CROSSTALK BETWEEN VASCULAR REDOX AND CALCIUM SIGNALING IN HYPERTENSION INVOLVES TRANSIENT RECEPTOR POTENTIAL MELASTATIN 2 CATION CHANNEL

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ABSTRACT

Increased generation of reactive oxygen species and (ROS) and altered Ca^{2+} handling cause vascular damage in hypertension. Molecular mechanisms linking these systems are unclear but TRPM2 could be important because it is a ROS sensor and regulates Ca^{2+} and Na^{+} entry. We hypothesized that TRPM2 is a point of cross-talk between redox and Ca^{2+} signaling in vascular smooth muscle cells (VSMC) and that in hypertension oxidative stress induces TRPM2 activation and increased $[Ca^{2+}]_i$ through processes involving NCX (Na^{+}/Ca^{2+} exchanger). VSMCs from hypertensive (HT) and normotensive individuals (NT) and arteries from wildtype (WT) and hypertensive mice (LinA3) were studied. Generation of O_2^- and H_2O_2 was increased in HT VSMCs. This was associated with activation of redox-sensitive PARP1, a TRPM2 regulator. AngII increased Ca^{2+} and Na^{+} influx with exaggerated responses in HT VSMCs. These effects were attenuated by PEG-catalase and 2-APB, 8-Br-cADPR and olaparib (TRPM2 inhibitors). In Na^{+}-depleted conditions increased Ca^{2+} in HT VSMCs was normalized. Benzamil, KB-R7943 and YM244769 (NCX inhibitors) ameliorated increased Ca^{2+} transients in HT. Increased phosphorylation of MLC20 in HT VSMACs was prevented by TRPM2 and NCX inhibitors. In isolated arteries from LinA3 mice, exaggerated agonist (U46619, AngII, phenylephrine)-induced vasoconstriction was attenuated by TRPM2 and NCX inhibitors. In conclusion activation of ROS-dependent PARP-1-regulated TRPM2 contributes to increased vascular Ca^{2+} and Na^{+} influx in part through NCX. We identify a novel pathway linking ROS to Ca^{2+} signalling through TRPM2/NCX in human VSMCs and suggest that oxidative stress-induced upregulation of this pathway is a new player in hypertension-associated vascular dysfunction.

INTRODUCTION

Hypertension is a multifactorial and complex disorder associated with abnormal vascular signaling[1]. Uncontrolled generation of reactive oxygen species (ROS), activation of redox-sensitive signalling pathways and increased intracellular free calcium concentration ($[Ca^{2+}]_i$) contribute to endothelial dysfunction, vascular hyperreactivity and structural remodeling in hypertension[2-5]. Signaling pathways involving ROS and Ca^{2+} may be interlinked through redox-sensitive cation channels.

The transient receptor potential (TRP) superfamily constitutes a large group of redox-regulated channels, including the TRP melastatin (TRPM) channels, of which there are 8 isoforms (TRPM1-TRPM8) [6-8]. Of these, TRPM2 is the most highly redox-sensitive. It is permeable to both Ca^{2+} and Na^{+} with a selectivity for Ca^{2+} over Na^{+} of 0.5–1.6 [9, 10] . TRPM2 is mainly activated by adenosine diphosphate ribose (ADPR), which has specific residues involved in binding to the NUDT9-H domain of TRPM2 to open the cation channel [11-13]. In addition to ADPR, Ca^{2+}, hydrogen peroxide (H_2O_2), calmodulin, NAADP and oxidation of cysteine residues (Cys549) can positively modulate TRPM2, while AMP, acidic pH and nitration of tyrosine 1485 are negative regulators [14-17]. H_2O_2 is the main ROS involved in TRPM2 activation, it can activate TRPM2 channel either directly via oxidation or indirectly via ADPR release after DNA damage [17-19]. DNA damage is linked with high and rapid PARylation activity, where Poly (ADP-ribose) polymerase (PARP) repeatedly catalyzes the transfer of successive units of ADPR to target proteins, leading to TRPM2 activation [20, 21]. Although TRPM2 channels are present in vascular smooth muscle cells (VSMCs) [22] and endothelial cells [23], there is a paucity of information on the functional role of TRPM2 in the vascular system.

Vascular smooth muscle cell handling of Ca^{2+} and Na^{+}, which are critically involved in vascular function, involve various transporters, channels and exchangers. Of these the

plasmalemmal sodium-calcium exchanger (NCX) is particularly important because its activity may be bimodal. In the forward mode NCX activation promotes Na^+ influx and Ca^{2+} extrusion, however positive membrane potential and increased intracellular Na^+ favour reverse mode NCX activation causing Ca^{2+} influx and increased $[Ca^{2+}]_i$ [24, 25]. Although reverse mode NCX has been demonstrated in endothelial cells [26], there has been debate regarding the influence of NCX operating in reverse mode in VSMCs and its role in vascular function is unclear [27, 28].

Here we tested the hypothesis that ROS regulate TRPM2-induced Ca^{2+} and Na^+ transport in VSMCs and that in hypertension oxidative stress causes increased activation of TRPM2 with augmented Ca^{2+} and Na^+ influx, processes that may in turn influence NCX activation further increasing Ca^{2+} influx, critically important in vascular contraction and function.

METHODS

Please see supplemental text for detailed methods. We confirm that all supporting data are available in the supplemental text and upon request.

