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RESEARCH PAPER

Hydrogen peroxide activates store-operated Ca^{2+} entry in coronary arteries

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BACKGROUND AND PURPOSE

Abnormal Ca^{2+} metabolism has been involved in the pathogenesis of vascular dysfunction associated with oxidative stress. Here, we have investigated the actions of H_2O_2 on store-operated Ca^{2+} (SOC) entry in coronary arteries and assessed whether it is impaired in arteries from a rat model of metabolic syndrome.

EXPERIMENTAL APPROACH

Simultaneous measurements of intracellular Ca^{2+} concentration and contractile responses were made in coronary arteries from Wistar and obese Zucker rats, mounted in microvascular myographs, and the effects of H_2O_2 were assessed.

KEY RESULTS

H_2O_2 raised intracellular Ca^{2+} concentrations, accompanied by simultaneous vasoconstriction that was markedly reduced in a Ca^{2+}-free medium. Upon Ca^{2+} re-addition, a nifedipine-resistant sustained Ca^{2+} entry, not coupled to contraction, was obtained in endothelium-denuded coronary arteries. The effect of H_2O_2 on this voltage-independent Ca^{2+} influx was concentration-dependent, and high micromolar H_2O_2 concentrations were inhibitory and reduced SOC entry evoked by inhibition of the sarcoplasmic reticulum ATPase (SERCA). H_2O_2-induced increases in Fura signals were mimicked by Ba^{2+} and reduced by heparin, Gd^{3+} ions and by Pyr6, a selective inhibitor of the Orai1-mediated Ca^{2+} entry,. In coronary arteries from obese Zucker rats, intracellular Ca^{2+} mobilization and SOC entry activated by acute exposure to H_2O_2 were augmented and associated with local oxidative stress.

CONCLUSION AND IMPLICATIONS

H_2O_2 exerted dual concentration-dependent stimulatory/inhibitory effects on store-operated, IP_3 receptor-mediated and Orai1-mediated Ca^{2+} entry, not coupled to vasoconstriction in coronary vascular smooth muscle. SOC entry activated by H_2O_2 was enhanced and associated with vascular oxidative stress in coronary arteries in metabolic syndrome.

Abbreviations

$[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CPA, cyclopiazonic acid; CRAC, Ca^{2+} release Ca^{2+}-activated; EDH, endothelium-derived hyperpolarization; ER, endoplasmic reticulum; IP_3, inositol trisphosphate; KPSS, 125 mM K^+ physiological saline solution; LZR, lean Zucker rat; OZR, obese Zucker rat; PSS, physiological saline solution; Pyr6, *N*-(4-(3,5-bis (trifluoromethyl)-1*H*-pyrazol-1-yl)phenyl)-3-fluorisonicotinamide; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; SOC, store-operated Ca^{2+}; SR, sarcoplasmic reticulum; STIM1, stromal interaction molecule 1

Tables of Links

TARGETS
Ligand-gated ion channels[a]
IP_3 receptor
Ion channels[b]
L-type Ca^{2+} channels ($Ca_V1.x$)
TRPC1
TRPM2

LIGANDS
CPA, cyclopiazonic acid
IP_3
Nifedipine
H_2O_2

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a b]Alexander *et al.*, 2013a, b).

Introduction

Reactive oxygen species (ROS) derived from various sources in the vascular wall are involved in the regulation of redox-sensitive physiological processes such as vasoconstriction, proliferation and migration. A role of ROS as intracellular signalling molecules in the regulation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of different cell types is now well established (Trebak *et al.*, 2010; Bogeski *et al.*, 2011). For instance, H_2O_2 and other ROS can activate Ca^{2+} mobilization from intracellular stores and stimulate Ca^{2+} entry through ion channels in the plasma membrane to increase the overall $[Ca^{2+}]_i$. Conversely, the activity of ROS-generating enzymes such as NADPH oxidase and NOS can be regulated by stimuli inducing changes in $[Ca^{2+}]_i$ (Touyz, 2005; Trebak *et al.*, 2010).

ROS have been reported to regulate Ca^{2+} influx through both voltage-dependent and non-voltage-dependent plasmalemmal Ca^{2+} channels (Trebak *et al.*, 2010). Voltage-gated L-type Ca^{2+} channels play a key role in increasing the global $[Ca^{2+}]_i$ during vascular smooth muscle (VSM) contraction, and both superoxide anion (O_2^-) and H_2O_2 exogenously applied or endogenously produced in response to vasoconstrictors and growth factors have been shown to activate L-type Ca^{2+} entry in VSM (Chaplin and Amberg, 2012; Santiago *et al.*, 2013), as well as in cardiac myocytes (Hool, 2000; Hool and Arthur, 2002; Viola *et al.*, 2007). Ca^{2+} entry through non-voltage-dependent channels is produced following receptor activation and/or falling of Ca^{2+} levels of the intracellular stores in the endoplasmic reticulum (ER), the latter known as *capacitative Ca^{2+} entry* that takes place through store-operated Ca^{2+} (SOC) channels (Parekh and Putney, 2005). Modulation of Ca^{2+} entry through non-voltage–gated channels by ROS has been characterized mainly in mammalian non-excitable cells including endothelial cells (Hu *et al.*, 1998; Az-ma *et al.*, 1999; Redondo *et al.*, 2004a,b) and to a lesser extent in excitable VSM cells, where information remains scarce (Lin *et al.*, 2007; Pourmahram *et al.*, 2008).

Accumulated evidence suggests that ROS are critically involved in the physiological regulation of coronary blood flow. H_2O_2 plays a role along with NO and adenosine in coronary autoregulation (Yada *et al.*, 2003) and is released by shear stress in coronary arterioles (Miura *et al.*, 2003) and involved in the pacing-induced metabolic coronary vasodilatation to couple coronary blood flow to myocardial O_2 consumption (Saitoh *et al.*, 2006; Yada *et al.*, 2007). H_2O_2 is an endogenous mediator of the endothelium-derived hyperpolarization (EDH) of coronary microvessels (Matoba *et al.*, 2003; Shimokawa, 2010), through activation of Ca^{2+}-activated K^+ channels (Barlow and White, 1998) and of the Na^+/K^+ pump (Wong *et al.*, 2014). We have recently demonstrated that H_2O_2 can also induce endothelium-dependent vasoconstriction tightly coupled to increased Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels in coronary arteries (Santiago *et al.*, 2013), a mechanism shared by cardiac myocytes where endogenous peroxide activates L-type Ca^{2+} currents and has been proposed to sensitize these Ca^{2+} channels and increase the responses to β-adrenoceptor stimulation (Hool, 2000; Hool and Arthur, 2002). However, H_2O_2 also induced a nifedipine-resistant $[Ca^{2+}]_i$ rise in coronary VSM (Santiago *et al.*, 2013) consistent with the fact that H_2O_2 might mobilize intracellular Ca^{2+} and subsequent Ca^{2+} entry through non-selective cation channels, as reported in pulmonary arterial myocytes (Lin *et al.*, 2007; Pourmahram *et al.*, 2008).

In the present study, we sought to determine whether H_2O_2 mobilized Ca^{2+} from intracellular stores and activated store depletion-dependent Ca^{2+} entry in coronary arteries. Furthermore, the effect of H_2O_2 on SOC entry in coronary arteries was also assessed under pathological conditions of high vascular oxidative stress such as metabolic syndrome (Furukawa *et al.*, 2004), a constellation of metabolic and cardiovascular abnormalities including obesity, insulin resistance, dyslipidemia and hypertension, which represent a risk condition for ischaemic heart disease (Arbel *et al.*, 2015).

Methods

Animals and tissue preparation

All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and were

approved by the IACUC at Complutense University (Madrid, Spain). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of N animals were used in the experiments described here.

Male Wistar rats 12–14 weeks old ($n = 55$) and Zucker rats 17–18 weeks old ($n = 20$, provided by Charles River Laboratories France, 69592 L'Arbresle Cedex France) were used. Animals were anesthetized with sodium pentobarbital (40 mg·kg^{-1}, i.p.) and killed by cervical dislocation and exsanguination. The heart was quickly removed and transferred into cold (4°C) physiological saline solution (PSS) of the following composition (mM): 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 1.5 $CaCl_2$, 24.9 $NaHCO_3$, 0.027 EDTA, and 11 glucose, pH = 7.4. Second-order branches of the left anterior descending coronary artery were dissected and mounted in microvascular myographs (Danish Myotechnology, Denmark) for isometric tension recording. The arteries were equilibrated for 30 min in PSS at 37°C while continuously gassed with a mixture of 95% O_2–5% CO_2. The internal circumference L_{100} corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ* was calculated, and the arteries were set to L_1 equal to 0.9 times L_{100} ($L_1 = 0.9 \times L_{100}$) (Mulvany and Halpern, 1977).

