Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Hypertension. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Hypertension. 2015 August ; 66(2): 374–381. doi:10.1161/HYPERTENSIONAHA.115.05274.

Superoxide Enhances Ca^{2+} Entry through L-Type Channels in the Renal Afferent Arteriole

Paul A. Vogel, **Xi Yang**, **Nicholas G. Moss**, and **William J. Arendshorst**

Department of Cell Biology and Physiology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545

Abstract

Reactive oxygen species regulate cardiovascular and renal function in health and disease. Superoxide participates in acute calcium signaling in afferent arterioles and renal vasoconstriction produced by angiotensin II, endothelin, thromboxane and pressure-induced myogenic tone. Known mechanisms by which superoxide acts include quenching of nitric oxide and increased ADP ribosyl cyclase/ryanodine-mediated calcium mobilization. The effect(s) of superoxide on other calcium signaling pathways in the renal microcirculation is poorly understood. The present experiments examined the acute effect of superoxide generated by paraquat on calcium entry pathways in isolated rat afferent arterioles. The peak increase in cytosolic calcium concentration caused by KCl (40 mM) was 99 ± 14 nM. The response to this membrane depolarization was mediated exclusively by L-type channels as it was abolished by nifedipine but was unaffected by the T-type channel blocker mibefradil. Paraquat increased superoxide production (dihydroethidium fluorescence), tripled the peak response to KCl to 314 ± 68 nM ($p<0.001$) and doubled the plateau response. These effects were abolished by tempol and nitroblue tetrazolium, but not by catalase, confirming actions of superoxide and not hydrogen peroxide. Unaffected by paraquat and superoxide was calcium entry through store-operated calcium channels activated by thapsigargin-induced calcium depletion of sarcoplasmic reticular stores. Also unresponsive to paraquat was ryanodine receptor-mediated calcium-induced calcium release from the sarcoplasmic reticulum. Our results provide new evidence that superoxide enhances calcium entry through L-type channels activated by membrane depolarization in rat cortical afferent arterioles, without affecting calcium entry through store operated entry or ryanodine receptor-mediated calcium mobilization.

Keywords

renal; nephrology; reactive oxygen species; superoxide; calcium signaling; calcium entry; hypertension

Editorial Correspondence: William J. Arendshorst, PhD, Department of Cell Biology and Physiology, Room 6341, Medical Biomedical Research Building, CB # 7545, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545, arends@med.unc.edu, Phone: 919-966-1067, FAX: 919-966-6927.

DISCLOSURES

None.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide $\left(\cdot O_2^-\right)$ and hydrogen peroxide (H_2O_2), influence vascular physiology and pathophysiology [1, 2]. $\cdot O_2^-$ in the renal vasculature and tubules is an important negative modulator of nitric oxide (NO), a vasodilator and natriuretic agent, by limiting its availability. Together these two opposing radicals provide an important balance in regulating the magnitude of vasoconstriction, sodium excretion and blood pressure (BP)[1]. In addition to reducing NO bioavailability, $\cdot O_2^-$ acts directly on vascular smooth muscle cells (VSMC) to augment calcium (Ca^{2+}) signaling and enhance vasoconstriction [1, 3, 4]. Our laboratory has observed that $\cdot O_2^-$ participates in and amplifies acute renal vasoconstrictor responses induced by angiotensin II (Ang II) [5], endothelin-1 (ET-1) [6], and thromboxane (TxA_2) [7] and stimulation of cytosolic calcium (Ca^{2+}) in the afferent arteriole by these agents [8, 9]. $\cdot O_2^-$ was implicated as the critical ROS, based on attenuation by dismutation of $\cdot O_2^-$ by tempol. Other investigators have reported that $\cdot O_2^-$ mediates the acute renal vasoconstriction produced by Ang II in normotensive and hypertensive animals with attenuation by antioxidants [5, 10-12]. Moreover, $\cdot O_2^-$ potentiates the strength of the myogenic response of cortical afferent arterioles [13-15].

Excessive vascular and renal $\cdot O_2^-$ lead to vascular dysfunction and/or disturbed salt and water homeostasis [1, 16, 17]. Oxidative stress caused by increased ROS levels and NO deficiency is associated with renal vasoconstriction and the development of Ang II-induced and salt-sensitive hypertension [1, 3, 18-21]. $\cdot O_2^-$ activity is enhanced in NO deficient rats, and contributes to abnormal renal function 1, 22. For example, increased $\cdot O_2^-$ activity is responsible for inducing salt-sensitive hypertension in endothelial NO synthase knockout mice [19, 22]. Administration of superoxide dismutase (SOD) effectively reduces BP in salt-sensitive and salt-independent models of hypertension [1, 23]. Knockout mice deficient in extracellular SOD-3 have a higher basal BP than wild-type mice, a phenotype attributed to higher $\cdot O_2^-$ and decreased NO levels in the kidney [24]. Moreover, chronic Ang II administration produces more pronounced hypertension in SOD-3 deficient mice than in wild-type controls [25].

Ang II-induced hypertension is coupled with oxidative stress in blood vessels [26, 27], and increased renal and non-renal vascular ROS is a common feature in both saltindependent and salt-sensitive hypertension [1, 20, 21, 28]. A ROS-dependent rise in renal vascular resistance (RVR) and BP is observed in Ang II-infused hypertensive mice and rats [29, 30]. Augmented oxidative stress in the spontaneously hypertensive rat (SHR) involves overexpression of NADPH oxidase and loss of extracellular SOD in the kidney [31]. The SOD mimetic tempol normalizes elevated basal RVR and BP and restores endothelial function of renal arteries in the SHR and in the 2 kidney, 1 clip Goldblatt model of renovascular hypertension [32, 33], further implicating $\cdot O_2^-$ in exaggerated renal vasoconstriction and the potentiation of hypertension during oxidative stress [34]. Therefore, increased intrarenal ROS or an abnormal $\cdot O_2^- / NO$ balance can alter renal hemodynamics and sodium excretion to cause hypertension [1].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

The precise mechanism(s) by which $\cdot O_2^-$ affects Ca^{2+} signaling and causes contraction of VSMC in the renal microcirculation is poorly understood. Ca^{2+} signaling studies have linked G-protein coupled receptors (GPCR) for Ang II, ET-1 and catecholamines, and TxA2 to rapid $\cdot O_2^-$ production and sensitization of RyR to mobilize Ca^{2+} from sarcoplasmic reticular stores in the renal vasculature [5, 7, 9, 35]. In cerebral arteries, ROS generation by hypoxanthine/xanthine oxidase (HX/XO), and by Ang II stimulation activates L-type channels to promote Ca^{2+} entry from the extracellular fluid [36]. Many GPCR ligands and perfusion pressure elicit contraction of afferent arterioles by stimulating Ca^{2+} entry through L-type channels [37]. Interactions between $\cdot O_2^-$ and L-type Ca^{2+} channel activity in the renal microcirculation have not been investigated.

METHODS

See details in Methods in the online-only Data Supplement. All animal studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of North Carolina.

Preparation of afferent arterioles

Afferent arterioles (<20 μm in diameter) were isolated from 3 to 6 wk-old (50–120 g) male Sprague-Dawley rats using a magnetized iron oxide-sieving technique as previously described in our laboratory [8, 9]. We elected to use young animals because the preparation and purification of single afferent arterioles with little advential tissue from young rats were technically much easier than from mature rats. Pilot studies established that the increase in cytosolic Ca^{2+} concentration produced by 40 mM KCl was similar in young and adult rat afferent arterioles (Online supplement, please see http://hyper.ahajournals.org). All animal research conducted adhered to the NIH Guide for the Care and Use of Laboratory Animals.

