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Direct measurement of nitroxide pharmacokinetics in isolated hearts situated in a low-frequency electron spin resonance spectrometer: Implications for spin trapping and *in vivo* oxymetry

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ABSTRACT **The pharmacokinetics of two nitroxides were investigated in isolated rat hearts situated in a low-frequency electron spin resonance spectrometer. The spin labels 2,2,3,3,5,5-hexamethyl-1-pyrrolidinyloxy and 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy were chosen for their physiochemical analogy to the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and its corresponding spin-trapped adduct, 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OH). The bioreductive rates of the two nitroxides were measured during constant perfusion as well as during ischemia and are discussed in terms of a two-compartment pharmacokinetic model. These data provide information necessary to the design and application of spin traps to detect oxy radicals during reperfusion of ischemic tissue and suggest the feasibility of monitoring free-radical processes in intact, functioning mammalian tissues by using a low-frequency electron spin resonance spectrometer.**

In recent years, a renewed interest in oxygen-centered free radicals has developed as these reactive species have been implicated in a variety of cellular responses, including ischemia/reperfusion injury (1–5). Despite enormous efforts, the preponderance of the data supporting the role of oxy radicals in these processes is indirect, relying upon the "specificity" of free-radical scavengers to significantly ameliorate the injury (6–12).

Of the available techniques for the identification of free radicals in biological milieu, spin trapping has received the most attention. This method consists of using a nitrone or nitroso compound to "trap" the initial unstable free radical as a "long-lived" nitroxide that can be detected at room temperature by a conventional electron spin resonance (ESR) spectrometer (13–15). While whole-organ preparations provide ideal models of the *in vivo* situation, current ESR technology limits detection of free radicals to intact cell suspensions, organ effluents, and frozen tissue samples (16–22). In spite of findings purporting to have identified free radicals generated as the result of ischemia/reperfusion injury (19–22), these data must be viewed critically to ensure that conclusions are not drawn on the basis of artifacts.

With the development of a low-frequency ESR spectrometer (23) capable of obtaining ESR spectra from isolated organs under physiologic conditions, we have the opportunity to study free-radical processes *in situ*. However, before such experiments can be undertaken, we must initially determine the dynamics of spin traps and their corresponding spin-trap adducts in an isolated organ preparation. Since nitrones are not free radicals, they are not observable by ESR spectroscopy. To circumvent this problem, we have synthesized two nitroxides, 2,2,3,3,5,5-hexamethyl-1-pyrrolidinyloxy (HMPO) and 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (CTMPO), that approximate the structure and lipophilicity of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and its spin trap adduct, 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OH), and determined the pharmacokinetics of these spin labels in isolated, perfused rat hearts situated in a low-frequency ESR spectrometer.

MATERIALS AND METHODS

Isolated Heart Preparation and Pharmacokinetic Studies. A Plexiglas cuvette (Fig. 1) was specially designed and constructed to house an isolated, perfused rat heart for the low-frequency ESR spectrometer. Polyethylene tubing for aortic perfusion, air venting, and effluent drainage was placed through holes drilled in the stopper of the cuvette. All other lines consisted of ⅛-inch (i.d.) silicone tubing (1 inch = 2.54×10^{-2} m).

All hearts were obtained from Sprague–Dawley rats (300–450 g) anesthetized with pentobarbital (50–75 mg/kg) i.p. and administered heparin (200 units) intravenously. After rapid extirpation and immersion in 0.15 M NaCl at 4°C, the aorta was cannulated and the heart was perfused in a retrograde fashion by way of the aorta with Krebs–Henseleit buffer (KHB, ref. 24) equilibrated with 95% O_2/5% CO_2 by bubbling. Excessive mediastinal tissue was excised. The heart was enclosed and secured horizontally in the cavity of the spectrometer. For perfusion, reservoirs were placed 80 cm above the level of the heart in the chamber. The aortic perfusion line contained a self-sealing injection port ≈15 cm proximal to the heart. The reservoirs and the aortic line were kept at 37°C by circulating water through jackets. All hearts continued to beat in an unrestricted manner.

