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Super-Resolution Ultrasound Imaging of Skeletal Muscle Microvascular Dysfunction in an Animal Model of Type 2 Diabetes

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Abstract

Objectives——To evaluate the use of super-resolution ultrasound (SR-US) imaging for quantifying microvascular changes in skeletal muscle using a mouse model of type 2 diabetes.

Methods—Study groups were young, standard chow–fed male C57BL/6J mice (lean group) and high fat diet–fed older mice (obese group). After an overnight fast, dynamic contrast-enhanced US imaging was performed on the proximal hind limb adductor muscle group for 10 minutes at baseline and again at 1 and 2 hours during administration of a hyperinsulinemic-euglycemic clamp. Dynamic contrast-enhanced US images were collected on a clinical US scanner (Acuson Sequoia 512; Siemens Healthcare, Mountain View, CA) equipped with a 15L8 linear array transducer. Dynamic contrast-enhanced US images were processed with a spatiotemporal filter to remove tissue clutter. Individual microbubbles were localized and counted to create an SR-US image. A frame-by-frame analysis of the microbubble count was generated (ie, time-microbubble count curve [TMC]) to estimate tissue perfusion and microvascular blood flow. The conventional time-intensity curve (TIC) was also generated for comparison.

Results—In vivo SR-US imaging could delineate microvascular structures in the mouse hind limb. Compared with lean animals, insulin-induced microvascular recruitment was attenuated in the obese group. The SR-US-based TMC analysis revealed differences between lean and obese animal data for select microvascular parameters ($P < .04$), which was not true for TIC-based measurements. Whereas the TMC and TIC microvascular parameters yielded similar temporal trends, there was less variance associated with the TMC-derived values.

Conclusions—Super-resolution US imaging is a new modality for measuring the microvascular properties of skeletal muscle and dysfunction from type 2 diabetes.

Keywords

diabetes; insulin resistance; microbubbles; microvascular recruitment; super-resolution ultrasound

Diabetes is a major cause of morbidity and mortality, resulting in high health care costs worldwide.^{1,2} There has been an alarming rise in the incidence of diabetes, with the number of adult diabetics globally rocketing from 108 million in 1980 to 422 million in 2014, and the bulk of the increase involves obesity-induced insulin resistance and type 2 diabetes.³ Despite recognition that weight control measures are critically important to type 2 diabetes prevention and management, long-term results from lifestyle-directed or pharmacologic interventions are generally disappointing.^{4,5} Although our understanding of the mechanisms by which obesity causes insulin resistance and type 2 diabetes has been improving,⁶ novel insights and therapeutic approaches are urgently needed to combat the diabetes that so commonly complicates obesity.

A major aspect of type 2 diabetes and obesity-induced insulin resistance is impaired insulin action in the skeletal muscle, where greater than 80% of wholebody glucose disposal normally occurs.⁷ Muscle glucose disposal is highly dependent on the actions of insulin on the endothelium in the skeletal muscle microvasculature, which normally stimulate microvascular recruitment and blood flow to enhance muscle glucose delivery. Signaling in response to insulin in endothelial cells also promotes the movement of insulin from the circulation to the underlying muscle cells.⁸ Studies in both humans and animal models indicate that attenuations in the skeletal muscle microvascular response to insulin play a critical role in type 2 diabetes and obesity-induced insulin resistance.^{9–15} However, the basis for impaired skeletal muscle microvascular function and how it contributes to type 2

diabetes remains enigmatic in various high-risk patient populations. As such, greater knowledge of the processes that regulate muscle microvascular function will increase our understanding of type 2 diabetes and lead to potential new therapeutic strategies.