Measurement of paraquat-induced cytosolic $\cdot O_2^-$ production

We used paraquatto generate $\cdot \mathbf{O_2^-}$ in fresh afferent arterioles. Paraquat (N,N'- dimethyl-4,4'-bipyridinium dichloride) is a classic and well established model for longterm oxidant-initiated toxicity due to its ability to generate $\cdot \mathbf{O_2^-}$. Paraquat redox cycles with cellular diaphorases and molecular oxygen to generate intracellular $\cdot \mathbf{O_2^-}$ at levels that do not acutely affect cell viability [39, 40]. We selected 1 mM paraquat for testing because it causes marked afferent arteriolar constriction that is reversed by tempol without adversely affecting short-term cell viability [35].

$\cdot O_2^-$ production was measured in isolated afferent arterioles using the oxidative-responsive fluorescent dye dihydroethidium (DHE) as previously described [7]. After a baseline recording of a 90 sec incubation period with paraquat (1mM) or PBS alone as a negative control, a final image was acquired. Some arterioles were incubated with tempol (1 mM) or catalase (250 U/ml) in PBS for 20 min before testing the response to paraquat.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Measurement of cytosolic free calcium concentration

$[Ca^{2+}]_i$ was measured in individual afferent arterioles as previously described [8, 41, 42]. Fura-2 fluorescence was detected using a CCD camera (Digital Video Camera Co. DVC 1500) after passing through a 510 nm emission filter. $[Ca^{2+}]_i$ was determined by ratiometric analysis of Fura-2 emission intensities at two excitation wavelengths (340 nm, 380 nm). Signal intensity was acquired and processed using InCytIm2 software (Intracellular Imaging, Cincinnati, OH).

Protocols

Values for each arteriole were averaged and analyzed to identify the peak and the plateau phases of the response. Peak values were the average of three data points corresponding to the highest measured $[Ca^{2+}]_i$ after stimulation. Plateau responses were defined as the average of the 5 data points occurring 45 sec (43-47 sec) after the peak response.

To determine the relative contributions of L-type and T-type channels to Ca^{2+} entry resulting from KCl-induced membrane depolarization, we used selective L-type and Ttype Ca^{2+} channel inhibitors nifedipine (1 μM) and mibefradil (1 μM). Arterioles were incubated for 10 min in HBSS containing either channel blocker prior to stimulation with 40 mM KCl.

To test the effects of $\cdot O_2^-$ on Ca^{2+} entry through L-type channels, paraquat was added 90 sec prior to stimulation with 40 mM KCl. The effect of paraquat on Ca^{2+} entry via SOC was determined using a protocol previously described by our laboratory [43]. Arterioles were incubated with Ca^{2+}-free HBSS containing 10 μM thapsigargin for 20 min and then the thapsigargin-containing HBSS was replaced with a bathing medium composed of Ca^{2+}-free PBS with 0.5 mM EGTA and 10 μM thapsigargin. Paraquat (1 mM) was added to the bathing medium 90 sec prior to switching the bath solution to PBS containing Ca^{2+} (1 mM).

To assess the contribution from Ca^{2+}-induced Ca^{2+} release (CICR), arterioles were incubated in HBSS containing 50 μM Ry for 20 min to block Ry receptors (RyR) and prevent CICR [41]. After incubation, arterioles were placed in a bathing medium composed of PBS with 1.1 mM Ca^{2+} and 50 μM Ry. In other experiments, paraquat (1 mM) was added 90 sec prior to KCl stimulation.

Statistics

A two-tailed Student's *t*-test was used to determine significance between groups (Prism Software). $P < 0.05$ was determined to be significant.

RESULTS

The results for $[Ca^{2+}]_i$ are presented as nM ± SEM change from baseline. Baseline $[Ca^{2+}]i$ for all afferent arterioles studied averaged 100 ± 4 nM (n=111). Basal values for each group of experiments did not differ statistically from the overall mean (p>0.1).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

High KCl stimulates Ca^{2+} entry through L-type channels

Fig. 1 (left panel) shows high KCl (40 mM)-induced depolarization of the plasma membrane of VSMC of afferent arterioles to stimulate Ca^{2+} influx to increase $[Ca^{2+}]_i$.. The initial peak increase in $[Ca^{2+}]_i$ averaged 99 ± 14 nM while the sustained plateau phase at 45 s after addition of KCl was 53 ± 10 nM (Fig. 1, right panel). Fig. 1 (right and left panels) shows the complete abolition of high KCl-induced Ca^{2+} entry when L-type channels were blocked by nifedipine (1 μM). Addition of the T-type inhibitor mibefradil (1 μM) did not alter Ca^{2+} responses to high KCl. These observations support previous reports that KCl-induced depolarization exclusively activates voltage-gated L-type Ca^{2+} channels in the rat cortical afferent arteriole [44-47].

Paraquat incresees $\cdot O_2^-$ production in afferent arteriolar VSMCs

Fig. 2 shows that paraquat (1 mM) induced a 50% increase in $\cdot O_2^-$ generation (DHE fluorescence) in these afferent arteriolar VSMCs. This increase in $\cdot O_2^-$ was abolished by tempol ($p<0.001$) and not affected by catalase.

Paraquat and $\cdot O_2^-$ generation increase Ca^{2+} influx induced by membrane depolarization

Fig. 3 (left panel) demonstrates that the addition of paraquat (10^{-3} M) to generate of $\cdot O_2^-$ markedly enhanced the Ca^{2+} entry through L-type Ca^{2+} channels stimulated by KCl-induced membrane depolarization but not during basal conditions before addition of KCl. Paraquat increased both the peak and the plateau phases of the response to KCl (Fig. 3, right panel). The peak was enhanced 3-fold to 314 ± 68 nM and the plateau phase was augmented nearly 2-fold to 92 ± 8 nM ($p<0.01$ for both).

Fig. 4 (left panel) illustrates the effect of $\cdot O_2^-$ dismutation by tempol on the $[Ca^{2+}]_i$ response to 40 mM KCl and paraquat stimulation. Tempol (10^{-3} M) had no effect on baseline $[Ca^{2+}]_i$ before KCl stimulation, but abolished the enhancement produced by paraquat, yielding results approximating those of KCl alone in the absence of paraquat. Fig. 4 (right panel) shows that the effect of paraquat induced $\cdot O_2^-$ production to increase both peak and plateau Ca^{2+} responses to KCl was eliminated by the antioxidant tempol. Similar inhibition of the paraquat stimulation was observed when NBT was used to scavenge $\cdot O_2^-$ (NBT results online supplement only, please see http://hyper.ahajournals.org).

Paraquat and $\cdot O_2^-$ generation does not increase Ca^{2+} entry through SOC channels

To verify that Ca^{2+} entry took place through SOC and not L-type Ca^{2+} channels, vessels were treated with nifedipine throughout the experiment. Fig. 5 (left panel) displays the magnitude of Ca^{2+} entry through SOC in the presence and absence of $\cdot O_2^-$ generated by paraquat. Fig. 5 (right panel) shows the peak and plateau values for $[Ca^{2+}]_i$ following activation of Ca^{2+} entry through SOC. Pretreatment with paraquat had no significant effect on Ca^{2+} entry through SOC during either phase ($P>0.5$).