Pharmacokinetic Studies. The pharmacokinetics and metabolism of two nitroxides were investigated. CTMPO was prepared as reported (25). The ESR spectrum of CTMPO in the low-frequency spectrometer is shown in Fig. 2. This spectrum is typical of a nitroxide having α carbons fully substituted with non-odd-numbered nuclei. HMPO was synthesized as described (26). These nitroxides were chosen because of structural similarity to DMPO and DMPO-OH.

Experiments were conducted by using one of the following three protocols: (*i*) continuous perfusion in a beating heart after a bolus injection of nitroxide (1 ml of a stock 1 mM

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Abbreviations: CTMPO, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-OH, 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy; HMPO, 2,2,3,3,5,5-hexamethyl-1-pyrrolidinyloxy.

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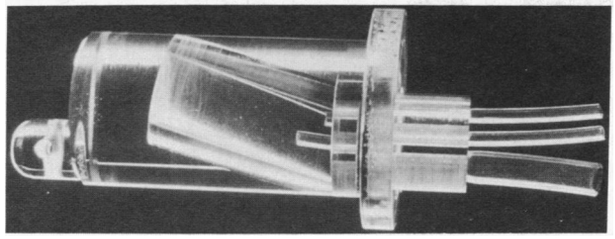

FIG. 1. Plexiglas cuvette, large enough to house a rat heart, is 2.5 cm in diameter and 5 cm long. A cylinder, 14 mm in diameter by 4 cm long, was removed from the cuvette. This cylindrical section was angled upward at 10° for better effluent drainage. A Plexiglas stopper (16 mm in diameter by 2 cm long) with an O-ring will seal the heart in the chamber.

solution) administered into the aortic perfusion line, (*ii*) initial perfusion of KHB followed by recirculation of nitroxide-containing KHB (a final concentration of 1 mM in a total volume of 40 ml) with an increased potassium concentration (25 mM) to arrest the heart, and (*iii*) ischemia produced by clamping the aorta after either 30 sec of recirculating KHB containing HMPO (a final concentration of 1 mM in a total volume of 40 ml) or 30 sec or 45 min of recirculating KHB containing CTMPO (a final concentration of 1 mM in a total volume of 40 ml). To keep the total volume of the recirculating system to a minimum, arrest was induced in this protocol to reduce metabolic demand so as not to exhaust the supply of glucose and oxygen. After administration of nitroxide in all the above protocols, the low-frequency ESR spectrometer was retuned and serial spectra were obtained. In a series of independent experiments, an isovolumic latex balloon was inserted into the heart to measure developed pressure in the left ventricle during retrograde aortic perfusion. At 1 mM of either spin label, temporary bradycardia and decreased developed heart pressure were observed. Within a minute after administration of either nitroxide, the heart completely recovered.

In addition to those experiments described above, parallel studies of the recirculating model were conducted with a Varian Associates (Palo Alto, CA) model E-9 spectrometer. Timed aliquots (0.5 ml) of recirculating KHB from the perfusion reservoir were assayed for nitroxide concentration. The initial concentration was 1 mM and the initial volume was 40 ml.

Partition Coefficient Studies. A stock solution (0.1 mM) of each nitroxide in KHB was prepared. Then 10 ml of each solution was combined with an equal volume of 1-octanol (Aldrich; gold label) and the biphasic mixture was shaken for 15 min. After separation, the concentration of the nitroxide was determined by ESR from a calibration curve generated from standards. The ratio of the concentration of the nitroxide in each phase gave the partition coefficient K_p. The partition coefficient for DMPO-OH, prepared as reported (27), was determined as described above, except water was used in place of KHB and the concentration of DMPO-OH in octanol was obtained by difference between control and extracted solutions (26).

