CROSSTALK BETWEEN VASCULAR REDOX AND CALCIUM SIGNALING IN HYPERTENSION INVOLVES TRPM2 CATION CHANNEL.

Rhéure Alves-Lopes[1], Karla B Neves[1], Aikaterini Anagnostopoulou[1], Francisco J Rios[1], Silvia Lacchini[2], Augusto C Montezano[1], Rhian M Touyz[1]

[1]Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK; [2]Biomedical Sciences Institute, University of São Paulo, Brazil.

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Corresponding authors:

Rhian M Touyz, MD, PhD/ Rhéure Alves-Lopes PhD

Institute of Cardiovascular and Medical Sciences

University of Glasgow

126 University Place

Glasgow, G12 8TA

Email: rhian.touyz@glasgow.ac.uk/Rheure.Lopes@glasgow.ac.uk

Phone: 014 1330-7775/7774

ABSTRACT

Increased generation of reactive oxygen species (ROS) and altered Ca^{2+} handling cause vascular damage in hypertension. Mechanisms linking these systems are unclear but transient receptor potential melastatin 2 (TRPM2) could be important because TRPM2 is a ROS sensor and a regulator of Ca^{2+} and Na^{+} transport. 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 ROS mediated-TRPM2 activation increases $[Ca^{2+}]_i$ through processes involving NCX (Na^{+}/Ca^{2+} exchanger). VSMCs from hypertensive (HT) and normotensive individuals (NT) and isolated arteries from wildtype (WT) and hypertensive mice (LinA3) were studied. Generation of superoxide anion and hydrogen peroxide was increased in HT VSMCs, effects associated with activation of redox-sensitive Poly (ADP-ribose) polymerase 1 (PARP1), a TRPM2 regulator. Angiotensin II (Ang II) increased Ca^{2+} and Na^{+} influx with exaggerated responses in HT. These effects were attenuated by catalase−polyethylene glycol (PEG-catalase) and TRPM2 inhibitors (2-APB, 8-Br-cADPR olaparib). TRPM2 siRNA decreased Ca^{2+} in HT VSMCs. NCX inhibitors (Benzamil, KB-R7943, YM244769) normalized Ca^{2+} hyper-responsiveness and MLC20 phosphorylation in HT VSMCs. In arteries from LinA3 mice, exaggerated agonist (U46619, AngII, phenylephrine)-induced vasoconstriction was decreased by TRPM2 and NCX inhibitors. In conclusion activation of ROS-dependent PARP-1-regulated TRPM2 contributes to vascular Ca^{2+} and Na^{+} influx in part through NCX. We identify a novel pathway linking ROS to Ca^{2+} signaling through TRPM2/NCX in human VSMCs and suggest that oxidative stress-induced upregulation of this pathway may be a new player in hypertension-associated vascular dysfunction.

Key words: calcium signaling; vascular biology; sodium channels; reactive oxygen species; hypertension.

INTRODUCTION

Hypertension is a multifactorial and complex disorder associated with abnormal vascular signaling[1]. Uncontrolled generation of ROS, activation of redox-sensitive signaling 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 [2, 3] . TRPM2 is mainly activated by adenosine diphosphate ribose (ADPR), which has specific residues involved in binding to the NUDT9 homology (NUDT9-H) domain of TRPM2 to open the cation channel [4-6]. In addition to adenosine diphosphate ribose (ADPR), Ca^{2+}, hydrogen peroxide (H_2O_2), calmodulin, nicotinic acid adenine dinucleotide phosphate (NAADP), and oxidation of cysteine residues (Cys549) can positively modulate TRPM2, while AMP, acidic pH and nitration of tyrosine 1485 are negative regulators [7-10]. 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 [10-12]. DNA damage is linked with high and rapid PolyADP-ribosylation activity, also known as PARylation, where Poly (ADP-ribose) polymerase (PARP) repeatedly catalyzes the transfer of successive units of ADPR to target proteins, leading to TRPM2 activation [13, 14]. Although TRPM2 channels are present in VSMCs [15] and endothelial cells [16], 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 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^+ favor reverse mode NCX activation causing Ca^{2+} influx and increased $[Ca^{2+}]_i$ [17, 18]. Although reverse mode NCX has been demonstrated in endothelial cells [19], there has been debate regarding the influence of NCX operating in reverse mode in VSMCs and its role in vascular function is unclear [20, 21].