Enhancement of Ca^{2+} influx by paraquat does not involve CICR

Increased $[Ca^{2+}]_i$ resulting from Ca^{2+} entry through L-type channels is known to increase CICR from the sarcoplasmic reticulum mediated by activation of the ryanodine receptor

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(RyR) [41]. To test for a possible effect of $\cdot O_2^-$ on RyR-mediated CICR, we used Ry (50 μM) to inactivate RyR and prevent CICR secondary to Ca^{2+} entry stimulated by high KCl. As Fig. 6 (left panel) shows, during RyR inactivation to prevent CICR, paraquat enhanced the peak and plateau $[Ca^{2+}]_i$ responses initiated by membrane depolarization ($p<0.05$), again demonstrating the ability of paraquat-derived $\cdot O_2^-$ to stimulate L-type channels. Also shown in Fig. 6, Ry decreased the $[Ca^{2+}]_i$ response to high KCl, reducing the peak by 50% and the plateau phase by 23%. Thus, RyR-mediated CICR contributes to the $[Ca^{2+}]_i$ response when Ca^{2+} entry is stimulated by membrane depolarization. Inactivation of RyR blunted the peak and plateau Ca^{2+} signals after paraquat to approximately the same extent (Fig. 6, right panel). $\cdot O_2^-$ enhanced KCl-induced Ca^{2+} influx during paraquat treatment, suggesting $\cdot O_2^-$ did not affect RyR-mediated Ca^{2+} mobilization in these experiments. Therefore, we conclude that the paraquat-induced increase in Ca^{2+} entry during membrane polarization is primarily due to L-type channel enhancement, and not by recruitment of SOC or augmented CICR.

DISCUSSION

This study evaluated the effects of $\cdot O_2^-$ generated by paraquat on Ca^{2+} signaling in VSMCs of rat renal afferent arterioles. $\cdot O_2^-$ was verified as the primary ROS responsible for increased Ca^{2+} influx based on DHE measurement and effective abolition of the paraquat effect on $[Ca^{2+}]_i$ by either tempol or NBT (NBT results online supplement only, please see http://hyper.ahajournals.org). Moreover, $\cdot O_2^-$ production was negated by tempol but not catalase. Our major new finding is that $\cdot O_2^-$ enhanced Ca^{2+} entry in response to membrane depolarization induced by high extracellular KCl. We conclude that $\cdot O_2^-$ acted to increase Ca^{2+} entry by increasing the activity of L-type Ca^{2+} channels. The stimulation was rapid, requiring at most 90 sec of exposure to paraquat and was effective upon stimulation with KCl, whereas basal $[Ca^{2+}]_i$ before stimulation was unaffected.

Our results extend previous studies showing that membrane depolarization by high KCl exclusively activates L-type Ca^{2+} channels to increase $[Ca^{2+}]_i$ and contract the rat cortical afferent arteriole with little to no participation of T-type channels sensitive to mibefradil [44-46]. Both responses were abolished by removal of extracellular Ca^{2+} or by pharmacological inhibition of voltage-gated L-type channels using nifedipine or nitrendipine [44-46]. Based on patch clamp studies of voltage-activated Ca^{2+} currents, freshly isolated VSMCs of the rat afferent arteriole have a high density of L-type channels but do not express functionally active voltage-dependent T-type Ca^{2+} channels [47]. In rat juxtamedullary afferent arterioles, it is reported that T-type channels are functionally expressed, but do not contribute to constrictor responses to KCl [48]. On the other hand, another study reports that high KCl-induced Ca^{2+} entry is attenuated in rat juxtamedullary afferent arterioles and rabbit cortical afferent arterioles during inhibition of T-type channels by mibefradil [49].

Our results provide important insight into physiological and pathophysiological mechanisms within the kidney as L-type Ca^{2+} channel activity is a critical determinant of contractile tone of the afferent arteriole, whether stimulated by GPCR agonists or increased renal perfusion

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

pressure [37]. $\cdot O_2^-$ is known to modulate renal hemodynamics 1 and vasoconstrictor responses to GPCR agonists such as Ang II, ET-1 and PE [5, 6]. Our finding of a direct action of $\cdot O_2^-$ on Ca^{2+} entry provides a mechanism to explain our previous demonstration that NADPH-derived $\cdot O_2^-$ mediates the afferent arteriolar $[Ca^{2+}]_i$ response to Ang II and ET-1 [8, 9]. The immediate increase in $[Ca^{2+}]_i$ stimulated by either agonist was attenuated by both tempol and apocynin, indicating participation of NADPH-derived $\cdot O_2^-$. Moreover, the actions of Ang II and ET-1 were attenuated by 8-Br-cyclic ADPR and nicotinamide, implicating involvement of ADPR cyclase and RyR in the Ca^{2+} signaling pathway, steps proposed to be downstream of $\cdot O_2^-$ production.

ADPR cyclase synthesis of cADPR can sensitize RyR to $[Ca^{2+}]_i$ to enhance CICR [50]. Our earlier studies of afferent arterioles showed that RyR-mediated CICR also contributes to the increased $[Ca^{2+}]_i$ following membrane depolarization induced by high KCl [41]. Locking the RyR in the closed position with a high concentration of ryanodine (50-100 μM) attenuated ~50% of the $[Ca^{2+}]_i$ response to Ca^{2+} entry through voltagegated L-type channels. Our current studies confirm this finding and extend it in that $\cdot O_2^-$ generated by paraquat had essentially no effect on the RyR-mediated CICR response to increased $[Ca^{2+}]_i$ secondary to KCl-induced depolarization.

In our studies of freshly isolated afferent arterioles, paraquat did not elicit a change in basal $[Ca^{2+}]_i$, suggesting no acute effect of $\cdot O_2^-$ on Ca^{2+} entry in the absence of KCl-induced membrane depolarization when L-type channels are quiescent. This contrasts with other published reports that ROS increased basal $[Ca^{2+}]_i$ in VSMC from nonrenal vessels [36, 51]. Differences among studies may stem from ROS exposure times and concentrations, intracellular vs extracellular ROS generation, and vascular beds. It is noteworthy that HX/XO primarily generates extracellular ROS, whereas paraquat stimulates intracellular $\cdot O_2^-$ production. Our 90 sec exposure time to paraquat / $\cdot O_2^-$ was considerably shorter than some previous studies. In cultured rat mesenteric arterial VSMCs, ROS generated by cell membrane permeable LY23583 increased Tempo-9AC florescence after 25 min, a response abolished by tempol, whereas $[Ca^{2+}]_i$ was increased after 15 min [50]. In these nonrenal VSMC of WKY but not SHR, the increase was attributed to augmented Ca^{2+} influx through both L-type and T-type Ca^{2+} channels as it was inhibited by putative selective antagonists verapamil/diltiazem (10 μM) and mibefradil (10 μM), respectively [51].

Amberg et al. investigated the role of ROS in activating Ca^{2+} entry via L-type Cav1.2 Ca^{2+} channels and the resultant constriction of pressurized, freshly isolated cerebral arteries [36]. Total internal reflection fluorescence microscopy revealed that Ang II and endogenous ROS rapidly stimulated Ca^{2+} entry by increasing L-type Ca^{2+} channel sparklet activity. Both the Ang II-induced increase in Ca^{2+} sparklets and arterial tone were abolished by apocynin inhibition of NADPH oxidase, implicating a stimulatory role of ROS, either $\cdot O_2^-$ or H_2O_2. Additionally, exogenous ROS generated by HX/XO increased PKCα and L-type Ca^{2+} channel activity and cerebral arterial vasomotor tone as a result of Ca^{2+} entry within 2 min of addition; the latter was abolished by inhibition of L-type channel activity with diltiazem [36]. Exogenous ROS increased both Ca^{2+} sparklet activity and sparklet site density

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

in voltage clamped arterial myocytes, further supporting the conclusion that ROS increases Ca^{2+} influx through L-type channels.

