Endothelin and Prostaglandin H₂/Thromboxane A₂ Enhance Myogenic Constriction in Hypertension by Increasing Ca²⁺ Sensitivity of Arteriolar Smooth Muscle

Zoltan Ungvari, Akos Koller

Abstract—The myogenic response of skeletal muscle arterioles is enhanced in hypertension because of the release of endothelin (ET) and prostaglandin H₂ (PGH₂)/thromboxane A₂ (TxA₂) from the endothelium. We hypothesized that ET and PGH₂/TxA₂ modulate Ca²⁺ signaling in arteriolar smooth muscle and thereby enhance myogenic constriction. Thus, simultaneous changes in intracellular Ca²⁺ concentration in smooth muscle ([Ca²⁺]), measured by fura 2 microfluorometry (expressed as Ca²⁺ fluorescence ratio [R Ca]), and diameter were obtained as a function of intraluminal pressure (Pᵢ) in isolated cannulated gracilis muscle arterioles (diameter ≈120 μm) of normotensive Wistar rats (WR) and spontaneously hypertensive rats (SHR). In the absence of extracellular Ca²⁺, increases in Pᵢ from 20 to 160 mm Hg increased the passive diameter of arterioles without changes in R Ca. In the presence of extracellular Ca²⁺ and endothelium, increases in Pᵢ elicited similar increases in R Ca (30±7% for control and 33±8% for SHR at 160 mm Hg) but a significantly (P<0.05) greater constriction of SHR arterioles compared with WR arterioles (at 160 mm Hg, 55±4% versus 38±2%, respectively, of passive diameter). In the absence of the endothelium, Pᵢ-induced changes in the R Ca and diameter of SHR and WR arterioles did not differ significantly. Also, a step increase in Pᵢ (from 80 to 140 mm Hg) elicited a similar increase in R Ca but greater constrictions in SHR versus WR arterioles. In the presence of the TxA₂ receptor inhibitor SQ29,548 and the ET A receptor inhibitor BQ123, there was no difference between responses of SHR and WR arterioles. In WR arterioles, increasing concentrations of KCl elicited a significant increase in R Ca (38±7% at 80 mmol/L) and completely constricted the arterioles. In contrast, constrictions to ET (52±7% at 3×10⁻¹² mol/L) and the TxA₂ agonist U46619 (40±8% at 3×10⁻⁹ mol/L) were not accompanied by increases in R Ca at submaximal concentrations. Collectively, these findings suggest that in hypertension, endothelium-derived ET and PGH₂/TxA₂ increase the Ca²⁺ sensitivity of the contractile apparatus of arteriolar smooth muscle; thus, the similar increases in [Ca²⁺], in response to the elevation of intraluminal pressure elicit greater myogenic constriction. (Hypertension. 2000;36:856-861.)

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signaling pathways (eg, protein kinase C [PKC] or Rho-kinase) that sensitize the smooth muscle contractile apparatus to Ca\(^{2+}\).\(^{1,26-33}\) Studies on ring preparations from aorta and conduit vessels suggest that ET and PGH\(_i\)/TxA\(_i\) increase force generation by increasing [Ca\(^{2+}\)].\(^{34,35}\) and/or Ca\(^{2+}\) sensitivity of the smooth muscle contractile apparatus,\(^{34,36}\) but these vessels do not exhibit active pressure-induced tone. Nevertheless, on the basis of these studies, it can be hypothesized that ET and PGH\(_i\)/TxA\(_i\) enhance the arteriolar myogenic response by upregulating the pressure-induced increase in [Ca\(^{2+}\)], in aSM. However, if ET and/or PGH\(_i\)/TxA\(_i\) decreases arteriolar diameter without a substantial elevation of [Ca\(^{2+}\)], then an increased Ca\(^{2+}\) sensitivity of aSM by these factors must be hypothesized to be responsible for the enhancement of arteriolar myogenic constriction in hypertension.

