J Physiol 590.15 (2012) pp 3431–3447

The Journal of Physiology

Protein *S*-glutathionylation enhances Ca^{2+}-induced Ca^{2+} release via the IP_3 receptor in cultured aortic endothelial cells

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Key points

- In non-excitable cells, oxidative stress increases inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) activity, which can cause Ca^{2+} oscillations under basal conditions and enhance agonist-stimulated changes in cytosolic free Ca^{2+} concentration.
- Protein *S*-glutathionylation, the reversible modification of cysteine thiols by glutathione, is elevated in response to oxidative stress, but the consequence of glutathionylation for IP_3R function is not known.
- In this study we provide evidence that Ca^{2+}-induced Ca^{2+}-release (CICR) via the IP_3R is enhanced by oxidant-induced glutathionylation in cultured aortic endothelial cells.
- Our results suggest glutathionylation may represent a fundamental mechanism for regulating IP_3R activity during physiological redox signalling and during pathological oxidative stress.

Abstract In non-excitable cells, thiol-oxidizing agents have been shown to evoke oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by increasing the sensitivity of the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) to IP_3. Although thiol modification of the IP_3R is implicated in this response, the molecular nature of the modification(s) responsible for changes in channel activity is still not well understood. Diamide is a chemical oxidant that selectively converts reduced glutathione (GSH) to its disulfide (GSSG) and promotes the formation of protein–glutathione (P-SSG) mixed disulfide, i.e. glutathionylation. In the present study, we examined the effect of diamide, and the model oxidant hydrogen peroxide (H_2O_2), on oscillations in $[Ca^{2+}]_i$ in fura-2-loaded bovine (BAECs) and human (HAECs) aortic endothelial cells using time-lapse fluorescence video microscopy. In the absence of extracellular Ca^{2+}, acute treatment with either diamide or H_2O_2 increased the number of BAECs exhibiting asynchronous Ca^{2+} oscillations, whereas HAECs were unexpectedly resistant. Diamide pre-treatment increased the sensitivity of HAECs to histamine-stimulated Ca^{2+} oscillations and BAECs to bradykinin-stimulated Ca^{2+} oscillations. Moreover, in both HAECs and BAECs, diamide dramatically increased both the rate and magnitude of the thapsigargin-induced Ca^{2+} transient suggesting that Ca^{2+}-induced Ca^{2+} release (CICR) via the IP_3R is enhanced by glutathionylation. Similar to diamide, H_2O_2 increased the sensitivity of HAECs to both histamine and thapsigargin. Lastly, biochemical studies showed that glutathionylation of native IP_3R_1 is increased in cells challenged with H_2O_2. Collectively our results reveal that thiol-oxidizing agents primarily increase the sensitivity of the IP_3R to Ca^{2+}, i.e. enhanced CICR, and suggest that

DOI: 10.1113/jphysiol.2012.230656

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(Submitted 15 February 2012; accepted after revision 25 May 2012; first published online 28 May 2012)
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Abbreviations 2-APB, 2-aminoethyl diphenyl borate; BAEC, bovine aortic endothelial cell; BK, bradykinin; BioGEE, biotinylated glutathione ethyl ester; CICR, Ca^{2+}-induce Ca^{2+} release; Cys, cysteine; EC, endothelial cell; ECS, extracellular solution; GSH, reduced glutathione; GSSG, oxidized glutathione; HAEC, human aortic endothelial cell; HIST, histamine; H_2O_2, hydrogen peroxide; IP_3R, inositol 1,4,5-trisphosphate receptor; IICR, IP_3-induced Ca^{2+} release; PLC, phospholipase C; P-SH, protein-thiol; P-SSG, protein-*S*-glutathione; RNS, reactive nitrogen species; ROS, reactive oxygen species; Ryn, ryanodine; TG, thapsigargin; XeC, xestospongin C.

Introduction

In most non-excitable cells, including vascular endothelial cells, the generation and propagation of oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) are predominantly driven by the release of Ca^{2+} from the endoplasmic reticulum (ER) via the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R; for reviews see Bootman & Berridge, 1995; Berridge *et al.* 2000; Foskett *et al.* 2007). At the cellular level, signal transduction commonly involves not just the generation of Ca^{2+} oscillations, but rather changes in both the frequency and the amplitude of the oscillatory response (Parekh, 2011), both of which are controlled to a great extent by the integrated regulation of the IP_3R by IP_3 and Ca^{2+}. Although the underlying molecular mechanism is quite complex (Marchant & Taylor, 1997; Mak *et al.* 1998), both IP_3 and Ca^{2+} are required for channel activation (Iino, 1990; Bezprozvanny *et al.* 1991). Furthermore, the interplay between IP_3 and Ca^{2+} is critical to the 'all-or-nothing' generation of a Ca^{2+} oscillation. At a fixed low concentration of Ca^{2+}, IP_3 increases IP_3R channel open probability which initiates IP_3-induced Ca^{2+} release (IICR). However, in the presence of a fixed low concentration of IP_3, Ca^{2+} activates the IP_3R through a process called Ca^{2+}-induced Ca^{2+} release (CICR). This feed-forward activation of the IP_3R by Ca^{2+} plays a critical role in the rising phase of a global Ca^{2+} oscillation. The effect of cytosolic Ca^{2+}, however, is biphasic as higher concentrations of Ca^{2+} inhibit channel activity. This feedback inhibition helps terminate Ca^{2+} release and contributes to the falling phase of a Ca^{2+} oscillation. Precise control of Ca^{2+} release via the IP_3R is further complicated at the cellular and tissue level by a differential distribution of IP_3R isoforms (Types 1–3) which exhibit different sensitivities to IP_3 and Ca^{2+} (Tu *et al.* 2005*a*,*b*).

Although the IP_3R is typically activated by a rise in IP_3 following receptor-initiated hydrolysis of phosphoinositol-4,5-bisphosphate (PIP_2) by phospholipase C (PLC; Berridge, 1993), Ca^{2+} release from IP_3-sensitive stores is reported to occur in the absence of an increase in IP_3 formation during periods of oxidative stress (Rooney *et al.* 1991; Bird *et al.* 1993). In non-excitable cells, including endothelial cells, reactive oxygen species and reactive nitrogen species (ROS/RNS), as well as a variety of pharmacological oxidants, cause an increase in Ca^{2+} oscillations under basal conditions and enhance agonist-stimulated changes in $[Ca^{2+}]_i$. Oxidant-induced changes in IP_3R activity can be reversed by reducing agents such as dithiothreitol (DTT), suggesting that alterations in thiol chemistry are responsible for this enhanced Ca^{2+} response (Bootman *et al.* 1992; Bird *et al.* 1993). Furthermore, studies examining the effects of oxidizing reagents on purified IP_3Rs indicate that this enhanced activation by IP_3 reflects direct modification of the channel protein (Kaplin *et al.* 1994; Thrower *et al.* 1996; Poirier *et al.* 2001). In this regard, a number of residues have been identified on the IP_3R as potential sites of redox modification (Joseph *et al.* 2006; Kang *et al.* 2008). However, despite the general consensus that oxidative stress sensitizes the IP_3R to activation by IP_3, the molecular basis for this response remains unknown.

Protein *S*-glutathionylation, the reversible modification of cysteine (Cys) thiol groups by glutathione (Dalle-Donne *et al.* 2008; Mieyal *et al.* 2008), represents a potential mechanism for regulating IP_3R activity during physiological redox signalling and during periods of oxidative stress. Glutathione is the most abundant reducing equivalent in mammalian cells and, along with its associative enzyme networks, serves as the primary cellular antioxidant defence system (Meister & Anderson, 1983). In endothelial cells, oxidative insults have been shown to increase the oxidation of reduced glutathione (GSH) to its disulfide (GSSG), and to promote the formation of protein–glutathione (P-SSG) mixed disulfides, i.e. glutathionylation (Schuppe *et al.* 1992). Redox regulated changes in Ca^{2+} signalling are attributed to perturbations in the cellular GSH:GSSG ratio (Elliott & Schilling, 1990; Bootman *et al.* 1992). Furthermore, changes in IP_3R activity have been observed following exogenous application of GSSG (Renard *et al.* 1992; Hilly *et al.* 1993; Renard-Rooney *et al.* 1995). However, in most studies investigating oxidant-induced changes in Ca^{2+} signalling, oxidative insults were initiated by either endogenous

generation or exogenous application of ROS/RNS. These reactive species have a variety of cellular targets including proteins, lipids and nucleic acids. Moreover, ROS/RNS can cause thiol modifications other than glutathionylation, such as sulfenation or nitrosylation. Thus, it is difficult to identify the changes in Ca^{2+} signalling in response to ROS/RNS that are solely attributable to protein glutathionylation.

Diamide, a cell-permeant thiol-oxidizing agent, preferentially and rapidly oxidizes intracellular GSH to GSSG and promotes the formation of P-SSG mixed disulfides without the added complexities associated with ROS/RNS (Kosower *et al.* 1969, 1972). Diamide is frequently used to identify protein targets of glutathionylation and to examine the subsequent effect of P-SSG formation on protein and/or cellular function (for examples see Barrett *et al.* 1999; Fratelli *et al.* 2002). Previously, we showed that low concentrations of diamide cause a dramatic increase in asynchronous single-cell Ca^{2+} oscillations in cultured aortic endothelial cells (Lock *et al.* 2011). Diamide, even at high concentrations, did not increase PIP_2 hydrolysis indicating that diamide does not activate PLC. However, the diamide response was observed even in the absence of extracellular Ca^{2+}, and was attenuated by inhibition of the IP_3R with 2-APB or by inhibition of PLC by U73122 suggesting that diamide sensitizes the IP_3R to basal levels of IP_3. Consistent with an important role for protein *S*-glutathionylation, the effect of diamide on Ca^{2+} dynamics was reversed by DTT, and was dependent upon intracellular GSH and the capacity of the cell to regenerate GSH from GSSG. Finally, biochemical studies showed that glutathionylation of the IP_3R was significantly increased following exposure of the cells to diamide. Together these results demonstrate that diamide mobilizes Ca^{2+} from IP_3-sensitive internal Ca^{2+} stores and suggest that oxidant-induced glutathionylation sensitizes the IP_3R. To test this hypothesis, the effect of diamide and H_2O_2 on IICR and CICR via the IP_3R was examined in the present study in two cultured aortic endothelial cell lines of bovine and human origin (BAECs and HAECs) using single-cell Ca^{2+} imaging. Our results show that both diamide and H_2O_2 primarily increase sensitivity of the IP_3R to Ca^{2+}, i.e. enhanced CICR.