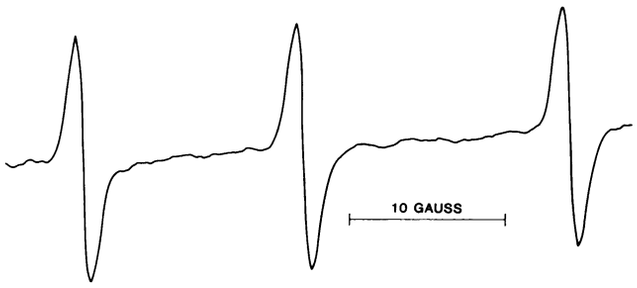

FIG. 2. ESR spectrum for CTMPO was recorded in the low-frequency ESR spectrometer at a microwave power of 10 mW, a central frequency of 257.6 MHz, a modulation amplitude of 0.55 G, a modulation frequency of 5.12 kHz, and a time constant of 0.15.

Low-Frequency ESR Spectrometer. A low-frequency ESR spectrometer (23) was used to measure *in situ* nitroxide concentration in the isolated rat heart preparation. The resonant cavity was a lumped L-C (inductor–capacitor) resonator with the inductive element acting as a sample holder. The spectrometer operates at frequencies of ≈250 MHz with magnetic fields of 90 G (1 G = 10^{-4} T) generated by Helmholtz configuration coils. Both applied magnetic field and radiofrequency magnetic field (H_i) were of high uniformity. Automatic frequency control was implemented locking the radiofrequency power (from a Hewlett–Packard model 8640B signal generator) to the cavity dispersion signal. The spin resonance signal was extracted from the cavity output by using a superheterodyne demodulation. The spectrometer output signal was fed to a lock-in amplifier (Princeton Applied Research, Princeton, NJ; model 5101), synchronized with the modulation field generated by oversized modulation coils, again in a Helmholtz configuration. Crucial to the operation of the spectrometer with a moving heart or flowing sample with air bubbles in the cavity was a coupling stabilization circuit. Power was coupled to the resonant circuit capacitively after passing through an electronically adjustable capacitor network. This allowed electronic adjustment of the circuit matching in response to the changes introduced to the circuit characteristics by sample motion (e.g., a beating heart). Spectra were recorded directly on a chart recorder (Moseley model 2D X–Y recorder) by using the following conditions: power, 0.1 mW; modulation amplitude, 2 G; time constant, 3 sec; and frequency, 247–254 MHz. Peak height was measured manually and recorded in cm. Peak heights for individual experiments were normalized by dividing each peak by the height of the initially recorded peak and multiplying by 100. Data are expressed as the natural logarithm of this corrected peak height.

Statistics. Data collected at standard times on the Varian Associates ESR spectrometer are expressed as the mean ± SEM for each time point. All other data were normalized as described above and subjected to linear regression analysis. Half-lives were calculated from the slope of the decay in a semilogarithmic plot.

RESULTS AND DISCUSSION

The identification of biologically generated free radicals is complicated, especially if one considers the dynamics associated with spin-trapping free radicals in a model more complex than isolated intact cells. Application of spin-trapping procedures to functional tissues, such as an isolated heart, requires understanding of the kinetics of distribution and elimination of the spin trap and the corresponding spin-trapped adduct(s) in the system to be studied. In contrast to cells, for which spin-trap equilibration between extra- and intracellular environments is rapid and uniform, the intravascularly administered spin trap in an intact heart must cross the vascular endothelium to reach the myocyte or whatever location represents the site of free-radical formation. In addition, dynamic factors, such as rate of perfusion as well as the metabolism of the nitrone and the corresponding nitroxide(s), must be considered.

We initially compared the lipophilic character of HMPO and CTMPO to DMPO and DMPO-OH, by measuring the octanol/KHB partition coefficient (K_p) of each compound (Table 1). We found that CTMPO has hydrophobic properties similar to DMPO, whereas the lipophilic character of HMPO parallels DMPO-OH. Numeric differences among the K_p values in each group, though substantial, do not alter the

Table 1. Partition coefficients for spin labels and a spin trap

Compound	K_p
HMPO	70
DMPO-OH	10*
CTMPO	0.005
DMPO	0.08†

*Partition coefficient was determined by using water instead of KHB.
†Partition coefficient was taken from ref. 26.

conclusions reached in the pharmacodynamic models developed later. Thus, the chosen nitroxides appear to reflect the relative behavior of the spin trap DMPO and its corresponding spin-trap adduct, DMPO-OH.

