British Journal of Pharmacology (2005) 145, 5–14
www.nature.com/bjp

ClC-3 chloride channel is upregulated by hypertrophy and inflammation in rat and canine pulmonary artery

[1,2]Yan-Ping Dai, [1,2]Shaner Bongalon, [1,2]William J. Hatton, [1,2]Joseph R. Hume & *[,1,2]Ilia A. Yamboliev

[1]Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557-0270, U.S.A. and
[2]Center of Biomedical Research Excellence, University of Nevada School of Medicine, Reno, NV 89557-0270, U.S.A.

1 Cl^- channels have been implicated in essential cellular functions including volume regulation, progression of cell cycle, cell proliferation and contraction, but the physiological functions of the ClC-3 channel are controversial. We tested the hypothesis that the ClC-3 gene (*ClCn-3*) is upregulated in hypertensive pulmonary arteries of monocrotaline-treated rats, and upregulated ClC-3 channel aids viability of pulmonary artery smooth muscle cells (PASMCs).

2 Experimental pulmonary hypertension was induced in rats by a single subcutaneous administration of monocrotaline (60 mg kg^{-1}). Injected animals developed characteristic features of pulmonary hypertension including medial hypertrophy of pulmonary arteries and right ventricular hypertrophy.

3 Reverse transcriptase–polymerase chain reaction (RT–PCR), immunohistochemistry and Western immunoblot analysis indicated that histopathological alterations were associated with upregulation of the ClC-3 mRNA and protein expression in both smooth muscle cells of hypertensive pulmonary arteries and in cardiac myocytes.

4 RT–PCR analysis of mRNA, extracted from canine cultured PASMCs, indicated that incubation with the inflammatory mediators endothelin-1 (ET-1), platelet-derived growth factor (PDGF), interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNFα), but not transforming growth factor beta (TGFβ), upregulated ClC-3 mRNA.

5 Adenovirus-mediated delivery and overexpression of ClC-3 in canine PASMCs improved cell viability against increasing concentrations of hydrogen peroxide (H_2O_2, range 50–250 μM).

6 In conclusion, upregulation of ClC-3 in rat hypertensive lung and heart is a novel observation. Our functional data suggest that upregulation of ClC-3 is an adaptive response of inflamed pulmonary artery, which enhances the viability of PASMCs against reactive oxygen species.
British Journal of Pharmacology (2005) **145,** 5–14. doi:10.1038/sj.bjp.0706135
Published online 21 February 2005

Keywords: Monocrotaline; rat pulmonary hypertension; inflammatory cytokines; hydrogen peroxide; cell viability; gene expression

Abbreviations: cDNA, complementary deoxyribonucleic acid; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ET-1, endothelin-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; IL-1β, interleukin-1beta; LV, left ventricle; NCS, newborn calf serum; PASMCs, pulmonary artery smooth muscle cells; PDGF, platelet-derived growth factor; RNA, ribonucleic acid; RT–PCR, reverse transcriptase–polymerase chain reaction; RV, right ventricle; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis; SMC, smooth muscle cell; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor alpha

Introduction

Diffusion of Cl^- across biological membranes is required for various essential physiological functions that include excitability of muscle cells and neurons, transmembrane movement of water and salts and regulation of cell volume (George *et al*., 2001). Cl^- channels have been implicated in cell proliferation, differentiation, migration and angiogenesis (Nilius *et al*., 1997). For example, the magnitude of Cl^- current in actively growing smooth muscle cells (SMCs) is higher than in growth-arrested or differentiated SMCs, suggesting that volume-sensitive Cl^- channels may be important for SMC proliferation (Voets *et al*., 1997). Recent evidence also suggests that Cl^- flux may regulate the cell cycle (Shen *et al*., 2000; Nilius, 2001; Furukawa *et al*., 2002; Rutledge *et al*., 2002). Regulation of the cell volume is another important homeostatic function of chloride channels, although the identity of the volume-sensitive Cl^- channels has yet to be conclusively identified. The ClC-3 channel, a member of the voltage-dependent ClC Cl^- channel family (Jentsch *et al*., 2002), has been proposed as a candidate protein (Duan *et al*., 1997). It has been demonstrated that ClC-3 is expressed in human aortic SMCs (Lamb *et al*., 1999) and pulmonary artery SMCs (PASMCs) (Yamazaki *et al*., 1998) and exhibits biophysical characteristics of a volume regulator; nonetheless, the role of ClC-3 as a

*Author for correspondence at: Department of Pharmacology, MS 318, University of Nevada School of Medicine, Reno, NV 89557-0270, U.S.A.; E-mail: yambo@med.unr.edu
Published online 21 February 2005

volume-regulated Cl^- channel remains controversial (Greenwood, 2004). More recently, it was demonstrated that antisense oligonucleotide-mediated downregulation of ClC-3 inhibits dramatically cell proliferation of rat aortic SMCs (Wang *et al*., 2002). These studies suggest that activation of ClC-3 channels may indeed play a role in proliferation, growth and volume regulation of vascular SMCs (VSMCs).

The majority of SMCs in healthy pulmonary arteries have a differentiated, contractile phenotype; proliferation and growth are not normal physiological functions of SMCs in the healthy pulmonary vasculature. However, pathophysiological conditions, including pulmonary hypertension and atherosclerosis, are associated with inflammation and increased local concentrations of vasoconstrictors, mitogens and inflammatory cytokines, which initiate phenotypic changes of VSMCs (Fujitani *et al*., 1995; Jeffery & Morrell, 2002; Runo & Loyd, 2003). Transition from contractile to proliferative phenotype is associated with changes of the gene expression profile and enhanced proliferation and growth of PASMCs. Since previous studies have suggested a role of ClC-3 channels in cell proliferation (Wang *et al*., 2002), we undertook this study to test the hypothesis that extensive proliferation and growth of PASMCs in inflamed pulmonary arteries is associated with upregulation of the *ClCn-3* gene. Our results indicate that indeed the *ClCn-3* gene is upregulated in rat pulmonary artery and heart in response to monocrotaline-induced pulmonary hypertension, and in canine cultured PASMCs incubated with inflammatory mediators. PASMCs infected to overexpress ClC-3 exhibited enhanced viability against hydrogen peroxide (H_2O_2), thus suggesting that ClC-3 may improve the resistance of VSMCs to reactive oxygen species in an environment of elevated inflammatory cytokine in hypertensive pulmonary arteries.

Methods

Monocrotaline model of rat pulmonary hypertension

Monocrotaline is a pyrrolizidine alkaloid and phytotoxin used to cause experimental pulmonary vascular syndrome in rats (Oho *et al*., 1995; Rabinovitch, 2001). Monocrotaline was dissolved in small volume of 1 N HCl, diluted with PBS and neutralized with 0.1 N NaOH (Chen & Lai, 2003). A total of 30 adult (8 weeks old) Sprague–Dawley rats were split into two groups: 15 animals received monocrotaline (60 mg kg^{-1} subcutaneously), and the others – the same volume of physiological saline. The use of rats was approved by the University of Nevada's Animal Care and Use Committee. Rats from each group were killed by an intraperitoneal overdose of sodium pentobarbital (300 mg kg^{-1}) at 7, 14 and 21 days after the monocrotaline injection, in conformity with the Panel on Euthanasia of the American Veterinary Medical Association. The heart and lung were excised *en bloc* and rinsed with PBS. The right ventricle (RV) was separated from the left ventricle with the septum (LV + S), and both fractions of the heart were weighed and stored frozen until used for extraction of ribonucleic acid (RNA) and total protein for analysis of ClC-3 mRNA and protein expression.

PASMC dispersion, culture and treatment protocol

Lobes of rat lungs were used to dissect main branches of pulmonary arteries. Blood vessels were cleaned of connective tissues, cut open to scrape endothelial cells with cotton swabs, and incubated with collagenase type II to disperse SMCs as described for canine pulmonary artery below. Acutely dispersed rat PASMCs were used for extraction of mRNA and assay of gene expression.

