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Two-photon imaging of endothelin-1-mediated intracellular Ca^{2+} handling in smooth muscle cells of rat renal resistance arteries

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ABSTRACT

Aims: Endothelin-1 (ET-1) is a potent vasoconstrictor which regulates the physiology of cardiorenal system. The aim of this study was to evaluate ET-1-mediated elevation of intracellular Ca^{2+} in smooth muscle cells (SMC) of renal resistance arteries.

Main methods: In vitro studies of primary SMC, which were isolated from rat renal microvessels, the levels of intracellular Ca^{2+} were calculated from the ratio of emissions at 340 and 380 nm after loading cells with Fura 2-AM dye. In ex vivo studies we used two-photon imaging of renal resistance arteries excised from rat kidneys and loaded with fluorescent Ca^{2+} indicator Fluo-4 AM.

Key findings: The two-photon imaging demonstrates that treatment of isolated rat renal resistance arteries with ET-1 causes a rapid increase of intracellular Ca^{2+} concentration in smooth muscle vasculature of these vessels. These ex vivo observations are in accordance with in vitro findings indicating that ET-1 mediates activation of TRPC channels and increases the level of intracellular Ca^{2+} in cultured SMC to 510 ± 83 nM.

Significance: ET-1-mediated elevation of intracellular Ca^{2+} is strongly linked to renal microvascular contraction and is crucial for ET-1-induced contraction of SMC. The two-photon imaging of intracellular Ca^{2+} in intact SMC of rat renal resistance arteries is a powerful technique which allows the detailed ex vivo analysis of intracellular Ca^{2+} handling by ET-1, an important player in hypertension-related kidney diseases.

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1. Introduction

Potent vasoconstrictor endothelin-1 (ET-1) is an important regulator of renal microcirculation which affects renal function through actions at multiple cell types in kidney, including vascular smooth muscle cells (SMC) [1,2]. Stimulation of SMC with ET-1 evokes a wide variety of signaling events, including mobilization of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), which plays a principal role in cellular contractility [3,4]. Even though primary SMC cultures are an exceptional resource for dissecting the activation mechanisms of SMC signaling, there is an unavoidable risk that the transfer to the status of cultured cells is accompanied by changes in the repertoire of expressed signaling proteins, including calcium channels and their regulatory proteins. To avoid this limitation, we have modified two-photon imaging technique previously used by us for analysis of Ca^{2+} handling in endothelial cells of an isolated rat aorta [5,6] to monitor the $[\text{Ca}^{2+}]_i$ changes in individual SMC in the native setting of the freshly isolated rat renal resistance arteries. Since autoregulation of renal vasculature is one of the most important mechanisms of maintaining the blood pressure at a

sustainable level, the ability to focus at small renal resistance arteries, such as afferent arterioles, is imperative for uncovering signaling mechanisms regulating the responsiveness of renal vasculature.

The aim of this study was to evaluate the feasibility of monitoring ET-1-mediated elevation of $[\text{Ca}^{2+}]_i$ in SMC of rat renal resistance arteries using two-photon imaging. Two-photon microscopy of isolated renal vessels is a very powerful and the least invasive method for the analysis of $[\text{Ca}^{2+}]_i$ handling ex vivo. For instance, recent studies utilized two-photon microscopy for the visualization of the binding of ET receptor agonists in isolated rat mesenteric resistance arteries [7]. Using ET-1 and L-ET-1 (linear ET-1) fluorescently labeled with an efficient donor-acceptor pair (OG488 and TAMRA, respectively) it was possible to visualize and analyze localization and fluorescent properties of the ET_A and ET_B receptor binding sites [7]. However, two-photon microscopy has never been used previously to monitor ET-1 action on changes in $[\text{Ca}^{2+}]_i$ in renal resistance arteries.