Methods

Cell culture

The isolation, culture and characterization of BAECs have been extensively described in previous reports (Colden-Stanfield *et al.* 1987; Schilling *et al.* 1988, 1989). Briefly, BAECs were cultured as monolayers on 100 mm plastic cell culture dishes in low glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing L-glutamine and supplemented with 10% heat inactivated fetal bovine serum (FBS), 15 mM Hepes, 28.6 mM sodium bicarbonate, and 1% penicillin–streptomycin–neomycin solution (PSN; Gibco). BAECs, cultured as confluent monolayers which exhibited a cobblestone appearance typical of contact-inhibited endothelial cell cultures, were used for experimentation between passages 12 and 17. HAECs were purchased from Lonza, and cultured on 100 mm plastic dishes in endothelial cell basal medium-2 (EMB-2; Lonza, cat. no. CC-3156) supplemented with growth factors, cytokines, and chemicals (2% FBS, hydrocortisone, human recombinant fibroblast growth factor-B, human recombinant vascular endothelial growth factor, recombinant Long R insulin like growth factor-1, ascorbic acid, human recombinant epidermal growth factor, gentamicin sulfate/amphotericin-B, and heparin) from Lonza (SingleQuots; cat. no. CC-4176). HAECs were cultured as sub-confluent monolayers according to the supplier's instructions, and used for experimentation between passages 2 and 5.

Ca^{2+} imaging solutions and reagents

Normal Ca^{2+} buffer (Ca^{2+}-extracellular solution (ECS)) contains (in mM): 10 Hepes, 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 10 glucose, pH 7.4 at 37°C. Zero Ca^{2+} buffer (zero Ca^{2+}-ECS) contains (in mM): 10 Hepes, 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.3 EGTA, and 10 glucose, pH 7.4 at 37°C. Diamide (diazene dicarboxylic acid bis(*N*,*N*-dimethylamide)), 30% hydrogen peroxide (H_2O_2), histamine (HIST), bradykinin (BK), poly-D-lysine (PDL), thapsigargin (TG), ionomycin, DTT, and DMSO were purchased from Sigma-Aldrich. Ryanodine (Ryn) and bafilomycin were from Calbiochem, and xestospongin C (XeC) was from Cayman Chemicals. Fura-2 acetoxymethyl ester (fura-2/AM), and pluronic F-127 were obtained from Invitrogen. Fura-2/AM was reconstituted using DMSO and 10% pluronic F-127 at a 1:1 ratio to yield a 1 mM stock solution. Diamide, H_2O_2, and ryanodine stock solutions were prepared in aqueous solution, whereas TG, ionomycin, bafilomycin, and XeC were reconstituted in 100% ethanol. All stock solutions were prepared at a final concentration of 10–100 mM.

Ca^{2+} imaging

Time-dependent changes in $[Ca^{2+}]_i$ were measured in both HAEC and BAEC monolayers, as previously described (Goel & Schilling, 2010). Briefly, cells were cultured on 12 mm glass coverslips for 2–5 days prior to experimentation. For HAECs, coverslips were charged with 1 mg ml^{-1} PDL to improve cell attachment. Oxidant and agonist responses did not differ in HAECs cultured on PDL-coated coverslips in comparison to cells grown

on untreated coverslips. BAECs and HAECs were loaded for 30 min at 37°C in normal Ca^{2+} containing solution (Ca^{2+}-ECS) with 6 μM and 3 μM fura-2/AM, respectively. Cells were then washed for 30 min at room temperature in Ca^{2+}-ECS, and mounted in a temperature-controlled perfusion chamber on the stage of a Leica DMIRE2 inverted microscope. All recordings were made in the absence of extracellular Ca^{2+} (zero Ca^{2+}-ECS) to specifically monitor changes in $[Ca^{2+}]_i$ related to the release of Ca^{2+} from internal stores. Solutions were perfused into the recording chamber via an in-line heater, and all fura-2 imaging experiments were performed at 37°C. At 6 s intervals, excitation wavelength alternated between 340 and 380 nm and emission was recorded at 510 nm using filters appropriate for fura-2. Epifluorescence was recorded using a SPOT-RT camera (Diagnostic Instruments, Sterling Heights, MI, USA) and images were acquired and analysed using SimplePCI imaging software (Compix Inc., Cranberry Township, PA, USA).

Data analysis

Over the course of these experiments, slight variation in the sensitivity of HAECs and BAECs to chemical oxidants and receptor agonists were noted. For this reason, controls were always performed in parallel for each experimental protocol. For example, the dose-dependent effect of histamine on Ca^{2+} oscillations in untreated HAECs is reported in Figs 2 and 9; these represent independent data sets. Traces show $[Ca^{2+}]_i$ responses from individual cells as greyscale and black lines. A range of 24–53 HAECs and 46–93 BAECs were monitored per experiment. The difference in the range of cells monitored per experiment in HAECs *versus* BAECs reflects the different density in which the cell lines are maintained in culture, i.e. ∼70% confluent *versus* fully confluent, respectively. The number of cells oscillating in response to oxidant or agonist challenge was quantified for each individual experiment as the percentage of the total cells monitored exhibiting at least one $[Ca^{2+}]_i$ oscillation during the indicated treatment period. Histograms and individual data points generating agonist dose–response curves represent the average values from multiple monolayers under each condition reported as means ± SEM with n equal to the number of monolayers examined under each condition. The total number of cells and the total number of monolayers tested for each condition is given in the figure legend. Statistical comparison of means was performed using Student's paired t test or ANOVA with *post hoc* Tukey's test for pairwise comparisons; a P value <0.05 was considered to be statistically significant. The phenotypic shift in the TG response depicted in Figs 5–7, and 10, and Figs S2 and S3 was quantified by cumulative frequency analysis of the peak change in fura-2 fluorescence ratio (peak ratio), and the length of time from TG exposure to the peak change (latency to peak). Differences were identified using the Kruskal–Wallis test and *post hoc* pairwise comparisons were made using the Mann–Whitney U test with Bonferonni's correction for multiple comparisons; $P < 0.01$ was considered significant.

Synthesis of BioGEE

GSH ethyl ester was labelled with sulfo-link-NHS-LC-biotin (Pierce) as previously described (Figtree *et al.* 2009). Essentially, the reaction was performed by combining 10 mM GSH ethyl ester with 10 mM of biotin reagent in phosphate buffered saline (PBS, pH 8.0). After 1 h at room temperature, 50 mM Tris was added to remove any excess sulfo-link reagent.

Isolation of membranes from BAECs

Membranes were isolated as previously described (Sinkins *et al.* 2009). Briefly, confluent BAEC monolayers were harvested from the culture dishes by scrapping, centrifuged at 500 *g* for 5 min, and resuspended in lysis buffer containing 20 mM Tris-Cl, 5 mM EDTA, 1 mM EGTA, and a protease inhibitor mixture. The cell suspension was sonicated on ice, three times for 10 s with a 10 s rest between pulses, using a sonic dismembranator (Fisher) on a power setting of 2.5. The cell lysate was centrifuged at 6000 *g* for 10 min at 4 °C, and the pellet was discarded. The remaining supernatant was subjected to additional centrifugation at 50,000 *g* for 30 min, and the resulting microsomal pellets were resuspended in lysis buffer at a protein concentration of 5–10 mg ml^{-1}.

Glutathionylation of IP_3R_1 *in vivo*

BAECs, harvested by scraping, were washed and re-suspended in 1.5 ml Ca^{2+}-ECS and incubated with 500 μM BioGEE for 180 min at 37°C. BioGEE-loaded cells were washed with Ca^{2+}-ECS and either left untreated or subjected to oxidative challenge with either 0.1 mM H_2O_2, or 1 mM H_2O_2 for 10 min at room temperature. Following treatment, the cells were washed with Ca^{2+}-ECS and membrane preparations were generated as described above. For each sample, membrane preparations were divided into two aliquots; one aliquot from each sample was incubated with 20 mM DTT. Membrane aliquots were then solubilized in PBS containing 1% Triton X-100 for 30 min on ice. Following solubilization membrane lysates were cleared by centrifugation, and biotinylated proteins were extracted with streptavidin-agarose beads by overnight incubation at 4°C.

Immunoblots

Following *in vivo* glutathionylation reactions, proteins captured on streptavidin–agarose beads were fractionated by SDS-PAGE and electrotransferred to PVDF membrane (100 V for 1 h) in CAPS/methanol buffer. Blots were probed with anti-IP_3 receptor type 1 antibody (Millipore; cat. no. 07-514) and detected, following incubation with HRP-conjugated IgG, by SuperSignal West Pico chemiluminescent substrate (Pierce).

Results

Effect of diamide on $[Ca^{2+}]_i$ in cultured aortic endothelial cells

The acute effect of diamide on $[Ca^{2+}]_i$ of two independent cultured endothelial cell lines – bovine (BAECs) and human (HAECs) aortic endothelial cells – was investigated by single-cell imaging of fura-2-loaded monolayers. Previously, we reported that diamide produced a concentration-dependent increase in asynchronous Ca^{2+} oscillations in BAECs by stimulating the release of Ca^{2+} from IP_3-sensitive stores (Lock *et al.* 2011). HAECs, however, were unexpectedly resistant to diamide-induced oscillations in $[Ca^{2+}]_i$. As shown in Fig. 1*A* and quantified in Fig. 1*C*, HAECs challenged with 100 μM diamide exhibited Ca^{2+} oscillations in only $1.5 \pm 0.9\%$ (mean $\pm$ SEM) of the total cells monitored, whereas oscillations were not observed in paired controls. Challenge with increasing concentrations of diamide (up to 1.0 mM) had no effect on the number of HAECs responding (data not shown). In comparison, BAECs challenged with 100 μM diamide displayed a significant increase in the number of cells exhibiting asynchronous Ca^{2+} oscillations when compared to paired untreated controls (Fig. 1*B* and quantified in Fig. 1*C*).