To test this hypothesis, first we aimed to characterize by fura 2 microfluorometry\(^{26,29-32}\) the pressure-induced changes in aSM [Ca\(^{2+}\)], and the decrease in the diameter of skeletal muscle arterioles isolated from normotensive Wistar rats (WR) and spontaneously hypertensive rats (SHR) in the presence and absence of endothelium or inhibition of ET and TxA\(_i\) receptors. We also investigated the effect of ET and a stable TxA\(_i\) analogue on aSM [Ca\(^{2+}\)], and the myogenic tone of arterioles.

**Methods**

**Simultaneous Measurement of aSM [Ca\(^{2+}\)], and Diameter of Isolated Arterioles**

The internal diameter of isolated gracilis muscle arterioles of 11-week-old normotensive WR (n=30) and SHR (n=30) was measured by videomicroscopy, as previously described.\(^{1,4,5,37}\) Changes in aSM [Ca\(^{2+}\)], were assessed by the ratiometric fura 2 fluorescence method\(^{26,30,32}\) with use of the Ionoptix Microfluorimeter System (Ionoptix Co) and were expressed as changes in Ca\(^{2+}\) fluorescence ratio (R\(_{Ca}\)).\(^{32}\) We used normotensive WR as a control strain for SHR because characteristics of pressure-diameter curves of gracilis muscle arterioles of WR and WKY do not differ significantly.\(^{5}\)

**Experimental Protocols**

Changes in aSM R\(_{Ca}\), and diameter of WR and SHR arterioles in response to increases in P\(_i\), (from 20 to 160 mm Hg in 20 mm Hg steps) were measured.\(^{4,5,32,37}\) In separate experiments, the endothelium of WR and SHR arterioles was removed.\(^{1,37}\) and arteriolar responses were assessed to increases in P\(_i\). In other experiments, R\(_{Ca}\) was increased from 80 to 140 mm Hg in one step, and the time course of the development of myogenic constriction was measured by videomicroscopy, as previously described.\(^{1,4,5,37}\) and the changes in R\(_{Ca}\) were recorded in the absence and presence of the TxA\(_i\) receptor inhibitor SQ29,548 (10\(^{-7}\) mol/L) and the ET\(_i\) receptor inhibitor BQ123 (10\(^{-7}\) mol/L). In other experiments, responses of endothelium-denuded WR arterioles to ET-1, the TxA\(_i\) analogue U46619, and KCl were obtained at 80 mm Hg. Next, pressure-induced responses of endothelium-denuded WR arterioles were measured in the absence and presence of ET-1 (3\times10\(^{-12}\) mol/L) or U46619 (3\times10\(^{-9}\) mol/L). At the conclusion of each experiment, the bath solution was changed to a Ca\(^{2+}\)-free physiological salt solution, which contained EGTA (10\(^{-7}\) mol/L), and the pressure steps were repeated to obtain the pressure-maximum passive diameter relationship.\(^{32}\)

**Materials and Data Analysis**

Fura-2 AM was purchased from Molecular Probes. All other chemicals were obtained from Sigma-Aldrich Co. Arteriolar diameters at each P\(_i\) were normalized to the passive diameter measured at 80 mm Hg. Myogenic constriction was expressed as a percentage of the maximal passive diameter of the vessel at the corresponding P\(_i\). \(^{1,26-30,37}\) Changes in R\(_{Ca}\) in response to increases in P\(_i\) were normalized to the R\(_{Ca}\) measured at 20 mm Hg P\(_i\). Drug-induced changes in arteriolar diameter and R\(_{Ca}\) were expressed as a percentage of the baseline values. All data are expressed as mean±SEM. Statistical analyses were performed by ANOVA, followed by the Tukey post hoc test or the Student t test, as appropriate. A value of P<0.05 was considered statistically significant.