Uptake of each nitroxide into the beating heart after a bolus injection (1 mM) in a continuous perfusion system was studied with the low-frequency ESR spectrometer. An ESR spectrum was recorded for both nitroxides within the initial 3 min after administration. By 6 min after injection, no measurable nitroxide remained in the heart. Coronary flow during these experiments was 8–10 ml/min.

These experiments suggested to us that coronary flow may be an important parameter to consider when conducting spin-trapping experiments. Thus, we examined the relationship between coronary flow and the distribution of our model spin trap, DMPO, after its bolus administration (0.1 M in KHB). When coronary flow was 8–10 ml/min, 90% of the spin trap appearing in the perfusate was observed within the first 30 sec. However, at flow rates of 3–4 ml/min, which would be expected upon reperfusion after 20 min of ischemia, it took 2 min to collect the same amount of DMPO. These results have significant implications for spin-trapping free radicals during ischemia and reperfusion, where perfusate flow rates may differ between experimental and control groups. For instance, short periods of myocardial ischemia result in reactive hyperemia and increased coronary flow rates upon reperfusion (28). Longer periods of ischemia are associated with increased tissue pressure (29) and vascular endothelial swelling (30), thus reducing coronary flow. Differences in perfusate flow rates may result in artifactual differences in spin-trap adduct generation and metabolism by several mechanisms. (*i*) Early sampling may be required so as not to underestimate spin-trap adduct formation in the group with higher perfusion rates. (*ii*) Decreased perfusate flow rates result in longer contact time between spin trap and heart tissue that can lead to altered levels of spin-trap adduct formation without a corresponding change in the real rate of

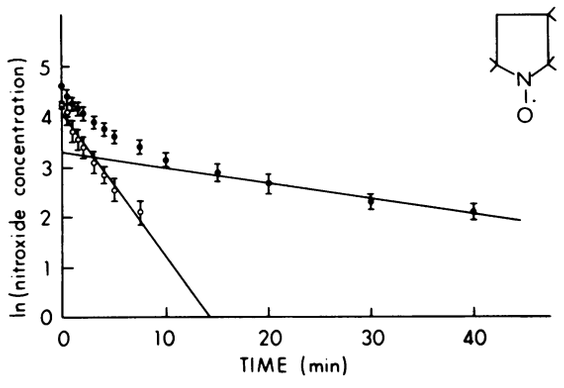

FIG. 3. Semilogarithmic plot of nitroxide concentration versus time after administration of HMPO to isolated rat hearts placed in a recirculating retrograde perfusion apparatus. Samples of the recirculating buffer were removed at defined times, added to a quartz flat cell, and assayed in a conventional ESR spectrometer. Each point represents the nitroxide concentration for six hearts (mean ± SEM). The distribution half-life (open circles) was 2.4 min, and the metabolic half-life (solid circles) was 22 min.

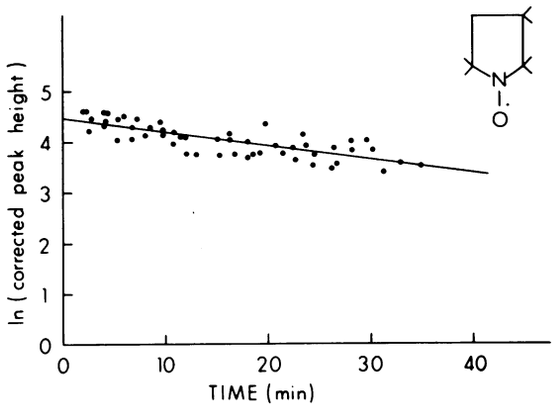

FIG. 4. Semilogarithmic plot of nitroxide concentration versus time after administration of HMPO to isolated rat hearts placed in the low-frequency ESR spectrometer. Each data point (four experiments) is plotted because of difficulties in obtaining ESR signals at the same time for each heart. The metabolic half-life was calculated to be 25 min.

free-radical generation. These factors assume increased importance when one considers that the spin trap DMPO readily oxidizes to DMPO-OH in oxygenated solutions (26), providing an ever-present, slowly increasing background level of spin-trap adduct in the perfusate effluent.