Diamide increases the sensitivity of cultured aortic endothelial cells to receptor-stimulated Ca^{2+} oscillations

Our previous studies showed that the diamide-induced change in $[Ca^{2+}]_i$ in BAECs is blocked by inhibition of PLC by U73122 or by inhibition of the IP_3R by 2-APB suggesting that diamide sensitizes the IP_3R to basal levels of IP_3. Thus, the lack of sensitivity of HAECs to diamide may reflect the lack of sufficient IP_3 to initiate an oscillation under resting conditions. If this is true, we reasoned that low concentrations of diamide that have little or no effect on Ca^{2+} oscillations, should enhance the response to receptor agonists that generate IP_3. To test this hypothesis, HAECs bathed in Ca^{2+} free medium, were left untreated or treated with diamide for 5 min immediately prior to stimulation with HIST in the continued absence or

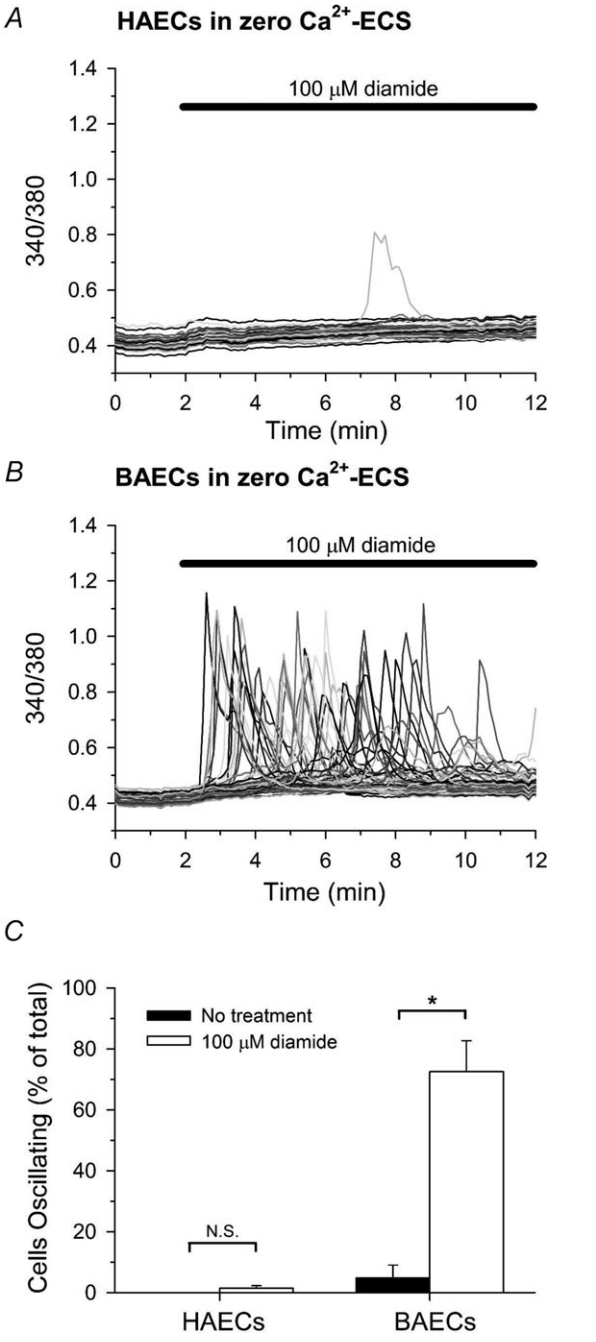

Figure 1. Effect of diamide on $[Ca^{2+}]_i$ in HAECs and BAECs
Fluorescence ratio (340/380) was recorded from single fura-2-loaded HAECs (*A*) and BAECs (*B*) bathed in zero Ca^{2+}-ECS during treatment with 100 μM diamide, as indicated at the top of each panel. In this and all subsequent figures, a representative experiment (i.e. a single coverslip) is shown with individual cells depicted as different grey scale traces. *C*, The number of cells oscillating in response to diamide was quantified for each individual experiment as the percent of the total cells monitored exhibiting at least one oscillation in $[Ca^{2+}]_i$ during the 10 min treatment period. A total of 118 (untreated) and 133 (100 μM diamide) HAECs, and 231 (untreated) and 202 (100 μM diamide) BAECs were analysed per experimental condition. Values represent the mean $\pm$ SEM of 3–4 experiments (coverslips) for each experimental condition. $^*P < 0.05$ compared to paired-untreated controls.

presence of diamide (Fig. 2). Under control conditions, 30 nM HIST had no effect on $[Ca^{2+}]_i$ (Fig. 2*A*). However, pretreatment with 100 μM diamide dramatically increased in the number cells oscillating in response to 30 nM HIST (Fig. 2*B*). Diamide pretreatment significantly increased the number of HAECs responding to every submaximal concentration of HIST tested, whereas neither untreated cells, nor cells treated with diamide alone exhibited Ca^{2+} oscillations in the absence of receptor stimulation. This increased sensitivity to HIST-stimulated Ca^{2+} oscillations is reflected in a 3- to 5-fold leftward shift in the HIST dose–response curve in HAECs pretreated with diamide relative to paired-untreated controls (Fig. 2*C*). In a parallel set of experiments (Fig. 3), we confirmed that this effect of diamide is related to a change in IP_3R function since the response was unaffected by Ryn (Fig. 3*C* and *D*) and significantly attenuated by XeC (Fig. 3*B* and *D*).

A similar effect of diamide on BK-mediated changes in $[Ca^{2+}]_i$ was observed in BAECs (Fig. 4). Cells, which did not respond to 100 pM BK under control conditions (Fig. 4*A*), exhibited a robust Ca^{2+} response to 100 pM BK when briefly pretreated with 20 μM diamide, a threshold concentration that has only modest affects on Ca^{2+} oscillations (Fig. 4*B*). Once again, diamide pretreatment significantly increased the number of cells oscillating in response to every submaximal agonist concentration tested, and altogether produced a 2- to 3-fold leftward shift in the BK dose–response relationship when compared to paired-untreated controls (Fig. 4*C*). Since BAECs do not express Ryn receptors (Schilling & Elliott, 1992), collectively these results demonstrate that diamide increases the sensitivity of cultured aortic endothelial cells to receptor-stimulated Ca^{2+} oscillations, and suggests that P-SSG formation enhances the sensitivity of the IP_3R to IICR.

Diamide enhances thapsigargin-mediated changes in the $[Ca^{2+}]_i$ of cultured aortic endothelial cells

Inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump by thapsigargin (TG) is a commonly used method for increasing $[Ca^{2+}]_i$ in the absence of receptor stimulation and in the absence of phosphoinositide hydrolysis (Lytton *et al.* 1991). It is thought that TG-induced rise in $[Ca^{2+}]_i$ reflects the passive leak of Ca^{2+} from the ER following SERCA inhibition. However, the results of our next experiments revealed a surprising finding. As previously reported in most non-excitable cell types, in the absence of extracellular Ca^{2+}, TG elicited a near homogeneous Ca^{2+} response in HAECs under control conditions (Fig. 5*A*, black traces). The response in each cell was characterized by a relatively slow rate of rise and small peak change in $[Ca^{2+}]_i$. In

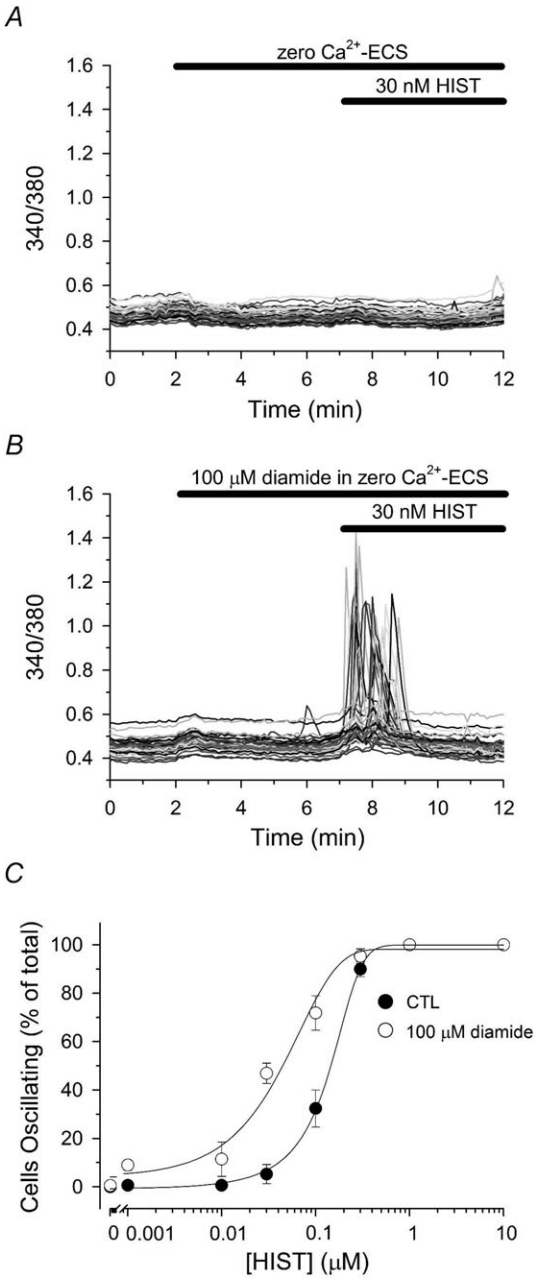

Figure 2. Diamide sensitizes HAECs to histamine-stimulated Ca^{2+} oscillations
Fura-2-loaded HAECs in zero Ca^{2+}-ECS were left untreated (*A*) or treated for 5 min with 100 μM diamide (*B*) prior to challenging with 30 nM histamine (HIST), as indicated by the bars at the top of each panel. *C*, HAECs were left untreated (filled circles) or treated with 100 μM diamide (open circles) for 5 min prior to stimulation with the indicated [HIST] for an additional 5 min in the continued absence or presence of 100 μM diamide. The number of cells oscillating was quantified as the percentage of the total cells monitored exhibiting at least one oscillation in $[Ca^{2+}]_i$ during the 5 min exposure to HIST. Values represents the means ± SEM of 3–5 experiments for each experimental condition. In untreated controls, and total of 156, 129, 153, 177, 218, 165, 137 and 115 cells were analysed for each [HIST] (0, 0.001, 0.01, 0.03, 0.1, 0.3, 1.0 and 10 μM, respectively), whereas 155, 133, 165, 172, 206, 143, 124 and 109 cells were analysed for each [HIST] in diamide-treated monolayers.

