Protein Carbonylation As a Novel Mechanism in Redox Signaling

Chi Ming Wong, Amrita K. Cheema, Lihua Zhang, Yuichiro J. Suzuki

Abstract—Reactive oxygen species serve as second messengers for signal transduction; however, molecular targets of oxidant signaling have not been defined. Here, we show that ligand–receptor–mediated signaling promotes reactive oxygen species–dependent protein carbonylation. Treatment of pulmonary artery smooth muscle cells with endothelin-1 increased protein carbonyls. Carbonylation of the majority of proteins occurred transiently, suggesting that there is also a mechanism for decarbonylation induced by endothelin-1. Decarbonylation was suppressed by inhibition of thioredoxin reductase, and cellular thioredoxin was upregulated during the decarbonylation phase. These results indicate that endothelin-1 promotes oxidant signaling as well as thioredoxin-mediated reductive signaling to regulate carbonylation and decarbonylation mechanisms. In cells treated with endothelin receptor antagonists, hydrogen peroxide scavengers, or an iron chelator, we identified, via mass spectrometry, proteins that are carbonylated in a receptor- and Fenton reaction–dependent manner, including annexin A1, which promotes apoptosis and suppresses cell growth. Carbonylation of annexin A1 by endothelin-1 was followed by proteasome-dependent degradation of this protein. We propose that carbonylation and subsequent degradation of annexin A1 may play a role in endothelin-mediated cell growth and survival, important events in pulmonary vascular remodeling. Protein carbonylation in response to ligand–receptor interactions represents a novel mechanism in redox signaling. (Circ Res. 2008;102:0-0.)

Key Words: endothelin-1 • protein carbonylation • oxidant signaling • pulmonary hypertension • smooth muscle

Reactive oxygen species (ROS) have been proposed to serve as second messengers for signal transduction processes. Numerous studies demonstrated that (1) ligand–receptor interactions produce ROS; (2) antioxidants block signal transduction; and (3) ROS can stimulate signaling events. ROS signaling is thought to play important roles in various diseases, including cancer, neurological disorders, immune diseases, and cardiovascular diseases. Although mechanisms of ROS actions during oxidant signaling have not been defined, protein thiols being the oxidation targets have been a popular concept. Recently, Lee and Hennemann described a regulatory mechanism for the Bacillus subtilis PerR transcription factor by metal-catalyzed oxidation. Thus, other types of protein oxidation such as metal-catalyzed protein carbonylation may also be important for cell signaling.

Endothelin (ET)-1 is produced by vascular endothelial cells and exerts potent vasoconstrictive and mitogenic actions on vascular smooth muscle cells (SMCs). In pulmonary circulation, ET-1 contributes to vasoconstriction and vascular remodeling, which occur in pulmonary hypertension. The ET-1 expression is increased in the lungs of patients with pulmonary hypertension, and ET receptor antagonists have been used to treat human pulmonary hypertension, indicating the clinical importance of ET-1 signal transduction.

ET-1 is a mitogen of pulmonary artery SMCs via the activation of either ET, or ET receptor. Signal transduction pathways induced by ET-1 in pulmonary artery SMCs, however, are not well understood. ET-1 can activate extracellular signal-regulated mitogen-activated protein kinase as well as GATA-4 transcription factor. ET-1 has also been shown to generate ROS, which promote pulmonary vascular SMC proliferation. Thus, ROS may play important roles in ET-1–mediated pulmonary artery SMC growth during the development of pulmonary hypertension.

The present study demonstrates that ET-1 promotes protein carbonylation in pulmonary artery SMCs in an ET receptor– and Fenton reaction–dependent fashion. We also identified the “decarbonylation” mechanism that is activated by ET-1 via thioredoxin. Proteins that are carbonylated in response to ET-1 were identified using 2D gel electrophoresis and mass spectrometry. Annexin A1, which inhibits cell growth, was found to be among the proteins that were carbonylated and subsequently degraded in response to ET-1 in a receptor and metal-catalyzed oxidation–dependent manner.

Materials and Methods

Cell Culture

Large to mid-sized bovine pulmonary artery and thoracic aorta were obtained from a local abattoir. Bovine pulmonary artery SMCs

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(BPASMCs) and aortic SMCs were isolated as described previously28 and cultured in RPMI medium 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.5% fungizone (Invitrogen, Carlsbad, Calif). Two to 8 passages were used for experiments. Cells were growth-arrested for 24 hours in medium supplemented with 0.01% FBS and then treated with ET-1 (Sigma-Aldrich, St Louis, Mo), H2O2 (Fisher Scientific, Hampton, NH), or 1-chloro-2,4-dinitrobenzene (DNCB) (Sigma-Aldrich) for 0, 5, 10, 15, 20, and 30 minutes. In some experiments, cells were pretreated for 30 minutes with BQ123, BQ788, ebselen, deferoxamine, MG132, or DNCB (Sigma-Aldrich).

