs). Following withdrawal of control samples, nitrite was added to an extracellular concentration of 3 mmol l^{-1} (calculated by taking into account the measured Hct), and further samples were then taken at predetermined times. Uptake of nitrite into the RBCs was assessed from the time-dependent decrease in extracellular [NO_2^-].

Measurements

The coronary pressure was continuously monitored through a saline-filled sidearm with a Uniflow Pressure Transducer (Baxter Uniflow, Bentley Laboratories Europe BV, Uden, Holland) connected to a computer for direct data acquisition. Pressure was expressed in kPa and was referenced to the saline level in the chamber and corrected for cannula resistance.

The coronary flow was determined by measuring the time it took 0.05 ml of perfusate to pass through the coronary artery, using a measuring system similar to that reported by Agnisola et al. (1994), modified for computer-driven automatic control and data acquisition (Fig. 1).

NO was measured with an ISO-NOP 2 mm sensor connected to an ISO-NO Mark II meter (World Precision Instruments), and the signal was sampled on a PC using the Duo.18^{TM} data recording system (World Precision Instruments) at a sampling rate of 3 samples s^{-1}. The electrode was calibrated by decomposition of the NO donor SNAP (*S*-nitroso-*N*-acetyl-penicillamine; Sigma) as described in the instruction manual. The reading from the electrode (pA) was converted to concentration (nmol l^{-1}) *via* the standard curve and multiplied by coronary flow to calculate rate of NO production.

Oxygen tension (P_{O_2}) and pH in saline and RBC suspension samples from the input and the atrium were measured at 15°C by Radiometer (Copenhagen, Denmark) electrodes in a BMS3 electrode set-up, with the signals displayed on Radiometer PHM 73 and PHM 84 meters and REC 80 recorders. Osmolality was measured with a Gonotec Osmomat 030 (Berlin, Germany). Nitrite was measured spectrophotometrically by a method based on the Gries reaction (Jensen, 1992). The fraction of metHb was evaluated by the three wavelength method of Benesch et al. (1973). Total Hb concentration was measured by the cyanmethaemoglobin method, using an extinction coefficient of 11 mmol l^{-1} cm^{-1} at 540 nm. Hct was determined by centrifugation (2 min at 13 700 ***g***) in glass capillaries. Oxygen content (C_{O_2}) in saline samples was calculated from the measured P_{O_2} and an O_2 solubility coefficient of 0.0408 μl O_2 ml^{-1} $torr^{-1}$. C_{O_2} of RBC suspension samples was measured by the Tucker (1967) method. Myocardial O_2 consumption ($\dot{V}_{O_2}$) was calculated by multiplying the difference in C_{O_2} between input and atrium with coronary flow and dividing by ventricle mass. Myocardial O_2 extraction (E_{O_2}) was determined as $(C_{O_2,input}–C_{O_2,atrium})/C_{O_2,input}$. The O_2 saturation (S_{O_2}) of functional Hb was calculated according to:

$$S_{O_2} = 100 \times (C_{O_2} - \beta_{O_2} \times P_{O_2}) / (C_{O_2,Hb} - C_{O_2,Hb} \times F_{metHb}) , \quad (1)$$

where β_{O_2} is the solubility coefficient of O_2 (Christoforides and Hedley-Whyte, 1969), $C_{O_2,Hb}$ is the O_2 capacity calculated from total [Hb], and F_{metHb} is the fractional metHb content.

Statistics

Results are expressed as means ± S.E.M., unless otherwise stated. Means were compared using one-way analysis of variance (ANOVA) with the Tukey *post hoc* test. One sample *t*-test was used to evaluate whether a mean was significantly different from zero. Results on nitrite uptake and metHb formation in oxygenated and deoxygenated RBCs *in vitro* were evaluated by two-way ANOVA for repeated measures. Differences were considered significant at $P<0.05$. Two-variable regression analysis and multiple regression analysis were used to evaluate the relationship between myocardial oxygen consumption and coronary flow, oxygen supply and oxygen extraction.

