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Eucapnic intermittent hypoxia augments endothelin-1 vasoconstriction in rats: role of PKC δ

Kyan J. Allahdadi, Laura C. Duling, Benjimen R. Walker, and Nancy L. Kanagy

Department of Cell Biology and Physiology, Vascular Physiology Group, University of New Mexico, Health Sciences Center, Albuquerque, New Mexico

Abstract

We reported previously that simulating sleep apnea by exposing rats to eucapnic intermittent hypoxia (E-IH) causes endothelin-dependent hypertension and increases constrictor sensitivity to endothelin-1 (ET-1). In addition, augmented ET-1-induced constriction in small mesenteric arteries (sMA) is mediated by increased Ca^{2+} sensitization independent of Rho-associated kinase. We hypothesized that exposing rats to E-IH augments ET-1-mediated vasoconstriction by increasing protein kinase C (PKC)-dependent Ca^{2+} sensitization. In sMA, the nonselective PKC inhibitor GF-109203x (3 μM) significantly inhibited ET-1-stimulated constriction in E-IH arteries but did not affect ET-1-stimulated constriction in sham arteries. Phospholipase C inhibitor U-73122 (1 μM) also inhibited constriction by ET-1 in E-IH but not sham sMA. In contrast, the classical PKC (cPKC) inhibitor Gö-6976 (1 μM) had no effect on ET-1-mediated vasoconstriction in either group, but a PKC δ -selective inhibitor (rottlerin, 3 μM) significantly decreased ET-1-mediated constriction in E-IH but not in sham sMA. ET-1 increased PKC δ phosphorylation in E-IH but not sham sMA. In contrast, ET-1 constriction in thoracic aorta from both sham and E-IH rats was inhibited by Gö-6976 but not by rottlerin. These observations support our hypothesis that E-IH exposure significantly increases ET-1-mediated constriction of sMA through PKC δ activation and modestly augments ET-1 contraction in thoracic aorta through activation of one or more cPKC isoforms. Therefore, upregulation of a PKC pathway may contribute to elevated ET-1-dependent vascular resistance in this model of hypertension.

Keywords

sleep apnea; intermittent hypoxia; hypercapnia; endothelin-1; protein kinase C δ ; vascular smooth muscle cell; mesenteric arteries

Sleep apnea, a common disease affecting up to 20% of Americans (3), is strongly associated with many cardiovascular diseases, including systemic and pulmonary hypertension, coronary artery disease, stroke, and cardiac arrhythmias (3,41). Several studies have found an association between increased circulating endothelin-1 (ET-1) and sleep apnea (15,34,42), suggesting this is one of the ways sleep apnea contributes to cardiovascular disease. In rats exposed to eucapnic intermittent hypoxia (E-IH) as a model of sleep apnea, circulating ET-1 and vasoconstrictor sensitivity to ET-1 are increased, whereas ET receptor antagonists lower blood pressure (4,16). Therefore, elevated levels of ET-1 combined with increased vasoconstrictor responses to this peptide may contribute to vascular pathologies in sleep apnea, and it is important to know how vascular pathologies contribute to the augmented constrictor sensitivity.

In a previous study, we observed that the augmented constriction to ET-1 in arteries from E-IH rats is not accompanied by greater increases in vascular wall Ca^{2+} concentration ($[\text{Ca}^{2+}]$) (1). This suggests that E-IH exposure increases ET-1-dependent vasoconstriction by elevating Ca^{2+} -sensitization rather than increased Ca^{2+} signaling.

Ca^{2+} sensitization increases vascular tone by elevating phosphorylation of the myosin regulatory light chain (MLC_{20}) independent of changes in intracellular $[\text{Ca}^{2+}]$. The degree of MLC_{20} phosphorylation is determined by the activities of both Ca^{2+} -activated MLC kinase (MLCK) and Ca^{2+} -independent MLC phosphatase (MLCP). Therefore, constriction can be initiated by phosphorylation of myosin by MLCK and/or by MLCP inhibition (38). Activation of both Rho-associated kinase (ROK) and protein kinase C (PKC) can lead to MLCP inhibition. Indeed some recent studies suggest elevated ROK activation contributes to augmented vasoconstriction in hypertension. However, we recently reported that ROK contributes to ET-1-induced vasoconstriction in the thoracic aorta but not in resistance-sized mesenteric arteries (2). Furthermore, most studies of ROK regulation of vasoconstriction have been conducted in large arteries or using agonists other than ET-1. In contrast, PKC may play a more important role than ROK in mediating ET-1-dependent Ca^{2+} sensitization and constriction in smaller arteries (5). Therefore, this study investigated the hypothesis that increased PKC-induced Ca^{2+} sensitization is responsible for augmented ET-1 vasoconstriction following exposure to E-IH.

