HEPATIC MICROCIRCULATORY FAILURE is a major determinant for the development of hepatocellular dysfunction in a number of conditions including trauma/hemorrhage, liver transplantation, endotoxemia, and sepsis. Our group has previously shown in a model of liver ischemia and reperfusion that the degree of microcirculatory failure determines the extent of lethal hepatocyte necrosis (6). Furthermore, microvascular perfusion failure in a model of hemorrhage/resuscitation affected the hepatic mitochondrial redox state (15) and was associated with an increase in hepatocellular enzyme release and hepatocyte necrosis (1, 18). Although the underlying mechanisms are not completely understood, accumulating evidence suggests a dysregulation of stress-inducible vasoactive mediators like endothelins, carbon monoxide synthase, or heme oxygenase (2, 3) as well as changes in the response of the effector cells to the mediators, which includes alteration in receptor expression or phenotypic transformation in cells (7). Microcirculatory failure in the liver after different stress events is characterized by a perfusion heterogeneity resulting in a mismatch between oxygen supply and demand. The impaired nutritive blood flow accompanied by a reduced oxygen availability decreases cellular levels of high-energy phosphates and contributes to early and late hepatocellular injury and dysfunction after several stress events. We recently demonstrated (16) in a model of prolonged and severe hemorrhagic shock that an improved oxygen supply through the use of an artificial oxygen carrier was associated with an increased restoration of hepatocellular ATP content. Studies of tissue oxygenation focusing on the relationship between microcirculatory disturbances and oxygen transport dynamics may contribute to a better understanding of the underlying pathophysiological mechanisms.

A number of methods have been reported during the last decade measuring the oxygen distribution in tissues; however, their applicability to investigate spatiotemporal changes in tissues is restricted due to technical limitations. For instance, the frequently used microelectrodes only measure tissue PO$_2$ at specific points and consume oxygen, and the measurement is invasive. Nuclear MRI approaches or electron paramagnetic resonance oximetry techniques are able to investigate spatiotemporal changes in tissue PO$_2$ (19) but their resolution is too low for imaging PO$_2$ changes within the liver microarchitecture e.g., on the level of individual hepatic sinusoids. Itoh and coworkers (11, 25) developed a fluorescent membrane on the basis of the oxygen-quenched fluorescent dye Tris(1,10-phenanthroline)ruthenium(II) chloride hydrate [Ru(phen)$_3^{2+}$], which allows in vivo visualization of the PO$_2$ distribution around mesenteric microvasculature. These investigators showed that the fluorescence of the indicator is a direct indicator of oxygen tension. A similar oxygen-sensitive membrane was recently used by our group to determine changes in the tissue surface oxygen distribution in livers of LPS-primed rats in response to endothelin-1 (4). This system was also demonstrated to respond linearly to changes in PO$_2$.

However, even if this technique allows a visualization of oxygen distribution with moderately high resolution on tissue surfaces, the method also possesses a number of technical shortcomings. For instance, the oxygen-sensitive membrane has to be under gastight and watertight conditions during microscopy to avoid interferences with environmental oxygen. Furthermore, after a short time of continuous photoactivation, the fluorescent membrane shows a photobleaching effect limiting a prolonged observation period. The method also does not...
allow visualization of changes in tissue $P_{O_2}$ directly, because the microscope has to be focused on the probe membrane. Subsequently, corresponding images of the tissue surface and the membrane have to be superimposed. Moreover, despite a higher resolution compared with other methods for oxygen mapping, the spatial resolution of oxygen-sensitive membranes is restricted by the diameter of the silica beads used to absorb the fluorescent dye and the thickness of the membrane. Quality of the obtained images is further restricted, because changes in fluorescence take place in the membrane and not on the tissue surface, thus requiring the membrane to be focused. Afterward, an editing of the membrane image and the corresponding tissue image is required. Therefore, we searched for a method that allows a direct visualization of the oxygen distribution in tissue combined with a high spatial resolution of the tissue being monitored.