2. Materials and methods

2.1. Calcium imaging and electrophysiological analysis in cultured cells

The fluorescent Ca^{2+} indicator Fura 2-AM was used to measure the $[\text{Ca}^{2+}]_i$ concentration changes during 100 nM ET-1 applications. SMC

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isolated from renal interlobar arteries of Dahl salt-sensitive (SS) rats were loaded with physiological saline solution (PSS) (in mM: 140 NaCl, 2 CaCl₂, 1.2 MgCl₂, 4.5 KCl, 6 D-glucose, 10 HEPES; pH 7.4) containing 2 μ M Fura 2-AM (Life Technologies) and 0.05% pluronic acid (F-127; Sigma-Aldrich) and stored for 40 min in the dark on a slow moving shaker at room temperature. After incubation cells were washed with PSS for 15 min to remove excess dye. All fluorescence imaging experiments using cultured SMC were performed in PSS solution. Fluorescence images of changes in the $[Ca^{2+}]_i$ level probed with Fura 2-AM were obtained using a Nikon TE-2000U inverted microscope equipped with a 40/1.3 DIC oil immersion objective lens (Nikon S Fluor) and a Zyla sCMOS camera (Andor Technologies). Excitation was provided by a Lambda XL xenon arc lamp at alternating wavelengths, and emission control was achieved using an optical filter changer with integrated shutter/filter wheel driver (Sutter Instrument, Novato, CA). Cells were alternately excited at 340 nm and 380 nm, and the respective 510 nm emission was acquired every 8 s. Changes in $[Ca^{2+}]_i$ were based on 340/380 ratios. To quantify $[Ca^{2+}]_i$ concentration, fluorescence intensity was recorded in the baseline and after addition of ionomycin and MnCl₂. Fluorescence signal changes in response to ionomycin (producing the maximum of the Fura 2-AM ratio) and MnCl₂, which quenches the dye and results in the lowest Fura 2-AM ratio. Intensity of fluorescence was translated into the actual calcium concentrations as previously described [8]. For electrophysiological analysis, standard patch clamp approach in the cell attached configuration was used as previously described [9]. Nicardipine (5 mM), niflumic acid (100 μ M) and DIDS (100 μ M) were added to the pipette solution directly before experiments to block N-type Ca²⁺ and Ca²⁺-activated Cl[−] channels [10].

2.2. Two-photon imaging of intracellular Ca²⁺ handling in renal resistance arteries

For isolation of renal resistance arteries, the kidneys were collected from male Dahl SS rats maintained on 1% salt Purina 5001 diet for 12 weeks. Animal use and welfare adhered to the NIH Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the IACUC of the Medical College of Wisconsin. Interlobar arteries of approximate 150–200 μ m diameter were manually dissected in an ice-cold low calcium PSS (PSS-LC; in mM: 140 NaCl, 0.1 CaCl₂, 1.2 MgCl₂, 4.5 KCl, 6 D-glucose, 10 HEPES; pH 7.4). The tunica externa was then carefully stripped from the vessels in order to reduce non-specific fluorescence. As seen in Fig. 3, the combination of dissecting and sieving steps allowed us to isolate the intact renal vascular tree for further analysis. The vascular tree was isolated by sectioning the kidney longitudinally and removing the medulla. The tissue was then gently pressed through a 150 μ m stainless steel mesh sieve with a spatula to carefully retain the vascular tree. The tissue was washed with PSS-LC to remove excess kidney tissue, and connective tissue was partially removed by manual dissection. The fluorescent Ca²⁺ indicator Fluo-4 AM (Life Technologies) was used to measure the $[Ca^{2+}]_i$ changes after 100 nM ET-1 applications. Dissected renal microvessels were loaded with PSS-LC, supplemented with 5 μ M Fluo-4 AM and 0.05% pluronic acid, and stored for 1 h on a rotating shaker at room temperature in the dark. After incubation, vessels were washed 2–3 times with PSS to remove extracellular dye and all further procedures were done in PSS. Renal vessels were transferred to a silicon-coated plate and fixed by anchor slice grid (Warner Instruments, Hamden, CT) for further application of ET-1. The plate was then transferred to the upright Olympus Fluoview FV1000 microscope equipped with Ti:sapphire lasers tuned to 820 nm and imaged with a 25 $\times$ (N.A. 1.05 and working distance 2 mm) water-immersion objective lens (XLPL25XWMP; Olympus). Once the cells were in focus, sequential collection of images in a fast scan mode was started (raster scan, 512 $\times$ 512 pixel window with a frame collection frequency of 1.1 s). In parallel with Fluo-4 AM fluorescence, transmitted light images were collected in order to detect changes in vessel contraction and better visualize the experimental