Primary culture human vascular smooth muscle cells

VSMCs from normotensive (NT) (n=9) and hypertensive (HT) subjects (n=7) were studied (supplemental table 1). Ethics approval was obtained from the West of Scotland Research Ethics Service (WS/12/0294). All subjects gave informed signed consent. Vascular tissue was obtained from NT and HT subjects undergoing elective maxillofacial surgery at the Craniofacial/Oral & Maxillofacial Unit, Queen Elizabeth University Hospital, Glasgow. Isolated small arteries were dissected and VSMCs cultured as we have previously decribed [29]. Hypertension was defined as blood pressure >140/90mmHg or a history of hypertension on antihypertensive treatment according to clinical notes.

Experimental protocols.

VSMCs were stimulated with Ang II in the absence and presence of pharmacological inhibitors of PARP1-TRPM2 (2-APB, olaparib, 8-Br-cADPR) and NCX (benzamil (forward/reverse mode) and KB-R7943, YM-244769 (reverse mode)). In some experiments, VSMC were pretreated with PEG-catalase.

Measurement of reactive oxygen species

NADPH-mediated ROS generation in VSMCs was measured by enhanced lucigenin chemiluminescence. ROS production was expressed as relative luminescence units (RLU)/µg protein. H_2O_2 was assessed with Amplex Red assay kit. H_2O_2 levels were corrected by protein concentration.

Calcium (Ca^{2+})and sodium (Na^{+}) influx

Intracellular Ca^{2+} and Na^{+} levels were measured in VSMCs using the fluorescent Ca^{2+} indicator, Cal-520 acetoxymethyl ester (Cal-520/AM; Abcam; 10 µmol/L) and Asante NaTRIUM Green-2, (Abcam; 10 µmol/L) respectively.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated. cDNA was generated from total RNA and real-time PCR was performed.

Immunoblotting

Total protein was extracted from VSMCs, separated by PAGE and transferred onto nitrocellulose membrane. Membranes were probed with primary antibodies (anti-myosin light chain (phospho S20), anti-TRPM2, anti-alpha tubulin, anti-β-actin). After incubation with secondary fluorescence-coupled antibodies, signals were visualized by an infrared laser scanner (Odyssey Clx, LICOR). Protein expression levels were normalized to loading controls and expressed as percentage (%) of the control.

PARP Activity

PARP activity was assessed based on the detection of biotinylated poly (ADP-ribose) deposited by PARP-1 onto immobilized histones.

Mouse vascular functional studies

Vascular functional studies were performed in isolated small arteries from male transgenic mice, which express human renin under the control of the transthyretin promoter (LinA3 mice) and their wild-type littermates on an C57BL/6 background (aged 4-5 months)[37]. LinA3 mice develop hypertension over the course of their lifespan as we previously described [37]. Second-order branches of mesenteric arteries were isolated from WT and LinA3 mice and mounted on a wire myograph. Contractile responses mediated by Ang II, U46619 and phenylephrine were evaluated in endothelium-intact arteries. In some experiments, vessels were pretreated with TRPM2 inhibitors (2-APB, olaparib, 8-Br-cADPR) and NCX inhibitors (benzamil, KB-R7943).

Statistical Analysis

Data are expressed as the means±standard error (SE). Statistical significance was determined by *t*-test or analysis of variance (ANOVA) and Tukey's post hoc test using GraphPad Prism 5 software, as appropriate. Two-way ANOVA with Bonferroni post-test was used to compare Emax and pD2 for concentration-response curves. $p<0.05$ was statistically significant. Using GraphPad Prism® our data passed in different normality (Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test, Kolmogorov-Smirnov test) and variance tests.

RESULTS

Ang II-stimulated Ca^{2+} influx involves H_2O_2 and TRPM2 in VSMCs from HT patients.

To establish whether VSMCs from HT individuals exhibit oxidative stress we measured ROS levels by assessing NADPH-dependent O_2- production and H_2O_2 levels in VSMCs. As demonstrated in figure 1, basal levels of O_2- and H_2O_2 are increased in VSMCs from HT

patients when compared to cells from NT subjects (Figure 1 A, B). This increase in ROS was associated with a significant increase in Ca^{2+} transients induced by Ang II in NT and HT VSMCs, with significantly enhanced responses in HT VSMCs (Figure 1 C). PEG-catalase, which catalyzes H_2O_2 to H_2O and O_2, reduced $[Ca^{2+}]_i$ in HT, without effect in NT VSMCs (Figure 1D), suggesting that Ca^{2+} transients are influenced by intracellular ROS. The Ca^{2+} selective ionophore ionomycin (10^{-6} mol/L) was used as a positive control in our experiments (supplemental figure 1A).

To assess whether TRPM2 and PARP1 play a role in Ang II-induced Ca^{2+} influx, cells were pretreated with 2-APB and 8-Br-cADPR, which inhibit TRPM2 activity or olaparib, a PARP inhibitor. Enhanced Ang-II induced Ca^{2+} influx in HT VSMCs was reduced in the presence of TRPM2/PARP inhibitors (Figures 1 E). In NT cells only 2-APB reduced Ca^{2+} influx, whereas in HT cells, Ang II-stimulated Ca^{2+} transients were reduced by 2-APB, 8-Br-cADPR and olaparib.

As demonstrated in figure 2A, TRPM2 was expressed in NT and HT VSMCs, but there was no difference in the expression profile observed between these groups. Basal activity of the key protein involved in TRPM2 activation, PARP was increased in HT VSMCs (Figure 2B). In the presence of Ang II, PARP activity was increased in NT and HT VSMCs. These effects were attenuated by PEG-catalase in HT but not NT cells.