Wilson et al. (24) used an intravenous phosphorescence oxygen probe to assess the effects of hyperventilation on oxygenation of the brain cortex of newborn piglets. A disadvantage of this method, which is based on measuring phosphorescence lifetimes, is that it requires highly specialized equipment. The use of fluorescence probes on the other hand needs only a standard fluorescence microscope.

The aim of the present study was to investigate 1) whether the intravenous infusion of the oxygen-quenching dye Ru(phen)$_{3}^{2+}$ allows visualization of the oxygen distribution on the liver tissue surface with a high spatial resolution but without toxicity and 2) whether this method allows for detection of changes in tissue $P_{O_2}$ under pathophysiological conditions.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) if not specified otherwise.

Animals. Male Sprague-Dawley rats (220–320 g body wt) were obtained from Charles River Laboratories (Wilmington, MA). The animals were fasted overnight before preparative surgery but were allowed free access to water. The protocol was approved by the University of North Carolina at Charlotte Institutional Animal Care and Use Committee.

Animal preparation. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). Anesthesia was maintained by intermittent intravenous pentobarbital application (1–3 mg/kg body wt). After rats were placed on a heating pad to maintain body temperature between 36 and 37°C, a tracheotomy was performed to facilitate spontaneous breathing of room air. The right jugular vein was cannulated for drug administration. The left carotid artery was cannulated for drug administration. The left carotid artery was cannulated for measurement of systemic arterial blood pressure with a standard pressure transducer (Digi-Med high-pressure analyzer; Micro-Med, Louisville, KY) and to allow withdrawal of blood samples. The transducer was connected to a Digi-Med blood pressure analyzer. A midline laparotomy was performed, and the gut was repositioned and covered with a saline-wetted cotton gauze to minimize evaporative loss during the procedure. The splenic arteries were ligated to prevent splenic congestion. The splenic vein was identified, and a PE-10 polyethylene catheter was inserted up to the portal vein for further drug administration. For intravital microscopy, animals were turned on their left sides, and left liver lobes were gently exteriorized with the lower surface uppermost. The specimen was then positioned on a micro coverglass window on a specially constructed stage. The liver was covered with plastic wrap to prevent surface drying.

Experimental protocol. The oxygen-sensitive fluorescent dye, Ru(phen)$_{3}^{2+}$ (Aldrich), was injected intravenously during intravital videomicroscopy for visualization of the hepatic tissue $P_{O_2}$. Little is known about possible side effects of Ru(phen)$_{3}^{2+}$ after systemic administration, because the chemical, physical, and toxicological properties have not been thoroughly investigated in vivo. Therefore, several pilot experiments were performed to determine a suitable dose of Ru(phen)$_{3}^{2+}$ that allows visualization of the tissue $P_{O_2}$ with a constant plasma concentration in the absence of any side effects or phototoxicity. Due to a high renal clearance rate, Ru(phen)$_{3}^{2+}$ was continuously infused via a syringe pump (model PHD 2000; Harvard Apparatus, Holliston, MA). The following infusion regimen resulted in a constant Ru(phen)$_{3}^{2+}$ plasma concentration and was chosen for all further experiments. The Ru(phen)$_{3}^{2+}$ infusion started at a high flow rate of 400 nmol·kg$^{-1}$·min$^{-1}$ for 5 min to rapidly achieve a steady-state plasma concentration. Subsequently, the infusion rate was reduced to 60 nmol·kg$^{-1}$·min$^{-1}$ for a further 10 min, followed by 40 nmol·kg$^{-1}$·min$^{-1}$ until the end of the experiment. While the infusion regimen was applied, blood samples were taken (0.2 ml) at different time points. After centrifugation, the Ru(phen)$_{3}^{2+}$ plasma concentration was determined fluorometrically at 480 nm (excitation), 580 nm (emission), and 590 nm with a CytoFluor multwell plate reader (PerSeptive Biosystems, Foster City, CA) using blank serum as a zero reference. The specific Ru(phen)$_{3}^{2+}$ plasma concentration was read against a standard curve.