Organ Culture
For organ culture of rat pulmonary artery, lungs, and hearts were obtained from male Sprague–Dawley rats (250 to 300 g) and placed in ice-cold PBS. Mid-to-large pulmonary arteries were dissected, and surrounding connective tissues were removed under a dissecting microscope. Blood vessels were cut into small pieces of ring segments and incubated in RPMI medium 1640 containing 0.01% FBS for 30 minutes at 37°C before treating with ET-1.

Statistical Analysis
Comparisons between 2 groups were analyzed by a 2-tailed Student’s t test, and comparisons among 3 or more groups were analyzed by ANOVA with a Student–Newman–Keuls post hoc test. P<0.05 was considered to be significant. Data are presented as means±SEM.

An expanded materials and methods section, as well as supplemental figures, can be found in the online data supplement at http://circres.ahajournals.org.

Results
ET-1 Promotes Protein Carbonylation
Treatment of BPASMCs with ET-1 (30 nmol/L) resulted in the production of ROS, as monitored by detecting green dichlorofluorescein fluorescence (supplemental Figure I). The production of ROS was apparent after 5 minutes of ET-1 treatment, and the level of ET-1 that produced dichlorofluorescein fluorescence was comparable to the level produced by adding 50 μmol/L hydrogen peroxide (H2O2) extracellularly (supplemental Figure I). Thus, ET-1 produces ROS in BPASMCs.

Immunoblotting of 2,4-dinitrophenylhydrazine (DNPH)-derivatized proteins revealed that various proteins were carbonylated in BPASMCs (Figure 1A). Without DNPH derivatization, no bands were detected (data not shown). Treatment of cells with ET-1 for 10 minutes increased protein carbonylation of specific proteins, as determined by 1D (Figure 1A) and 2D gel electrophoresis (supplemental Figure II A). Coomassie blue staining of the membranes showed no changes in total protein content (Figure 1A, bottom). We assigned numbers for each of carbonylated protein (CP) bands from 1D gel electrophoresis experiments to be CP1 to CP17 (Figure 1B). The ET-1–mediated promotion of carbonylation of these proteins was also found in the tissue culture of bovine pulmonary artery smooth muscle (supplemental Figure II B) and in the organ culture of rat pulmonary artery (supplemental Figure IIC).

Roles of ET Receptors
To determine whether ET-1–mediated protein carbonylation is dependent on ET receptors, BPASMCs were treated with BQ123 (ETa receptor antagonist)29 or BQ788 (ETb receptor antagonist)30 before treatment with ET-1. Results show that BQ123 inhibited ET-1–mediated carbonylation of CP8, -10, -11, -12, and -17 (Figure 2A and supplemental Figure IIIA); and BQ788 inhibited that of CP3, -5, -7, -10, -11, -12, -13, -15, and -17 (Figure 2B and supplemental Figure IIIB). Neither of the ET receptor antagonists influenced basal levels of protein carbonylation. This is the first demonstration of receptor-mediated signaling promoting protein carbonylation.

Roles of H2O2 and Metal-Catalyzed Oxidation
CPs are often formed via peroxide-dependent Fenton reaction,31 and H2O2 has been shown to play integral roles in oxidant-mediated signal transduction.8,32 Consistently, pretreatment of BPASMCs with ebselen,3 a mimic of glutathione peroxidase that decomposes H2O2, effectively inhibited all of the ET receptor–dependent protein carbonylation (Figure 2C and supplemental Figure III C). Similar results were obtained by catalase overexpression via adenovirus-mediated gene transfer (data not shown). Furthermore, H2O2 at the concentration as low as 0.5 μmol/L mimicked ET-1–
mediated protein carbonylation (Figure 2D and supplemental Figure IIID). These results suggest that ET-1 promotes protein carbonylation via H$_2$O$_2$.

Recently, a transcription factor PerR was found to be regulated by metal-catalyzed oxidation.$^{15}$ Thus, we tested the effects of deferoxamine, and we found that this iron chelator inhibited ET-1–mediated carbonylation of CP3, -7, -13, -15, and -17 (Figure 2E and supplemental Figure IIIE), suggesting the role of metal-catalyzed oxidation.