For the arrested, recirculating heart model, pharmacokinetic curves for HMPO and CTMPO were constructed by measuring the nitroxide concentration either from the recirculating line or *in situ* by using the standard and low-frequency ESR spectrometers, respectively. These experimental protocols were used to compare studies conducted with both instruments. For HMPO by using the standard ESR spectrometer, data in Fig. 3 are consistent with a two-compartment open-system model that has an initial distribution half-life of 2.4 min and a metabolic half-life of 22 min. Separate experiments conducted in the low-frequency spectrometer are shown in Fig. 4. Because it required ≈3 min to retune the instrument after administration of the nitroxide into the perfusion system, we were unable to record early time points. This along with scatter prevented us from observing the distribution phase. However, the metabolic half-life for HMPO in the low-frequency spectrometer was found to be 25 min, in excellent agreement with the value obtained with the standard spectrometer. For CTMPO, no bioreduction was observed regardless of the technique for measuring nitroxide concentration.

Experiments described above demonstrate the equivalence of the two spectrometric techniques for studying

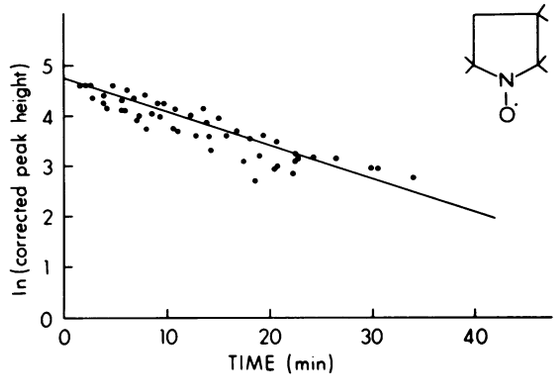

FIG. 5. Semilogarithmic plot of nitroxide concentration versus time after administration of HMPO to rat hearts placed in the low-frequency ESR spectrometer and then made ischemic. The metabolic half-life was found to be 10 min.

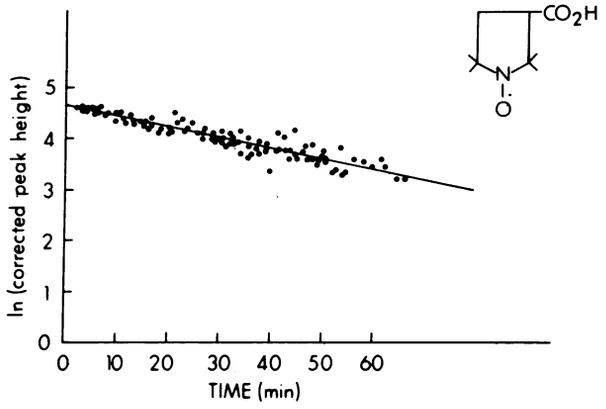

FIG. 6. Semilogarithmic plot of nitroxide concentration versus time after administration of CTMPO under experimental conditions identical to those described in Fig. 5. The metabolic half-life was calculated to be 33 min.

pharmacokinetics of nitroxides in an isolated rat heart perfusion system. However, in the ischemic model, only the low-frequency ESR spectrometer can be used to obtain pharmacokinetic data. From these studies, the calculated half-lives for HMPO and CTMPO were found to be 10.5 and 33 min, respectively (Figs. 5 and 6). Although these findings suggest an increased rate of nitroxide bioreduction as the result of ischemia, these data must be interpreted with extreme care considering differences in experimental model between the ischemic and the perfused states. In the ischemic heart preparation, the heart can act on only those spin-label molecules in the perfusate contained in the heart vasculature and parenchymal tissues. For the perfused heart preparation, the heart is exposed to all 40 ml of perfusate containing the nitroxide. Thus, one expects disparity between experimental states based on changes in volume and total number of molecules in the perfusate.