Mongrel dogs of either sex (averaging 15 kg) were obtained from vendors licensed by the United States Department of Agriculture. The University of Nevada's Animal Care and Use Committee approved the use of dogs for experimental studies. Animals were euthanized by barbiturate overdose, lung was dissected and used to isolate second pulmonary artery branches. Blood vessels were cleaned from connective tissue and placed in Ca^{2+}-free Hank's solution, containing 125 mM NaCl, 5.36 mM KCl, 15.5 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 10 mM glucose, 2.9 mM sucrose, 10 mM HEPES, pH 7.4, at 37°C. Arteries were sliced longitudinally, the endothelium was removed by a cotton swab, the smooth muscle layer was minced and digested with 1 mg ml^{-1} type II collagenase, 0.1 mg ml^{-1} protease (P5147, Sigma, St Louis, MO, U.S.A.), 2 mg ml^{-1} bovine serum albumin (BSA), 2 mg ml^{-1} trypsin inhibitor and 0.3 mg ml^{-1} Na_2ATP at 37°C for 1.5 h. SMCs were recovered by three washes of the partially digested tissue with Ca^{2+}-free Hank's solution at 37°C. Dispersed cells were sedimented by centrifugation and resuspended in M199 cell culture medium supplemented with 10% newborn calf serum (NCS), 0.2 mM glutamine and antibiotics. SMCs were plated onto 75 cm^2 cell culture flasks coated with type I rat tail collagen, and grown to 80% confluence. These primary cells were trypsinized, passaged onto culture dishes and grown to 90–95% confluence in M199 supplemented with 6.25 μg ml^{-1} insulin, 6.25 μg ml^{-1} transferrin, 6.25 ng ml^{-1} selenious acid, 5.35 μg ml^{-1} linoleic acid (ITS +) and 10% NCS. Unless otherwise specified, confluent cells were growth-arrested (M199 supplemented with 0.1% NCS) for 36–48 h prior to treatment. ClC-3 mRNA and protein expression was studied in canine PASMCs incubated for 5 and 24 h with 100 nM ET-1, or 10 ng ml^{-1} platelet-derived growth factor (PDGF), tumor necrosis factor alpha (TNFα), interleukin-1beta (IL-1β) or transforming growth factor beta (TGFβ).

Assay of ClC-3 gene transcription by quantitative relative reverse transcriptase–polymerase chain reaction (RT–PCR)

After acute dispersion (rat PASMCs) or incubation with growth factors (canine PASMCs), cells were rinsed with PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.6 mM KCl and 137 mM NaCl, pH 7.4) and total RNA was extracted with Trizol reagent, following the manufacturer's instructions. RNA samples were dissolved in nuclease-free water and treated with 5 U DNase I for 10 min at 37°C and the reaction was stopped by addition of 25 mM ethylenediaminetetraacetic acid (EDTA) and a 15-min incubation at 65°C. RNA concentrations were quantitated by measuring absorbance at 260 nm. First strand complementary deoxyribonucleic acid (cDNA) was synthesized with the Superscript II RNAse H$^-$ Reverse Transcriptase kit (Gibco BRL) at 42°C from 1 μg total RNA using 250 ng random hexamers, 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.125 mM dATP, dithiotheritol (dTTP), dGTP and cCTP and 1 U SuperScript II RT. Following cDNA synthesis, 20 U of RNAse H was added

to remove RNA, complementary to the cDNA (Bongalon *et al*., 2004). Gene-specific primers for ClC-3 were designed from published sequence (Acc. AF172729) using Lasergene DNAStar software and the primers were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.). The forward primer, 5′-AAAGAATCAGCATGGGAAATGA CAAAA-3′ and the reverse primer, 5′-TCCTACCTCTTTC CAAAGTATAGCACA-3′ were designed to amplify RT–PCR products of 584 bp. Quantitative relative RT–PCR was performed with the Quantum RNA 18S internal standards, according to the manufacturer's protocol (Ambion, Inc., Austin, TX, U.S.A.). An optimized ratio of 18S rRNA primers were added to the reaction as an endogenous standard along with 18S competimers allowing modulation of the 18S amplification without affecting the performance of the gene-specific PCR targets in the reaction. RT–PCR amplification was carried out within the linear range of each individual cDNA preparation, which was determined by plotting the density of ethidium bromide-stained ClC-3 PCR products *versus* the number of cycles. RT–PCR amplification was carried out in a Gene Amp 2400 Thermal Cycler (Perkin–Elmer) under the following conditions: 94°C for 1 min; optimal annealing temperature of 58°C of the ClC-3 primer set for 30 s; and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Reaction mixtures contained 60 mM Tris–HCl (pH 8.5), 15 mM $(NH_4)SO_4$, 1.5 mM $MgCl_2$, 0.25 mM dATP, dCTP, dGTP and dTTP, 10% dimethylsulfoxide (DMSO), 50 μM of each primer, 5 μl template cDNA, and 2.5 U *Taq* polymerase. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. ClC-3 bands were quantitated by densitometry and normalized by the densities of the respective 18S rRNA bands, amplified in parallel from each sample. Nucleotide sequencing was performed by the Nevada Genomics Center.

Histological analysis

Rats were euthanized as described above. Both right and left lobes of the lung were removed and fixed with 4% paraformaldehyde in PBS for 1 h, washed 3×15 min with PBS and cryoprotected in increasing gradients of sucrose in PBS (5, 10 and 15%) for 30 min each, and in 20% sucrose overnight. The fixed sections of lung tissue were then rapidly frozen by immersion in liquid N_2, and embedded in a 1 : 1 solution containing OCT compound (Miles Inc., Elkhart, IN, U.S.A.) and 20% sucrose in PBS. Frozen sections were cut at 8 μm on a cryostat (Leica CM 3050) and stained with Masson trichrome (Sigma, MO, U.S.A.) as per the manufacturer's instructions. Slides were then washed 3×15 min with PBS and mounted on vectabond-coated microscope slides. Sections were viewed and photomicrographed with a Nikon Eclipse E600 microscope equipped with a Spot RT Slider digital camera.

Immunofluorescence analysis

Immunofluorescence analysis of ClC-3 expression was carried out on frozen sections, prepared as described above. Sections were washed 3×10 min in PBS and blocked in 1% BSA/PBS for 1 h at room temperature. Excess blocking solution was removed and sections were incubated with a primary rabbit polyclonal ClC-3 antibody (dilution 1 : 100, for 24 h at 4°C). The primary antibody was omitted for staining of control sections. All sections were then labeled with a secondary anti-rabbit antibody conjugated to fluorescent marker Alexa Fluor 488 (Molecular Probes, OR, U.S.A.) for 30 min at room temperature. Sections were then washed 3×15 min in PBS, mounted on vectabond-coated microscope slides and examined with a BioRad Radiance 2100 confocal microscope. Confocal micrographs were obtained and *Z*-series were constructed with Bio-Rad Lasersharp software. Final images were analyzed using Compix Simple PCI image analysis software.

