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A Microdialysis Method to Measure *in vivo* Hydrogen Peroxide and Superoxide in Various Rodent Tissues

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Abstract

Reactive oxygen species (ROS) play a critical role in cell signaling and disease pathogenesis. Despite their biological importance, assessment of ROS often involves measurement of indirect byproducts or measurement of ROS from excised tissue. Herein, we describe a microdialysis technique that utilizes the Amplex Ultrared assay to directly measure hydrogen peroxide (H_2O_2) and superoxide in tissue of living, anesthetized rats and mice. We demonstrate the application of this methodology in the penis, adipose tissue, skeletal muscle, kidney, and liver. We provide data demonstrating the impact of important methodological considerations such as membrane length, perfusion rate, and time-dependence upon probe insertion. In this report, we provide a complete list of equipment, troubleshooting tips, and suggestions for implementing this technique in a new system. The data herein demonstrate the feasibility of measuring both *in vivo* H_2O_2 and superoxide in the extracellular environment of various rodent tissues, providing a technique with potential application to a vast array of disease states which are subject to oxidative stress.

Keywords

H_2O_2 ; oxidative stress; ROS detection; Amplex Red; Redox; Liver

1. INTRODUCTION

The free radical theory of aging was first proposed in the mid 1950's [1]. Since then, the role of reactive oxygen species (ROS) in cell signaling, physiology, health, and disease has been an intense area of investigation in a wide range of fields [2, 3]. Despite these important physiological roles for ROS and the vast study of oxidative stress in numerous disease pathologies, assessment of oxidative stress at the human level often relies on measurement of indirect byproducts of lipid peroxidation from plasma, serum, or urine [4].

Malondialdehyde (MDA), often measured in the 2-thiobarbituric acid (TBARS) assay, and 8-isoprostanes are two commonly measured lipid peroxidation byproducts measured in such

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studies [5]. Several technical issues have been reported with these assays, such as the non-specific nature of the assays for these lipid peroxidation byproducts and that these byproducts may be produced by means other than lipid peroxidation [4, 6]. In addition, there is high potential for MDA formation during sample processing, while high variance between different labs has been reported for similar healthy control subjects [6]. These limitations invoke technological advancements for more accurate and direct ROS measurement.

We have gained immense knowledge of redox biology and disease pathology through the advent of chemiluminescent and fluorescent probes that offer more direct ROS measurement. Luminol was one of the first chemiluminescent probes available, and has served as a useful probe for cellular oxidant stress [7]. Luminol however, lacks specificity, as it is oxidized by superoxide, hydrogen peroxide (H_2O_2), peroxynitrite, hypochlorous acid, and the hydroxyl radical [8, 9]. Lucigenin-enhanced chemiluminescence is another commonly used assay for superoxide detection, although reports that lucigenin undergoes redox cycling have raised questions about its utility in biological systems [10, 11]. Dichlorodihydrofluorescein is a commonly used fluorescent dye, but has similar limitations to luminol in that it is oxidized by H_2O_2 , the hydroxyl radical, peroxynitrite, and nitric oxide [12]. Amplex Red is a relatively new fluorogenic substrate with high specificity and high sensitivity toward H_2O_2 , with the ability to detect as little as 50 nM H_2O_2 [13, 14]. Amplex Red has been utilized in several contexts, including measurement of H_2O_2 in the extracellular environment in cell culture models [15, 16] and from excised tissue [17, 18], as well as for measurement of mitochondrial H_2O_2 release in permeabilized muscle fibers [19, 20].

Research utilizing animal models has led us to knowledge implicating oxidative stress in the pathogenesis of a myriad of diseases in multiple organ systems. These studies often measure ROS *in vitro* from excised tissue or from tissue markers of lipid peroxidation. Oxidative stress, both systemically and in the penis specifically, has been linked to erectile dysfunction by our lab and others [21–24], while penile oxidative stress has been linked to priapism in a mouse model of sickle cell disease [25]. Adipose tissue oxidative stress has been linked to adipose tissue dysfunction and adipose tissue insulin resistance, while adipose tissue oxidative stress has specifically been implicated in the development of the metabolic syndrome in animal obesity models [26–28]. Similarly, excessive ROS production in skeletal muscle has been causally linked to skeletal muscle insulin resistance, a critical hallmark of Type II diabetes pathogenesis [19, 29]. Oxidative stress in the kidney, particularly in the renal cortex and renal medulla, has been linked to hypertension [30, 31]. Finally, animal models have revealed the important role of liver oxidative stress in the pathogenesis of nonalcoholic steatohepatitis, alcoholic liver disease, cirrhosis, and hepatocellular carcinoma [32–35].