The obese Zucker rat (OZR) was used as a well-established model of insulin resistance and the metabolic syndrome, caused by a dysfunctional gene of the leptin receptor. Male OZR (*fa/fa*, $n = 13$)) and their control counterparts, lean Zucker rats (LZR) (*fa*/-, $n = 7$)), were purchased from Charles River Laboratories (Barcelona, Spain) at 8–10 weeks of age, housed at the Pharmacy School animal care facility and maintained on standard chow and water *ad libitum*, until they were used for study, at 16–18 weeks of age (Villalba *et al.*, 2009).

Simultaneous measurements of $[Ca^{2+}]_i$ and tension

Simultaneous measurements of $[Ca^{2+}]_i$ and tension were performed in intact arterial segments using Fura-2-acetoxymethylester (Fura-2-AM) fluorescence as described previously (Villalba *et al.*, 2008; Santiago *et al.*, 2013). Coronary arteries were incubated in the dark at 37°C in PSS containing the indicator 4 μM Fura-2-AM and 0.05% Cremophor EL for a 2 h period. They were washed three times in PSS to remove remaining Fura-2-AM, and the solution was changed to PSS with fresh Fura-2-AM after 1 h. After Fura-2-AM loading, arteries were washed for 45 min in PSS. Experiments were performed in PSS (37°C) continuously gassed with a mixture of 95% O_2–5% CO_2 to maintain pH at 7.4. The myograph was mounted on an inverted microscope (Zeiss Axiovert S100 TV) equipped for dual excitation wavelength microfluorimetry (Deltascan, Photon Technology International).The coronary artery was alternately illuminated at two different wavelengths, 340 and 380 nm, and the intensity of emitted fluorescence was collected through a 510 nm filter using a photomultiplier and monitored together with the tension. At the end of each experiment, Ca^{2+}-insensitive signals determined by quenching of Fura-2-AM with Mn^{2+} were subtracted from the measured emission levels at 340 (F_{340}) and 380 nm (F_{380}). The ratio (R) F_{340}/F_{380} corrected for autofluorescence was taken as a measure of $[Ca^{2+}]_i$.

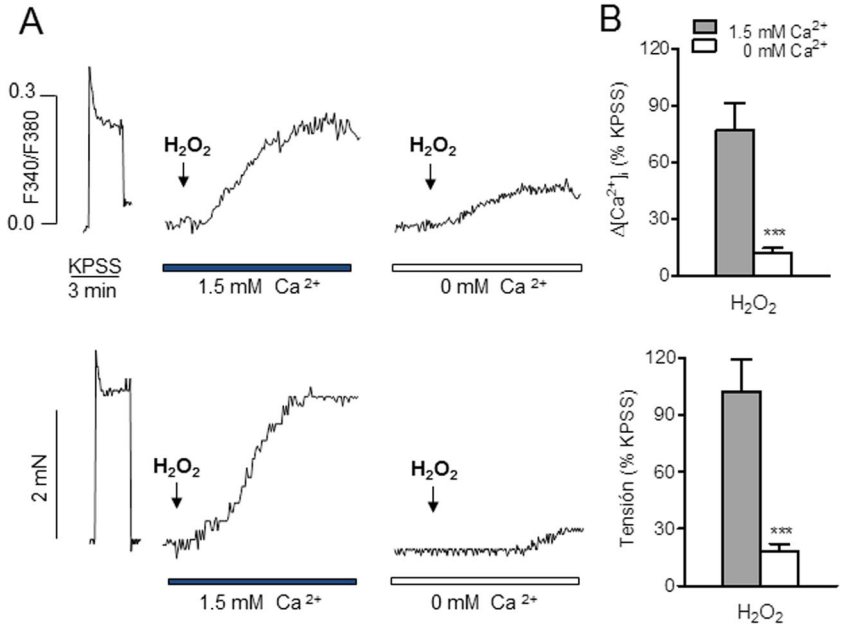

Figure 1

Rise in $[Ca^{2+}]_i$ and vasoconstriction induced by H_2O_2 are in part to due to mobilization of Ca^{2+} from intracellular stores in coronary arteries. (A) Simultaneous recordings showing changes in $[Ca^{2+}]_i$ (top) and tension (bottom) induced by KPSS and H_2O_2 in the presence and absence of extracellular Ca^{2+} in coronary arteries with intact endothelium. (B) Summarized data showing the changes in $[Ca^{2+}]_i$ (top) and tension (bottom) in response to H_2O_2 (100 μM) in the presence and absence of extracellular Ca^{2+}. Results are expressed as a percentage of the KPSS-induced contraction and are means ± SEM of eight animals. ***$P < 0.001$; significantly different from control; Student's *t*-test.

Experimental procedures for the functional experiments

At the beginning of each experiment, the viability of each artery was tested by stimulating it twice with 124 mM K^+ PSS (KPSS), similar to PSS except that NaCl was replaced by KCl on an equimolar basis. In some experiments, the endothelial cell layer was mechanically removed by guiding a human hair through the vessel lumen and gently moving it back and forward as described previously. Absence of functional endothelium was assessed by the lack of relaxation to ACh (10 μM). The role of intracellular Ca^{2+} in the H_2O_2-induced vasoconstriction was assessed by stimulating the arteries with a single dose of H_2O_2 (100 μM) in the absence or presence of extracellular Ca^{2+}. In order to evaluate whether store depletion by H_2O_2 induces 'capacitative Ca^{2+} entry', the bath solution was exchanged for Ca^{2+}-free PSS for 5 min and then for nominally Ca^{2+}-free PSS with nifedipine (1 μM), a selective blocker of L-type voltage-operated Ca^{2+} channels, for a period of 10 min. H_2O_2 (30, 100 μM and 300 μM) or cyclopiazonic acid (CPA; 20 μM), a selective inhibitor of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) was then added for a period of 8 min, after which 1.5 mM Ca^{2+} or Ba^{2+} was re-introduced into the bath solution and kept for 20 min.

The effects of H_2O_2 on the SOC entry in coronary VSM were characterized in endothelium-denuded coronary arteries. The inositol trisphosphate receptor (IP_3R) antagonist heparin (200 μg·mL^{-1}) (Saleem *et al.*, 2014), Gd^{3+} ions (50 μM), as a non-selective cation channel blocker and Pyr6 (3 μM; *N*-(4-(3,5-bis(trifluoromethyl)-1*H*-pyrazol-1-yl)phenyl)-3-fluorisonicotinamide), a new selective blocker of the stromal interaction molecule 1 (STIM1) and Orai1-coupled Ca^{2+} release Ca^{2+}-activated (CRAC)-mediated SOC entry (Schleifer *et al.*, 2012) were tested on the non-L-type Ca^{2+} entry induced by store depletion with either H_2O_2 or CPA. In order to assess thiol oxidation-dependency of H_2O_2 actions on Ca^{2+} entry, the effect of H_2O_2 on the SOC entry in coronary arteries was assessed in arteries treated with the reducing agent DTT (100 μM).

Measurement of O_2^- production by chemiluminescence

Levels of production O_2^- by coronary arteries and myocardial tissue from control (LZR) and metabolic syndrome (OZR) rats under basal conditions were detected by lucigenin-enhanced chemiluminescence as previously described in heart tissue (Santiago *et al.*, 2013). Briefly, segments of coronary arteries and samples of myocardial tissue were dissected and equilibrated in PSS for 30 min at room temperature and then incubated in the presence or absence of tempol (30 μM) at 37°C. Samples were then transferred to microtiter plate wells containing 5 μM lucigenin in air-equilibrated Krebs solution buffered with 10 mM HEPES-NaOH, in the absence and presence of tempol, and H_2O_2 (100 μM) was then applied.