L-type channels are sensitive to oxidants, potentially due to direct redox modification of cysteines on the channel, or by redox modification of regulatory proteins involved in channel function [52-54]. $\cdot O_2^-$ may amplify Ca^{2+} entry through L-type Ca^{2+} channels by promoting clustering that leads to cooperative gating [55]. In this manner, increased $\cdot O_2^-$ production during ANG II-induced hypertension may explain increased L-type Ca^{2+} channel sparklet activity in arterial VSMC [56, 57] and the increased density of L-type channels [58]. Another new finding of our present studies is that $\cdot O_2^-$ does not influence Ca^{2+} entry mediated by SOC in freshly isolated afferent arterioles. In cultured VSMC of porcine coronary artery and bovine pulmonary artery, ROS generated by HX/XO was reported to stimulate $[Ca^{2+}]_i$ by inhibiting plasma membrane Ca^{2+}-ATPase and SERCA [59-62]. Such inhibition is predicted to deplete intracellular Ca^{2+} stores and thereby indirectly enhance SOC entry. Both TRPC and STIM-Orai channels have been proposed to be the primary SOC channels in other vascular beds [63, 64]. AVP stimulation of $\cdot O_2^-$ and H_2O_2 production is reported to increase TRPC6 channel activity and Ca^{2+} influx in the A7r5 line of cultured VSMC and cultured mouse aortic VSMC [65]. Presently, the molecular identity of SOC channels in the renal microcirculation is unknown.

In summary, our major novel finding is that $\cdot O_2^-$ enhances Ca^{2+} entry through L-type channels in VSMCs of freshly isolated afferent arterioles. We found no evidence for potentiation by paraquat of either Ca^{2+} entry through SOC or Ca^{2+} mobilization and CICR mediated by RyR.

PERSPECTIVES

It is well established that ROS play a pathophysiological role in the development of hypertension, however the specific mechanisms by which ROS alter renal hemodynamics in health and disease are poorly understood. $\cdot O_2^-$ interacts with NO and participates in rapid, acute constriction of the afferent arteriole and increased renal vascular resistance, but the effects on Ca^{2+} signaling pathways in the renal microcirculation are not known. Our studies provide new information that $\cdot O_2^-$ acts to enhance Ca^{2+} influx through L-type Ca^{2+} channels in the afferent arteriole, the major preglomerular resistance vessel in the kidney where Ca^{2+} entry through voltage-gated channels is a predominant Ca^{2+} signaling pathway. This stimulatory effect can be reversed both by $\cdot O_2^-$ dismutation with tempol and by $\cdot O_2^-$ scavenging with NBT. Ca^{2+} entry through store-operated channels resulting from thapsigargin-induced intracellular Ca^{2+} depletion of SR stores is not markedly influenced by cellular $\cdot O_2^-$ levels.

During renal autoregulation, changes in tone of the cortical radial arteries and afferent arterioles result from pressure-induced activation of L-type Ca^{2+} channels to maintain RBF, glomerular filtration rate, and buffer pressure-natriuresis. Acute increases in $\cdot O_2^-$ modulate the efficiency of renal autoregulation by augmenting the myogenic response of afferent arterioles [13, 15]. An enhancement in L-type channel activity by $\cdot O_2^-$ may provide a

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

mechanistic explanation for these observations. Increased $\cdot O_2^-$ production participates in renal vasoconstriction and sodium retention during the development of hypertension [1, 10, 11, 34, 66]. The ability of $\cdot O_2^-$ to increase Ca^{2+} entry through L-type channels is likely to play an important role in the vasoconstriction of the preglomerular vasculature often associated with sodium retention and the development and maintenance of hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCE OF FUNDING

This research was supported by NIH research grant HL-02334.

REFERENCES

1. Araujo M, Wilcox CS. Oxidative stress in hypertension: Role of the kidney. Antioxidants & redox signaling. 2014; 20:74–101. [PubMed: 23472618]
2. Touyz RM, Briones AM. Reactive oxygen species and vascular biology: Implications in human hypertension. Hypertension research : official journal of the Japanese Society of Hypertension. 2011; 34:5–14. [PubMed: 20981034]
3. Schnackenberg CG. Physiological and pathophysiological roles of oxygen radicals in the renal microvasculature. American journal of physiology. Regulatory, integrative and comparative physiology. 2002; 282:R335–R342.
4. Lob HE, Vinh A, Li L, Blinder Y, Offermanns S, Harrison DG. Role of vascular extracellular superoxide dismutase in hypertension. Hypertension. 2011; 58:232–239. [PubMed: 21730294]
5. Just A, Olson AJ, Whitten CL, Arendshorst WJ. Superoxide mediates acute renal vasoconstriction produced by Angiotensin II and catecholamines by a mechanism independent of nitric oxide. American journal of physiology. Heart and circulatory physiology. 2007; 292:H83–H92. [PubMed: 16951043]
6. Just A, Whitten CL, Arendshorst WJ. Reactive oxygen species participate in acute renal vasoconstrictor responses induced by ETA and ETB receptors. American journal of physiology. Renal physiology. 2008; 294:F719–F728. [PubMed: 18256310]
7. Moss NG, Vogel PA, Kopple TE, Arendshorst WJ. Thromboxane-induced renal vasoconstriction is mediated by the adp-ribosyl cyclase CD38 and superoxide anion. American journal of physiology. Renal physiology. 2013; 305:F830–F838. [PubMed: 23884143]
8. Fellner SK, Arendshorst W. Endothelin-a and -b receptors, superoxide, and Ca2+ signaling in afferent arterioles. American journal of physiology. Renal physiology. 2007; 292:F175–F184. [PubMed: 16788136]
9. Fellner SK, Arendshorst WJ. Angiotensin II, reactive oxygen species, and Ca2+ signaling in afferent arterioles. American journal of physiology. Renal physiology. 2005; 289:F1012–F1019. [PubMed: 15942049]
10. de Richelieu LT, Sorensen CM, Holstein-Rathlou NH, Salomonsson M. NO-independent mechanism mediates tempol-induced renal vasodilation in SHR. American journal of physiology. Renal physiology. 2005; 289:F1227–F1234. [PubMed: 16033921]
11. Feng MG, Dukacz SA, Kline RL. Selective effect of tempol on renal medullary hemodynamics in spontaneously hypertensive rats. American journal of physiology. Regulatory, integrative and comparative physiology. 2001; 281:R1420–R1425.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