The purpose of this report is to describe in detail our newly developed technique to measure *in vivo* H_2O_2 and superoxide and to demonstrate its utility in a variety of rodent tissue beds subject to oxidative stress, including the penis, adipose tissue, skeletal muscle, kidney, and liver. This technique utilizes Amplex Ultrared, a more sensitive and more stable version of the original Amplex Red reagent for H_2O_2 detection, and capitalizes on the high specificity of superoxide dismutase (SOD) for superoxide [36]. This technique has the added advantage

of being a microdialysis based technique, offering the ability to measure H_2O_2 and superoxide in live, anesthetized animals. We feel that this is an important technological advantage over measuring oxidative stress in excised tissue, as it allows the investigator to directly measure ROS in real time under physiological conditions with the complete oxidant detoxification system intact. Furthermore, the traditional lipid peroxidation byproducts may require prolonged periods of redox imbalance in order to detect changes, whereas this technique affords the investigator the ability to measure ROS under more acute physiologic or environmental stressors. In addition, the highly reactive nature of ROS infers that transient changes in ROS levels can have major physiological consequences. We test several important variables to consider when implementing a microdialysis study with respect to this technique, and provide troubleshooting tips and general advice for implementation of this methodology throughout the report.

2. MATERIALS AND METHODS

2.1 Animals

Young adult (3–4 months) male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Young adult (3–4 months) male C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a pathogen-free, temperature controlled facility with *ad libitum* access to standard rodent chow and drinking water. All animals were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine prior to microdialysis testing, and supplemented with an intramuscular injection as needed throughout experimentation. Following microdialysis testing, animals were euthanized while under anesthesia by exsanguination and double pneumothorax. All experimental procedures were conducted in accordance with the ethical standards of the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

2.2 Microdialysis Probe Insertion

Microdialysis involves embedding a thin, flexible, biocompatible fiber, termed microdialysis probe, into tissue [37, 38]. The fibers utilized herein had a length of 30 cm and a diameter of 218 μm . Pictures of the microdialysis probe both overlain on top of a rat hind limb and embedded in the hind limb skeletal muscle are provided in Figure 1. A portion of the fiber contains a semi-permeable membrane (the white portion of the probe in Fig. 1), allowing for bidirectional diffusion of metabolites and small molecules between the microdialysis probe and the interstitium. Probes are perfused with saline, Ringer's solution, or other physiological salt solutions and may contain drugs or other small molecule pharmacologic agents for investigation of drugs in the immediate vicinity of the semi-permeable membrane. When embedded into tissue, the semi-permeable membrane will rest in the interstitium, effectively mimicking a capillary by allowing exchange between the perfusion media and extracellular environment, and extracellular environment and the cell. The inflowing perfusion media is termed the perfusate, and the post-membrane outflowing solution from which measurements are taken is termed dialysate. A schematic of microdialysis sampling in the mouse liver is illustrated in Figure 2.

Insertion of microdialysis probes involves piercing the organ of interest with a needle, passing the linear probe through the lumen of the needle, pulling the needle out of the organ whilst leaving the probe in the organ, with the semi-permeable membrane positioned in the area of interest. The probes used in the present study had a tubing outer diameter of 218 μm , with a membrane outer diameter of 320 μm . Because the membrane is thicker than the tubing, it is recommended to pass the thicker, membranous portion of the probe into the needle, and to hold the pre-membrane side of the probe in place while pulling the needle out of the tissue. A 22-gauge needle was used for probe implantation in the present study in order to retain some maneuverability of the probe within the needle. A 20-gauge needle may be recommended, tissue permitting, if re-using probes several times in order to avoid tearing the probe at the glue points, the strength of which may be compromised with repeated uses.

For penile microdialysis probe insertion, the penis was freed of skin and fascia. For probe insertion into the rat penis, the needle was positioned perpendicularly to the center of the shaft and transversely driven through the corpus cavernosum, positioning the membrane in a cross-section of the corpus cavernosum in a transverse plane. For probe insertion into the mouse penis, the needle was inserted at the base of the shaft, distal to the left crus, driven through the corpus cavernosum and exiting the penis proximal to the right glans, positioning the membrane in the cavernous body. For liver microdialysis probe insertion, the liver was exposed through a midline abdominal incision, followed by a “y-shaped” incision to expose the median lobe and left lateral lobe of the liver. The left lateral lobe was pierced laterally through the center of the lobe in a frontal plane. For kidney microdialysis probe insertion, a paramedian incision through the abdomen was made to expose the right kidney. The kidney was pierced from the inferior to superior renal capsule in a sagittal plane, positioning the membrane in place at the depth of the renal medulla. For probe insertion into the retroperitoneal fat pad, the same incision used to expose the kidney was used to expose the fat pad. The fat pad was pierced inferior to superior, positioning the membrane embedded in the center of the fat pad. For probe insertion into the skeletal muscle, skin and fascia were trimmed from the anterior left hind limb, and the biceps femoris was isolated with forceps. The belly of the biceps femoris was pierced inferior to superior, positioning the membrane in the belly of the muscle.