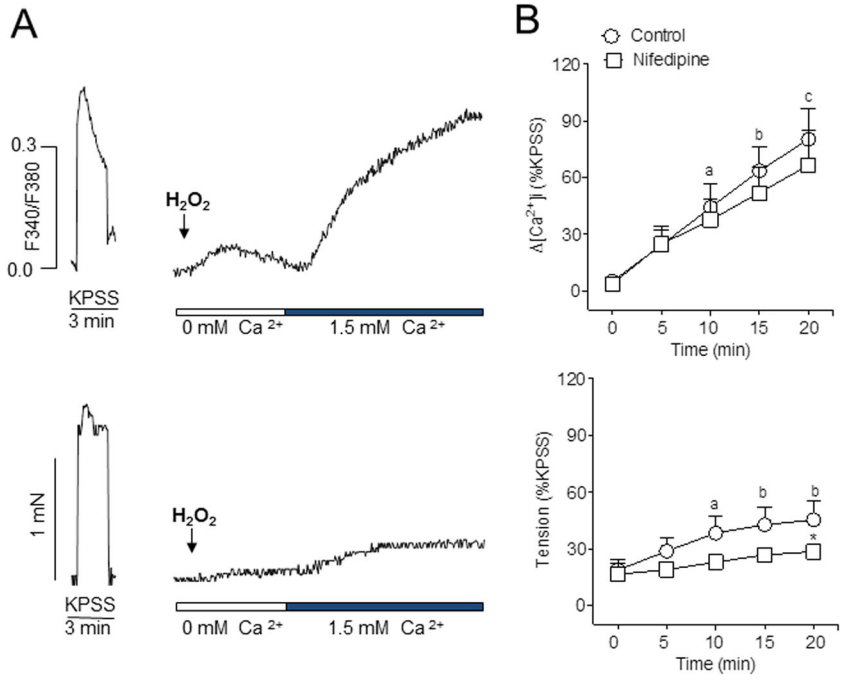

Figure 2

H_2O_2 induces L-type voltage channel-independent Ca^{2+} entry in coronary arteries. (A) Simultaneous recordings of $[Ca^{2+}]_i$ (A, top) and tension (A, bottom) showing the marked increase in $[Ca^{2+}]_i$ and moderate vasoconstriction produced by H_2O_2 (100 μM) in a Ca^{2+}-free medium after the re-addition of 1.5 mM Ca^{2+} in endothelium-intact coronary arteries. (B) Summarized data showing the changes in $[Ca^{2+}]_i$ (top) and tension (bottom) induced by H_2O_2 (100 μM) in response to the re-addition of Ca^{2+} to the extracellular medium, in the absence and presence of the L-type channel blocker nifedipine (1 μM). Results are expressed as a percentage of the KPSS-induced contraction and are means ± SEM of five animals. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$; significantly different from $[Ca^{2+}]_i$ or tension at time 0 (Ca^{2+} re-addition); one-way ANOVA. $^*P < 0.05$; significantly different from control (absence of nifedipine); Student's *t*-test.

Chemiluminescence was measured in a luminometer (BMG Fluostar Optima). Baseline values were subtracted from the observed values under the different experimental conditions, and O_2^- production was normalized to the weight of the tissue samples.

Data analysis

Mechanical responses of the arteries were measured as force and expressed as active wall tension, which is the increase in force, ΔF, divided by twice the segment length (Mulvany and Halpern, 1977). Changes in $[Ca^{2+}]_i$ were measured as the increase in the Fura-2-AM ratio taking as baseline the average value of the baseline in PSS and baseline in Ca^{2+}-free PSS (ΔF340/F380). The results are expressed as either absolute values of tension (as N m^{-1}) or in units of ratio of fluorescence (F340/F380), or as a percentage of the responses induced by KPSS. All the results are reported as means ± SEM; *n* represents the number of animals. One arterial segment per animal was used in the calcium experiments, and one to two arterial or myocardial samples per animal were used in the chemiluminescence experiments for O_2^- measurement. Statistical differences between means were analyzed by unpaired Student's *t*-test for comparison between two groups when assessing the effect of various treatments on the changes in $[Ca^{2+}]_i$ and tension activated by H_2O_2 or CPA. Two-factor repeated measures one-way ANOVA followed by a Bonferroni *post hoc* test was applied to assess statistically significant differences in changes in either $[Ca^{2+}]_i$ or tension activated by H_2O_2 or CPA (factor 1) over time (factor 2). The time at which Ca^{2+} re-addition was made was taken as time 0. $P < 0.05$ was considered significant. All calculations were made using a standard software package (Prism 5.0; GraphPad Software).

Materials

The sources of the compounds used were as follows: barium chloride, gadolinium chloride, H_2O_2, indomethacin and lucigenin were obtained from Sigma Aldrich (Madrid, Spain), Fura-2-AM and ionomycin from Invitrogen (Life Technologies SA, Madrid, Spain), CPA, heparin and nifedipine from Tocris (Bristol, UK) and Pyr6 from Calbiochem (Merck-Millipore, Madrid, Spain).

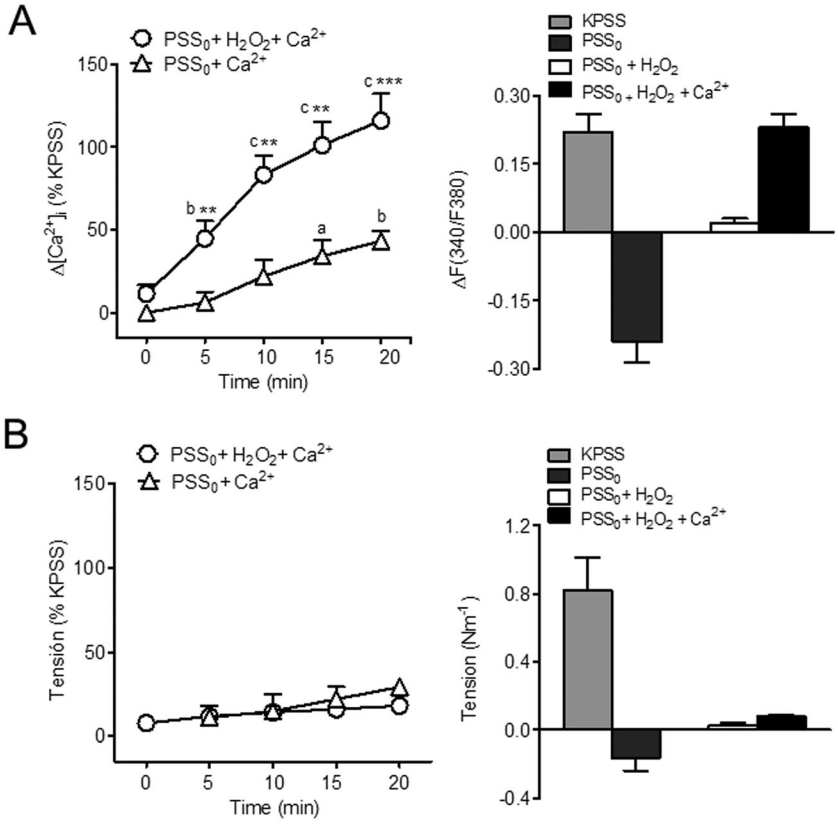

Figure 3

H_2O_2 promotes store-operated Ca^{2+} entry not coupled to contraction in coronary vascular smooth muscle. Average changes in (A) $[Ca^{2+}]_i$ and (B) contraction evoked by Ca^{2+} re-addition to endothelium-denuded coronary arteries kept in Ca^{2+}-free medium (PSS_0) and activated by H_2O_2 under conditions of L-type channel blockade. In the presence of nifedipine (1 μM) and 100 μM H_2O_2, re-admission of 1.5 mM Ca^{2+} to the extracellular medium caused a large rise in $[Ca^{2+}]_i$ (A, left) similar to the one induced by KPSS (A, right), which was not accompanied by significant changes in tension (B). In control experiments, Ca^{2+} re-addition was conducted in arteries kept in Ca^{2+}-free PSS in the absence of activating H_2O_2. Results are expressed as a percentage of the KPSS-induced responses (A, B right) or as absolute values of F340/380 (A, left) and of tension (as N m^{-1}: B, left) and are means ± SEM of four to nine animals. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$; significantly different from $[Ca^{2+}]_i$ or tension at time 0 (Ca^{2+} re-addition); one-way ANOVA.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from control (absence of H_2O_2); Student's *t*-test.

Results

H_2O_2 stimulates Ca^{2+} entry and mobilizes Ca^{2+} from intracellular stores in coronary arteries

To assess whether H_2O_2 might induce Ca^{2+} mobilization from intracellular stores thus activating SOC entry, coronary arteries were stimulated with a single submaximal vasoconstrictor concentration of H_2O_2 (100 μM) (Santiago *et al.*, 2013), in the absence and presence of extracelullar Ca^{2+}. In PSS containing 1.5 mM Ca^{2+}, H_2O_2 induced an increase in $[Ca^{2+}]_i$ ($\Delta F_{340}/F_{380}$ = 0.33 ± 0.06, n = 8) that was accompanied by a simultaneous sustained vasoconstriction on baseline tension (0.67 ± 0.11 Nm^{-1}, n = 8) (Figure 1). Both the $[Ca^{2+}]_i$ rise and the increase in tension induced by H_2O_2 were markedly reduced after removal of Ca^{2+} from the extracellular medium, although a small fraction of the $[Ca^{2+}]_i$ rise ($\Delta F_{340}/F_{380}$ = 0.05 ± 0.01, n = 8) and contraction (0.11 ± 0.02 Nm^{-1}, n = 8) still persisted in Ca^{2+}-free medium (Figure 1). These data suggest that H_2O_2 induces Ca^{2+} mobilization from intracellular stores.

H_2O_2 stimulates SOC entry not coupled to contraction in coronary arteries

In Ca^{2+}-free PSS, addition of H_2O_2 produced a small Ca^{2+} mobilization and further re-addition of Ca^{2+} (1.5 mM) to the medium resulted in a marked and sustained increase in $[Ca^{2+}]_i$ after 20 min ($\Delta F_{340}/F_{380}$ = 0.48 ± 0.16, n = 5) similar in magnitude to the increase in $[Ca^{2+}]_i$ produced by stimulation with KPSS in the same arteries ($\Delta F_{340}/F_{380}$ = 0.52 ± 0.16, n = 5) (Figure 2A, top). However, vasoconstriction induced by H_2O_2 under these conditions (0.29 ± 0.07 Nm^{-1}) was about one-third of the KPSS-elicited contraction (0.88 ± 0.22, n = 5) (Figure 2A, bottom). The changes in $[Ca^{2+}]_i$ induced by H_2O_2 were not affected by the presence of the voltage-dependent L-type Ca^{2+} channel blocker nifedipine (Figure 2B, top), suggesting that Ca^{2+} entry occurs through voltage-independent Ca^{2+} channels activated by emptying of intracellular Ca^{2+} stores induced by H_2O_2. Nifedipine blunted the small contraction induced by H_2O_2 (Figure 2B, bottom).

