SHORT COMMUNICATION

Novel Method for Detection of Reactive Oxygen Species In Vivo in Human Skeletal Muscle

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Summary

Excessive production of reactive oxygen species (ROS) are implicated in the pathogenesis of numerous disease states. However, direct measurement of *in vivo* ROS in humans has remained elusive due to limited access to appropriate tissue beds and the inherently short half-lives and high reactivity of ROS. Herein, we describe a novel technique by which to measure *in vivo* ROS in human skeletal muscle. Microdialysis probes were inserted into the vastus lateralis of eight healthy volunteers. Amplex Ultrared, a highly specific fluorogenic substrate for hydrogen peroxide (H_2O_2), and horseradish peroxidase (HRP), were perfused through microdialysis probes, and outflowing dialysate was collected and fluorescence was measured. Extracellular H_2O_2 that crossed the microdialysis membrane was measured *via* fluorescence of the dialysate. Superoxide dismutase (SOD) was then added to the inflowing perfusion media to convert any superoxide crossing the microdialysis membrane to H_2O_2 within the microdialysis probe. Fluorescence significantly increased (P=0.005) upon SOD addition. These data demonstrate the feasibility of measuring both *in vivo* H_2O_2 and superoxide in the extracellular environment of human skeletal muscle, providing a technique with a potential application to a wide range of circulatory and metabolic studies of oxidative stress.

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Oxidative stress is traditionally used to describe situations in which the generation of reactive oxygen species (ROS) overwhelms antioxidant defense systems, resulting in oxidative damage to tissue and macromolecules. Superoxide ($O_2^{\bullet-}$) is considered the parent ROS molecule, which is a highly reactive molecule that can be dismutated either enzymatically by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2), or spontaneously into H_2O_2, peroxynitrite ($ONOO^-$), or the hydroperoxyl radical (HOO^-) (Murrant and Reid 2001). ROS act as intracellular signaling molecules that modulate many biological processes (Poli *et al.* 2004); however, in the state of excessive ROS levels the signaling process acts to cause damage to lipids, proteins, and DNA. As a consequence, apoptosis and increased cellular permeability may occur, which

lead to inflammation, endothelial dysfunction, and vascular remodeling, all of which contribute to cardiovascular and metabolic disease pathogenesis (Weseler and Bast 2010).

Oxidative stress has been implicated in the pathogenesis of several diseases and detrimental processes, such as hypertension, atherosclerosis, heart failure, diabetes mellitus, neurodegeneration, cancer, and aging (Dikalov 2011). Despite the important physiological and pathological roles of ROS, assessment of oxidative stress in humans often relies on measurement of indirect byproducts of lipid peroxidation in serum or plasma (Janero 1990). Direct measurement of *in vivo* ROS levels in humans has remained elusive due to limited access to appropriate tissue beds and the inherently short half-lives and high reactivity of ROS. In the present study, we describe a novel method in which to measure *in vivo* H_2O_2 and $O_2^{\bullet-}$ production in human skeletal muscle interstitial space. Importantly, H_2O_2 and $O_2^{\bullet-}$ appear to be the predominant ROS molecules in skeletal muscle (Fisher-Wellman and Neufer 2012).

Young (age 21-29), healthy men were recruited for participation in this study. All subjects were non-smokers with no known history of cardiovascular disease, and were not taking medications for hypertension, hypercholesterolemia, or insulin resistance. All subjects abstained from antioxidant supplementation for at least two weeks prior to testing. All subjects provided written informed consent, and all procedures were approved by the University and Medical Center Institutional Review Board of East Carolina University.

A microdialysis probe was inserted under sterile technique into the left vastus lateralis of each subject as previously described (Hickner *et al.* 1994), while the subject was resting in a hospital bed. Following administration of local anesthesia (1 ml of 1 % Lidocaine HCl) above the muscle fascia, an 18-gauge catheter (Jelco, Smiths Medical, Southington, CT) surrounded by plastic introducer tubing (CMA Microdialysis AB, Solna, Sweden) was inserted into the vastus lateralis. The catheter was withdrawn, while the introducer tubing was left in the muscle. A microdialysis probe (CMA 20 Elite, CMA Microdialysis) was inserted into the introducer, and the splittable tubing was pulled out of the thigh, leaving the probes in place in the muscle bed.