To more precisely quantify microvascular function and recruitment in skeletal muscle, spatial visualization of the microvascular network is a critically important prerequisite. Considering the different medical imaging modalities that populate most hospitals worldwide, x-ray computed tomography has a blood vessel detection limit of 400 μm ,¹⁶ and magnetic resonance imaging has a limit of about 300 μm ,¹⁷ which is comparable with ultrasound (US) at clinical imaging frequencies.¹⁸ Although the use of an intravascular microbubble (MB) contrast agent during US imaging (termed dynamic contrast-enhanced ultrasound [DCE-US]) improves the detection of small blood vessels,¹⁹ it still lacks the spatial resolution necessary to differentiate vessels at the capillary level, where a bulk of the skeletal muscle microvascular recruitment manifests.²⁰ To overcome this technological limitation, our research group and others have been actively working on the development of a new modality termed super-resolution ultrasound (SR-US) imaging.^{21–27} Adapted from an optical imaging technique that won the 2014 Nobel Prize in chemistry,^{28,29} SR-US imaging allows detection of individual MB contrast agents that are only a few micrometers in size and, more importantly, located at the capillary level. Detailed microvascular images are then constructed by creating a spatial density (count) map of the MB positions after processing a sequence of US frames. Our findings presented herein reveal that SR-US imaging with MB counting has great potential for quantifying insulin-mediated microvascular changes in skeletal muscle, and the approach is an improvement over a conventional time-intensity curve (TIC)-based perfusion measurement due to reduced variance in the parameters obtained. Super-resolution US imaging with MB counting can now be applied to both preclinical work in mice, in which the processes that govern the skeletal muscle microvasculature responses to insulin can be interrogated genetically, and clinical studies of type 2 diabetes risk and pathogenesis.

Materials and Methods

Super-Resolution US Imaging

The SR-US system used for our in vivo imaging study consisted in part of a clinical US scanner (Acuson Sequoia 512; Siemens Healthcare, Mountain View, CA) equipped with a 15L8 linear transducer array and operating in a nonlinear contrast mode with a center frequency of 10 MHz. For all imaging, a low transmit power was used (mechanical index <0.2) to minimize the possibility of MB destruction. Dynamic contrast-enhanced US images were collected for 10 minutes at 15 frames per second for each US imaging sequence. All scanner settings were held constant, and US cine sequences were saved in a grayscale 8-bit format. Custom software was developed in MATLAB (The MathWorks, Natick, MA) for all image processing. There was minimal motion in this study because the site of US scanning was the fixed hind limb, which was confirmed by a cross-correlation analysis of a muscle region containing a constant scatter signal from a prior reference. Any motion artifacts were removed by excluding frames with an empirically determined cross-correlation value of less than 0.98. Spatiotemporal filtering was applied by using a singular value decomposition

method to remove the background tissue signal as well as noise.³⁰ The image stack size for singular value decomposition processing was the entire 10-minute frame sequence. The number of singular values removed after decomposition was determined by maximizing the local contrast-to-noise ratio (*CNR*)²⁷:

$$CNR = \frac{\mu_{MB} - \mu_B}{\sigma_B},$$

where μ and σ denote the mean and standard deviation of intensity values in maximum-intensity projection images, respectively, and the subscripts *MB* and *B* indicate image regions of the MBs and known background, respectively. Regions of predominantly MBs or background were chosen by comparing the maximum-intensity projection (MIP) image with the SR-US vascular map. The first several singular values, representing stationary tissue signal, were removed until the maximum local CNR was achieved. After spatiotemporal filtering, frames with potential MBs were binarized by using an intensity threshold, and the sizes of any connected regions in an image were compared with the expected point spread function to reject signals from image regions containing multiple closely spaced MBs or noise. This step was necessary, as one cannot assume that the center of the mass of signals from multiple separated unresolved MBs with a point spread function-sized volume is representative of the true MB location. The accumulated frame of all spatially isolated MB signals (ie, centroid enumerations) were overlaid on a coregistered grayscale US image for anatomic reference. A detailed diagram of the image-processing sequence is shown in Figure 1. Here we note that this approach for in vivo SR-US imaging was previously shown to resolve microvessels in skeletal muscle with diameters as small as 70 μm .²⁷

Animal Preparation and Imaging Protocol

This study used male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) fed a normal diet (D12329; Research Diets, Inc, New Brunswick, NJ) and studied at 13 to 16 weeks of age (lean group; $n = 14$) or fed a high-fat western diet (D12331; Research Diets, Inc) beginning at 5 weeks of age and studied at 24 to 31 weeks (obese group; $n = 10$). Mice were imaged by the SR-US method detailed above and a custom lipid-shelled, perfluorocarbon gas-filled MB contrast agent.^{31,32}

After a period of overnight fasting, the mice were anesthetized by isoflurane inhalation. Normal body temperature was maintained throughout the procedure by a heating pad with a rectal temperature monitor and homeothermic controller (Kent Scientific Corp, Torrington, CT). The skin overlying the proximal hind limb adductor muscle group (adductor magnus and semimembranosus) was shaved to minimize imaging artifacts. A jugular venous catheter with a 4-way connector was inserted, allowing administration of insulin, glucose, and the MB contrast agent.