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. Studies were performed using a multidisciplinary approach including human vascular tissue from normotensive and hypertensive subjects and LinA3 hypertensive mice. LinA3 mice express human prorenin in the liver and consequently have chronic activation of the renin angiotensin aldosterone system (RAAS). This is associated with a progressive increase in blood pressure and by adulthood (16-20 weeks) mice have established hypertension, with associated vascular dysfunction, cardiac hypertrophy and impaired renal function. In humans, essential hypertension develops gradually over many years, with associated Ang II-related cardiovascular damage, effects that are also seen in LinA3 mice. Accordingly, LinA3 mice are useful experimental models that recapitulate human essential hypertension.

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 (n=9) and hypertensive subjects (n=7) were studied (Table S1). 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 described [22, 23]. Hypertension was defined as blood pressure >140/90mmHg or a history of hypertension on antihypertensive treatment according to clinical notes. The definition of hypertension of >140/90 mmHg was based on JNC8 (when the study was commenced) and major guidelines (NICE, European Society of Cardiology/European Society of Hypertension, International Society of Hypertension and American College of Physicians/American Academy of Family Physicians) as recently reviewed [24].

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, VSMCs were pretreated with PEG-catalase to reduce levels of ROS (H_2O_2). In some experiments, TRPM2 was down regulated by siRNA.

Measurement of ROS

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-α 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 tansthyretin promoter (LinA3 mice) and their WT littermates on an C57BL/6 background (aged 4-5 months) [25]. LinA3 mice develop hypertension over the course of their lifespan as we previously described [25]. Systolic blood pressure measured by tail cuff methodology [25] was significantly higher in LinA3 mice versus WT counterparts at 16 weeks (Figure S1). Second-order branches (diameter of 150 – 300 μm) of mesenteric arteries were isolated from WT and LinA3 mice and mounted on a wire myograph. Contractile responses mediated by different vasoactive agonists, 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 maximum response (Emax) and negative logarithm to base 10 of the half maximal effective concentration (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. PEG-catalase did not completely abolish Ang II-induced effects, suggesting that other systems also play a role in enhanced Ca^{2+} responses in hypertension. The Ca^{2+} selective ionophore ionomycin (10^{-6} mol/L) was used as a positive control in our experiments (Figure S2A).

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 and olaparib, a PARP inhibitor. TRPM2 was also downregulated with siRNA. Enhanced Ang II induced Ca^{2+} influx in HT VSMCs was reduced in the presence of TRPM2/PARP inhibitors (Figure 1 E) and in VSMCs in which TRPM2 was downregulated by siRNA (Figure S3). 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.

Multiple TRPM2 isoforms have been identified, including TRPM2-L (full-length functional TRPM2, 171 kDa) and several short splice variants (TRPM2-S, 95 kDa). To assess the TRPM2 isoforms in VSMCs, we evaluated mRNA expression by qPCR and found that the predominant form is TRPM2-L (Figure 2A), corresponding to a molecular size of 171 kDa (Figure 2B).

As shown in figure 2B, TRPM2 was expressed in NT and HT VSMCs, with no difference in the expression profile between groups. Basal activity of the key protein involved in TRPM2 activation, PARP, was increased in HT VSMCs (Figure 2C) and in NT VSMCs in the presence of Ang II. 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 human embryonic kidney (HEK) cells overexpressing TRPM2 (TRPM2-HEK cells) (Figure S4). H_2O_2 stimulated Ca^{2+} influx in TRPM2-HEK cells with no effect in control HEK cells (Figure S4A). 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 (Figure S4B).

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). In these experiments we only used olaparib and 8-Br-cADPR because they more selectively target PARP-TRPM2 than 2-APB.

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 (Figure S4B). 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 (Figure S5A). 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 (Figure S5B), but significantly reduced Na^+ responses in HT VSMCs (Figure S5C). Na^+ responses in the presence of inhibitors in cells from HT subjects (Figure S5C) were similar to responses in cells from NT individuals (Figure S5B).

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 [26]. 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 signaling

Phosphorylation of MLC is an important step involved in VSMC contraction, migration and cytoskeletal organization and is dependent on increased $[Ca^{2+}]_i$ [27]. 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 [25, 28]. Similar to human cells, basal ROS generation was higher in VSMCs from LinA3 mice versus WT (Figure S6). Ang II (60 min) increased ROS production to a greater extent in VSMCs from wildtype than LinA3 mice. Reasons for this may relate to the fact that in LinA3 mice, ROS generation and oxidative stress are already significantly increased in basal conditions, and perhaps the pro-oxidant system is saturated and the Ang II challenge is not able to further stimulate the system, at least at the time points studied.

