Contrast-enhanced ultrasound measurement of microvascular perfusion relevant to nutrient and hormone delivery in skeletal muscle: A model study in vitro

Renee M. Ross*, Kathleen Downey, John M.B. Newman, Stephen M. Richards, Michael G. Clark, Stephen Rattigan

Menzies Research Institute, University of Tasmania, Hobart, 7001, Australia

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Abstract

Contrast-enhanced ultrasound (CEU) has been used to measure muscle microvascular perfusion in vivo in response to exercise and insulin. In the present study we address whether CEU measurement of capillary volume is influenced by bulk flow and if measured capillary filling rate allows discrimination of different flow pattern changes within muscle. Three in vitro models were used: (i) bulk flow rate was varied within a single length of capillary tubing; (ii) at constant bulk flow, capillary volume was increased 3-fold by joining lengths of capillary in series, and compared to a single length; and (iii) at constant bulk flow, capillary volume was increased by sharing flow between a number of lengths of identical capillaries in parallel. The contrast medium for CEU was gas-filled albumin microbubbles. Pulsing interval (time) versus acoustic–intensity curves were constructed and from these, capillary volume and capillary filling rate were calculated. CEU estimates of capillary volume were not affected by changes in bulk flow. Furthermore, as CEU estimates of capillary volume increased, measures of capillary filling rate decreased, regardless of whether capillaries were connected in series or parallel. Therefore, CEU can detect a change in filling rate of the microvascular volume under measurement, but it cannot be used to discriminate between different flow patterns within muscle that might account for capillary recruitment in vivo.

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Introduction

Microvascular delivery of nutrients and hormones to skeletal muscle involving changes in microvascular perfusion may be a key regulatory step in insulin action. Impairments associated with vascular dysfunction can contribute to the metabolic syndrome and insulin resistance. Recently, using an ultrasound imaging technique, we reported that insulin action and muscle contraction each resulted in a marked increase in microvascular perfusion (capillary recruitment) of hind leg muscles of the rat (Dawson et al., 2002; Rattigan et al., 1997) and of human forearm (Coggins et al., 2001). We also observed that insulin’s control of this process was impaired in insulin resistant states (Vincent et al., 2006; Wallis et al., 2002). The imaging technique is based on contrast-enhanced ultrasound (CEU) where gas-filled microbubbles served as the contrast medium. The microbubbles are infused intravenously to reach a steady-state arterial concentration in the blood and the ultrasound beam is focused on a region of interest with parameters set to both destroy the microbubbles and capture the echoed signal. From these it is then possible to measure both muscle microvascular volume and microvascular filling rate in the region of interest. However, there are two important aspects in the application of CEU for the measurement of capillary recruitment in muscle. First, it is essential that the signal is not influenced by the increase in bulk flow associated with muscle contraction (Shoemaker and Hughson, 1999) or the increase in bulk flow that occurs during hyperinsulinemic clamps (Yki-Jarvinen and

* Corresponding author. Menzies Research Institute, University of Tasmania, Private Bag 58, Hobart, 7001, Australia. Fax: +61 3 6226 2703.
E-mail address: rmross@utas.edu.au (R.M. Ross).

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Second, there is the issue of whether CEU has the potential to discriminate between increased microvascular perfusion resulting from either flow redistribution from short (possibly non-nutritive) to tortuous (nutritive) capillaries, where capillary blood flow rate would not be expected to change and/or by flow sharing into capillaries of similar properties, where capillary blood flow rate might be expected to decrease. These studies can not be conducted in vivo and only one previous modeling study of this kind has been reported (Wei et al., 1998) where bulk flow was shown to correlate well with measured filling rate, but capillary volume was not determined. Accordingly, in the present study, CEU is used with constructed capillary tubing models in vitro to assess the effect of bulk flow and its potential for discriminating between different flow patterns within muscle that might explain capillary recruitment reported in vivo (Dawson et al., 2002; Rattigan et al., 1997; Vincent et al., 2006).