After a 60-minute stabilization period following catheter placement, baseline SR-US imaging was performed both before and after a 1-minute controlled intravenous infusion of the MB contrast agent (2.5×10^7 MBs in 100 μL of saline) administered by an infusion pump. The concentration of the MB contrast agent was measured by conventional means

(Multisizer 3 Coulter counter; Beckman Coulter, Brea, CA). To maintain a consistent plane for all image sequences, the US transducer was mounted and secured over the tissue of interest. A 2 hour hyperinsulinemic-euglycemic clamp, which is used to invoke a muscle microvascular response to insulin, was applied with a continuous infusion of insulin (20 mU/kg/min) and a variable infusion of glucose to maintain a constant blood glucose level of 120 ± 5 mg/dL as determined every 5 minutes by a glucometer. Imaging sessions were also performed after 1 hour and again near the end of the 2 hour clamp. Figure 2 shows the experimental setup and time line. All animal experiments were approved by the Institutional Animal Care and Utilization Committee at the University of Texas Southwestern Medical Center.

Image Processing

A region of interest (ROI) identified for further processing was manually marked with a polygon placed to encompass the entire relevant muscle tissue. A frame-by-frame summary of MB enumeration within the ROI generated a time-microbubble count curve (TMC) to describe the temporal history of contrast agent circulation. Through careful selection of the ROI, larger superficial blood vessels were eliminated from the TMC curve, making the quantitative analysis more sensitive to microvascular changes at the capillary level. A smoothing spline curve was fit to the TMC data to minimize noise and improve the subsequent perfusion analysis. From the resulting curves, tissue perfusion parameters, such as the peak microbubble count (I_{PK}) and area under the curve (AUC), were estimated. Also estimated were the microvascular blood flow rate parameters, namely, the time to peak microbubble count (T_{PK}), wash-in rate (WIR), and wash-out rate (WOR).^{19,33} A measure of skeletal muscle microvascular density was computed as the percentage of the SR-US image area detailing microvasculature compared with the total hind limb image ROI area. Together these parameters may allow the overall assessment of skeletal microvascular function. For comparison, conventional TIC data sets were generated from the same ROIs and processing steps used during the TMC analysis.

Statistics

All experimental data were summarized as mean $\pm$ standard error and percent changes from baseline measurements when applicable. An unpaired *t*-test was used to compare the lean and obese group data when applicable. Due to a non-normal distribution of the US data, a Wilcoxon signed rank test was used to compare the absolute parametric estimates of the TMC and TIC curves derived from the lean and obese experimental group data. The coefficient of variation was computed for all parametric data, which shows the extent of variability in relation to the group mean. A repeated-measures analysis of variance test was used to analyze the 1- and 2-hour parametric measurements relative to absolute baseline values. $P < .05$ was considered statistically significant.

Results and Discussion

Dynamic contrast-enhance US imaging has evolved considerably over the last decade, and clinical procedures are slowly being accepted, as the MB contrast agents used are safe with a very low incidence of side effects. An inherent advantage of DCE-US is the opportunity to

assess the contrast enhancement patterns in real time, with a much higher temporal resolution than is possible with other imaging modalities. There are two established methods for intravascular administration of the MB contrast agent: ie, continuous infusion and a slow bolus injection followed by a saline flush. The former has been explored during preclinical DCE-US studies of insulin-induced microvascular recruitment in skeletal muscle.^{7,20} Although both MB administration approaches can be used to yield comparable information on tissue perfusion, a manual bolus injection is the more popular method of choice clinically in part because of its ease of use.^{34–36} Given this consideration and others, we used a controlled-rate bolus injection of MBs for our in vivo DCE-US imaging study.