2.3 Microdialysis and the Amplex Ultrared Assay

We have recently described the use of microdialysis with the amplex ultrared assay to detect H_2O_2 and superoxide in human skeletal muscle [39]. Microdialysis probes were perfused with a 0.9% saline solution containing 100 μM Amplex Ultrared (Molecular Probes, Eugene, OR) and 1.0 U/ml horseradish peroxidase (HRP; Sigma Aldrich, St. Louis, MO), whereby these reagents are continuously flowing through the microdialysis probe. Amplex Ultrared is a fluorogenic substrate with a very low background fluorescence which, in the presence of HRP, reacts with H_2O_2 with a 1:1 stoichiometry to produce the highly fluorescent resorufin [13, 14]. Perfusion of these substrates allows interstitial H_2O_2 to passively diffuse into the microdialysis membrane, producing the fluorescent resorufin inside the microdialysis probe. Linear microdialysis probes (Bioanalytical Systems, Inc., West Lafayette, IN) with a 20 kilodalton (kDa) maximal pore size were used in this study. The molecular weight of HRP is much greater than 20 kDa, ensuring that all HRP-mediated resorufin production takes place

within the microdialysis probe and limiting the H_2O_2 detected to that both in the interstitium and capable of cell-to-cell signal transduction. 10 U/ml superoxide dismutase (SOD; Sigma Aldrich, St. Louis, MO) was added to the perfusion media (where indicated in the text), allowing for conversion of superoxide that crosses over the microdialysis membrane into H_2O_2 , which then stimulates the production of resorufin [39]. Like HRP, the molecular weight of SOD is also much greater than the pore-size cut-off, ensuring that only superoxide diffusing into the microdialysis membrane is converted to H_2O_2 , and that SOD is not diffusing into the cell. Adding SOD to the Amplex Red reaction mixture and subtracting values attained without SOD in the mixture is a commonly used method to measure superoxide using the Amplex Red assay [40]. A schematic of this reaction is illustrated in Figure 3.

The perfusion media was made in 1.6 ml centrifuge tubes wrapped in aluminum foil, and immediately transferred to a 1 ml syringe wrapped in aluminum foil. The syringe was fit with a 22-gauge needle, which connected to the microdialysis probe *via* a tubing adapter (CMA3409500, Harvard Apparatus, Holliston, MA). The syringe was then secured to a CMA 102 microdialysis pump (Harvard Apparatus, Holliston, MA), allowing for continuous control of the perfusion flow rate. The outflowing end of the microdialysis probe was capped by piercing a polyethylene vial cap (Top-450, Milian USA, Gahanna, OH) with a 25-gauge needle, threading the microdialysis fiber through the needle, sliding the cap onto the fiber, and removing the needle. Outflowing dialysate was collected in a polyethylene collection vial (EWS-27-07, Milian USA, Gahanna, OH) wrapped in aluminum foil and snapped onto the vial cap. Amplex Ultrared can undergo photochemical oxidation mediated by room light [41]; however, shielding the perfusate and dialysate solutions from light with aluminum foil at all possible points of light exposure will prevent photochemical oxidation.

At least 15 μl of dialysate was collected and dialysate fluorescence was measured with a BioRad VersaFlour Fluorometer (BioRad, Hercules, CA) with a 510 nm excitation filter and 590 nm emission filter. The fluorometer cuvette was replaced with a quartz sub-micro fluorometer cell (16.10F-Q-10/Z8.5, Starna Cells, Atascadero, CA) to decrease the volume of the optical window to 10 μl . A gel loading pipet tip (#02-707-181, Fisher Scientific, Waltham, MA) was used to pipet 15 μl into the 2mm x 10 mm x 8 mm sample chamber of the sub-micro cell cuvette. Although 10 μl is the listed minimum requirement to fill the optical window of the sub-micro cell cuvette, we have found that using 15 μl of sample volume greatly increases the user ability to achieve stable fluorescence readings. Sample fluorescence was recorded five seconds after inserting the sub-micro cell cuvette into the fluorometer and closing the fluorometer lid. In the case that a stable fluorescence reading is not achieved within five seconds, it is likely that an air bubble has been introduced into sample chamber. If a stable fluorescence reading was not attained within five seconds, sample was aspirated out of the sample chamber and re-inserted until a stable reading was observed. After a successful reading, the sample was aspirated out of the sample chamber with a gel loading pipet tip and discarded. The sub-micro cell cuvette was washed between sample readings by pipetting 30 μl of deionized water into the sample chamber, aspirating the deionized water out, and repeating this process twice with excessive aspiration following the final rinse to ensure removal of all of the water.