Methods

Capillary models

In preliminary experiments a number of different commercially available capillary tubing were tested. Wall thickness was found to contribute significantly to the reflected ultrasound signal and thus masked the signal from the microbubbles. Accordingly, we chose to use thin-walled microdialysis tubing (Ultrafiltration Probes, Catalogue No. MF-7023/UF-3-12, BAS Bioanalytical Systems, Inc., West Lafayette, IN, USA). This had minimal reflectance and proved to be suitable for construction of the models. Each ultrafiltration probe consists of three 14-mm loops of capillary tubing (320 μm external diameter and 280 μm internal diameter) with the six ends inserted into a single larger non-permeable length of conducting tubing. For the construction of the single tube model 60 mm of one of the capillary loops was cut from the probe and one end inserted into 23-gauge stainless steel needle (10 mm in length, that had been blunted and removed from a hypodermic needle adapter). This was in turn joined to a length of non-permeable conducting tubing (PE 20, Becton Dickinson, Parsippany, NJ, USA), by a short sleeve (1.5 cm) of PE 50. All junctions...
Capillary tubing filling rate constant ($QLAB$ images a pulsing interval (time) versus acoustic intensity and after background subtraction using continuous ultrasound intensity in decibels within the region of interest was converted to pulses (Fig. 3). Data were transferred to an offline computer and analyzed using progressively greater replenishment of the ultrasound beam between destructive at pulse intervals of 0.2, 0.3, 0.5, 1, 2, 3, 5, 8, 12 and 15 s, thus allowing destroy all microbubbles in the region of interest. Once the microbubble rotation and the delivery line from the syringe vibrated. CEU measurements were suspension of the microbubbles the syringe and pump were continuously mixed by solution. Delivery of the microbubbles was by syringe pump. To maintain an even isotonic saline (previously gassed with perfluorocarbon) to make the infusion with perfluorocarbon). One milliliter of this solution was then diluted with 4 ml of suspension which was then diluted with 4.5 ml of isotonic saline (previously gassed 5 ml plastic syringe was used to draw up approx. 0.5 ml of the microbubble suspension which was then diluted with 4.5 ml of isotonic saline (previously gassed with perfluorocarbon). One milliliter of this solution was then diluted with 4 ml of isotonic saline (previously gassed with perfluorocarbon) to make the infusion solution. Delivery of the microbubbles was by syringe pump. To maintain an even suspension of the microbubbles the syringe and pump were continuously mixed by rotation and the delivery line from the syringe vibrated. CEU measurements were made using an HDI 5000 (Philips Medical Systems, Australasia), in harmonic imaging mode with 7–4 linear MHz transducer at a mechanical index of 1.0 to destroy all microbubbles in the region of interest. Once the microbubble concentration reached steady state in the field of interest, images were captured at pulse intervals of 0.2, 0.3, 0.5, 1, 2, 3, 5, 8, 12 and 15 s, thus allowing progressively greater replenishment of the ultrasound beam between destructive pulses (Fig. 3). Data were transferred to an offline computer and analyzed using QLAB™ software (Version 2.0, Philips Ultrasound, Bothwell, USA). The ultrasound intensity in decibels within the region of interest was converted to acoustic intensity and after background subtraction using continuous ultrasound images a pulsing interval (time) versus acoustic–intensity curve was plotted (see typical trace, Fig. 3) to allow calculation of capillary tubing volume ($A$) as well as capillary tubing filling rate constant ($\beta$) according to the equation $y = A(1 - e^{-\beta t})$. Capillary tubing filling rate is the product of $A \times \beta$.

**Experimental design**

Initial experiments focused on establishing a concentration curve for the microbubbles to be used by plotting acoustic intensity at a pulsing interval of 8 s as a function of microbubble concentration. The concentration infused was varied by diluting the commercial Optison™ suspension at 1:10; 1:20; 1:40; 1:60; 1:80; 1:100; and 1:120 into perfluorocarbon-gassed 0.9% saline (Fig. 4), where 1:10 dilution corresponded to ~2400 microbubbles $\mu l^{-1}$. A dilution of 1:50 was used for the three different experiments described below.

