

Endothelin-1, superoxide and adeninediphosphate ribose cyclase in shark vascular smooth muscle

Susan K. Fellner^{1,2,*} and Laurel Parker¹

¹Mount Desert Island Biological Laboratory, Salisbury Maine 04672 USA and ²Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545, USA

*Author for correspondence (e-mail: sfellner@med.unc.edu)

Accepted 24 January 2005

Summary

In vascular smooth muscle (VSM) of *Squalus acanthias*, endothelin-1 (ET-1) signals via the ET_B receptor. In both shark and mammalian VSM, ET-1 induces a rise in cytosolic Ca²⁺ concentration ([Ca²⁺]_i) via activation of the inositol trisphosphate (IP₃) receptor (IP₃R) and subsequent release of Ca²⁺ from the sarcoplasmic reticulum (SR). IP₃R-mediated release of SR Ca²⁺ causes calcium-induced calcium release (CICR) via the ryanodine receptor (RyR), which can be sensitized by cyclic adeninediphosphate ribose (cADPR). cADPR is synthesized from NAD⁺ by a membrane-bound bifunctional enzyme, ADPR cyclase. We have previously shown that the antagonists of the RyR, Ruthenium Red, high concentrations of ryanodine and 8-Br cADPR, diminish the [Ca²⁺]_i response to ET-1 in shark VSM. To investigate how ET-1 might influence the activity of the ADPR cyclase, we employed inhibitors of the cyclase. To explore the possibility that ET-1-induced production of

superoxide (O₂⁻) might activate the cyclase, we used an inhibitor of NAD(P)H oxidase (NOX), DPI and a scavenger of O₂⁻, TEMPOL. Anterior mesenteric artery VSM was loaded with fura-2AM to measure [Ca²⁺]_i. In Ca²⁺-free shark Ringers, ET-1 increased [Ca²⁺]_i by 104±8 nmol l⁻¹. The VSM ADPR cyclase inhibitors, nicotinamide and Zn²⁺, diminished the response by 62% and 72%, respectively. Both DPI and TEMPOL reduced the response by 63%. The combination of the IP₃R antagonists, 2-APB or TMB-8, with DPI or TEMPOL further reduced the response by 83%. We show for the first time that in shark VSM, inhibition of the ADPR cyclase reduces the [Ca²⁺]_i response to ET-1 and that superoxide may be involved in the activation of the cyclase.

Key words: NAD(P)H oxidase, nicotinamide, CICR, ryanodine, calcium, shark, *Squalus acanthias*.

Introduction

It is becoming well accepted that peptide agonist stimulation of G protein-coupled receptors, generation of inositol trisphosphate (IP₃) and activation of the IP₃ receptor (IP₃R) causes a release of Ca²⁺ of short duration from the endoplasmic/sarcoplasmic reticulum (ER/SR; Berridge, 1993; Galione and Churchill, 2002; Guse et al., 1999). This Ca²⁺ signal is augmented by activation of the ryanodine receptor (RyR) by the increase in cytosolic Ca²⁺ concentration ([Ca²⁺]_i); a process known as calcium-induced calcium release, CICR, by cyclic adenine-diphosphoribose (cADPR) and possibly by IP₃ as well (Galione and Churchill, 2002; Yusufi et al., 2002; Guse et al., 1999). cADPR, first discovered in sea urchin eggs (Lee et al., 1989), is synthesized from β-nicotinamide adenine dinucleotide (NAD⁺) by a bifunctional membrane bound enzyme, CD38, in a wide variety of eukaryotic cells (Guse, 1999; Lee, 2001). cADPR, in concert with calmodulin, sensitizes the ryanodine receptor (RyR) to Ca²⁺, thereby amplifying the process of CICR (Galione et al., 1991; Lee, 1993; Dousa et al., 1996)

and can directly stimulate the RyR (Yusufi et al., 2001; Li et al., 2001).

Evidence for the presence of an ADPR cyclase and for a role of cADPR in Ca²⁺ signaling has been established in a large number of mammalian cell types (Guse, 1999; Lee, 2001). There are only two reports of cADPR activity in fish, both in oocytes (Polzonetti et al., 2002; Fluck et al., 1999). Recently we demonstrated a role for cADPR in endothelin B receptor (ET_BR)-mediated Ca²⁺ signaling in the anterior mesenteric artery of *Squalus acanthias* (Fellner and Parker, 2004). Participation of cADPR in Ca²⁺ signaling has been demonstrated from a variety of mammalian vascular smooth muscle (VSM) sources: membrane preparations of rat aorta (Yusufi et al., 2002; de Toledo et al., 2000), renal microvessels (Li et al., 2000), bovine and porcine coronary arteries (Zhang et al., 2004; Yu et al., 2000; Kannan et al., 1996), and pulmonary artery (Wilson et al., 2001). The VSM ADPR cyclase has several unique properties that distinguish it from CD38. In contrast to the ADPR cyclase of sea urchin eggs,

Aplysia and HL-60 cells, in which Zn^{2+} enhances the activity of the enzyme, Zn^{2+} inhibits the cyclase of rat aortic VSM cells (de Toledo et al., 2000). Furthermore, the VSM cyclase has a specific activity 20-fold greater than the CD38 of HL 60 cells (de Toledo et al., 2000), suggesting that the enzyme may play an important role in normal vascular physiology. Recently, it has been shown that oxidative stress increases $[Ca^{2+}]_i$ in fresh bovine coronary VSM cells (Zhang et al., 2004) and that nitric oxide (NO) inhibits ADPR cyclase in bovine coronary artery. These findings contrast with the stimulatory effect of NO in non-vascular cells such as macrophages, pancreatic cells, neurons and sea urchin eggs (Yu et al., 2000).

Evidence for participation of cADPR in endothelin-1 (ET-1) Ca^{2+} signaling has been demonstrated in porcine airway smooth muscle (White et al., 2003), rat seminiferous peritubular smooth muscle (Barone et al., 2002), rat mesenteric artery (Giuliumian et al., 2000) and shark mesenteric artery VSM (Fellner and Parker, 2004). None of these studies has explored the mechanism(s) by which ET-1 might activate the ADPR cyclase. A link between ET-1 and the generation of superoxide ($O_2^{\cdot-}$) has been demonstrated in cultured A-10 cells (Sedeek et al., 2003) and human gluteal arterial VSM cells (Touyz et al., 2004). We hypothesized that ET-1 might activate VSM NAD(P)H oxidases (NOX) causing the generation of $O_2^{\cdot-}$ leading to the activation of the VSM ADPR cyclase. Therefore, in the current study we utilized inhibitors of the ADPR cyclase, an inhibitor of NOX and a superoxide dismutase mimetic to investigate how ET-1 might activate the cyclase to generate cADPR. Because our previous investigations of Ca^{2+} signaling pathways in shark VSM have demonstrated remarkable concordance with those of mammalian VSM (Fellner and Parker, 2004), we believe that the current study can give insight into the role of superoxide, preserved during evolution, in the responses of VSM to peptide agonists.