PKC is a large family of serine/threonine kinases activated by many agonists, including ET-1 (10,33). The 13 isoforms of PKC are grouped into three subclasses; classical PKC (cPKC) isoforms (α , βI , βII , and γ) requiring both Ca^{2+} and diacylglycerol (DAG) for activation; novel PKC (nPKC) isoforms (δ , ϵ , η , and θ) requiring only DAG for activation; and atypical PKC isoforms (λ , μ , and ζ) that are activated by binding phosphatidylserine but not DAG or Ca^{2+} (17). Multiple PKC isoforms can inhibit MLCP indirectly by phosphorylating and activating PKC-potentiated inhibitory protein (CPI-17) (6). Based on our previous observation that augmented ET-1 vasoconstriction in E-IH arteries is not accompanied by increased intracellular $[\text{Ca}^{2+}]$ (1) or ROK activation (2), we hypothesized that one or more nPKC isoform mediate augmented ET-1 constriction in small mesenteric arteries from E-IH-exposed rats. To test this hypothesis, endothelium-disrupted mesenteric arteries or thoracic aorta segments were exposed to increasing concentrations of ET-1 (10^{-10} to 10^{-8} M) in the presence or absence of PKC inhibitors. The contribution of phospholipase C (PLC) to ET-1-mediated constriction was also evaluated because PLC liberates DAG, the activator of nPKC isoforms. Finally, we determined expression levels and ET-1-stimulated phosphorylation of PKC in small mesenteric arteries from E-IH and sham rats.

METHODS

Animals

Male Sprague Dawley rats (weighing 250–300 g) were exposed to E-IH as described previously (1,16). Briefly, animals were housed in Plexiglas boxes with free access to food and water and exposed 7 h/day for 14 days to either E-IH (20 brief exposures/h to 5% O_2 -5% CO_2) or air-air cycling (sham, constant stream of room air). We have demonstrated that this protocol lowers P_{O_2} to ~35 mmHg with no change in P_{CO_2} (37), simulating moderate to severe sleep apnea where O_2 saturation falls as low as 70% (22,23). Body weight, systolic blood pressure (SBP), and heart rate were recorded on *days 0* and *14* before the start of the daily E-IH or air-air exposure using a standard tail-cuff apparatus (IITC). Approximately 16 h after the final E-IH exposure, animals were deeply anesthetized with pentobarbital sodium (150 mg/kg), and the mesenteric arterial arcade or the thoracic aorta was collected for constrictor studies. All animal protocols were reviewed and approved by

the Institutional Animal Care and Use Committee of the University of New Mexico Health Science Center and conform to National Institutes of Health guidelines for animal use.

Isolated Mesenteric Arteriole Preparation

Isolation—The intestinal arcade was removed and placed in a Silastic-coated petri dish containing chilled physiological salt solution [PSS (in mmol/l): 129.8 NaCl, 5.4 KCl, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose]. Small mesenteric artery (sMA) segments (4th to 5th order, diameter <250 μm) were dissected from the vascular arcade and placed in fresh PSS oxygenated with normoxic gas (21% O₂-6% CO₂-73% N₂). Cleaned arterioles were transferred to a vessel chamber (Living Systems), cannulated with glass micropipettes, and secured with silk ligatures. The vessels were slowly pressurized to 60 mmHg with PSS using a servocontrolled peristaltic pump (Living Systems) and superfused with oxygenated 37°C PSS at a rate of 5 ml/min.

Endothelium disruption—The endothelium was disabled in all mesenteric artery experiments by passing 1 ml of air through the lumen. Disruption of the endothelium was assessed by exposing phenylephrine (PE, 10 μmol/l)-constricted arterioles to ACh (1 μmol/l), and only arteries where ACh-mediated vasodilation was eradicated were used.