contrast, the TG response of HAECs briefly pretreated with a low concentration of diamide could be segregated into two phenotypic profiles. The first profile was that seen in controls, i.e. slow rate of rise and low amplitude, whereas the second profile was characterized by a rapid rate to rise and large peak change in $[Ca^{2+}]_i$ (Fig. 5*B*; grey-scale traces), suggesting that diamide may sensitize the IP_3 receptor to CICR. If this is true we reasoned that a low concentration of HIST, which would increase IP_3 but not produce oscillations, would also sensitize HAECs to TG. As seen in Fig. 5*C*, the TG-induced Ca^{2+} release in HIST pre-treated cells was phenotypically similar both in magnitude and time course to that observed in the diamide-treated group, consistent with enhanced CICR. In HAECs, TG evoked a CICR response in 87.1 ± 3.5% of the total cells pretreated with 100 μM diamide and 58.8% ± 9.3% of cells pretreated with 30 nM HIST, whereas a CICR profile in response to TG was observed in only 1 out of 121 cells analysed under control conditions. The phenotypic change in the TG response (i.e. enhanced CICR) was quantified in an unbiased manner using cumulative frequency analysis of the peak ratio and the latency to peak. As seen in Fig. 5*D* and *E*, both diamide and HIST produced a significant increase in the peak ratio and a significant decrease in the latency to peak following TG exposure in HAECs.

Similar to the Ca^{2+} response to TG observed in HAECs, BAECs briefly pretreated with a sub-threshold concentration of either diamide (Fig. 6*B*) or BK (Fig. 6*C*) also exhibited an enhanced Ca^{2+} response to TG. However, unlike HAECs, BAECs exhibited a heterogeneous response, consisting of both slow (black

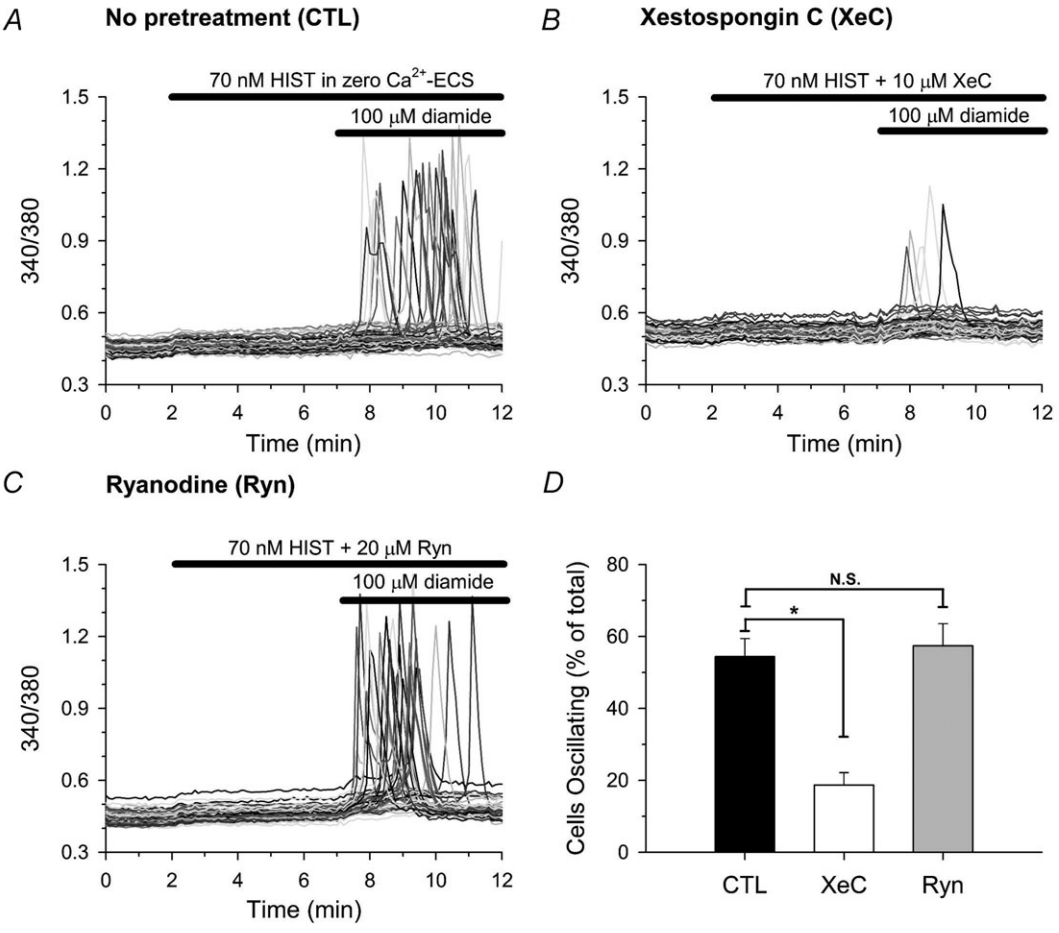

Figure 3. Effects of diamide are attenuated by xestospongin C but not ryanodine
Fura-2-loaded HAECs were left untreated (*A*), or pretreated with either 10 μM xestospongin C (XeC) for 30 min (*B*), or 20 μM ryanodine (Ryn) for 10 min (*C*) immediately prior to sequential exposure, in zero Ca^{2+}-ECS, to 70 nM HIST for 5 min followed by 100 μM diamide for an additional 5 min in the continued presence of HIST, as indicated by the bars at the top of each panel. All recordings in panels *B* and *C* were made in the continued presence of the indicated inhibitor. *D*, the number of cells oscillating in response to diamide was quantified as described in the legend to Fig. 1. A total of 139 (untreated), 132 (XeC pretreated), and 118 (Ryn pretreated) cells were analysed per experimental condition. Values represents the mean ± SEM of 3 experiments for each condition. $^*P < 0.01$ compared to paired-untreated controls.

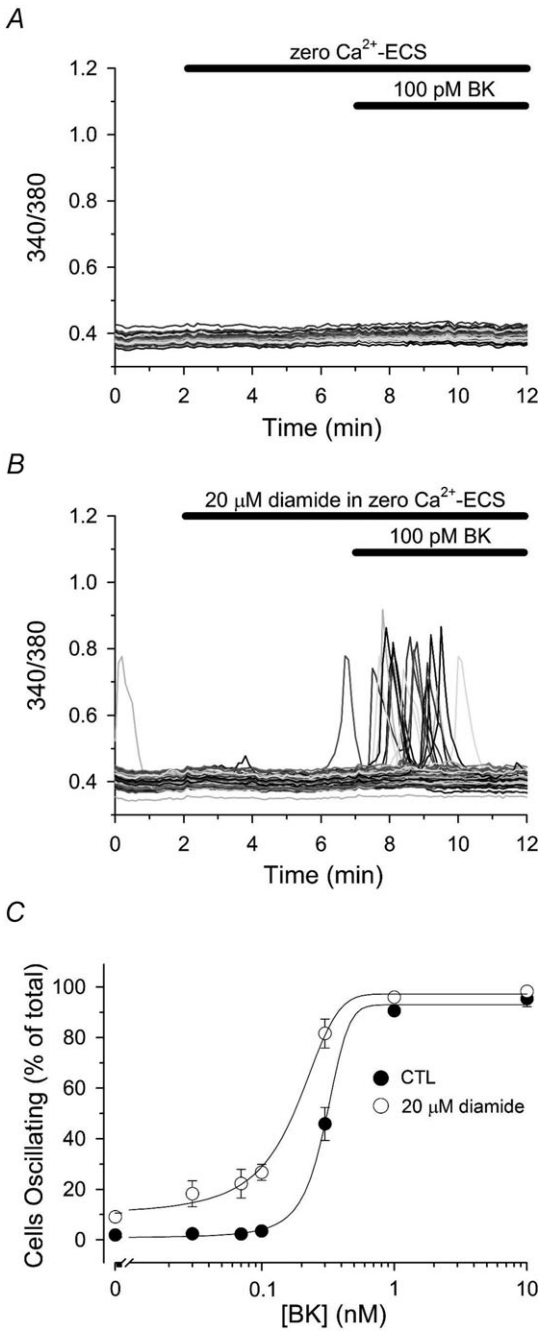

Figure 4. Diamide sensitizes BAECs to bradykinin-stimulated Ca^{2+} oscillations
Fura-2-loaded BAECs in zero Ca^{2+}-ECS were left untreated (*A*) or treated for 5 min with 20 μM diamide (*B*) prior to challenging with 100 pM bradykinin (BK), as indicated by the bars at the top of each panel. *C*, BAECs were left untreated (filled circles) or treated with 20 μM diamide (open circles) for 5 min prior to stimulation with the indicated [BK] for an additional 5 min in the continued absence or presence of diamide. Values represent the mean ± SEM of 3–8 experiments for each experimental condition. In untreated controls, a total of 401, 371, 530, 468, 345, 193 and 196 cells were analysed for each [BK] (0, 0.03, 0.07, 0.1, 0.3, 1.0 and 10 nM, respectively), whereas 340, 329, 436, 471, 316, 225, 180, cells were analysed for each [BK] in diamide-treated monolayers.

traces) and fast (grey traces) Ca^{2+} profiles, even under control conditions (Fig. 6*A*). In some cells, a rapid release of Ca^{2+} was observed after a slow rise in $[Ca^{2+}]_i$, providing further evidence that the fast response reflects all-or-nothing CICR from stores (inset Fig. 6*A*). Altogether, a CICR profile in response to TG was observed in 74.4 ± 4.9% of diamide-treated and 52.4% ± 9.3% of BK-treated cells, whereas only 32.3% ± 4.5% of untreated BAECs exhibited a CICR response to TG. As seen in Fig. 6*D* and *E*, both diamide and BK significantly increased the peak ratio and significantly decrease the latency to peak in response to TG in BAECs.