Results

Myocardial O_2 supply

Oxygen content (C_{O_2}) was measured on both input samples and atrium samples in most experiments. This allowed calculation of myocardial O_2 consumption and an analysis of its dependence upon input C_{O_2}, coronary flow and myocardial O_2 extraction during both saline perfusion and RBC perfusion. When all determinations of myocardial O_2 consumption were plotted as a function of input C_{O_2}, it was evident that the higher input C_{O_2} associated with RBC perfusion significantly increased O_2 consumption compared with saline perfusion (Fig. 2A). Most saline experiments were associated with relatively low input C_{O_2}, but points from experiments with saline equilibrated to high P_{O_2} (resulting in elevated C_{O_2}) overlapped with points obtained from RBC perfusion, whereby the relationship between $\dot{V}_{O_2}$ and input C_{O_2} could be approximated by one overall linear regression (Fig. 2A). When $\dot{V}_{O_2}$ was plotted as a function of coronary flow, it was seen that the low $\dot{V}_{O_2}$ during saline perfusion pertained to a large range of flows (Fig. 2B). With RBC perfusion, on the other hand, there was a significant linear increase in myocardial O_2 consumption with coronary flow (Fig. 2B). $\dot{V}_{O_2}$ also correlated positively with the O_2 extraction coefficient (Fig. 2C). The mean extraction coefficient was 0.60±0.11 (mean ± S.D., $N=17$) during RBC perfusion, which was significantly higher than the value of 0.34±0.11 (mean ± S.D., $N=23$) during saline perfusion. When all data were analysed simultaneously by multiple linear regression, each of the assigned independent variables [input C_{O_2}, coronary flow ($\dot{F}$) and E_{O_2}] contributed significantly to the assigned dependent variable $\dot{V}_{O_2}$, resulting in the following relationship ($r=0.91$, $F=57.4$, $P<0.0001$):

$$\dot{V}_{O_2} = 0.4034 \times C_{O_2} + 2.9195 \times \dot{F} + 5.6585 \times E_{O_2} - 5.2375 . \quad (2)$$

Corresponding values of S_{O_2} and P_{O_2} measured at the inflow to the coronary circulation and in the atrium outflow are shown in Fig. 3. It is evident that a significant desaturation of Hb occurred in the coronary circulation and that O_2 offloading took place at the steep portion of the O_2 equilibrium curve (OEC). Because metabolic CO_2 was added as O_2 was extracted in the microvasculature, a right shift of the OEC (Bohr effect) can be expected in the atrium. This increased the steepness of the *in situ* OEC compared with an *in vitro* OEC at constant

P_{CO_2}. To take this into account, the data were fitted to the rearranged Hill equation with n (Hills coefficient) fixed at 2.8 (compared with ~2 for *in vitro* OECs):

$$S_{O_2} = 100 \times (P_{O_2}/P_{50})^n / [1 + (P_{O_2}/P_{50})^n]. \quad (3)$$

This produced the overall *in situ* OEC shown in Fig. 3 and a fitted P_{50} (O_2 tension at half saturation) of 32.8±1.2 mmHg. The measured pH values were 7.772±0.018 at the input and 7.742±0.022 in the atrium.