Vessel wall intracellular [Ca²⁺] detection—After endothelium disruption, pressurized mesenteric arteries (inner diameter: sham = 144.0 ± 3.4 μm; E-IH = 148.2 ± 3.4 μm) were loaded with the cell-permeable ratiometric Ca²⁺-sensitive fluorescent dye fura 2-AM (Molecular Probes). Fura 2-AM was dissolved in anhydrous dimethyl sulfoxide (DMSO; 1 mmol/l) with 20% pluronic acid and then added to PSS for a final concentration of 2 μmol/l fura 2-AM and 0.05% pluronic acid. Pressurized arteries were incubated 45 min in the dark at room temperature in fura 2-AM solution receiving normoxic gas. After incubation, arteries were washed with 37°C PSS for 15 min to remove excess dye and allow complete deesterification of the compound. Fura 2-loaded vessels were alternately excited at 340 and 380 nm at a frequency of 10 Hz with an IonOptix Hyperswitch dual-excitation light source, and the respective 510-nm emissions were collected with a photomultiplier tube [ratio of fluorescence at 340 nm (F₃₄₀) to that at 380 nm (F₃₈₀)]. Background-subtracted F₃₄₀/F₃₈₀ emission ratios were calculated with Ion Wizard software (IonOptix) and recorded continuously throughout the experiment with simultaneous measurement of inner diameter from bright-field images as described previously (28).

Constrictor studies—After determining baseline internal diameter and F₃₄₀/F₃₈₀, sMA were pretreated for 10 min with one of the following in the superfusion media; the nonselective PKC inhibitor GF-109203x (3 μM; Sigma), the cPKC inhibitor Gö-6976 (1 μM; Calbiochem), the nPKC inhibitor rottlerin (3 μM; Calbiochem), the PLC inhibitor U-73122 (1 μM; Calbiochem), or vehicle (DMSO, 2.9 mM; Sigma). Effective concentrations of inhibitors were determined from concentration-response curves with each inhibitor on sMA constricted 50% with either ET-1 or phorbol dibutyrate (3 μM). Concentrations used in studies were maximally effective at reversing constriction. After inhibitor incubation, arteries were exposed to increasing concentrations of ET-1 (10⁻¹⁰ to 10⁻⁸ mol/l) in the superfusion media. Arteries were exposed to each concentration of agonist for 5 min in the continued presence of the inhibitors, and each artery was used for only one concentration-response curve. After ET-1 exposure, sMA were superfused with Ca²⁺-free PSS to cause complete dilation and demonstrate that the constriction was caused by active tone. Only sMA demonstrating 100% or greater dilation were used. Constrictions are expressed as percent baseline diameter. Vessel wall [Ca²⁺] is expressed as the F₃₄₀/F₃₈₀ ratio because the ratio is linearly related to the true molar [Ca²⁺] when the dissociation constant of fura 2 does not differ between treatment groups (11).

Permeabilized studies—sMA were prepared as described above. After fura 2 loading, arteries were superfused with Ca^{2+} -free PSS to achieve complete dilation. Once completely dilated, 3 μM ionomycin (Calbiochem) was added to the Ca^{2+} -free superfusate to permeabilize sMA to Ca^{2+} as described previously (2). After permeabilization, arteries were equilibrated in PSS with a calculated free $[\text{Ca}^{2+}]$ of 300 nM to hold intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) constant at approximate basal levels throughout the study. This maintained diameter and fura 2 ratios near baseline [diameters: sham $98.1 \pm 3.3\%$, E-IH $100.6 \pm 2.4\%$; ratios: sham (basal vs. ionomycin + Ca^{2+}) 1.18 ± 0.05 vs. 1.02 ± 0.06 F_{340}/F_{380} , E-IH 1.08 ± 0.08 vs. 0.86 ± 0.04 F_{340}/F_{380}].

Aortic Ring Contraction Studies

Isolation, denuding, and contractile studies—Thoracic aortas were collected from E-IH and sham rats and placed in chilled PSS. Arteries were cleaned of blood, connective tissue, and surrounding fat and cut into 3-mm segments. Endothelial cells were removed by gently rubbing the lumen with fine-tipped forceps. Aortic rings were suspended between two stainless steel hooks in a water-jacketed tissue bath containing PSS maintained at 37°C and bubbled with 21% O_2 -6% CO_2 -73% N_2 . The hooks were attached on one side to a rigid Plexiglas support and on the other side to a force transducer (Grass FT03) connected to an amplifier (Gould) that fed into a data acquisition program (version 3.01; Dataq). Rings were stretched with 2,500 mg of passive tension and equilibrated for 60 min. After equilibration, viability was confirmed by contraction to PE (10^{-6} mol/l), and endothelial integrity was assessed by relaxation to ACh (10^{-6} mol/l) in rings contracted with PE. Segments generating >1 g tension and relaxing $<5\%$ were used. Tissues were washed repeatedly over 60 min to remove PE and ACh and then exposed to increasing concentrations of ET-1 (10^{-10} to 10^{-7} mol/l) in the presence or absence of the inhibitors GF-109203X (3 $\mu\text{mol/l}$), Gö-6976 (1 $\mu\text{mol/l}$) or rottlerin (3 $\mu\text{mol/l}$). Each ring was used to generate only one concentration-response curve. Contractions are expressed as the percentage of PE (10^{-6} M) contraction (7).

