Muscle Interstitial Calcium During Head-Up Tilt in Humans

Soraya Samii, MD, PhD; Mazhar H. Khan, MD; David A. MacLean, PhD; Nicholas King, BSc; Michael D. Herr, PhD; Lawrence I. Sinoway, MD

Background—During head-up tilt (HUT), peripheral vasoconstriction occurs. This response requires appropriate communication between the sympathetic nerve terminal and vascular smooth muscle cell in the neurovascular space. Both of these cell types require extracellular calcium ([Ca\(^{2+}\)]\text{o}) for proper activation and function. We hypothesize that [Ca\(^{2+}\)]\text{o} increases with tilt and in the process contributes to vasoconstriction.

Methods and Results—We used microdialysis techniques in the lower-limb skeletal muscle to measure [Ca\(^{2+}\)]\text{o} changes in this space with HUT. [Ca\(^{2+}\)]\text{o} was measured in 10 healthy subjects during HUT. We found a 62% increase in the dialysate [Ca\(^{2+}\)]\text{o} (0.223±0.018 to 0.353±0.028 mmol/L) with HUT.

Conclusions—This result implies a significant increase in [Ca\(^{2+}\)]\text{o} in the neurovascular space during HUT. This represents the first report of such in situ [Ca\(^{2+}\)]\text{o} measurements in humans. This rise in [Ca\(^{2+}\)]\text{o} may provide a mechanism for proper cell-cell interaction, helping to promote peripheral vasoconstriction during HUT. How this [Ca\(^{2+}\)]\text{o} transient affects the nerve terminal, vascular smooth muscle cells, or both remains to be determined. (Circulation, 2004;109:215-219.)

Key Words: calcium • muscle, smooth • vasoconstriction • vessels

The normal physiological response to the standing position includes an increased heart rate (HR) and peripheral vascular resistance. The peripheral vascular changes help preserve blood pressure and cerebral and cardiac perfusion in the upright position. The sympathetic nervous system contributes to this response. Because striated muscle makes up ≈40% of total body mass, muscle sympathetic nerve activity plays a significant role in signaling this appropriate response. When proper peripheral vasoconstriction does not take place in response to upright posture, subjects suffer from hypotension that often leads to syncopal or presyncopal symptoms. Orthostatic intolerance is common in patients with autonomic failure, but subjects with an intact sympathetic nervous system can also have these symptoms. In fact, neurally mediated syncope is the most common cause of syncope in adults.\(^1\)

In subjects with orthostatic intolerance, there is altered signaling involving muscle sympathetic nerve activity and the microvasculature. The end effect is that the vasconstrictor response is diminished\(^2,3\); however, the specific changes in this signaling have not been completely elucidated. Both neurons and vascular smooth muscle cells (VSMCs) are dependent on extracellular calcium for their signaling. Thus, altered sympathetic nerve and vascular responses may be due to changes in the extracellular calcium concentration ([Ca\(^{2+}\)]\text{o}). We hypothesize that [Ca\(^{2+}\)]\text{o} is an important factor in this sympathetic-mediated reflex and that changes in [Ca\(^{2+}\)]\text{o} dynamics (in this space) may result in orthostatic intolerance.

Specifically, it is well known that the sympathetic nerve terminal requires influx of calcium from the extracellular space in order for norepinephrine (NE) to be released.\(^4,5\) Once calcium enters the cytosol, it binds and activates presynaptic proteins such as synaptotagmins. These activated calcium-dependent proteins trigger neurotransmitter exocytosis.\(^6\) This process cannot take place unless there is sufficient [Ca\(^{2+}\)]\text{o}, available to enter through the activated channels.