**Kinetics of ET-1–Mediated Protein Carbonylation**

As shown in Figure 1, the increases in protein carbonyls by ET-1 appear transient in cell culture of BPASMCs (Figure 1A) and in tissue culture of bovine pulmonary artery smooth muscle (supplemental Figure IIB). Analyses of individual bands further revealed that the levels of protein carbonyls were transiently increased. In BPASMCs, carbonylation of CP10, -11, -12, -13, and -15 increased by 5 minutes, peaked at 10 minutes, and decreased to the basal level by 30 minutes of ET-1 treatment (Figure 3A). In contrast, carbonylation of these CPs in bovine aortic SMCs increased rapidly by 5 minutes and was sustained for 20 to 30 minutes (Figure 3B), differing from transient kinetics as seen in SMCs of pulmonary circulation. These experiments identified that the ET-1–mediated stimulation of protein carbonylation is followed by normalization of CPs (we termed “decarbonylation”) in pulmonary artery SMCs, whereas in aortic SMCs, such decarbonylation mechanism is slower.

**Mechanism of Decarbonylation**

A decrease in CPs may be attributable to the proteasomal degradation of oxidatively modified proteins.$^{34,35}$ To test this, BPASMCs were pretreated with MG132 (proteasome inhibitor) for 30 minutes before the ET-1 treatment. Figure 4 shows that ET-1–mediated increases in the majority of CPs occurred transiently even after the treatment with MG132 (30 µmol/L) for 30 minutes. MG132 can effectively inhibit proteolytic degradation in BPASMCs, because it inhibited the degradation of IκB-α induced by tumor necrosis factor-α (supplemental Figure IV). Thus, the decarbonylation mechanism appears to be independent of the proteasomal degradation of oxidatively modified proteins.

We hypothesized that reductive signaling may be promoted by ET-1, subsequently to oxidant signaling, perhaps to serve as a negative-feedback mechanism. Consistently, CPs in BPASMCs are reduced by β-mercaptoethanol (Figure 5). In
these experiments, β-mercaptoethanol was added to BPASMC lysates before derivatization with DNPH. Also, an inhibitor of thioredoxin reductase, DNCB, effectively promoted protein carbonylation (Figure 6A and supplemental Figure VA), suggesting the possible role of thioredoxin in reductive signaling. Unlike transient carbonylation induced by ET-1 in BPASMCs, DNCB-mediated carbonylation was sustained for at least 30 minutes without exhibiting decarbonylation. Treatment of BPASMCs with ET-1 after DNCB pretreatment further promoted some degree of protein carbonylation; however, ET-1 signaling did not elicit decarbonylation mechanism under these conditions (Figure 6B and supplemental Figure VB), demonstrating that the thioredoxin system is required for ET-1-mediated decarbonylation.

Western blotting revealed that ET-1 time-dependently increased the levels of thioredoxin protein in BPASMCs (Figure 6C). Interestingly, not only did ET-1 fail to increase thioredoxin in aortic SMCs, but the basal level of aortic SMC thioredoxin was found to be substantially lower compared with that in pulmonary artery SMCs (Figure 6C), providing a possible mechanism for sustained kinetics of ET-1-mediated protein carbonylation and the lack of the decarbonylation mechanism in aortic SMCs. Actinomycin D, a general inhibitor of gene transcription, inhibited ET-1-mediated increase in thioredoxin expression (supplemental Figure VC), suggesting that ET-1 activates thioredoxin gene transcription. These results suggest that, in pulmonary artery SMCs, thioredoxin may play a role in ET-1 signal transduction and the regulation of decarbonylation.

To provide direct evidence that thioredoxin regulates protein carbonylation in pulmonary artery SMCs, the hypothesis that ET-1 promotes protein–protein interactions between thioredoxin and CPs was tested. Lysate samples of BPASMCs with or without ET-1 treatment were derivatized with DNPH and immunoprecipitated with the 2,4-dinitrophenol (DNP) antibody, electrophoresed, and blotted with the thioredoxin antibody. Thioredoxin–CP interactions appear intact even in the presence of DNPH derivatization. Thioredoxin interactions with CP were found to be increased during the decarbonylation phase (ie, 20 to 30 minutes after ET-1 treatment) (Figure 6D). Thioredoxin itself is not carbonylated, because the thioredoxin band resides between CP16 and CP17 (data not shown). These results suggest that thioredoxin–carbonyl group interactions may regulate decarbonylation.

Figure 3. Kinetics of ET-1–induced protein carbonylation. Growth-arrested BPASMCs (A) and bovine aortic SMCs (B) were treated with ET-1 (30 nmol/L) and harvested at the indicated time points. Cell lysates were prepared and derivatized with DNPH. CP levels were monitored by Western blot with the DNP antibody. Bar graphs show means±SEM (n=6) of the percentage of carbonyl content relative to untreated control, as determined by densitometry. The symbols a and b denote values that are significantly different from untreated control and the value from cells treated with ET-1 for 10 minutes, respectively, at P<0.05.
Discussion

In summary, here, we describe novel redox signaling events that occur in response to ligand–receptor interactions. In pulmonary artery SMCs, ET-1 promotes protein carbonylation via an ET receptor–dependent mechanism, followed by the reduction of protein carbonylation events, which may play important functional roles.