**Results**

**Pressure-Induced Changes in R\(_{Ca}\) and Arteriolar Diameter**

In the absence of Ca\(^{2+}\) in the bath solution, increases in P\(_i\), (from 20 to 160 mm Hg) elicited similar increases in the diameters of WR (from 115±3 to 180±4 μm) and SHR (from 111±5 to 177±5 μm, P=NS) arterioles without changes in R\(_{Ca}\). In the presence of Ca\(^{2+}\) in the bath solution, increases in P\(_i\) elicited significant increases in R\(_{Ca}\) in WR arterioles that were not significantly different from those in SHR arterioles. The pressure-induced rise in R\(_{Ca}\) was accompanied by arteriolar myogenic constrictions that were significantly greater in SHR than in WR arterioles (Figure 1). In the absence of endothelium, pressure-induced increases in R\(_{Ca}\) were not significantly different between SHR and WR arterioles. Also, there was no significant difference between pressure-induced increases in R\(_{Ca}\) in the presence or in the absence of the endothelium in either group. However, removal of the endothelium significantly attenuated pressure-induced constrictions in SHR arterioles but had no significant effect on myogenic constriction of WR arterioles, thus eliminating the difference in responses between WR and SHR arterioles (Figure 1). A step increase in pressure from 80 to 140 mm Hg elicited a similar increase in R\(_{Ca}\) but significantly greater constriction in SHR than in WR intact arterioles. In the presence of SQ29,548 and BQ123, there was no significant difference between responses of SHR and WR arterioles.
(Figure 2). In Figure 3A and 3B, changes in R_Ca to increases in P_i were plotted against the corresponding constriction, which yielded a relationship between aSM_R_Ca and myogenic constriction in the presence and absence of the endothelium. On the linear portion of these curves, regression lines were fitted. In the presence of endothelium, a given increase in aSM_R_Ca resulted in a significantly greater myogenic constriction (Figure 3A), showing that the aSM_R_Ca–myogenic constriction relationship is significantly steeper in SHR than in WR arterioles (in WR, slope 0.7±0.1, r²=0.91; in SHR, slope 1.7±0.2, r²=0.96; P<0.05). In contrast, in the absence of endothelium, the aSM_R_Ca–myogenic tone relationships were not significantly different in SHR and WR arterioles (Figure 3B). Also, changes in R_Ca to a step increase in P_i (from 80 to 140 mm Hg) were plotted against the amplitudes of the corresponding myogenic constriction. Although the increases in R_Ca were similar, a significantly greater myogenic constriction was observed in SHR than in WR arterioles. SQ29,548 and BQ123 did not significantly affect the increases in R_Ca, but they attenuated the myogenic constriction in SHR arterioles, eliminating the difference between the responses of 2 groups (Figure 3C and 3D).

Changes in R_Ca and Arteriolar Diameter to ET, U46619, and KCl

Although ET (from 10⁻¹² mol/L) and the TxA₂ analogue U46619 (from 3×10⁻¹⁰ mol/L) constricted endothelium-denuded arterioles (EC₅₀ 3×10⁻¹² and 6×10⁻⁹ mol/L, respectively), only submaximal and maximal constrictions were accompanied by significant increases in aSM_R_Ca (Figure 4A and 4B). Increases in KCl concentration (from 25 to 80 mmol/L) elicited significant increases in aSM_R_Ca and simultaneous constriction of arterioles (Figure 4C). To characterize the agonist-induced changes in aSM_Ca²⁺ sensitivity, changes in R_Ca to ET, U46619, and KCl were plotted against the simultaneous changes in diameter, yielding a relationship between aSM_R_Ca and arteriolar constriction for each vasoactive substance. On the linear portion of these curves, regression lines were fitted. In the presence of ET or U46619, the slope of the lines (slope 16±2, r²=0.96, and slope 13±2, r²=0.90, respectively) was significantly steeper than in the presence of KCl (slope 3±1, r²=0.98), demonstrating that these agonists increased R_Ca only at very high concentrations. In contrast, KCl, in a concentration-dependent manner, significantly and linearly elevated R_Ca and decreased the diameter of arterioles (Figure 4D). Next, we measured arteriolar responses to increases in P_i in the absence and presence of ET (3×10⁻¹² mol/L) or U46619 (3×10⁻⁹ mol/L), and changes in R_Ca were plotted against the corresponding constrictions (Figure 5A). In the presence of ET or U46619, pressure-induced arteriolar constrictions, but not increases in R_Ca, were significantly enhanced, and a given increase in aSM_R_Ca resulted in a significantly greater myogenic constriction.
Discussion