The perfusate forms one compartment, having a volume of V_p and a total number of nitroxide molecules at time t of $D_p(t)$. The spin-label concentration in the perfusate is then $D_p(t)/V_p$. The heart is the other compartment with an unknown volume V_h and a total number of spin-label molecules of $D_h(t)$. In the latter case the compartment consists of a vascular bed, which acts as a barrier to diffusion without any metabolic activity toward the nitroxide, and a lipid component consisting of endothelial and parenchymal cells. Thus, the transport of nitroxide across the vascular membrane is governed by the rate constant k_{ph} and in the reverse direction by k_{hp}. The ratio of the rate constants is the partition coefficient for a particular nitroxide. Bioreduction of the nitroxide takes place only in the lipid compartment with a rate constant of k_m. The three rate constants involve reactions taking place in the heart or at the heart perfusate interface, so if experiments are conducted with hearts of equal size, the pharmacodynamics can be modeled by using pseudo-first-order kinetics for the transport and metabolism of the spin label and ignoring the heart concentration. We express the kinetic equations in terms of numbers of spin-label molecules or spin-label amounts, since the low-frequency spectrometer measures a constant fraction of nitroxide in the perfusate compartment. Thus, the dynamics are driven by concentration gradients:

$$dD_p/dt = -(k_{ph}D_p/V_p) + (k_{hp}D_h/V_h)$$

and

$$dD_h/dt = (k_{ph}D_p/V_p) - (k_{hp}D_h/V_h) - (k_mD_h/V_h).$$

The solution to these equations is well known, with the number of molecules in the various compartments changing with time as the sum of exponentials. If spin label is introduced into the perfusate at time zero, then

$$D_p(t) = D_p(0)/(a - b) \times \{[k_{ph}/(V_p - b)]e^{-at} + [(a - k_{ph})/V_p]e^{-bt}\}$$

and

$$D_h(t) = D_p(0)\,(k_{ph}/V_p)/(a - b).$$

The a and b are the measured rates in the kinetic curves and they are related to the rate constants by

$$a + b = (k_{ph}/V_p) + (k_{hp}/V_h)\,(k_m/V_h)$$

and

$$ab = k_{ph}k_m/V_pV_h.$$

These equations predict two types of behavior for the two nitroxides. For CTMPO, $k_{hp} \gtrsim k_m \gg k_{ph}$. Under these conditions, transport into the heart is the rate-limiting step for the disappearance of the spin label. Only one of the two exponential terms in the decay of CTMPO will have any amplitude. The small component has a rapid exponential decay, while the exponential rate constant for the large component becomes

$$a \simeq -(k_{ph}/k_{hp})\,(k_m/V_p)t.$$

Then,

$$D_p(t) = D_p(0)\exp[-(k_{ph}/k_{hp})\,(k_m/V_p)t]$$

and

$$D_h(t) \approx 0.$$

Two aspects of the relationship between the metabolic rate (as reflected in the rate constant k_m) and the measured rate are worth noting. (*i*) The metabolic rate constant is multiplied by the partition coefficient k_{ph}/k_{hp}. For CTMPO, this is a small number (1/200) yielding a very slow measured rate. (*ii*) The measured rate is inversely proportional to the perfusate volume. Thus, even equal metabolic rate constants (k_m) for the ischemic and perfused conditions would have measured rates that would differ by the inverse of the ratio of the perfusate volume—a factor of ≈20.