Western analysis—To determine whether E-IH alters ET-1 activation of PKC in mesenteric arteries, PKC δ translocation and levels of phosphorylated PKC δ (P-PKC δ) were evaluated as described previously (1). Briefly, mesenteric artery cascades (2nd to 5th order inclusive) from sham and E-IH rats were cleaned of connective and adipose tissue, incubated with either vehicle or ET-1 (10^{-8} mol/l) for 30 min at 37°C , frozen in liquid nitrogen, and homogenized on ice in Tris-HCl buffer (pH 7.4) containing protease inhibitors. Homogenates were centrifuged at 1,500 g at 4°C for 10 min to remove insoluble material. For the translocation studies, homogenates were then separated into cytosolic and particulate fractions by high-speed centrifugation (100,000 g at 4°C for 60 min). The cytosolic fraction was removed, and the particulate fraction was reconstituted in 100 μl of Laemmli buffer. For the phosphorylation study, total tissue homogenates were solubilized in Laemmli buffer. Proteins were separated in 4–20% gradient polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. After being blocked, membranes were incubated 2 h at 25°C and then overnight at 4°C with a mouse monoclonal antibody specific for rat total PKC δ (1:1,000, translocation study; Santa Cruz) or for P-PKC δ (1:500; Santa Cruz). After being washed, blots were incubated with peroxidase-labeled goat anti-mouse IgG (1:5,000; Bio-Rad), followed by chemiluminescence labeling (Amersham). Band densities (SigmaGel; SPSS) were normalized to the total protein loaded per lane as determined by Coomassie blue staining of the membranes.

Statistical analysis—Constriction and $[\text{Ca}^{2+}]$ -response curves were analyzed using two-way repeated-measures ANOVA with Student-Newman Keul's post hoc analysis for differences between groups and treatments. Percent data were transformed to the square root

of the arcsin before analysis to approximate a normal distribution (SigmaStat Software; SPSS). $P < 0.05$ was considered statistically significant for all analyses.

RESULTS

Table 1 shows that, in rats, sham treatment does not affect SBP (mmHg), but SBP is significantly increased after 14 days of E-IH exposure. Additionally, body weight (g) is significantly increased in both sham and E-IH rats. However, E-IH rats gained less weight than sham rats.

ET-1-mediated Constriction in sMA

ET-1-mediated constriction was greater in sMA from vehicle-treated E-IH rats than in sMA from vehicle-treated sham rats and was not accompanied by an increase in vessel wall $[Ca^{2+}]_i$, which is consistent with our previous observations (1). Therefore, DMSO does not appear to affect ET-1-mediated constriction or vessel wall $[Ca^{2+}]_i$ (Fig. 1 and Table 2).

Effects of Nonselective PKC Inhibitor on ET-1-mediated sMA Constriction

The PKC inhibitor GF-109203x (3 μ M) reduced ET-1 constriction in endothelium-disrupted sMA from E-IH rats (Fig. 2B). GF-109203x-treatment increased the calculated EC_{50} (effective concentration to constrict to 50% of the maximum) and decreased the calculated maximal response (E_{max} , Table 2). GF-109203x had no significant effect on ET-1-dependent constriction in sham sMA (Fig. 2A). Because GF-109203x (3 μ M) inhibits both classical Ca^{2+} -dependent (cPKC) and novel Ca^{2+} -independent (nPKC) isoforms of PKC, specific cPKC and nPKC inhibitors were also used to determine the contribution of different PKC isoforms to ET-1-mediated vasoconstriction.