Protein Carbonylation As a Novel Mechanism in Redox Signaling

The field of ROS has undergone a paradigm shift when the idea had emerged that these species are not merely damage-causing...
but can also serve as second messengers for signal transduction.\textsuperscript{1,2,4} Subsequent to the reports by Herzenberg and colleagues showing that antioxidants such as N-acetylcysteine can inhibit nuclear factor \( \kappa \)-B and HIV activation,\textsuperscript{42,43} Baeuerle and colleagues proposed that \( \text{H}_2\text{O}_2 \) is a widely used second messenger for nuclear factor \( \kappa \)-B activation in T cells.\textsuperscript{8} In vascular smooth muscle, early work demonstrated that ROS promoted cell growth, protooncogene expression,\textsuperscript{44} and \( \text{Ca}^{2+} \) signaling.\textsuperscript{10} Subsequently, ROS were reported to mediate signal transduction induced by angiotensin II\textsuperscript{6} and platelet-derived growth factor\textsuperscript{9} in aortic SMCs. In pulmonary artery SMCs, Fanburg and colleagues found that serotonin activates the production of superoxide\textsuperscript{45,46} and \( \text{H}_2\text{O}_2 \),\textsuperscript{47} presumably via NAD(P)H oxidase. We have shown that GATA-4 transcription factor plays an important role in pulmonary artery SMC growth and that antioxidants can inhibit serotonin-induced GATA-4 activation.\textsuperscript{26} Lawrie et al\textsuperscript{48} reported that, in human pulmonary artery SMCs, serotonin activates GATA-4 via ROS produced by monoamine oxidase. ET-1 has also been shown to produce ROS in fetal sheep pulmonary artery SMCs via NAD(P)H oxidase, and antioxidants block ET-1–induced cell proliferation,\textsuperscript{27} suggesting that ROS may play a role in ET-1–mediated pulmonary vascular thickening. The targets of ROS produced by ET-1 as well as other mediators of pulmonary hypertension, however, have not been defined. For the mechanisms of ROS signaling, ligand–receptor interactions producing ROS via NAD(P)H oxidase\textsuperscript{6,49} and ROS targeting protein thiols\textsuperscript{12,14} are currently popular proposed mechanisms.

During various oxidative stress conditions, protein oxidation results in the inactivation of protein functions. Carbonylation is one of oxidation processes, which can occur on protein molecules. Protein carbonyls are quite stable products formed on proline, arginine, lysine or threonine residues, often in response to metal-catalyzed Fenton reaction.\textsuperscript{31} Protein carbonyl groups react with DNPH, and various techniques have been developed to detect their interactions; these groups also have been used effectively as markers of oxidative stress. To our knowledge, the present study is the first demonstration of the protein carbonyl formation in response to ligand–receptor interactions and, therefore, the first dem-

\begin{figure}
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Effects of thiol reductant on protein carbonylation. BPASMCs were treated with ET-1, and cell lysates were prepared with buffer with or without 2% \( \beta \)-mercaptoethanol (\( \beta \)-ME). Lysates were then derivatized with DNPH to monitor CP content. The bar graph represents means±SEM (\( n=4 \)) of the percentage of total carbonyl content relative to untreated control without \( \beta \)-mercaptoethanol treatment. The symbol * denotes that 2 groups are significantly different from each other.}
\end{figure}
onstration of such formation in signal transduction processes. Protein carbonylation could offer a specific targeting mechanism for oxidant-mediated signal transduction, because site-directed Fenton reaction may occur at certain metal-binding sites within protein molecules. Further understanding of the roles of protein carbonylation in signal transduction pathways may yield invaluable information for the identification of targeting mechanisms for ROS signaling.

**Discovery of the Decarbonylation Mechanism**

It was interesting to note that carbonylation of the majority of the proteins were formed transiently in response to ET-1 in pulmonary artery SMCs. This led us to discover a process in which carbonylation can be eliminated in the cell. We termed this process “decarbonylation.” Apparent decarbonylation could be observed if CPs were degraded, because oxidized proteins have been shown to be susceptible to proteolytic degradation. However, our experiments show that decarbonylation occurs even when proteasomes are inhibited, suggesting that the degradation mechanism may not explain our observations. It is plausible that CPs may be aggregated; however, because our cell lysis solution contains detergent, it is expected that these proteins remain in the supernatant after centrifugation. We propose that decarbonylation is dependent on reduction reactions through plausible that CPs may be aggregated; however, because our cell lysis solution contains detergent, it is expected that these proteins remain in the supernatant after centrifugation. We propose that decarbonylation is dependent on reduction reactions through thioredoxin reductase inhibitor -mercaptoethanol. Second, a thioredoxin reductase inhibitor can promote protein carbonylation without the occurrence of decarbonylation. Thirdly, when thioredoxin reductase is inhibited, ET-1 promotes protein carbonylation in a sustained fashion without the occurrence of decarbonylation. Also, it is interesting to note that thioredoxin is upregulated in pulmonary artery SMCs in response to ET-1, and its expression is substantially lower in aortic SMCs, where the decarbonylation event is not apparent. These results indicate that thioredoxin, which has been shown to play integral roles in redox signaling, may also
regulate signal transduction mechanism involving protein carbonylation and decarbonylation. Because protein carbonylation is not chemically reversible, the nature of decarbonylation is not yet known.

