Nitric oxide (NO) synthase in the vasculature (endothelial NO synthase [eNOS]) is constantly activated by the shear stress generated by circulating blood. The resulting NO, which activates soluble guanylate cyclase (sGC), maintains a vasodilator tone which is determinant in the regulation of blood flow and pressure. NO also interacts with the terminal enzyme of the electron transport chain, cytochrome c oxidase (CcO), in a manner that is competitive with oxygen (O2), leading to an increase in the Km of this enzyme for O2 and the consequent inhibition of respiration. A number of studies in vascular endothelial cells and in tissues have shown that this results in a decrease in consumption of O2 and in its redistribution away from mitochondria and toward other oxygen-dependent targets. Thus NO plays a role in the subcellular profile of O2 distribution. In this study, we have investigated this phenomenon by monitoring the dynamics of O2 consumption and its distribution both throughout the thickness of conductance vessels and in the microcirculation. Furthermore, we have shown that such modulation regulates O2 distribution to the surrounding tissues. We have demonstrated these effects by measuring O2 consumption in blood vessels in a hypoxic chamber and O2 distribution in the microcirculation using the fluorescent oxygen-probe Ru(phen)3²⁺. Removal of NO by physical or pharmacological means, or in eNOS⁻/⁻ mice, abolishes this regulatory mechanism. Our results indicate that, in addition to its well-known effect on the regulation of vascular tone, endothelial NO plays a major role in facilitating the distribution of O2, an action which is crucial for the adaptation of tissues, including the vessel wall itself, to hypoxia. It is possible that changes in the distribution of O2 throughout the vessel wall may be implicated in the origin of vascular pathologies such as atherosclerosis. (Circ Res. 2009;104:1178-1183.)

Key Words: nitric oxide  endothelium  oxygen consumption

Materials and Methods

Tissue Preparation

Human umbilical cords were obtained from the Department of Gynaecology and Obstetrics of the Hospital Doctor Peset. Vascular rings, 5 mm in length, were extracted from the middle portion of the cords. Male Sprague–Dawley rats (200 to 250 g, Harlan Laboratories, Barcelona, Spain) or wild-type (WT) and eNOS knockout (eNOS⁻/⁻) mice (WT, C57BL/6Jx129, 20 to 25 g, UCL, London, UK) were decapitated and their thoracic aortas and pulmonary and mesenteric arteries were removed, cleaned of adhering tissues, and cut into 5-mm (rat vessels) or 2-mm (mouse vessels) rings. When necessary, the endothelium was disrupted by gently rubbing the luminal surface. For the experiments with fluorescence microscopy, a portion of the rat or mouse mesenteric artery tree was isolated and cleaned of surrounding fat. All experiments were performed in Krebs solution (in 10⁻³ mol/L) (NaCl 118, KCl 4.75, CaCl₂ 1.9, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 10.1). All protocols complied with the European Community guidelines for the use of experimental animals and were approved by the Ethics Committee of the University of Valencia.

Contractility Studies

These experiments were performed with rat vascular rings suspended in a 5-mL organ bath (37°C) gassed with 12% O2, 5% CO₂, and 83% N₂, which produced an O2 concentration of ~130×10⁻⁶ mol/L, similar to that present in the aortic blood, and monitored with a dissolved O2 meter (ISO₂; World Precision Instruments, Stevenage, Herts, UK). Tension was measured isometrically with Grass FTO3

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force-displacement transducers and recorded (Power Laboratory). An initial load of 2 g (aorta and human umbilical artery) or 1 g (pulmonary and mesenteric arteries) was applied to each preparation and maintained throughout the experiment. Following a 75- to 90-minute equilibration period, rings were contracted with phenylephrine (Phe) \((1 \times 10^{-5}\) mol/L in mesenteric artery or \(1 \times 10^{-6}\) mol/L in pulmonary artery, aorta, and human umbilical artery), and the presence of a functional endothelium was confirmed by eliciting a relaxant response (>95%) to acetylcholine (ACH) \((1 \times 10^{-5}\) mol/L), a dose that induces maximal SGC-mediated vasodilatation.7