For HMPO, we take $k_{ph} \gg k_m \gg k_{hp}$. Both exponential components of $D_p(t)$ have measurable amplitudes with:

$$a \simeq (k_{ph}/V_p) + (k_{hp}/V_h) \approx (k_{ph}/V_p)$$

and

$$b \simeq k_{ph}k_m/\{V_pV_h[(k_{ph}/V_p) + (k_m/V_h) + (k_{hp}/V_h)]\} \approx k_m/V_h.$$

For the distribution coefficient estimated here, the number of spin-label molecules in the perfusate decays rapidly with a rate equal to $a \simeq k_{ph}/V_p$, until it is roughly in equilibrium with nitroxide in the heart. It then decays at a rate approaching $b \simeq k_m/V_h$. For HMPO with a large partition coefficient, the measured rate of disappearance of spin label is independent of perfusate volume (at least for the volumes used in the present work). This is because the effective volume of the lipid compartment (an estimated volume of 2.5 ml multiplied

by a partition coefficient of 70) is so much larger than that of the perfusate that changes in the perfusate volume V_p between ischemic (2 ml) and recirculating (40 ml) experiments still yield volumes that are small with respect to the effective volume of the lipid compartment.

One of our goals is to relate measured time dependence of the ESR signal to bioreduction changes reflected in the metabolic rate constant k_m. This analysis, we believe, clarifies the interpretation of the time-dependence data we observe with both nitroxides under conditions of recirculation and ischemia. We note, from the foregoing results for CTMPO under conditions of recirculation, no reduction was observed, as measured with either a conventional or a low-frequency ESR spectrometer. Under ischemic conditions, in which only the low-frequency ESR spectrometer can be used, the calculated half-life was 33 min. The overall rate constant for CTMPO is inversely proportional to the perfusate volume. This, in turn, changes from ≈2 ml to 40 ml when we change from ischemia to recirculation. The overall rate constant, then, even if the metabolic rate constant k_m were to remain unchanged, would be reduced by a factor of 20. The half-life would increase to ≈10 hr, which is well beyond the 1 hr the isolated heart was in the spectrometer.

The situation is very different for HMPO. Here the partition coefficient, which is equal to k_{ph}/k_{hp}, is ≈70. As noted in the analysis, under these conditions there is no dependence of the measured rate constant on the volume of the perfusate compartment. A two-component time dependence is expected from the analysis and is observed where there is sensitivity at short times after injection of the nitroxide. The overall rate constant of the longer half-life component directly reflects the metabolic rate constant with no factors changing, shifting from recirculation to ischemia. Thus, a decrease in observed half-life for HMPO from 25 min (for recirculation) to 10 min (for ischemia) suggests an increase in bioreduction rate between the two states of the heart under these experimental conditions.

Finally, an additional comparison might be made between the bioreduction rates of CTMPO and HMPO. This is difficult given the disparate pharmacokinetics of the two nitroxides that in turn is the result of their very different lipophilicities. For CTMPO in the recirculating perfusion experiments, the absence of nitroxide bioreduction can be understood simply on the basis of pharmacokinetics. The large perfusate volume (V_p) and the small partition coefficient (k_{ph}/k_{hp}) multiplied by the bioreduction rate constant (k_m) would lead to a very slow apparent rate of reduction. However, under ischemia, the half-life of CTMPO is only three times that of HMPO. We say only because, in relation to HMPO, the rate constant k_m is multiplied by $k_{ph}V_p/k_{hp}V_h \simeq 0.005$. One may infer that the actual k_m for CTMPO might be considerably larger than for HMPO. Studies with isolated cells and cell fractions should yield more definitive information on this subject.

The ability to obtain ESR spectra from living tissues has implications beyond the field of free-radical biology. For example, nitroxide linewidth is very sensitive to oxygen tension. This is of major importance to the field of radiation oncology in which administered nitroxides could determine oxygen concentration in a tumor and thus aid in killing the tumor. Again, nitroxides are currently under extensive investigation as contrast agents for nuclear magnetic resonance imaging. Elucidation of the pharmacokinetics of these materials requires highly controlled conditions that retain much of the complexity of an *in vivo* model. The use of whole-organ preparations in combination with the low-frequency ESR spectrometer provides an ideal and accurate evaluation of these agents.

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