Characterization of SOC entry activated by H_2O_2 in coronary VSM

Because vascular endothelium is involved in the Ca^{2+} mobilization induced by H_2O_2 in the coronary arterial wall (Santiago *et al.*, 2013), experiments were performed in coronary arteries in which endothelium was mechanically removed in order to assess the SOC entry activated by H_2O_2 in coronary VSM. In endothelium-denuded arteries kept in a Ca^{2+}-free medium under conditions of L-type channel blockade, H_2O_2 (100 μM) mobilized intracellular Ca^{2+}, and readmission of Ca^{2+} to the medium evoked a large and sustained increase in $[Ca^{2+}]_i$ (Figure 3A) similar to that obtained in endothelium-intact arteries and not significantly different from the one induced by depolarization with KPSS in the same arteries. This $[Ca^{2+}]_i$ increase was not accompanied by significant increases in tension (Figure 3B). These data suggest that H_2O_2 activates SOC entry in VSM not coupled to contraction. Re-introduction of Ca^{2+} in the absence of peroxide evoked a minor $[Ca^{2+}]_i$ increase in endothelium-denuded coronary arteries (Figure 3A).

SOC entry activated by H_2O_2 in coronary VSM was compared with the Ca^{2+} entry stimulated by passive sarcoplasmic reticulum (SR) Ca^{2+} depletion by SERCA inhibition with CPA. In endothelium-denuded coronary arteries kept in Ca^{2+}-free PSS, CPA (20 μM) induced a moderate increase in $[Ca^{2+}]_i$ significantly larger than that evoked by 100 μM H_2O_2 (Figure 4A–C). Re-addition of Ca^{2+} to the medium resulted in a marked rise in $[Ca^{2+}]_i$ in the presence of CPA (Figure 4B) that was about 1.5-fold larger than the one induced by H_2O_2 (Figure 4C and D).

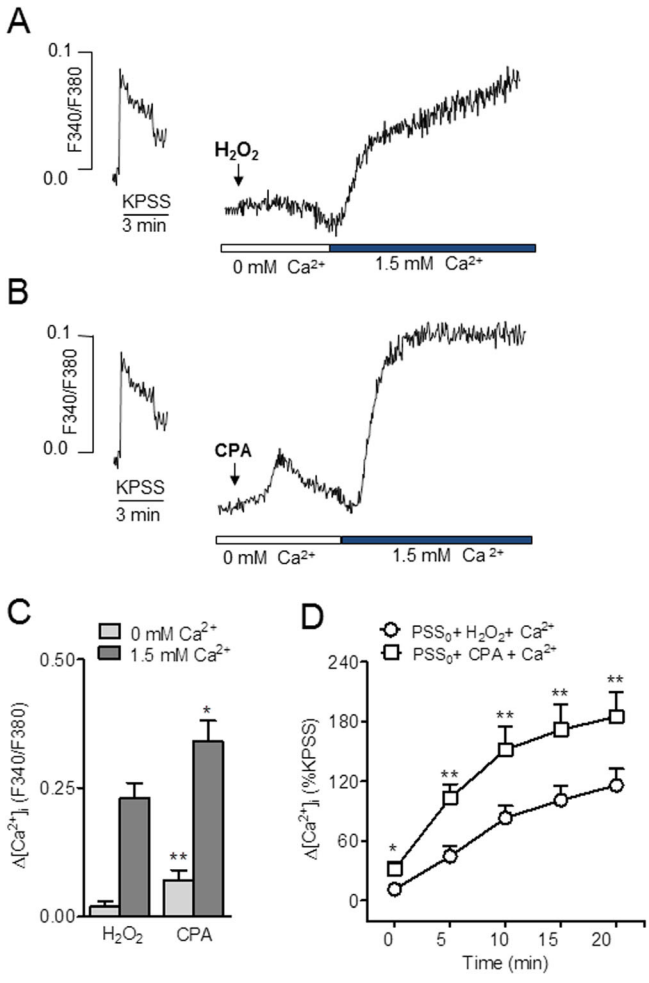

Figure 4

Store-operated Ca^{2+} entry induced by SERCA inhibition and by H_2O_2 in coronary vascular smooth muscle. (A, B) Representative traces showing changes in $[Ca^{2+}]_i$ induced by H_2O_2 (100 μM) (A) and by the SERCA nhibitor CPA (20 μM) (B) in response to the re-addition of Ca^{2+} to the extracellular Ca^{2+}-free medium in an endothelium-denuded coronary artery. (C) Comparative average effects of CPA and 100 μM H_2O_2, on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} and after Ca^{2+} re-addition. (D) Average time-dependent changes in $[Ca^{2+}]_i$ in response to the re-addition of Ca^{2+} to the extracellular medium in endothelium-denuded arteries activated with CPA or H_2O_2. Results are expressed as absolute values of F340/380 (C) or as a percentage of the KPSS-induced ΔF340/380 (D) and are means ± SEM of four to nine animals. *$P < 0.05$, **$P < 0.01$; significantly different from control (100 μM H_2O_2); Student's t-test.

To assess whether the action of H_2O_2 on SOC entry was concentration dependent, the effect of three different concentrations of H_2O_2 on the Ca^{2+} entry upon Ca^{2+} re-addition to the extracellular medium was compared. While 100 μM H_2O_2 increased $[Ca^{2+}]_i$ about 116% of the KPPS-induced response, 30 μM H_2O_2 induced about half this response, while 300 μM H_2O_2 had an inhibitory effect compared with the effect at 100 μM (Figure 5A–D). Moreover, treatment of endothelium-denuded coronary arteries with 300 μM H_2O_2 for 30 min reduced by half SOC entry induced by SERCA inhibition with CPA (Figure 5E). At 1 mM H_2O_2, SOC entry was nearly abolished (not shown). Subsequent experiments were performed at 100 μM H_2O_2.

Activation of IP_3Rs and Orai1 is involved in the H_2O_2-induced Ca^{2+} entry of coronary VSM

To investigate whether stimulation of IP_3Rs and the subsequent intracellular Ca^{2+} release underlie H_2O_2-induced Ca^{2+} entry, coronary arteries were treated with the IP_3R antagonist heparin (200 $\mu g \cdot mL^{-1}$). Acute exposure to heparin reduced the nifedipine-resistant Ca^{2+} influx activated by H_2O_2 (Figure 6A) and caused a large inhibition of SOC entry obtained by SERCA inhibition with CPA (Figure 6B). Heparin also reduced the rises in intracellular Ca^{2+} induced by CPA but not by H_2O_2 in the absence of extracellular Ca^{2+} (Figure 6C).

The contribution of non-selective cation channels to the Ca^{2+} entry stimulated by H_2O_2 in coronary VSM was evaluated by using Ba^{2+} fluorimetry instead of Ca^{2+} to investigate the influx of divalent cations activated by peroxide treatment without the intervention of Ca^{2+} extrusion and of Ca^{2+} storage mechanisms (Villalba *et al.*, 2008). In endothelium-denuded coronary arteries kept in Ca^{2+}-free PSS under conditions of L-type channel blockade and stimulated with H_2O_2 (100 μM), addition of Ba^{2+} (1.5 mM) to the Ca^{2+}-free solution increased the Fura-2-AM ratio to a similar extent to the rise elicited by Ca^{2+} re-addition (Figure 7A, B and D). Moreover, treatment of coronary arteries with Gd^{3+} (50 μM), a