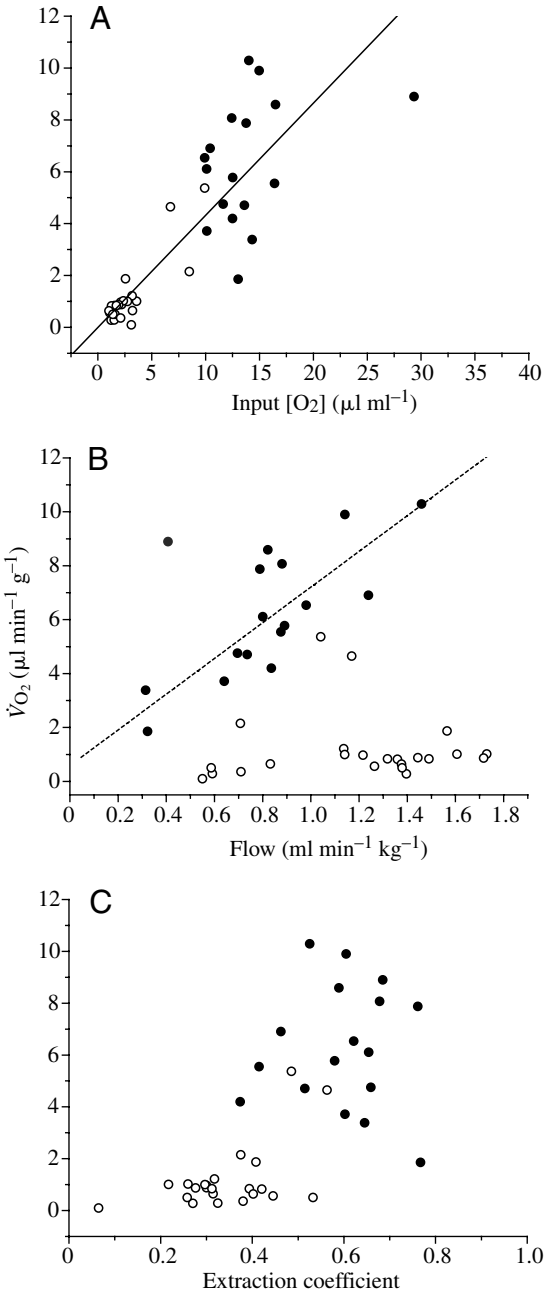

Fig. 2. Relationship between myocardial O_2 consumption ($\dot{V}_{O_2}$) and (A) input O_2 concentration, (B) coronary flow or (C) the O_2 extraction coefficient during perfusion of the isolated trout heart coronary circulation with saline (open circles) and red blood cell (RBC) suspensions (filled circles). The solid line in A is the linear regression between $\dot{V}_{O_2}$ and input C_{O_2}, using all saline and RBC perfusion points (y=0.432x; R=0.85, N=40, P<0.0001), and the broken line in B shows the linear relationship between $\dot{V}_{O_2}$ and coronary flow during RBC perfusion (y=6.6x+0.6; R=0.81, N=17, P=0.00013).

NO production

A representative NO trace from the experiments is shown in Fig. 4. The signal included significant noise as well as artefacts from the sampling procedure and measurements of flow, but the underlying NO signal could be effectively isolated by performing a 400-point adjacent-averaging smoothing of the raw data. After stabilisation of the signal during saline perfusion, a switch to RBC perfusion was performed. This typically caused an increased signal that subsequently tended to stabilise. Nitrite addition caused a further increase. Usually, the increase in the signal occurred after a delay (Fig. 4), which reflected the time needed for the perfusate to travel the

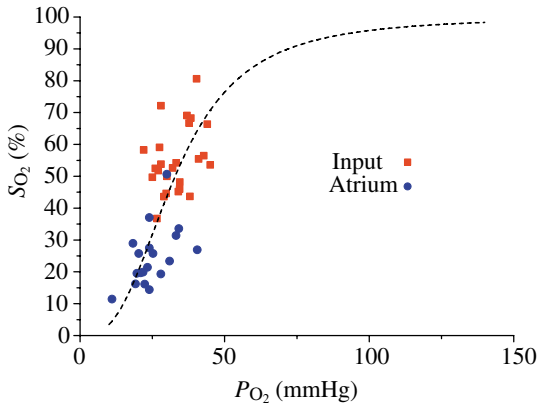

Fig. 3. Relationship between Hb O_2 saturation (S_{O_2}) and oxygen tension (P_{O_2}) in samples taken from the input and the atrium. The broken curve shows the overall *in situ* O_2 equilibrium curve. See text for details. (1 mmHg=133.3 Pa.)

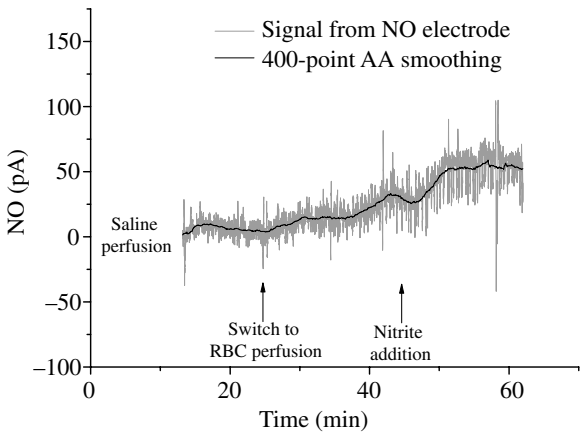

Fig. 4. Representative NO trace showing the NO signal as a function of time during saline perfusion, after switching to red blood cell (RBC) perfusion and after nitrite addition. The grey curve is the raw signal (3 measurements s^{-1}), and the black curve is the isolated NO signal, resulting from a 400-point adjacent averaging smoothing of the raw data.

coronary circulation and reach the measurement position at the atrial level. These changes to a new quasi-steady plateau indicated that a new balance between NO production and degradation was established and probably reflected an increased NO production. The NO signal change also depends on the prevailing coronary flow, which can change with treatment. The NO current at a given plateau was therefore converted to NO concentration *via* the standard curve, and the change in NO production rate related to a given experimental change was obtained as the difference between the multiplication product of concentration and flow after and before the change.