2.4 Time course response of ROS production following probe insertion

Microdialysis probes were implanted in the penis and liver of mice ($n = 5$) as described in section 2.2. Custom designed linear microdialysis probes with a 3 mm semi-permeable membrane were purchased from Bioanalytical Systems Inc. (BASi, West Lafayette, IN). Upon implantation in both organs, probes were connected to perfusion syringes and flushed for 30 seconds. The perfusion rate was then set at 1.0 $\mu\text{l}/\text{min}$ and perfusion proceeded for two minutes before sample collection to ensure complete flushing of the probe dead space distal to the membrane. Dialysate was then collected for the following three hours, with collection vials changed out and fluorescence measured every 15 minutes. These experiments were designed to test the time response to perfusion and to test whether or not insertion of probes induces a traumatic stress resulting in a short term elevation of ROS. To further test whether or not probe insertion causes an acute burst of ROS, a second probe was inserted into the rat penis ($n = 4$) within 2 mm of the original probe. The original microdialysis probe with a 3 mm membrane was implanted into the rat penis and perfused with Amplex Ultrared, HRP, and SOD at 1.0 $\mu\text{l}/\text{min}$ as described above for 45 minutes to allow for stabilization of ROS levels to be established. Immediately after collection of the 45 minute sample, the penis was pierced with a 22-gauge needle within 1–2 mm of the original probe, and a second microdialysis probe was implanted in the tissue. Dialysate was collected from the original microdialysis probe at 15 minute intervals over the next 45 minutes.

2.5 Impact of perfusion flow rate

Rats ($n = 9$) were utilized to test the effect of perfusion flow rate on ROS recovery, with the liver chosen because it is large enough to implant three microdialysis probes into simultaneously while maintaining a distance of at least 1 cm between probes. All probes used had a 3 mm membrane length. One probe each was perfused at 0.5, 1.0, or 2.0 $\mu\text{l}/\text{min}$ within each experiment. Probes were perfused with saline containing amplex ultrared and HRP for 45 minutes to achieve stable ROS readings (due to results from section 2.4, Figure 3), whereafter dialysate was collected for the next hour, with fluorescence readings taken every 30 minutes. SOD was then added to the perfusate. Probes were again perfused for 45 minutes prior to dialysate collection over the following hour.

2.6 Impact of membrane length

Microdialysis was again performed in the liver of rats ($n = 9$) for the same reasons described in section 2.5. One 3 mm membrane probe was again used, with one 5 mm membrane probe and one 10 mm membrane probe used in each rat. Linear probes with 5 mm (LM-5; product # MD-2005) and 10 mm (LM-10; product # MD-2000) membrane lengths are regularly available from BASi without the need for custom design. All probes were perfused at 1.0 $\mu\text{l}/\text{min}$ with saline containing amplex ultrared and HRP for 45 minutes prior to dialysate collection. Three samples were collected thereafter every 15 minutes and fluorescence read. SOD was then added to the perfusate. Probes were again perfused for 45 minutes prior to dialysate collection every 15 minutes over the next 45 minutes.

2.7 Measurement of endogenous ROS in different organs

Probes were inserted into the penis, retroperitoneal fat pad, biceps femoris, kidney, and liver of rats ($n = 7$), and the penis, kidney, and liver of mice ($n = 5$). Probes with 3 mm membranes were perfused with saline containing amplex ultrared, HRP, and SOD at 1.0 $\mu\text{l}/\text{min}$ for 45 minutes prior to dialysate collection in order to achieve consistence across all tissue beds. Three-15 minute samples were collected over the subsequent 45 minutes and fluorescence measured. The purpose of these experiments was to compare the total ROS levels in these organs of two commonly used rodent strains.

2.8 Stimulation of ROS

A microdialysis probe with a 3 mm membrane was inserted into the kidney of rats ($n = 4$) as described above. Probes were perfused at 1.0 $\mu\text{l}/\text{min}$ with saline containing Amplex Ultrared, HRP, and SOD for 45 minutes. Three-15 minute samples were collected over the subsequent 45 minutes and fluorescence measured to monitor basal ROS levels. In order to stimulate ROS production, 100 μM angiotensin II (Ang II) was added to the perfusate, and perfusion ensued in the same probe. Fluorescence from dialysate collected over minutes 30–75 of Ang II stimulation were collected, from which three replicate data points were attained.

2.9 Assessment of catalase-sensitive fluorescence

A microdialysis probe with a 5 mm membrane was inserted into the rat liver to test the catalase-sensitive fluorescence. The probe was perfused with Amplex Ultrared and 0.05 U/ml HRP for 45 minutes, whereafter fluorescence was measured from three-15 minute dialysate samples. 200 U/ml catalase was then added to the perfusate, and fluorescence was measured from three samples over the subsequent 45 minutes. As the molecular weights of HRP and catalase are too large to allow for escape from the probe, the concentration of HRP was lowered in order to set up a competition between HRP and catalase for H_2O_2 , as described [42].