Materials and methods

Sharks *Squalus acanthias* L. (2–3 kg) of both sexes were caught in the coastal waters of Maine, USA and kept in running seawater tanks (11–15°C, early to late summer) until the animals were pithed through the snout and sacrificed. This protocol was approved by the Institutional Animal Care Committee at Mount Desert Island Biological Laboratory, Salisbury Cove, ME, USA.

The anterior mesenteric artery was dissected and placed in ice-cold Ca^{2+} -free shark Ringers, pH 7.7, containing, in $mmol\ l^{-1}$, NaCl, 275; KCl, 4; $MgCl_2$, 3; Na_2SO_4 , 0.5; KH_2PO_4 , 1.0; $NaHCO_3$, 8; urea, 350; D-glucose, 5; HEPES, ~5, and trimethylamine oxide (TMAO), 72 (Fellner and Parker, 2002). Calcium buffer contained $2.5\ mmol\ l^{-1}$ calcium (normal concentration in the shark; Prosser and Kirschner, 1973), whereas no $CaCl_2$ was added to the calcium-free buffer. The anterior mesenteric artery was minced into pieces <0.5 mm in size and then loaded with the Ca^{2+} -sensitive

fluorescent dye, fura-2AM at 13°C for 30 min in the dark in Ca^{2+} -Ringers containing 0.5% bovine serum albumin (BSA); the sample was washed three times with Ca^{2+} -free buffer and incubated for another 30 min at 18°C. Subsequent experiments were conducted in a temperature-controlled room kept at 18°C.

Measurement of cytosolic free calcium concentration

$[Ca^{2+}]_i$ was measured as previously described (Fellner and Parker, 2004; Fellner and Parker, 2002). Arterial tissue was placed in an open static chamber and examined in a small window of the optical field of a $\times 40$ oil-immersion fluorescence objective of an inverted microscope (Olympus IX70). All experiments were conducted in a room maintained at 18°C. Approximately 5–6 typical elongated vascular smooth muscle cells were selected for analysis. There were no visible endothelial cells in the study sample. The tissue was excited alternately with light of 340 and 380 nm wavelengths from a dual-excitation wavelength Delta-Scan equipped with dual monochromators and a chopper (Photon Technology International (PTI), New Jersey, USA). After passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. The calibration of $[Ca^{2+}]_i$ was based on the signal ratio at 340/380 nm and known concentrations of Ca^{2+} (Grynkiewicz et al., 1985) and was performed with a calibration kit purchased from Molecular Probes (Eugene, OR, USA).

Experimental protocol

The concentrations of ET-1 that we employed in each experiment was $2 \times 10^{-7}\ mol\ l^{-1}$, a concentration at least twice the maximal stimulatory concentrations reported in the literature (Just et al., 2004; Touyz et al., 1995; Shimoda et al., 2000; Yanagisawa et al., 1988; Cavarape et al., 2003; Batra et al., 1993). The concentrations of antagonists were also chosen on the basis of values reported in the literature: Zn^{2+} ($3\ mmol\ l^{-1}$) (de Toledo et al., 2000), nicotinamide ($3\ mmol\ l^{-1}$) (Sethi et al., 1996), 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (TEMPOL; $1\ mmol\ l^{-1}$) (Zhang et al., 2004; Touyz et al., 2004) and diphenyl iodonium (DPI; $1\ \mu mol\ l^{-1}$) (Touyz et al., 2004; Rodriguez-Puyol et al., 2002). Tissue was preincubated with antagonists for at least 2 min before adding ET-1.

Mesenteric VSM cells were analyzed only once and then discarded. All experiments were conducted initially in Ca^{2+} -free Ringers. After responses to ET-1 in the presence or absence of inhibitors had concluded, we added Ca^{2+} (final concentration, $2.5\ mmol\ l^{-1}$), to confirm tissue viability. Calcium entry *via* store-operated channels or voltage-gated channels as well as operation of the calcium sensing receptor (Fellner and Parker, 2002) should increase $[Ca^{2+}]_i$. In the case of the Zn^{2+} experiments however, because Zn^{2+} inhibits voltage-gated Ca^{2+} entry (Kerchner et al., 2000) and possibly store-operated Ca^{2+} entry (Uehara et al., 2002), the effect of adding Ca^{2+} was markedly diminished. If there was no Ca^{2+} response, that sample was discarded.

Reagents

Trimethylamine oxide (TMAO), nicotinamide, DPI, 2-aminoethoxy diphenyl borate (2-APB) and TEMPOL were purchased from Sigma (St Louis, MO, USA), endothelin-1 from California Peptide Research, Inc (Napa, CA, USA), fura-2-AM from Teflab (Austin, TX, USA) and 3,4,5-trimethoxybenzoic acid-8-(diethylamino) octyl ester (TMB-8; CalBiochem, La Jolla, CA, USA).

Statistics and graphics

The data are presented as means ± S.E.M. Each data set is derived from tissue originating from at least three different sharks. For representative tracings of original data with ET-1 and antagonists, we selected data pairs from the same experimental day. Paired data sets were tested with Student's paired *t*-test. Multiple comparisons were analyzed using one-way analysis of variance for repeated measures followed by Student–Neuman–Kuels *post hoc* test. *P* < 0.05 was considered statistically significant.

Results

[Ca²⁺]_i response to ET-1

Mesenteric artery VSM cells in Ca²⁺-free Ringers responded to ET-1 with a peak response in 5–25 s. As we reported previously, the response of shark VSM cells to ET-1 at cool temperatures is characterized by a broad peak, which falls back to baseline values in approximately 100 s (Fellner and Parker, 2004). Based on the techniques employed in the present study, we found that the mean baseline [Ca²⁺]_i was 116 ± 6 and the peak response to ET-1 was 220 ± 9 nmol l⁻¹ (*N* = 53, *P* < 0.01; Fig. 1). (The apparent [Ca²⁺]_i is denoted simply as [Ca²⁺]_i.) Thus the net increase in [Ca²⁺]_i after ET-1 stimulation for all experiments was 104 ± 8 nmol l⁻¹. Addition of Ca²⁺ at the nadir of the ET-1 response caused a further increase in [Ca²⁺]_i of 115 ± 9 nmol l⁻¹.