Perfusion of the coronary circulation with saline alone was associated with an NO production that was significantly different from zero (Fig. 5A). The NOS inhibitor L-NA significantly obliterated this NO production (Fig. 5A), suggesting that it resulted from endothelial NOS activity. Switching to RBC perfusion appeared to increase the NO production but, due to a large variability between preparations, the ΔNO production rate was not significantly different from zero (Fig. 5B). Interestingly, there was a significant linear decrease in ΔNO production with ventricle mass that seemed to explain the variability between preparations (Fig. 6). L-NA

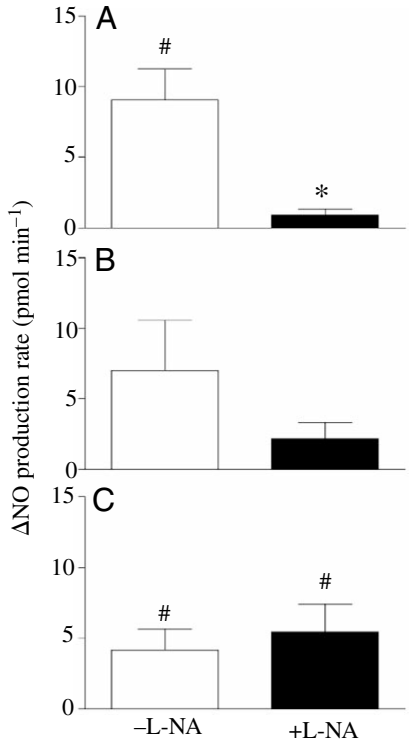

Fig. 5. NO production in the isolated, non-working trout heart preparation. Open bars: perfusion in absence of L-NA; filled bars: perfusion in presence of L-NA. (A) NO production rate during saline perfusion; (B) change in the NO production rate during RBC perfusion; (C) change in the NO production rate after addition of nitrite to RBC perfusate. # signifies that NO production or ΔNO production is significantly different from zero. * signifies a significant effect of L-NA.

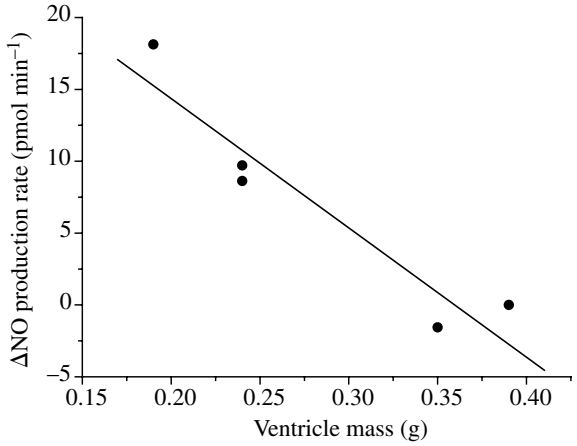

Fig. 6. Relationship between ΔNO production rate and ventricle mass following the switch from saline perfusion to RBC perfusion in the absence of L-NA (y=–90x+32.4; R=–0.95, P=0.015).

tended to inhibit the ΔNO production (Fig. 5B). When nitrite was added during RBC perfusion, the ΔNO production rate increased to values that were significantly different from zero, but in this case there was no difference between absence and presence of L-NA (Fig. 5C). There was no sign of haemolysis in centrifuged samples of the RBC perfusates, which is important because extracellular Hb would have scavenged the produced NO (Gladwin et al., 2004).

Nitrite was added to a nominal extracellular concentration of approximately 400 μmol l^{-1} during RBC perfusion. Values measured at the input were lower (Fig. 7), suggesting a rapid initial influx of NO_2^- to the RBCs, which was confirmed by *in vitro* experiments (see below). As the RBC passed through the coronary circulation, a further slight decrease in extracellular [NO_2^-] occurred, and this was paralleled by a significant increase in metHb, as measured in the atrium output (Fig. 7). Hct values during these experiments were 6.3±0.6 for input samples and 5.6±0.7 for atrium samples (N=12 in each case), which were not statistically different.

Effects of treatments on coronary flow

RBC perfusion (with or without nitrite) was associated with significantly higher O_2 content and myocardial O_2 consumption than during saline perfusion, but it did not affect coronary flow (and coronary resistance) significantly (Fig. 8). The presence of L-NA caused a significant decrease in coronary flow, when compared with the absence of L-NA, both during saline perfusion and during RBC perfusion (compare Fig. 8C and Fig. 8F), and it decreased $\dot{V}_{O_2}$ (Fig. 8B,E) during saline perfusion but not C_{O_2} (Fig. 8A,D).

Nitrite addition during RBC perfusion did not change coronary flow in either the absence or presence of L-NA (Fig. 8C,F). Nitrite was also without significant influence on coronary flow when added during saline perfusion in the absence of L-NA (flow values, 1.34±0.11 and 1.28±0.05 ml min^{-1} kg^{-1} before and after nitrite addition, respectively; N=3).