Probes were perfused with a 0.9 % saline solution with a microinfusion pump (CMA 107, CMA

Microdialysis) at a flow rate of 2.0 µl/min for the remainder of the experiment. The distal 10 mm of the microdialysis probe contains a semi-permeable membrane allowing for bi-directional diffusion of small molecules (<20 kDa). Probes were perfused for 90 min to allow for recovery from trauma induced by probe insertion. Amplex Ultrared (100 µM final concentration; Molecular Probes, Eugene, OR) and horseradish peroxidase (HRP; 1.0 U/ml final concentration; Sigma Aldrich, St. Louis, MO) were added to the perfusate. 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 (Mohanty *et al.* 1997, Zhou *et al.* 1997). Perfusion of these substrates allows H_2O_2 in the muscle extracellular environment to cross over the membrane to produce the fluorescent resorufin within the microdialysis probe. The molecular size of HRP is much greater than the pore size of the microdialysis probes, ensuring that all resorufin production takes place within the microdialysis probe. Three 20 min dialysate (probe outflow) samples were collected in 150 µl polyethylene collection vials and analyzed immediately upon collection. 30 µl of dialysate was added to a 250 µl borosilicate cuvette (Wheaton Industries, Millville, NJ) with a gel-loading pipet tip, and fluorescence intensity was measured with a TD-700 laboratory fluorometer (Turner Designs, Sunnyvale, CA) fit with a minicell adaptor kit (Turner Designs), at an excitation wavelength of 550 nm and an emission wavelength of 570 nm. A platform was inserted into the minicell to raise the cuvette position such that 30 µl of sample covered the optical window of the fluorometer. SOD (10 U/ml final concentration; Sigma Aldrich) was then added to the perfusate allowing for the conversion of $O_2^{\bullet-}$ that crosses over the membrane to H_2O_2, which reacts with the fluorogenic substrate. Like HRP, the molecular size of SOD is much greater than the pore size of the microdialysis probes, ensuring that all conversion of $O_2^{\bullet-}$ to H_2O_2 occurs within the microdialysis probe. Three 20 min dialysate samples were collected and fluorescence intensity was measured. The infusion pump and all collection vials were wrapped in aluminum foil throughout the duration of the experiment to prevent room light-mediated photochemical oxidation of Amplex Ultrared at any time or site throughout experimentation.
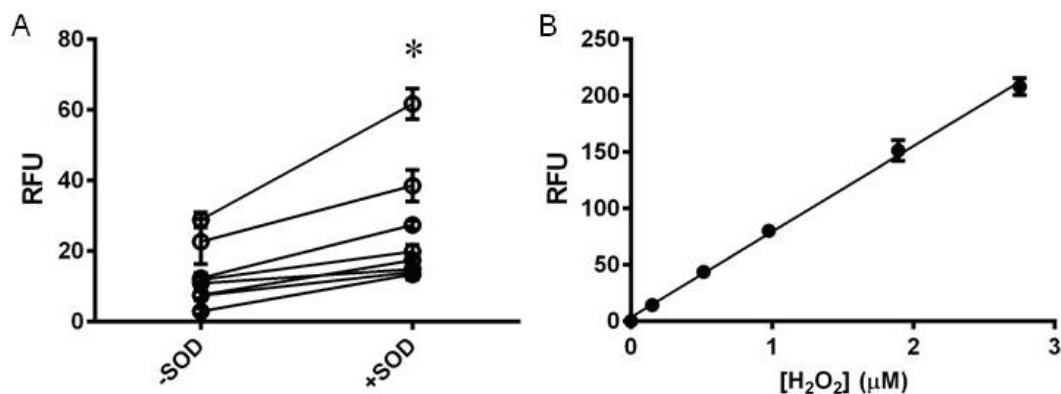
Fig. 1. (A) Fluorometric analysis of *in vivo* reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2) was measured in the skeletal muscle of n=8 individuals in the absence (-SOD) and presence (+SOD) of superoxide dismutase (SOD) in the perfusate. Background fluorescence of the perfusate ranged from 1-3 relative fluorescence units (RFU), and was subtracted from fluorescence of the dialysate, expressed in RFU. -SOD values strictly reflect H_2O_2 that diffuses into the probe, while the increased fluorescence upon SOD addition reflects the additional contribution by superoxide. * Fluorescence was increased upon addition of SOD to the perfusate (P=0.005). Connecting lines indicate results from the same individual. Values are expressed as mean ± SEM of three replicates of each measurement. **(B)** H_2O_2 standard curve. Perfusates were subjected to varying concentrations of H_2O_2 prepared from a stock solution. The background fluorescence of the perfusate was used as the zero standard and subtracted off of the fluorescence of the H_2O_2 standards. Values are means ± SEM for n=8 trials.