To verify the ability of these drugs to inhibit TRPM2, we assessed effects of pharmacological inhibitors in HEK cells overexpressing TRPM2 (TRPM2-HEK cells) (Supplemental figure 2C). H_2O_2 stimulated Ca^{2+} influx in TRPM2-HEK cells with no effect in control HEK cells (Supplemental figure 2A). The increase in Ca^{2+} influx in TRPM2-HEK cells was reduced in the presence of TRPM2 inhibitors 2-APB, 8-Br-cADPR and olaparib (Supplemental figure 2B).

Increased Na^+ influx in VSMCs from HT subjects involves TRPM2

TRPM2 is also permeable to Na^+ which in turn may influence Ca^{2+} influx by altering NCX function. Na^+ influx was measured in live VSMCs by FACS after stimulation with Ang II (10^{-7} mol/L). In cells isolated from NT patients no difference was observed in Na^+ influx after Ang II stimulation (Figure 3A). On the other hand, Ang II increased Na^+ influx in cells isolated from HT patients, effect not observed in the presence of the TRPM2 inhibitors olaparib and 8-Br-cADPR (Figure 3B).

Na^+ influx was also assessed by fluorescence microscopy and live cell imaging. The Na^+ selective ionophore SQI-Pr 40 ($4x10^{-5}$ mol/L) was used as a positive control (supplemental figure 1B). Na^+ influx was assessed by measuring $[Na^+]_i$ in the absence (0 to 1 min) and presence of 150 mM Na^+ (1 to 10 min). The switch in Na^+ concentration (from low to high) induces a slow and sustained increase in Na^+ influx. Using this approach we measured the magnitude of Na^+ influx in cells from NT and HT patients in basal conditions and in the presence of TRPM2 inhibitors. Addition of extracellular Na^+ induced Na^+ influx in cells from NT and HT subjects (Supplemental figure 3A). Maximal responses/AUC were higher in HT versus NT cells. TRPM2 and PARP inhibitors did not significantly alter Ang II-induced $[Na^+]_i$ in NT cells (Supplemental figure 3B), but significantly reduced Na^+ responses in HT VSMCs to levels similar to those in control VSMCs (Supplemental figure 3C).

Since NCX operation depends on the intracellular levels of Na^+, we questioned if TRPM2-induced Na^+ influx influences NCX function in reverse mode, which promotes Ca^{2+} influx [39]. To address this, Ang II-stimulated Ca^{2+} influx was measured in VSMCs in the presence and absence of extracellular Na^+. As shown in Figure 4A, increased Ca^{2+} transients in HT VSMCs were reduced in Na^+-free conditions.

To investigate the role of NCX in increased Ang II-stimulated Ca^{2+} influx in HT cells, Ca^{2+} was measured in the presence of NCX inhibitors. Figures 4B-C demonstrate that the non-

specific NCX inhibitor benzamil and inhibitors of reverse mode of NCX, KB-R7993 and YM-244769, reduced Ca^{2+} responses only in HT VSMCs.

Redox-sensitive TRPM2 and NCX influence vascular signalling

Phosphorylation of MLC is an important step involved in VSMC contraction, migration and cytoskeletal organization and is dependent on increased $[Ca^{2+}]_i$ [40]. Considering the involvement of TRPM2/NCX in enhanced Ca^{2+} influx in VSMCs, we next evaluated whether MLC phosphorylation in cells stimulated with Ang II involves TRPM2 and NCX. Ang II induced a significant increase in MLC20 phosphorylation, with maximal responses at 5 minutes. Ang II-induced MLC20 phosphorylation was significantly greater in HT versus NT VSMCs (Figure 5A). Pretreatment of cells with 8-Br-cADPR, 2-APB or KB-R7943 attenuated Ang II-stimulated phosphorylation of MLC20, especially in HT VSMCs (Figures 5 B-D).

Vascular dysfunction in LinA3 HT mice involves TRPM2 and NCX

To evaluate whether our cell-based findings are recapitulated in whole vessels, we studied intact small arteries from LinA3 mice, an experimental model of human hypertension as we previously reported [37, 41]. Figure 6A demonstrates that TRPM2 and NCX are present in mouse vessels, with greater expression in LinA3 mice versus WT controls. Similar to human cells, ROS generation was higher in VSMCs cells from LinA3 mice versus WT (Supplemental figure 4).

Vascular function was assessed by wire myography and showed that contractile responses to U46619 (Figure 6B), Ang II (Supplemental figure 5A) and phenylephrine (Supplemental figure 6A) were increased in LinA3 mice versus controls. Exposure of vessels to 2-APB (Figure 6B, supplemental figure 5B, 6B), olaparib (Figure 6C, supplemental figure 6C) and 8-Br-cADPR (Figure 6D, supplemental figure 5C) attenuated agonist-stimulated hypercontractile responses in LinA3 mice. Inhibition of NCX (benzamil) (Figure 6E,

supplemental figure 5D) and NCX operating in reverse mode (KB-R7943) (Figure 6F) and YM-244769 (supplemental figure 5E) reversed vascular dysfunction in HT mice.

DISCUSSION

Major findings from the present study demonstrate that vascular oxidative stress in hypertension is associated with increased ROS-regulated influx of Ca^{2+} and Na^{+} through TRPM2- and NCX-dependent mechanisms. These molecular processes influenced signaling in VSMCs from hypertensive patients and were associated with increased vascular contraction in experimental models of human hypertension. Our findings, in clinically-relevant tissue, identify a novel pathway involving redox-sensitive TRPM2, which influences cellular Ca^{2+} and Na^{+} homeostasis in part through NCX, important in the regulation of vascular function in hypertension (Supplemental figure 7).