**Variable bulk flow rate**

The flow rate of delivery of a microbubble suspension (1:50 dilution) was varied using a single length of capillary tubing (Model 1, Fig. 2). The capillary tubing was positioned under the L7-4 transducer along the longer axis. Parameters on the CEU were set as so as to obtain a clear image on screen of the capillary tubing (depth=4.1 cm) and the CEU set on continuous imaging (13 Hz) to capture background images. The initial flow rate was 20 $\mu l$ min$^{-1}$ and an image set consisting of 5 images, captured at each intermittent pulsing interval (see above) was collected. The flow rate was then increased to 40 and finally to 80 $\mu l$ min$^{-1}$ and the above procedure repeated for each rate. In subsequent replicate experiments the order of flow rate change was randomized. The image set at each flow rate was transferred to a network computer for analysis in QLAB™ (Philips Medical Systems), construction of pulsing interval curves and the calculation of capillary volume and filling rate.

**Flow pattern change from a single short capillary tube to a long capillary tube crossing the field of measurement three times**

In essence, the capillary surface area or volume was increased by connecting three 60-mm lengths of capillary tubing ‘in series’ in a zigzag pattern (Model 2, Fig. 2). Thus the model comprised a longer length of capillary tubing that crossed the field of measurement three times (by doubling back on itself twice) contained within the same region of interest. All three lengths of tubing were arranged so as to pass under the transducer along the longer axis. They were one above the other, with the entry point nearest the transducer and the exit point most distant from the transducer and the spacing between each length was approximately 4 mm. A single 60-mm capillary tubing, separate to the tubing connected in series, with its own inflow and outflow tube, was positioned in the same region of interest to allow direct comparison with the longer in series model (Model 2, Fig. 2). The flow rate was constant at 80 $\mu l$ min$^{-1}$ and image sets were captured as described above in a randomized manner between the single short tube and longer ‘in series’ arrangements.

**Flow pattern change involving the sharing of flow from a single capillary to multiple similar capillaries**

Capillary surface area or volume was increased by connecting lengths of capillary tubing to a manifold so that flow sharing could be manipulated from one to four capillaries. The manifold of four identical 60-mm capillaries was arranged horizontally under the transducer with each length ‘in parallel’ and positioned one above the other along the longer axis of the transducer (Model 3, Fig. 2). The

![Fig. 3. A typical pulsing interval curve. Microbubbles were infused at a distal site to achieve steady-state concentration at time=zero. High mechanical index sound was used to rupture the microbubbles which refill the capillary tubing volume in the field of measurement as the interval between progressive destructions increased. The equation describes the shape of the curve, where $A=$ capillary volume and $\beta=$ filling rate constant.](image)

![Fig. 4. Plot of acoustic intensity as a function of microbubble (MB) concentration. Increasing dilutions of Optison™ microbubbles were infused into a single capillary and the acoustic intensity at a pulsing interval of 8 s recorded. The lowest dilution was 1:10 (~2400 microbubbles $\mu l^{-1}$) and the acoustic intensity signal was saturated at a dilution of 1:40. Values are means±SE.](image)
spacing between each length was approximately 4 mm. Initially flow was confined to only one capillary. The image sets were captured as described above. Blockade of the second capillary tube was then removed and flow allowed to be shared between two capillaries and a second set of images captured. Flow was then allowed to pass through three then four capillaries and at each the image sets were captured and pulsing interval curves constructed for the determination of capillary volume and filling rate. Flow was maintained at 80 μl min⁻¹ throughout and the pattern was in the field of interest as shown in Fig. 2.

Statistics

A one-way repeated measures ANOVA was used to determine differences between groups in the variable VDR model and the flow sharing model. A paired t-test was used to determine differences between the short versus long capillary tubing model. Significance was accepted at a level of P<0.05. All tests were performed using SigmaStat software (Systat Software Inc., California, USA).

Results

Effect of flow rate on capillary volume and filling rate

The presence of microbubbles in aqueous suspension can be detected by their characteristic property to oscillate in high-frequency sound and reflect back an intense signal. Providing
the microbubbles are thoroughly mixed and delivered uniformly then any change in volume through which the microbubbles are moving will be reflected by a change in acoustic signal. The volume occupied by the microbubbles is determined by using a procedure that initially completely destroys the microbubbles in the region of interest and then progressively monitors their refill by varying the pulsing interval between successive pulses of ultrasound. This approach also allows measurement of the rate of refill by the microbubbles as they replenish the capillary tubing volume in the designated field of interest. The microbubble flow rate is reflected by the product of $A$ (the plateau acoustic intensity that represents the volume) and $\beta$ (the rate constant), which is the slope of the pulsing interval curve versus acoustic intensity at $t=0$. Units for $A$ are arbitrary and values are dependent on the individual microbubble preparation and the settings used to capture the image.