12. Majid DS, Nishiyama A, Jackson KE, Castillo A. Superoxide scavenging attenuates renal responses to Ang II during nitric oxide synthase inhibition in anesthetized dogs. American journal of physiology. Renal physiology. 2005; 288:F412–F419. [PubMed: 15467005]
13. Lai EY, Wellstein A, Welch WJ, Wilcox CS. Superoxide modulates myogenic contractions of mouse afferent arterioles. Hypertension. 2011; 58:650–656. [PubMed: 21859962]
14. Ozawa Y, Hayashi K, Wakino S, Kanda T, Homma K, Takamatsu I, Tatematsu S, Yoshioka K, Saruta T. Free radical activity depends on underlying vasoconstrictors in renal microcirculation. Clinical and Experimental Hypertension. 2004; 26:219–229. [PubMed: 15132300]
15. Ren Y, D'Ambrosio MA, Liu R, Pagano PJ, Garvin JL, Carretero OA. Enhanced myogenic response in the afferent arteriole of spontaneously hypertensive rats. American journal of physiology. Heart and circulatory physiology. 2010; 298:H1769–H1775. [PubMed: 20363886]
16. Harrison DG, Gongora MC, Guzik TJ, Widder J. Oxidative stress and hypertension. Journal of the American Society of Hypertension : JASH. 2007; 1:30–44. [PubMed: 20409831]
17. Montezano AC, Touyz RM. Oxidative stress, NOXs, and hypertension: Experimental evidence and clinical controversies. Annals of medicine. 2012; 44(Suppl 1):S2–16. [PubMed: 22713144]
18. Schulman IH, Zhou MS, Raij L. Nitric oxide, Angiotensin II, and reactive oxygen species in hypertension and atherogenesis. Current hypertension reports. 2005; 7:61–67. [PubMed: 15683588]
19. Kopkan L, Majid DS. Superoxide contributes to development of salt sensitivity and hypertension induced by nitric oxide deficiency. Hypertension. 2005; 46:1026–1031. [PubMed: 16103275]
20. Manning RD Jr. Meng S, Tian N. Renal and vascular oxidative stress and salt-sensitivity of arterial pressure. Acta physiologica Scandinavica. 2003; 179:243–250. [PubMed: 14616240]
21. Wilcox CS. Reactive oxygen species: Roles in blood pressure and kidney function. Current hypertension reports. 2002; 4:160–166. [PubMed: 11884272]
22. Kopkan L, Majid DSA. Enhanced superoxide activity modulates renal function in NO-deficient hypertensive rats. Hypertension. 2006; 47:568–572. [PubMed: 16401762]
23. Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. Role of superoxide in Angiotensin II-induced but not catecholamine-induced hypertension. Circulation. 1997; 95:588–593. [PubMed: 9024144]
24. Welch WJ, Chabrashvili T, Solis G, Chen Y, Gill PS, Aslam S, Wang X, Ji H, Sandberg K, Jose P, Wilcox CS. Role of extracellular superoxide dismutase in the mouse angiotensin slow pressor response. Hypertension. 2006; 48:934–941. [PubMed: 17015770]
25. Gongora MC, Qin Z, Laude K, Kim HW, McCann L, Folz JR, Dikalov S, Fukai T, Harrison DG. Role of extracellular superoxide dismutase in hypertension. Hypertension. 2006; 48:473–481. [PubMed: 16864745]
26. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. The Journal of clinical investigation. 2000; 105:1631–1639. [PubMed: 10841522]
27. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. The Journal of clinical investigation. 1996; 97:1916–1923. [PubMed: 8621776]
28. Kitiyakara C, Chabrashvili T, Chen Y, Blau J, Karber A, Aslam S, Welch WJ, Wilcox CS. Salt intake, oxidative stress, and renal expression of NADPH oxidase and superoxide dismutase. Journal of the American Society of Nephrology : JASN. 2003; 14:2775–2782. [PubMed: 14569087]
29. Kawada N, Imai E, Karber A, Welch WJ, Wilcox CS. A mouse model of Angiotensin II slow pressor response: Role of oxidative stress. Journal of the American Society of Nephrology : JASN. 2002; 13:2860–2868. [PubMed: 12444204]
30. Welch WJ, Blau J, Xie H, Chabrashvili T, Wilcox CS. Angiotensin-induced defects in renal oxygenation: Role of oxidative stress. American journal of physiology. Heart and circulatory physiology. 2005; 288:H22–H28. [PubMed: 15598867]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

31. Adler S, Huang H. Oxidant stress in kidneys of spontaneously hypertensive rats involves both oxidase overexpression and loss of extracellular superoxide dismutase. American journal of physiology. Renal physiology. 2004; 287:F907–F913. [PubMed: 15475543]
32. Welch WJ, Mendonca M, Aslam S, Wilcox CS. Roles of oxidative stress and AT1 receptors in renal hemodynamics and oxygenation in the postclipped 2k,1c kidney. Hypertension. 2003; 41:692–696. [PubMed: 12623981]
33. Schnackenberg CG, Wilcox CS. Two-week administration of tempol attenuates both hypertension and renal excretion of 8-iso prostaglandin F-2 alpha. Hypertension. 1999; 33:424–428. [PubMed: 9931141]
34. Schnackenberg CG, Welch WJ, Wilcox CS. Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic: Role of nitric oxide. Hypertension. 1998; 32:59–64. [PubMed: 9674638]
35. Schnackenberg CG, Welch WJ, Wilcox CS. TP receptor-mediated vasoconstriction in microperfused afferent arterioles: Roles of O(2)(−) and NO. American journal of physiology. Renal physiology. 2000; 279:F302–F308. [PubMed: 10919850]
36. Amberg GC, Earley S, Glapa SA. Local regulation of arterial L-type calcium channels by reactive oxygen species. Circulation research. 2010; 107:U1002–U1123.
37. Navar LG, Arendshorst WJ, Pallone TL, Inscho EW, Imig JD, Bell PD. The renal microcirculation. Handbook of Physiology: Microcirculation (2nd Edition.). 2008:550–683.
38. Bus JS, Gibson JE. Paraquat: Model for oxidant-initiated toxicity. Environmental Health Perspectives. 1984; 55:37–46. [PubMed: 6329674]
39. Ody C, Junod AF. Direct toxic effects of paraquat and oxygen on cultured endothelial cells. Laboratory investigation; a journal of technical methods and pathology. 1985; 52:77–84.
40. Gobbel GT, Chan TY, Chan PH. Nitric oxide- and superoxide-mediated toxicity in cerebral endothelial cells. The Journal of pharmacology and experimental therapeutics. 1997; 282:1600–1607. [PubMed: 9316877]
41. Fellner SK, Arendshorst WJ. Voltage-gated Ca2+ entry and ryanodine receptor Ca2+-induced Ca2+ release in preglomerular arterioles. American journal of physiology. Renal physiology. 2007; 292:F1568–1572. [PubMed: 17190906]
42. Fellner SK, Arendshorst WJ. Angiotensin II Ca2+ signaling in rat afferent arterioles: Stimulation of cyclic ADP ribose and IP3 pathways. American journal of physiology. Renal physiology. 2005; 288:F785–F791. [PubMed: 15598842]
43. Fellner SK, Arendshorst WJ. Store-operated Ca2+ entry is exaggerated in fresh preglomerular vascular smooth muscle cells of SHR. Kidney international. 2002; 61:2132–2141. [PubMed: 12028453]
44. Carmines PK, Fowler BC, Bell PD. Segmentally distinct effects of depolarization on intracellular [Ca2+] in renal arterioles. American journal of physiology. Renal physiology. 1993; 265:F677–F685.
45. Loutzenhiser R, Epstein M. Activation mechanisms of human renal artery: Effects of KCl, norepinephrine and nitrendipine upon tension development and 45Ca influx. European journal of pharmacology. 1984; 106:47–52. [PubMed: 6529973]
46. Loutzenhiser R, Hayashi K, Epstein M. Divergent effects of KCl-induced depolarization on afferent and efferent arterioles. American journal of physiology. Renal physiology. 1989; 257:F561–F564.
47. Smirnov SV, Loutzenhiser K, Loutzenhiser R. Voltage-activated Ca(2+) channels in rat renal afferent and efferent myocytes: No evidence for the T-type Ca(2+) current. Cardiovascular research. 2013; 97:293–301. [PubMed: 23042470]
48. Feng MG, Li M, Navar LG. T-type calcium channels in the regulation of afferent and efferent arterioles in rats. American journal of physiology. Renal physiology. 2004; 286:F331–337. [PubMed: 14583435]
49. Hansen PB, Jensen BL, Andreasen D, Skott O. Differential expression of T- and L-type voltage-dependent calcium channels in renal resistance vessels. Circulation research. 2001; 89:630–638. [PubMed: 11577029]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