Reactive oxygen species are increasingly being recognized as second messengers that influence various downstream signalling molecules including Ca^{2+}. On the other hand, Ca^{2+} regulates mitochondrial- and Nox-derived ROS generation, indicating important interplay between Ca^{2+} and redox signalling [42]. Furthermore cross-talk between mitochondrial ROS and endoplasmic reticular Ca^{2+} form positive reciprocal loops involved in vascular injury and dysfunction [43]. Oxidative stress promotes Ca^{2+} influx and intracellular Ca^{2+} mobilization, leading to increased $[Ca^{2+}]_i$ and activation of Ca^{2+}-dependent processes including contraction. Many molecular mechanisms have been implicated in ROS-regulated Ca^{2+} and vascular function, including activation of L- and T-type Ca^{2+} channels, Ca^{2+}/Mg^{2+} ATPase, SERCA, Ca^{2+} exchangers and members of the TRP channel family[44-49].

Of the many types of Ca^{2+} channels regulated by ROS, TRPM2 is particularly important because it is highly sensitive to changes in intracellular levels of H_2O_2. However there is a paucity of information regarding molecular mechanisms linking ROS, TRPM2 and $[Ca^{2+}]_i$ and

the role of TRPM2 in vascular (dys)function in hypertension is unknown. In the present study we unravel some of these processes and show that in VSMCs from hypertensive patients, enhanced Ang II-induced Ca^{2+} influx is ameliorated by PEG-catalase, 2-APB, olaparib and 8-Br-cADPR, suggesting that H_2O_2, TRPM2, PARP and ADPR contribute to increased $[Ca^{2+}]_i$ in hypertension. These phenomena were associated with activation of pro-contractile signalling pathways, as demonstrated by increased phosphorylation of MLC20, effects reversed by TRPM2 inhibitors.

Associated with oxidative stress and enhanced Ca^{2+} transients in HT VSMCs, was an increase in activation of redox-sensitive PARP1, a key regulator of TRPM2. This was ameliorated by PEG-catalase, indicating the importance of H_2O_2 in PARP1-related processes. To assess the functional significance of these molecular systems, we studied intact arteries from mouse models that recapitulate human hypertension. Vascular contraction was enhanced in LinA3 hypertensive mice, similar to what has been previously described in other models of Ang II-induced hypertension [50, 51]. Vascular hypercontractility in LinA3 mice was attenuated by 2-APB, 8-br and olaparib. Together our human *in vitro* and experimental *ex vivo* studies highlight an important role for redox-regulated PARP1-TRPM2 modulation of Ca^{2+} that contributes to vascular hypercontractility in hypertension. In this context PARP1-regulated TRPM2 may be an important point of crosstalk between vascular redox and Ca^{2+} signalling.

Although TRPM2 is typically characterized as a Ca^{2+} channel, it also regulates transmembrane Na^+ transport. This was confirmed in our studies where increased $[Ca^{2+}]_i$ was associated with enhanced Na^+ influx in VSMCs from HT patients, an effect that was repressed by TRPM2 inhibitors. Moreover, changes in Ca^{2+} transients are dependent on Na^+, because Na^+ depletion prevented TRPM2-induced Ca^{2+} influx. These findings demonstrate tight coupling between VSMC Na^+ and Ca^{2+} homeostasis. Mechanisms linking these processes may involve NCX, an antiporter that can operate in forward or reverse mode, depending on the

combined effects of Na^+ and Ca^{2+} gradients [24, 25]. Increased $[Na^+]_i$ activates the reverse mode of NCX, allowing Ca^{2+} entry via the exchanger into the VSMCs [52, 53]. We found that inhibition of reverse-mode NCX prevented an increase in Ca^{2+} influx and phosphorylation of MLC20 in HT VSMCs, suggesting that ROS-regulated TRPM2-mediated Ca^{2+} and Na^+ influx may promote reverse-mode activation of NCX, which further increases Ca^{2+} influx in hypertension. In support of this notion, we observed that vessels from LinA3 hypertensive mice have increased RNA levels of NCX and that inhibition of reverse-mode NCX attenuated vascular hypercontractility. These processes only become evident in pathological conditions, possibly when oxidative stress is increased, because VSMCs from NT subjects and vessels from wildtype control mice did not exhibit NCX- regulated Ca^{2+} changes.

Supporting our paradigm, others have shown in dendritic cells that NCX is a link between Na^+ and Ca^{2+} influx [54]. In addition, recent studies demonstrated that Na^+ accumulates in the instertitium and promotes inflammation in part through NCX-related mechanisms [55-57]. In dendritic cells, Na^+ entry is mediated through an amiloride-inhibitable Na^+ channel leading to Ca^{2+} influx via NCX operating in reverse mode. This leads to protein kinase C activation, phosphorylation of $p47^{phox}$ and ROS production, effects prevented by NCX inhibition [54]. These findings corroborate our results and provide some new insights into how elevated $[Na^+]_i$ contributes to enhanced Ca^{2+} influx and consequent vascular dysfunction in hypertension.

To probe TRPM2 in our study, we used a number of pharmacological agents that inhibit activation of TRPM2 at multiple levels: 2-APB is a channel blocker, olaparib is a PARP inhibitor and 8-Br-cADPR is a cyclic ADP-ribose inhibitor. While these agents may have some non-specificity, we verified in TRPM2 overexpressing HEK cells that they do inhibit ROS-induced Ca^{2+} influx in a TRPM2-dependent manner. Accordingly, notwithstanding the limitations of pharmacological inhibitors, we believe that targeting TRPM2 using a multipronged drug approach, as we have done in the present study, is an acceptable model to

interrogate TRPM2 in human VSMCs. However, we cannot exclude the possibility that a component of TRPM2-independent processes may also contribute to the findings in our study.