2.10 Data and Statistical Analysis

With the exception of the time course experiments outlined in section 2.4, all fluorescence measurements were taken in duplicate or triplicate and averaged to control for both technical and biologic variability. Background fluorescence of the perfusate was measured following collection of replicate samples and subtracted from all dialysate fluorescence readings. Relative fluorescence units were converted to $[\text{H}_2\text{O}_2]$ based on H_2O_2 standard curves constructed in the perfusate of each experiment. Superoxide concentrations were determined by subtracting $[\text{H}_2\text{O}_2]$ measured without SOD in the perfusate from the $[\text{H}_2\text{O}_2]$ measured with SOD in the perfusate and expressed as $[\text{H}_2\text{O}_2]$, as the concentrations are based on the H_2O_2 standard curve. Differences in time course responses and differences in ROS detection comparing varying flow rates or membrane lengths were determined by two-way repeated measures ANOVA with Tukey's posthoc analysis. Differences in superoxide concentrations and differences in ROS detected between different organs were determined by one-way repeated measures ANOVA with Tukey's posthoc analysis. Data were presented as means $\pm$

SEM. Statistical analyses were performed with GraphPad Prism (Prism 6.0, GraphPad, San Diego, CA), with an alpha level of 0.05.

3. RESULTS AND DISCUSSION

3.1 Impact of time and probe insertion on ROS measurement

ROS were measured immediately following probe insertion in the mouse penis and liver (Figure 4A). Penile ROS measured over the first 15 minutes of dialysate collection were significantly lower than all subsequent measurements ($P < 0.023$ for all time points), whereas ROS measurements were remarkably stable in all dialysate collected following 15 minutes ($P > 0.999$ at all time points). In the liver, ROS measured over the first 15 minutes of dialysate collection was lower than that collected over minutes 15–30 ($P < 0.001$), where ROS from minutes 15–30 were lower than all measurements taken following 45 minutes of collection ($P < 0.035$ for all time points). ROS measurements in all dialysate collected following 45 minutes of perfusion were stable ($P > 0.98$ at all time points). Perfusion of microdialysis probes for one hour following probe insertion is customary in microdialysis studies to allow for equilibration of metabolites across the semi-permeable membrane [43, 44]. However, the time to equilibration is dependent upon many variables, including the molecular weight of the analyte of interest and the concentration of the analyte in the extracellular environment surrounding the microdialysis probe [37, 38]. In animal studies, it is desirable to minimize the duration of time that the animal is under anesthesia, particularly if a surgical incision exposes the body cavity to ambient air and potential for dehydration. Depending on the study design, perfusion flow rate chosen, and minimum volume of fluid required for a particular fluorometer, it will be optimal to minimize the time of the equilibration period. This last point is especially valid if multiple replicates are desired for each measurement, which is recommended. These data highlight the need to test the time response of ROS detected following probe insertion in each experimental system to ensure stable readings and reproducible results. The stability of these data with no decrease at later time points suggests that insertion of the probes is not causing a traumatic stress to the tissue that manifests in excessive ROS generation in the early phase of dialysate collection. To more directly test the possibility of an acute burst of ROS upon probe insertion, an additional probe was inserted into the rat penis within 2 mm of a probe from which ROS had been measured for 45 minutes (Figure 4B). There was no discernible change in ROS detection upon insertion of the new probe.

3.2 Impact of perfusion flow rate on H_2O_2 and superoxide detection

Determining the perfusion rate is another important consideration for designing a microdialysis study. A slower perfusion rate allows the perfused reagents greater time in the membrane in which they are subject to interaction with the extracellular environment, and simple diffusion kinetics dictate that more analytes will cross over the semi-permeable membrane in that timespan [37, 38]. The results presented in Figure 5A clearly demonstrate a perfusion rate dependence on $[H_2O_2]$ captured, with 0.5 $\mu\text{L}/\text{min}$ capturing more H_2O_2 than 1.0 $\mu\text{L}/\text{min}$ ($P < 0.0001$), and 1.0 $\mu\text{L}/\text{min}$ capturing more H_2O_2 than 2.0 $\mu\text{L}/\text{min}$ ($P < 0.0001$). There was a general trend (one-way ANOVA $P = 0.19$) toward decreased superoxide capture with increased perfusion rate (Figure 4B), though this trend was clearly not as pervasive as

that of H₂O₂ capture. The presence of endogenous extracellular SOD could be partly responsible for this, as the increased time allotted for diffusion could be partly negated by extracellular SOD converting extracellular superoxide to H₂O₂ in that additional time period. These potential effects of endogenous extracellular SOD would manifest as increased extracellular H₂O₂, which was clearly detected with decreased perfusion rates.