Clinical Implications
Because ET receptor antagonists are used for the treatment of human pulmonary hypertension, these redox regulatory mechanisms promoted by ET-1 may offer important insights to therapeutic strategies. Our laboratory has previously demonstrated that ET-1 can promote anti-apoptotic signaling in pulmonary vascular SMCs, suggesting that ET-1 may exert multiple actions to contribute to the development of pulmonary hypertension. Because proteins that were found to be carbonylated in response to ET-1 signaling include annexin A1, which has been shown to promote apoptosis and inhibit cell proliferation, protein carbonylation of this protein by ET-1 may increase cell growth. In fact, we found that ET-1-mediated carbonylation of annexin A1 was followed by proteasome-dependent degradation, consistent with the idea that ET-1 alleviates apoptotic and antiproliferative actions of annexin A1 for promoting survival signaling and increasing SMC number. Further studies of carbonylated annexin A1, as well as other identified proteins, should provide important information for determining the mechanism of redox regulation of signal transduction, as well as for identifying effective therapeutic interventions against pulmonary hypertension.

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Disclosures
None.

References


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Expanded Materials and Methods

Measurement of ROS production

BPASMC were incubated with 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCF-DA; 10 µmol/L; Invitrogen) for 30 min at 37°C. Cells were washed with PBS, treated with ET-1 or H2O2 for indicated times, and examined using an Olympus fluorescence microscope. Fluorescence images were captured using a digital camera, and the intensity of fluorescence signals was quantified using IPLab imaging software (Scanalytics Inc, Fairfax, VA, USA).

Lysate preparations

To prepare lysates from cultured cells, cells were washed in PBS and solubilized with lysis buffer containing 50 mmol/L Hepes (pH 7.4), 1% (v/v) Triton X-100, 4 mmol/L EDTA, 1 mmol/L sodium fluoride, 0.1 mmol/L sodium orthovanadate, 1 mmol/L tetrasodium pyrophosphate, 2 mmol/L PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Samples were then centrifuged at 16,000g for 10 min at 4°C, supernatants collected, and protein concentrations determined. To prepare lysates from blood vessels, vessels were snap-frozen in liquid nitrogen and homogenized with lysis buffer. The homogenates were centrifuged at 16,000g for 20 min at 4°C and supernatants were collected.
Western blot analysis

To detect carbonylated proteins, carbonyl groups in protein side chains were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to form the 2,4-dinitrophenyl (DNP) hydrazone derivative (Oxyblot Protein Oxidation Detection Kit, Millipore, Billerica, MA, USA). 20 µg of lysate protein were denatured with 12% sodium dodecylsulfate (SDS), incubated with DNPH for 15 min, and mixed with neutralization solution and β-mercaptoethanol. In some experiments, cell lysates were pre-treated with β-mercaptoethanol before derivatization with DNPH. To evaluate the selectivity of carbonyl measurements, some protein samples underwent the protein carbonyl detection procedure without the derivatization step (negative control). DNP-derivatized proteins were electrophoresed through a reducing 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature and incubated overnight at 4°C with rabbit anti-DNP antibody (1:150 dilution). The levels of carbonylated proteins were detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:300 dilution) for 1 h at room temperature and the enhanced chemiluminescence (ECL) System (Amersham Biosciences, Piscataway, NJ, USA). The developed films were scanned with an imaging densitometer and optical densities (OD) of protein bands were quantified using NIH ImageJ version 1.43.

For immunoprecipitation experiments, cell lysates were derivatized with DNPH and incubated with anti-DNP IgG and GammaBind G-Sepharose (Amersham) overnight at 4°C with gently shaking. After washing twice, the pellet was boiled in Laemmli buffer and centrifuged, and the supernatant was electrophoresed. Western blot was performed using anti-thioredoxin-1
IgG, anti-peroxiredoxin-6 IgG, anti-annexin A1 IgG, anti-annexin A2 IgG, or anti-cofilin-1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Two-dimensional gel electrophoresis