TRPM2 and NCX are present in mouse vessels, with greater expression in LinA3 mice versus WT controls (Figure S7A). To investigate whether TRPM2 and NCX influence vascular function, we assessed vascular functional responses to various vasoconstrictors in the absence and presence of pharmacological modulators. Vascular function was assessed by wire myography and showed that contractile responses to U46619 (Figure 6A), Ang II (Figure S7B)

and phenylephrine (Figure S8A) were increased in LinA3 mice versus controls. Exposure of vessels to 2-APB (Figure 6B, Figure S7C, 8B), olaparib (Figure 6C, Figure S8C) and 8-Br-cADPR (Figure 6D, Figure S7D) attenuated agonist-stimulated hypercontractile responses in LinA3 mice. Inhibition of NCX (benzamil) (Figure 6E, Figure S7E) and NCX operating in reverse mode (KB-R7943) (Figure 6F) and YM-244769 (Figure S7F) 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 modulates cellular Ca^{2+} and Na^{+} homeostasis in part through NCX, important in the regulation of vascular function in hypertension.

ROS are increasingly being recognized as second messengers that regulate various downstream signaling molecules including Ca^{2+}. On the other hand, Ca^{2+} controls mitochondrial- and Nox-derived ROS generation, indicating important interplay between Ca^{2+} and redox signaling [29]. Furthermore cross-talk between mitochondrial ROS and endoplasmic reticulum Ca^{2+} form positive reciprocal loops involved in vascular injury and dysfunction [30]. 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 [30].

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 signaling 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. Catalase did not abolish effects in NT, suggesting that other systems also influence PARP-1 activity. Additionally, Ang II did not increase PARP activity in HT VSMCs, probably due to the already activated PARP in basal condition relative to the NT VSMCs. To assess the functional significance of these molecular processes, 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 [31, 32]. 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

298 an important point of crosstalk between vascular redox and Ca^{2+} signaling. In addition to
299 influencing vascular function, redox-regulated TRPM2 plays a role in Ang II-induced insulin
300 resistance through processes that involve CaMKII/JNK-dependent signaling pathway[33].
301 Hence, inhibition of TRPM2, besides improving vascular function in hypertension, may also
302 ameliorate hypertension-associated insulin resistance.

303 While results from our study suggest that activation of TRPM2 is involved in vascular
304 damage, TRPM2 effects in myocardial ischemia/reperfusion (I/R) injury are less clear. Hiroi
305 and colleagues reported that knocking out TRPM2 protects the heart against I/R injury [34],
306 whereas Miller and colleagues demonstrated that TRPM2 protects against tissue damage
307 following oxidative stress I/R injury, through processes involving FOXO3, Pyk2
308 phosphorylation and inhibition of ROS production [35, 36]. Reasons for these discrepancies are
309 unclear but may be due to involvement of other TRPM isoforms given that both groups used
310 global TRPM-2 KO mice. In particular, TRPM7 and TRPM8 have been shown to have
311 cardiovascular protective anti-inflammatory and anti-fibrotic effects through processes that
312 decrease ROS production [37-39]. TRPM4, another TRPM isoform has been linked to NCX and
313 Ca^{2+} transport in goblet cells [40].

314 Although TRPM2 is typically characterized as a Ca^{2+} channel, it also regulates
315 transmembrane Na^+ transport. This was confirmed in our studies where increased $[Ca^{2+}]_i$ was
316 associated with enhanced Na^+ influx in VSMCs from HT patients, an effect that was repressed
317 by TRPM2 inhibitors. Moreover, changes in Ca^{2+} transients are dependent on Na^+, because
318 Na^+ depletion prevented TRPM2-induced Ca^{2+} influx. These findings demonstrate tight
319 coupling between VSMC Na^+ and Ca^{2+} homeostasis. Mechanisms linking these processes may
320 involve NCX, an antiporter that can operate in forward or reverse mode, depending on the
321 combined effects of Na^+ and Ca^{2+} gradients [17, 18]. Increased $[Na^+]_i$ activates the reverse mode
322 of NCX, allowing Ca^{2+} entry via the exchanger into the VSMCs [41, 42]. 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 WT 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 [43]. In addition, recent studies demonstrated that Na^{+} accumulates in the interstitium and promotes inflammation in part through NCX-related mechanisms [44-46]. 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[41]. These findings suggest that in dendritic cells NCX is upstream of ROS generation. In our paradigm, NCX was downstream of ROS generation. Together these findings indicate important cross-talk between ROS, NCX, Ca^{2+} and Na^{+}, but suggest that regulatory mechanisms differ in different cell types. It may also be possible that there is a feedforward system where redox-sensitive NCX induces ROS production, which further promotes NCX activation [43]. A potential mediator of this system is TRPM2.