Diamide is expected to increase glutathionylation of a number of cellular proteins, some of which may impact Ca^{2+} homeostasis. For example, diamide may affect the Ca^{2+} buffer capacity of the cytoplasm and/or alter the Ca^{2+} load of the internal stores by stimulation of SERCA, both of which may contribute to the enhanced response to TG. However, at the concentrations used, diamide had no significant effect on basal $[Ca^{2+}]_i$ or on the peak response to maximum concentrations of HIST, BK, or ionomycin (Fig. S1). Furthermore, diamide had no significant effect on the kinetics of these responses. Thus, alterations in stored Ca^{2+} or in the buffer capacity of the cytoplasm seem unlikely. Additionally, the diamide-induced change in TG response was unaffected by blockade of the Ryn receptor, or by inhibition of lysosmes by bafilomycin, but could be partially (but significantly) attenuated by XeC (Figs S2 and S3). To further demonstrate the impact of diamide on CICR via the IP_3R, we examined a near threshold concentration of TG. As seen in Fig. 7, 3 nM TG produced a slow gradual rise in $[Ca^{2+}]_i$ in HAECs when measured in the absence of extracellular Ca^{2+} under control conditions. However, in the presence of diamide, CICR was seen in 57.0 ± 2.1% of cells examined (Fig. 7*B* and *D*) and 14.1 ± 1.3% exhibited repetitive Ca^{2+} oscillations over the recording period (Fig. 7*C*). Collectively these results demonstrate IP_3R activation can shape the change in $[Ca^{2+}]_i$ following depletion of the ER Ca^{2+} store by a process of CICR, and suggest that diamide increases the sensitivity of the IP_3R to cytosolic Ca^{2+}. Moreover, these results indicate that the stimulatory effect of diamide on IP_3R activity is down-stream of receptor activation.

Effect of H_2O_2 on $[Ca^{2+}]_i$ of cultured aortic endothelial cells

To determine if a physiologically relevant oxidant produces similar changes in IP_3R activity, the effect of hydrogen peroxide (H_2O_2) on the $[Ca^{2+}]_i$ of HAECs and BAECs was investigated under identical conditions to those described above for diamide. In the absence of extracellular Ca^{2+}, HAECs challenged with 100 μM

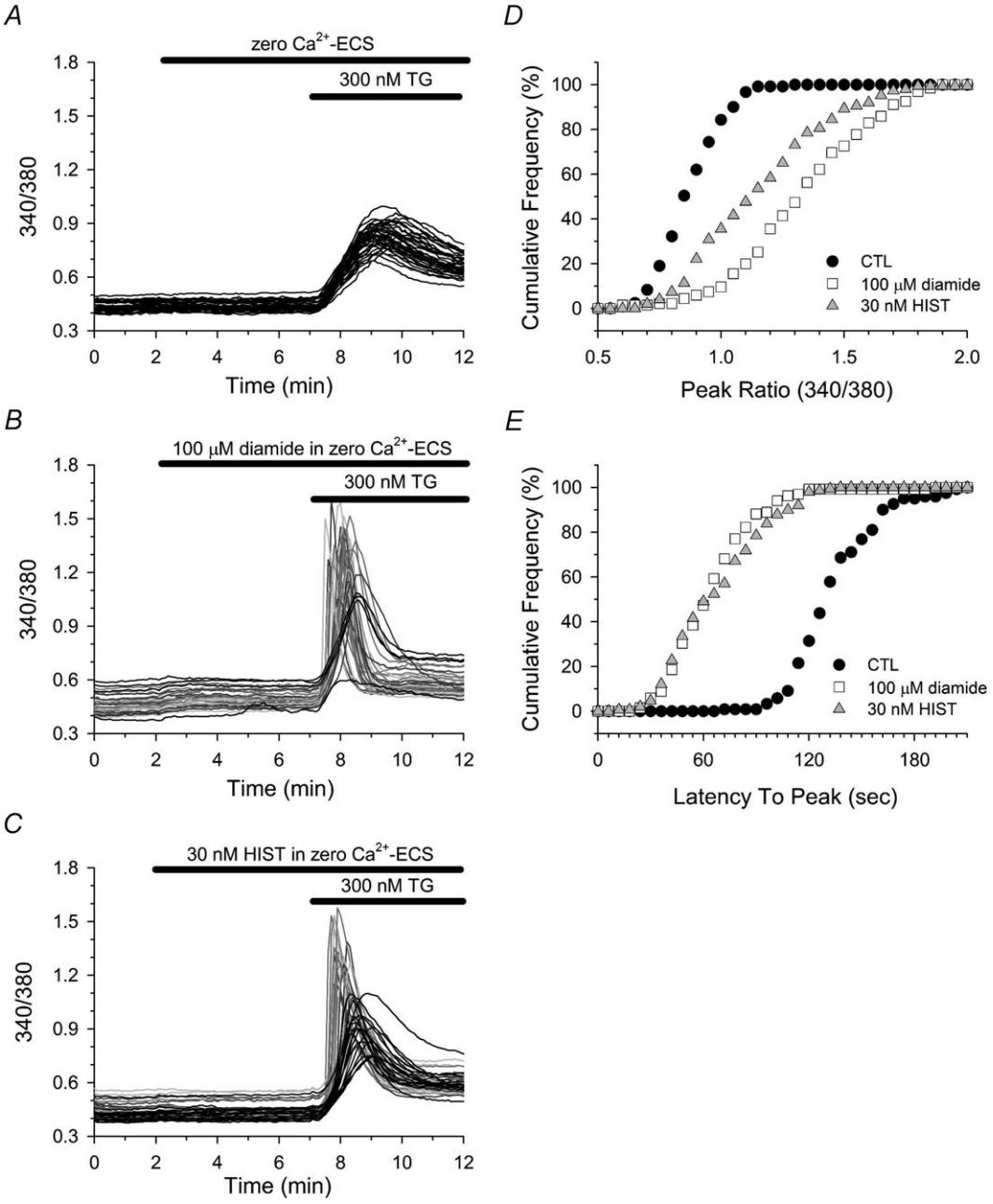

Figure 5. Diamide enhances thapsigargin-mediated changes in $[Ca^{2+}]_i$ of HAECs
Fura-2-loaded HAECs in zero Ca^{2+}-ECS were left untreated (*A*), or treated with either 30 nM HIST (*C*) or with 100 μM diamide (*B*) for 5 min immediately prior to challenging with 300 nM thapsigargin (TG). *A–C*, cells were segregated into two phenotypic profiles based on both the rate and the magnitude of the TG-mediated change in $[Ca^{2+}]_i$. Cells represented by black lines displayed a slow rate of rise and a small peak change in $[Ca^{2+}]_i$, whereas cells represented by greyscale lines exhibited an enhanced response characterized by a rapid rate of rise and a large change in $[Ca^{2+}]_i$. *D*, cumulative frequency of the maximal change in fura-2 fluorescence (peak ratio) observed in response to TG in control (filled circles), diamide (open squares) and HIST (grey triangles) treated cells. *E*, cumulative frequency of the time from TG exposure to the maximal change in fura-2 fluorescence (latency to peak) for control (filled circles), diamide (open squares), and HIST (grey triangles) treated cells. A total of 121 (control), 135 (diamide) and 149 (HIST) cells were analysed from 4 experiments for each experimental condition. *D* and *E*, the TG-induced peak ratio and latency to peak for both diamide- and HIST-treated cells were significantly different from untreated controls; $P < 0.001$.

H_2O_2 did not exhibit oscillations in $[Ca^{2+}]_i$, nor were Ca^{2+} oscillations observed in paired-untreated controls ($0 \pm 0\%$ *vs.* $0 \pm 0\%$, respectively; Fig. 8*A* and quantified in Fig. 8*C*). BAECs, on the other hand, exhibited a significant increase in the number of cells oscillating in response to 100 μM H_2O_2 when compared to paired-untreated controls ($10.3 \pm 0.8\%$ *vs.* $1.2 \pm 0.7\%$, respectively; Fig. 8*B* and quantified in Fig. 8*C*).