4 volumes of 10 mmol/L DNPH (in 2 mol/L HCl) were added to 200 µg protein per sample and incubated for 30 min at room temperature. Ice-cold trichloroacetic acid (TCA) was then added to yield a final concentration of 15% and samples were then incubated for 10 min on ice. Samples were centrifuged for 10 min at 16,000g, the pellet was washed three times with ethanol ethyl acetate, and samples were centrifuged at 16,000g for 15 min. The pellet was resuspended in 2-D re-hydration buffer containing 8 mol/L urea, 2% CHAPS and 50 mmol/L dithiothreitol (DTT). First-dimension of protein separation was performed with the Protean Isoelectric Focusing (IEF) Cell (Bio-Rad Laboratories, Hercules, CA, USA). Samples were applied to immobilized pH gradient strips (nonlinear pH 3 - 10 or pH 5 - 8) for 1 h at room temperature. Strips were then covered with mineral oil and isoelectric focusing was performed at 20˚C. For the second dimension, IEF strips were equilibrated in 6 mol/L urea, 0.375 mol/L Tris-HCl (pH 8.8), 2% SDS, 20% glycerol and 2% DTT for 10 min, followed by incubation for an additional 10 min with a 2.5% iodoacetamide-containing solution. The strips were embedded in 0.7% agarose on top of 4 - 20% or 10.5 - 14% acrylamide gels for the second dimension SDS-PAGE, followed by electrotransfer to nitrocellulose membranes and immunoblotting with the anti-DNP IgG.
In-gel tryptic digestion and protein identification by mass spectrometry

Carbonylated protein spots of interest were excised from a Coomassie Blue stained gel and transferred to a 96 well ZipPlate (Millipore) and destained with 50% acetonitrile in 25 mmol/L ammonium bicarbonate, dehydrated with acetonitrile for 15 min, and vacuum dried. Gel pieces were then rehydrated with 15 µl of ammonium bicarbonate (25 mmol/L) supplemented with trypsin (5 ng/µl, Promega, Madison, WI, USA) at 30°C for 16 h. Tryptic peptides were then extracted in 0.2% TFA and captured by C18 resin at the bottom of each well. Peptides were then eluted in 0.1% TFA/50% acetonitrile, which contains 2.5 mg/ml CHCA (Acros Organics, Morris Plains, NJ, USA). Mass spectra were recorded with a matrix assisted laser desorption/ionization–time of flight, time of flight (MALDI-TOF-TOF) spectrometer (4800 Proteomics Analyzer, Framingham, MA, USA) set in reflector positive mode by spotting the samples onto a MALDI plate. The samples were ionized with a fixed laser intensity of 3,500 J, and 1,000 laser shots were collected per spectrum. The detector voltage was 2.1 kvolts, the bin size was set at 0.5 nsec and the signal/noise threshold was set at 10. The spectra were collected with a specified mass range of 799 – 4,000 daltons with a focus mass of 2,000 daltons. Peptide masses were compared with the theoretical masses derived from the sequences contained in SWISS-PROT/NCBI databases using MASCOT. The search parameters were set as follows: cysteines as carbamidomethyl derivative, allowed peptide mass error 50 ppm up to one missed cleavage and methionine oxidized form. Protein carbonylation and DNPH derivatization were assumed to occur on lysine, proline, threonine and arginine residues with added mass of 179, 196, 179 and 137, respectively.
Transfection with annexin A1 DNA

The day before transfection, cells were plated in a 12-well plate. 2 µg annexin A1 DNA/well was transfected using the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) in serum-free, antibiotic-free RPMI. Cells were transfected for 6 h, and then medium was replaced with RPMI containing 0.01% FBS with antibiotics for growth-arrest for 2 days before treatment with PDGF or FBS.

Figure Legends for Online Supplements

Fig. S1  ET-1 activates ROS generation in BPASMC. Growth arrested BPASMC were incubated with CM-H₂DCF-DA for 30 min, then treated with ET-1 (30 nmol/L). Green fluorescence signals indicating the production of ROS were captured by a digital camera on a fluorescence microscope. The line graph represents means ± SEM (n = 5) of % control fluorescence intensity at indicated time points after ET-1 treatment. The bar graph represents means ± SEM (n = 5) of % control of fluorescence intensity after treatment with ET-1 (30 nmol/L) or a positive control H₂O₂ (50 µmol/L) for 5 min. Symbol * denotes values that are significantly different from untreated control at p < 0.05.

Fig. S2  ET-1 promotes protein carbonylation in BPASMC. (A) Growth-arrested BPASMC were treated with ET-1 (30 nmol/L) for 10 min. Cell lysates were derivatized with DNPH and subjected to 2 dimensional gel electrophoresis. (B) Freshly isolated bovine pulmonary artery medial smooth muscle tissues and (C) rat pulmonary arteries were cut into 1 mm segments, and
treated with ET-1 (30 nmol/L) in 0.01% FBS containing RPMI medium and harvested at
indicated time points. Cell lysates were prepared after homogenization and derivatized with
DNPH. Carbonylated proteins were monitored by Western blot.