Furthermore, mean arterial blood pressure (MAP) and heart rate were recorded at several time points during infusion to investigate the effects of Ru(phen)$_{3}^{2+}$ on central hemodynamics. Before and after Ru(phen)$_{3}^{2+}$ infusion, additional blood samples (0.4 ml) were taken and serum enzyme levels of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were determined to exclude early organ damage during infusion of Ru(phen)$_{3}^{2+}$. On illumination, Ru(phen)$_{3}^{2+}$ complexes can generate oxygen free radicals as they dissipate absorbed energy by transferring an electron to oxygen. Thus in a further series of experiments, possible phototoxic effects of Ru(phen)$_{3}^{2+}$ during intravital microscopy were investigated. Several liver areas were continuously exposed to epi-illumination at 460 nm for a period of 5 min per high-power field. Propidium iodide (50 μg/100 g body wt) was injected intravenously before and after light exposure to
metabolic response. In the same preparations, cellular redox potential was determined by using NADH autofluorescence.

In the first set of experiments, hepatic oxygen demand was increased and oxygen supply was decreased by administration of phenylephrine (PE), a short-acting selective α₁-adrenoceptor agonist that augments the metabolic rate in the liver and simultaneously attenuates the hepatic blood flow. Twenty minutes after the start of Ru(phen)³⁺ infusion, PE was injected at a flow rate of 40 μmol·kg⁻¹·min⁻¹ via a splenic vein catheter for 10 min. Images were recorded at baseline and at several time points during and after the end of PE infusion. In a second set of experiments, tissue Po₂ was decreased through reduction of the circulating blood volume. Hemorrhagic hypotension was induced by arterial blood withdrawal to a MAP of 35 ± 5 mmHg within 30 s and was maintained for 10 min. If necessary, additional blood drawings were taken to maintain low blood pressure. Shed blood was collected in syringes containing citrate-phosphate-dextrose solution (0.14/1.0 ml shed blood). After 10 min of hemorrhage, shed blood was reinjected. Intravital microscopy images were also recorded at baseline and at several time points during hemorrhage and after reinfusion of the shed blood. Both treatments, PE infusion and acute hemorrhage, were expected to cause parallel changes in Ru(phen)³⁺ fluorescence as well as NADH autofluorescence due to an attenuation in tissue Po₂. Therefore, we used ethanol to affect the mitochondrial redox potential without altering the hepatic Po₂. After baseline microscopy images were taken, 500 mg/kg body wt ethanol was intravenously administered as a bolus injection and further images were recorded after 10 and 20 min.

**Intravital videomicroscopy.** The prepared rats were placed on the stage of an Olympus IX70 inverted microscope (Olympus America, Melville, NY). The liver surface was epi-illuminated with a 100-W mercury lamp using 366 nm excitation and 450 nm emission band-pass filters for NADH autofluorescence and 480 nm excitation and 625 emission band-pass filters to visualize Ru(phen)³⁺ fluorescence. Images were viewed at either low power (×4 objective) or high power (×20 objective) recorded by a Dage-MTI charge-coupled device integrating camera (CCD 300 Camera Systems; IPS, North Reading, MA) connected to an S-VHS video recorder (JVC HR-S3911U; JVC-East Coast, Wayne, NJ) and were analyzed during video playback. These objectives produced fields on the monitor representing 1,050 × 1,270 and 220 × 250 μm, respectively. Gate time (number of frames integrated) was identical in all experiments [NADH, 8; Ru(phen)³⁺, 64]. For real-time digital contrast enhancement, an Argus-20 image processor (Hamamatsu, Bridgewater, NJ) was used. Gain, black level, and the enhancement settings were also identical in all experiments. At each recorded time point, the liver was exposed to epi-illumination for 15 s at most. Between time points, the microscope shutter was closed to prevent photobleaching and photodamage. Fluorescence intensity was densitometrically assessed by using a commercially available software program (MetaMorph; Universal Scientific Software, West Chester, PA).