Similarly, VSMCs require extracellular calcium for contraction.\(^7\) Vessels dilate when incubated in Ca\(^{2+}\)-free media. Harder\(^7\) showed that in cerebral arteries, contractility increased with increasing levels of [Ca\(^{2+}\)]\text{i}. Multiple mechanisms have been proposed to explain the relationship between [Ca\(^{2+}\)]\text{i}, and intracellular events that lead to smooth muscle contraction. Perhaps the most commonly mentioned mechanism suggests that calcium influx is mediated via voltage-gated calcium channels (VGCCs).\(^8\) VSMC contraction is not completely abolished in the presence of calcium channel blockers. Additionally, there are a variety of receptors other than VGCCs that have been identified in VSMC plasma membranes, such as calcium-sensing receptors, receptor-operated channels,\(^10\) and stretch-activated channels.\(^11\) These different channel types all require [Ca\(^{2+}\)]\text{o}, for proper function, either for binding (such as in calcium-sensing receptors) or for proper current through the opened channel (VGCC, receptor-operated channel, and stretch-activated channel).
Thus, [Ca\(^{2+}\)], may be very important in evoking both sympathetic and vascular constrictor responses. In our laboratory, we have developed a novel technique to examine the dynamics of this extracellular space to help us better understand signaling mechanisms. Using microdialysis methods in the lower-limb skeletal muscle, we are able to measure calcium in the dialysate to determine whether [Ca\(^{2+}\)] changes with head-up tilt (HUT). In this report, we examined calcium dynamics in the extracellular space during HUT to determine whether this peripheral regulation involves changes in [Ca\(^{2+}\)].

**Methods**

We studied a total of 10 healthy subjects (8 men and 2 women) with a mean age of 25±3 years. We performed microdialysis data acquisition on all subjects. All volunteers were nonsmokers and in good health as assessed by a prestudy history and physical. All subjects provided an informed consent approved by the Institutional Review Board of The Milton S. Hershey Medical Center. No subjects had a history of orthostatic intolerance or syncope.

**Experimental Paradigm**

Subjects reported to the General Clinical Research Center in the morning after an overnight fast. They were instructed to refrain from caffeine intake for 24 hours before the experiment. Continuous measures of HR, ECG, arterial blood pressure (finger plethysmograph, Finapres: Ohmeda) were collected at baseline, during instrumentation of microdialysis probes (see below), and with tilt and recovery. After microdialysis probes were placed, continuous measurements of [Ca\(^{2+}\)] changes were collected. After 20 minutes of baseline data collection, HUT was performed to 60° for 20 minutes, and data collection continued for 20 minutes into recovery in the supine position.

**Tilt Protocol**

Subjects were passively tilted to 60° HUT. Subjects supported their own weight on the right leg. The left leg remained flexed and was used for microdialysis data collection. After 20 minutes in the HUT position, subjects were returned to the supine position, and data collection continued for 20 minutes.

**Microdialysis**

The microdialysis probes were inserted in the vastus lateralis muscle of the subject’s left leg. This muscle has no important nerves or vessels passing through it and has low risk of neurovascular damage. An area measuring ∼25×20 cm over the subject’s thigh was shaved, prepared with povidone-iodine solution, and then draped with sterile sheets. A skin marker was used to mark the probe insertion and exit sites. The skin and subcutaneous sites at the probe entry and exit sites were anesthetized locally with lidocaine (0.5 to 1.0 mL) by a strict aseptic technique. Four to 6 probes were placed 2 to 3 cm apart with the data from each subject, the leading edge midpoint [Ca\(^{2+}\)] peak during HUT was readily identified and used as the timing marker. This marker was subsequently used to time shift the waveform to align its peak midpoint time to the nominal HUT starting time of 20 minutes. After this synchronization procedure, waveforms from all subjects were averaged into a composite waveform (Figure 2A).