The new findings of the present study are that (1) in arterioles of hypertensive rats, the enhanced myogenic constriction is not accompanied by a greater increase in aSM $[\text{Ca}^{2+}]_{i}$; (2) in the absence of the endothelium or in the presence of BQ123 and SQ29,548, pressure-induced increases in aSM $[\text{Ca}^{2+}]_{i}$ and decreases in diameter are not different in SHR and WR arterioles; and (3) ET and the TxA$_2$ agonist U46619 enhance myogenic constriction without significantly altering increases in aSM $[\text{Ca}^{2+}]_{i}$.

In the present study, we confirmed that increases in $P_i$ elicit substantial increases in smooth muscle $[\text{Ca}^{2+}]_{i}$, followed by constriction in arterioles (Figures 1 and 2). This is consistent with the idea that cellular mechanisms responsible for the development of myogenic response include an increase in $[\text{Ca}^{2+}]_{i}$ that is likely due to pressure-induced depolarization of aSM followed by an entry of extracellular $\text{Ca}^{2+}$ via opening of voltage-operated $\text{Ca}^{2+}$ channels.1–3,26–32,38,39 We confirmed that in the presence of endothelium, pressure-induced myogenic constriction is enhanced in isolated skeletal muscle SHR arterioles compared with vessels from normotensive rats (Figure 1) and that the difference between myogenic constriction of SHR and WR arterioles increases as $P_i$ is increased from 40 to 160 mm Hg.4,5 However, assessment of $[\text{Ca}^{2+}]_{i}$ in the present study showed that there is no difference in pressure-induced increases in $[\text{Ca}^{2+}]_{i}$ between WR and SHR arterioles (Figure 1), suggesting that in hypertension the enhanced myogenic constriction of skeletal muscle arterioles is not associated with a greater increase in aSM $[\text{Ca}^{2+}]_{i}$.

The pressure–passive diameter relationship of arterioles of SHR and WR did not differ significantly (Figure 1); thus, changes in the mechanical properties of the arteriolar wall are unlikely to alter the myogenic response in hypertension.4–6 Also, changes in diameter per se do not affect $R_{Ca}$ in a $\text{Ca}^{2+}$-free physiological solution, suggesting that increases in $P_i$ are unlikely to elicit release of $\text{Ca}^{2+}$ from intracellular stores.

Removal of the endothelium (Figure 1) or inhibition of the TxA$_2$ and ET$_{A}$ receptors (Figure 2) decreased myogenic constriction in arterioles (Figures 1 and 2). This is consistent with the idea that cellular mechanisms responsible for the development of myogenic response include an increase in $[\text{Ca}^{2+}]_{i}$, that is likely due to pressure-induced depolarization of aSM followed by an entry of extracellular $\text{Ca}^{2+}$ via opening of voltage-operated $\text{Ca}^{2+}$ channels.1–3,26–32,38,39 We confirmed that in the presence of endothelium, pressure-induced myogenic constriction is enhanced in isolated skeletal muscle SHR arterioles compared with vessels from normotensive rats (Figure 1) and that the difference between myogenic constriction of SHR and WR arterioles increases as $P_i$ is increased from 40 to 160 mm Hg.4,5 However, assessment of $[\text{Ca}^{2+}]_{i}$, in the present study showed that there is no difference in pressure-induced increases in $[\text{Ca}^{2+}]_{i}$ between WR and SHR arterioles (Figure 1), suggesting that in hypertension the enhanced myogenic constriction of skeletal muscle arterioles is not associated with a greater increase in aSM $[\text{Ca}^{2+}]_{i}$, in response to elevations in $P_i$.