In conclusion, we define a novel molecular pathway involving redox-sensitive TRPM2 and NCX, which influence VSMC Na^+ and Ca^{2+} homeostasis, important in the regulation of vascular function in hypertension. We suggest that TRPM2 may be an important point of cross-talk between redox and cation (Ca^{2+}/Na^+) signaling in VSMCs and that in hypertension oxidative stress promotes activation of the TRPM2/NCX axis leading to perturbed Ca^{2+} handling and altered vascular function.

PERSPECTIVES

We demonstrate important interplay between redox and Ca^{2+} signaling through TRPM2 in VSMCs. In pathological conditions associated with oxidative stress, such as hypertension, ROS-regulated TRPM2 is activated leading to perturbed Ca^{2+} and Na^+ handling in part through NCX. We define a novel TRPM2/NCX pathway that links key molecular players (ROS, Ca^{2+} and Na^+) involved in vascular dysfunction in hypertension. Targeting dysregulated redox-sensitive TRPM2 may ameliorate vascular dysfunction in hypertension. Our findings have clinical relevance because unlike most molecular studies that rely on cell lines or rodent VSMCs, we examined human VSMCs from clinically phenotyped patients.

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DISCLOSURES

None.

REFERENCES

1. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jimenez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P, American Heart Association Statistics C, Stroke Statistics S. Heart disease and stroke statistics-2017 update: A report from the american heart association. *Circulation*. 2017;135:e146-e603
2. Lopes RA, Neves KB, Tostes RC, Montezano AC, Touyz RM. Downregulation of nuclear factor erythroid 2-related factor and associated antioxidant genes contributes to redox-sensitive vascular dysfunction in hypertension. *Hypertension*. 2015;66:1240-1250

3. Bruder-Nascimento T, Ferreira NS, Zanotto CZ, Ramalho F, Pequeno IO, Olivon VC, Neves KB, Alves-Lopes R, Campos E, Silva CA, Fazan R, Carlos D, Mestriner FL, Prado D, Pereira FV, Braga T, Luiz JP, Cau SB, Elias PC, Moreira AC, Camara NO, Zamboni DS, Alves-Filho JC, Tostes RC. Nlrp3 inflammasome mediates aldosterone-induced vascular damage. *Circulation*. 2016;134:1866-1880

4. Fisher SA. Smooth muscle phenotypic diversity: Effect on vascular function and drug responses. *Adv Pharmacol*. 2017;78:383-415

5. Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A, Montezano AC. Vascular smooth muscle contraction in hypertension. *Cardiovasc Res*. 2018

6. Nagamine K, Kudoh J, Minoshima S, Kawasaki K, Asakawa S, Ito F, Shimizu N. Molecular cloning of a novel putative ca2+ channel protein (trpc7) highly expressed in brain. *Genomics*. 1998;54:124-131

7. Miller BA, Wang J, Hirschler-Laszkiewicz I, Gao E, Song J, Zhang XQ, Koch WJ, Madesh M, Mallilankaraman K, Gu T, Chen SJ, Keefer K, Conrad K, Feldman AM, Cheung JY. The second member of transient receptor potential-melastatin channel family protects hearts from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2013;304:H1010-1022

8. Venkatachalam K, Montell C. Trp channels. *Annu Rev Biochem*. 2007;76:387-417

9. Uemura T, Kudoh J, Noda S, Kanba S, Shimizu N. Characterization of human and mouse trpm2 genes: Identification of a novel n-terminal truncated protein specifically expressed in human striatum. *Biochem Biophys Res Commun*. 2005;328:1232-1243

10. Owsianik G, Talavera K, Voets T, Nilius B. Permeation and selectivity of trp channels. *Annu Rev Physiol*. 2006;68:685-717

11. Yu P, Xue X, Zhang J, Hu X, Wu Y, Jiang LH, Jin H, Luo J, Zhang L, Liu Z, Yang W. Identification of the adpr binding pocket in the nudt9 homology domain of trpm2. *J Gen Physiol*. 2017;149:219-235

12. Perraud AL, Fleig A, Dunn CA, Bagley LA, Launay P, Schmitz C, Stokes AJ, Zhu Q, Bessman MJ, Penner R, Kinet JP, Scharenberg AM. Adp-ribose gating of the calcium-permeable ltrpc2 channel revealed by nudix motif homology. *Nature*. 2001;411:595-599

13. Kuhn F, Kuhn C, Luckhoff A. Different principles of adp-ribose-mediated activation and opposite roles of the nudt9 homology domain in the trpm2 orthologs of man and sea anemone. *Front Physiol*. 2017;8:879

14. Magnone M, Bauer I, Poggi A, Mannino E, Sturla L, Brini M, Zocchi E, De Flora A, Nencioni A, Bruzzone S. Nad+ levels control ca2+ store replenishment and mitogen-induced increase of cytosolic ca2+ by cyclic adp-ribose-dependent trpm2 channel gating in human t lymphocytes. *J Biol Chem*. 2012;287:21067-21081

15. Wang Q, Huang L, Yue J. Oxidative stress activates the trpm2-ca(2+)-camkii-ros signaling loop to induce cell death in cancer cells. *Biochim Biophys Acta*. 2017;1864:957-967