Before the hyperinsulinemic-euglycemic clamp procedure and the SR-US study, the body weights (ages) of the lean and obese animals were 25.1 ± 0.78 g (12.8 ± 0.35 weeks) and 51.8 ± 1.07 g (27.0 ± 0.67 weeks), respectively ($P < .001$). The fasting and midclamp blood glucose levels and glucose infusion rates during the hyperinsulinemic-euglycemic clamps are summarized in Figure 3. In the obese animals, there was a higher fasting blood glucose level and a lower glucose infusion rate than in the lean mice ($P < .001$). As expected, these results indicate that the obese mice had glucose intolerance and insulin resistance. This resistance to insulin-stimulated glucose uptake is present in many patients with type 2 diabetes.³⁷

Super-resolution US is a new modality that shows subwavelength resolution (on a scale of tens of micrometers) and the ability to resolve microvascular structures in vivo at the tissue depth.^{24,25,27,38,39} Representative SR-US images from hind limb skeletal muscle in lean mice are presented in Figure 4. Both the traditional DCE-US MIP images (Figure 4, A–C) and the SR-US images (Figure 4, D–F) reveal that there was insulin-induced microvascular recruitment in the lower-limb skeletal muscle during the hyperinsulinemic-euglycemic clamp. The response to insulin is more evident in the SR-US images. Previous in vivo DCE-US imaging studies have demonstrated that insulin-mediated microvascular recruitment in skeletal muscle occurs within 5 to 10 minutes of applying a hyperinsulinemic-euglycemic clamp, and this preceded both the activation of insulin signaling in muscle and increases in muscle glucose disposal.²⁰

In Figure 5, example individual TMC (Figure 5A) and TIC curves (Figure 5B) from the same imaging session at 0, 1, and 2 hours of clamping also highlight the marked difference in the response to insulin in the lean and obese mice. Overall, there were increases in the I_{PK} of the TMC curves at 1 and 2 hours after the clamp procedure in lean animals ($78.6\% \pm 20.6\%$ and $101.2\% \pm 20.7\%$, respectively), whereas only modest increases were observed in the obese animal group ($46.8\% \pm 16.0\%$ and $56.3\% \pm 20.3\%$).

Likewise, there were 2- to 3-fold increases in the peak intensity of the TIC curves at 1 and 2 hours in the lean animals ($237.4\% \pm 86.0\%$ and $301.8\% \pm 84.8\%$) and more modest increases in the obese animals ($123.2\% \pm 59.2\%$ and $108.8\% \pm 34.5\%$, respectively). Although the timing of the imaging differed, these traditional DCE-US-based results in lean control mice were in agreement with previous findings.²⁰

Figure 6 summarizes the absolute TMC- and TIC-derived parametric perfusion measures and further highlights lean-versus-obese animal group differences during the hyperinsulinemic-euglycemic clamp procedure. The absolute SR-US-based measurements at baseline and in response to insulin at 1 and 2 hours for both lean and obese TMC-derived parameters are summarized in Table 1. The repeated-measures analysis of variance of the lean data revealed statistically significant increases for the AUC, I_{PK} , WIR, WOR, and microvasculature parameters ($P < .001$). As for that found in the lean animals, there were increases in the obese animals in the AUC, I_{PK} , WOR, and microvasculature parameters that were also statistically significant ($P < .05$). In the comparison of the lean and obese SR-US measures, there were no significant differences in any of the baseline parameters ($P > .28$). Conversely, there were significant differences between the lean and obese groups in the WIR and microvasculature at 1 hour ($P < .05$) and I_{PK} and microvasculature at 2 hours ($P < .04$). This difference was likely due to the microvascular recruitment in lean animals, which was the focus of this study. The data collected and analyzed by the more traditional DCE-US TIC-based approach for lean and obese animals are presented in Table 2. The repeated-measures analysis of variance of these data also revealed statistically significant increases for the AUC, I_{PK} , WIR, and WOR parameters ($P < .05$). Here, significant increases were found in the obese animals after 2 hours for the AUC, I_{PK} , WIR, and WOR parameters ($P < .04$). Despite noticeable changes, it is important to note that there were no statistically significant differences between the lean and obese DCE-US-based parametric measurements at either baseline or 1 or 2 hours ($P > .07$). The online supplemental material depicts the SR-US parametric data after normalizing by baseline measurements, which helps visualize these temporal changes in the experimental group data.