Effects of Selective PKC Inhibition on ET-1-mediated sMA Constriction

Gö-6976 is a potent inhibitor of cPKC isoforms α , $\beta 1$, $\beta 2$, and μ with an inhibitory constant of 0.1 μ M (12). Gö-6976 (1 μ M) did not affect ET-1-mediated constriction in either group of sMA (Fig. 2, C and D, and Table 2). To verify that the concentration used was effective, we administered increasing concentrations of Gö-6976 to reverse phorbol 12,13-dibutyrate (PDBu, 3 μ M)-mediated constriction. PDBu-mediated constriction in endothelium-disabled mesenteric arteries was completely reversed with 1.0 μ M Gö-6976 (data not shown). Therefore, cPKC isoforms do not appear to contribute to ET-1-mediated vasoconstriction in sMA.

Rottlerin (3 μ M) greatly inhibited ET-1-mediated constriction in E-IH sMA (Fig. 3B) similar to the effect of the nonselective PKC inhibitor (Fig. 2B). This was seen as an increase in EC_{50} and a decrease in E_{max} (Table 2). At this concentration, rottlerin specifically inhibits PKC δ and only affects other PKC isoforms at 10-fold greater concentrations (13). This suggests the effects of GF-109023x (Fig. 2B) were primarily through PKC δ inhibition. In contrast, rottlerin had no effect on ET-1-mediated constriction in sham sMA (Fig. 3A), suggesting that ET-1 does not normally signal through PKC δ to constrict sMA.

Permeabilized arteries were used as another approach to determine if PKC inhibition prevented constriction in the E-IH arteries independent of decreases in $[Ca^{2+}]_i$. In Ca^{2+} -permeabilized arteries, where changes in Ca^{2+} levels were not observed, ET-1-induced constriction occurred without alterations in vessel wall F_{340}/F_{380} (data not shown). In this study, sMA were permeabilized with ionomycin (3 μ M) and maintained in buffer with a calculated free $[Ca^{2+}]$ of 300 nM. Again, rottlerin (3 μ M) profoundly decreased ET-1-mediated constriction in E-IH but not sham sMA (Fig. 3, C and D). This confirms that, in E-IH arteries, ET-1-stimulated constriction requires PKC δ and suggests that activation of this

nPKC isoform mediates augmented ET-1-mediated vasoconstriction independent of increases in $[Ca^{2+}]_i$.

Effects of PLC Inhibition on ET-1-mediated sMA Constriction

Both cPKC and nPKC require DAG for activation. Therefore, we investigated the contribution of the DAG-liberating enzyme PLC to ET-1-mediated constriction. U-73122 (1 μ M), a phosphatidylinositol-PLC inhibitor, significantly reduced ET-1-mediated constriction in E-IH sMA (Fig. 4B) but not in sham arteries (Fig. 4A). This suggests that ET-1 activates PLC upstream of PKC activation in the E-IH sMA.

PKC δ Western Blots

Western blots revealed that PKC δ is present in both E-IH and sham mesenteric arteries (Fig. 5A) and that a fraction of it is phosphorylated (Fig. 5B). Although ET-1 increased the amount of P-PKC δ in E-IH arteries but not in sham arteries, it did not cause translocation of PKC δ in any of the sMA (Fig. 5A). This suggests that ET-1 activates PKC δ in sMA from E-IH rats but not in those from sham rats.

Effects of PKC Inhibition on ET-1-mediated Contraction in Thoracic Aorta

PKC inhibition with GF-109203x (3 μ mol/l) diminished contraction in arteries from both sham (Fig. 6A) and E-IH (Fig. 6B) rats, but the inhibition appeared to be greater in the E-IH arteries (Table 2).

Effects of cPKC and nPKC Inhibition on ET-1-mediated Contraction in Thoracic Aorta

In contrast to the observations in sMA, rottlerin (3 μ M) did not affect ET-1-induced contraction in aortic rings from either sham (Fig. 6C) or E-IH (Fig. 6D) rats. However, cPKC inhibition with Gö-6976 (1 μ M) significantly inhibited contraction in aorta from both sham (Fig. 6C) and E-IH (Fig. 6D) rats.