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Removal of the endothelium (Figure 1) or inhibition of the TxA$_2$ and ET$_{A}$ receptors (Figure 2) decreased myogenic constriction in SHR but not in WR arterioles and eliminated the difference between responses of WR and SHR vessels without affecting pressure-induced increases in aSM $[\text{Ca}^{2+}]_{i}$. Responses of WR arterioles were not significantly affected by either removal of the endothelium or inhibition of the TxA$_2$ and ET$_{A}$ receptors. Collectively, these findings confirmed that the augmented myogenic constriction of SHR arterioles is likely due to an increased release of ET and PGH$_2$/TxA$_2$ from the endothelium.4,5,7

Figure 4. A through C, Changes in aSM $R_{Ca}$ and diameter of endothelium-denuded fura 2-loaded skeletal muscle arterioles of WR in response to ET (A), U46619 (B), and KCl (C). D, Constriction of arterioles as a function of changes in $R_{Ca}$ to ET, U46619, or KCl. Data are mean±SEM. *$P<0.05$ (n=5 to 10).

Figure 5. A, Myogenic tone of endothelium-denuded fura 2-loaded skeletal muscle arterioles of WR as a function of changes in aSM $R_{Ca}$ in the presence or absence of ET (3×10$^{-12}$mol/L) or U46619 (3×10$^{-6}$ mol/L). Data are mean±SEM. *$P<0.05$ (n=4 to 10). B, Proposed scheme for endothelial modulation of smooth muscle $\text{Ca}^{2+}$ signaling and myogenic constriction of arterioles in hypertension.
Construction and analysis of the aSM R_{Ca}—myogenic constriction curves, an indicator of Ca^{2+} sensitivity, demonstrate that in the presence of endothelium a given change in [Ca^{2+}], elicits greater constriction in SHR than in WR arterioles (Figure 3), suggesting that the Ca^{2+} sensitivity of the aSM contractile apparatus is increased in hypertension. The crucial role of endothelial release of ET and PGH_{2}/TxA_{2} is indicated by the findings that both in the absence of the endothelium and in the presence of ET and TxA_{2} receptor inhibitors, there was no difference between the Ca^{2+} sensitivity of the myogenic mechanism of WR and SHR arterioles (Figure 3). Thus, in hypertension, ET and PGH_{2}/TxA_{2} released from the arteriolar endothelium\textsuperscript{4,7} increase the Ca^{2+} sensitivity of the contractile apparatus rather than [Ca^{2+}], in the aSM. To further test this hypothesis, we investigated the effects of ET and the TxA_{2} analogue U46619 on smooth muscle [Ca^{2+}], and diameter of endothelium-denuded arterioles. Indeed, we found that low concentrations of ET and U46619 elicited significant and substantial (>50%) constrictions that were not accompanied by significant increases in aSM [Ca^{2+}], (Figure 4). Also, we have confirmed the findings of studies of isolated smooth muscle cells and large conduit vessels that reported that high concentrations of ET and U46619\textsuperscript{34,35} increase smooth muscle [Ca^{2+}], (Figure 4). However, at these concentrations, isolated skeletal muscle arterioles were almost completely constricted.

To test the relationship between increases in aSM [Ca^{2+}], and decreases in diameter, we investigated arteriolar constriction to KCl, which elicits increases in [Ca^{2+}], via depolarization-induced Ca^{2+} influx through voltage-operated Ca^{2+} channels.\textsuperscript{28} Increases in K\textsuperscript{+} concentration elicited increases in smooth muscle [Ca^{2+}], and arteriolar constriction (Figure 4C), confirming that increases in [Ca^{2+}], lead to proportional constriction of skeletal muscle arterioles. Analysis of aSM R_{Ca}—arteriolar constriction relationships demonstrates that ET and U46619 elicited significantly greater constrictions for a given increase in [Ca^{2+}], than did a rise in extracellular KCl concentration (Figure 4D). Also, we found that in the presence of ET or U46619, similar pressure-induced increases in aSM R_{Ca} elicited greater arteriolar constrictions (Figure 5). On the basis of our findings, we concluded that enhancement of myogenic constriction of skeletal muscle arterioles by ET and PGH_{2}/TxA_{2}, in a concentration range likely to be present in hypertension,\textsuperscript{15,40} depends primarily on an increase in Ca^{2+} sensitivity of the contractile apparatus rather than altering pressure-induced increase in [Ca^{2+}], in aSM. ET and PGH_{2}/TxA_{2} may activate the phospholipase C–diacylglycerol–PKC pathway and thus increase the Ca^{2+} sensitivity of vascular smooth muscle as shown in large vessels and aorta.\textsuperscript{30,34–36,41} Also, in renal afferent arterioles of rats, ET-induced enhancement of myogenic constriction can be prevented by inhibition of PKC.\textsuperscript{42} Activation of the PKC pathway (with phorbol esters or synthetic diacylglycerol analogues) was shown to increase myogenic constriction of cerebral arteries,\textsuperscript{30,43} renal afferent arterioles,\textsuperscript{42} and skeletal muscle arterioles,\textsuperscript{38} likely by altering the Ca^{2+} sensitivity of the contractile apparatus in aSM.\textsuperscript{30} Furthermore, in hypertension, an enhanced Ca^{2+} sensitivity of the smooth muscle contractile apparatus to pharmacological stimuli has been suggested by several studies\textsuperscript{44–47} and attributed to the increased activity of the PKC pathway, although participation of other newly suggested mechanisms cannot be excluded.\textsuperscript{33}