**Fig. S3** ET-1 activates protein carbonylation via endothelin receptors, \( \mathrm{H}_2\mathrm{O}_2 \) and metal-
catalyzed oxidation. Growth-arrested BPASMC were pre-treated with (A) BQ123, (B) BQ788,
(C) ebselen and (E) 50 µmol/L deferoxamine for 30 min and then treated with ET-1 (30 nmol/L)
for 10 min. (D) BPASMC were treated with \( \mathrm{H}_2\mathrm{O}_2 \) (0.5 µmol/L) and harvested at indicated time
points. Cell lysates were prepared and derivatized with DNPH. Carbonylated protein levels
were monitored by Western blot. Bar graphs show means ± SEM (n = 6) of percent of carbonyl
content relative to untreated control as determined by densitometry. In panels A, B C and E,
symbols a and b denote that the values are significantly different from untreated control and the
value from cells treated with ET-1, respectively, at \( p < 0.05 \). In panel D, symbols a and b denote
values that are significantly different from untreated control and the value from cells treated with
\( \mathrm{H}_2\mathrm{O}_2 \) for 5 or 10 min, respectively, at \( p < 0.05 \).

**Fig. S4** Effects of proteasome inhibitor on I\( \kappa \)B-\( \alpha \) degradation. As a control experiment to
assess the cellular effectiveness of MG132, BPASMC were pre-treated with MG132 (30 µmol/L)
for 30 min, then treated with TNF-\( \alpha \) (20 ng/ml). Cell lysates were prepared and the levels of
I\( \kappa \)B-\( \alpha \) and ERK proteins (showing equal protein loading) were monitored by Western blot. The
bar graph shows the percent of I\( \kappa \)B-\( \alpha \) protein level relative to untreated control.
Fig. S5  Role of thioredoxin in de-carbonylation mechanism. (A) BPASMC were treated with DNCB (30 µmol/L). Cell lysates were prepared and derivatized with DNPH. Carbonylated protein levels were monitored by Western blot with the DNP antibody. Bar graphs show means ± SEM (n = 5) of percent of carbonyl content relative to untreated control. The symbol a denotes values that are significantly different from untreated control. (B) BPASMC were pre-treated with DNCB (30 µmol/L) for 30 min before ET-1 treatment and harvested at indicated time points. The groups treated with ET-1 alone or DNCB alone act as controls. Cell lysates were prepared and derivatized with DNPH. Carbonylated protein levels were monitored by Western blot with the DNP antibody. Line graphs show means ± SEM (n = 5) of percent of carbonyl content relative to the level at 0 min. Bar graphs show means ± SEM (n = 5) of rates of carbonylation (0 – 10 min ET-1 treatment) and de-carbonylation (10 – 30 min ET-1 treatment). The symbol a denotes values that are significantly different from the group of ET-1 alone. (C) BPASMC were pre-treated with actinomycin D (5 µg/ml) for 30 min, then treated with ET-1. Cell lysates were prepared and thioredoxin expression was monitored by Western blot. The bar graph represents means ± SEM (n = 6) of percent of thioredoxin expression relative to untreated control. The symbol * denotes the value that is significantly different from untreated control.

Fig. S6  Two-dimensional gel electrophoresis analyses of ET-1 responsive carbonylated proteins. (A) Lysates from untreated cells and cells treated with ET-1 (30 nmol/L) for 10 min were derivatized with DNPH and subjected to isoelectric focusing, followed by SDS-PAGE with a 10.5 - 14% gel. Each ET-1-activated carbonylated protein spot was assigned a number from 1 to 26. (B) Immunoblot images showing the changes in carbonyl contents of protein Spot 10 with treatment of BPASMC with ET-1 for 10 min after pre-treatment with inhibitors indicated. (C)
Bar graphs show means ± SEM (n = 3) of percent of carbonyl content of protein spots relative to untreated control as determined by densitometry. Symbols a and b denote that the values are significantly different from untreated control and the value from cells treated with ET-1, respectively, at $p < 0.05$. (D) Top. Immunoblot showing spots defined as proteins that are carbonylated in response to ET-1 in an endothelin receptor and Fenton reaction-dependent manner. Bottom. Coomassie Blue staining of the corresponding two dimensional gel from which spots of interest were excised for mass spectrometry analyses.

**Fig. S7** Mass spectrometry analyses to identify carbonylated proteins. Corresponding carbonylated protein spots of interest were excised from Coomassie Blue stained gel for mass spectrometry analyses. Spectral masses (in mass per charge unit, M/z) obtained by MALDI-TOF mass spectrometry were queried to the entire theoretical, mammalian peptide masses in the protein databases using Mascot search engine. Matrix mass (less than 799 M/z) was excluded while trypsin autolytic mass was included (2,211 & 842.5 M/z). Proteins with MOWSE scores greater than 58 were considered significantly matched. Mass spectra of 11 spots are shown.