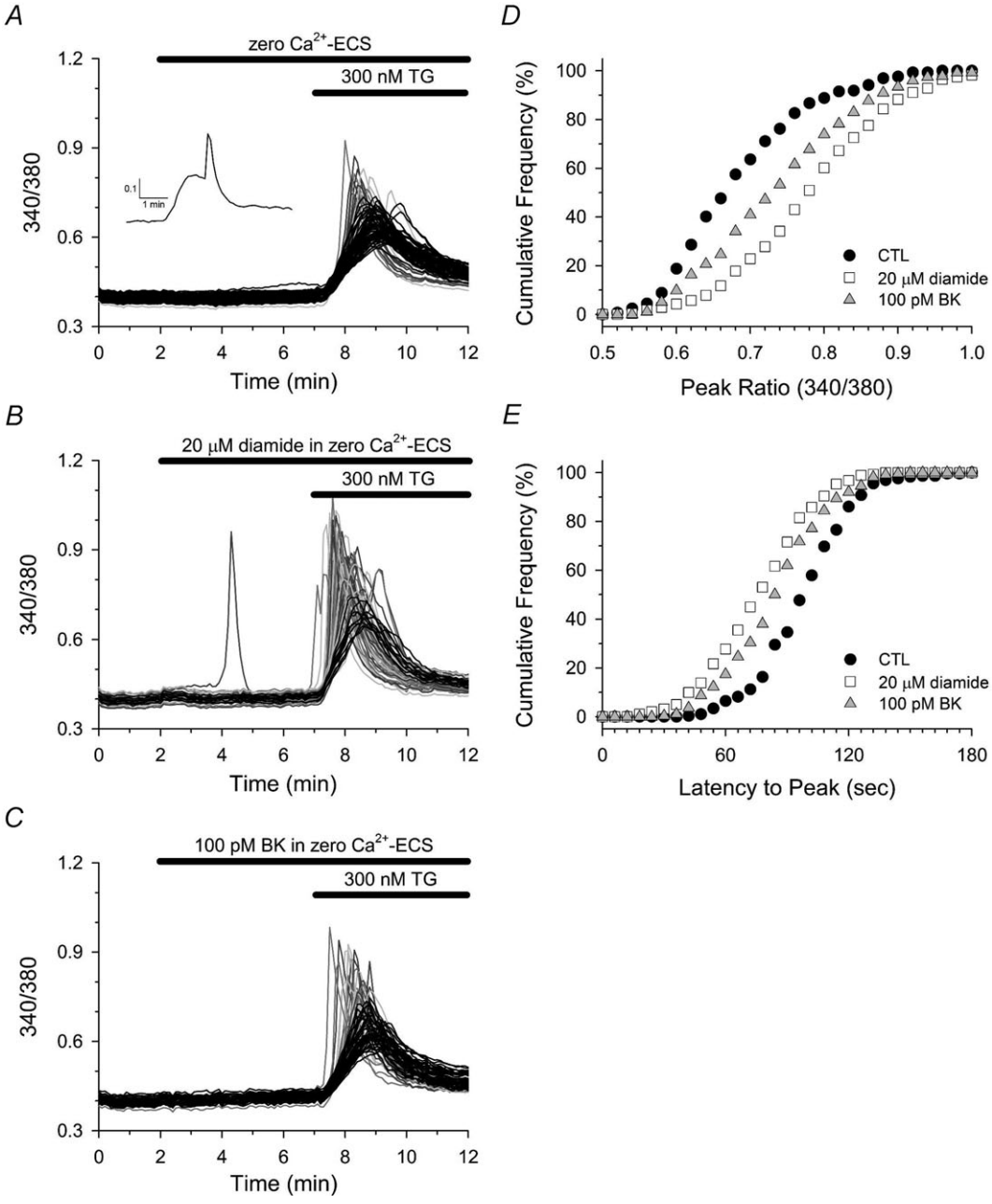

Figure 6. Diamide enhances TG-mediated changes in $[Ca^{2+}]_i$ of BAECs
Fura-2-loaded BAECs in zero Ca^{2+}-ECS were left untreated (*A*), or treated with either 100 pM BK (*C*) or 20 μM diamide (*B*) for 5 min immediately prior to challenging with 300 nM TG. *A*–*C*, individual cell traces are segregated into two phenotypic profiles as described in the legend of Fig. 5. Inset in *A* shows a single-cell trace exhibiting a delayed CICR response to TG that was sometimes observed. *D*, cumulative frequency of the peak ratio (*D*) and the latency to peak (*E*) observed in response to TG in control (filled circles), diamide- (open squares) and BK-treated (grey triangles) cells. A total of 294 (control), 281 (diamide) and 276 (BK) cells were analysed from 4 experiments for each experimental condition. *D* and *E*, the TG-induced peak ratio and latency to peak of both diamide- and BK-treated cells were significantly different from untreated controls; $P < 0.001$.

H_2O_2 increases the sensitivity of HAECs to HIST- and TG- stimulated changes in $[Ca^{2+}]_i$

To determine if H_2O_2-induced Ca^{2+} oscillations reflect an increased sensitivity of the IP_3R, the effect of H_2O_2 on HIST- and TG-mediated changes in $[Ca^{2+}]_i$ was examined in HAECs. Similar to the effects seen with diamide, brief pretreatment of HAECs with 100 μM H_2O_2 significantly increased the number of cells oscillating in response to a submaximal concentration of HIST, and altogether resulted in a 2- to 3-fold shift in the HIST dose–response relationship when compared to paired-untreated controls (Fig. 9). Again, analogous to effect of diamide, HAECs pre-treated with H_2O_2 exhibited an enhanced response to TG, indicative of IP_3R activation by CICR, when compared to paired-untreated controls (Fig. 10). Collectively, these results demonstrate that H_2O_2, like diamide, can increase the sensitivity of the IP_3R to activation by IICR and CICR.

Oxidative stress promotes glutathionylation of the IP_3R_1 *in vivo*

Despite substantial evidence that thiol-oxidizing agents directly modify the IP_3R, the molecular nature of the modification(s) responsible for changes in IP_3R activity are still not well understood. The type 1 IP_3R (IP_3R_1) is expressed in vascular endothelial cells (Grayson *et al.* 2004) and we previously reported that glutathionylation of native type 1 IP_3R (IP_3R_1) is increased in BAECs treated with diamide (Lock *et al.* 2011). To determine if glutathionylation occurs in response to a physiological oxidant, the effect of H_2O_2 on glutathionylation of native IP_3R_1 was investigated in BAECs loaded with biotin-GSH ethyl ester (BioGEE). BioGEE is a membrane-permeant form of biotin-labelled GSH that is trapped within the cell by the action of cellular esterases. Following 10 min treatment with H_2O_2, glutathionylated proteins from BioGEE-loaded

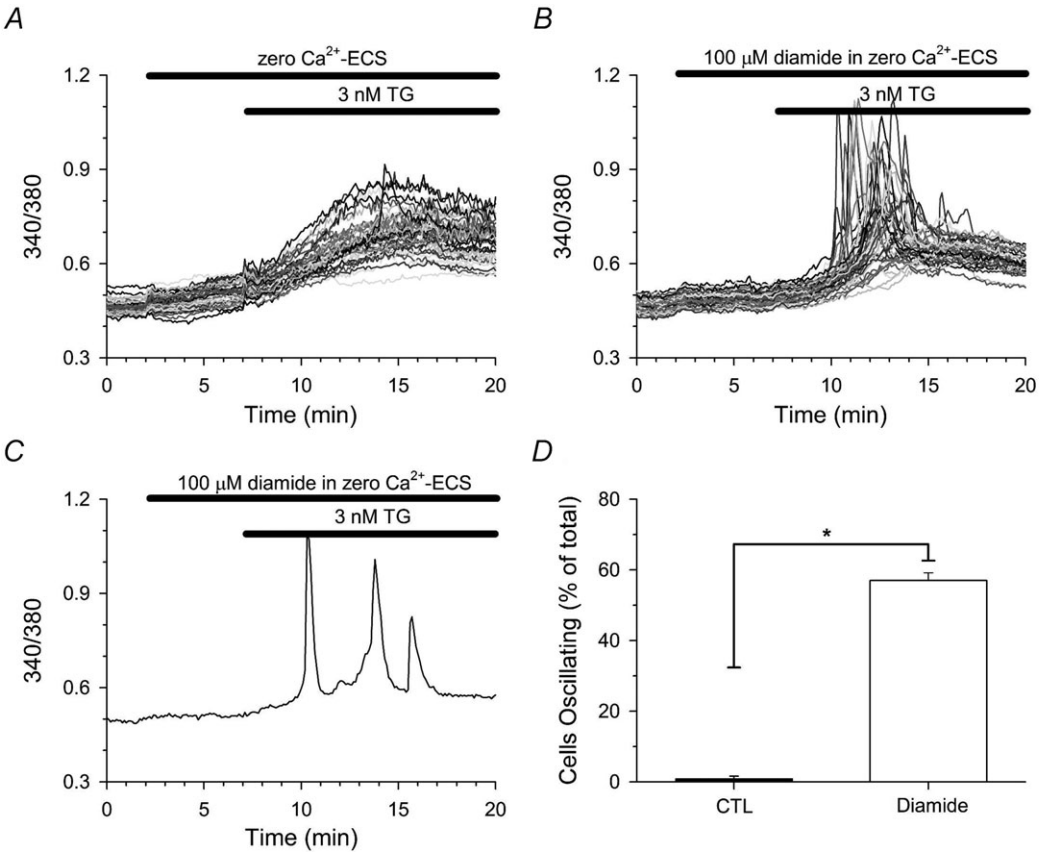

Figure 7. Diamide stimulates Ca^{2+} oscillations in response to 3 nM TG in HAECs
Fura-2 loaded HAECs in zero Ca^{2+}-ECS were left untreated (*A*) or treated for 5 min with 100 μM diamide (*B*) prior to stimulation with 3 nM TG in the continued absence or presence of diamide, as indicated by the bars at the top of each panel. *C*, a representative single-cell trace demonstrating multiple Ca^{2+} oscillations in response to 3 nM TG in the presence of diamide. *D*, the number of cells oscillating in response to TG was quantified for each individual experiment as the percentage of the total cells monitored exhibiting at least one oscillation in $[Ca^{2+}]_i$ during the 13 min treatment period. A total of 101 (control) and 112 (diamide) cells were analysed for each experimental condition. Values represent the mean ± SEM of 3 experiments per condition. $^*P < 0.001$ compared to paired controls.