The coefficients of variation of the SR-US TMC-derived parametric measurements ($42.5\% \pm 11.3\%$; range, $22.1\%–67.9\%$) were considerably less than those of the TIC-derived parameters ($88.0\% \pm 33.8\%$; range, $22.1\%–165.5\%$). These results help explain in part how statistically significant differences between lean and obese groups were only found with the SR-US imaging measurements. By reducing measurement variability, this led to parametric estimates from skeletal muscle microvasculature that showed statistically significant differences for the SR-US imaging measurements. This observation suggests that future preclinical studies incorporating SR-US imaging of skeletal muscle microvasculature may require a smaller sample size than those same experiments conducted using traditional DCE-US imaging techniques. Furthermore, a reduction in measurement variability will help in clinical and translational studies. It is also important to highlight that the SR-US-derived TMC is based on a quantitative enumeration of individual MB contrast agents flowing in the microvasculature and based on their detection above a filtered background signal but not their relative intensity. The spatiotemporal filtering of the background signal in the TMC-based method removes sources of variation and thus reduces variability. Although closely spaced MBs may impede an ability to localize the individual agents and compromise the absolute MB count, this represents a completely new approach for the study of skeletal muscle tissue perfusion and is unique to SR-US imaging.²⁷ In comparison, traditional DCE-US measurements are based on image intensity values, which are known to be more affected by several US scanner-level settings (eg, nonlinear imaging mode and dynamic range)²⁴ and properties of the MB contrast agent used (eg, size and concentration).⁴⁰

A potential limitation of this study was the use of anesthetized animals, which was required to obtain stable images. However, US imaging of conscious animals for extended periods (ie, 10 minutes) is not feasible because of the inevitable artifacts that would occur due to animal movement. Although the absolute parametric estimates of microvascular perfusion may have been altered from what would be seen in conscious animals, the relative changes and group comparisons remain valid, since experimental conditions were matched. Future work could use SR-US imaging to study the impact of pharmacologic interventions. Although no motion artifacts were identified from the cross-correlation analysis of the DCE-US frame sequences before SR-US image formation, a more detailed motion detection and compensation approach might improve our findings. Although a comparison with a point spread function estimate was capable of rejecting MB clusters during the automated MB localization step, this simplistic approach may not produce the most accurate result. Any MB miscount will compromise both the spatial resolution of the SR-US images and any TMC-based quantification. To that end, a more complex strategy, such as a true point spread function deconvolution, might improve this critical image-processing step. Development of a 3-dimensional SR-US imaging system and method may also help sensitize this new modality to microvascular changes at the capillary level due to quantification in the volume space (ie, increased tissue sample size), thereby improving the capacity to detect microvascular dysfunction that contributes to type 2 diabetes pathogenesis.

In conclusion, the findings reported here demonstrate that an analysis of skeletal muscle perfusion with SR-US imaging readily reveals microvascular recruitment in response to insulin in the skeletal muscle of young, lean mice. The recruitment of skeletal muscle capillaries promotes insulin and glucose delivery to muscle, thus augmenting muscle glucose uptake. Super-resolution US-based measurements also revealed impaired microvascular responses to insulin in older obese mice. Overall, SR-US imaging represents a promising new imaging modality for characterizing microvascular function in the skeletal muscle and its contribution to metabolic health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AUC	area under the curve
DCE-US	dynamic contrast-enhanced ultrasound
I_{PK}	peak microbubble count
MB	microbubble
ROI	region of interest

SR-US	super-resolution ultrasound
TIC	time-intensity curve
TMC	timemicrobubble count curve
T_{PK}	time to peak microbubble count
WIR	wash-in rate
WOR	wash-out rate

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Figure 1.

Image-processing flowchart detailing the procedure for creating a density map of MB contrast agent locations, termed an SR-US image, from a length n temporal sequence of DCE-US images. The color map for the SR-US image denotes the MB count.

Figure 2. Illustration of the experimental setup for the hyperinsulinemic-euglycemic clamp procedure, with the time line showing administration of insulin and glucose, as well as US imaging points.

Figure 3.