Inhibitors of the ADPR cyclase

A major question is what is the relationship between agonist stimulation of a G-protein-coupled receptor to initiate the sequence of IP₃ generation, activation of the IP₃R, release of Ca²⁺ from the SR and participation of the RyR to augment the Ca²⁺ signal? And further, what is the communication between ET-1 and the membrane ADPR cyclase to direct formation of cADPR? If inhibitors of the ADPR cyclase diminish the response of VSM cells to ET-1, one might infer that ET-1 is somehow sending a message to the cyclase to increase the formation of cADPR. Both nicotinamide and Zn²⁺ are well-studied inhibitors of the ADPR cyclase in vascular smooth muscle (de Toledo et al., 2000; Sethi et al., 1996). Neither of these antagonists is known to have an effect on the IP₃ receptor (IP₃R). Nicotinamide (3 mmol l⁻¹) pretreatment of arterioles reduced the [Ca²⁺]_i response to ET-1 by 62% (40 ± 6 nmol l⁻¹; *N* = 15, *P* < 0.01 for ET-1 alone vs ET-1 + nicotinamide; Fig. 2A,C). In the presence of Zn²⁺ (3 mmol l⁻¹), the net response to ET-1 was decreased to 30 ± 3 nmol l⁻¹ (72% inhibition; *N* = 11, *P* < 0.01; Fig. 2B,C). Together, the

nicotinamide and Zn²⁺ experimental data suggest that there is a pathway utilized by ET-1 that increases the activity of the ADPR cyclase and that is independent of the IP₃ pathway.

Blockade of superoxide generation or effect

To address the question of whether there is a connection between ET-1, O₂⁻ generation and ET-1-induced elevation of [Ca²⁺]_i, we employed the NOX inhibitor DPI (Babior, 1999; Touyz et al., 2004). In the presence of DPI (1 μmol l⁻¹), the increase in [Ca²⁺]_i following addition of ET-1 was 39 ± 7 (*N* = 19, *P* < 0.01 for ET-1 + DPI vs baseline, and 0.05 for ET alone vs ET + DPI; Fig. 3A,C). To further examine the role of O₂⁻ in ET-1-mediated Ca²⁺ signaling in shark VSM, we utilized TEMPOL, a superoxide dismutase mimetic (Evans et al., 2004; Sedeek et al., 2003). When the VSM was preincubated with TEMPOL (1 mmol l⁻¹), the increase in [Ca²⁺]_i was 38 ± 3 (*N* = 19, *P* < 0.05 for ET-1 + TEMPOL vs baseline, and < 0.01 for ET-1 vs ET-1 + TEMPOL; Fig. 3B,C). These data, showing 63% inhibition of the ET-1-induced [Ca²⁺]_i response by DPI and TEMPOL, suggest that when the production or duration of elevated O₂⁻ is diminished, the ability of ET-1 to mobilize Ca²⁺ from the SR and increase [Ca²⁺]_i is markedly reduced.

Simultaneous blockade of the IP₃R and O₂⁻ generation or effect

To substantiate the premise that ET-1 signals *via* two independent pathways, namely the classic IP₃ pathway and perhaps a NOX, O₂⁻, ADPR cyclase pathway, we measured the [Ca²⁺]_i response to ET-1 in the presence of added TEMPOL or of DPI plus 2-APB (33 μmol l⁻¹) or DPI plus TMB-8 (1 μmol l⁻¹). We were unable to test TEMPOL plus TMB-8 because of precipitation when the two reagents were combined. The [Ca²⁺]_i response to ET-1 in the presence of TEMPOL plus 2-APB was 19 ± 6 nmol l⁻¹ (82% inhibition, *N* = 6, *P* < 0.01 vs TEMPOL alone). For DPI plus 2-APB, the [Ca²⁺]_i response was 17 ± 6 nmol l⁻¹ (84% inhibition, *N* = 8, *P* = 0.02 vs DPI

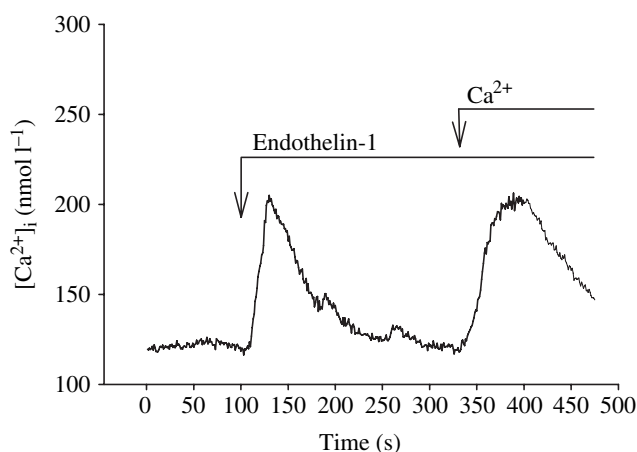


Fig. 1. Representative tracing of cytosolic Ca²⁺ ([Ca²⁺]_i) response of shark anterior mesenteric artery vascular smooth muscle (VSM) to endothelin-1 (ET-1) in Ca²⁺-free shark Ringers followed by the addition of Ca²⁺ (2.5 mmol l⁻¹).

alone). For DPI plus TMB-8, the response was 18 ± 4 nmol l⁻¹ (83% inhibition, $N=9$, $P=0.01$ vs DPI alone; Fig. 4).

Discussion

We have previously shown that G-protein-coupled activation of the ET_BR, leading to IP₃ formation and