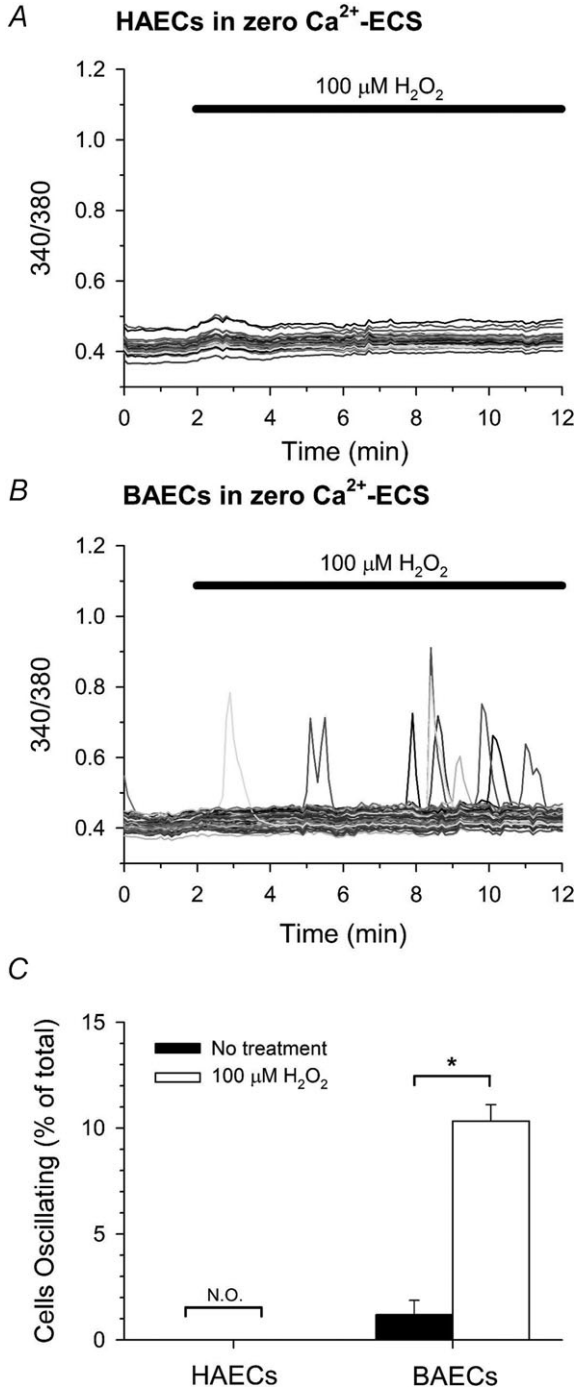

Figure 8. Effect of H_2O_2 on $[Ca^{2+}]_i$ in HAECs and BAECs
Fura-2 fluorescence ratio was recorded from single HAECs (*A*) and BAECs (*B*) in zero Ca^{2+}-ECS during treatment with 100 μM H_2O_2, as indicated at the top of each panel. *C*, the number of cells oscillating in response to H_2O_2 was quantified as described in the legend of Fig. 1. A total of 111 (untreated) and 151 (H_2O_2) HAECs, and 217 (untreated) and 248 (H_2O_2) BAECs were analysed. Values represent the mean ± SEM of 3–4 experiments for each experimental condition. *$P < 0.001$ compared to paired-untreated controls; N.O., not observed.

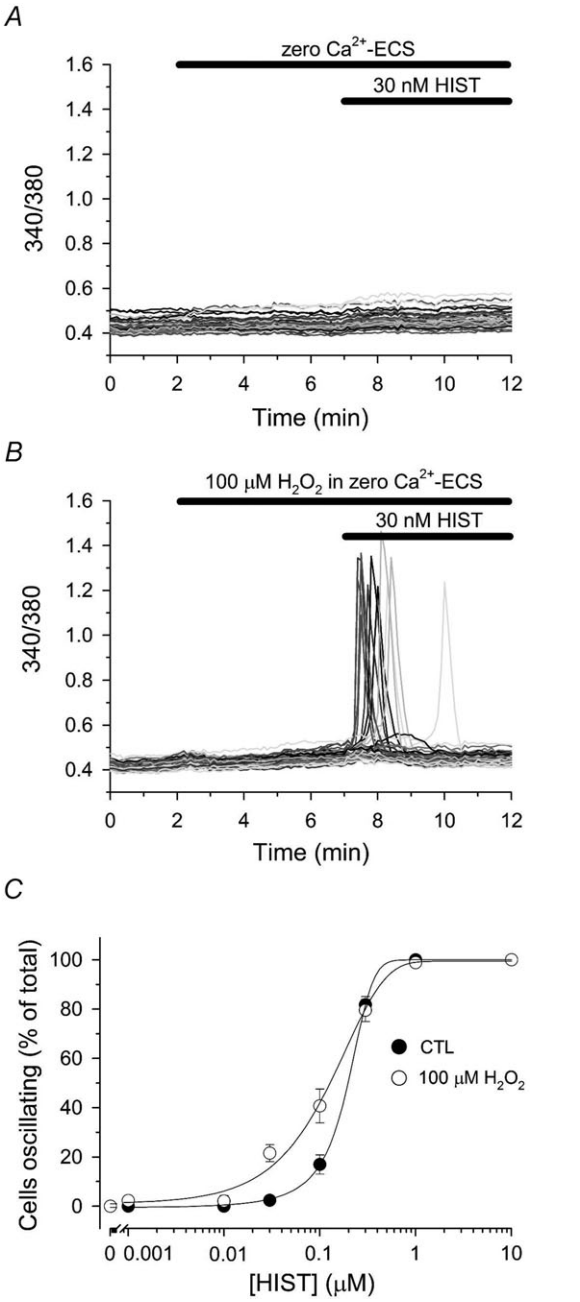

Figure 9. H_2O_2 sensitizes HAECs to histamine-stimulated Ca^{2+} oscillations
Fura-2-loaded HAECs in zero Ca^{2+}-ECS were left untreated (*A*) or treated for 5 min with 100 μM H_2O_2 (*B*) prior to challenging with 30 nM histamine (HIST) for an additional 5 min, as indicated by the bars at the top of each panel. *C*, HAECs were left untreated (filled circles) or treated with 100 μM H_2O_2 (open circles) for 5 min prior to stimulation with the indicated [HIST] for an additional 5 min in the continued absence or presence of H_2O_2. The number of cells oscillating in response to HIST stimulation was quantified as described in the legend of Fig. 2. Values represent the mean ± SEM of 3–6 experiments for each experimental condition. In untreated controls, a total of 117, 157, 155, 133, 208, 225, 94 and 104 cells were analysed for each [HIST] (0, 0.001, 0.01, 0.03, 0.1, 0.3, 1.0 and 10 μM, respectively), whereas 116, 95, 141, 135, 225, 171, 97 and 104 cells were analysed for each [HIST] in H_2O_2-treated monolayers.

cells were captured using streptavidin–agarose beads and probed for IP_3R_1 by Western blot as described in Methods. As seen in Fig. 11, IP_3R_1 glutathionylation was increased in response to H_2O_2. Consistent with thiol modification, oxidant-induced glutathionylation of the IP_3R was reversed by addition of excess DTT prior to avidin pull-down. These results suggest that glutathionylation of IP_3R_1 may be a common response to oxidative stress.

Discussion

In the present study, we utilized the thiol-oxidant diamide to investigate the consequences of protein *S*-glutathionylation on IP_3R function in intact cultured aortic ECs. Diamide reacts with GSH and promotes P-SSG formation in a well-established two step reaction (Kosower *et al.* 1969; Kosower & Kosower, 1995). Intracellularly, diamide rapidly and preferentially reacts with GSH producing a diamide-GS intermediate which can either react with another molecule of GSH (producing GSSG) or react with a protein thiol (P-SH) giving rise to P-SSG. Protein de-glutathionylation is achieved primarily through the enzymatic action of glutaredoxin, which uses GSH to produce P-SH and GSSG (Shelton *et al.* 2005). Reduced GSH is regenerated from GSSG by glutathione reductase at the expense of NADPH. Since the concentration of GSH in the cytosol is generally 1–10 mM (Meister & Anderson, 1983), low concentrations of diamide (e.g. 20–100 μM) are thought to shift the steady-state equilibrium in favour of P-SSG. Previously we found that diamide produced asynchronous Ca^{2+} oscillations in BAECs (Lock *et al.* 2011). This effect of diamide was prevented by inhibition of PLC or by blockade of the IP_3R, but diamide did not increase hydrolysis of PIP_2. Together these results suggested that diamide sensitizes the IP_3R to basal levels of IP_3. However, the extent to which this reflects enhanced sensitivity to IP_3 or to cytosolic Ca^{2+} is difficult to distinguish at the cellular level. Since IP_3 is not changing during challenge with diamide, an increase in Ca^{2+} oscillations likely reflects an increased sensitivity of the IP_3R to Ca^{2+}, i.e. enhanced

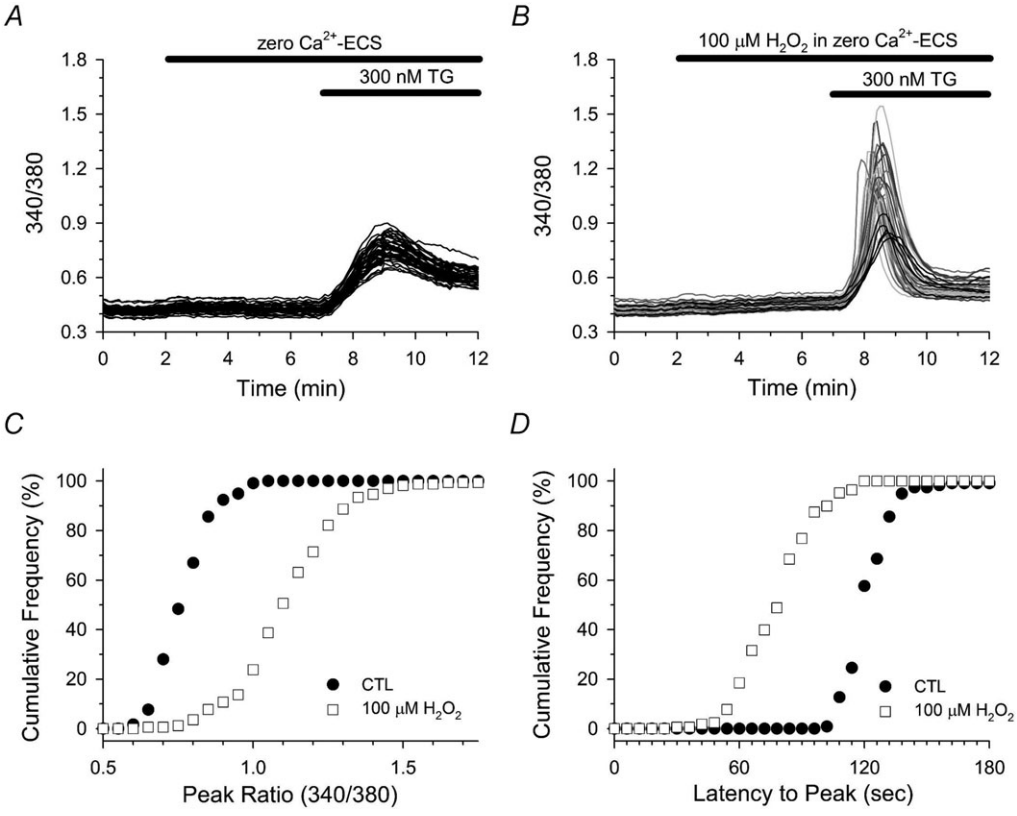

Figure 10. H_2O_2 enhanced TG-mediated changes in $[Ca^{2+}]_i$ of HAECs
Fura-2-loaded HAECs in zero Ca^{2+}-ECS were left untreated (*A*), or treated with 100 μM H_2O_2 (*B*) for 5 min immediately prior to challenging with 300 nM TG. *A* and *B*, individual cell traces are segregated into two phenotypic profiles as described in the legend of Fig. 5. *C* and *D*, cumulative frequency analysis of the peak ratio (*C*) and the latency to peak (*D*) in response to TG in control (filled circles) and H_2O_2 (open squares) treated cells. A total of 118 (control) and 168 (H_2O_2) cells were analysed from 3–4 experiments for each experimental condition. *C* and *D*, the TG-induced peak ratio and latency to peak of H_2O_2-treated cells was significantly different from untreated controls; $P < 0.001$.