After a further equilibration period of 30 to 45 minutes, vessels were contracted with a depolarizing solution \((60 \times \text{mol}^{-1}\) L\(^{-1}\) KCl) to obtain the maximal level of contraction. After further washing, the rings were allowed to equilibrate for at least 1 hour. Thereafter, they were again stimulated with Phe and concentration–response curves of relaxation to ACh \((1 \times 10^{-7}\) to \(1 \times 10^{-5}\) mol/L), bradykinin (Bk) \((1 \times 10^{-6}\) to \(1 \times 10^{-5}\) mol/L), or the slow-releasing NO donor diethylenetriamine (DETA-NO) \((1 \times 10^{-8}\) to \(1 \times 10^{-5}\) mol/L) were carried out. In another set of experiments concentration-response curves of contraction to Phe \((1 \times 10^{-10}\) to \(10 \times 10^{-6}\) mol/L) were performed. The concentration \((-\log[\text{mol/L}]\) that produced 95% of the maximal effect \((\text{pEC}_{95})\) was obtained by a nonlinear regression analysis with GraphPad software.

**Electrochemical Measurement of O\(_2\) Consumption**

Aortic rings from 2 rats (80 to 100 mg total wet weight) or 3 mice (20 to 30 mg wet weight), pulmonary and mesenteric rings from 3 rats (15 to 25 mg wet weight), or human umbilical arteries (190 ± 5 mg total wet weight) were placed in gas-tight chambers containing 1 mL of Krebs solution and gently agitated at 37°C. The O\(_2\) consumption by the tissue was measured with a Clark-type O\(_2\) electrode (Rankin Brothers, Bottisham, UK) calibrated with an air-saturated Krebs solution, assuming an \([\text{O}_2]\) of 200 \(\times 10^{-6}\) mol/L. Experiments were performed in the presence of the NO synthase inhibitor \(N^\text{\textsuperscript{G}}\)-nitro-l-arginine (L-NA) \((1 \times 10^{-4}\) mol/L), the sGC inhibitor \(1H-1,2,4\text{-oxadiazolo[4,3-}a\text{-quinazolin-1-one (ODQ)}\), \((5 \times 10^{-6}\) mol/L, ACh \((1 \times 10^{-5}\) mol/L), Bk \((1 \times 10^{-5}\) mol/L), or Phe \((1 \times 10^{-5}\) mol/L) in mesenteric artery or \(1 \times 10^{-6}\) mol/L in pulmonary artery, aorta, and human umbilical artery) in intact or endothelium-denuded vessels. Sodium cyanide \((1 \times 10^{-5}\) mol/L) was used to confirm that O\(_2\) consumption was mainly mitochondrial \((>95\% \text{ to } 99\%)\). In some experiments, the reversibility of NO-induced inhibition of respiration was assessed by adding oxyHb \((1 \times 10^{-5}\) mol/L) when the \([\text{O}_2]\) in the chamber was 100 \(\times 10^{-6}\) mol/L. Measurements were obtained using a data acquisition device, Duolab (World Precision Instruments, Stevenage, UK). A hyperbolic function was used to describe the relationship between \([\text{O}_2]\) and the rate of O\(_2\) consumption \((\text{VO}_2)\). The apparent O\(_2\) affinity (Michaelis–Menten constant \([K_m]\)) \((1 \times 10^{-6}\) mol/L) was calculated using the program GraphPad, and the results are shown in the Table. \(\text{VO}_2\) was calculated at 2 specific concentrations \([O_2]\), \(130 \times 10^{-6}\) mol/L and \(30 \times 10^{-6}\) mol/L, which were selected as representing those \([O_2]\) present in arterial blood or within tissues, respectively.8 \(\text{VO}_2\) was expressed as 1 mL of Krebs solution and gently agitated at 37°C. This system was then placed under a fluorescence microscope (Axiovert 200M Zeiss inverted). Following equilibration for 10 minutes at atmospheric \([O_2]\) \((200 \times 10^{-6}\) mol/L), the O\(_2\) concentration in the tissue medium, however, was \(130 \times 10^{-6}\) mol/L, as measured with a dissolved O\(_2\) meter \((\text{ISO}_2\); World Precision Instruments, Stevenage, Herts, UK). Samples were subjected to 10 minutes of incubation. Samples of mesentery between 2 vessel branches were then mounted on a purpose-built chamber for oxygen measurements with the Oxy-100 device (Oxy-100, Saint-Germain-en-Laye, France). The vital cell nuclear stain Hoechst 33342 \((1 \times 10^{-6}\) mol/L) exhibits high O\(_2\) sensitivity, a large Stoke shift, and high photo-chemical stability in the cellular environment. Because of its long emission wavelength the fluorescence signal of the indicator is shifted away from cellular autofluorescence, which causes serious perturbation when UV sensing indicators are used. In our experimental conditions, ruthenium is taken up by living cells and maintains its O\(_2\)-sensing properties for at least 5 hours after loading.