conditions. Fluorescent layer of SMC was identified using two-photon microscopy. After collection of baseline signal images, ET-1 was carefully applied while imaging. The increase of the fluorescent signal was observed within the loaded cells and measured as arbitrary units of fluorescence intensity (a.u.). In contrast to measurements in cultured cells, it is not possible to calculate the exact $[Ca^{2+}]_i$ in intact vessels, because the calibration can't be accurately done when cells are in the native setting of renal microvessels. The narrow focus of two-photon excitation significantly reduces auto fluorescence from collagen tissue but allows focusing only on small part of vessel. Due to round shape of vessel the longitudinal SMC could be located at upper or lower focus planes, which allowed monitoring of Ca²⁺ transient only in visible part of the SMC. Application of ET-1 produces rapid Ca²⁺ transient in SMC, detected by increase in fluorescence on non-ratiometric calcium indicator Fluo-4 AM. ROI (region of interest) for each cell (or cell fragment) was chosen using Image J software and changes in fluorescence were analyzed as was described previously [5].

3. Results

In primary SMC isolated from rat renal microvessels exposure to ET-1 (100 nM) caused rapid activation of TRPC channels (Fig. 1A) followed by dramatic intracellular $[Ca^{2+}]_i$ changes (Fig. 1B,C). The quantification of the SMC data revealed that ET-1 increases the basal calcium from 77 ± 17 nM to 510 ± 83 nM.

The basic strategy for isolation and imaging of rat renal resistance arteries is depicted in Fig. 2. It is of note, that two-photon imaging technique allows the efficient separation of fluorescent signal coming from other distinct layers of renal microvessels. As seen in Fig. 2C, the interference caused by fibroblast and collagen layer could be an extremely difficult problem to deal with, if it is not negated by the key features of the two-photon imaging: low background fluorescence and ability of fine focusing upon the SMC layer within the microvessel.

Isolation of intact vascular tree (Fig. 3) enabled us to evaluate ET-1 mediated $[Ca^{2+}]_i$ changes in individual SMC ex vivo. Addition of ET-1 to isolated renal resistance arteries caused an influx of Ca²⁺ in individual SMC in the native setting of renal microvessel (Fig. 4). As shown in Fig. 4B, two-photon imaging allows quantification of $[Ca^{2+}]_i$ changes in individual SMC of renal vasculature. ET-1 induces rapid initial increase in $[Ca^{2+}]_i$, most likely due to mobilization of Ca²⁺ from intracellular sources, followed by a prolonged extracellular influx of Ca²⁺, which is presumably mediated via TRPC channels activity. The quantitative analysis of 39 individual SMC, embedded in 6 vessels which were excised from 4 rats, allowed us to measure the ET-1-triggered increase of $[Ca^{2+}]_i$ from baseline (zero) to a peak of 577 ± 197 a.u. which is directly related to increase of $[Ca^{2+}]_i$. Fig. 4 demonstrates the representative trace obtained from one vessel.

4. Discussion

Native vascular SMC contain diverse TRPC-mediated channels, which upon stimulation allow influx of Ca²⁺ ions contributing to SMC contractility [11]. Many of the effects of ET-1 are due to an increase in $[Ca^{2+}]_i$ concentration [12]. ET-1 is inducing rapid changes in $[Ca^{2+}]_i$ in a variety of cultured renal cells including glomerular mesangial cells, SMC and others [4,13,14]. These and other studies provided crucial information about the ET-1-mediated Ca²⁺ handling in cultured cells of different origin. However, the responsiveness of individual cells to ET-1 in their native settings has not been sufficiently studied. We demonstrate here the dynamics of ET-1-triggered changes in $[Ca^{2+}]_i$ in individual SMC of small renal resistance arteries isolated from rat tissues. The handling of $[Ca^{2+}]_i$ by ET-1 in rat arteries is in a good accordance with results obtained using primary SMC isolated from renal tissues, indicating that two-photon imaging of individual SMC from renal vasculature is a feasible approach. Some difference in kinetic response to ET-1 between cultured SMC and SMC in their native setting of microvessels is

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