16. Jiang Q, Gao Y, Wang C, Tao R, Wu Y, Zhan K, Liao M, Lu N, Lu Y, Wilcox CS, Luo J, Jiang LH, Yang W, Han F. Nitration of trpm2 as a molecular switch induces autophagy during brain pericyte injury. *Antioxid Redox Signal*. 2017;27:1297-1316

17. Wang G, Cao L, Liu X, Sieracki NA, Di A, Wen X, Chen Y, Taylor S, Huang X, Tiruppathi C, Zhao YY, Song Y, Gao X, Jin T, Bai C, Malik AB, Xu J. Oxidant sensing by trpm2 inhibits neutrophil migration and mitigates inflammation. *Dev Cell*. 2016;38:453-462

18. Ru X, Yao X. Trpm2: A multifunctional ion channel for oxidative stress sensing. *Sheng Li Xue Bao*. 2014;66:7-15

19. Keckeis S, Wernecke L, Salchow DJ, Reichhart N, Strauss O. Activation of a ca(2+)-dependent cation conductance with properties of trpm2 by reactive oxygen species in lens epithelial cells. *Exp Eye Res*. 2017;161:61-70

20. Sousa FG, Matuo R, Soares DG, Escargueil AE, Henriques JA, Larsen AK, Saffi J. Parps and the DNA damage response. *Carcinogenesis*. 2012;33:1433-1440

21. Wei H, Yu X. Functions of parylation in DNA damage repair pathways. *Genomics Proteomics Bioinformatics*. 2016;14:131-139

22. Alonso-Carbajo L, Kecskes M, Jacobs G, Pironet A, Syam N, Talavera K, Vennekens R. Muscling in on trp channels in vascular smooth muscle cells and cardiomyocytes. *Cell Calcium*. 2017;66:48-61

23. Hecquet CM, Ahmmed GU, Vogel SM, Malik AB. Role of trpm2 channel in mediating h2o2-induced ca2+ entry and endothelial hyperpermeability. *Circ Res*. 2008;102:347-355

24. Lillo MA, Gaete PS, Puebla M, Ardiles NM, Poblete I, Becerra A, Simon F, Figueroa XF. Critical contribution of na(+)-ca(2+) exchanger to the ca(2+)-mediated vasodilation activated in endothelial cells of resistance arteries. *FASEB J*. 2017

25. Moriguchi S, Kita S, Fukaya M, Osanai M, Inagaki R, Sasaki Y, Izumi H, Horie K, Takeda J, Saito T, Sakagami H, Saido TC, Iwamoto T, Fukunaga K. Reduced expression of na(+)/ca(2+) exchangers is associated with cognitive deficits seen in alzheimer's disease model mice. *Neuropharmacology*. 2017

26. Szewczyk MM, Davis KA, Samson SE, Simpson F, Rangachari PK, Grover AK. Ca2+-pumps and na2+-ca2+-exchangers in coronary artery endothelium versus smooth muscle. *J Cell Mol Med*. 2007;11:129-138

27. Viatchenko-Karpinski S, Terentyev D, Jenkins LA, Lutherer LO, Gyorke S. Synergistic interactions between ca2+ entries through l-type ca2+ channels and na+-ca2+ exchanger in normal and failing rat heart. *J Physiol*. 2005;567:493-504

28. Sobie EA, Cannell MB, Bridge JH. Allosteric activation of na+-ca2+ exchange by l-type ca2+ current augments the trigger flux for sr ca2+ release in ventricular myocytes. *Biophys J*. 2008;94:L54-56

29. Montezano AC, Lopes RA, Neves KB, Rios F, Touyz RM. Isolation and culture of vascular smooth muscle cells from small and large vessels. *Methods Mol Biol*. 2017;1527:349-354

30. Hermosura MC, Cui AM, Go RC, Davenport B, Shetler CM, Heizer JW, Schmitz C, Mocz G, Garruto RM, Perraud AL. Altered functional properties of a trpm2 variant in guamanian als and pd. *Proc Natl Acad Sci U S A*. 2008;105:18029-18034

31. Togashi K, Inada H, Tominaga M. Inhibition of the transient receptor potential cation channel trpm2 by 2-aminoethoxydiphenyl borate (2-apb). *Br J Pharmacol*. 2008;153:1324-1330

32. Bian C, Zhang C, Luo T, Vyas A, Chen SH, Liu C, Kassab MA, Yang Y, Kong M, Yu X. Nadp(+) is an endogenous parp inhibitor in DNA damage response and tumor suppression. *Nat Commun*. 2019;10:693

33. Wang X, Takeya K, Aaronson PI, Loutzenhiser K, Loutzenhiser R. Effects of amiloride, benzamil, and alterations in extracellular na+ on the rat afferent arteriole and its myogenic response. *Am J Physiol Renal Physiol*. 2008;295:F272-282

34. Hoyt KR, Arden SR, Aizenman E, Reynolds IJ. Reverse na+/ca2+ exchange contributes to glutamate-induced intracellular ca2+ concentration increases in cultured rat forebrain neurons. *Mol Pharmacol*. 1998;53:742-749

35. Iwamoto T, Kita S. Ym-244769, a novel na+/ca2+ exchange inhibitor that preferentially inhibits ncx3, efficiently protects against hypoxia/reoxygenation-induced sh-sy5y neuronal cell damage. *Mol Pharmacol*. 2006;70:2075-2083

36. Eraslan E, Tanyeli A, Polat E, Polat E. 8-br-cadpr, a trpm2 ion channel antagonist, inhibits renal ischemia-reperfusion injury. *J Cell Physiol*. 2019;234:4572-4581