The vital cell nuclear stain Hoechst 33342 \((1 \times 10^{-6}\) mol/L) was added for the last 30 minutes of incubation. Samples of mesentery were excited at 37°C. This system was then placed under a fluorescence microscope (Axiovert 200M Zeiss inverted), following equilibration for 10 minutes at atmospheric \([O_2]\) \((200 \times 10^{-6}\) mol/L). The O\(_2\) concentration in the tissue medium, however, was \(130 \times 10^{-6}\) mol/L, as measured with a dissolved O\(_2\) meter \((\text{ISO}_2\); World Precision Instruments, Stevenage, Herts, UK). Samples were subjected to hypoxia \((15 \times 10^{-6}\) mol/L) for 20 minutes by the addition of nitrogen in the presence or absence of the NO synthase inhibitor L-NA \((1 \times 10^{-4}\) mol/L), ACh \((1 \times 10^{-5}\) mol/L), or DETA-NO \((1 \times 10^{-5}\) mol/L). Images \((\times 10\) magnification) were obtained every 2 minutes throughout the entire experimental period, and changes in Ru(phen)

\(^{2+}\) fluorescence were analyzed using a 543-nm He-Ne laser to excite the probe. A 405-nm diode laser was used to excite Hoechst 33342. Fluorescence was detected through 440/20 BP and 604 LP filters for Hoechst 33342 and Ru(phen)

\(^{2+}\), respectively, and the fluorescence microscope settings were adjusted to produce the optimum signal to noise ratio and maximum signal detection. An analysis of the ruthenium fluorescence in individual cells was obtained with Leica image analysis software that processed the image stacks acquired in the region of interest. The ratio of fluorescence intensity was calculated for each cell using imaging software (Leica Confocal Software, version 2.61, Leica Microsys-

| **Table. Effect of NO on the Apparent \(K_m\) for \(\text{O}_2\) in Rings From Rat and Human Vessels** |
|-----------------|-----------------|-----------------|-----------------|
|                  | Aorta           | Pulmonary       | Mesenteric      |
| **Control**      | 23.2 ± 1.3      | 20.7 ± 3.1      | 29.3 ± 3.1      |
| **L-NA**         | 7.3 ± 0.4*      | 10.9 ± 1.0*     | 12.5 ± 1.8*     |
| **ODQ**          | 8.3 ± 0.6*      | 9.9 ± 0.7*      | 12.9 ± 1.2*     |
| **ACh**          | 7.59 ± 2.6*     | 42.7 ± 2.3*     | 76.6 ± 10.6*    |
| **L-NA + ACh**   | 7.3 ± 0.4*      | 9.9 ± 0.7*      | 12.9 ± 1.2*     |
| **E(-)**         | 8.3 ± 0.5*      | 9.9 ± 0.7*      | 12.9 ± 1.2*     |
| **Bk**           | 79.7 ± 3.8*     | 46.4 ± 2.8*     | 72.3 ± 10.5*    |
| **L-NA + Bk**    | 8.3 ± 0.5*      | 8.1 ± 0.3*      | 12.6 ± 1.3*     |
| **E(-)**         | 6.5 ± 0.5*      | 9.2 ± 0.2*      | 11.7 ± 1.1*     |
| **DETA**         | 113.1 ± 5.8*    | 78.3 ± 5.2*     | 83.4 ± 6.1*     |
| **L-NA + DETA**  | 93.8 ± 2.6*     | 69.4 ± 4.1*     | 78.5 ± 5.1*     |
| **E(-)**         | 83.1 ± 2.4*     | 66.5 ± 5.3*     | 75.3 ± 6.5*     |
| **Phe**          | 71.6 ± 3.1*     | 38.1 ± 3.6*     | 56.5 ± 5.1*     |
| **L-NA + Phe**   | 6.6 ± 0.5*      | 8.3 ± 0.5*      | 9.3 ± 0.4*      |
| **E(-)**         | 7.8 ± 0.5*      | 7.6 ± 0.6*      | 9.9 ± 0.6*      |

\(^*\text{P}<0.05\) vs control.
tems GmbH, Heidelberg, Germany). At least 70 cells were analyzed for each sample.