Western immunoblot analysis for assay of ClC-3 protein expression

Control and treated PASMCs were rinsed twice with cold PBS, and lysed with a buffer composed of 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 100 μM AEBSF and 1 μM leupeptin. RVs and LVs of control and monocrotaline-treated rat hearts were glass–glass homogenized with the same buffer. Cell and tissue homogenates were centrifuged at $1000 \times g$ for 3 min at 4°C to remove cell nuclei and other debris. Supernatants were transferred into clean tubes and centrifuged at $22{,}000 \times g$ for 30 min at 4°C. Pellets, containing enriched cellular membranes, were resuspended in Ripa extraction buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM Na_2EDTA, 0.5% (v v^{-1}) NP40, 0.5% (v v^{-1}) Triton X-100, 1 mM NaF, 1 μM leupeptin and 1 μM AEBSF) and incubated on ice for 1 h to solubilize membrane protein. The total protein concentrations were assayed by the Micro BCA Protein Assay (Pierce, Rockford, IL, U.S.A.). Equal amounts of total protein (usually 80 μg) were resolved by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes for 1.5 h at 24 V, 4°C (Genie blotter, Idea Scientific Company, Minneapolis, MN, U.S.A.). The membranes were blocked for 1 h with LI-COR Blocking solution (LI-COR, Inc., Lincoln, NE, U.S.A.) and were probed with a rabbit polyclonal ClC-3 antibody, diluted in LI-COR blocking buffer (Wang *et al*., 2003). The membranes were simultaneously probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – a noninducible gene, used for translation control (goat, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), diluted 200 times in LI-COR buffer. Incubation with primary antibodies took place for 2 h at room temperature, or overnight at 4°C. Excess primary antibody was removed by 2×5 min washes with TNT buffer (100 mM Tris, pH 7.5, 0.1% Tween-20, 150 mM NaCl), and 2×5 min washes with PBS. Membranes were then simultaneously incubated with two secondary antibodies: one antibody coupled to an infrared fluorescence marker with emission wavelength of 800 nm (IR800, Rockland Immunochemicals, PA, U.S.A.), and the other antibody coupled to an infrared fluorescence marker with emission wavelength of 680 nm (Alexa Fluor 680, Molecular Probes, OR, U.S.A.). Both secondary antibodies were diluted 100,000 times with LI-COR buffer. Incubation was carried out for 45 min at room temperature. Excess secondary antibody was removed as with the primary antibodies. Immunoblots were scanned to obtain double-color fluorescent images with an Odyssey scanner (LI-COR, Inc., NE, U.S.A.). Immunoreactive band densities

of ClC-3 were normalized to GAPDH, and stimulus-dependent changes were expressed relative to nontreated controls.

Cell viability assay

Viability was assayed by incubation of PASMCs with the tetrazolium salt MTT and quantitation of the produced formazan by UV absorbance (Mosmann, 1983; Cao *et al*., 2003). Briefly, 1.5×10^5 canine PASMCs were seeded and allowed to plate in 12-well plates overnight. On the next day, cells were infected with an adenovirus construct carrying the guinea pig ClC-3 gene (Acc. U83464). For production of the adenovirus vector, the ClC-3 gene, cloned into the mammalian expression vector pcDNA3.1-V5-His-Topo (Invitrogen, CA, U.S.A.), was released and subcloned into the *Hind*III and *Xba*I sites of the pAdTrack shuttle vector. The latter was ligated into pAdEasy backbone by homologous recombination, following published protocols (Graham & Prevec, 1995; He *et al*., 1998). Production of viral stock took place in HEK 293-AD cells (Stratagene, CA, U.S.A.) and the ClC-3 transgenes were delivered into PASMCs as described elsewhere (He *et al*., 1998). The shuttle vector carries a green fluorescence protein (GFP) gene, whose expression indicates successful infection of target cells. Control cells were infected with an empty adenovirus construct. On an average, about 90% of the cells developed green fluorescence within 3–4 days of infection, without changes in cell morphology. Control and ClC-3-infected cells were then incubated for 24 h with increasing concentrations of H_2O_2 in the range 50–250 μM, prior to incubation with MTT reagent (1 mg ml^{-1}, for 2 h at 37°C). Cells were rinsed twice with PBS and formazan crystals were solubilized with a mixture of DMSO, isopropanol and distilled water in a volume ratio of 1 : 4 : 5. Absorption was read at 595 nm on an automated microplate reader Bio-Tek EL800, using 96-well plate format. Infected cells were lysed for assay of overexpression of the ClC-3 transgene and GAPDH by Western immunoblot analysis. For quantitation of protein overexpression, the ClC-3 immunoreactive bands were normalized to GAPDH and then to controls, infected with empty pAdEasy vector.

Statistical methods

Results are presented as the mean ± s.e.m. The *n* values refer to the number of parallel experiments. Student's *t*-test for paired and unpaired data, or one-way ANOVA for multiple group comparisons to a single control (Dunnett's test) were applied to test for differences between treatment means, using the Sigma Stat software (Jandel Corporation, San Rafael, CA, U.S.A.). Values of $P < 0.05$ were considered statistically significant.

Results

ClC-3 is upregulated in PASMCs of rat hypertensive lung

To test whether the *ClCn-3* gene is upregulated in hypertensive lung, we injected rats with monocrotaline to induce pulmonary hypertension. The condition is characterized by proliferative pulmonary vasculitis, increased pulmonary resistance and right ventricular hypertrophy (Goto *et al*., 2002). To confirm that monocrotaline-treated rats develop pulmonary hypertension, we dissected lungs and stained 8-μm thick cryosections with Masson trichrome for examination by light microscopy. Pulmonary arteries possess a relatively thin muscularis of 10–15 μm and the average thickness of the smooth muscle layer in control rats injected with physiological saline remained unchanged through day 21 post injection (Figure 1a). While there was no significant change of the endothelial cell layer thickness, monocrotaline-treated rats developed mild medial hypertrophy by day 7 with an average thickness of the muscularis of 15–20 μm. The thickness of the smooth muscle layer further increased to 25–30 μm by day 14, and further to more than 40 μm by day 21 (Figure 1a). These histopathological alterations are consistent with the features of monocrotaline-dependent pulmonary hypertension in rats, as shown previously by other investigators (e.g. Jones & Rabinovitch, 1996; Nakazawa *et al*., 2001).

To assay whether smooth muscle hypertrophy was associated with upregulation of the *ClCn-3* gene, we dissected

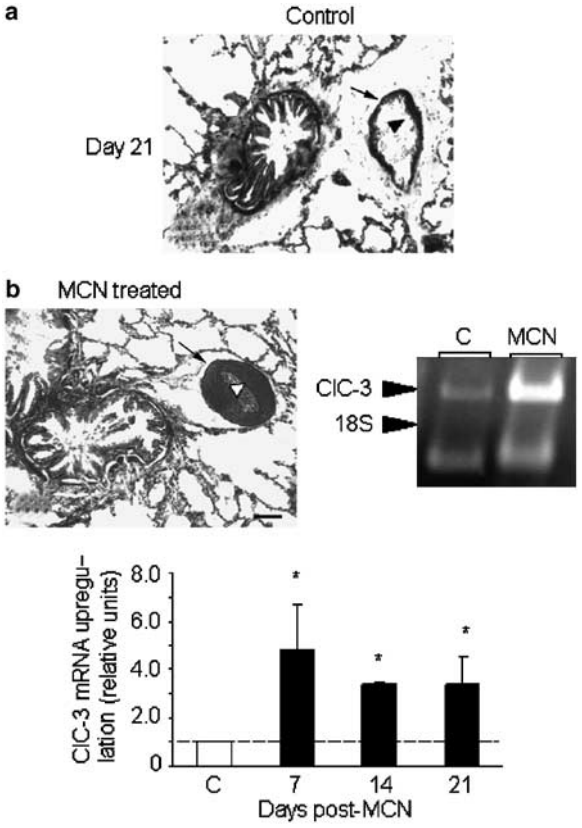

Figure 1 Monocrotaline (MCN)-induced rat pulmonary hypertension is associated with hypertrophy of the smooth muscle layer of pulmonary arteries (a) and upregulation of ClC-3 mRNA (b). Control rat pulmonary arteries (arrows, a) have normal endothelial (black arrowhead) and medial thickness (10–15 μm). Pulmonary arteries from monocrotaline-treated rats lack noticeable changes of the endothelial cell layer (white arrowhead), but exhibit progressive medial hypertrophy (a), with average thickness of the SMC layer of over 40 μm at day 21. Scale bar = 50 μm. The expression of ClC-3 mRNA was quantitated by PCR and densitometry, followed by normalization of the ClC-3 bands (584 bp) to the housekeeping 18S rRNA bands (324 bp), and changes in ClC-3 mRNA at days 7, 14 and 21 after the monocrotaline administration were expressed relative to control (C) rats, injected with physiological saline (bar graph, b). $^*P < 0.05$ compared to nontreated controls, $n = 5$.

pulmonary arteries, digested with collagenase type II and collected SMCs. Total RNA was extracted with Trizol reagent and reverse-transcribed to cDNA. RT–PCR amplification revealed a potent upregulation of ClC-3 mRNA at day 7 after the monocrotaline injection to 4.86 ± 1.84-fold above basal. The ClC-3 mRNA levels slightly decreased and remained at about 3.4-fold the basal by days 14 and 21 (Figure 1b). These data suggest that the *ClCn-3* gene is upregulated during pulmonary artery smooth muscle hypertrophy. It appears that effective upregulation of ClC-3 occurs at early stages and may contribute to the progression of pulmonary artery hypertrophy.