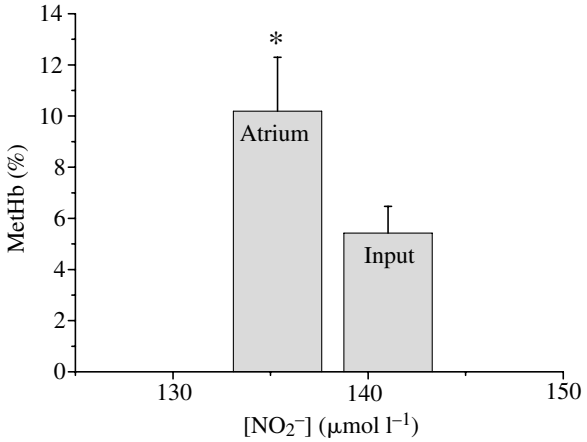

Fig. 7. Methaemoglobin (metHb) content and extracellular $[NO_2^-]$ in input and atrium samples during RBC + nitrite perfusion of the coronary circulation. * signifies a significant difference in metHb.

In vitro *RBC nitrite uptake*

When nitrite was added to an RBC suspension in the tonometer under conditions similar to those prevailing during RBC perfusion of the coronaries (i.e. with respect to $[NO_2^-]$, S_{O_2}, Hct and pH), extracellular $[NO_2^-]$ showed a major and rapid decline below the added value (400 μmol l^{-1}) and then continued to decrease at a slower rate (Fig. 9A). This reflected a rapid initial entry of nitrite into the RBCs followed by a slower continued entry. The entry of nitrite caused a moderate increase in RBC metHb content (Fig. 9C). A change in Hb O_2 saturation from about 50% to full deoxygenation, followed by a subsequent full oxygenation, had no major influence on $[NO_2^-]$ and metHb (Fig. 9A,C). To further study a possible oxygenation dependency of nitrite uptake, nitrite was added to oxygenated and deoxygenated RBC suspensions at a higher extracellular concentration (3 mmol l^{-1}) and haematocrit (Hct ~20%). This was followed by a rapid initial decrease in extracellular $[NO_2^-]$, with a subsequent slower continued decrease, and there was no significant difference between oxygenated and deoxygenated RBC suspensions (Fig. 9B). The entry of nitrite into the RBCs caused a significant increase in metHb with time, and metHb values tended to be higher in oxygenated than deoxygenated RBCs, but this difference was not significant (Fig. 9D).

Discussion

The coronary circulation of the isolated trout heart is a useful model in studies of endocrine and paracrine mechanisms involved in regulation of microcirculation in fish (Agnisola, in press). Such studies have traditionally been performed with a physiological saline as the perfusion medium. The low O_2 capacitance of saline, however, represents a problem, which can be compensated to some extent by equilibration with high P_{O_2}; but when hypoxia is studied, P_{O_2} inherently needs to be low. This leads to a compromised O_2 delivery to the myocardium that can be overcome by perfusing with an RBC suspension instead. Indeed, perfusion of the coronary circulation with RBCs significantly increased myocardial O_2 consumption, even at relatively low haematocrit (Fig. 2). The present upgrading of the isolated non-working trout heart set-up to include perfusion of the coronary circulation with RBC