Fluorescence of all dialysate samples were above the detection limit of the fluorometer (Fig. 1A), demonstrating the utility of the Amplex Ultrared fluorescent probe for use in this manner. A paired t-test reveals that fluorescence intensity was significantly elevated when SOD was added to the perfusate, indicating that $O_2^{\cdot-}$ is capable of crossing the microdialysis membrane. An H_2O_2 standard curve was conducted following each experiment by addition of known concentrations of H_2O_2 to 30 μl aliquots of the remaining perfusate, allowing for conversion of relative fluorescence units to [H_2O_2]. The H_2O_2 standard curve (Fig. 1B) demonstrates the linearity of resorufin production in response to H_2O_2, and demonstrates the ability to accurately measure H_2O_2 in the low micromolar range with the concentrations of Amplex Ultrared and HRP utilized in this study. This standard curve indicates that the [H_2O_2] detected by this system in these subjects is ~0.16 μM without addition of SOD, and ~0.32 μM with the addition of SOD. It should be acknowledged that recovery of H_2O_2 from the interstitial fluid is likely to be less than 100 % at the perfusion flow rate and membrane size used, indicating that the [H_2O_2] determined by this method is not equal to the absolute interstitial [H_2O_2]. Preliminary studies were performed with SOD in the perfusate, but without Amplex Ultrared or HRP added to the perfusate. In these preliminary studies, dialysate was added directly to a saline solution containing HRP and Amplex Ultrared, from which fluorescence was measured. All of these experiments failed to produce a

fluorescence reading above the detection limit, suggesting that H_2O_2 may diffuse out of the membrane after entering the membrane, or that H_2O_2 may spontaneously decompose into H_2O during the 20 min collection period. These preliminary studies demonstrate the necessity of addition of Amplex Ultrared and HRP to the perfusate to form resorufin rapidly upon H_2O_2 diffusion into the membrane.

It has previously been reported that reduced glutathione (GSH) and β-nicotinamide adenine dinucleotide (NADH) may induce auto-oxidation of the Amplex Red/HRP assay (Votyakova and Reynolds 2004). Because both GSH and NADH are small enough to cross over the microdialysis membrane, we performed *in vitro* experiments to test their auto-oxidative capacity in the microdialysis system. Microdialysis probes were perfused as described above, with the microdialysis membrane submerged in a beaker containing varying concentrations of GSH (Sigma Aldrich) or NADH (Agros Organics) mixed in saline, and fluorescence of the dialysate was measured. GSH concentrations of 0.5, 1.0, 5.0, and 10 mM were tested, which did not induce any detectable fluorescence (data not shown). NADH concentrations below 10 μM did not induce an appreciable amount of auto-oxidation (Fig. 2). The cellular NADH concentration has been estimated at 110 nM (Zhang *et al.* 2002), thus physiological NADH is unlikely to influence ROS detection with this method. However, supraphysiological concentrations of NADH did induce apparent auto-oxidation, which was partially