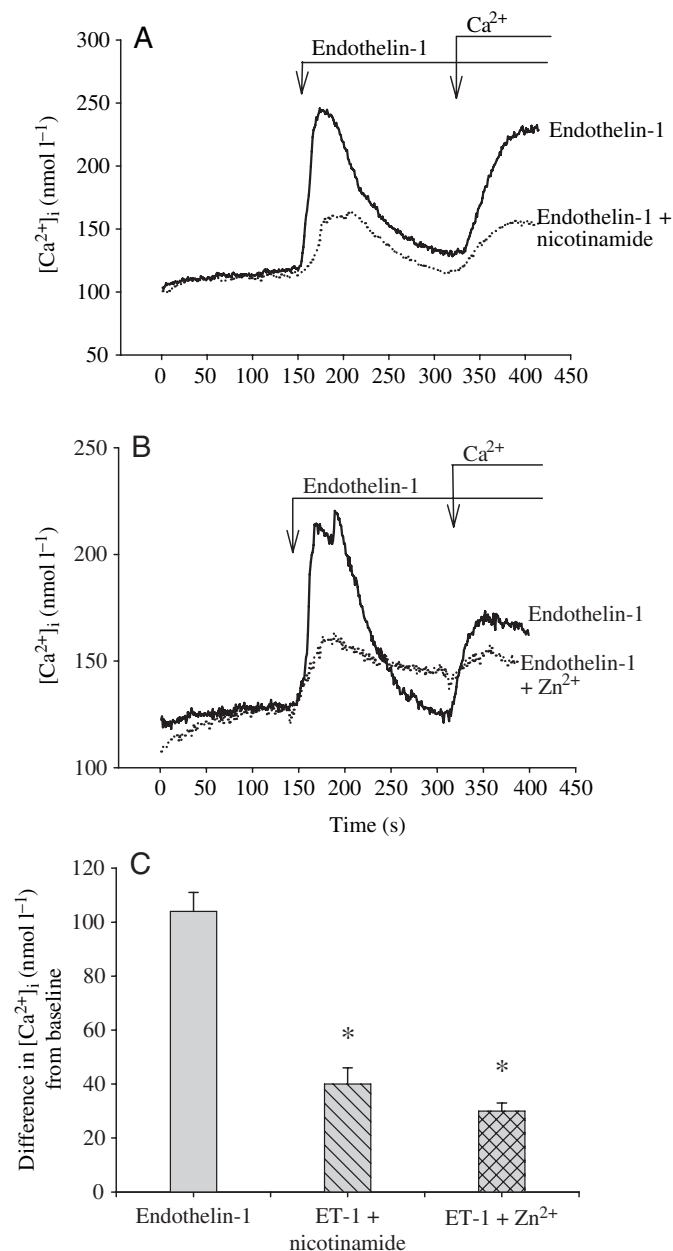


Fig. 2. Effect of inhibitors of ADPR cyclase on endothelin-1 (ET-1)-stimulated increases in $[Ca^{2+}]_i$ in vascular smooth muscle (VSM). (A) Representative tracing of the $[Ca^{2+}]_i$ response to ET-1 in the presence and absence of nicotinamide. (B) Representative tracing of the response to ET-1 in the presence and absence of Zn²⁺. (C) Summary data of the effects of nicotinamide ($N=15$) and Zn²⁺ ($N=11$) on the ET-1-induced peak elevation of $[Ca^{2+}]_i$. * $P<0.01$ for both inhibitors vs ET-1 alone.

stimulation of the IP₃R, is a major signaling pathway for increasing $[Ca^{2+}]_i$ in VSM cells of the anterior mesenteric artery of *Squalus acanthias* (Fellner and Parker, 2004). Furthermore, we demonstrated that three antagonists of the RyR, Ruthenium Red, high concentrations of ryanodine and 8-Br cADPR inhibited the response by a mean 39% (Fellner and Parker, 2004). In the present study, we have investigated ET-1-stimulated participation in the activation of the ADPR cyclase and show that ET-1 somehow has an effect on the formation of cADPR. To investigate the possibility that ET-1 influences the activity of the ADPR cyclase, we utilized two inhibitors of this plasmalemmal membrane enzyme, nicotinamide (Sethi et al., 1996) and Zn²⁺ (de Toledo et al., 2000). Both nicotinamide and Zn²⁺ inhibited the $[Ca^{2+}]_i$ response to ET by about 60%. Nicotinamide does not actually inhibit the cyclase, but rather forces the reaction in the direction of forming NAD⁺ rather than cADPR (Kim et al., 1993). Nicotinamide is not known to influence any other components of ET-1 signaling pathways (Geiger et al., 2000). Although Zn²⁺ can block voltage-gated Ca²⁺ entry (Kerchner et al., 2000) and possibly store-operated Ca²⁺ entry (Uehara et al., 2002), these pathways are not operative in Ca²⁺-free buffer. Zn²⁺ can also inhibit the plasma membrane Ca²⁺ ATPases of fish gill (Hogstrand et al., 1996), and in so doing, reduces Ca²⁺ efflux from the cell. If this were a significant effect in VSM cells, one would expect an enhancement of the $[Ca^{2+}]_i$ response to ET-1, rather than inhibition. Zn²⁺ may inhibit proton currents associated with NOX in phagocytic cells (DeCoursey et al., 2003). Whether or not Zn²⁺ interacts with the novel isoforms of VSM NOX (Bengtsson et al., 2003) has not been studied. The strong inhibitory effects of both nicotinamide and Zn²⁺ provide firm evidence that ET-1 is involved in the activation of the ADPR cyclase to form cADPR in VSM of *S. acanthias*.

An enzyme capable of forming cADPR was first described in homogenates of sea urchin eggs (Lee et al., 1989) and subsequently has been shown to be present in a wide variety of cell types (Lee, 1997; Guse, 1999, 2004). In mammals, a single bifunctional protein, CD38, can act as a cyclase or hydrolase for cADPR (Lee et al., 1997; reviewed by Schuber and Lund, 2004). This unusual membrane-bound enzyme is stimulated by a number of different agonists in specific mammalian cell types: for example, estrogen in myometrium (Barata et al., 2004); glucose in pancreatic beta cells (Takasawa et al., 1993); retinoic acid and triiodothyronine (T₃) in aortic VSM cells (de Toledo et al., 1997); reactive oxygen species (ROS) in bovine coronary VSM (Zhang et al., 2004); angiotensin II in neonatal cardiac myocytes (Higashida et al., 2000), tumor necrosis factor- α and interleukin 1- β in glomerular mesangial cell (Yusufi et al., 2001), and acetylcholine and ET-1 in airway smooth muscle (White et al., 2003). How this structurally diverse group of molecules mediates the same process, namely activation of the ADPR cyclase, has not yet been elucidated with certainty.