**Microdialysis Probes**

The microdialysis probes were constructed from semipermeable fibers (GFS Plus 12, Gambro) with a molecular mass cutoff of 3000 kDa (0.20 mm inner diameter, 0.22 mm outer diameter). Briefly, each end of the single fiber was inserted ∼1 cm into a hollow polyamide tube (0.25 mm inner diameter, 0.36 mm outer diameter) and glued. The actual diffusible portion was 4 cm. A complete description of microdialysis probe construction has been published previously.\(^{12}\)

**Extracellular Calcium Measurements**

All microdialysis probes were connected in-line to calcium electrodes (Microelectrodes, Inc), and data were recorded from these at 0.2 Hz. Before each experiment, the microelectrodes were calibrated by a 2-point method with solutions containing 1.125 and 2.25 mmol/L of CaCl\(_2\). Each microdialysis probe was perfused at a rate of 5 μL/min with a normal saline solution. Probes were equilibrated for 1 hour after the last probe was inserted.

**Extracellular Sodium Measurements**

In 3 other subjects, microdialysis probes were connected in-line to sodium electrodes (Microelectrodes, Inc) and data were recorded every minute. Each microdialysis probe was perfused at a rate of 5 μL/min with a normal saline solution.

**Fluid Dynamics Measurements**

In 3 of the 10 original subjects, additional measurements of tritiated NE, dialysate volume, and lactate were performed. In these 3 subjects, NE-H3 (PerkinElmer NET-377) 7.2 mmol/L was added to the perfusate. Five-microliter aliquots of the perfusate and the dialysate were counted on a beta counter, and the percentage remaining in the dialysate was calculated. Samples were collected in the baseline supine, HUT, and supine recovery positions.

**Microdialysis Data Collection**

Dialysate was collected in the baseline supine, HUT, and supine recovery positions for 20 minutes. Dialysate volume was measured by weighing the collection tubes before and after collection. Volume was calculated assuming the specific gravity was 1.00.

**Data Analysis**

Ten subjects underwent HUT during this protocol in which [Ca\(^{2+}\)] was measured via microdialysis at 5-second intervals for up to 60 minutes. Differences between baseline and HUT dialysate [Ca\(^{2+}\)] were assessed by simple paired t-test analysis.

**Signal Averaging**

A second analysis involved signal averaging and integration. Excel templates processed all dialysate [Ca\(^{2+}\)] data as follows. Initially, for each subject, time base information was accompanied by raw [Ca\(^{2+}\)] measurements from 6 individual microdialysis probes. The date from each probe typically contained large spike artifacts that were removed by median smoothing. After smoothing, [Ca\(^{2+}\)] traces were averaged into a composite waveform for that subject. To synchronize the data from each subject, the leading edge midpoint [Ca\(^{2+}\)] peak during HUT was readily identified and used as the timing marker. This marker was subsequently used to time shift the waveform to align its peak midpoint time to the nominal HUT starting time of 20 minutes. After this synchronization procedure, waveforms from all subjects were averaged into a composite waveform (Figure 2B).

**Signal Integration**

From the above-generated [Ca\(^{2+}\)] waveform, 3 intervals were numerically integrated by a trapezoidal algorithm: baseline (5 to 15 minutes), early HUT (20 to 30 minutes), and recovery (40 to 50 minutes). The baseline integral was normalized to a final value of 1.0, and the HUT and recovery integrals were rescaled with respect to baseline. These integrals are plotted as a function of time (Figure 2B) or, with the total area value at the end of each interval, may be shown in column format (Figure 2C).

**Results**

Effects of HUT on HR and Blood Pressure

A total of 10 subjects participated in this study. HR and blood pressure responses to tilt were as expected and are demonstrated in the Table.
Effects of HUT on \([\text{Ca}^{2+}]_o\)

Measurements of dialysate \([\text{Ca}^{2+}]\) are expressed in millimoles per liter and were recorded in a total of 10 patients with a total of 45 probes. Figure 1 shows the average calcium concentration in each dialysate probe at baseline and then with HUT. There was a significant increase in dialysate \([\text{Ca}^{2+}]\) with HUT (from 0.223±0.018 to 0.353±0.028 mmol/L, \(P<0.0001\)). This represents a 62% increase in the concentration of calcium in the dialysate.