3.3 Impact of membrane length on H₂O₂ and superoxide detection

Another important factor to consider in design of a microdialysis study is the length of semi-permeable membrane to use. By using microdialysis, extracellular fluid is continuously sampled from the microenvironment within approximately a 2 mm radius of the microdialysis probe [45]. Thus, by increasing the length of the membrane, a larger volume of extracellular fluid is sampled. We demonstrate that increasing the membrane length results in increased H₂O₂ capture (Figure 6A), as 10 mm membranes captured more H₂O₂ than 5 mm membranes ($p = 0.001$), and 5 mm membranes captured more H₂O₂ than 3 mm membranes ($P < 0.0001$). Additionally, there was a clear effect of membrane length on superoxide detection (Figure 6B), with significantly more superoxide detected using 10 mm membranes compared to 3 mm membranes ($P = 0.039$). Comparing and contrasting figures 5 and 6, it would appear that perfusion rate has a greater effect on H₂O₂ detection, whereas membrane length has a greater effect on superoxide detection. However, maximizing membrane length while minimizing perfusion rate will allow for maximal detection of both ROS, thereby increasing the overall signal and likely increasing the investigator's ability to detect differences between groups when biological differences in ROS levels are present. The tradeoff with using slower perfusion rates is of course either increasing the duration of experimentation or decreasing the number of replicate samples. The membrane length will naturally be limited to the organ size or to the size of the compartment within the organ from which measurement is desired.

3.4 Comparison of ROS across different organs

Total ROS detected in the rat penis, retroperitoneal fat, hindlimb skeletal muscle, kidney, and liver are presented in Figure 7A. ROS levels in the kidney were higher than that in the penis ($P = 0.005$) and retroperitoneal fat ($P < 0.0001$). ROS levels in the liver were higher than that of all other organs, with stark differences observed in all cases ($P < 0.0001$ vs. all other organs). Total ROS detected in the mouse penis, kidney, and liver are presented in Figure 7B. Similarly, ROS levels in the kidney were higher than that of the penis ($P = 0.008$), while ROS levels in the liver were higher than that for both other organs ($P < 0.05$). These results further demonstrate similar ROS production in comparable tissue beds between Sprague-Dawley rats and C57Bl/6 mice, two of the most commonly used strains of their respective species. It should be noted these animals were young and subjected only to "control" conditions, and do not reflect how each tissue bed would respond to a pathologic or environmental stressor.

Given the pronounced differences in liver ROS production relative to the other organs investigated and the high rate of blood perfusion through the liver, it is tempting to speculate that the liver represents a primary driver of systemic oxidative stress. However, correlations between liver and blood markers of oxidative stress are dependent on the specific marker,

while these correlations are variable and dependent upon the environmental condition to which the rats are exposed [46]. Furthermore, previous studies comparing oxidative stress byproducts across organs have produced variable results. Liver MDA levels have been shown to be dramatically higher in control animals than kidney and skeletal muscle MDA levels [47]. Similar, albeit less dramatic results have been observed for tissue concentrations of lipid hydroperoxides [48]. However, plenty of contradictory results exist, as kidney and skeletal muscle MDA and conjugated dienes have been found to be higher than that in the liver [49–51]. Given the potential for artifactual MDA signal resulting from sample processing [4, 6] and potential differences in sample processing across tissues, some of these contrasting results could be the mere product of differences in sample processing. Furthermore, differences in cellular lipid dynamics and tissue architecture dictate susceptibility to lipid peroxidation by reactive aldehydes, such as MDA [52]. These considerations suggest that technical advancements in measurement of ROS are needed. We believe that direct *in vivo* measurement of H₂O₂ and superoxide as outlined in this report represents a clear advancement in comparing redox environment across different tissues.

3.5 Stimulation of ROS

ROS were detected in the kidney both before and during microdialysis perfusion of Ang II (Figure 8), revealing a 35% increase in ROS upon Ang II stimulation ($P < 0.001$). Ang II is a well documented stimulator of NADPH oxidase activity [53, 54]. Ang II has previously been perfused through microdialysis probes embedded in human skin, where Ang II attenuated the nitric oxide-dependent increase in cutaneous blood flow upon local heating, effects which were abolished by NADPH oxidase inhibition [55]. The finding in the present study that demonstrates an increase in ROS upon Ang II perfusion provides proof of principle that microdialysis can be used to deliver drugs to the local environment that can alter ROS generation, which is detectable with the methodology described herein.

3.6 Determination of catalase-sensitive fluorescence

Pulse radiolysis experiments investigating various one electron oxidants have demonstrated a potential for Amplex Red to be oxidized by peroxynitrite [56]. In order to test the specificity of the fluorescent signal to H₂O₂ achieved with microdialysis, we reduced the concentration of HRP and co-perfused catalase through microdialysis probes in the rat liver (Figure 9A). As the molecular weights of HRP and catalase are both much larger than the pore size of the membrane, neither HRP nor catalase are able to escape the microdialysis probe to act in the extracellular environment. As such, this experiment effectively sets up competition between HRP and catalase for H₂O₂ within the microdialysis probe. This experiment revealed that 76% of the fluorescent signal was inhibited by catalase. However, performing H₂O₂ standard curves in the perfusates (Figure 9B) reveals that low concentration HRP is still able to compete with catalase for some of the H₂O₂. When the fluorescent signals observed in the absence and presence of catalase were converted to [H₂O₂] based on the standard curve constructed in each of the perfusates, the mean [H₂O₂] of each condition were within 5% of each other (– Cat: $4.05 \pm 0.37 \mu\text{M}$; + Cat: $3.88 \pm 0.79 \mu\text{M}$). That the fluorescent signal was dampened by catalase and that the calculated H₂O₂ concentrations observed in the presence and absence of catalase were comparable, we are confident that the fluorescent signal observed in the microdialysis system is predominantly H₂O₂ driven. Amplex red