prevented by addition of SOD to the perfusate (Fig. 2). These results suggest that it is unlikely that Amplex Ultrared is auto-oxidized by GSH or NADH in an *in vivo* setting, but indicate that the methods described herein are not suitable to detect ROS in a system where exogenous NADH is added.
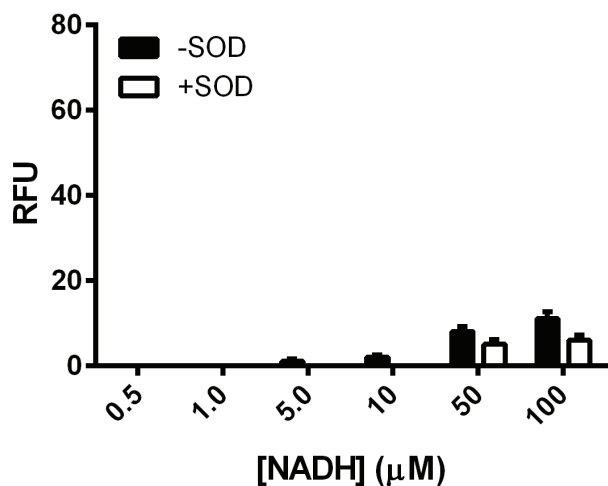

Fig. 2. Auto-oxidation of Amplex Ultrared by *in vitro* exposure to NADH. Microdialysis probes were perfused with the Amplex Ultrared/HRP mixture, in the absence (-SOD) and presence (+SOD) of superoxide dismutase (SOD) in the perfusate. The microdialysis membrane was submerged in a beaker containing the indicated concentration of NADH, and fluorescence of dialysate measured. The background fluorescence of the perfusate of 2 relative fluorescence units (RFU) was subtracted from the fluorescence of each sample. Values are expressed as mean ± SEM of three replicates of each measurement.

Microdialysis has previously been utilized to measure extracellular $O_2^{\cdot-}$ in rodent (Close *et al.* 2007, McArdle *et al.* 2001, 2004, Pattwell *et al.* 2001) and human (Hellsten *et al.* 2007) skeletal muscle, particularly in response to electrically stimulated muscle contractions or exercise. These investigations have utilized cytochrome c reduction for $O_2^{\cdot-}$ detection. We believe that the Amplex Ultrared technique described in the present study affords several advantages over the cytochrome c reduction assay. Particularly, the low molecular weight of cytochrome c allows for passage of cytochrome c from the microdialysis membrane into the interstitial fluid, where it may be reduced outside of the probe. Additionally, the high sample volume requirement necessitates a need for either a very high perfusion flow rate, ultimately limiting the potential for diffusion; or a need for long duration collection times, which places a greater time constraint on the study protocol design.

Importantly, the specificity of cytochrome c has been questioned (Münzel *et al.* 2002, Murrant and Reid 2001, Thomson *et al.* 1995). Cytochrome c has been found to be reduced by the hydroxyl radical or nitric oxide (Murrant and Reid 2001) and can be oxidized by $ONOO^-$ (Thomson *et al.* 1995). Since high levels of $O_2^{\cdot-}$ are associated with low nitric oxide bioavailability and potentially with elevated $ONOO^-$ production, the cytochrome c reduction assay may not be appropriate for comparing ROS production levels in a pathological state relative to a control group. In addition, it has recently been suggested that cytochrome c reduction is appropriate for quantifying $O_2^{\cdot-}$ released during the respiratory burst of neutrophils or by isolated enzymes, but the assay is likely not sensitive enough to detect small quantities of $O_2^{\cdot-}$ produced by nonphagocytic cells (Dikalov and Harrison 2014). As HRP is too large to pass out of the membrane, the methods utilized in the present study strictly detect ROS that diffuse into the microdialysis membrane with high specificity to H_2O_2, and can be measured quickly and easily in non-contracting skeletal muscle of healthy individuals that likely produce levels of ROS that are on the low end of what is to be expected in a human population.

The technique described in the present study has a wide range of utility, as it can be used safely in humans with minimally invasive techniques. This technique could be used to measure ROS in pathological states, and could be used to test pharmacological efficacy of oral antioxidant therapies or drugs targeted to improvement of components of the metabolic syndrome. An additional advantage to the microdialysis technique is that it can be utilized with pharmacologic agents or enzyme targeted antioxidants delivered directly to the muscle *via* microdialysis to test the localized effect of these agents on extracellular ROS levels. Furthermore, these measures could be performed with simultaneous monitoring of blood flow (Hickner *et al.* 1992) and metabolism (Hershberger *et al.* 2004) *via* microdialysis.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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