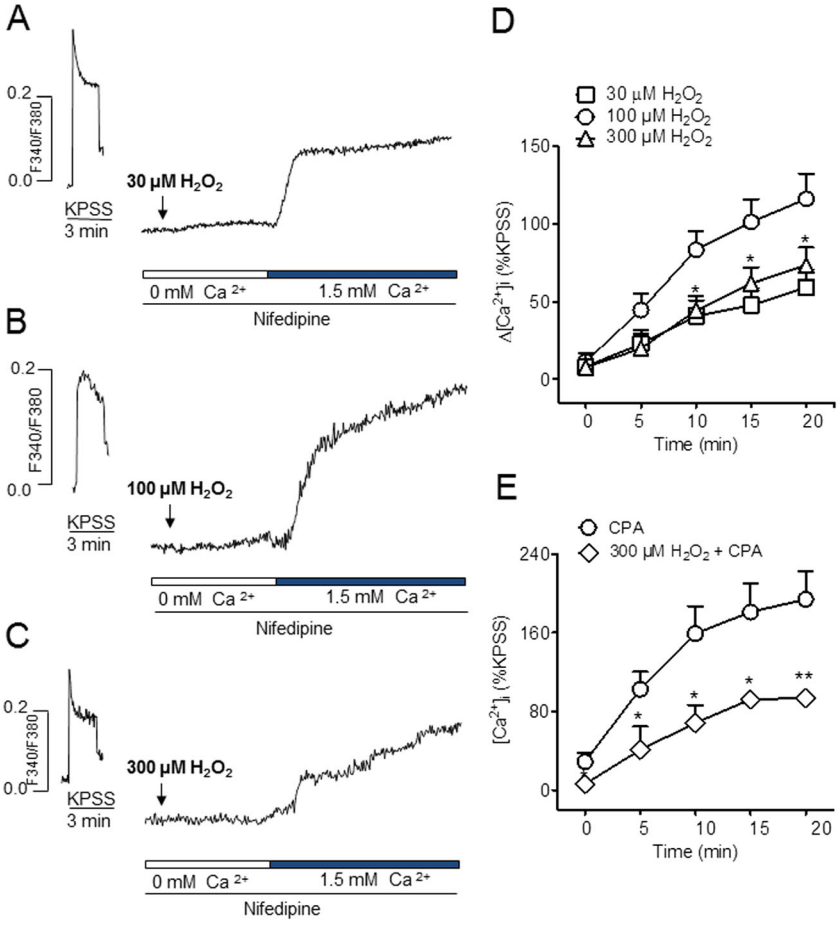

Figure 5

H_2O_2 has dual concentration-dependent effects on non-L-type Ca^{2+} entry in coronary vascular smooth muscle. (A, B, C) Representative traces showing the increase in $[Ca^{2+}]_i$ produced by the re-addition of Ca^{2+} (1.5 mM) to the extracellular medium after stimulation with 30 (A), 100 (B) or 300 μM H_2O_2 (C) in endothelium-denuded coronary arteries. (D) Mean values of the changes in $[Ca^{2+}]_i$ in response to the re-addition of Ca^{2+} (1.5 mM) into the extracellular medium after stimulation 30, 100 and 300 μM H_2O_2. (E) Mean values of the changes in $[Ca^{2+}]_i$ in response to the re-addition of Ca^{2+} (1.5 mM) into the extracellular medium in the presence of CPA (20 μM) in control conditions and after 30 min treatment with 300 μM H_2O_2. Results are expressed as a percentage of the KPSS-induced ΔF340/380 and are means ± SEM of three to nine animals. $*P < 0.05$, $**P < 0.01$, significantly different from control (D, 100 μm H_2O_2) (E, 20 μM CPA); Student's *t*-test.

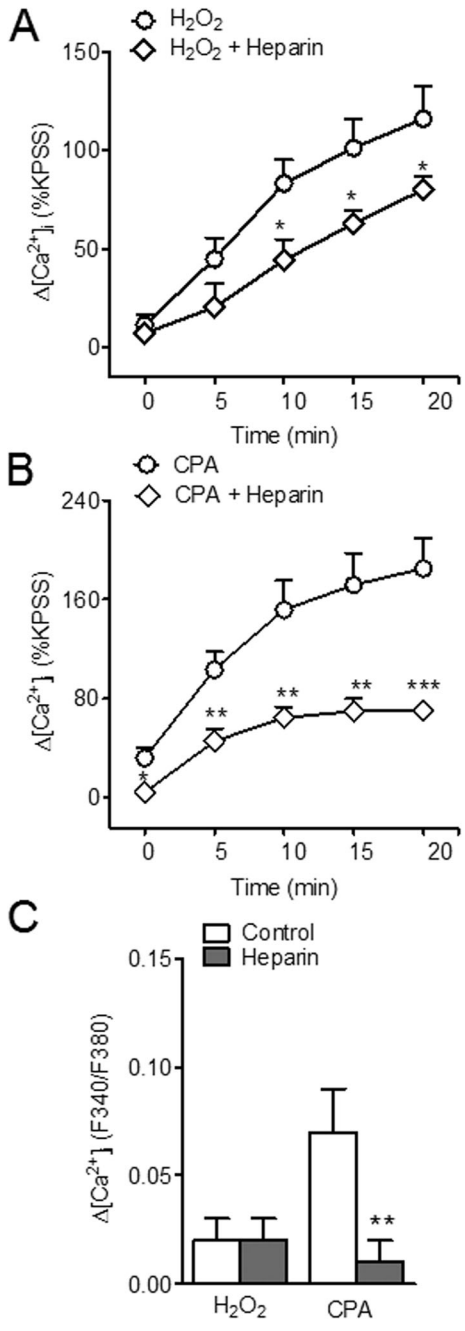

Figure 6

Non-L-type Ca^{2+} entry induced by H_2O_2 is inhibited by the IP_3R antagonist heparin. (A, B) Average values of the changes in $[Ca^{2+}]_i$ induced by H_2O_2 (100 μM) (A) and CPA (20 μM) (B) in response to the re-addition of Ca^{2+} to the extracellular medium in the absence and after 10 min exposure to heparin (200 μg•mL^{-1}) in coronary arteries. (C) Comparative average effects of heparin on the CPA-induced and H_2O_2-induced rises $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Results are expressed as a percentage of the KPSS-induced ΔF340/380 and are means ± SEM of four animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant effects of heparin; Student's *t*-test.

non-selective blocker of cation channels, significantly reduced the increase in $[Ca^{2+}]_i$ activated by H_2O_2 upon extracellular Ca^{2+} re-addition (Figure 7C and E). These results suggest the involvement of non-selective cation channels in the capacitative Ca^{2+} influx activated by H_2O_2 in coronary VSM.

The role of Orai1 channels in the nifedipine-resistant Ca^{2+} entry induced by H_2O_2 was further assessed by treatment of coronary arteries with the new selective inhibitor of the Orai1-mediated SOC entry Pyr6. In the presence of Pyr6 (3 μM), Ca^{2+} influx activated by the re-addition of Ca^{2+} in coronary arteries activated with either H_2O_2 (Figure 8A, C) or CPA (Figure 8 B,D) was abolished and reduced to levels similar to those obtained by Ca^{2+} re-addition in the absence of either H_2O_2 or CPA in control experiments (Figure 3A).

Thiol oxidation-dependent effects of H_2O_2 on SOC entry in coronary VSM

Treatment of endothelium-denuded coronary arteries with the thiol-specific reducing agent DTT (100 μM) induced a marked inhibition of the voltage-independent Ca^{2+} entry stimulated by H_2O_2 (Figure 9A) and to a lesser but not significant extent that induced by SERCA inhibition with CPA (Figure 9B), which suggests that the action of H_2O_2 on SOC entry in VSM is due to oxidation of thiol groups.

Effect of H_2O_2 on SOC entry in coronary VSM in metabolic syndrome

Figure 10 shows that basal O_2^- production was markedly enhanced in coronary arteries (Figure 10A) but not myocardium (Figure 10B) from insulin resistant OZR compared with their lean counterparts and was quenched by the O_2^- scavenger tempol. These data suggest higher levels of vascular oxidative stress in animals with metabolic syndrome.

In order to determine whether the increase in $[Ca^{2+}]_i$ and vasoconstriction elicited by H_2O_2 in coronary VSM was affected by the conditions of metabolic syndrome, endothelium-denuded coronary arteries from LZR and OZR were stimulated with a single concentration of H_2O_2 (100 μM). H_2O_2 induced a sustained increase in $[Ca^{2+}]_i$ (Figure 10C) that was accompanied by a simultaneous and sustained vasoconstriction (Figure 10D) of similar magnitude in coronary arteries from LZR and OZR. Ca^{2+} responses to H_2O_2 were largely inhibited upon removal of extracellular Ca^{2+}, and mobilization of intracellular Ca^{2+} by H_2O_2 was higher in arteries from OZR compared with LZR (Figure 10E). Furthermore, SOC entry activated by H_2O_2 in coronary VSM from OZR was impaired. Thus, in a Ca^{2+}-free medium in the presence of nifedipine, Ca^{2+} entry stimulated by H_2O_2 upon Ca^{2+} re-addition after 20 min was significantly larger in coronary VSM from OZR than in that from LZR (Figure 10F). However, increases in $[Ca^{2+}]_i$ induced by depolarization with KPSS were unchanged in OZR ($\Delta F_{340}/F_{380}$ 0.20 ± 0.02, n = 5) compared with LZR ($\Delta F_{340}/F_{380}$ = 0.21 ± 0.05, n = 6).