Further experiments were performed to exclude interference with Ru(phen)$_3^2+/H_11001$ fluorescence by free radicals (H$_2$O$_2$) and NO (DETA-NO). Different concentrations (1$\times$10$^{-4}$ mol/L to 1$\times$10$^{-3}$ mol/L) of H$_2$O$_2$ and DETA-NO were incubated in the presence of Ru(phen)$_3^2+$ (1$\times$10$^{-4}$ mol/L) in a cell-free Krebs solution. Fluorescence was measured with a Fluoroskan plate reader (Thermo Labsystems). Basal fluorescence was not modified by incubation with DETA-NO or H$_2$O$_2$, or either of the compounds in normoxia and hypoxia, except at a maximal concentration of H$_2$O$_2$ (1$\times$10$^{-3}$ mol/L) (n=4 to 5 experiments; results not shown).

Data Analysis

Unless otherwise stated, all results are the means±SEM of at least 5 experiments from different animals. Statistical analysis was performed by 1-way ANOVA (GraphPad Software) and Student–Newman–Keuls as a post hoc test. Significance was defined as $P<0.05$.

Drugs and Solutions

Phe, ACh, Bk, L-NA, sodium cyanide, ODQ, meth(a)emoglobin, Ru(phen)$_3^{2+}$, and Hoechst 33342 were acquired from Sigma-Aldrich (St Louis, Mo). DETA-NO was from Alexis (Lausen, Switzerland). OxyHb was prepared by reduction of human meth(a)emoglobin with a 10-fold molar excess of sodium dithionite, followed by dialysis against PBS. All drugs were dissolved in ultrapure water. Ru(phen)$_3^{2+}$ and Hoechst 33342 were dissolved in Krebs solution and ODQ was dissolved in DMSO (1:5000 vol:vol).

Results

Consumption of O$_2$ by Blood Vessels

First we monitored Vo$_2$ in several isolated blood vessels when placed in an O$_2$-tight chamber, as previously described.7,9,10 Figure 1A and 1B show how the rat aorta gradually reduced its rate of O$_2$ consumption as the [O$_2$] decreased. The requirement of the tissue for O$_2$ was mainly mitochondrial because addition of sodium cyanide resulted in almost complete (95% to 99%) inhibition of O$_2$ consumption (data not shown). The reduction in the rate of consumption at low [O$_2$] was attenuated both by blocking NO synthesis with L-NA and by removal of the endothelium, indicating that NO was responsible for this effect and therefore for the observed increase in the apparent $K_m$ for O$_2$ (Figure 1B and the Table). This action of NO was independent of an effect on sGC because it was unaffected by treatment with the specific sGC inhibitor ODQ (Table).

Figure 1C shows the consumption of O$_2$ at 2 specific [O$_2$] that approximate to those of arterial blood (130$\times$10$^{-6}$ mol/L) and tissue (30$\times$10$^{-6}$ mol/L), respectively.7,8 Aortas were found to consume $\approx$50% less O$_2$ at the lower of the 2 [O$_2$]. This NO-dependent adaptive response was not observed only in the aorta but in all the vessels studied, independent of species (human, rat, mouse) or anatomic origin (placental, systemic, or pulmonary circulation) (Table).

In aortas from eNOS knockout mice, we observed a reduced apparent $K_m$ for O$_2$ and similar rates of O$_2$ consumption at the high and low [O$_2$] studied. This contrasted with the response of tissues from WT mice, which was similar to that of control rat aortas. The responses of tissues from the eNOS knockout mice were similar to those exhibited by WT controls after treatment with L-NA or endothelium denudation (Figure 1D through 1F).

Figure 1E shows the consumption of O$_2$ at 2 specific [O$_2$] that approximate to those of arterial blood (130$\times$10$^{-6}$ mol/L) and tissue (30$\times$10$^{-6}$ mol/L), respectively.7,8 Aortas were found to consume $\approx$50% less O$_2$ at the lower of the 2 [O$_2$]. This NO-dependent adaptive response was not observed only in the aorta but in all the vessels studied, independent of species (human, rat, mouse) or anatomic origin (placental, systemic, or pulmonary circulation) (Table).