NAD(P)H oxidases are plasmalemmal enzymes that catalyze the production of (O₂⁻) from two molecules of O₂

(reviewed by Babior, 1999). Although widely studied in phagocytic cells, NOX has been more recently found to be present in VSM and to be activated by peptide agonists such as angiotensin II (Rajagopalan et al., 1996). There is now evidence for ET-1-induced activation of NOX and formation

of superoxide (O₂⁻) and reactive oxygen species (ROS) in VSM (Sedeek et al., 2003; Touyz et al., 2004; Li et al., 2003; Galle et al., 2000; Wedgwood et al., 2001). In cultured rat aortic VSM cells, ET-1 dose dependently (10⁻⁸ to 10⁻⁶ mol l⁻¹) increased the formation of O₂⁻ (Sedeek et al., 2003) and, in human gluteal VSM cells, similar results were noted (Touyz et al., 2004). DPI, an inhibitor of NOX, diminished the ET-1-induced production of O₂⁻ only at high concentrations of ET (10⁻⁶ mol l⁻¹) whereas thenotrifluoroacetone (TIFT), a mitochondrial electron chain inhibitor, reduced the production of O₂⁻ at concentrations of ET-1 between 10⁻⁹ and 10⁻⁶ mol l⁻¹ (Touyz et al., 2004). To our knowledge, there have been no reports of a vascular smooth muscle NOX, or NOX of any non-phagocytic cell origin, in fish. Our finding of inhibition of the [Ca²⁺]_i response to ET-1 by the NOX inhibitor DPI and by the superoxide dismutase mimetic, TEMPOL, lends support to the presence of generation of O₂⁻ by NOX on VSM of *S. acanthias*.

Recent studies have investigated a possible linkage between O₂⁻ generation, cADPR and changes in [Ca²⁺]_i, and vascular contraction in small bovine coronary arteries (Zhang et al., 2004). Xanthine/xanthine oxidase (X/XO), a O₂⁻ generating system, increased the activity of ADPR cyclase and increased [Ca²⁺]_i in fresh coronary artery VSM cells. The elevation in [Ca²⁺]_i was partially blocked by 8-Br cADPR, nicotinamide, high concentrations of ryanodine and tetracaine (Zhang et al., 2004). Other studies have likewise suggested that O₂⁻ increases that activity of ADPR cyclases (Xie et al., 2003; Okabe et al., 2000). In cardiac myocytes, nearly nanomolar concentrations of O₂⁻ stimulated the synthesis of cADPR and Ca²⁺ release (Okabe et al., 2000). Oxidation of cysteine residues of the cyclase results in the formation of disulfide bond and dimers of the enzyme, which have much greater activity than the monomer (Tohgo et al., 1994; Chidambaram et al., 1998). Taken together, these studies suggest that ET-1-

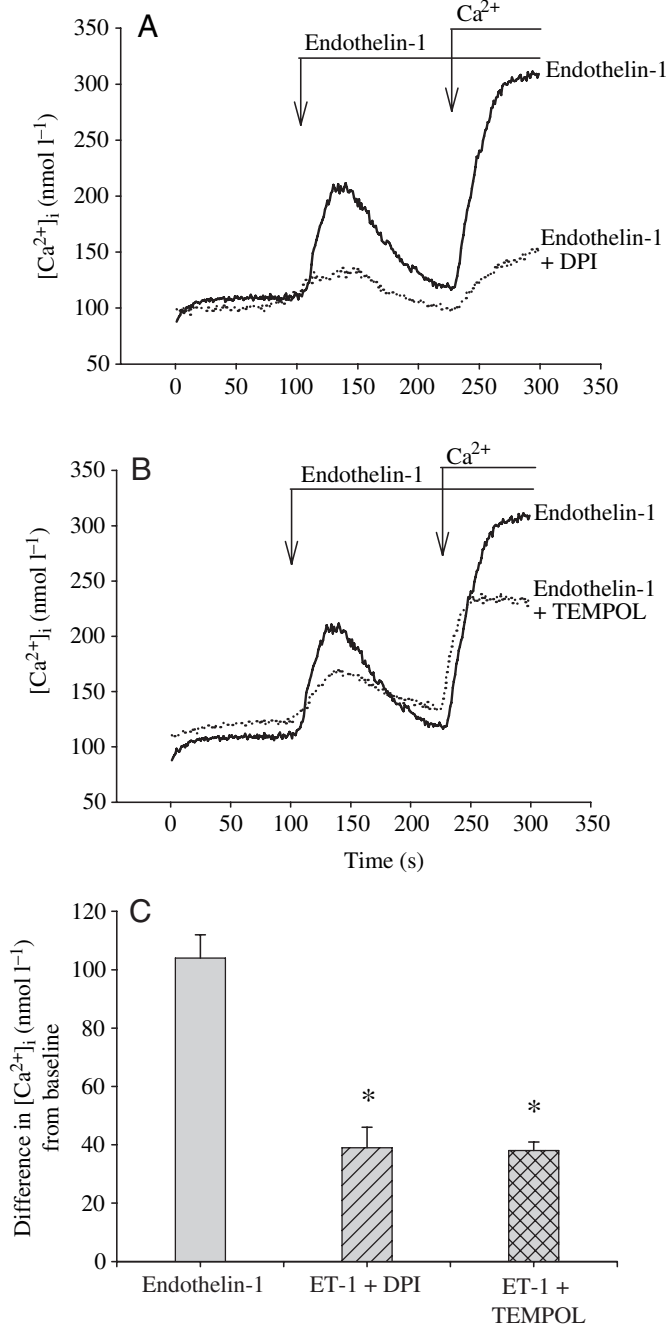


Fig. 3. Effect of NAD(P)H oxidase inhibitor (DPI) and superoxide dismutase mimetic (TEMPOL) on endothelin-1 (ET-1) stimulation of [Ca²⁺]_i in shark vascular smooth muscle (VSM). (A) Typical tracing of the [Ca²⁺]_i response to ET-1 in the presence and absence of DPI. (B) Typical tracing of the ET-1 in the presence and absence of TEMPOL. (C) Summary data showing the inhibitory effects of DPI (N=19) and TEMPOL (N=19) on the ET-1 induced elevation of [Ca²⁺]_i. *P<0.01 for ET-1 plus DPI or TEMPOL vs ET-1 alone.

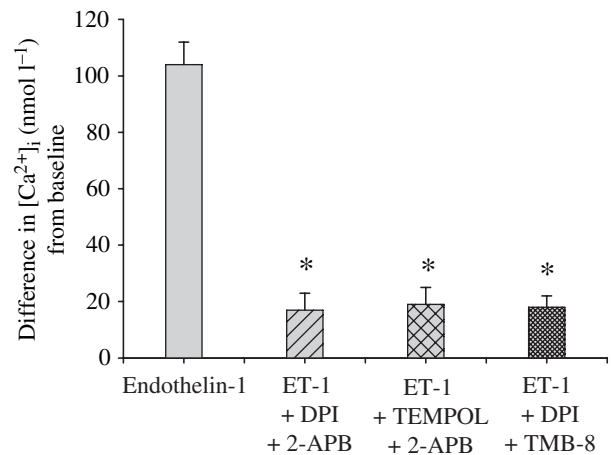


Fig. 4. Effects of combining inhibitors of the IP₃ receptor with an inhibitor of NAD(P)H oxidase (DPI) or a superoxide scavenger (TEMPOL) in Ca²⁺-free shark Ringers. In each case, the combination of antagonists blocked the [Ca²⁺]_i response to endothelin-1 (ET-1) by 83±1%. *P<0.01.

induced formation of O_2^- may acutely increase the activity of ADPR cyclase, possibly *via* dimerization of the enzyme.