**Fig. 2.** Effects of intravenous Ru(phen)³⁺ administration on hemodynamics. Intravenous infusion of Ru(phen)³⁺ resulted in a negligible decrease in mean arterial blood pressure (MAP) (Fig. 2A) and heart rate during the highest dose of 400 nmol·kg⁻¹·min⁻¹ compared with baseline values. Both parameters returned to baseline levels after lowering the dose to 60 nmol·kg⁻¹·min⁻¹. Blood pressure and heart rate varied in the physiological range without any statistically significant differences and was not affected by intravenously administered Ru(phen)³⁺.

In another series of experiments, the Ru(phen)³⁺ infusion was used for visualization of the tissue Po₂ in the liver under conditions of altered oxygen supply or changes in cellular redox state resulting from identify damaged cells. The number of propidium iodide-positive cells per high-power field (×40) were counted before and after epi-illumination using offline video analysis.

**Fig. 3.** Visualization of tissue Po₂ by intravenous Ru(phen)³⁺ administration. Intravenous infusion of Ru(phen)³⁺ resulted in a marked fluorescence within the liver tissue. The heterogeneous fluorescence intensity reflects the physiological distribution of oxygen tension in the liver acinus characterized by a higher Po₂ in periportal regions with a continuous decrease toward pericentral areas (A). These differences in fluorescence intensity were also evident by using a high resolution (B). Dark areas represent liver regions with a high Po₂ due to an inhibition of fluorescence by molecular oxygen. Magnification, ×40 (A) and ×100 (B).
Imaging, Downingtown, PA) and was analyzed as average intensity per liver acinus.

Quantitative determination of serum enzyme levels. To assess whether intravenously injected Ru(phen)$_3^{2+}$ leads to early organ damage resulting in serum enzyme release, blood samples were taken at baseline and after 1 h of Ru(phen)$_3^{2+}$ infusion. Serum was prepared, and aliquots were stored at $-80^\circ$C until analysis. ALT and LDH were photometrically analyzed with commercially available kits.

Statistical analysis. Data are presented as means $\pm$ SE. Differences were evaluated by ANOVA followed by post hoc multiple comparison according to the Student-Newman-Keuls method. According to the study design, either a one-way or repeated-measures ANOVA was performed by using the SigmaStat software package (Jandel Scientific, San Rafael, CA). When criteria for parametric testing were violated, the appropriate nonparametric test, i.e., Kruskal-Wallis ANOVA on ranks and Friedman test, were used. $P < 0.05$ was considered significant.

RESULTS

Plasma concentration and hemodynamic response during Ru(phen)$_3^{2+}$ infusion. Preliminary experiments showed that Ru(phen)$_3^{2+}$ was rapidly excreted in the urine (data not shown). Therefore, a continuous infusion using a syringe pump was necessary. First, several pilot experiments were performed to determine an infusion regimen resulting in a steady state of Ru(phen)$_3^{2+}$ plasma concentration within an acceptable time period. An infusion regimen started at a flow rate of 400 nmol·kg body wt$^{-1}$·min$^{-1}$ within the first 5 min, then 60 nmol·kg body wt$^{-1}$·min$^{-1}$ during the next 10 min, followed by 40 nmol·kg body wt$^{-1}$·min$^{-1}$ for a further 45 min, resulted in a peak in Ru(phen)$_3^{2+}$ plasma concentration of 6.6 $\pm$ 0.5 $\mu$mol/l after 5 min. Ten minutes after start of the infusion, Ru(phen)$_3^{2+}$ plasma concentration declined to 2.1 $\pm$ 0.1 $\mu$mol/l.