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Figure 1F shows the consumption of O$_2$ at 2 specific [O$_2$] that approximate to those of arterial blood (130$\times$10$^{-6}$ mol/L) and tissue (30$\times$10$^{-6}$ mol/L), respectively.7,8 Aortas were found to consume $\approx$50% less O$_2$ at the lower of the 2 [O$_2$]. This NO-dependent adaptive response was not observed only in the aorta but in all the vessels studied, independent of species (human, rat, mouse) or anatomic origin (placental, systemic, or pulmonary circulation) (Table).

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Treatment of rat arteries with vasodilators such as ACh (1$\times$10$^{-4}$ mol/L) or Bk (1$\times$10$^{-5}$ mol/L) (EC$_{50}$) (see Materials and Methods) that stimulate endothelial NO generation resulted in a greater inhibition of O$_2$ consumption at the low [O$_2$] and, in addition, an inhibition of O$_2$ consumption at the
Figure 2. Effect of ACh and DETA-NO on VO₂ in rat aorta. Vessels were treated with ACh (1×10⁻⁵ mol/L) or DETA-NO (1×10⁻⁶ mol/L) in endothelium-denuded vessels (E-) or following incubation with L-NA (1×10⁻⁴ mol/L). A shows the continuous measurement of VO₂, and B shows the VO₂ measured at 130×10⁻⁶ mol/L and 30×10⁻⁶ mol/L O₂. Data are means±SEM from n=4 independent experiments, with *P<0.05 vs control at the corresponding O₂, and #P<0.05 vs the corresponding value at 130×10⁻⁶ mol/L O₂.

Figure 3. Oxygen consumption during hypoxia in the mesenteric arteries. A: Typical images of fluorescent tissue stained with Ru(phen)₃Cl₃ and showing the effect of hypoxia on VO₂ in the mesenteric artery of WT mice. B: Time course of VO₂ reduction following control (control) or hypoxia (Hypo) treatment. The time to reach 50% VO₂ reduction is shown. C: Effect of inhibition of NO synthesis on VO₂ during hypoxia. Data are means±SEM from n=6 independent experiments. *P<0.05 vs control. **P<0.01 vs control.

Discussion
Our results indicate that both basal and stimulated release of NO from the vascular endothelium is responsible not only for the well-known regulation of vascular tone but also for the adjustment of VO₂ consumption. In our experiments using isolated blood vessels, although we studied the consumption of VO₂ over a wide range of [O₂], we chose to compare the behavior of the vessels at 2 [O₂], namely 130×10⁻⁶ mol/L and 30×10⁻⁶ mol/L, which approximate the [O₂] in arterial blood and tissues, respectively. Our results demonstrate that the [O₂] decreased so did the consumption of VO₂. In the absence of endothelium-derived NO, following inhibition of its synthesis with L-NA or removal of the endothelium, this adaptive change disappeared and the vascular consumption of O₂ remained constant over a wide range of [O₂]. There was eventually a drop in consumption in the absence of NO; however, this occurred at much lower [O₂].

This modulatory effect of NO was observed in a variety of vessels of different anatomic origin and sources, including human, and is consistent with its known effect in reducing the vascular consumption of O₂ (Figure 3A and 3B and Online Movies).
affinity of CcO for O$_2$ in a competitive manner, i.e., increasing its $K_m$ and adjusting mitochondrial O$_2$ consumption.$^8,15$ We found that aortas from eNOS knockout mice exhibited a reduced $K_m$ for O$_2$ and consumed equal amounts of O$_2$ independently of the [O$_2$] in the medium, further indicating that endothelium-derived NO is responsible for regulating O$_2$ consumption.