DISCUSSION

ET-1 is a major regulator of vascular smooth muscle tone, and recent data from our laboratory and others suggest that augmented ET-1 vasoconstriction contributes to sleep apnea-induced hypertension (16,30,31). ET-1 initiates vasoconstriction through increases in both $[Ca^{2+}]_i$ and Ca^{2+} sensitization (1). Thus the purpose of this study was to determine if one or more PKC isoforms are responsible for augmented ET-1 activation of Ca^{2+} sensitization in arteries from E-IH rats compared with arteries from sham rats. The novel findings of this study are: 1) PKC activation contributes to ET-1 constriction in sMA from E-IH but not sham rats. Conversely, PKC participates in contraction of aorta in both groups. 2) Only the nPKC isoform, PKC δ , appears to be activated by and contribute to ET-1 vasoconstriction in E-IH sMA while only cPKC participates in contraction of aorta. 3) Inhibition of the PKC upstream activator PLC attenuates ET-1 vasoconstriction in E-IH but not in sham sMA. 4) When $[Ca^{2+}]_i$ is held constant, PKC δ mediates constriction in E-IH sMA, but some other mechanism leads to Ca^{2+} sensitization-mediated constriction in sham sMA.

ET-1, like many other G protein-coupled receptor agonists, causes Ca^{2+} -independent contraction of vascular smooth muscle cells by activating Ca^{2+} -sensitizing pathways (20). PKC can increase Ca^{2+} sensitivity in smooth muscle by phosphorylating CPI-17, which inhibits MLCP thus maintaining MLC₂₀ in a phosphorylated state (38). ET-1 can activate both Ca^{2+} -dependent PKC isoforms (cPKC) and Ca^{2+} -independent PKC isoforms (nPKC) in coronary smooth muscle cells (27), and PKC inhibition significantly reduced ET-1-stimulated constriction in the superior mesenteric artery of portal-hypertensive rats (40).

Thus ET-1 can cause Ca^{2+} sensitization and constriction through the activation of PKC. Our previous studies showed that, in sMA from E-IH rats, the ET-1 constrictor responses were augmented without an apparent increase in vessel wall $[\text{Ca}^{2+}]_i$ and mostly independent of ROK activation. These data suggested that ET-1 increases Ca^{2+} -sensitivity in E-IH arteries more than in sham arteries (1). The current studies demonstrate that this augmented Ca^{2+} sensitization is at least in part through activation of PKC, a pathway that does not appear to contribute to ET-1 constriction in sham sMA. Therefore, E-IH exposure appears to enable ET-1 activation of PKC δ -dependent constriction in resistance-size arteries via a pathway that is completely inactive in the sham arteries. Interestingly, the same inhibitor had no effect in aorta from either sham or E-IH rats.

In contrast, the cPKC inhibitor Gö-6976, which potently inactivates PKC α and PKC β 1 isoforms (26), did not reduce ET-1-mediated constriction in either E-IH or sham sMA but was the most effective inhibitor of ET-1 contraction in aortic rings. This supports our hypothesis that cPKC isoforms are not involved in ET-1 constriction in mesenteric arteries and demonstrates that ET-1-dependent vasoconstriction is mediated by very different signaling pathways in these two arteries. Additionally, this suggests that the inhibition of ET-1-mediated constriction in sMA by GF-109203x is through its effects on one or more nPKC isoforms. Other studies have shown that cPKC inhibition with Gö-6976 selectively reduces norepinephrine-mediated contraction of thoracic aorta from deoxycorticosterone acetate-salt hypertensive rats (21) and UK-14304 contraction in aorta from rats made hypertensive with a nitric oxide synthase inhibitor (7). Therefore, increased activity of cPKC isoforms appears to contribute to elevated constrictor responses in large arteries in many models of hypertension. However, these PKC isoforms do not appear to be a component of ET-1 signaling in sMA nor to contribute to the E-IH-induced augmented Ca^{2+} sensitization in this vascular bed.

In contrast to the results with Gö-6976, the PKC δ inhibitor rottlerin significantly reduced ET-1-mediated constriction in E-IH (Fig. 4B) but not in sham sMA (Fig. 4A), similar to the effects of GF-109203x (Fig. 2). This same concentration of rottlerin, which is quite selective for PKC δ (13), had no effect on ET-1 contraction of thoracic aorta from either group, suggesting E-IH exposure bed specifically facilitates ET-1 activation of PKC δ -dependent vasoconstriction. This observation was confirmed using ionomycin-permeabilized sMA to evaluate Ca^{2+} sensitization when $[\text{Ca}^{2+}]_i$ was held constant. In these studies, rottlerin almost completely inhibited ET-1-mediated constriction in E-IH sMA (Fig. 5B), supporting the hypothesis that PKC δ mediates ET-1 vasoconstriction in small arteries only after exposure to simulated sleep apnea. We have previously shown that E-IH exposure results in an increase in oxidative stress (39), and others have shown that intermittent hypoxia-induced oxidative stress can activation PKC δ in cardiomyocytes (19). Thus elevated oxidative stress may underlie the observed PKC δ upregulation.