Fig. 4. Effect of phenylephrine on hepatic tissue $P_O^2$ and NADH autofluorescence. Fifteen minutes after the initial Ru(phen)$_3^{2+}$ infusion, the short-acting $\alpha_1$-adrenoceptor agonist phenylephrine was administered for 10 min at a dose of 40 $\mu$mol·kg$^{-1}$·min$^{-1}$. A, C, and E: Ru(phen)$_3^{2+}$ fluorescence. B, D, and F: NADH autofluorescence. A and B: baseline fluorescence. The outlined area corresponds to the approximate extent of zone 3 around one terminal hepatic venule for sake of reference. The decrease in tissue $P_O^2$ due to phenylephrine resulted in a significant increase in Ru(phen)$_3^{2+}$ fluorescence intensity (C) and NADH autofluorescence (D) compared with respective baseline values [Ru(phen)$_3^{2+}$ (A), NADH (B)]. After phenylephrine infusion, fluorescence intensity continuously returned toward baseline levels (E and F), consistent with an increase in tissue $P_O^2$. G: average brightness in Ru(phen)$_3^{2+}$ fluorescence in 3–5 liver acini of 8 individual experiments. H: corresponding intensity in NADH autofluorescence of the identical liver areas. Data are means $\pm$ SE. *$P < 0.05$ vs. baseline levels; $\#P < 0.05$ vs. 5 min phenylephrine infusion; $\$P < 0.05$ vs. 10 min phenylephrine infusion.
and reached nearly constant values between 15 and 60 min varying between 1.5 ± 0.2 and 1.7 ± 0.2 μmol/l (Fig. 1). During Ru(phen)$_3^{2+}$ infusion, MAP and heart rate were continuously monitored and recorded every 5 min. Infusion at the highest flow rate (400 nmol-kg body wt$^{-1}$-min$^{-1}$) at the beginning resulted in a slight decrease in MAP (Fig. 2A) and heart rate (Fig. 2B) without statistical significance and full recovery after 5 min. MAP and heart rate showed almost constant levels during the whole infusion period and were not affected by Ru(phen)$_3^{2+}$.

Intravitral microscopy during Ru(phen)$_3^{2+}$ infusion. Approximately 3–4 min after starting the Ru(phen)$_3^{2+}$ infusion regimen, Ru(phen)$_3^{2+}$ fluorescence became visible within liver tissue by intravitral microscopy (Fig. 3A) and allowed visualization of differences in tissue PO$_2$ at both low and high magnification (Fig. 3B). The intensity of fluorescence was most pronounced in pericentral areas of the liver acinus, reflecting the low PO$_2$ of this region, and showed a gradual decrease toward periportal regions, reflecting the physiological distribution of oxygen tension in the liver acini with a decrease in PO$_2$ from periportal to pericentral areas. The Ru(phen)$_3^{2+}$ dye presented a homogenous distribution within the extracellular fluid compartment and was not restricted to the intravascular space. In contrast, Ru(phen)$_3^{2+}$ fluorescence was attenuated in hepatic sinusoids or venules due to the light-absorbing effect of hemoglobin.

Organ damage and phototoxicity due to Ru(phen)$_3^{2+}$ administration. Before starting the Ru(phen)$_3^{2+}$ administration and 1 h after continuous infusion, blood samples were taken; serum enzyme release of LDH as a parameter of nonspecific organ damage and ALT as a specific parameter for hepatocellular injury were determined. Serum activity of both enzymes showed no significant differences compared with baseline levels (LDH: baseline 270.1 ± 36.3 U/l, 60 min 265 ± 37 U/l; ALT: baseline 15.2 ± 0.8 U/l, 60 min 15.3 ± 1.3 U/l). Ru(phen)$_3^{2+}$ complexes have been shown to generate oxygen free radicals on illumination, causing cell damage during illumination in vitro. We then tested whether the lower concentration of Ru(phen)$_3^{2+}$ used in our experiments caused photodamage during intravitral microscopy. Randomly selected liver areas continuously exposed to epi-illumination for 5 min during Ru(phen)$_3^{2+}$ infusion showed no increase in the number of damaged liver cells as assessed by propidium iodide injection before and after epi-illumination (propidium iodide-positive cells/high power field: baseline 1.3 ± 0.9; after 5 min epi-illumination 1.3 ± 0.9; data are means ± SE of 4 high-power fields in 6 individual experiments).