When endothelial NO generation was increased with ACh or Bk, at concentrations at which these agonists produced potent sGC-mediated vasodilation,$^{16}$ there was an enhanced reduction in VO$_2$. This occurred even at the higher [O$_2$] and was also prevented by treatment with L-NA or removal of the endothelium. Administration of exogenous NO with DETA-NO also induced a greater inhibition of O$_2$ consumption and increase in $K_m$, which were not affected either by inhibition of NO generation or by removal of the endothelium. These results are in accordance with previous observations in cells$^{3,9}$ and in tissues.$^4,7,17$ This NO-dependent effect on consumption of O$_2$ was also observed in the presence of the vasoconstrictor Phe, which is known to release NO following activation of endothelial $\alpha_1$ adrenoreceptors.$^{18}$

Inhibition of CcO by NO (a phenomenon that has been called “metabolic hypoxia”$^8$) leads to a major redistribution of O$_2$, which has been demonstrated in cells in culture.$^6,19$ Our results, obtained with fluorescence microscopy show that this redistribution also occurs in vascular tissues in vitro. Indeed, these experiments show that the stimulation of endothelial NO production with ACh resulted in an increase in the intracellular [O$_2$] of the mesenteric cells surrounding a microvessel, as shown by the decreased fluorescence of Ru(phen)$_3^{2+}$.
effect of NO. The reason for the paradoxical increase in cellular [O₂] when exposed to hypoxia is that in normoxia, where O₂ consumption is high, there is a steep O₂ gradient toward O₂-consuming mitochondria, leaving a relatively low level of spare O₂ (hence high ruthenium fluorescence). In hypoxia, NO-dependent inhibition of respiration leads to decreased O₂ consumption and thus a paradoxical increase in the levels of available O₂ (hence a lower number of ruthenium fluorescent cells).

In conclusion, our results indicate that the basal release of endothelial NO adjusts vascular O₂ consumption. This may occur at the level of the microcirculation to facilitate the distribution of O₂ to the tissues, especially at low [O₂], as has occurred at the level of the microcirculation to facilitate the distribution of O₂ to the tissues, especially at low [O₂], as has been indicated before.⁶,²⁰,²³ It may also occur in the larger vessels, where the requirement for O₂ by the vascular smooth muscle may vary as a result of the action of hormones such as ACh and adrenaline, as well as the activity of the different sets of nerves that innervate the vasculature. This remains to be studied. In addition, it is possible that changes in the distribution of O₂ throughout the vessel wall, because of a decrease in NO production in endothelial dysfunction, may play a role in the development of vascular disease.

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Disclosures
None.

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Regulation of Oxygen Distribution in Tissues by Endothelial Nitric Oxide
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Hypoxia-induced changes in intracellular \([O_2]\) in rat and mouse mesenteric cells in close proximity to a blood vessel. The preparation was loaded with Ru(phen)\(_3\)^{2+} (3h, 1X10^{-6} \text{ mol/L \textmu M, red fluorescence}), whose fluorescence is inversely proportional to the \([O_2]\). Fluorescence was filmed for a 20 min period in normoxia or hypoxia, recording one frame every 2 min. When tissues were subjected to hypoxia following normoxia, the first frame was taken in normoxia and the subsequent frames in hypoxia. (A) Control normoxia, (B) control hypoxia, (C) hypoxia in the presence of L-NA, (D) hypoxia in the presence of ACh, (E) hypoxia in the presence of DETA-NO, (F) normoxia in the presence of cyanide, (G) hypoxia in cells from eNOS \(^{-/-}\) mice, (H) addition of ACh in the presence of normoxia, and (I) hypoxia in the presence of ACh.

Online Figure I.

Representative images of cells from the mesenterium showing the changes that took place in intracellular \([O_2]\) when exposed to hypoxia (nucleus stained with Hoescht (blue), \(O_2\) stained with Ru(phen)\(_3\)^{2+} (red) in the cytosol of the cells). The preparation was loaded with Ru(phen)\(_3\)^{2+} (3h, 1X10^{-4} \text{ mol/L, red fluorescence}), whose fluorescence is inversely proportional to the \([O_2]\). The vital cell nuclear stain Hoechst 33342 (1X10^{-6} \text{ mol/L}) was added for the last 30 min of incubation. Following a 10 min equilibration period at atmospheric \([O_2]\) (200X10^{-6} \text{ mol/L O}_2), samples were subjected to hypoxia (15X10^{-6} \text{ mol/L O}_2) for a 20 min period by the
addition of nitrogen. The panels on the left show the merged fluorescence of Hoescht (blue) and Ru(phen)$_3^{2+}$ (red) while the panels on the right show Ru(phen)$_3^{2+}$ alone.