**Fig. S8** Confirmation of ET-1 responsive carbonylated proteins. (A) BPASMC were treated with ET-1 (30 nmol/L) for 10 min. Cell lysates were prepared, derivatized with DNPH, immunoprecipitated with the DNP antibody, and subjected to Western blotting with the annexin A2 antibody. The bar graph represents means ± SEM (n = 3) of percent of band density relative to untreated control. Symbol * denotes value that is significantly different from untreated control. (B) BPASMC lysates were derivatized with DNPH, immunoprecipitated with the DNP antibody, and was subjected to Western blotting with the cofilin-1 antibody.
Wong et al. Fig. S2.

A - pH 3 to 10

kDa
191 → 125 → 82 → 40 → 31 → 17 → 7

(4-20% gel)

B - Immunoblot

kDa
191 → 125 → 82 → 40 → 31 → 17 → 7

ET-1 (min)

0 5 10 20 30 60

C - Immunoblot

kDa
191 → 125 → 82 → 40 → 31 → 17 → 7

ET-1 (min)

0 10 0 10 0 10

Coomassie staining
Wong et al. Fig. S3.
Wong et al. Fig. S3.

B

CP3 (129kDa)  CP5 (74kDa)  CP7 (52kDa)

Carbonyl content (% control)

Untreated  ET-1  1 µM BQ788  10 µM BQ788  1 µM BQ788 + ET-1  10 µM BQ788 + ET-1

CP10 (33kDa)  CP11 (30kDa)  CP12 (27kDa)

Carbonyl content (% control)

Untreated  ET-1  1 µM BQ788  10 µM BQ788  1 µM BQ788 + ET-1  10 µM BQ788 + ET-1

CP13 (24kDa)  CP15 (14kDa)  CP17 (9kDa)

Carbonyl content (% control)

Untreated  ET-1  1 µM BQ788  10 µM BQ788  1 µM BQ788 + ET-1  10 µM BQ788 + ET-1
Wong et al. Fig. S3.

**E**

- **CP3 (129kDa)**
  - Untreated
  - ET-1
  - Deferoxamine
  - Deferoxamine + ET-1

- **CP7 (52kDa)**
  - Untreated
  - ET-1
  - Deferoxamine
  - Deferoxamine + ET-1

- **CP13 (24kDa)**
  - Untreated
  - ET-1
  - Deferoxamine
  - Deferoxamine + ET-1

- **CP15 (14kDa)**
  - Untreated
  - ET-1
  - Deferoxamine
  - Deferoxamine + ET-1

- **CP17 (9kDa)**
  - Untreated
  - ET-1
  - Deferoxamine
  - Deferoxamine + ET-1
Wong et al. Fig. S4.
Wong et al. Fig. S5.

A

CP3 (129kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP5 (74kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP7 (52kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP8 (47kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP10 (33kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP11 (30kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP12 (27kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP13 (24kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP15 (14kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)

CP17 (9kDa)

Carbonyl content (% control)

0 5 10 15 20 30

DNCB (min)
Wong et al. Fig. S5.
C

Thioredoxin

Actin

0 5 10 15 20 30
ET-1 (min)

0 100 200
Thioredoxin expression (% control)

0 5 10 15 20 30
ET-1 (min)
Figure S6. A: Untreated and ET-1 treated samples are shown. The gel is labeled with molecular weight markers in kDa and pH values are indicated. The samples show distinct protein bands labeled from 1 to 26.
CPS 20

Carbonyl content (% control)

Untreated
ET-1
BQ123+ET-1
BQ788+ET-1
Ebselen+ET-1
Deferoxamine+ET-1

Wong et al. Fig. S6.
Spot 8: heat-shock protein beta-1

Spot 10: peroxiredoxin 6
Spot 11: peroxiredoxin 6

Mass (m/z)  

Relative intensity (%)

Spot 12: annexin A2

Mass (m/z)  

Relative intensity (%)

Wong et al. Fig. S7.
Spot 13: annexin A2

Spot 14: phosphoglycerate mutase 1
Spot 16: heat-shock protein beta-1

Spot 17: phosphoglycerate dehydrogenase
Spot 20: cofilin-1

Spot 21: annexin A1
Spot 26: DJ-1 protein

Relative intensity (%) vs. Mass (m/z)
Figure S8.

(A) Untreated vs. ET-1

IP-DNP Blot-Annexin-A2
Annexin-A2

IP-DNP with blot-Annexin-A2 (% control)

Untreated ET-1

(B) Untreated vs. ET-1

IP-DNP Blot-Cofilin-1
Cofilin-1

IP-DNP with blot-Cofilin-1 (% control)

Untreated ET-1