Superoxide generation may have other effects on $[Ca^{2+}]_i$ and the activity of ADPR cyclase. Superoxide rapidly combines with nitric oxide (NO) to form peroxynitrate (Gryglewski et al., 1986). Nitric oxide has been shown to inhibit the ADPR cyclase of bovine coronary arterial VSM cells (Yu et al., 2000). Thus, ET-1-stimulated O_2^- production may diminish available NO, thereby abolishing the usual inhibitory effect of NO on the cyclase, and increasing both the ability of the cyclase to form cADPR and its participation in CICR. There are vascular effects of O_2^- that are independent of cADPR. Nitrosylation of tyrosine residues may impair the synthesis of prostacyclin, which is vasodilatory (Zou et al., 1997). Superoxide may combine non-enzymatically with arachidonate, generating isoprostanes, which can then activate thromboxane receptors (Seshiah et al., 2002). Thus there are several possible mechanisms by which ET-1 stimulated O_2^- formation could have an effect on Ca^{2+} in VSM.

Our finding that DPI or TEMPOL plus 2-APB or TMB-8 inhibited the $[Ca^{2+}]_i$ response to ET-1 in afferent arterioles by 83% raises the question of why there was not 100% inhibition. It is possible that neither the IP_3R nor O_2^- blockers were given at maximal inhibitory concentrations. There may be other pathways of ET-1-induced calcium signaling such as receptor-operated mechanisms, working through diacyl glycerol rather than IP_3 . Our data suggest that blockade of the IP_3R represents the sum of IP_3R -mediated release of Ca^{2+} from the SR and that obtained from CICR. Anything that interferes with generation or disposition of O_2^- , will reduce activation of the ADPR cyclase, production of cADPR and sensitization of the RyR to Ca^{2+} . The inhibitors of the ADPR cyclase, nicotinamide and Zn^{2+} , diminished the $[Ca^{2+}]_i$ response to ET-1 by two thirds. Similarly, both DPI and TEMPOL reduced the response by about two thirds, suggesting that the effect of cADPR on the RyR is a major component of the global response of afferent arteriolar VSM to ET-1.

Complex control systems have developed in animals to ensure homeostasis in response to intermittent feeding conditions and to environmental changes. The well-known initiating event in contraction of vascular smooth muscle is a change in $[Ca^{2+}]_i$ caused by hormones, autocrine or paracrine substances or stretch of the vascular wall. Endothelin-1, which is produced by the vascular endothelium, acts locally to cause vasoconstriction. In all animals, the ability to regulate systemic blood flow in the face of environmental stresses has great survival benefit. The elasmobranch, *Squalus acanthias*, controls plasma osmolality and blood volume by secreting hypertonic fluid from its rectal gland. We have previously proposed that changes in blood flow to the rectal gland are important modulators of salt excretion from the rectal gland (Fellner and Parker, 2002). As well, ET-1 is thought to have an important role in influencing gill function in the shark (Evans and Gunderson, 1999).

In summary, we have shown that ET-1 stimulation of the anterior mesenteric artery VSM of the shark increases $[Ca^{2+}]_i$

via several distinct pathways. The classic G-protein-coupled receptor activation that results in IP_3 generation and release of $[Ca^{2+}]_i$ from the SR probably provides an initial increase of $[Ca^{2+}]_i$. The IP_3R -stimulated increase in $[Ca^{2+}]_i$ can initiate CICR. Our data suggest that in *S. acanthias*, ET-1 activates NOX to produce O_2^- which, in turn, activates VSM ADPR cyclase to increase the formation of cADPR. cADPR, with its interaction with the RyRs, further amplifies the Ca^{2+} signal. These findings demonstrate that vascular NOX and ADPR cyclase are enzymes that have been preserved for millions of years during evolution.

This work was supported in part by a grant from the Thomas H. Maren Foundation.

References

- Babior, B. M. (1999). NADPH oxidase: an update. *Blood* **93**, 1464-1476.
- Barata, H., Thompson, M., Zielinska, W., Han, Y. S., Mantilla, C. B., Prakash, Y. S., Feitoza, S., Sieck, G. and Chini, E. N. (2004). The role of cyclic-ADP-ribose-signaling pathway in oxytocin-induced Ca^{2+} transients in human myometrium cells. *Endocrinol.* **145**, 881-889.
- Barone, F., Genazzani, A. A., Conti, A., Churchill, G. C., Palombi, F., Ziparo, E., Sorrentino, V., Galione, A. and Filippini, A. (2002). A pivotal role for cADPR-mediated Ca^{2+} signaling: regulation of endothelin-induced contraction in peritubular smooth muscle cells. *FASEB J.* **16**, 697-705.
- Batra, V. K., McNeill, J. R., Xu, Y., Wilson, T. W. and Gopalakrishnan, V. (1993). ETB receptors on aortic smooth muscle cells of spontaneously hypertensive rats. *Am. J. Physiol.* **264**, C479-C484.
- Bengtsson, S. H., Gulluyan, L. M., Dusting, G. J. and Drummond, G. R. (2003). Novel isoforms of NADPH oxidase in vascular physiology and pathophysiology. *Clin. Exp. Pharmacol. Physiol.* **30**, 849-854.
- Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315-325.
- Cavarape, A., Endlich, N., Assaloni, R., Bartoli, E., Steinhausen, M., Parekh, N. and Endlich, K. (2003). Rho-kinase inhibition blunts renal vasoconstriction induced by distinct signaling pathways in vivo. *J. Am. Soc. Nephrol.* **14**, 37-45.
- Chidambaram, N., Wong, E. T. and Chang, C. F. (1998). Differential oligomerization of membrane-bound CD38/ADP-ribosyl cyclase in porcine heart microsomes. *Biochem. Mol. Biol. Int.* **44**, 1225-1233.
- de Toledo, F. G., Cheng, J. and Dousa, T. P. (1997). Retinoic acid and triiodothyronine stimulate ADP-ribosyl cyclase activity in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **238**, 847-850.
- de Toledo, F. G., Cheng, J., Liang, M., Chini, E. N. and Dousa, T. P. (2000). ADP-Ribosyl cyclase in rat vascular smooth muscle cells: properties and regulation. *Circ. Res.* **86**, 1153-1159.
- DeCoursey, T. E., Morgan, D. and Cherny, V. V. (2003). The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. *Nature* **422**, 531-534.
- Dousa, T. P., Chini, E. N. and Beers, K. W. (1996). Adenine nucleotide diphosphates: emerging second messengers acting *via* intracellular Ca^{2+} release. *Am. J. Physiol.* **271**, C1007-C1024.
- Evans, D. and Gunderson, M. P. (1999). Characterization of an endothelin ET(B) receptor in the gill of the dogfish shark *Squalus acanthias*. *J. Exp. Biol.* **202**, 3605-3610.
- Evans, D., Rose, R. E., Roeser, J. M. and Stidham, J. D. (2004). NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. *Am. J. Physiol.* **286**, R560-R568.
- Fellner, S. K. and Parker, L. (2002). A Ca^{2+} -sensing receptor modulates shark rectal gland function. *J. Exp. Biol.* **205**, 1889-1897.
- Fellner, S. K. and Parker, L. A. (2004). Endothelin B receptor Ca^{2+} signaling in shark vascular smooth muscle: participation of inositol trisphosphate and ryanodine receptors. *J. Exp. Biol.* **207**, 3411-3417.
- Fluck, R., Abraham, V., Miller, A. and Galione, A. (1999). Microinjection of cyclic ADP-ribose triggers a regenerative wave of Ca^{2+} release and exocytosis of cortical alveoli in medaka eggs. *Zygot.* **7**, 285-292.
- Galione, A. and Churchill, G. C. (2002). Interactions between calcium