The small size of rat pulmonary arteries was a limiting factor for assay of ClC-3 channel protein levels by Western immunoblot analysis. As an alternative, we stained lung cryosections with a polyclonal anti-ClC-3 antibody and assessed the intensity of fluorescence in the smooth muscle layers of pulmonary arteries. Our result revealed a significant progressive increase of ClC-3-like fluorescence in smooth muscle layers of hypertensive pulmonary arteries, reaching values more than four-fold greater than fluorescence of control rat arteries at day 21 after the injections (Figure 2). Thus, monocrotaline-induced pulmonary hypertension of rats is associated with both transcriptional and translational upregulation of the *ClCn-3* gene.

ClC-3 channel is upregulated in cardiac myocytes of hypertensive rats

RV hypertrophy is another characteristic feature of the monocrotaline-induced hypertension. To test whether monocrotaline treatment induced RV hypertrophy, we dissected and weighed RV and LV + S of control and monocrotaline-treated rats at days 7, 14 and 21. On an average, the weight ratio of RV/(LV + S) remained unchanged by day 7, but increased by 25.4% at day 14, and by 33.8% at day 21 following monocrotaline injection (Figure 3a).

To test whether the *ClCn-3* gene was upregulated in the heart, we homogenized and extracted mRNA from RV and LV sections of control and monocrotaline-treated rats. RT–PCR amplification indicated that the ClC-3 mRNA was noticeably upregulated in RV, but only moderately enhanced in LV (Figure 3b). To further study whether the upregulated ClC-3 mRNA in hypertrophied RV is translated into protein, we homogenized RV and LV + S from control and monocrotaline-treated rats and probed with a polyclonal ClC-3 antibody. For quantification, the densities of ClC-3 immunoreactive bands were normalized to the densities of the respective GAPDH bands. The relative upregulation of the ClC-3 protein in the RV *versus* the LV at day 7 was indistinguishable between control and monocrotaline-treated rats (not shown). However at day 14, and particularly at day 21, the ClC-3 channel protein was significantly upregulated in RVs, but was only modestly enhanced in the LVs (Figure 4). Together, the quantitative analysis of ClC-3 mRNA and protein in rat pulmonary arteries and hearts suggest that upregulation of ClC-3 Cl^- channel is an early event that precedes the progression of vascular smooth muscle and cardiac hypertrophy.

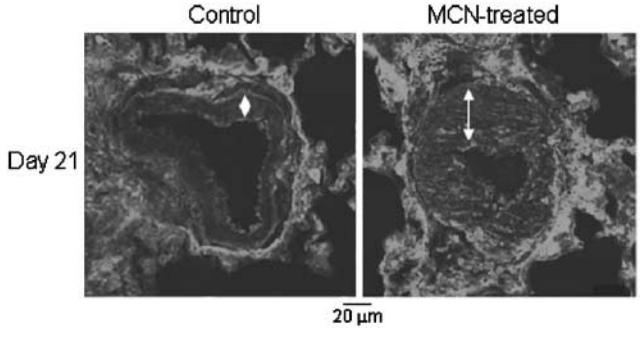

Figure 2 Monocrotaline (MCN)-induced rat pulmonary artery hypertrophy is associated with enhanced ClC-3-like fluorescence in the SMC layer. Cryostat sections from control and monocrotaline-treated rats were stained with a rabbit polyclonal ClC-3 antibody. The fluorescence per unit of SMC layer (arrows) surface areas was analyzed using Compix Simple PCI image analysis software.

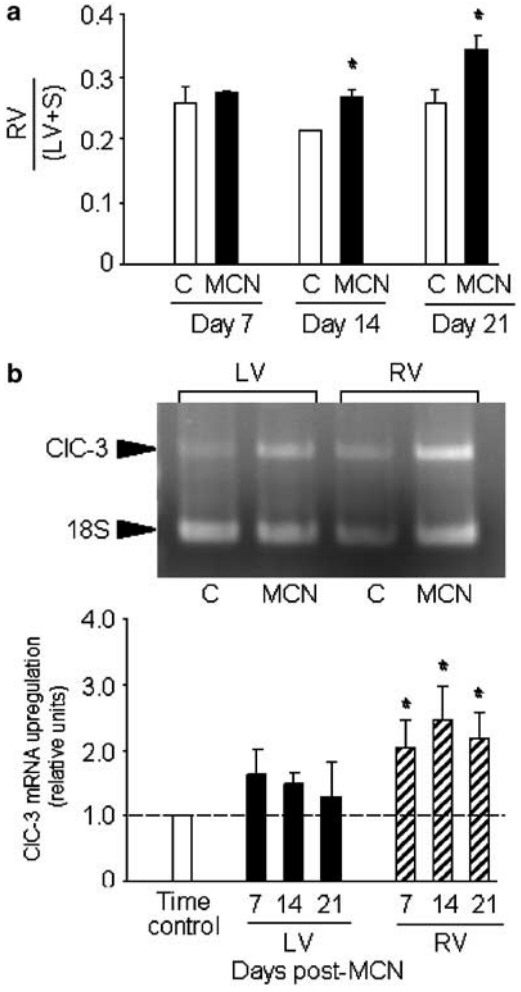

Figure 3 Monocrotaline (MCN)-induced rat pulmonary artery hypertrophy is associated with RV hypertrophy and upregulation of *ClCn-3* gene. (a) Hearts from control and MCN-treated rats were dissected into right ventricles (RV) and left ventricles plus septum (LV + S), and the ratios of RV/(LV + S) of MCN-treated and control rats were calculated for days 7, 14 and 21. (b) RT–PCR amplification of the ClC-3 mRNA was performed as described in the Methods section. Densities of ClC-3 bands (584 bp) were normalized to 18S rRNA bands (324 bp), and changes in ClC-3 mRNA expression at days 7, 14 and 21 following the monocrotaline injections were expressed relative to control (C) rats, injected with physiological saline (bar graph, open bar). $^*P < 0.05$ compared to nontreated controls, $n = 5$.

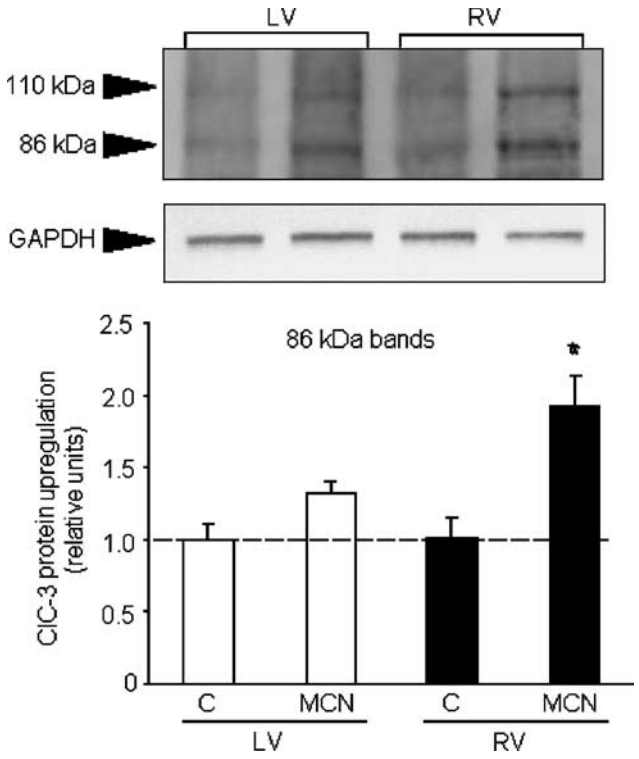

Figure 4 Protein levels of ClC-3 are significantly upregulated in right ventricles (RV), but only moderately in left ventricles (LV) of monocrotaline (MCN)-treated rats at day 21. Hearts from control and monocrotaline-treated rats were dissected to separate RV from LV. Total protein was analyzed by Western immunoblot analysis. ClC-3 and GAPDH (housekeeping gene) were simultaneously labeled with polyclonal rabbit and goat antibodies, respectively (insets). Densities of immunoreactive ClC-3 bands were normalized to the respective band densities of GAPDH, and changes in ClC-3 protein expression at day 21 following the injections were expressed relative to control (C) rats, injected with physiological saline (bar graph).