Discussion

Interactions between ROS and the molecular machinery involved in non-L-type Ca^{2+} entry in VSM are relatively little explored. The present study provides evidence for a redox regulation of SOC entry in coronary VSM, as well as oxidative stress-associated changes in this store depletion-dependent

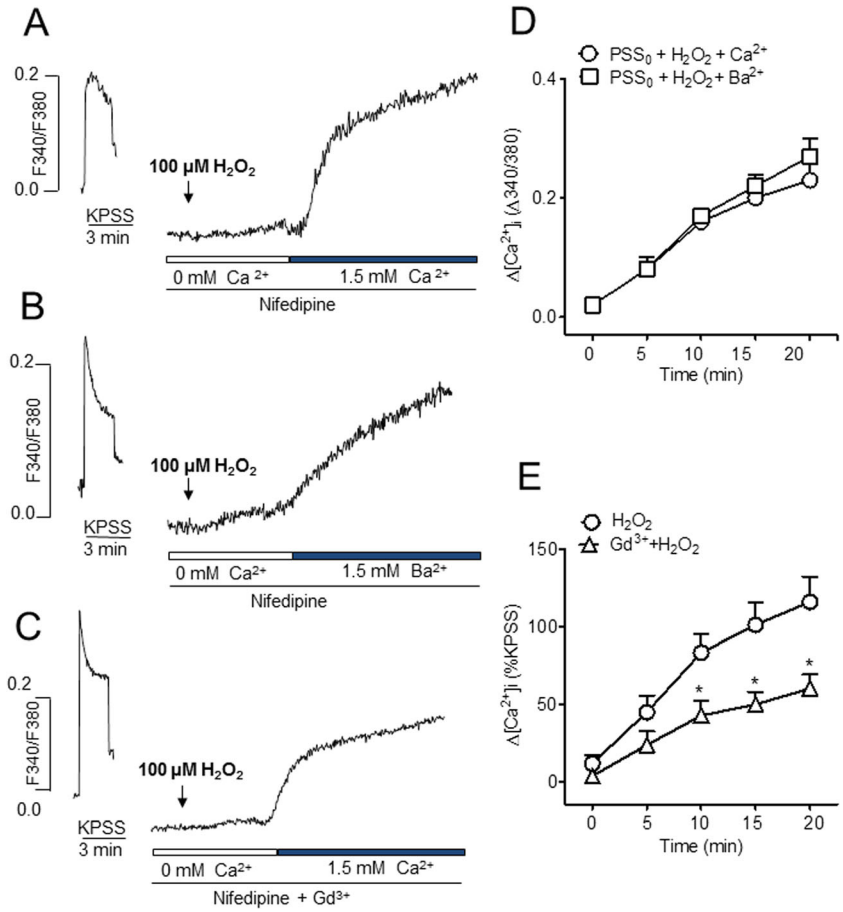

Figure 7

Non-L-type Ca^{2+} entry induced by H_2O_2 is mediated by non-selective cation channels. (A, B, C) Representative traces showing (A) the increase in $[Ca^{2+}]_i$ induced by 100 μM H_2O_2 in a Ca^{2+}-free medium upon Ca^{2+} re-addition, (B) the increase in Fura-2-AM fluorescence in arteries stimulated with 100 μM H_2O_2 in a Ca^{2+}-free medium after re-addition of 1.5 mM Ba^{2+} and (C) the increase in $[Ca^{2+}]_i$ induced by 100 μM H_2O_2 in a Ca^{2+}-free medium upon Ca^{2+} re-addition after treatment with 50 μM Gd^{3+} in endothelium-denuded coronary arteries. (D) Average comparative effects on the increase in F340/380 upon Ca^{2+} and Ba^{2+} re-addition in coronary arteries activated with 100 μM H_2O_2 in a Ca^{2+}-free medium. (E) Mean values of changes in $[Ca^{2+}]_i$ in response to H_2O_2 in the presence and absence of the non-selective cation channel inhibitor Gd^{3+} (50 μM). Results are expressed as a percentage of the KPSS-induced ΔF340/380 and are means ± SEM of three to four animals. *$P < 0.05$, **$P < 0.01$, significant effects of Gd^{3+}; Student's *t*-test).

Ca^{2+} influx under conditions of vascular dysfunction in the metabolic syndrome.

ROS regulation of voltage-independent Ca^{2+} entry has previously been reported mainly in non-excitable cells including blood and endothelial cells, where SOC entry is a major route for Ca^{2+} entry (Hu *et al.*, 1998, 2000; Az-ma *et al.*, 1999; Redondo *et al.*, 2004a,b; Florea and Blatter, 2008; Sun *et al.*, 2011). In the present study, we show that H_2O_2 mobilizes Ca^{2+} from intracellular stores and activates a significant non-L-type Ca^{2+} entry in intact coronary arteries. Because H_2O_2-induced $[Ca^{2+}]_i$ rises in coronary arteries are partially dependent on the endothelium (Santiago *et al.*, 2013), SOC entry was further characterized in endothelium-denuded coronary arteries. Our data confirmed a nifedipine-resistant Ca^{2+} entry activated by H_2O_2 in coronary VSM of similar magnitude to the one obtained by maximum high-K^+ depolarization leading to Ca^{2+} entry through L-type channels and about two-thirds the SOC entry obtained by inhibition of SERCA and passive depletion of the SR with CPA.

Although H_2O_2 is known to produce vasoconstriction via activation of Ca^{2+} entry through L-type Ca^{2+} channels in coronary arteries (Santiago *et al.*, 2013), the current results reveal an uncoupling between the nifedipine-resistant Ca^{2+} entry and contraction activated by H_2O_2 in coronary VSM. H_2O_2 is a well-recognized endogenous mediator of the EDH response in coronary arteries (Matoba *et al.*, 2003; Miura *et al.*, 2003; Yada *et al.*, 2003; Shimokawa, 2010), and therefore, the possibility that H_2O_2 activates vasodilator mechanisms involving K_{Ca} channels (Barlow and White, 1998) or the Na^+/K^+ pump (Wong *et al.*, 2014) cannot be ruled out. However, uncoupling of the nifedipine-resistant Ca^{2+} entry stimulated by H_2O_2 and contraction in coronary arteries, as shown in the present study, is consistent with the uncoupling between the *capacitative Ca^{2+} entry* induced by SERCA inhibition with CPA and vasoconstriction in the same arteries. This dissociation between $[Ca^{2+}]_i$ and contraction is in agreement with that earlier observed in other small arteries and arterioles and suggests that Ca^{2+} entering through SOC channels

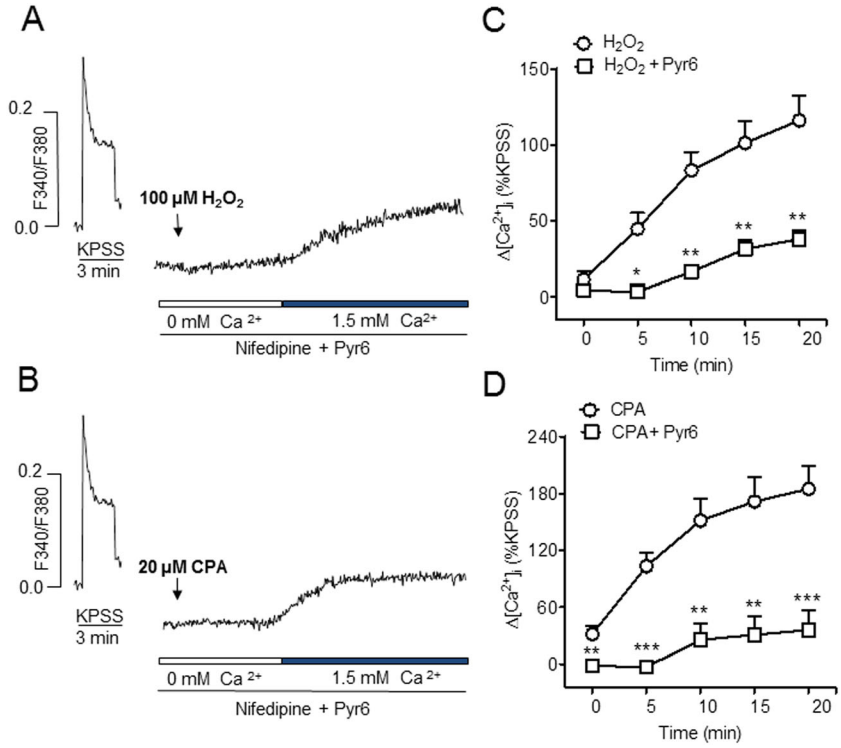

Figure 8

Non-L-type Ca^{2+} entry induced by H_2O_2 is inhibited by Pyr6, a selective blocker of Orai1-mediated Ca^{2+} entry. (A, B) Representative traces showing the increase in $[Ca^{2+}]_i$ induced by H_2O_2 (A) and by CPA (B) in the presence of Pyr6 (3 μM) in response to re-addition of Ca^{2+} to the extracellular medium. (C, D) Mean values of the changes in $[Ca^{2+}]_i$ induced by H_2O_2 (100 μM) (C) and CPA (20 μM) (D) in response to the re-addition of Ca^{2+} to the extracellular medium in the absence and presence of Pyr6 (3 μM) in coronary arteries. Results are expressed as a percentage of the KPSS-induced ΔF340/380 and are means ± SEM of four animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant effects of Pyr6; Student's *t*-test).

is confined to a subcellular compartment that has limited access to the contractile apparatus (Flemming *et al.*, 2002; Snetkov *et al.*, 2003; Villalba *et al.*, 2007). In fact, SOC entry, CRAC currents and channel proteins involved in SOC entry have been reported to be up-regulated in synthetic/proliferative VSM cells compared with quiescent/contractile vascular myocytes and associated to gene expression, proliferation and migration (Berra-Romani *et al.*, 2008; Potier *et al.*, 2009; Li *et al*, 2011).