- release pathways: multiple messengers and multiple stores. *Cell Calcium* **32**, 343-354.
- Galione, A., Lee, H. C. and Busa, W. B.** (1991). Ca(2+)-induced Ca²⁺ release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. *Science* **253**, 1143-1146.
- Galle, J., Lehmann-Bodem, C., Hubner, U., Heinloth, A. and Wanner, C.** (2000). CyA and OxLDL cause endothelial dysfunction in isolated arteries through endothelin-mediated stimulation of O(2)(-) formation. *Nephrol. Dial. Transplant.* **15**, 339-346.
- Geiger, J., Zou, A. P., Campbell, W. B. and Li, P. L.** (2000). Inhibition of cADP-ribose formation produces vasodilation in bovine coronary arteries. *Hypertension* **35**, 397-402.
- Giuliumian, A. D., Meszaros, L. G. and Fuchs, L. C.** (2000). Endothelin-1-induced contraction of mesenteric small arteries is mediated by ryanodine receptor Ca²⁺ channels and cyclic ADP-ribose. *J. Cardiovasc. Pharmacol.* **36**, 758-763.
- Gryglewski, R. J., Palmer, R. M. and Moncada, S.** (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* **320**, 454-456.
- Grynkiewicz, G., Poenie, M. and Tsien, R. Y.** (1985). A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.
- Guse, A. H.** (1999). Cyclic ADP-ribose: a novel Ca²⁺-mobilising second messenger. *Cell Signal.* **11**, 309-316.
- Guse, A. H.** (2004). Regulation of calcium signaling by the second messenger cyclic adenosine diphosphoribose (cADPR). *Curr. Mol. Med.* **4**, 239-248.
- Guse, A. H., da Silva, C. P., Berg, I., Skapenko, A. L., Weber, K., Heyer, P., Hohenegger, M., Ashamu, G. A., Schulze-Koops, H., Potter, B. V. and Mayr, G. W.** (1999). Regulation of calcium signalling in T lymphocytes by the second messenger cyclic ADP-ribose. *Nature* **398**, 70-73.
- Higashida, H., Zhang, J., Hashii, M., Shintaku, M., Higashida, C. and Takeda, Y.** (2000). Angiotensin II stimulates cyclic ADP-ribose formation in neonatal rat cardiac myocytes. *Biochem. J.* **352**, 197-202.
- Hogstrand, C., Verboost, P. M., Bonga, S. E. and Wood, C. M.** (1996). Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. *Am. J. Physiol.* **270**, R1141-R1147.
- Just, A., Olson, A. J. and Arendshorst, W. J.** (2004). Dual constrictor and dilator actions of ETB receptors in the rat renal microcirculation: interactions with ETA receptors. *Am. J. Physiol. Renal Physiol.* **286**, F660-F668.
- Kannan, M. S., Fenton, A. M., Prakash, Y. S. and Sieck, G. C.** (1996). Cyclic ADP-ribose stimulates sarcoplasmic reticulum calcium release in porcine coronary artery smooth muscle. *Am. J. Physiol.* **270**, H801-H806.
- Kerchner, G. A., Canzoniero, L. M., Yu, S. P., Ling, C. and Choi, D. W.** (2000). Zn²⁺ current is mediated by voltage-gated Ca²⁺ channels and enhanced by extracellular acidity in mouse cortical neurones. *J. Physiol.* **528**, 39-52.
- Kim, H., Jacobson, E. L. and Jacobson, M. K.** (1993). Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases. *Science* **261**, 1330-1333.
- Lee, H. C.** (1993). Potentiation of calcium- and caffeine-induced calcium release by cyclic ADP-ribose. *J. Biol. Chem.* **268**, 293-299.
- Lee, H. C.** (1997). Mechanisms of calcium signaling by cyclic ADP-ribose and NAADP. *Physiol. Rev.* **77**, 1133-1164.
- Lee, H. C.** (2001). Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. *Annu. Rev. Pharmacol. Toxicol.* **41**, 317-345.
- Lee, H. C., Graeff, R. M. and Walseth, T. F.** (1997). ADP-ribosyl cyclase and CD38. Multi-functional enzymes in Ca²⁺ signaling. *Adv. Exp. Med. Biol.* **419**, 411-419.
- Lee, H. C., Walseth, T. F., Bratt, G. T., Hayes, R. N. and Clapper, D. L.** (1989). Structural determination of a cyclic metabolite of NAD⁺ with intracellular Ca²⁺-mobilizing activity. *J. Biol. Chem.* **264**, 1608-1615.
- Li, L., Watts, S. W., Banas, A. K., Galligan, J. J., Fink, G. D. and Chen, A. F.** (2003). NADPH oxidase-derived superoxide augments endothelin-1-induced vasoconstriction in mineralocorticoid hypertension. *Hypertension* **42**, 316-321.
- Li, N., Teggatz, E. G., Li, P. L., Allaire, R. and Zou, A. P.** (2000). Formation and actions of cyclic ADP-ribose in renal microvessels. *Microvasc. Res.* **60**, 149-159.
- Li, P. L., Tang, W. X., Valdivia, H. H., Zou, A. P. and Campbell, W. B.** (2001). cADP-ribose activates reconstituted ryanodine receptors from coronary arterial smooth muscle. *Am. J. Physiol. Heart Circ. Physiol.* **280**, H208-H215.
- Okabe, E., Tsujimoto, Y. and Kobayashi, Y.** (2000). Calmodulin and cyclic ADP-ribose interaction in Ca²⁺ signaling related to cardiac sarcoplasmic reticulum: superoxide anion radical-triggered Ca²⁺ release. *Antioxid. Redox. Signal.* **2**, 47-54.
- Polzonetti, V., Cardinali, M., Mosconi, G., Natalini, P., Meiri, I. and Carnevali, O.** (2002). Cyclic ADPR and calcium signaling in sea bream (*Sparus aurata*) egg fertilization. *Mol. Reprod. Dev.* **61**, 213-217.
- Prosser, C. and Kirschner, L.** (1973). *Comparative Animal Physiology. Inorganic ions*, pp. 84-87. Philadelphia, PA: Saunders College Publishing, Holt Rinehart and Winston.
- Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, B. A., Griending, K. K. and Harrison, D. G.** (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest.* **97**, 1916-1923.
- Rodriguez-Puyol, M., Griera-Merino, M., Perez-Rivero, G., Diez-Marques, M. L., Ruiz-Torres, M. P. and Rodriguez-Puyol, D.** (2002). Angiotensin II induces a rapid and transient increase of reactive oxygen species. *Antioxid. Redox. Signal.* **4**, 869-875.
- Schuber, F. and Lund, F. E.** (2004). Structure and enzymology of ADP-ribosyl cyclases: conserved enzymes that produce multiple calcium mobilizing metabolites. *Curr. Mol. Med.* **4**, 249-261.
- Sedeek, M. H., Llinas, M. T., Drummond, H., Fortepiani, L., Abram, S. R., Alexander, B. T., Reckelhoff, J. F. and Granger, J. P.** (2003). Role of reactive oxygen species in endothelin-induced hypertension. *Hypertension* **42**, 806-810.
- Seshiah, P. N., Weber, D. S., Rocic, P., Valppu, L., Taniyama, Y. and Griending, K. K.** (2002). Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ. Res.* **91**, 406-413.
- Sethi, J. K., Empson, R. M. and Galione, A.** (1996). Nicotinamide inhibits cyclic ADP-ribose-mediated calcium signalling in sea urchin eggs. *Biochem. J.* **319**, 613-617.
- Shimoda, L. A., Sylvester, J. T. and Sham, J. S.** (2000). Mobilization of intracellular Ca(2+) by endothelin-1 in rat intrapulmonary arterial smooth muscle cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **278**, L157-L164.
- Takasawa, S., Tohgo, A., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Yonekura, H. and Okamoto, H.** (1993). Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J. Biol. Chem.* **268**, 26052-26054.
- Tohgo, A., Takasawa, S., Noguchi, N., Koguma, T., Nata, K., Sugimoto, T., Furuya, Y., Yonekura, H. and Okamoto, H.** (1994). Essential cysteine residues for cyclic ADP-ribose synthesis and hydrolysis by CD38. *J. Biol. Chem.* **269**, 28555-28557.
- Touyz, R. M., Deng, L. Y. and Schiffrin, E. L.** (1995). Endothelin subtype B receptor-mediated calcium and contractile responses in small arteries of hypertensive rats. *Hypertension* **26**, 1041-1045.
- Touyz, R. M., Yao, G., Viel, E., Amiri, F. and Schiffrin, E. L.** (2004). Angiotensin II and endothelin-1 regulate MAP kinases through different redox-dependent mechanisms in human vascular smooth muscle cells. *J. Hypertens.* **22**, 1141-1149.
- Uehara, A., Yasukochi, M., Imanaga, I., Nishi, M. and Takeshima, H.** (2002). Store-operated Ca²⁺ entry uncoupled with ryanodine receptor and junctional membrane complex in heart muscle cells. *Cell Calcium* **31**, 89-96.
- Wedgwood, S., McMullan, D. M., Bekker, J. M., Fineman, J. R. and Black, S. M.** (2001). Role for endothelin-1-induced superoxide and peroxynitrite production in rebound pulmonary hypertension associated with inhaled nitric oxide therapy. *Circ. Res.* **89**, 357-364.
- White, T. A., Kannan, M. S. and Walseth, T. F.** (2003). Intracellular calcium signaling through the cADPR pathway is agonist specific in porcine airway smooth muscle. *FASEB J.* **17**, 482-484.
- Wilson, H. L., Dipp, M., Thomas, J. M., Lad, C., Galione, A. and Evans, A. M.** (2001). Adp-ribosyl cyclase and cyclic ADP-ribose hydrolase act as a redox sensor: a primary role for cyclic ADP-ribose in hypoxic pulmonary vasoconstriction. *J. Biol. Chem.* **276**, 11180-11188.
- Xie, G. H., Rah, S. Y., Yi, K. S., Han, M. K., Chae, S. W., Im, M. J. and Kim, U. H.** (2003). Increase of intracellular Ca(2+) during ischemia/reperfusion injury of heart is mediated by cyclic ADP-ribose. *Biochem. Biophys. Res. Commun.* **307**, 713-718.
- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T.** (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411-415.