Summary of the age (A), body weight (B), fasting blood glucose level (C), average blood glucose level during the hyperinsulinemic-euglycemic clamp (D) and glucose infusion rate (GIR) used during the hyperinsulinemic-euglycemic clamp procedure (E). Note the lower glucose infusion rate for the obese animals indicates insulin resistance. * $P < .05$ between bar graph data.

Figure 4.

In vivo SR-US imaging of insulin-induced skeletal muscle microvascular recruitment in the mouse hind limb. Results depict SR-US images collected from the same animal and image plane at baseline (**A**) and 1 hour (**B**) and 2 hours (**C**) after insulin/glucose infusion, followed by images based on a maximum-intensity projection (MIP) at baseline (**D**) and 1 hour (**E**) and 2 hours (**F**) after insulin/glucose infusion. Scale bars denote 1 mm. Note the clear microvascular network in the SR-US images (**A–C**) that is obscured in the traditional DCE-US–based maximum-intensity projection (MIP) images (**D–F**). Also note the increases in image intensity with insulin compared to baseline (**B** and **C** compared with **A** and **E** and **F** compared with **D**, respectively).

Figure 5.

In **A**, representative TMCs reveal an increase in perfusion (I_{PK}) in response to insulin during the hyperinsulinemic-euglycemic clamp in the lean animal (left), which was negligible in the obese animal (right). In **B**, TICs reveal a similar obvious increase in perfusion (peak intensity) in response to the clamp in the lean animal and a blunted response in the obese animal.

Figure 6.

Summary of skeletal muscle parametric measurements characterizing the insulin-induced microvascular response from SR-US– derived TMC data acquired in lean and obese animals (A–F) and parametric measurements derived from the traditional DCE-US–derived TIC data (G–K). Bar graphs detail the mean parametric values at baseline and 1 and 2 hours after infusion of insulin/glucose. * $\dagger P < .05$ versus baseline and lean data, respectively.

Table 1.

Summary of TMC-Derived Parameters for Lean and Obese Animals

Parameter	Lean Animals			Obese Animals		
	Baseline	1 h	2 h	Baseline	1 h	2 h
AUC, AU	453.7 ± 55	759.9 ± 89.1	861.3 ± 85.6	450.8 ± 58.8	699.0 ± 95.3	701.5 ± 118.2
I _{PK}	45.1 ± 4.9	73.7 ± 7.9	82.4 ± 7.5	42.7 ± 3.7	64.1 ± 8.9	66.0 ± 9.0
T _{PK} , s	54.5 ± 4.9	50.9 ± 4.0	48.2 ± 3.0	50.1 ± 4.4	64.9 ± 5.6	47.1 ± 3.5
WIR, AU/s	0.8 ± 0.1	1.3 ± 0.2	1.6 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	1.3 ± 0.2
WOR, AU/s	0.08 ± 0.01	0.12 ± 0.02	0.13 ± 0.01	0.06 ± 0.01	0.10 ± 0.02	0.10 ± 0.02
Microvasculature, %	16.4 ± 2.4	29.0 ± 3.4	34.2 ± 3.7	13.6 ± 1.0	20.5 ± 4.2	21.9 ± 3.3

Data are summarized as mean ± SE. AU indicates arbitrary units.

Table 2.

Summary of TIC-Derived Parameters for Lean and Obese Animals

Parameter	Lean Animals			Obese Animals		
	Baseline	1 h	2 h	Baseline	1 h	2 h
AUC, AU	30.2 ± 10.9	52.1 ± 8.8	65.8 ± 12.5	40.9 ± 16.1	74.1 ± 25.3	67.5 ± 21.1
I _{PK} , AU	4.5 ± 1.4	9.4 ± 1.6	11.6 ± 1.9	5.8 ± 2.1	10.3 ± 3.2	10.4 ± 2.9
T _{PK} , s	48.5 ± 2.7	48.3 ± 3.7	46.8 ± 2.9	46.3 ± 2.8	51.6 ± 3.9	56.9 ± 11.3
WIR, AU/s	0.10 ± 0.3	0.24 ± 0.05	0.3 ± 0.05	0.13 ± 0.06	0.25 ± 0.08	0.26 ± 0.08
WOR, AU/s	0.01 ± 0.001	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.002	0.02 ± 0.01	0.03 ± 0.01

Data are summarized as mean ± SE. AU indicates arbitrary units.