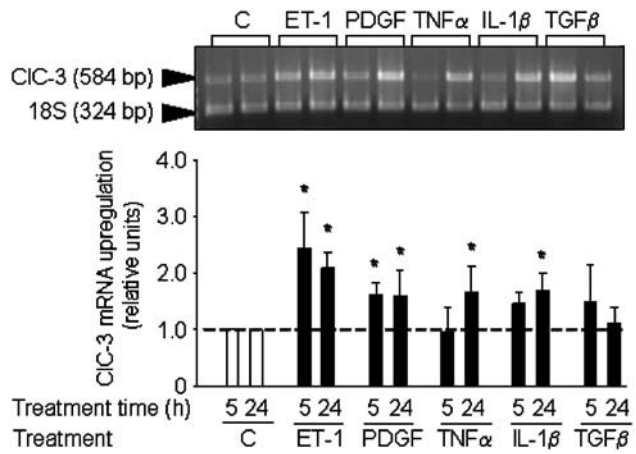

Figure 5 Bioactive peptides upregulate the *ClCn-3* gene in canine cultured PASMCs. First passage canine PASMCs were incubated for 5 and 24 h with ET-1 (100 nM), or PDGF, TNFα, TGFβ and IL-1β (10 ng ml^{-1}). Changes of mRNA were assessed by quantitative relative RT–PCR, using 18S rRNA as an internal standard. ClC-3 bands (584 bp) were normalized to the respective 18S rRNA bands (324 bp), and changes in ClC-3 mRNA expression were expressed relative to nontreated cell controls (C, bar graph). $*P<0.05$ compared to nontreated controls, $n=5$.

Inflammatory mediators differentially upregulate the ClC-3 mRNA

To identify inflammatory mediators involved in upregulation of ClC-3 mRNA, we incubated cultured PASMCs with recognized bioactive peptides and mitogens including ET-1, PDGF, TNFα, IL-1β and TGFβ. Although culturing of rat PASMCs proved feasible, dispersion and growth of sufficient number of cells for all treatment groups was a limiting factor for use of these cells. To resolve this problem, we cultured SMCs from canine pulmonary artery, following the same general procedure for cell dispersion and growth. Cells were growth-arrested for 48 h prior to 5 and 24 h incubations with 100 nM ET-1 or 10 ng ml^{-1} of each PDGF, TNFα, IL-1β and TGFβ. mRNA was extracted with Trizol reagent and reverse transcribed to obtain cDNA, which was then used as a template to amplify 18S rRNA and ClC-3 mRNA. The amplification conditions of all RT–PCR reactions were optimized to produce comparable band densities of the internal standard 18S rRNA among all samples (Figure 5). Among all stimulants, TGFβ was the only one that failed to stimulate a significant upregulation of the *ClCn-3* gene (Figure 5). With upregulation of the ClC-3 mRNA to 2.46 ± 0.63- and 2.10 ± 0.28-fold the basal at 5 and 24 h, respectively, ET-1 ranked the most potent stimulant in the group (Figure 5). PDGF and IL-1β also stimulated significant and comparable upregulation of ClC-3 mRNA at 5 and 24 h, while TNFα enhanced ClC-3 mRNA only after 24-h stimulation (1.67 ± 0.46-fold the basal, Figure 5). These results indicate that *ClCn-3* is an inducible gene in PASMCs, and that upregulation could be stimulated by some recognized bioactive peptides, that is, ET-1, PDGF and IL-1β.

Overexpression of ClC-3 channel in PASMCs improves cell resistance to oxidative stress

Inflammation of pulmonary artery is associated with increased concentrations of H_2O_2, produced endogenously by local cells or by recruited neutrophils and macrophages in response to high partial pressure of oxygen. Exposure to H_2O_2 causes injury of endothelial cells and increased vascular permeability, and at high concentrations may lead to decreased cell viability and to necrotic cell death (Lewis *et al*., 1988; Lelli *et al*., 1998). Since necrosis is characterized by cell swelling, mechanisms leading to compensatory volume decrease may prove beneficial for cell survival. ClC-3 has been proposed as a molecular candidate responsible for volume-sensitive Cl^- channels, which are activated upon cell swelling and contribute to cell volume homeostasis by promoting regulatory volume decrease (Duan *et al*., 1997). Therefore, we tested the hypothesis that overexpression of ClC-3 may reverse H_2O_2-induced cell swelling and thus improve cell survival. For these experiments, we used canine PASMCs, which were infected with adenovirus vector carrying wild-type ClC-3 gene. Infected cells overexpressed ClC-3 protein to about 2.3-fold its basal level, while expression of the housekeeping gene GAPDH was not affected (Figure 6 inset). The expression of ClC-3 in control PASMCs, infected with an empty adenovirus (sham infection), remained unchanged. Both control and ClC-3-overexpressing PASMCs were incubated with increasing concentrations of H_2O_2,

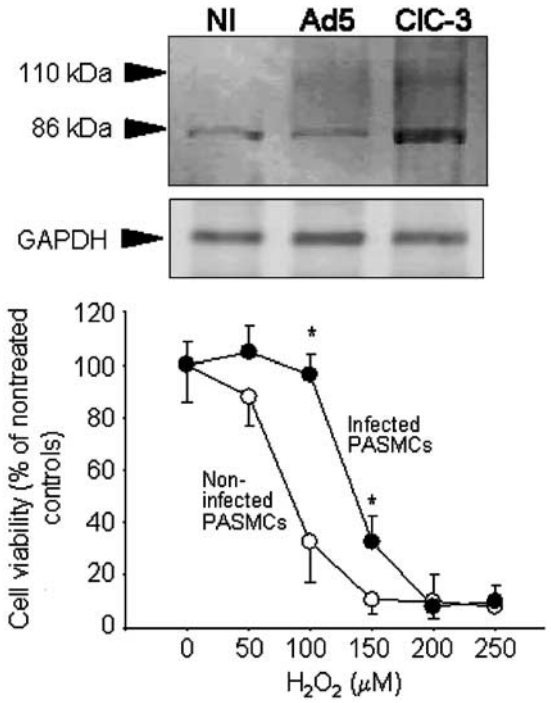

Figure 6 Adenovirus-mediated overexpression of ClC-3 improves viability of canine cultured PASMCs. Increase of ClC-3 protein was assayed by Western immunoblot analysis (top inset) and expressed relative to immunoreactive bands of GAPDH in the same samples (bottom inset). For assay of viability, cells were incubated with increasing concentrations of H_2O_2 and then with MTT reagent. Formazan crystals were solubilized and quantitated by the absorbance at 595 nm (bar graph). $^*P<0.05$ compared to non-treated controls, $n=3$. NI = non-infected cell control, Ad5 = empty adenovirus vector control.

ranging between 50 and 250 μM. The control cells tolerated concentrations of H_2O_2 lower than 50 μM, cell viability decreased at higher concentrations (Figure 6, open circles), while concentrations above 150 μM caused severe toxicity comprised of changes in cell morphology, cell rounding and ultimately detachment. The extent of cell toxicity was markedly reduced in cells overexpressing the ClC-3 transgene and the increased cell viability is demonstrated by the rightward shift of the cell viability/H_2O_2 concentration curves (Figure 6, closed circles). These concentration/response curves demonstrate that while only about 35% of the control noninfected cells were viable at 100 μM H_2O_2 (Figure 6, open circles), overexpression of ClC-3 almost completely restored cell viability at this concentration (Figure 6, closed circles). Although cell viability in ClC-3-overexpressing cells was significantly improved at 150 μM H_2O_2, the overexpression of ClC-3 failed to rescue cells incubated at concentrations higher than 200 μM (Figure 6). Overall, these results indicate that overexpression of ClC-3 may lead to a more efficient regulation of the cell volume and hence improve cell viability of canine PASMCs during oxidative stress.