Changes in tissue PO$_2$, redox state, and hemodynamics during PE infusion. The reduction in oxygen supply and elevation in oxygen demand by continuous infusion of 40 μmol-kg$^{-1}$-min$^{-1}$ PE via a splenic vein catheter for 10 min resulted in a significant increase in intensity in both Ru(phen)$_3^{2+}$ fluorescence (Fig. 4, C and G) and NADH autofluorescence (Fig. 4, D and H) compared with baseline intensity (Fig. 4, A and B). An increase in brightness in either method can result from a decrease in tissue PO$_2$. After the PE infusion had stopped, Ru(phen)$_3^{2+}$ fluorescence intensity continuously decreased within the observation period (Fig. 4G). After 20 min of recovery, Ru(phen)$_3^{2+}$ fluorescence in the liver tissue was slightly brighter compared with baseline images but without statistical significance. In contrast, NADH autofluorescence presented a faster decline in fluorescence intensity after the end of the short-acting PE infusion and returned to baseline intensity at the end of the observation period (Fig. 4, F and H). The administration of PE led to a significant increase in systemic MAP compared with baseline values (Fig. 5) accompanied by a slight decrease in heart rate without statistical significance (data not shown). Blood pressure and heart rate immediately returned to baseline values at the end of PE infusion and showed constant levels until the end of the experiment. A decrease in hepatic oxygen supply was induced by blood withdrawal to a MAP of 35 ± 5 mmHg for 10 min, resulting in a profound increase in Ru(phen)$_3^{2+}$ fluorescence (Fig. 6C) and NADH autofluorescence intensity (Fig. 6D) with a maximum at 10 min compared with respective baseline value (Fig. 6, A and B). Retransfusion of the anticoagulated shed blood led to a continuous decrease in Ru(phen)$_3^{2+}$ fluorescence (Fig. 6G). Twenty minutes after retransfusion, the intensity of Ru(phen)$_3^{2+}$ fluorescence was slightly increased compared with baseline levels but without statistical significance (Fig. 6E). At all time points, the intensity of fluorescence showed a heterogeneous distribution within the liver acinus with brighter pericentral areas and a gradual attenuation in brightness toward periportal liver regions. However, the intensity of NADH autofluorescence demonstrated a faster decrease after reinfusion of the shed blood and reached baseline levels 5 min after the end of hemorrhage (Fig. 6, F and H).

Effect of ethanol injection on Ru(phen)$_3^{2+}$ fluorescence and NADH autofluorescence. Because hemorrhage and PE administration led to an increase in both Ru(phen)$_3^{2+}$ fluorescence and NADH autofluorescence, ethanol (500 mg/kg body wt) was intravenously injected to change the mitochondrial redox potential by increasing the NADH-to-NAD$^+$ ratio without altering the hepatic tissue PO$_2$. Ru(phen)$_3^{2+}$ fluorescence showed almost no detectable changes in intensity 10 min after ethanol injection (Fig. 7A and H).

![Fig. 5. Changes in MAP in response to phenylephrine infusion. The α₁-selective adrenoceptor agonist phenylephrine was continuously infused at a rate of 40 μmol-kg$^{-1}$-min$^{-1}$ via a splenic vein catheter for 10 min. Phenylephrine infusion resulted in a significant increase in MAP, which returned to baseline values immediately after infusion. Data represent means ± SE for n = 8 individual experiments; *P < 0.05 vs. baseline.](http://ajpgi.physiology.org/Downloadedfrom)
Fig. 6. Changes in Ru(phen)$_2^{3+}$ fluorescence and NADH autofluorescence during hemorrhage. MAP was reduced to 35 ± 5 mmHg by acute blood withdrawal within 30 s and was maintained for 10 min. The decrease in oxygen supply due to hemorrhage was accompanied by an increased intensity of Ru(phen)$_2^{3+}$ fluorescence (C) and NADH autofluorescence (D) compared with baseline images [Ru(phen)$_2^{3+}$ (A); NADH (B)]. This effect was reversible in both Ru(phen)$_2^{3+}$ fluorescence (E) and NADH autofluorescence (F) by reinjection of the anticoagulated shed blood. G: average fluorescence intensity of Ru(phen)$_2^{3+}$ in 3–5 liver acini of 8 individual experiments. H: demonstrates the intensity in NADH autofluorescence of the identical liver areas. Data are means ± SE. *P < 0.05 vs. baseline levels; $P < 0.05$ vs. 5 min hemorrhage; $\#P < 0.05$ vs. 10 min hemorrhage.