Studies on the role of ROS in SOC entry in non-excitable cells have often provided controversial data, and both enhanced and attenuated ROS-induced *capacitative Ca^{2+} entry* have been reported, these reciprocal effects being ascribed to the concentration, the time of exposure and the type of oxidant species used in acute experimental protocols. In human platelets (Redondo *et al.*, 2004a), endothelial cells (Elliott *et al.*, 1989; Hu *et al.*, 1998; Florea and Blatter, 2008) and pulmonary artery myocytes (Lin *et al.*, 2007), concentrations of H_2O_2 in the micromolar range up to 500 μM, dose-dependently stimulated intracellular Ca^{2+} release and store depletion-dependent Ca^{2+} entry, while higher millimolar concentrations had an inhibitory effect on SOC entry that was also time-dependent (Florea and Blatter, 2008). In agreement with these observations, the present results indicate that redox regulation of SOC entry in coronary VSM is also dependent on the local levels of ROS and, whereas a maximum nifedipine-resistant Ca^{2+} entry was obtained upon exposure to 100 μM H_2O_2 for 30 min, this Ca^{2+} response and also SOC entry elicited by SERCA inhibition with CPA were decreased at higher concentrations and practically abolished at millimolar concentrations of H_2O_2 in coronary arteries, which suggests an inhibition of store-dependent Ca^{2+} entry under conditions of elevated vascular oxidative stress. Concentrations of H_2O_2 up to 100 μM have been reported in inflammatory microenvironments surrounding macrophages (Dröge, 2003). The inhibitory effects of high levels of H_2O_2 are currently explained on the basis of irreversible oxidations of thiol groups of proteins at higher oxidant conditions, considered as oxidative damage (Nunes and Demaurex, 2014). Consistent with this view, concentrations of H_2O_2 up to 100 μM have been shown to activate the Na^+/K^+ ATPase and the EDH-type relaxant responses in porcine coronary arteries, while exposure to millimolar concentrations of H_2O_2 inhibited the pump and caused non-specific relaxations by impairing VSM contraction (Ellis *et al.*, 2003; Wong *et al.*, 2014). The present findings further suggest that high oxidant stress might induce coronary relaxation by reducing Ca^{2+} entry in VSM.

ROS can influence SOC entry via either direct effects on the molecular machinery of SOC including Orai, the STIM1 and transient receptor potential canonic (TRPC) channels or indirectly by influencing the content of the intracellular SR Ca^{2+} stores (Trebak *et al.*, 2010; Nunes and Demaurex,

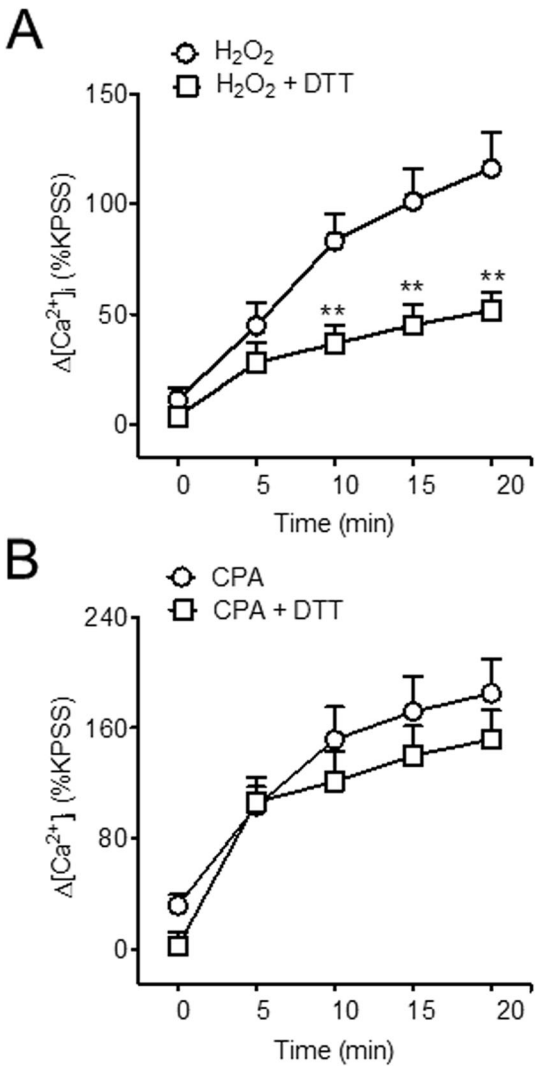

Figure 9

H_2O_2-induced SOC entry is due to thiol oxidation. Average changes in $[Ca^{2+}]_i$ induced by H_2O_2 (100 μM) (A) and CPA (B) in response to the re-addition of Ca^{2+} to the extracellular medium in the absence and after treatment with the thiol specific reducing agent DTT (100 μM) in endothelium-denuded coronary arteries. Results are expressed as a percentage of the KPSS-induced ΔF340/380 and are means ± SEM of four to eight animals. *$P < 0.05$, **$P < 0.01$, significant effects of DTT; Student's *t*-test.

2014). H_2O_2 has been reported to induce intracellular Ca^{2+} release via IP_3R activation in blood cells (Parekh and Penner, 1995; Redondo *et al.*, 2004b), and NADPH-derived endothelial H_2O_2 has been shown to increase the sensitivity of intracellular Ca^{2+} stores to IP_3 in human endothelial cells (Hu *et al.*, 2000; Zheng and Shen, 2005). In the present study, the inhibitory effect of the IP_3R antagonist heparin suggests that H_2O_2-induced SOC Ca^{2+} entry in coronary VSM is partially mediated by store depletion through activation of IP_3R. These findings are consistent with data obtained in DT40 B-lymphocytes where both pharmacological inhibition of IP_3R and molecular knock-out of all three IP_3Rs strongly reduced H_2O_2-induced CRAC currents (Grupe *et al.*, 2010). Heparin also significantly reduced intracellular Ca^{2+} mobilization and SOC entry activated by CPA in coronary arteries, which suggests that passive depletion of the SR by SERCA inhibition is blunted upon blockade of the IP_3R.

The molecular identity of the non-voltage-gated Ca^{2+} channels mediating CRAC currents and SOC entry has been clarified during the last decade, and the ER Ca^{2+} sensor STIM1 (Roos *et al.*, 2005), the plasma membrane Ca^{2+} selective pore-forming subunit Orai1 (Vig *et al.*, 2006) and also the channel proteins of the TRPC subfamily, including TRPC1 (Ong *et al.*, 2007; Jardin *et al.*, 2008), are known to interact to allow SOC entry. In the present study, the nifedipine-resistant Ca^{2+} entry activated by H_2O_2 in coronary VSM exhibited classical pharmacological features of SOC entry in other cells, namely, inhibition by low concentrations of lanthanides and reproduction by Ba^{2+} fluorescence, indicative of Ba^{2+} influx through non-selective cation channels (Parekh, 2006). Furthermore, the selective inhibitor of Orai1-mediated Ca^{2+} entry, Pyr6 (Schleifer *et al.*, 2012), abolished Ca^{2+} entry obtained by SERCA inhibition and also the non-L-type-dependent Ca^{2+} entry activated in the presence of H_2O_2, thus indicating that H_2O_2 activates SOC entry in a Orai1-dependent manner in coronary VSM. These data are consistent with recent observations in several native cell lines demonstrating that at micromolar concentrations, H_2O_2 activates Orai1-mediated Ca^{2+} influx in a STIM1-dependent manner, also dependent on activation of IP_3R activity rather than modification of Orai1/STIM1 itself (Grupe *et al.*, 2010). On the other hand, the present data showing that H_2O_2 mobilizes Ca^{2+} from intracellular stores and activates a non-L-type Ca^{2+} entry in coronary VSM agreed in part with that earlier reported in vascular myocytes from rat intralobar pulmonary arteries, where H_2O_2 releases Ca^{2+} from IP_3-sensitive and ryanodine-sensitive intracellular stores and further activates a nifedipine-resistant Ca^{2+} entry and a Ni^{2+}-sensitive cation current (Lin *et al.*, 2007). However, this voltage-independent Ca^{2+} entry was not inhibited by either lanthanides or TRPC blockers, and hence, TRPM2 channels were proposed to be involved in Ca^{2+} influx activated by H_2O_2 in pulmonary arterial myocytes.