50. Arendshorst WJ, Thai TL. Regulation of the renal microcirculation by ryanodine receptors and calcium-induced calcium release. Current opinion in nephrology and hypertension. 2009; 18:40–49. [PubMed: 19077688]
51. 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. Journal of cardiovascular pharmacology. 2004; 44:200–208. [PubMed: 15243301]
52. Hool LC. Evidence for the regulation of L-type Ca2+ channels in the heart by reactive oxygen species: Mechanism for mediating pathology. Clinical and experimental pharmacology & physiology. 2008; 35:229–234. [PubMed: 18197892]
53. Campbell DL, Stamler JS, Strauss HC. Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and s-nitrosothiols. The Journal of general physiology. 1996; 108:277–293. [PubMed: 8894977]
54. Gupte SA, Wolin MS. Oxidant and redox signaling in vascular oxygen sensing: Implications for systemic and pulmonary hypertension. Antioxidants & redox signaling. 2008; 10:1137–1152. [PubMed: 18315496]
55. Navedo MF, Amberg GC. Local regulation of L-type Ca2+ channel sparklets in arterial smooth muscle. Microcirculation. 2013; 20:290–298. [PubMed: 23116449]
56. Navedo MF, Nieves-Cintron M, Amberg GC, Yuan C, Votaw VS, Lederer WJ, McKnight GS, Santana LF. AKAP150 is required for stuttering persistent Ca2+ sparklets and Angiotensin II-induced hypertension. Circulation research. 2008; 102:e1–e11. [PubMed: 18174462]
57. Gulia J, Navedo MF, Gui PC, Chao JT, Mercado JL, Santana LF, Davis MJ. Regulation of L-type calcium channel sparklet activity by c-Src and PKC-alpha. American Journal of Physiology. Cell Physiology. 2013; 305:C568–C577. [PubMed: 23804206]
58. Pesic A, Madden JA, Pesic M, Rusch NJ. High blood pressure upregulates arterial L-type Ca2+ channels: Is membrane depolarization the signal? Circulation research. 2004; 94:e97–e104. [PubMed: 15131006]
59. Suzuki YJ, Ford GD. Inhibition of Ca(2+)-ATPase of vascular smooth muscle sarcoplasmic reticulum by reactive oxygen intermediates. American journal of physiology. Heart and circulatory physiology. 1991; 261:H568–H574.
60. Grover AK, Samson SE, Fomin VP, Werstiuk ES. Effects of peroxide and superoxide on coronary artery: Ang II response and sarcoplasmic reticulum Ca2+ pump. American Journal of Physiology. Cell Physiology. 1995; 269:C546–C553.
61. Grover AK, Kwan CY, Samson SE. Effects of peroxynitrite on sarco/endoplasmic reticulum Ca2+ pump isoforms SERCA2b and SERCA3a. American journal of physiology. Cell physiology. 2003; 285:C1537–C1543. [PubMed: 14600079]
62. Lounsbury KM, Hu Q, Ziegelstein RC. Calcium signaling and oxidant stress in the vasculature. Free Radical Biology and Medicine. 2000; 28:1362–1369. [PubMed: 10924855]
63. Wang Y, Deng X, Gill DL. Calcium signaling by STIM and Orai: Intimate coupling details revealed. Science Signaling. 2010; 3:pe42. [PubMed: 21081752]
64. Smyth JT, Dehaven WI, Jones BF, Mercer JC, Trebak M, Vazquez G, Putney JW. Jr. Emerging perspectives in store-operated Ca2+ entry: Roles of Orai, STIM and TRP. Biochimica et biophysica acta. 2006; 1763:1147–1160. [PubMed: 17034882]
65. Ding Y, Winters A, Ding M, Graham S, Akopova I, Muallem S, Wang Y, Hong JH, Gryczynski Z, Yang SH, Birnbaumer L, Ma R. Reactive oxygen species-mediated TRPC6 protein activation in vascular myocytes, a mechanism for vasoconstrictor-regulated vascular tone. The Journal of biological chemistry. 2011; 286:31799–31809. [PubMed: 21768109]
66. Ichihara A, Hayashi M, Hirota N, Saruta T. Superoxide inhibits neuronal nitric oxide synthase influences on afferent arterioles in spontaneously hypertensive rats. Hypertension. 2001; 37:630–634. [PubMed: 11230347]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

NOVELTY AND SIGNIFICANCE

What is new?

- Our studies provide new information that $\cdot O_2^-$ acts to enhance Ca^{2+} influx through L-type Ca^{2+} channels in the afferent arteriole, the major preglomerular resistance vessel in the kidney.
- We also show that superoxide does not participate in other Ca^{2+} signaling mechanisms we investigated, such as store operated calcium entry and ryanodine receptor-mediated calcium mobilization and subsequent calcium-induced-calcium-release.

What is relevant?

- The ability of $\cdot O_2^-$ to increase Ca^{2+} entry through L-type channels is likely to play an important role in vasoconstriction the preglomerular vasculature often associated with sodium retention and the development of hypertension.

Summary

- Our studies address a lack of understanding of the mechanisms by which superoxide affects Ca^{2+} signaling and causes contraction of VSMC of renal resistance arterioles.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

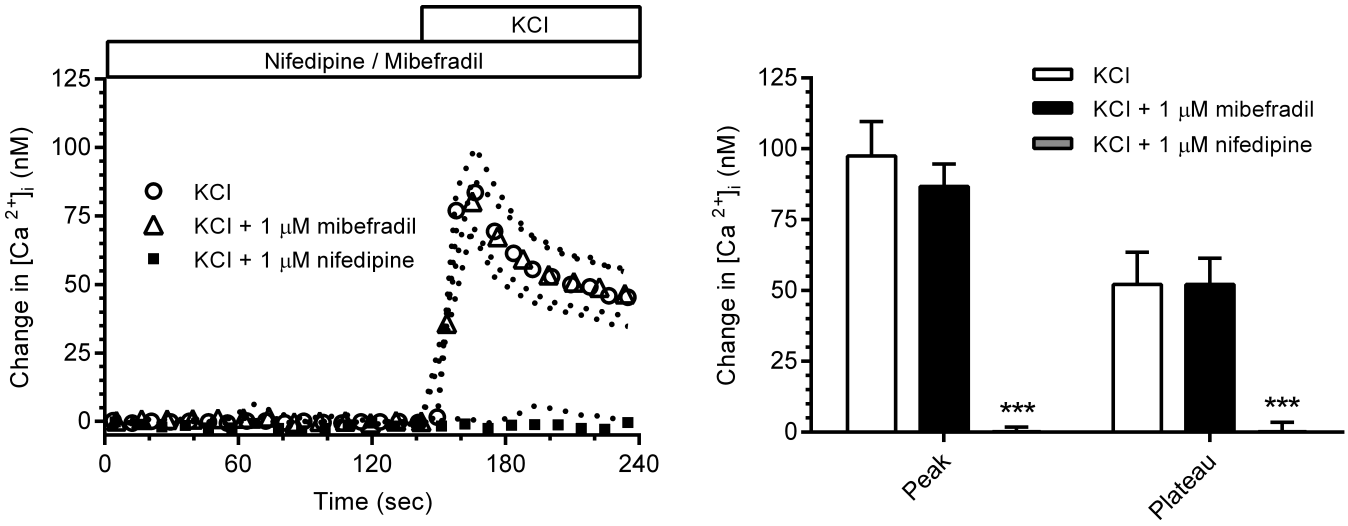

Fig. 1.