DISCUSSION

In the present study, we demonstrate a new method for visualizing the tissue oxygen distribution on the liver surface under several pathophysiological conditions in vivo. Oxygen is the essential final electron acceptor for aerobic energy metabolism. Especially in the liver, oxygen regulates metabolic zonation under normal conditions and it can be a modulator of liver disease under pathophysiological conditions (13). Perivenous hypoxia is considered a major cause for several secondary liver diseases. The microcirculatory failure in the liver after different stress events may lead to a perfusion heterogeneity resulting in an impaired nutritive blood flow and a mismatch between oxygen supply and demand (3, 7, 20). Understanding the role of oxygen in physiology and pathophysiology may be helpful in evaluating the underlying mechanisms leading to cell death and organ dysfunction after ischemia-reperfusion or in septic conditions.
A recently published study (8) investigated the interaction of the oxygen-sensitive fluorescent probe Ru(phen)$_2^{2+}$ with animal cells. This report demonstrated that ruthenium(II) complexes cannot freely penetrate intact biological membranes and do not cause measurable photodamage to plasma membranes at a concentration of ≤0.2 mM. Apart from this study in cultured cells, little is known about chemical, physical, and toxicological properties of Ru(phen)$_3^{2+}$ and potential side effects after systemic administration in living organisms. In pilot experiments, Ru(phen)$_3^{2+}$ bolus injections between 4 and 16 mM/kg body wt resulting in a theoretical Ru(phen)$_3^{2+}$ plasma concentration of 0.025–0.1 mM frequently led to a drop in heart rate and respiratory arrest and was accompanied by a rapid decrease in fluorescence due to a high renal clearance rate (unpublished results). These observations led to the described Ru(phen)$_3^{2+}$ infusion regimen that resulted in a constant Ru(phen)$_3^{2+}$ plasma concentration in the absence of any significant hemodynamic or respiratory side effects. Phototoxicity studies of Ru(II) complexes in cultured macrophages have shown photodamage to plasma membranes in a dose-dependent manner. Whereas Ru(phen)$_3^{2+}$ concentrations of 1 or 2 mM in the culture medium were accompanied by a profound increase in photodamage with extended illumination, a Ru(phen)$_3^{2+}$ concentration of 0.2 mM caused no measurable photodamage (8). This is in line with our observations demonstrating no detectable photodamage during continuous epifluorescence for 5 min. In addition, the risk of Ru(phen)$_3^{2+}$-induced photodamage in our method is unlikely due to the fact that the continuous infusion resulted in a Ru(phen)$_3^{2+}$ plasma concentration of ~2.0 μM.

In the present study, the Ru(phen)$_3^{2+}$ fluorescence was not restricted to the vascular space, indicating a sinusoid permeability of the dye that allows an assessment of the PO$_2$ gradient outside the microvessels in the interstitial space. In contrast, due to the light-absorbing effect of hemoglobin, the vascular space showed a negative contrast compared with the surrounding tissue. The unidirectional blood flow in the liver acinus is characterized by a continuous decrease in PO$_2$ that is ~60–65 mmHg in the periporal blood and attenuates to ~30–35 mmHg in the perivenous blood (12). In the present study, the fluorescence images obtained under baseline conditions, demonstrating a continuous increase in fluorescence intensity from periporal to pericentral regions, indicate that the described method is sensitive enough to detect even small differences in tissue PO$_2$.