On the basis of our present and previous findings\textsuperscript{4,5} and the aforementioned studies, we developed a model for describing the endothelial modulation of smooth muscle Ca^{2+} signaling leading to an enhanced myogenic constriction in skeletal muscle arterioles in hypertension (Figure 5B). Accordingly, we propose the following scheme: (1) In normotension, increases in P_{i} elicit an increase in aSM [Ca^{2+}], that is due to an influx of extracellular Ca^{2+}.\textsuperscript{29,30,32} that activates the contractile apparatus, resulting in an arteriolar constriction. (2) The magnitude of myogenic constriction also depends on the activity of signaling pathways (eg, PKC) that sensitize the contractile apparatus to Ca^{2+}.\textsuperscript{29,30,39} In normotension, aSM Ca^{2+} sensitivity and myogenic constriction\textsuperscript{4,5} are independent of the endothelium. (3) In hypertension, increases in P_{i} elicit increases in smooth muscle [Ca^{2+}], comparable to those in normotension. (4) However, in response to higher P_{i}, ET and PGH_{2}/TxA_{2} are released from the endothelium.\textsuperscript{4,7} These increase the Ca^{2+} sensitivity of the contractile apparatus by activating signaling pathways (such as PKC).\textsuperscript{34–36,41} Thus, the same increases in P_{i} elicit an enhanced myogenic constriction.

The main feature of hypertension is a sustained elevation of intravascular pressure; thus, to reduce arteriolar wall tension requires a chronic decrease in arteriolar diameter according to the law of Laplace.\textsuperscript{48} In theory, it seems more specific and efficient to maintain an enhanced myogenic constriction by increasing the Ca^{2+} sensitivity of contractile apparatus than by increasing [Ca^{2+}], in aSM, because higher [Ca^{2+}], may also affect other cellular functions, such as the activity of various enzymes that are not related to the myogenic mechanism. The present findings reveal a possible mechanism by which arterioles adapt differently from the aorta and other conduit vessels in response to chronic elevation of P_{i}. The large vessels do not possess myogenic mechanism; thus, in hypertension, their wall thickness increases to normalize wall tension,\textsuperscript{49,50} whereas arterioles decrease their diameter to a great extent. The importance of the present findings is underscored by recent studies showing that ET-converting enzyme blockers and ET and TxA_{2} receptor antagonists effectively lower peripheral resistance and blood pressure in hypertension,\textsuperscript{12,21,22,24} suggesting that modulation of pressure-induced aSM constriction by endothelium-derived constrictor factors and aSM Ca^{2+} sensitivity can be a new target of the pharmacological treatment of hypertension.

In summary, our findings suggest that ET and PGH_{2}/TxA_{2}, released from the endothelium, increase the Ca^{2+} sensitivity of the contractile apparatus in smooth muscle, a mechanism that may be responsible for the enhanced pressure-induced myogenic constriction in skeletal muscle arterioles in hypertension.

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