- Yu, J. Z., Zhang, D. X., Zou, A. P., Campbell, W. B. and Li, P. L.** (2000). Nitric oxide inhibits Ca(2+) mobilization through cADP-ribose signaling in coronary arterial smooth muscle cells. *Am. J. Physiol.* **279**, H873-H881.
- Yusufi, A. N., Cheng, J., Thompson, M. A., Burnett, J. C. and Grande, J. P.** (2002). Differential mechanisms of Ca(2+) release from vascular smooth muscle cell microsomes. *Exp. Biol. Med.* **227**, 36-44.
- Yusufi, A. N., Cheng, J., Thompson, M. A., Dousa, T. P., Warner, G. M., Walker, H. J. and Grande, J. P.** (2001). cADP-ribose/ryanodine channel/Ca²⁺-release signal transduction pathway in mesangial cells. *Am. J. Physiol.* **281**, F91-F102.
- Zhang, A. Y., Yi, F., Teggatz, E. G., Zou, A. P. and Li, P. L.** (2004). Enhanced production and action of cyclic ADP-ribose during oxidative stress in small bovine coronary arterial smooth muscle. *Microvasc. Res.* **67**, 159-167.
- Zou, M., Martin, C. and Ullrich, V.** (1997). Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol. Chem.* **378**, 707-713.