Left panel: $[Ca^{2+}]_i$ responses to KCl in the presence of the L-type and T-type Ca^{2+} channel inhibitors, nifedipine (n=7) and mibefradil (n=8), respectively, are compared to a control KCl response (n=8). Right Panel: Effects of nifedipine and mibefradil on average peak and plateau $[Ca^{2+}]_i$ responses. (*** $p<0.005$)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

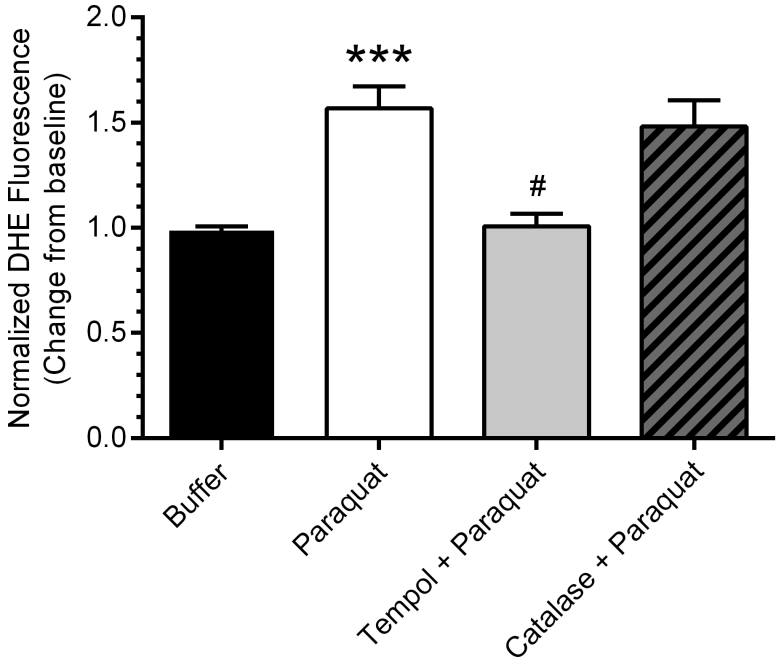

Fig. 2.

Paraquat elicited a 50% increase in cytosolic $\cdot O_2^-$ (DHE fluorescence, n=7, filled bar) as compared to buffer as a negative control (n=7, open bar) and paraquat (n=7, filled bar). The paraquat-induced increase in $\cdot O_2^-$ production was abolished by tempol (n=7, $p<0.005$, gray bar), but was not significantly influenced by catalase (n=7, patterned bar) (*** $p<0.005$ vs. control, # $p<0.005$ vs. paraquat).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

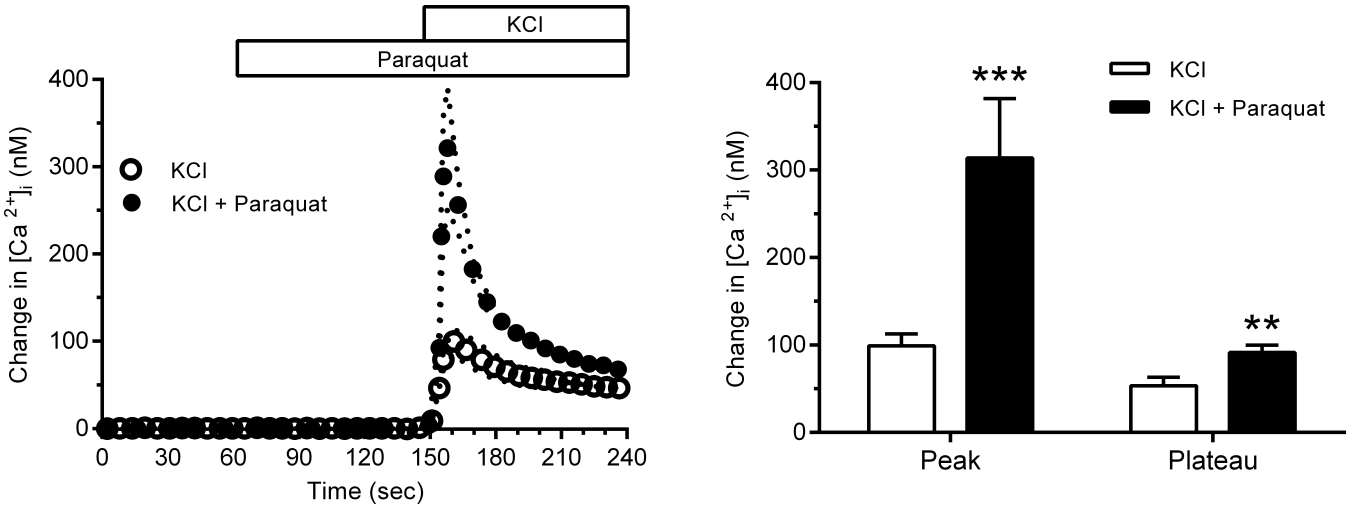

Fig. 3.

Left panel: $[Ca^{2+}]_i$ response of freshly isolated afferent arterioles to KCl-induced (40 mM) membrane depolarization before (n=14, open circles) and after paraquat stimulation (n=10, filled circles). Note that paraquat did not affect basal $[Ca^{2+}]_i$ prior to stimulation with KCl. Right panel: Average peak and plateau (45 sec after the peak) responses to KCl in the presence and absence of paraquat (*** $p<0.005$, ** $p<0.01$).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