Figure 2A demonstrates the average continuous signal-averaged dialysate \([\text{Ca}^{2+}]\) measurements before, during, and after HUT. Figure 2B is the time integral of the (signal-averaged) dialysate \([\text{Ca}^{2+}]\). This figure suggests the increases in dialysate \([\text{Ca}^{2+}]\) occur quite rapidly with HUT. The \([\text{Ca}^{2+}]\), then returns to baseline levels when the subjects return to the supine position during the recovery period. Figure 2C demonstrates this increase in \([\text{Ca}^{2+}]\), during HUT by expressing the sum of the time-integrated \([\text{Ca}^{2+}]\) baseline, HUT, and recovery. With HUT, the integrated \([\text{Ca}^{2+}]\), increased =20% and then returned to baseline values with recovery.

Effect of HUT on Dialysate Fluid Dynamics

To exclude the possibility of alterations in fluid dynamics as a cause of the calcium changes seen, further measurements of the dialysate were made. There was no change in the dialysate sodium concentration with HUT (from 137.33±2.53 mEq at baseline to 136±1.31 mEq 5 minutes into HUT). In addition, dialysate volume did not change with HUT (from 96.5±5.1 to 102.1±2.5 \(\mu\)L during tilt to 102.8±0.9 \(\mu\)L in recovery). These findings demonstrate that the calcium changes seen were not secondary to nonspecific ion or volume changes with HUT. Dialysate lactate levels did not significantly change with HUT (from 0.51±0.9 to 0.47±0.05 mmol/L during tilt and to 0.56±0.56 mmol/L in recovery). Similarly the percentage of tritiated NE retained in the dialysate did not change significantly with HUT (from 75.25±3.04% to 77.75±2.66% with tilt and to 81.0±1.96% in recovery).

Discussion

The signal trafficking between the nerve terminal and VSMC is still not well understood. Both the nerve terminal and VSMC require \([\text{Ca}^{2+}]\), for proper function. Thus, we hypothesized that if \([\text{Ca}^{2+}]\), played an important physiological role, as has been demonstrated in other vascular tissue, then interstitial lower-limb \(\text{Ca}^{2+}\) should rise with tilt. With our techniques, we have demonstrated that there is a rapid elevation in \([\text{Ca}^{2+}]\), with HUT. Thus, we believe that \([\text{Ca}^{2+}]\), may serve as a cellular activator (or messenger) and may be involved in neurovascular regulation.

\([\text{Ca}^{2+}]_o\) and the VSMC

Intact arteries are unable to vasoconstrict when incubated in calcium-free media. Furthermore, the strength of contraction of these vessels is dependent on the \([\text{Ca}^{2+}]_o\). How \([\text{Ca}^{2+}]_o\) exerts its effects on the VSMC is not fully understood, although a number of mechanisms are possible.

One potential mechanism for extracellular calcium signaling is through the receptor-operated channel and nonselcetive cation channel. NE has been shown to bind the receptor-operated channel. This leads to opening of nonselcetive
cation channels. These channels have been shown to have significant calcium permeability and can allow sufficient Ca\(^{2+}\) ions to enter the cell to activate contraction (independent of VGCCs).\(^{10,13}\) Albert and Large\(^{14}\) demonstrated that current through this channel is dependent on the [Ca\(^{2+}\)]\(_{i}\) concentration. The extracellular calcium transient seen in the present study may be required for proper VSMC activation and contraction.

The stretch-activated channel is another [Ca\(^{2+}\)]\(_{i}\)-dependent channel that has been found in smooth muscle.\(^{11}\) External forces activate this type of channel.\(^{15}\) A possible means of activation may be through alterations in gravitational forces on these cells, as occurs with postural changes. The current through this activated channel depends in part on the [Ca\(^{2+}\)]\(_{i}\). This influx leads to significant increases in intracellular calcium concentration that result in either calcium-induced calcium release or activation of VGCCs.\(^{16}\) In the presence of an elevated [Ca\(^{2+}\)]\(_{o}\), the activated stretch-activated channel will have increased Ca\(^{2+}\) current and resultant faster activation of the cell.