CICR. The results of the present study demonstrate that CICR is indeed enhanced following diamide treatment. Furthermore, we also found that exogenous application of H_2O_2 produced similar changes in IP_3R activity and, like diamide, H_2O_2 increased glutathionylation of native IP_3R_1 in BAECs. Although an increased sensitivity of the IP_3R to IP_3 in response to oxidative stress has been reported previously (Rooney *et al.* 1991; Bird *et al.* 1993), to our knowledge this is the first demonstration that thiol-oxidizing agents enhance the sensitivity of the IP_3R to CICR. This may reflect a direct modification of the IP_3R by glutathionylation.

The mobilization of intracellular Ca^{2+} stores in response to thiol-oxidizing agents has been reported in a wide range of non-excitable cell types including hepatocytes (Rooney *et al.* 1991; Bird *et al.* 1993), Hela cells (Bootman *et al.* 1992), hamster eggs (Miyazaki *et al.* 1992), pancreatic acinar cells (Klonowski-Stumpe *et al.* 1997), platelets (vanGorp *et al.* 1997), and aortic ECs (Hu *et al.* 1998). Although H_2O_2 was shown to stimulate single-cell Ca^{2+} oscillations in cultured HAECs in a prior study, in our hands neither H_2O_2, nor diamide evoked an oscillatory response in HAECs under basal conditions. In contrast, both diamide and H_2O_2 were effective in eliciting Ca^{2+} oscillations in BAECs. These results are reminiscent of a study by Bird *et al.*, in which they observed Ca^{2+} oscillations in response to tert-butyl hydroperoxide (t-BOOH) in primary rat hepatocytes, but not in hepatocytes isolated from guinea pig (Bird *et al.* 1993). In their study, if the intracellular IP_3 was elevated by a low concentration of receptor agonist or by microinjection of IP_3, t-BOOH-induced Ca^{2+} oscillations were then observed in guinea pig hepatocytes. Similarly, we found that brief (5 min) pretreatment of either HAECs or BAECs with diamide, at a concentration which did not stimulate Ca^{2+} oscillations, significantly increased the number of cells oscillating in response to either HIST or BK, respectively. Moreover, H_2O_2 pretreatment increased the sensitivity of HAECs to HIST-induced oscillations. Since both HIST and BK initiate Ca^{2+} oscillations by activating PLC and elevating IP_3, both diamide and H_2O_2 may decrease the threshold for activation of the IP_3R by IP_3. However, since a local rise in $[Ca^{2+}]_i$ in response to IP_3 binding can stimulate Ca^{2+} release through a feed-forward process involving CICR, the increased IP_3R activity during an oxidative challenge could also reflect changes in the Ca^{2+} sensitivity of the receptor.

It is well established that IP_3R activity is regulated by cytosolic Ca^{2+} (Taylor, 1998). In both single channel studies (Bezprozvanny *et al.* 1991; Tu *et al.* 2005*a*; Ionescu *et al.* 2006) and permeabilized cell models (Iino, 1990; Marshall & Taylor, 1993; Bootman *et al.* 1995; Marchant & Taylor, 1997) low concentrations of cytosolic Ca^{2+} are required for IP_3-mediated activation of the IP_3R, and have been shown to stimulate IP_3R activity at a fixed concentration of IP_3. Moreover, increasing $[Ca^{2+}]_i$ has been shown to augment IP_3 binding to the IP_3R (Hilly *et al.* 1993; Cardy *et al.* 1997). However, despite a clear role for Ca^{2+} in the regulation of IP_3R activity, the effect of thiol-oxidants on the Ca^{2+} sensitivity of the IP_3R has not been investigated. We took advantage of the SERCA inhibitor TG to address whether changes in $[Ca^{2+}]_i$ can influence IP_3R activity during an oxidative challenge in intact cells. TG-mediated inhibition of SERCA leads to a transient rise in $[Ca^{2+}]_i$ due to passive leak of Ca^{2+} from the ER (Lytton *et al.* 1991). Our results reveal that both diamide and H_2O_2 increased the sensitivity of the IP_3R to a rise in $[Ca^{2+}]_i$ induced by TG. This increased sensitivity toward cytosolic Ca^{2+} resulted in a rapid Ca^{2+} transient when cells were challenged with TG suggestive of CICR via the IP_3R. Moreover, regenerative Ca^{2+} oscillations, a well-established characteristic of IP_3-sensitive stores, were observed in response to near threshold concentrations of TG in diamide-treated cells. Concordant with a role for the IP_3R in the Ca^{2+} response to TG, low concentrations of HIST or BK produced a similar shift in the Ca^{2+} profile following TG exposure. A role for CICR via the IP_3R in shaping the kinetics of the Ca^{2+} transient induced by TG is consistent with the observations that TG-mediated changes $[Ca^{2+}]_i$ are dependent upon the basal level of IP_3 (Smith & Gallacher, 1994), and can be attenuated by the IP_3R blocker 2-APB (Luo *et al.* 2001). Taken together, our results suggest thiol-oxidizing agents increase the sensitivity of the IP_3R to activation by cytosolic Ca^{2+}. Additionally, our results demonstrate that the stimulatory

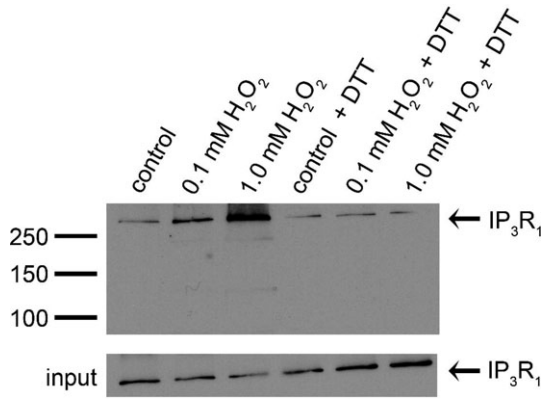

Figure 11. Glutathionylation of native IP_3R_1 *in vivo*
BioGEE-loaded BAECs were suspended in Ca^{2+}-ECS and cells were divided equally into aliquots as indicated above each lane. Cells were left untreated (control) or incubated for 10 min with 0.1 mM or 1.0 mM H_2O_2. Following treatment, the membrane lysates from one sample of each of the indicated conditions tested was incubated with 20 mM DTT prior to pull-down. Biotinylated proteins were extracted from cleared lysates with avidin–agarose beads and probed for the IP_3R_1 by Western Blot. Aliquots of cell lysates prior to pull-down are shown as *input* controls. Result shown is representative of 3 independent experiments.

effects of both diamide and H_2O_2 on IP_3R activation are downstream of receptor stimulation and are independent of PLC activity and IP_3 formation.

Oxidant-induced changes in IP_3R activity are thought to reflect a direct thiol modification of the receptor/channel. Functional IP_3Rs contain over 200 Cys residues (~60 Cys per monomer), and although a number of Cys have been identified as potential sites of redox modification (Joseph *et al.* 2006; Kang *et al.* 2008), a role for glutathionylation has not been defined. Our results demonstrate that, in addition to diamide (Lock *et al.* 2011), glutathionylation of native IP_3R_1 in cultured aortic ECs occurs in response to H_2O_2. The increase in IP_3R glutathionylation in response to thiol-oxidizing agents may be indirect, and we cannot unequivocally exclude the possibility that glutathionylation of a tightly bound accessory/regulatory protein is responsible for the increase in IP_3R_1 detected by Western blot following avidin pull-down. However, given the evidence that thiol-oxidizing agents can directly modify the IP_3R (Kaplin *et al.* 1994; Thrower *et al.* 1996; Poirier *et al.* 2001), a direct modification by glutathionylation seems plausible. In preliminary studies using BAECs permabilized with saponin, we found that IP_3R_1 is not glutathionylated in its cytoplasmic domain (unpublished observation) whereas robust glutathionylation of IP_3R_1 was observed in isolated BAEC microsomes under similar conditions (Lock *et al.* 2011). This would suggest that the IP_3R_1 is glutathionylated in an ER luminal domain. The IP_3R monomer has six membrane spanning segment; the NH_2- and COOH-termini are cytoplasmic and there are three luminal loops, L1–L3. L3 has four Cys residues that are conserved in type-1, -2, and -3 IP_3R. Previous studies by Mikoshiba's group showed that the ER resident protein, ERp44, binds tightly to the type-1 receptor, but not to type-2 or type-3 (Higo *et al.* 2005). Binding of ERp44 to the IP_3R is sensitive to redox potential and is favoured under reducing conditions and inhibited under oxidizing conditions. Binding of ERp44 to the IP_3R requires reduced Cys residues, and binding inhibits channel activity. Importantly, oxidized conditions will also favour protein *S*-glutathionylation. Thus, glutathionylation of one or more of these Cys residues may block interaction of ERp44 with IP_3R and hence prevent inhibition of the channel activity by ERp44. Further studies will be necessary to determine the role of these residues in IP_3R glutathionylation and the subsequent effect on IP_3R function.

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Author contributions

All experiments were performed in the laboratory of W.P.S. Conception and design of research by J.T.L., W.G.S. and W.P.S. Data were collected by J.T.L. and W.G.S., and analysed by J.T.L., W.G.S. and W.P.S. The manuscript was written by J.T.L and W.P.S with assistance from W.G.S. All authors have read and approved the final version of the manuscript.

Acknowledgements

This work was supported by grant HL097355, and J.T.L. was supported in part by training grant HL007887, both from the National Heart Lung and Blood Institute (USA).