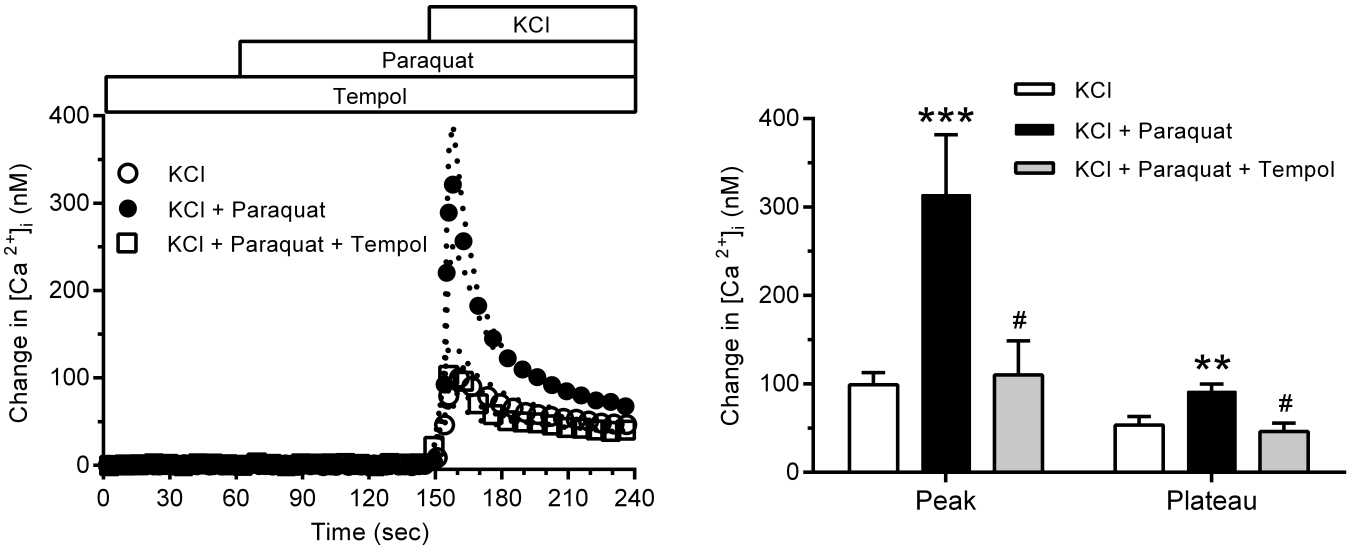

Fig. 4.

Left panel: The $[Ca^{2+}]_i$ responses to KCl in the presence and absence of paraquat (closed and filled circles, respectively) (same as in Fig. 4), and the effect of tempol dismutation of paraquat-generated $\cdot O_2^-$ (n=8, open squares). Right panel: Effect of tempol on the average peak and plateau $[Ca^{2+}]_i$ values. (*** $p<0.005$ vs. control, # $p<0.05$ vs. paraquat).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

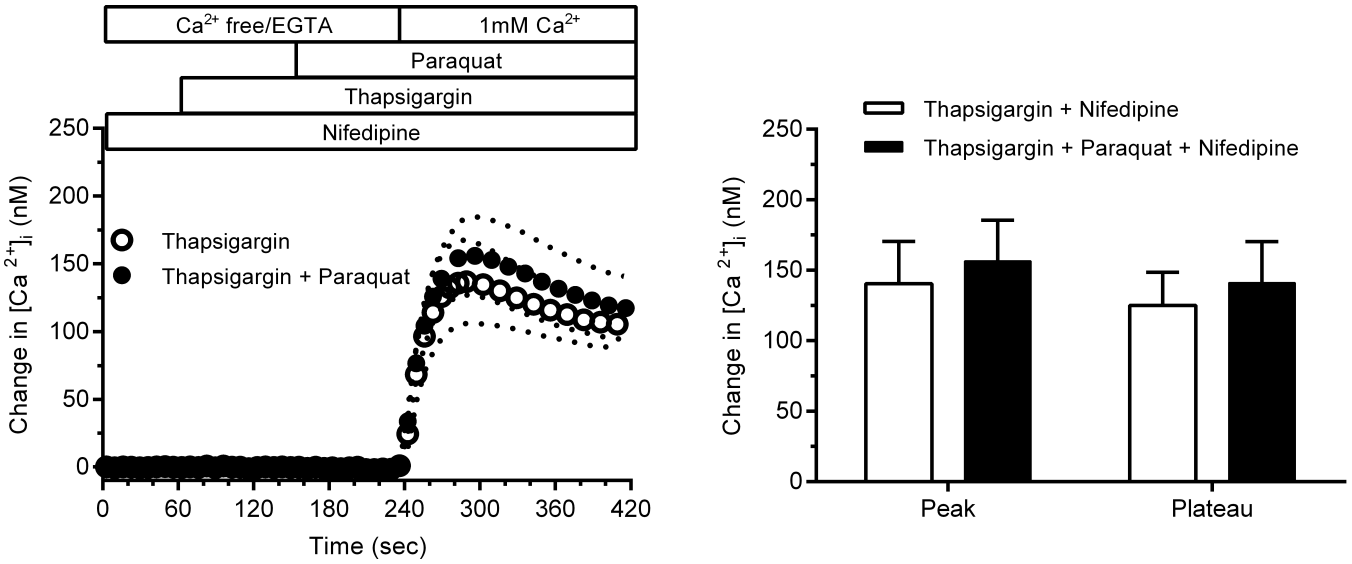

Fig. 5.

Left panel: The magnitude of Ca^{2+} entry through SOC after thapsigargin-induced depletion of sarcoplasmic reticular Ca^{2+} stores and the lack of effect of paraquat (n=8 for both). Right panel: Peak and plateau values for $[Ca^{2+}]_i$ as a result of Ca^{2+}entry through SOC before and after addition of paraquat.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

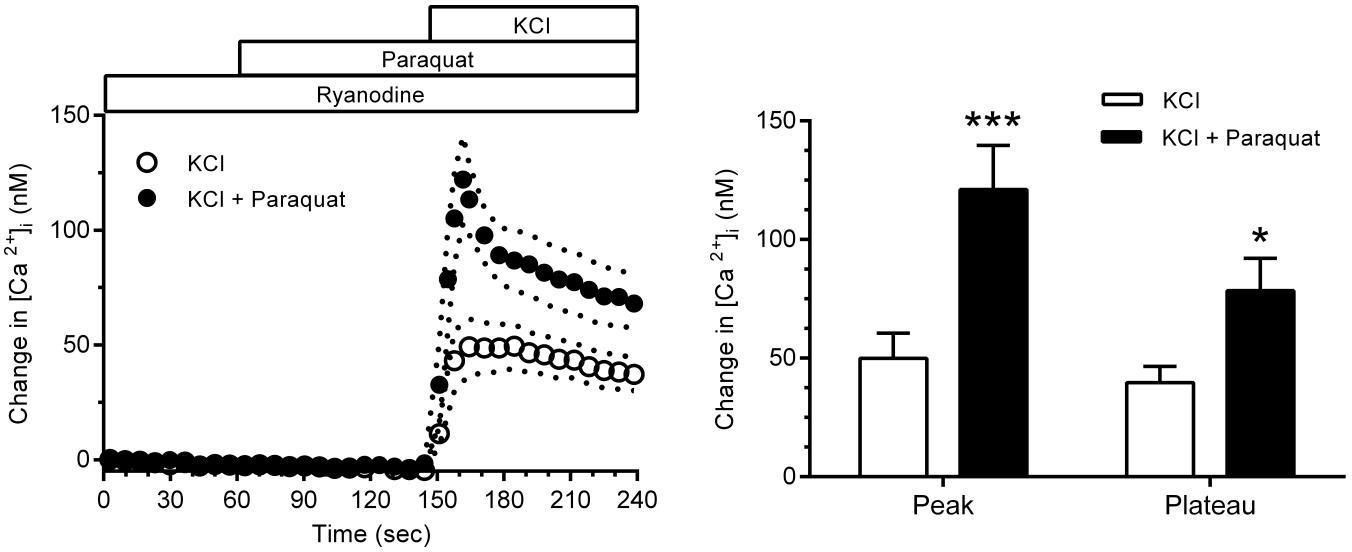

Fig. 6.

Left panel: $[Ca^{2+}]_i$ responses to KCl during inactivation of RyR-induced CICR in the presence (n=9) and absence (n=8) of paraquat. Right panel: Effect of paraquat on the average peak and plateau $[Ca^{2+}]_i$ responses (*** $p<0.005$, * $p<0.05$).