fluorescence driven by peroxynitrite has previously been detected in macrophages stimulated with phorbol 12-myristate 13-acetate to stimulate NADPH oxidase activity and lipopolysaccharide to stimulate inducible nitric oxide synthase (iNOS) activity [57]. However, the peroxynitrite driven fluorescence accounted for approximately 5% of the total fluorescence and was only observable when NADPH oxidase and iNOS activity were both stimulated, and not when macrophages were exposed to only one of the stimulants [57]. It is unclear how comparable peroxynitrite levels are from pure macrophages subjected to concurrent NADPH oxidase and iNOS stimulation to physiological peroxynitrite levels observed at the tissue level, although it remains possible that peroxynitrite contributes to the fluorescent signal in the microdialysis system to some degree. Recent research utilizing purified mitochondria has demonstrated that Amplex red is capable of being converted to resorufin in the mitochondrial matrix via carboxylesterase [42]. While Amplex red reagents are generally described as being highly sensitive and specific for H_2O_2 [13, 14], it is important to recognize that some degree of H_2O_2 -independent fluorescence is possible. Therefore, it is important to stress that the calculated concentrations of H_2O_2 and superoxide detected by this microdialysis technique are not to be stated as the absolute concentrations of the interstitium or the tissue.

3.7 General Discussion

This technique affords the ability to directly measure H_2O_2 and superoxide in the extracellular space of living animals, which we believe represents a major physiological advantage over measuring ROS or accumulated oxidative stress byproducts in excised tissue. The rapid oxidation of glutathione under *ex vivo* conditions has been well described as a confounding factor in redox biology investigations [58]. Thus, the ability to maintain physiological blood flow to the organ of interest and to prevent excessive oxidation of a major endogenous antioxidant provides a significant advantage in investigating the ROS balance and redox state of the organ. The primary limitation cited for the Amplex Red reagents is their susceptibility to oxidation upon light exposure [41]. However, we have observed very low background fluorescence when shielding the reagents from light with aluminum foil at all possible points of exposure, while any background fluorescence is accounted for by subtracting the fluorescence of the perfusate from that of the dialysate. Furthermore, the ability to measure interstitial superoxide by addition of SOD to the perfusate [40] adds another layer of applicability to this technique, as H_2O_2 and superoxide are two of the most biologically relevant ROS with distinctly different biological functions. In addition, microdialysis offers the ability to co-perfuse small molecule drugs or inhibitors to test their local effect on ROS levels. Drugs perfused through microdialysis probes have been found to affect an area reaching the length of the microdialysis membrane with an approximate radius of 2 mm surrounding the membrane [45]. Testing drugs or inhibitors in this manner avoids the potential compensatory effects associated with systemic administration. An additional advantage of this technique is that it utilizes common, relatively inexpensive laboratory equipment (a bench top fluorometer) for sample testing. There is no need for high-performance liquid chromatography [59] or other such equipment that is less commonly found in basic physiology laboratories.

4. CONCLUSION

In conclusion, we have scaled down previous methodology [39] used to detect H₂O₂ and superoxide in human skeletal muscle to the rat and mouse models. We have described the protocol and microdialysis set-up in extensive detail to allow a novice to microdialysis to implement these techniques in their own model. We have worked out the major methodological considerations for implementing this technique and provide data utilizing this assay to allow a new investigator to optimize this technique in their own animal model. We have further demonstrated the utility of these methods in the penis, adipose tissue, skeletal muscle, kidney, and liver, where we demonstrate markedly higher ROS levels in the liver under “control” conditions compared to all other tissues investigated. This technique is not meant to measure the absolute concentration of H₂O₂ or superoxide in the tissue, but rather to be used as a tool to compare interstitial H₂O₂ and superoxide levels between groups or treatment conditions. We believe that this methodology of measuring H₂O₂ and superoxide directly in the tissue as they are produced in live animals represents a physiologically relevant advantage over previous technology.

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Abbreviations

ROS	reactive oxygen species
MDA	malondialdehyde
TBARS	2-thiobarbituric acid
H₂O₂	hydrogen peroxide
SOD	superoxide dismutase
HRP	horseradish peroxidase
Ang II	angiotensin II
Cat	catalase

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Figure 1.