Discussion

Inflammation of pulmonary arteries can be initiated by hypoxia, atherosclerosis, mechanical injury of branching blood vessels or increased blood flow (shear stress) (Rabinovitch, 1999; Jeffery & Morrell, 2002). Damage of the endothelial cell layer is an early event that triggers vascular remodeling by inducing cell proliferation or by altering homeostatic functions, secondary to increased endothelial permeability, degradation of extracellular matrix and production of vasoactive peptides (ET-1, angiotensin II), growth factors (e.g. PDGF, TGFβ and VEGF) and inflammatory cytokines (IL-1β and TNFα) (Oho *et al*., 1995; Nicod, 2003). Chronic simultaneous presence of these soluble extracellular factors can induce changes of the gene expression profile (Jones *et al*., 1997; Boudreau & Jones, 1999; Barillari *et al*., 2001). The magnitude of gene expression, therefore, is the result of interactive nuclear signaling of various stimulants, and may include potentiation and/or suppression of gene expression. Monocrotaline-induced pulmonary hypertension is an established model of vascular inflammation, which is initiated by damage of the endothelial cell layer of pulmonary arteries and exposure of underlying cells to blood plasma mitogens and inflammatory cytokines. Consistent with previous reports (Rosenberg & Rabinovitch, 1988), in the present study, monocrotaline-treated rats developed characteristic symptoms of pulmonary hypertension that included medial arterial hypertrophy. Our major novel observation, however, is that pulmonary artery hypertrophy was associated with a potent upregulation of ClC-3 mRNA (Figure 1) and protein expression, as suggested by the increased ClC-3-like immunoreactivity of the SMC layer (Figure 2). Another characteristic feature of the monocrotaline-induced pulmonary hypertension is RV hypertrophy, which develops secondary to increased pulmonary artery resistance and ventricular load. As observed in the hypertrophic pulmonary artery, the *ClCn-3* gene was significantly upregulated in the right, but not in the LV of monocrotaline-treated rats (Figures 3 and 4). Therefore, upregulation of ClC-3 and hypertrophy seem to progress in a correlative manner in both vascular SMCs and cardiac myocytes. Moreover, because ClC-3 was strongly upregulated 7 days after the monocrotaline administration (Figure 1), while PASMC and RV hypertrophy were yet in early stages (Figures 1 and 3), one could speculate that upregulation of ClC-3 precedes and may contribute to the development of the hypertrophic response.

One major goal of our experiments in canine cultured PASMCs was to assess the contribution of some recognized bioactive peptides and inflammatory mediators in the upregulation of ClC-3. The mitogenic properties of ET-1 (Malarkey *et al*., 1995; Simonson *et al*., 1996; Ortega & de Artinano, 1997), PDGF (Bornfeldt *et al*., 1995; Hughes *et al*., 1996; Conway *et al*., 1999) and IL-1β (Barillari *et al*., 2001; Shabahang *et al*., 2002) have long been recognized, but the information regarding their role in the expression of the *ClCn-3* gene in the pulmonary circulation is limited. ET-1, and to a lesser extent PDGF and IL-1β, caused a potent upregulation of ClC-3 mRNA in canine cultured PASMCs, which was readily detectable at 5 and 24 h of incubation. In contrast, TGFβ failed to upregulate the *ClCn-3* gene, while incubation of PASMCs with TNFα for 24 h, but not for 5 h, caused upregulation of the *ClCn-3* gene comparable to the effects of PDGF and IL-1β. The lack of effect of TGFβ indicates that upregulation of the *ClCn-3* gene is not a general, but is a ligand-specific event. Together, our observations suggest that *ClCn-3* is an inducible gene in PASMCs, cultured under conditions of experimental inflammation. Moreover, since

the upregulation of the ClC-3 mRNA in rat hypertensive pulmonary arteries (Figure 1) was several folds greater that the upregulation elicited by any of the bioactive peptides used alone in cultured PASMCs (Figure 5), the *ClCn-3* gene appears to be under a redundant and perhaps synergistic control by various signal transduction pathways in rat pulmonary arteries *in vivo*. This type of complex gene regulation is indicative of important and perhaps indispensable functions of ClC-3 in VSMC physiology. To our knowledge, this is the first report for upregulation of the *ClCn-3* gene in hypertensive pulmonary circulation and in cultured PASMCs, incubated with mitogens and mediators of vascular inflammation.

The physiological role of ClC-3 in VSMCs is poorly understood. ClC-3 is a candidate for a cell volume-sensitive anion channel in freshly dispersed VSMCs and in growth-arrested and differentiated cultured PASMCs (Yamazaki *et al*., 1998; Wang *et al*., 2003). Cell swelling-dependent activation of Cl^- current may be required for regulatory volume decrease and recovery of the physiological cell volume and functions. Although these channels are also activated or modulated by mechanical cell stretch (Browe & Baumgarten, 2003), CaMKII (Huang *et al*., 2001; Robinson *et al*., 2004), H_2O_2 (Varela *et al*., 2004) and by the serum and glucocorticoid-dependent kinase, SGK (Wang *et al*., 2004a), the physiological significance of ClC-3 activation by these stimuli remains to be determined. Pharmacological inhibition of Cl^- channels has been shown to cause arrest of human cervical cancer cells in G0/G1 stage, thus preventing G1/S checkpoint progression (Shen *et al*., 2000; Wang *et al*., 2004b). The role of Cl^- channels in cell growth is further supported by the expression of large Cl^- currents in proliferating mouse muscle cell lines (Voets *et al*., 1997). It is presently unknown whether Cl^- channels are important for regulation of the cell volume during cell cycle progression or cell growth, or Cl^- channels are involved in other activities. Previous data have provided evidence that ClC-3 may be necessary for cell proliferation by the demonstration that antisense oligonucleotide-mediated down-regulation of ClC-3 causes a substantial reduction of the ET-1-stimulated thymidine incorporation and delays proliferation of ClC-3-defficient rat aortic SMCs (Wang *et al*., 2002). These results provide strong support of the notion that ClC-3 has important intracellular functions for cell cycle and growth and, on a tissue level, for remodeling of vascular walls (Wang *et al*., 2002).

The present study provides novel functional information suggesting that ClC-3 channels may protect PASMCs in an environment of increased reactive oxygen species. H_2O_2 is abundant at lesion sites of the vasculature (Lewis *et al*., 1988; Lelli *et al*., 1998) and particularly in advanced atherosclerotic plaques, and is a major cause of vascular cell death (Mayr & Xu, 2001). Cell exposure to high concentrations of H_2O_2 has been shown to cause irreversible cell swelling and necrotic cell death, characterized by disintegration of cell membranes and denaturation of proteins and nucleic acids (Davies, 1999; Bergamini *et al*., 2004). Efficient maintenance of physiological cell volume, therefore, may be beneficial for cell resistance against H_2O_2-induced cell swelling and for cell survival. Our previous studies have identified ClC-3 as a volume-sensitive Cl^- channel, which is expressed in canine PASMCs and is activated upon hypotonic cell swelling (Yamazaki *et al*., 1998; Wang *et al*., 2003). Since our present results indicate that overexpression of ClC-3 channels improves cell viability during exposure of PASMCs to H_2O_2, one likely explanation is that ClC-3 promotes regulatory volume decrease, which is of vital importance during oxidative stress-induced cell swelling. Alternatively, overexpression of ClC-3 may be coupled to NADPH activity (Varela *et al*., 2004) or be involved in signal transduction events initiated by reactive oxygen species (Hamilton *et al*., 2004). Since oxidative stress may play a causative role in the pathogenesis of cardiovascular diseases (e.g. hypertension) and in heart failure, overexpression of ClC-3 channels may be an adaptive response to reduce the cytotoxic effects of H_2O_2 and thus improve cell survival.