Activation of PKC requires binding of DAG to facilitate translocation from the cytosol to the particulate fraction (32). However, the duration of the translocation appears to be isoform, stimulus, and tissue specific (25). In ventricular myocytes, PKC δ and PKC ϵ are both translocated to the particulate fraction for a prolonged period by hypoxia in cultured cells (8,29) and by intermittent hypoxia in vivo (19). However, PKC δ was equally distributed between the particulate and cytosolic fractions under resting conditions for both sham and E-IH arteries, and neither E-IH nor ET-1 appeared to stimulate translocation (Fig. 5). This is similar to a previous report from Sirous and coworkers (36) that in cultured coronary artery smooth muscle cells most PKC δ is in the particulate fraction under basal conditions and ET-1 causes little translocation. Our constrictor studies, however, strongly indicate that ET-1 signals through PKC δ in sMA from E-IH-exposed rats. The apparent lack of ET-1-mediated PKC δ translocation may thus be because of high basal levels in the

membrane or very rapid, reversible translocation as has previously been shown in cultured cardiac myocytes. Because PKC δ remains activated after it returns to the soluble fraction as long as it is phosphorylated (25), the ET-1-stimulated increase in Ser⁶⁴³-P-PKC δ in the E-IH sMA suggests that this is also the pattern in mesenteric arteries and supports the conclusion that ET-1-dependent contraction is dependent on PKC δ -mediated Ca²⁺ sensitization. Future studies to find the target(s) of the activated PKC will determine if this is through the well-described phosphorylation of CPI-17 (14,18) or some other target.

Although we did not investigate targets downstream of PKC, we did determine that the inhibition of PLC greatly diminished ET-1-mediated constriction in E-IH but not sham sMA. These data suggest that E-IH augmentation of ET-1 activation of PKC is in part dependent on increased PLC activation. Because PLC inhibition did not reduce ET-1-mediated constriction as effectively as PKC inhibition, PKC may be activated in part through other pathways such as phosphatidylcholine-PLC or PLD (9,24,35).

Overall, this study has established that E-IH exposure increases ET-1 activation of Ca²⁺ sensitization to augment constriction in isolated endothelium-disabled sMA. It also demonstrates that ET-1-induced vasoconstriction is PKC dependent in both mesenteric arteries and thoracic aorta following E-IH but the PKC δ pathway is not activated in sMA from sham rats. This suggests that exposure to E-IH uniquely couples ET-1 receptors with PLC and PKC δ to augment sMA constriction. Furthermore, two different isoforms of PKC appear to mediate ET-1-dependent contraction in aorta and sMA. Therefore, these data suggest that ET-1 signaling and PKC activation are vascular targets of E-IH and these two pathways are potential sites of intervention for controlling hypertension and vascular pathologies in sleep apnea and other states with intermittent exposures to hypoxia.

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Fig. 1.

Eucapnic intermittent hypoxia (E-IH) augments endothelin (ET)-1-mediated constriction in endothelium-disrupted mesenteric arteries. Simultaneous measurements of concentration-dependent changes in inner diameter (constriction) and vessel wall Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) to ET-1 in pressurized, endothelium-disabled mesenteric arteries from sham and E-IH rats. Constrictions are expressed as % vasoconstriction with 100% equivalent to a closed lumen, and vessel wall intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) is expressed as a ratio of fura 2 fluorescence [ratio of fluorescence at 340 nm (F_{340}) to that at 380 nm (F_{380})]. $P < 0.05$, significant difference from sham (*).

Fig. 2.

Protein kinase C (PKC) inhibition reduces ET-1-stimulated constriction of E-IH mesenteric arteries. Concentration-dependent constriction to ET-1 in sham (A and C) and E-IH (B and D) endothelium-disabled mesenteric arteries in the presence of vehicle (○) or PKC inhibitors (●). The nonselective PKC inhibitor GF-103209x reduced constriction in E-IH arteries (B) but not in sham arteries (A). The classical PKC (cPKC) selective inhibitor Gö-6976 (1 μ mol/l) did not affect constriction in either group (C and D). *Significant difference from vehicle for $P < 0.05$.

Fig. 3.