To probe TRPM2 in our study, we used various pharmacological agents that inhibit TRPM2 activation at multiple levels. In particular, 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 inhibit ROS- induced Ca^{2+} influx in a TRPM2-dependent manner. We also found that downregulation

of TRPM2 by siRNA ameliorated Ca^{2+} responses in HT VSMCs. Accordingly, notwithstanding the limitations of pharmacological inhibitors, we believe that targeting TRPM2 using a multipronged 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 our findings.

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.

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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.

562 **FIGURE LEGENDS**

563 **Figure 1. Increased Ang II-induced Ca^{2+} influx in VSMCs from HT subjects involves**
564 **TRPM2 signaling.** ROS generation was measured in VSMCs from NT and HT subjects using
565 lucigenin assay (A) and Amplex Red (B). Ca^{2+} influx (Cal-520 AM) (C-E) was measured in
566 VSMCs in the presence of vehicle (1min) and Ang II 10^{-7} mol/l (2min). The area under the
567 curve (AUC) was used for statistical analysis (C, D, E). Cells were pre-treated with 2-APB
568 ($3x10^{-5}$ mol/L), 8-Br-cADPR (10^{-6} mol/L), olaparib (10^{-6} mol/L) and PEG-Catalase (1000
569 U/ml) for 30mins. Figures 1A, 1B - data are normalized by control, considered as 100 %. Bars
570 represent the mean±SEM (n=6–9). *P<0.05 NT *vs* HT (A-C) and drug *vs* other groups (D-E).
571 **Figure 2. TRPM2 expression and PARP activity in HT VSMCs-role of H_2O_2.** (A) mRNA
572 expression of TRPM2 isoforms in human VSMCs. (B) TRPM2 expression in VSMCs from
573 NT and HT subjects. (B) PARP activity, assessed by incorporation of biotinylated ADP-ribose
574 to histone proteins, in VSMCs in basal and Ang II-stimulated conditions in the presence or
575 absence of PEG-Catalase (1000 U/ml, 30min pre-treatment). Bars are mean±SEM (n=6–9).
576 *P<0.05. NT: Normotensive. HT: Hypertensive
577 **Figure 3. Angiotensin-II induced Na^+ influx in VSMCs from hypertensive subjects**
578 **involves TRPM2 channel.** Na^+ influx was measured using the cytosolic Na^+ indicator
579 NaTRIUM Green™-2 AM in FACS. Cells were pre-treated (30mins) with 8-Br-cADPR (10^{-6}
580 mol/L) and olaparib (10^{-6} mol/L). Bars represent mean±SEM (n=6). *P<0.05.
581 **Figure 4. Increased Ang II-induced Ca^{2+} influx in VSMCs from HT subjects is not**
582 **observed in Na^+-free medium and is reversed by NCX inhibitors.** Ca^{2+} influx (Cal-520
583 AM) (A-C) was measured in VSMCs. Influx of Ca^{2+} was assessed by measuring $[Ca^{2+}]_i$ in the
584 absence (1min) and presence of 150 mM Na^+ (2min) (A) or in the presence of vehicle (1 min)
585 or Ang II 10^{-7} mol/l (2min). To control the osmolarity, in the absence of sodium, choline
586 chloride 150 mM was added to the HEPES. Bar graphs are presented as the area under the

curve (AUC). Cells were pre-treated with benzamil (10^{-6} mol/L), KB-R7943 (10^{-6} mol/L) and YM 244769 (10^{-6} mol/L) for 30min. Bars represent mean±SEM (n=6–8). *P<0.05. NT: Normotensive. HT: Hypertensive.

Figure 5. Enhanced Ang II-induced phosphorylation of myosin light chain in VSMCs from HT subjects is reversed by TRPM2 inhibition. Myosin light chain (MLC) phosphorylation at serine 20 (PMLC(S20)) was evaluated by immunoblotting in VSMCs (A). VSMCs were pre-treated with 8-Br-cADPR (B), 2-APB (C) and KB-R7943 (D) for 30 min prior to addition of Ang II. Values express MLC phosphorylation and represent the mean±SEM (n=5-6). *P<0.05. # 5 min Ang II *vs* 5 min Ang II with inhibitor. NT: Normotensive. HT: Hypertensive.

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