**Models of capillary recruitment**

Two approaches were used to simulate capillary recruitment. In the first, capillary surface area or volume was increased by switching flow from a short single pass capillary tubing to a long tortuous capillary that crossed the field of interest three times. Fig. 6 shows data from the pulsing interval curves measured from the two patterns and a representative ultrasound image of this model. Bulk flow was held constant at 80 $\mu$l min$^{-1}$ for each and the data were collected as acoustic intensity and plotted as a function of the pulsing interval. Values for $A$ and $\beta$ were calculated as above and are shown. As can be seen from Fig. 6, the calculated volume ($A$) of the capillary that passed three times across the field of interest was approximately 3-fold that of the capillary that passed only once. The calculated filling rate constant ($\beta$ value) decreased with the switching of flow from the short to the long tortuous capillary tube. As a consequence, the product ($\beta \times A$) was unchanged, reflecting the constant flow rate.

In the second model intended to simulate capillary recruitment, flow was shared progressively from one to four capillaries. Fig. 7 shows data from the pulsing interval curves measured for the situations where flow was carried by one, two, three or four capillaries. A representative ultrasound image of this model is also shown in Fig. 7. Bulk flow was constant at 80 $\mu$l min$^{-1}$ throughout and the data were collected as acoustic intensity and plotted as a function of the pulsing interval. Values for $A$ and $\beta$ were calculated and are shown. As can be seen from Fig. 7, the calculated volume ($A$) increased as the flow was shared progressively between one and four capillaries. This was accompanied by a decrease in filling rate constant ($\beta$ value), essentially in proportion to the expected effect of sharing the...
flow. Again the constant flow rate is confirmed by the unaltered flow rate \((β \times A)\).

**Discussion**

In the present study we have used contrast-enhanced ultrasound with the intention of capturing an image of the fluid volume of capillary tubing models *in vitro* as well as to compute the flow rate of filling. To achieve these objectives we have used a gas-encapsulated microbubble suspension as a contrast agent that *in vivo* is retained in the vasculature (Forsberg et al., 1997). Microbubbles are the most widely used contrast agents in myocardial studies, where the technique was developed (Kaul, 1997). Microbubbles have a number of properties that makes them ideal for use as flow tracers. Some of these properties include rheological properties that are similar to blood in both large vessels and the microcirculation (Lindner et al., 2002). In addition, the contrast agent is stable, mixes uniformly and rapidly, is hemodynamically inert, shows no extravasation (Lindner and Kaul, 1995; Lindner and Wei, 2002), and is small enough to fit the physical dimensions of the microcirculation (Lindner and Kaul, 1995; Schutt et al., 2003). Moreover, an added feature of the microbubbles is that they are compressible. The compressibility of the encapsulated gas is five times that of tissue or water (Chomas et al., 2001). The average microbubble size is smaller than the ultrasound wave produced and this allows microbubbles to undergo volumetric oscillations within the generated acoustic field (Blomley et al., 2001). Thus, during pressure peaks the microbubbles can be compressed, while under low pressure they can expand (Blomley et al., 2001). These oscillations cause backscattering of the acoustic signal and the microbubbles themselves become the source of an acoustic signal. If the incident ultrasound signal is sufficiently strong (i.e., set at a high mechanical index), the microbubbles will burst and produce a strong acoustic signal. Consequently, when the ultrasound wave passes through the area where the microbubbles are flowing, such as the capillary tubing models herein, the acoustic signal given off by the microbubbles can be captured by the ultrasound machine and the area of interest can be assayed for strength of signal using computational methods. The basis of the computational method is the pulsing interval curve (Fig. 3) (Wei et al., 1998), where intermittent bursts of high mechanical index ultrasound are used to destroy the microbubbles within the region of the beam. By increasing the time interval between successive bursts and allowing replenishment, it is possible to compute the vessel volume and rate of refill. The ultrasound signal obtained from destruction of the microbubbles is in pixels of certain brightness (Kaul, 1997). The acoustic intensity is calculated from the arithmetic mean of the pixel intensity. At low concentrations a linear relationship between the microbubbles and acoustic intensity exists, and this relationship plateaus as the concentration is increased (Wei et al., 1998). The extent of perfusion (capillaries recruited) can be evaluated using quantification software which calculates the acoustic intensity for user-defined regions of interest on the image provided the dose of microbubbles is within the linear portion of the relationship between microbubble concentration and acoustic intensity (Wei et al., 1998).