An earlier study established that pulmonary arteries from monocrotaline-treated rats exhibited higher basal tone compared to blood vessels from vehicle-treated rats (Nakazawa *et al*., 2001). The elevated spontaneous muscular tone was sensitive to DIDS, thus implicating Cl^- channels as regulators of this effect. Interestingly, however, the authors failed to observe differences of the ClC-3 mRNA levels between the control and monocrotaline-treated rats (Nakazawa *et al*., 2001), data at odds with our present results. This discrepancy could perhaps be explained by differences in the experimental approaches. For example, Nakazawa *et al*. extracted RNA by homogenization of intact pulmonary arteries. In contrast, our approach included thorough initial removal of connective tissue and endothelial cells, which was then followed by proteolytic digestion of smooth muscle cell layer and removal of residual nondigested debris prior to RNA extraction. By using this method, we extracted RNA from preparations enriched of PASMC, and hence we largely eliminated other potential sources of total RNA. Since monocrotaline-induced pulmonary hypertension causes hypertrophy primarily of smooth muscle, upregulation of ClC-3 is likely to be most pronounced in extensively growing PASMCs. Moreover, because cells within the smooth muscle layer of the vascular wall are the major producer of mechanical force, upregulation of ClC-3 in the PASMC layer is likely to have most significant impact on smooth muscle tone. Therefore, we believe that our approach for studying rat pulmonary arteries is likely to provide reliable molecular and biochemical information related to the upregulation of the *ClCn-3* gene in PASMCs. Also, this information may be helpful in explaining the functional behavior of hypertensive pulmonary arteries described by Nakazawa *et al*. (1999).

In summary, the results of our study establish that ClC-3 is an inducible gene in rat pulmonary arteries and cardiac myocytes under conditions of experimental monocrotaline-induced pulmonary hypertrophy. Further, ClC-3 is upregulated in canine cultured PASMCs, incubated with inflammatory cytokines and growth factors. Overexpression of ClC-3 channels in canine PASMCs increased cell resistance against H_2O_2-induced oxidative stress. Therefore, upregulation of ClC-3 channels may be a survival mechanism for cells in inflammation sites of the vasculature. Moreover, by improving cell viability in an environment of increased background of inflammatory mediators, ClC-3 channels may contribute to proliferation and motility of VSMCs, and to the global remodeling of inflamed vascular wall.

This study was supported by NIH NCRR Grants P20RR 15581 and HL49254.

References

BARILLARI, G., ALBONICI, L., INCERPI, S., BOGETTO, L., PISTRITTO, G., VOLPI, A., ENSOLI, B. & MANZARI, V. (2001). Inflammatory cytokines stimulate vascular smooth muscle cells locomotion and growth by enhancing alpha5beta1 integrin expression and function. *Atherosclerosis,* **154,** 377–385.

BERGAMINI, C.M., GAMBETTI, S., DONDI, A. & CERVELLATI, C. (2004). Oxygen, reactive oxygen species and tissue damage. *Curr. Pharm. Des.,* **10,** 1611–1626.

BONGALON, S., DAI, Y.P., SINGER, C.A. & YAMBOLIEV, I.A. (2004). PDGF and IL-1 upregulate cofilin and LIMK2 in canine cultured pulmonary artery smooth muscle cells. *J. Vasc. Res.,* **41,** 412–421.

BORNFELDT, K.E., RAINES, E.W., GRAVES, L.M., SKINNER, M.P., KREBS, E.G. & ROSS, R. (1995). Platelet-derived growth factor. Distinct signal transduction pathways associated with migration *versus* proliferation. *Ann. N. Y. Acad. Sci.,* **766,** 416–430.

BOUDREAU, N.J. & JONES, P.L. (1999). Extracellular matrix and integrin signalling: the shape of things to come. *Biochem. J.,* **339** (Part 3), 481–488.

BROWE, D.M. & BAUMGARTEN, C.M. (2003). Stretch of {beta}1 integrin activates an outwardly rectifying chloride current *via* FAK and Src in rabbit ventricular myocytes. *J. Gen. Physiol.,* **122,** 689–702.

CAO, Z., HARDEJ, D., TROMBETTA, L.D., TRUSH, M.A. & LI, Y. (2003). Induction of cellular glutathione and glutathione *S*-transferase by 3*H*-1,2-dithiole-3-thione in rat aortic smooth muscle A10 cells: protection against acrolein-induced toxicity. *Atherosclerosis,* **166,** 291–301.

CHEN, M.J. & LAI, Y.L. (2003). Tachykinin dysfunction attenuates monocrotaline-induced pulmonary hypertension. *Toxicol. Appl. Pharmacol.,* **187,** 178–185.

CONWAY, A.M., RAKHIT, S., PYNE, S. & PYNE, N.J. (1999). Platelet-derived-growth-factor stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle: role of pertussis-toxin-sensitive G-proteins, c-Src tyrosine kinases and phosphoinositide 3-kinase. *Biochem. J.,* **337,** 171–177.

DAVIES, K.J. (1999). The broad spectrum of responses to oxidants in proliferating cells: a new paradigm for oxidative stress. *IUBMB Life,* **48,** 41–47.

DUAN, D., WINTER, C., COWLEY, S., HUME, J.R. & HOROWITZ, B. (1997). Molecular identification of a volume-regulated chloride channel. *Nature,* **390,** 417–421.

FUJITANI, Y., NINOMIYA, H., OKADA, T., URADE, Y. & MASAKI, T. (1995). Suppression of endothelin-1-induced mitogenic responses of human aortic smooth muscle cells by interleukin-1 beta. *J. Clin. Invest.,* **95,** 2474–2482.

FURUKAWA, T., OGURA, T., ZHENG, Y.J., TSUCHIYA, H., NAKAYA, H., KATAYAMA, Y. & INAGAKI, N. (2002). Phosphorylation and functional regulation of ClC-2 chloride channels expressed in Xenopus oocytes by M cyclin-dependent protein kinase. *J. Physiol.,* **540,** 883–893.

GEORGE JR, A.L., BIANCHI, L., LINK, E.M. & VANOYE, C.G. (2001). From stones to bones: the biology of ClC chloride channels. *Curr. Biol.,* **11,** R620–R628.

GOTO, J., ISHIKAWA, K., KAWAMURA, K., WATANABE, Y., MATUMOTO, H., SUGAWARA, D. & MARUYAMA, Y. (2002). Heme oxygenase-1 reduces murine monocrotaline-induced pulmonary inflammatory responses and resultant right ventricular overload. *Antioxid. Redox. Signal.,* **4,** 563–568.

GRAHAM, F.L. & PREVEC, L. (1995). Methods for construction of adenovirus vectors. *Mol. Biotechnol.,* **3,** 207–220.

GREENWOOD, I.A. (2004). CLC-3 knockout hints at swelling-activated chloride channel complexity. *J. Physiol.,* **557,** 343.

HAMILTON, C.A., MILLER, W.H., AL BENNA, S., BROSNAN, M.J., DRUMMOND, R.D., MCBRIDE, M.W. & DOMINICZAK, A.F. (2004). Strategies to reduce oxidative stress in cardiovascular disease. *Clin. Sci. (London),* **106,** 219–234.

HE, T.C., ZHOU, S., DA COSTA, L.T., YU, J., KINZLER, K.W. & VOGELSTEIN, B. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 2509–2514.

HUANG, P., LIU, J., DI, A., ROBINSON, N.C., MUSCH, M.W., KAETZEL, M.A. & NELSON, D.J. (2001). Regulation of human CLC-3 channels by multifunctional Ca2+/calmodulin-dependent protein kinase. *J. Biol. Chem.,* **276,** 20093–20100.

HUGHES, A.D., CLUNN, G.F., REFSON, J. & DEMOLIOU-MASON, C. (1996). Platelet-derived growth factor (PDGF): actions and mechanisms in vascular smooth muscle. *Gen. Pharmacol.,* **27,** 1079–1089.