It is well established that H_2O_2 regulates intracellular signalling pathways through reactions with the highly reactive cysteine residues in target proteins, these oxidative modifications including the reversible formation of disulfide bridges or oxidation products such as sulphenic acid, or the irreversible production of sulphinic or sulphonic acids (Trebak *et al.*, 2010; Bogeski *et al.*, 2011). The data provided here suggest that thiol oxidation produced store depletion-dependent Ca^{2+} entry in coronary VSM, because the nifedipine-resistant $[Ca^{2+}]_i$ rise induced by H_2O_2, but not by SERCA inhibition with CPA, was abolished by the oxidant thiol reductant DTT. These findings are in line with functional studies demonstrating redox-dependent regulation of coronary blood flow (Saitoh *et al.*, 2006, 2007) and H_2O_2-mediated EDH-dependent relaxation (Wong *et al.*, 2014) through oxidation of thiol groups in the redox sensitive p38 MAPK and the Na^+/K^+ ATPase respectively.

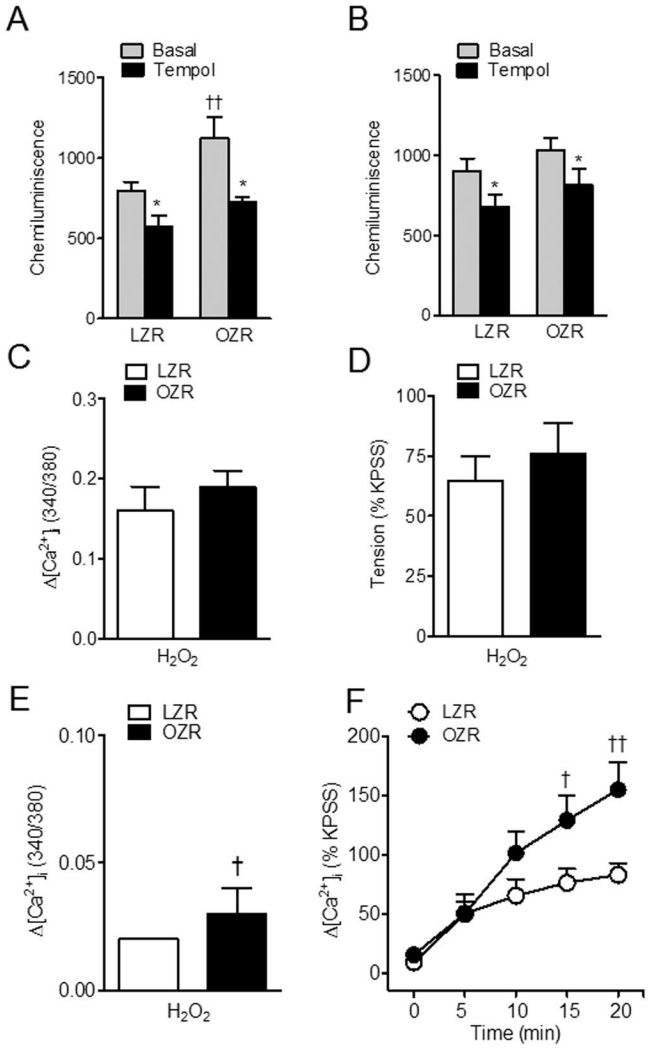

Figure 10

ROS generation and effects of H_2O_2 on $[Ca^{2+}]_i$ and vasoconstriction in coronary arteries from LZR and OZR. (A, B) Basal O_2^- production is increased in coronary arteries (A) but not myocardium (B) from OZR. Effect of the free radical scavenger tempol (30 μM) on basal superoxide production in coronary arteries (A) and myocardium (B) from LZR and OZR. (C, D) H_2O_2 induces rises in $[Ca^{2+}]_i$ and vasoconstriction of similar magnitude in LZR and OZR. Changes induced by H_2O_2 (100 μM) in resting $[Ca^{2+}]_i$ (C) and basal tension (D) in the presence of Ca^{2+} in the extracellular medium in endothelium-denuded coronary arteries from LZR and OZR. (E) Intracellular Ca^{2+} mobilization induced by H_2O_2 in a Ca^{2+}-free medium was larger in coronary arteries from OZR. (F) Increases in $[Ca^{2+}]_i$ induced by H_2O_2 (100 μM) in response to the re-addition of Ca^{2+} (1,5 mM) to the extracellular medium in the presence of nifedipine (1 μM) was enhanced in coronary arteries from OZR compared with LZR. (A, B) Results are expressed as cpm (mg tissue)$^{-1}$ and bars represent mean ± SEM of 4–13 animals. (C–F) Results are expressed either as absolute values of ratio F340/380 (C, E) or as a percentage of the KPSS-induced responses (D, F) and are means ± SEM of three to four animals. *$P < 0.05$, significantly different from basal; †$P < 0.05$, ††$P < 0.01$, significantly different from LZR; one-way ANOVA.

The fact that H_2O_2 mobilizes Ca^{2+} from intracellular stores through IP_3R activation along with the inhibitory effect of DTT on H_2O_2-induced SOC entry in coronary arteries further suggests that H_2O_2 might regulate IP_3R activity through thiol oxidation to induce store depletion, as earlier shown in blood (Parekh and Penner, 1995; Redondo *et al.*, 2004a) and endothelial cells where Ca^{2+}-induced Ca^{2+} release via the IP_3R is enhanced by oxidant-induced glutathionylation (Lock *et al.*, 2012). However, involvement of other Ca^{2+} transport protein oxidation in the activation of SOC entry induced by H_2O_2 in coronary VSM, such as ryanodine receptors (Du *et al.*, 2005), SERCA (Lancel *et al.*, 2010), the Na^+–Ca^{2+} exchanger (Kuster *et al.*, 2010), Orai1 (Bogeski *et al.*, 2010) and/or STIM1 Hawkins *et al.*, 2010), cannot be ruled out.

Oxidative stress underlies the pathogenesis of vascular disease in diabetes, obesity and other insulin resistant states (Furukawa *et al.*, 2004; Muñoz *et al.*, 2015). On the other hand, accumulated evidence suggests a critical involvement of abnormal VSM $[Ca^{2+}]_i$ homeostasis in the augmented vasoconstriction, remodelling and vascular dysfunction in insulin-resistant states (Touyz, 2005; Villalba *et al.*, 2008; Linde *et al.*, 2012; Contreras *et al.*, 2013). The present findings confirm high levels of oxidative stress in coronary arteries from OZR, an experimental model of the metabolic syndrome and insulin resistance, consistent with the high levels of ROS reported in the myocardium of these rats (Serpillon *et al.*, 2009), despite the compensatory increase in antioxidant defences including the H_2O_2-generating enzymes superoxide dismutases (SOD) (Conti *et al.*, 2004). In our study, we found that mobilization of Ca^{2+} from intracellular stores and SOC entry obtained upon acute exposure to H_2O_2 were enhanced in arteries from obese compared with lean rats, as shown by the higher $[Ca^{2+}]_i$ rise in a Ca^{2+}-free medium and by the larger nifedipine-resistant Ca^{2+} entry induced by H_2O_2, and in contrast to the preserved L-type-mediated Ca^{2+} entry elicited by KPSS depolarization. These observations suggest that SR Ca^{2+} release and SOC entry activated by exogenous H_2O_2 are sensitized under conditions of increased vascular levels of ROS and would be consistent with the increased sensitivity of the intracellular Ca^{2+} stores to IP_3R activation by H_2O_2 reported in human endothelial cells (Hu *et al.*, 2000). Likewise, enhanced intracellular Ca^{2+} mobilization and Ca^{2+} entry have been found along with increased IP_3R and SERCA2 expression in mesenteric artery myocytes from genetic hypertensive rats (Linde *et al.*, 2012), where high levels of vascular oxidative stress have been reported (Touyz, 2005). Augmented Ca^{2+} signalling was associated with increased vasoconstriction in hypertensive rats, in contrast to the augmented H_2O_2-induced SR Ca^{2+} release and entry not coupled to contraction in coronary arteries from obese rats. The latter would be consistent with the increased SOC entry and CRAC currents found in proliferative VSM myocytes (Berra-Romani *et al.*, 2008; Potier *et al.*, 2009; Li *et al*, 2011).

In conclusion, we provide evidence that Orai1-mediated SOC entry in coronary VSM is redox sensitive and can be activated by micromolar concentrations of H_2O_2 in part through the release of Ca^{2+} from IP_3-sensitive intracellular stores, whereas higher H_2O_2 concentrations are inhibitory and blunt SOC entry. Under conditions of vascular oxidative stress in the metabolic syndrome, SR Ca^{2+} release and store-dependent Ca^{2+} entry responses to acute exposure to H_2O_2 are sensitized, which suggests abnormal Ca^{2+} handling, not related to augmented coronary vasoconstriction.

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Author contributions

E. S. and D. P. conceived and designed the experiments. E. S., B. C. and M. M. performed the experiments. E. S., B. C., M. M. and D. P. analysed the data. B. C., A. G. S., L. R. and D. P. contributed reagents/materials/analysis tools. E. S. and D. P. wrote the manuscript. E. S., B. C., A. G. S., L. R. and D. P. revised the article critically for important intellectual content.

Conflict of interest

None declared.

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