PKC δ inhibition reduces ET-1-stimulated constriction of E-IH mesenteric arteries. Concentration-dependent constriction to ET-1 in endothelium-disabled mesenteric arteries from sham (A and C) and E-IH (B and D) rats in the presence of vehicle ($\circ$) or rottlerin (3 μM , $\bullet$). Arteries on *bottom* were permeabilized to Ca^{2+} with ionomycin (1 $\mu\text{mol/l}$). *Significant difference from vehicle for $P < 0.05$.

Fig. 4. Phospholipase C (PLC) inhibition reduces ET-1-initiated constriction in E-IH arteries. Concentration-dependent constriction to ET-1 in sham and E-IH endothelium-disabled mesenteric arteries in the presence of vehicle (○) or U73122 (1 μ M, ●). *Significant difference from vehicle for $P < 0.05$.

Fig. 5.

ET-1 stimulation phosphorylates but does not translocate PKC δ to particulate fraction in mesenteric arteries. PKC δ protein expression from mesenteric arteries stimulated with vehicle (white bar) or ET-1 (10^{-8} mol/l for 30 min at 37°C; black bar). **A**: arbitrary densitometry units are reported as (particulate fraction)/(cytosolic fraction); $n = 4$. **B**: whole tissue homogenates of mesenteric arteries stimulated as above with vehicle ($n = 4$) or ET-1 ($n = 5$) were probed with an antibody to phospho-PKC (P-Ser⁶⁴³) and demonstrate a significant increase in phosphorylation following ET-1 stimulation of mesenteric arteries from E-IH but not sham rats. *Significant difference from vehicle, and #significant difference from sham.

Fig. 6.

PKC inhibition reduces ET-1-stimulated contraction of sham and E-IH aortic segments. Concentration-dependent contraction to ET-1 in sham (A and C) and E-IH (B and D) endothelium-denuded aortic segments (3-mm rings) in the presence of vehicle (○) or PKC inhibitors (●). PKC δ inhibition (■) does not alter ET-1-stimulated contraction in either group, but cPKC-selective inhibition reduces ET-1-induced contraction of aortic segments from both sham and E-IH rats (gray squares). *Significant difference from vehicle for $P < 0.05$.

Table 1

Baseline SBP and body weight for E-IH and sham rats

Group	SBP, mmHg	Body Wt, g
Sham		
<i>Day 1</i>	138.13 ± 0.98	271.25 ± 2.42
<i>Day 14</i>	138.75 ± 0.87	323.75 ± 1.96 ^{*†}
E-IH		
<i>Day 1</i>	139.38 ± 0.67	267.50 ± 3.35
<i>Day 14</i>	161.25 ± 2.20 ^{*†}	292.50 ± 4.84 ^{*†}

Values are means ± SE. SBP, systolic blood pressure; E-IH, eucapnic intermittent hypoxia.

^{*} Significant difference from sham for $P < 0.05$.

[†] Difference from *day 1* within group.

Table 2EC₅₀ and E_{max} for endothelin-1 constriction of mesenteric arteries and contraction of aortic rings

Treatment	Sham		E-IH	
	EC ₅₀ , nmol/l	E _{max} , %max	EC ₅₀ , nmol/l	E _{max} , %max
Small mesenteric arteries				
Vehicle	2.03 ± 0.07	89.5 ± 8	1.56 ± 0.08*	100 ± 6
GF-109203x	1.94 ± 0.07	75.6 ± 7	3.57 ± 0.16*	59.9 ± 8* [†]
Gö-6976	2.84 ± 0.16	97.4 ± 8	1.59 ± 0.11*	100 ± 9
Rottlerin	1.95 ± 0.06	87.4 ± 7	5.73 ± 0.22* [†]	37.9 ± 8* [†]
Treatment	Sham		E-IH	
	EC ₅₀ , -log M	E _{max} , mg	EC ₅₀ , -log M	E _{max} , mg
Aorta				
Vehicle	-8.8 ± 0.2	2,003 ± 177	-8.2 ± 0.2	1,717 ± 89
GF-109203x	-10.2 ± 1.2	1,135 ± 41 [†]	-8.5 ± 1.4 [†]	1,060 ± 56 [†]
Gö-6976	-7.7 ± 0.3		-7.7 ± 0.1	
Rottlerin	-8.2 ± 0.2	1,475 ± 280	-8.0 ± 0.3	1,867 ± 197

Values are means ± SE. Units for E_{max} for aorta are mg.

* Different from sham.

[†] Different from vehicle for *P* < 0.05.