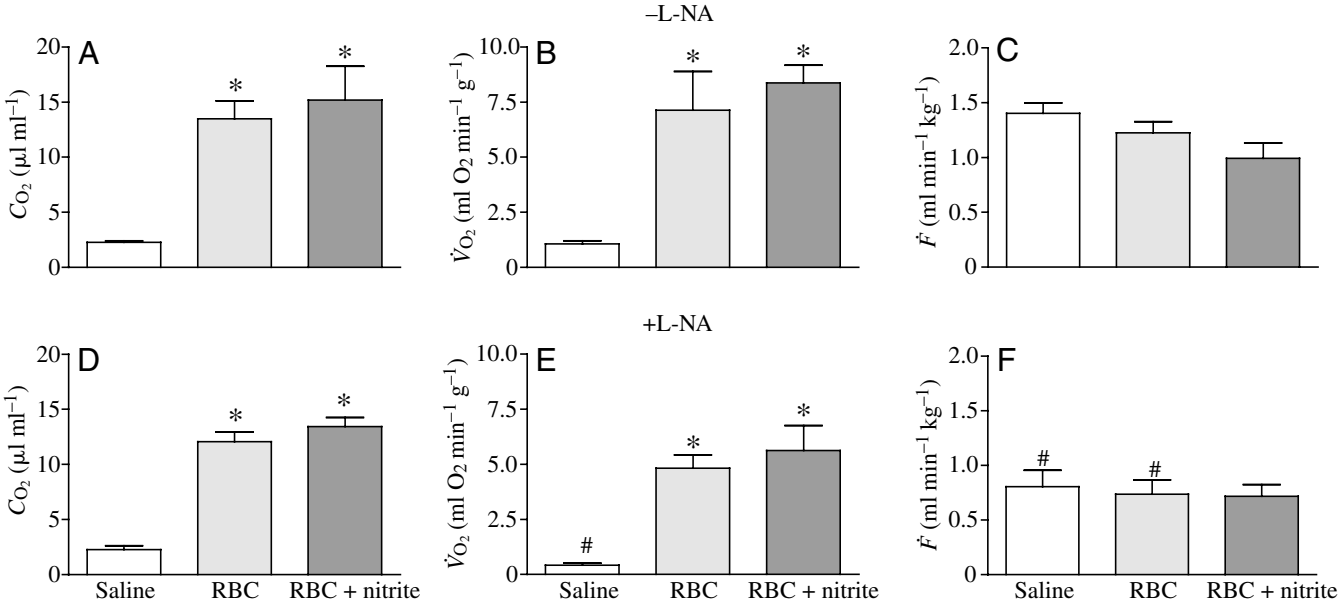

Fig. 8. Comparison of mean values for (A,D) input oxygen concentration (C_{O_2}), (B,E) myocardial oxygen consumption ($\dot{V}_{O_2}$) and (C,F) coronary flow ($\dot{F}$) during saline perfusion, red blood cell (RBC) perfusion and RBC + nitrite perfusion in the absence (A–C) and presence (panels D–F) of L-NA. # signifies significant effects of L-NA; * signifies significant effects of RBC perfusion or RBC + nitrite perfusion compared with saline perfusion.

suspensions therefore greatly enhances the applicability of this preparation.

Basically, $\dot{V}_{O_2}$ is the product of the ingoing O_2 concentration, the flow and the O_2 extraction coefficient ($\dot{V}_{O_2}=C_{O_2,input}\times\dot{F}\times E_{O_2}$). With saline as the perfusion medium, both $C_{O_2,input}$ and E_{O_2} are low (Fig. 2A,C), and changes in flow have little impact on $\dot{V}_{O_2}$ (Fig. 2B). Perfusion with RBCs elevates the O_2 capacitance of the medium, which elevates $C_{O_2,input}$ (Fig. 2A), increases E_{O_2} (Fig. 2C) and effects a large influence of myocardial flow on O_2 delivery (Fig. 2B). Thus, RBC perfusion of the coronaries comes closer to the *in vivo* situation where C_{O_2} and E_{O_2} are high and where the major method to increase $\dot{V}_{O_2}$ is *via* increased myocardial flow. A parallel can be drawn to the human heart, where coronary blood flow and myocardial $\dot{V}_{O_2}$ are closely matched (Tune et al., 2002). In rainbow trout, coronary blood flow increases *in vivo* both during hypoxia and during exercise, which improves myocardial O_2 supply and cardiac performance (Gamperl et al., 1995).

Through the measurements of corresponding values of S_{O_2} and P_{O_2} before and after the coronary circulation, we constructed the overall *in situ* O_2 equilibrium curve (Fig. 3). The P_{50} was slightly higher than reported in normoxic rainbow trout (Tetens and Lykkeboe, 1981). This is explained by the Bohr effect (increase in P_{50} with pH decrease), because we used an equilibration P_{CO_2} (3.74 mmHg) that was slightly higher than the normal arterial P_{CO_2} value (2–3 mmHg), and our input pH (7.772) was therefore lower than the arterial pH above 7.9 in rainbow trout at 15°C (Wang et al., 1998). The input and atrium S_{O_2}–P_{O_2} values show that there was a major decrease in S_{O_2} as result of the RBC transit through the microvasculature. Thus, the condition of low S_{O_2}, which is required for deoxyHb-mediated reduction of nitrite to NO to take place, was present.

NO production

There was a significant NO production when the coronary vessels were perfused with hypoxic saline, and this NO production was fully inhibited by L-NA (Fig. 5A). This shows that the trout coronaries produce NO under hypoxic conditions and that inhibition of NO synthase obliterates this production. The parallel recordings of coronary flow furthermore attest a vasodilatory role to this NO production (cf. below). The NO probably resulted from endothelial NOS activity in the endothelium lining the vessels. A contribution from inducible NOS cannot be excluded, although this isoform usually mediates responses that are slower and wider than those observed here (Mershon et al., 2002).