NADH, a naturally occurring fluorophore, transfers electrons to oxygen by means of an electron transport chain located in the inner membrane of mitochondria (5). Under hypoxic conditions, with no oxygen available to accept electrons from cytochrome $a$, intracellular NADH accumulates. Unlike the oxidized form NAD$^+$, NADH is highly fluorescent (10). Therefore, we compared the changes in NADH fluorescence, which reflect the extent of tissue hypoxia, with the results obtained by the Ru(phen)$_3^{2+}$ approach under pathophysiological conditions. Both methods showed a profound increase in fluorescence intensity in response to decreased tissue oxygen supply either by the administration of PE or due to acute hemorrhage. After restoration of baseline conditions, intensity of Ru(phen)$_3^{2+}$ fluorescence and NADH autofluorescence diminished. The decrease in NADH fluorescence intensity was quicker compared with Ru(phen)$_3^{2+}$ fluorescence and reached baseline levels 20 min after the intervention had finished. In contrast, the Ru(phen)$_3^{2+}$ fluorescence presented a slower decrease in intensity, and the densitometric analysis demonstrated a higher intensity compared with the baseline levels, albeit without statistical significance. Several factors may contribute to these results. The tissue PO$_2$ in the liver could be lower compared with baseline conditions due to microcirculatory disturbances without affecting the mitochondrial redox state even after restoration of normal conditions after finishing the PE infusion or retransfusion of the shed blood. In contrast, NADH autofluorescence is subjected to a photobleaching effect on illumination. Even if the specific liver region was subjected to illumination for only 15 s at each time point, this may contribute to the quick attenuation in NADH fluorescence and the lower intensity at the end of the experiment compared with Ru(phen)$_3^{2+}$ fluorescence. Although Ru(phen)$_3^{2+}$ shows a photobleaching effect during illumination, it is negligible under continuous intravenous infusion, because the illuminated Ru(phen)$_3^{2+}$ is constantly replaced. Despite the constant plasma
concentration during infusion of Ru(phen)$_3^{2+}$ as determined by plasma fluorescence activity, it has been shown that after illumination, Ru(phen)$_3^{2+}$ complexes can accumulate in the regions of plasma membranes, although ruthenium(II) complexes themselves have no affinity to plasma membranes (8). Moreover, the preparation of the liver for intravital microscopy can damage capillar cells. Furthermore, during microscopy, the liver surface is in contact with the micro coverglass and may cause additional damage to the liver capsule, thus allowing Ru(phen)$_3^{2+}$ to enter damaged cells and stain nucleic acids. On binding to DNA, the fluorescence of Ru(phen)$_3^{2+}$ increases by 50% compared with unbound Ru(phen)$_3^{2+}$ (9).

Both Ru(phen)$_3^{2+}$ fluorescence and NADH autofluorescence provide information on the metabolic state in the liver tissue. Whereas the NADH fluorescence reflects the mitochondrial redox state and the activity of the mitochondrial electron transport chain, the Ru(phen)$_3^{2+}$ fluorescence is directly dependent on the tissue Po$_2$. To demonstrate the independence of these measurements, a high dose of ethanol was injected to increase the NADH-to-NAD$^+$ ratio without affecting the tissue Po$_2$. Oxidation of ethanol to acetaldehyde is NAD$^+$ dependent and is catalyzed by alcohol dehydrogenase resulting in an increase in the NADH/NAD$^+$ redox potential within the cytosol and mitochondria, with subsequent alteration in several tissue metabolites (17). Although hypoxia seems to be involved in alcohol-induced liver injury (22), the present study merely shows that the NADH-to-NAD$^+$ ratio was affected during the first 10 min after ethanol administration without any significant changes in tissue Po$_2$. This confirms our conclusion that the simultaneous use of both fluorometric techniques in studies of tissue oxygenation/microcirculatory failure provide additional information regarding underlying pathophysiological mechanisms. It allows differentiation between disturbances in oxygen supply and oxygen utilization. For instance, a normal tissue oxygen supply assessed by Ru(phen)$_3^{2+}$ fluorescence accompanied by an increased NADH fluorescence suggests a rate of NAD$^+$ reduction that exceeds energy demand.

In conclusion, the intravenous administration of Ru(phen)$_3^{2+}$ for intravital videomicroscopy represents a new and simple method for visualizing the hepatic tissue Po$_2$ with a high resolution in vivo. In combination with NADH autofluorescence, the developed method provides information on the oxygen distribution, the metabolic state, and the mitochondrial redox potential within tissue.

GRANTS

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-38201 and Deutsche Forschungsgemeinschaft Grant PA-864 1-1.

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