37. Touyz RM, Mercure C, He Y, Javeshghani D, Yao G, Callera GE, Yogi A, Lochard N, Reudelhuber TL. Angiotensin ii-dependent chronic hypertension and cardiac hypertrophy are unaffected by gp91phox-containing nadph oxidase. *Hypertension*. 2005;45:530-537

38. Group NCRRGW. Animal research: Reporting in vivo experiments: The arrive guidelines. *J Physiol*. 2010;588:2519-2521

39. Andrikopoulos P, Kieswich J, Harwood SM, Baba A, Matsuda T, Barbeau O, Jones K, Eccles SA, Yaqoob MM. Endothelial angiogenesis and barrier function in response to thrombin require ca2+ influx through the Na+/Ca2+ exchanger. *J Biol Chem*. 2015;290:18412-18428

40. Takashima S. Phosphorylation of myosin regulatory light chain by myosin light chain kinase, and muscle contraction. *Circ J*. 2009;73:208-213

41. Burger D, Reudelhuber TL, Mahajan A, Chibale K, Sturrock ED, Touyz RM. Effects of a domain-selective ace inhibitor in a mouse model of chronic angiotensin ii-dependent hypertension. *Clin Sci (Lond)*. 2014;127:57-63

42. Bertero E, Maack C. Calcium signaling and reactive oxygen species in mitochondria. *Circ Res*. 2018;122:1460-1478

43. Song T, Zheng YM, Wang YX. Cross talk between mitochondrial reactive oxygen species and sarcoplasmic reticulum calcium in pulmonary arterial smooth muscle cells. *Adv Exp Med Biol*. 2017;967:289-298

44. Olschewski A, Weir EK. Redox regulation of ion channels in the pulmonary circulation. *Antioxid Redox Signal*. 2015;22:465-485

45. Ma R, Chaudhari S, Li W. Canonical transient receptor potential 6 channel: A new target of reactive oxygen species in renal physiology and pathology. *Antioxid Redox Signal*. 2016;25:732-748

46. Touyz RM. Reactive oxygen species as mediators of calcium signaling by angiotensin ii: Implications in vascular physiology and pathophysiology. *Antioxid Redox Signal*. 2005;7:1302-1314

47. Suresh K, Servinsky L, Reyes J, Baksh S, Undem C, Caterina M, Pearse DB, Shimoda LA. Hydrogen peroxide-induced calcium influx in lung microvascular endothelial cells involves trpv4. *Am J Physiol Lung Cell Mol Physiol*. 2015;309:L1467-1477

48. Tabet F, Savoia C, Schiffrin EL, Touyz RM. Differential calcium regulation by hydrogen peroxide and superoxide in vascular smooth muscle cells from spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 2004;44:200-208

49. Gao YJ, Lee RM. Hydrogen peroxide induces a greater contraction in mesenteric arteries of spontaneously hypertensive rats through thromboxane a(2) production. *Br J Pharmacol*. 2001;134:1639-1646

50. Bressan AF, Fonseca GA, Tostes RC, Webb RC, Lima VV, Giachini FR. Interleukin-10 negatively modulates extracellular signal-regulated kinases 1 and 2 in aorta from hypertensive mouse induced by angiotensin ii infusion. *Fundam Clin Pharmacol*. 2019;33:31-40

51. Lima VV, Zemse SM, Chiao CW, Bomfim GF, Tostes RC, Clinton Webb R, Giachini FR. Interleukin-10 limits increased blood pressure and vascular rhoa/rho-kinase signaling in angiotensin ii-infused mice. *Life Sci*. 2016;145:137-143

52. Larbig R, Torres N, Bridge JH, Goldhaber JI, Philipson KD. Activation of reverse na+-ca2+ exchange by the na+ current augments the cardiac ca2+ transient: Evidence from ncx knockout mice. *J Physiol*. 2010;588:3267-3276

53. Shattock MJ, Ottolia M, Bers DM, Blaustein MP, Boguslavskyi A, Bossuyt J, Bridge JH, Chen-Izu Y, Clancy CE, Edwards A, Goldhaber J, Kaplan J, Lingrel JB, Pavlovic D, Philipson K, Sipido KR, Xie ZJ. Na+/ca2+ exchange and na+/k+-atpase in the heart. *J Physiol*. 2015;593:1361-1382

54. Barbaro NR, Foss JD, Kryshtal DO, Tsyba N, Kumaresan S, Xiao L, Mernaugh RL, Itani HA, Loperena R, Chen W, Dikalov S, Titze JM, Knollmann BC, Harrison DG, Kirabo A. Dendritic cell amiloride-sensitive channels mediate sodium-induced inflammation and hypertension. *Cell Rep*. 2017;21:1009-1020

55. Machnik A, Neuhofer W, Jantsch J, Dahlmann A, Tammela T, Machura K, Park JK, Beck FX, Muller DN, Derer W, Goss J, Ziomber A, Dietsch P, Wagner H, van Rooijen N, Kurtz A, Hilgers KF, Alitalo K, Eckardt KU, Luft FC, Kerjaschki D, Titze J. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-c-dependent buffering mechanism. *Nat Med*. 2009;15:545-552

56. Titze J, Shakibaei M, Schafflhuber M, Schulze-Tanzil G, Porst M, Schwind KH, Dietsch P, Hilgers KF. Glycosaminoglycan polymerization may enable osmotically inactive na+ storage in the skin. *Am J Physiol Heart Circ Physiol*. 2004;287:H203-208

57. Titze J, Machnik A. Sodium sensing in the interstitium and relationship to hypertension. *Curr Opin Nephrol Hypertens*. 2010;19:385-392

NOVELTY AND SIGNIFICANCE

What Is New?