In the presence of RBCs, NO produced in the endothelium and in the RBCs runs the risk of being scavenged by Hb (Gladwin et al., 2004). On the other hand, if an NO signal is produced in the blood to be picked up by vascular smooth muscle, then it should also be possible to harvest that signal at the NO electrode membrane. Indeed, we registered an increased signal after switch to RBC perfusion and after NO_2^- addition (Fig. 4). The amperometric NO sensor method was also found to be reliable for recordings of NO production in human RBC suspensions (Carvalho et al., 2004). The ΔNO production

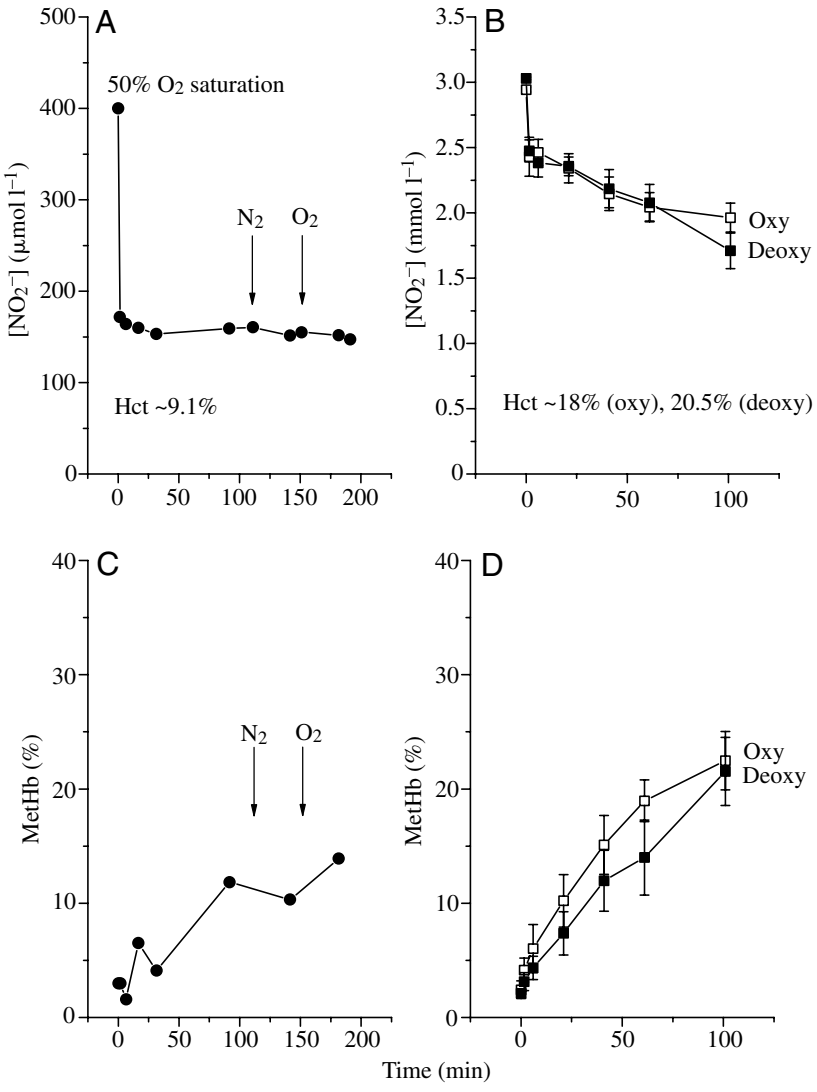

Fig. 9. Time-dependent changes in extracellular $[NO_2^-]$ after addition of nitrite to red blood cell (RBC) suspensions in a tonometer (A,B). The decrease in extracellular $[NO_2^-]$ reflects an influx of nitrite into the RBCs. Note the different $[NO_2^-]$ axes, haematocrits and O_2 saturations. N_2 and O_2 indicate full deoxygenation and oxygenation, respectively. C and D show the corresponding methaemoglobin (metHb) values. See text for further details.

from RBC perfusion *per se* showed large variation (Fig. 5B), which appeared accounted for by differences in ventricle mass (Fig. 6). One possible explanation is that RBC perfusion causes a higher shear stress in smaller hearts due to their smaller vessel dimensions, which might effect a compensatory increase in endothelial NO production. Sheer stress is known to increase NO release from the endothelium in the mammalian coronary circulation (Stepp et al., 1999), and sheer stress-mediated NO-release has been implicated in the vasodilatory response of the trout coronaries to adenosine (Mustafa and Agnisola, 1998).

Addition of nitrite during RBC perfusion increased the ΔNO production rate, and, importantly, this NO production was unaffected by L-NA (Fig. 5C). Thus, in contrast to the NO production during saline perfusion, the nitrite-induced NO production was not due to endothelial NOS activity, suggesting that it occurred *via* the RBCs or cardiac myocytes. The potential role of RBCs in blood flow regulation *via* deoxyHb-mediated reduction of nitrite to NO (Cosby et al., 2003) implies a sequence of events. First, nitrite should enter the RBCs. Secondly, because nitrite reacts with oxyHb to form nitrate and metHb and with deoxyHb to form NO and metHb, a significant desaturation of Hb is required, and some metHb should be formed. Thirdly, to exert an effect on the vasculature, the NO formed should escape the RBCs. All these prerequisites appeared to be fulfilled: there was a rapid entry of nitrite into the RBCs (Fig. 9), a gradient in $[NO_2^-]$ and rise in metHb between input and atrium (Fig. 7), a significant decrease in HbO_2 saturation in the coronary circulation (Fig. 3), and an NO signal was registered (Fig. 5C). Thus, the data support the idea that NO is produced from nitrite in the RBCs. It cannot be excluded, however, that the heart itself may generate NO from nitrite by means of xanthine oxidoreductase activity, as reported in human and rat hearts (Webb et al., 2004).