JEFFERY, T.K. & MORRELL, N.W. (2002). Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension. *Prog. Cardiovasc. Dis.,* **45,** 173–202.

JENTSCH, T.J., STEIN, V., WEINREICH, F. & ZDEBIK, A.A. (2002). Molecular structure and physiological function of chloride channels. *Physiol. Rev.,* **82,** 503–568.

JONES, P.L., COWAN, K.N. & RABINOVITCH, M. (1997). Tenascin-C, proliferation and subendothelial fibronectin in progressive pulmonary vascular disease. *Am. J. Pathol.,* **150,** 1349–1360.

JONES, P.L. & RABINOVITCH, M. (1996). Tenascin-C is induced with progressive pulmonary vascular disease in rats and is functionally related to increased smooth muscle cell proliferation. *Circ. Res.,* **79,** 1131–1142.

LAMB, F.S., CLAYTON, G.H., LIU, B.X., SMITH, R.L., BARNA, T.J. & SCHUTTE, B.C. (1999). Expression of CLCN voltage-gated chloride channel genes in human blood vessels. *J. Mol. Cell. Cardiol.,* **31,** 657–666.

LELLI JR, J.L., BECKS, L.L., DABROWSKA, M.I. & HINSHAW, D.B. (1998). ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. *Free Radic. Biol. Med.,* **25,** 694–702.

LEWIS, M.S., WHATLEY, R.E., CAIN, P., MCINTYRE, T.M., PRESCOTT, S.M. & ZIMMERMAN, G.A. (1988). Hydrogen peroxide stimulates the synthesis of platelet-activating factor by endothelium and induces endothelial cell-dependent neutrophil adhesion. *J. Clin. Invest.,* **82,** 2045–2055.

MALARKEY, K., CHILVERS, E.R., LAWSON, M.F. & PLEVIN, R. (1995). Stimulation by endothelin-1 of mitogen-activated protein kinases and DNA synthesis in bovine tracheal smooth muscle cells. *Br. J. Pharmacol.,* **116,** 2267–2273.

MAYR, M. & XU, Q. (2001). Smooth muscle cell apoptosis in arteriosclerosis. *Exp. Gerontol.,* **36,** 969–987.

MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* **65,** 55–63.

NAKAZAWA, H., HORI, M., OZAKI, H. & KARAKI, H. (1999). Mechanisms underlying the impairment of endothelium-dependent relaxation in the pulmonary artery of monocrotaline-induced pulmonary hypertensive rats. *Br. J. Pharmacol.,* **128,** 1098–1104.

NAKAZAWA, H., HORI, M., MURATA, T., OZAKI, H. & KARAKI, H. (2001). Contribution of chloride channel activation to the elevated muscular tone of the pulmonary artery in monocrotaline-induced pulmonary hypertensive rats. *Jpn. J. Pharmacol.,* **86,** 310–315.

NICOD, L.P. (2003). Pulmonary hypertension. *Swiss. Med. Wkly.,* **133,** 103–110.

NILIUS, B. (2001). Chloride channels go cell cycling. *J. Physiol.,* **532,** 581.

NILIUS, B., EGGERMONT, J., VOETS, T., BUYSE, G., MANOLOPOULOS, V. & DROOGMANS, G. (1997). Properties of volume-regulated anion channels in mammalian cells. *Prog. Biophys. Mol. Biol.,* **68,** 69–119.

OHO, S., DALEY, S.J., KOO, E.W., CHILDS, T., GOTLIEB, A.I. & RABINOVITCH, M. (1995). Increased elastin-degrading activity and neointimal formation in porcine aortic organ culture. Reduction of both features with a serine proteinase inhibitor. *Arterioscler. Thromb. Vasc. Biol.,* **15,** 2200–2206.

ORTEGA, M.A. & DE ARTINANO, A.A. (1997). Highlights on endothelins: a review. *Pharmacol. Res.,* **36,** 339–351.

RABINOVITCH, M. (1999). Pulmonary hypertension: pathophysiology as a basis for clinical decision making. *J. Heart Lung Transplant.,* **18,** 1041–1053.

RABINOVITCH, M. (2001). Pathobiology of pulmonary hypertension. *Extracellular matrix. Clin. Chest Med.,* **22,** 433–449.

ROBINSON, N.C., HUANG, P., KAETZEL, M.A., LAMB, F.S. & NELSON, D.J. (2004). Identification of an N-terminal amino acid of CLC-3 critical in phosphorylation-dependent activation of ICl, CaMKII. *J. Physiol.,* **556,** 353–368.

ROSENBERG, H.C. & RABINOVITCH, M. (1988). Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. *Am. J. Physiol. Heart Circ. Physiol.,* **255,** H1484–H1491.

RUNO, J.R. & LOYD, J.E. (2003). Primary pulmonary hypertension. *Lancet,* **361,** 1533–1544.

RUTLEDGE, E., DENTON, J. & STRANGE, K. (2002). Cell cycle- and swelling-induced activation of a *Caenorhabditis elegans* ClC channel is mediated by CeGLC-7{alpha}/{beta} phosphatases. *J. Cell Biol.,* **158,** 435–444.

SHABAHANG, S., HUWILER, A. & PFEILSCHIFTER, J. (2002). Identification of the LIM kinase-1 as a ceramide-regulated gene in renal mesangial cells. *Biochem. Biophys. Res. Comm.,* **298,** 408–413.

SHEN, M.R., DROOGMANS, G., EGGERMONT, J., VOETS, T., ELLORY, J.C. & NILIUS, B. (2000). Differential expression of volume-regulated anion channels during cell cycle progression of human cervical cancer cells. *J. Physiol.,* **529,** 385–394.

SIMONSON, M.S., WANG, Y. & HERMAN, W.H. (1996). Nuclear signaling by endothelin-1 requires Src protein-tyrosine kinases. *J. Biol. Chem.,* **271,** 77–82.

VARELA, D., SIMON, F., RIVEROS, A., JORGENSEN, F. & STUTZIN, A. (2004). NAD(P)H oxidase-derived H_2O_2 signals chloride channel activation in cell volume regulation and cell proliferation. *J. Biol. Chem.,* **279,** 13301–13304.

VOETS, T., WEI, L., DE SMET, P., VAN DRIESSCHE, W., EGGERMONT, J., DROOGMANS, G. & NILIUS, B. (1997). Downregulation of volume-activated Cl− currents during muscle differentiation. *Am. J. Physiol.,* **272,** C667–C674.

WANG, G.L., WANG, X.R., LIN, M.J., HE, H., LAN, X.J. & GUAN, Y.Y. (2002). Deficiency in ClC-3 chloride channels prevents rat aortic smooth muscle cell proliferation. *Circ. Res.,* **91,** e28–e32.

WANG, G.X., HATTON, W.J., WANG, G.L., ZHONG, J., YAMBOLIEV, I.A., DUAN, D. & HUME, J.R. (2003). Functional effects of novel anti-ClC-3 antibodies on native volume-sensitive osmolyte and anion channels (VSOACs) in cardiac and smooth muscle cells. *Am. J. Physiol. Heart Circ. Physiol.,* **284,** H1453–H1463.

WANG, G.X., MC CRUDDEN, C., DAY, Y.P., HOROWITZ, B., HUME, J.R. & YAMBOLIEV, I.A. (2004a). Hypotonic activation of volume-sensitive outwardly rectifying chloride channels in cultured PASMCs is modulated by SGK. *Am. J. Physiol. Heart Circ. Physiol.,* **287,** H533–H544.

WANG, L.W., CHEN, L.X. & JACOB, T. (2004b). ClC-3 expression in the cell cycle of nasopharyngeal carcinoma cells. *Sheng Li Xue. Bao.,* **56,** 230–236.

YAMAZAKI, J., DUAN, D., JANIAK, R., KUENZLI, K., HOROWITZ, B. & HUME, J.R. (1998). Functional and molecular expression of volume-regulated chloride channels in canine vascular smooth muscle cells. *J. Physiol. (London),* **507,** 729–736.

(Received July 8, 2004
Revised October 21, 2004
Accepted October 28, 2004)