The principal finding emerging from this study is that the CEU/microbubble measured microvascular volume, when conducted under controlled conditions using a capillary tubing model *in vitro*, was not influenced by an increase in bulk flow. Thus a four-fold increase in flow gave identical measures of microvascular volume. The implications of this are that when applied *in vivo*, the increase in microvascular volume (capillary recruitment) as has been reported for muscle contraction (Dawson et al., 2002) and for insulin action (Coggins et al., 2001; Rattigan et al., 1997) could not have been influenced by the accompanying increase in bulk flow. Independence has been assumed but not directly shown previously. Thus the CEU/microbubble technique contrasts with laser Doppler flowmetry where the signal predominantly reflects non-vectorial motion rather than particle number (Clark et al., 2000) and measurements of capillary recruitment by laser Doppler flowmetry can only be made when bulk blood flow does not change.

The second major finding from this study was that the CEU/microbubble method was not able to discriminate between the two flow pattern changes within muscle thought most likely to occur as a consequence of capillary recruitment *in vivo*. The first of these flow pattern changes is particularly relevant to insulin where it has been proposed that insulin action on the muscle microvasculature involves a combination of vasodilatation and vasconstriction possibly involving nitric oxide and endothelin (Erina et al., 2004), respectively. Such a combined action is proposed by us to result in blood flow being switched from relatively short low-resistance, high-capacitance non-nutritive vessels to longer tortuous nutritive vessels (Clark et al., 2003). In this scenario active vasoconstriction, if it occurs, is proposed to restrict flow into the non-nutritive and vasodilatation to access the nutritive route (Clark et al., 2003). Were this to be the consequence of insulin action then mean red cell velocity in the capillaries would not be expected to change. The second flow pattern change that may also apply to insulin action involves recruitment of similar previously unperfused capillaries by flow sharing. In this case because of flow sharing the mean red cell velocity would be expected to decrease. For the two flow pattern changes in this study, one involving flow switching from short linear to long tortuous capillary and the second involving flow sharing, each showed the expected increase in volume \((A)\) value hence capillary surface area. However, both models showed a decrease in refill rate constant \((β)\) to match the increased volume and there was no change in the filling flow rate \((β \times A)\). Thus the CEU/microbubble technique cannot deduce the type of flow pattern that occurs *in vivo* as it measures volume filling rate and not red cell velocity.

There are four studies, three involving human forearm (Clerk et al., 2006; Coggins et al., 2001; Vincent et al., 2006) and one on rat hindlimb muscle (Dawson et al., 2002) in which CEU/microbubbles have been used *in vivo* to determine changes in capillary recruitment. For the rat, the stimuli that gave rise to capillary recruitment were muscle contraction and insulin. Because the contraction stimulus was quite intense there were large increases in both limb blood flow and the microvascular
In the insulin study in rats (Dawson et al., 2002), two doses of insulin were used and although there was an increase in limb blood flow and capillary recruitment for each dose the β value did not change. Such an outcome would occur whether capillary recruitment has arisen from flow sharing or flow redistribution from short to long tortuous capillaries. Similarly, in the human forearm studies of normal healthy individuals (Coggins et al., 2001) a physiologic dose of insulin increased A value by 54% had no change on forearm blood flow but decreased β value by 42%. This outcome is again as expected and does not allow discrimination between the two possibilities. Similarly, a more recent study (Clerk et al., 2006) comparing insulin response in lean and obese individuals shows for the lean subjects that insulin increased brachial artery blood flow, A value, and tended to decrease β value, although the latter was not significant. Interestingly, at low-intensity handgrip exercise brachial artery blood flow and A value each increased but β value again tended to decrease (Vincent et al., 2006).

In summary, CEU measures changes in capillary recruitment and is not affected by flow. Although CEU can detect changes in both capillary volume and filling rate, the two are related inversely and thus CEU is unable to discriminate between capillary recruitment resulting from either flow redistribution from short to tortuous capillaries and/or by flow sharing into capillaries of similar properties.

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