Finally, the extracellular calcium receptor (calcium-sensing receptor) could also be involved in extracellular calcium signaling. This receptor, first discovered in the parathyroid gland, is an important component in systemic calcium regulation.\(^{17,18}\) The calcium-sensing receptor has been demonstrated to communicate signals from one cell to another in in vitro models.\(^{19}\) Recently, extracellular calcium-sensing proteins have been identified in VSMCs.\(^{20,21}\) These cells have been shown to vasoconstrict in response to activation of this receptor.\(^{9}\)

**[Ca\(^{2+}\)]\(_{o}\) and the Nerve Terminal**

The nerve terminal also requires extracellular calcium for NE release. Specifically, calcium inward current into the sympathetic nerve terminal is required for NE release. Calcium binds to specific proteins, synaptotagmin, to trigger NE exocytosis.\(^{4,5}\) How calcium enters the nerve is a matter of debate, but 1 or a variety of different channels may be involved. The influx is classically thought to be through VGCCs. However, other channels have been described to also permit calcium influx, such as the nonselective cation channel and sodium-calcium exchanger.\(^{22,23}\)

**Sources of [Ca\(^{2+}\)]\(_{o}\)**

Interstitial calcium must be released from localized calcium stores. Potential regulated compartments include intracellular stores such as the sarcoplasmic reticulum and mitochondria\(^{24}\) or the extracellular matrix itself. Hofer et al\(^{10}\) showed that intracellular Ca\(^{2+}\) signaling events associated with efflux of Ca\(^{2+}\) in 1 cell can activate the calcium-sensing receptor on nearby cells and result in intracellular signaling in that neighboring cell. Thus, extracellular release of cytosolic or stored calcium may provide a source to trigger such intercellular events.

The cytoskeleton in VSMCs has been described to maintain a functional arrangement of these calcium-sequestering organelles and the plasma membrane (for review, see Lee et al\(^{25}\)). This intricate organization is necessary for tight regulation of intracellular calcium levels. Because of the close proximity of the cytoskeleton to the extracellular matrix, this microstructure may also serve to regulate extracellular calcium levels. Integrins (plasma membrane-associated glycoproteins) have been described to transmit signals from the extracellular matrix to the cytoskeleton.\(^{26}\) These glycoproteins have been shown in in vitro studies to regulate VGCC (L-type calcium channel) activity and hence intracellular calcium levels in isolated arterioles.\(^{27}\) Thus, it is possible that extracellular calcium is regulated by similar matrix-integrin-channel interactions. Such interactions present a potential way that mechanical forces associated with the change in posture can signal vasomotor control.

**Study Limitations**

It is possible that the increases in [Ca\(^{2+}\)]\(_{o}\) are due to increases in venous pressure with HUT. In this way, changes in venous pressure could serve to evoke arterial vasoconstriction. This possibility cannot be excluded.

In summary, we have demonstrated that the calcium concentration in the interstitial space changes in response to HUT. Fluctuations in [Ca\(^{2+}\)]\(_{o}\) may be expected given the relatively small volume of extracellular matrix and the very dynamic calcium fluxes that occur across the plasma membrane in both smooth muscle and nerve terminals. To the best of our knowledge, this is the first report of in situ extracellular calcium measurements. We propose that the changes in [Ca\(^{2+}\)]\(_{o}\) may serve as a signal in response to postural changes. Such a signal would provide a mechanism of linking the myogenic response to the sympathetic response during postural changes. Whether this signaling is mediated in the neuron, VSMC, or both remains to be determined. How this rise in [Ca\(^{2+}\)]\(_{o}\) alters the function of the nerve terminal and VSMC also remains unknown; however, [Ca\(^{2+}\)]\(_{o}\) may provide another signal for proper vasoconstrictor responses.

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**References**