Photographs of the microdialysis probe. A microdialysis probe against the hind limb of a 280 gram rat in the upper left panel. A zoomed in picture of the probe outside of the muscle is shown in the upper right panel. The white section of the fiber is the 3 mm semi-permeable membrane. The picture in the lower right panel shows the probe embedded in the skeletal muscle, which shows the entirety of the white semi-permeable membrane in the muscle bed.

Figure 2.

Illustration of the microdialysis set-up. The microdialysis fiber is depicted as implanted in the mouse liver and connected to the pump system on the left and a collection vial on the right. The zoomed in panel depicts a view inside the liver, with the semi-permeable membrane, depicted in white, firmly entrenched in the tissue. The further zoomed in panel illustrates the membrane interacting with the interstitium. Molecules are produced by the cell which diffuse into the interstitium and ultimately diffuse into the microdialysis probe through the semi-permeable membrane, where they can be analyzed in the outflowing dialysate.

Figure 3.

Schematic of ROS measurement with microdialysis. Amplex Ultrared, HRP, and SOD are continuously perfused through the microdialysis probe. H_2O_2 in the extracellular space may diffuse through the semi-permeable membrane to react with HRP, producing resorufin inside the microdialysis probe. Extracellular superoxide may diffuse through the semi-permeable membrane where it will be converted to H_2O_2 by the perfused SOD, or it may be converted to H_2O_2 by extracellular SOD (ecSOD) in the extracellular space. Increased H_2O_2 within the microdialysis probe will result in more resorufin production and consequent brighter fluorescence, which is measured in the outflowing dialysate. NADPH oxidase (red), uncoupled nitric oxide synthase (violet), and the mitochondria (blue) are depicted as potential sources of ROS in the interstitium.

Figure 4.

Impact of time and probe insertion on ROS measurements. Probes with 3 mm membranes were perfused with Amplex Ultrared, horseradish peroxidase, and superoxide dismutase for 3 hours following probe insertion in (A) the penis and liver of mice, and (B) the penis of rats, with measurements taken every 15 minutes. A second microdialysis probe was inserted into the rat penis at minute 45 to test the acute impact of probe insertion on ROS generation. $n = 5$ for mice, $n = 4$ for rats. * $P < 0.05$ vs. minutes 30–180. † $P < 0.05$ vs. minutes 60–180.

Figure 5.

Impact of perfusion flow rate on hydrogen peroxide and superoxide detection. Three – 3mm membrane probes were inserted into the liver of the same rat, and perfused at 0.5, 1.0, or 2.0 $\mu l/min$. A.) Probes were perfused without SOD (–SOD) and with SOD (+SOD). B.)

Superoxide concentrations captured were calculated as [–SOD] subtracted from [+SOD]. *

$P < 0.05$ vs. 2.0 $\mu l/min$, † $P < 0.05$ vs. 1.0 and 2.0 $\mu l/min$. $n = 9$.

Figure 6.

Impact of membrane length on hydrogen peroxide and superoxide detection. Microdialysis probes with membrane lengths of 3, 5, and 10 mm were simultaneously inserted in into the liver of the same rat. A.) Probes were perfused without SOD (-SOD) and with SOD (+SOD). B.) Superoxide concentrations captured were calculated as [-SOD] subtracted from [+SOD]. * $P < 0.05$ 5 mm vs, 3 mm, † $P < 0.05$ 10 mm vs. 5 mm and 3 mm. $n = 9$.

Figure 7.

Measurement of combined hydrogen peroxide and superoxide detected across various organs in rats and mice. Probes with 3 mm membranes were used for all comparisons with a perfusion rate of 1.0 μ l/min. A.) ROS were measured in the penis, retroperitoneal fat, skeletal muscle, kidney, liver of the same rats. B.) ROS were measured in the penis, kidney, and liver of the same mice. * $P < 0.05$ vs Retro Fat, † $P < 0.05$ vs. Penis, ‡ $P < 0.05$ vs. Sk Muscle, § $P < 0.05$ vs. Kidney. $n = 7$ for rat, $n = 5$ for mouse.

Figure 8. Stimulation of ROS generation by angiotensin II. Probes with 3 mm membranes were perfused with Amplex Ultrared, HRP, and SOD in the rat kidney (Control). Local ROS generation was then stimulated by addition of 100 μM angiotensin II (Ang II) to the perfusate and perfused through the same probe. * P < 0.05 vs. Control. n = 4.

Figure 9.

Investigation of catalase-sensitive fluorescence. A.) The HRP concentration was reduced to 0.05 U/ml and probes in the rat liver were perfused both without (– Cat) and with (+ Cat) 200 U/ml catalase in order to set up competition between HRP and catalase for H_2O_2 . B.) H_2O_2 standard curves for HRP-diluted perfusates with and without catalase. RFU, relative fluorescence units. * $P < 0.05$ against the opposing condition within the same organ. $n = 4$.