This study defines a novel molecular pathway involving redox-sensitive TRPM2 and NCX, which influence VSMC Na^+ and Ca^{2+} homeostasis, important in the regulation of vascular function in hypertension.

What Is Relevant?

- Redox-sensitive TRPM2 and NCX play a role in the regulation of Ca^{2+} and Na^+ influx in human vascular smooth muscle cells.
- Increased vascular oxidative stress in hypertension promotes activation of redox-regulated TRPM2, increased influx of Ca^{2+} and Na^+ and activation of reverse mode NCX, which further increases $[Ca^{2+}]_i$.
- We define a novel mechanism linking ROS, Ca^{2+} and Na^+ through TRPM2 and NCX, which when perturbed, such as in hypertension, leads to vascular dysfunction.

Summary

TRPM2 may be an important point of cross-talk between redox and cation (Ca^{2+}/Na^+) signaling in VSMCs and that in hypertension oxidative stress promotes activation of the TRPM2/NCX axis leading to abnormal Ca^{2+} handling and altered vascular contraction.

FIGURE LEGENDS

Figure 1. Increased Ang II-induced Ca^{2+} influx in VSMCs from HT subjects involves TRPM2 signaling. ROS generation was measured in VSMCs from NT and HT subjects using lucigenin assay (A) and Amplex Red (B). Ca^{2+} influx (Cal-520 AM) (C-E) was measured in VSMCs in the presence of vehicle (1min) and Ang II 10^{-7} mol/l (2min). The area under the curve (AUC) was used for statistical analysis (C,D,E). Cells were pre-traeted with 2-APB

547 ($3x10^{-5}$ mol/L), 8-Br-cADPR (10^{-6} mol/L), olaparib (10^{-6} mol/L) and PEG-Catalase (1000
548 U/ml) for 30mins. Figures 1A, 1B - data are normalized by control, considered as 100 %. Bars
549 represent the mean±SEM (n=7–9). *P<0.05 NT *vs* HT (A-C) and drug *vs* vehicle (D-E).

550 **Figure 2. Increased PARP activity in HT VSMCs involves H_2O_2 signaling.** (A) TRPM2
551 expression in VSMCs from NT and HT subjects. (B) PARP activity, assessed by incorporation
552 of biotinylated ADP-ribose to histone proteins, in VSMCs in basal and Ang II-stimulated
553 conditions in the presence or absence of PEG-Catalase (1000 U/ml, 30min pre-treatment). Bars
554 are mean±SEM (n=7–9). *P<0.05. NT: Normotensive. HT: Hypertensive

555 **Figure 3. Angiotensin-II induced Na^+ influx in VSMCs from hypertensive subjects**
556 **involves TRPM2 channel.** Na^+ influx was measured using the cytosolic Na^+ indicator
557 NaTRIUM Green™-2 AM in FACS. Cells were pre-treated (30mins) with 8-Br-cADPR (10^{-6}
558 mol/L) and olaparib (10^{-6} mol/L). Bars represent mean±SEM (n=6). *P<0.05.

559 **Figure 4. Increased Ang II-induced Ca^{2+} influx in VSMCs from HT subjects is not**
560 **observed in Na^+-free medium and is reversed by NCX inhibitors.** Ca^{2+} influx (Cal-520
561 AM) (A-C) was measured in VSMCs. Influx of Ca^{2+} was assessed by measuring $[Ca^{2+}]_i$ in the
562 absence (1min) and presence of 150 mM Na^+ (2min) (A) or in the presence of vehicle (1 min)
563 or Ang II 10^{-7} mol/l (2min). Bar graphs are presented as the area under the curve (AUC). Cells
564 were pre-treated with benzamil (10^{-6} mol/L), KB-R7943 (10^{-6} mol/L) and YM 244769 (10^{-6}
565 mol/L) for 30min. Bars represent mean±SEM (n=6–8). *P<0.05. NT: Normotensive. HT:
566 Hypertensive.

567 **Figure 5. Enhanced Ang II-induced phosphorylation of myosin light chain in VSMCs**
568 **from HT subjects is reversed by TRPM2 inhibition.** Myosin light chain (MLC)
569 phosphorylation at serine 20 (PMLC(S20)) was evaluated by immunoblotting in VSMCs (A).
570 VSMCs were pre-treated with 8-Br-cADPR (B), 2-APB (C) and KB-R7943 (D) for 30 min
571 prior to addition of Ang II. Values express MLC phosphorylation and represent the mean±SEM

572 (n=6). *P<0.05. # 5 min Ang II *vs* 5 min Ang II with inhibitor. NT: Normotensive. HT:
573 Hypertensive.

574 **Figure 6. TRPM2 and NCX inhibitors reverse hypertension-associated hypercontractility**
575 **in mesenteric arteries.** mRNA expression (A) was evaluated by real-time PCR in mesenteric
576 arteries from WT and LinA3 mice (B-F). Concentration-response curves to U46619 were
577 performed in mesenteric arteries from wildtype (WT) and hypertensive (LinA3) mice and
578 studied by myography in the presence and absence of 2-APB ($3x10^{-5}$ mol/L), olaparib (10^{-6}
579 mol/L), 8-Br-cADPR (10^{-6} mol/L), benzamil (10^{-6} mol/L) and KB-R7943 (10^{-6} mol/L) (30 min
580 pretreatment). U46619 tension curves (contraction) are expressed in mN and represent the
581 mean±SEM (n=6). *P<0.05 WT *vs* LinA3. # LinA3 *vs* LinA3 with inhibitor.