The entry of nitrite into the RBCs deserves some attention. In carp, tench and whitefish, nitrite preferentially permeates the RBC membrane at low O_2 saturation (Jensen, 1992, 2003), which appears to supply nitrite for subsequent deoxyHb-mediated reduction to NO in an appropriate way. In rainbow trout, on the other hand, there was no significant oxygenation dependency of nitrite transport (Fig. 9). This contrasts sharply with the other teleost fishes examined but compares with the situation in pig erythrocytes (Jensen, 2005). It appears that in some species (carp, tench, whitefish) nitrite transport is governed by a major oxygenation-dependent change in membrane permeability, whereas in others (pig, trout) nitrite quickly equilibrates across the membrane, after which nitrite entry is governed by intracellular nitrite removal *via* its reactions with Hb (cf. Jensen, 2005).

Effects on coronary flow

Coronary flow was significantly higher in the absence of L-NA than in the presence of L-NA during both saline perfusion and RBC perfusion (Fig. 8C,F). This shows that the NO released from the coronary endothelium produced vasodilation in the absence of L-NA, whereas inhibition of NOS-catalyzed NO production by L-NA induced vasoconstriction. The data therefore corroborate that endothelial NO plays a role in regulating flow in the saline perfused trout coronary circulation (Mustafa et al., 1997) and extends the conclusion to RBC perfusions, where C_{O_2} is higher. The mean values for coronary flow (Fig. 8C) are some 3–4% of the *in vivo* cardiac output in resting normoxic trout at 15°C (~38 ml min^{-1} kg^{-1}; Wood and Shelton, 1980) and are accordingly higher than *in vivo* coronary flow in normoxic salmonids (~1% of cardiac output; Axelsson and Farrell, 1993; Gamperl et al., 1994). This is not surprising given the hypoxic conditions and the lower viscosity and oxygen capacitance in saline and low-Hct erythrocyte media than in whole blood (Farrell, 1987). In particular, as shown in the present study, hypoxia will induce NO release and vasodilation.

Addition of nitrite during RBC perfusion did not change coronary flow, and this was the case both in the absence and the presence of L-NA (Fig. 8C,F). Thus, the NO produced from nitrite was unable to induce further vasodilation in the absence of L-NA or to overcome the relative vasoconstriction in L-NA-treated preparations. The formation of NO from nitrite in RBCs with subsequent vasodilation may require that P_{O_2} is at or below P_{50}, i.e. that $S_{O_2} \leqslant 50\%$ (Gladwin et al., 2004). In our experiments, S_{O_2} in the inflowing RBC suspension was around or above 50% (Fig. 3). One possible explanation for the absent vasodilation may therefore be that most NO was produced from nitrite following deoxygenation in the capillaries, whereby the NO was found in capillaries, post-capillary vessels and the atrium, where it had little likelihood of acting on coronary arterioles. If the RBCs function as a sensor of O_2 conditions (*via* degree of deoxygenation) and mediator of increased blood flow (*via* NO release) in the normal arterial–venous circulation, then an essential question is how the signal is propagated to the major resistance vessels. In the microcirculation, RBCs may experience a decline in P_{O_2} before reaching the capillaries, in which case NO released from RBCs could exert an effect on arterioles. It is also possible that a signal generated in the capillaries can be conducted to upstream arterioles, as in skeletal muscle microvasculature (Murrant and Sarelius, 2000). The present data do not support this happening in the trout coronary circulation, but this could be a characteristic of the particular microvascular bed. It may be rewarding to test other microcirculations in fish, such as skeletal muscle vasculature, where nitrite-induced vasodilation is known to occur in mammals (Cosby et al., 2003), or cerebral microcirculation, where nitric oxide-dependent, acetylcholine vasodilation has been demonstrated in rainbow trout and crucian carp (Söderström et al., 1995; Hylland and Nilsson, 1995).

Thus, the RBC perfused trout coronary circulation did not respond to nitrite under normal and moderately hypoxic conditions, probably because deoxyHb-mediated NO generation occurred after the resistance vessels. During severe hypoxia, NO production from nitrite can be predicted to occur before the arterioles. In this situation, vasodilation may occur,

because our results show that NO (of endothelial origin) is vasoactive in the trout coronary circulation.

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