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Original research article

ET-1 mediates the release of reactive oxygen species and TNF-α in lung tissue by protein kinase C α and β1

Anna Gorąca [a,*], Paulina Kleniewska [b], Beata Skibska [c]

[a] Experimental and Clinical Physiology, Department of Cardiovascular Physiology, Medical University of Lodz, Łódź, Poland
[b] Department of Immunopathology, Faculty of Biomedical Sciences and Postgraduate Training, Medical University of Lodz, Łódź, Poland
[c] Department of Applied Pharmacy, Department of Pharmacy, Medical University of Lodz, Łódź, Poland

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ABSTRACT

Background: The aim of this study was to determine the involvement of protein kinase C (PKC) in the ET-1 induced generation of reactive oxygen species and TNF-α in rat lungs.
Methods: Experiments were performed on 6 groups of rats: Group I: saline-treated control; Group II: saline followed by endothelin-1 (ET-1) (3 μg/kg); Group III: saline followed by selective PKC αβ1 inhibitor (Gö6976) (2 μg/kg); Group IV: Gö6976 (2 μg/kg) administered 30 min before ET-1 (3 μg/kg); Group V: saline followed by the PKC activator phorbol 12-myristate 13-acetate (PMA) (50 μg/kg); Group VI: Gö6976 (2 μg/kg) administered 30 min before PMA (50 μg/kg). After 5 h, the animals were euthanized and their lungs were isolated for measurements.
Results: ET-1 resulted in increase in thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide (H_2O_2) levels and lung edema, as well as a decrease in GSH/GSSG ratio compared to the controls. The level of TNF-α also was elevated in the presence of ET-1. Administration of Gö6976 30 min before ET-1 injection significantly decreased lung edema, as well as the concentrations of TBARS, H_2O_2 and TNF-α, but increased the GSH/GSSG redox ratio compared to ET-1. Conversely, PMA elevated lung edema and TBARS, H_2O_2 and TNF-α concentrations, but decreased the GSH/GSSG redox ratio compared to the control group. Treatment with Gö6976 significantly ameliorated the PMA-induced oxidative stress parameters, decreased tissue TNF-α level, and lung edema.
Conclusion: Endothelin-1 induces ROS generation, increases TNF-α level and lung edema *via* activation of PKC αβ1.

Introduction

Reactive oxygen species (ROS) are chemically reactive molecules derived from molecular oxygen and are formed as a natural by-product of the normal metabolism of oxygen. During energy conversion, ROS are produced as a by-product of oxidative phosphorylation in mitochondria, which is presumed to be the major source of superoxide ($O_2^{\bullet-}$) production [1]. The $O_2^{\bullet-}$ can subsequently be converted to more the stable H_2O_2, either spontaneously or by superoxide dismutase. H_2O_2 may be converted to hydroxyl radicals (OH^-) in the presence of transition metal ions (Fe^{2+}, Cu^{2+}). In endothelial cells, H_2O_2 also can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), another highly reactive form of ROS [2]. Moreover, ROS can also be produced by a variety of enzymes including nitric oxide synthase, cyclooxygenase, xanthine oxidase, and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) [3].

In normal circumstance, concentrations of ROS are tightly controlled by endogenous cellular antioxidant mechanisms such as superoxide dismutase, which rapidly converts $O_2^{\bullet-}$ into H_2O_2 and O_2, catalase, glutathione peroxidase and peroxiredoxins, keeping them in the picomolar range [4]. These low concentrations of ROS enable them to act as secondary messengers in signal transduction for vascular homeostasis and cell signaling. When produced in excessive amounts, or when antioxidants are depleted, the presence of ROS can result in damage to lipids, proteins and DNA. An imbalance between ROS production and the cellular defense system leads to oxidative stress, which can subsequently contribute to the development and/or progression of many diseases.

ET-1 was discovered by Yanagisawa in 1988 [5] as a peptide with vasoconstrictive effects and high expression in the intima of the aorta. ET-1 is synthesized from the precursor "big endothelin",

* Corresponding author.
E-mail address: anna.goraca@umed.lodz.pl (A. Gorąca).

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which is cleaved by the ET converting enzyme (ECE) to form the active 21-amino acid peptide. The physiological and pathophysiological effects of ET-1 are mediated *via* two receptor subtypes, ET_A and ET_B receptors, both of which are G protein-coupled receptors. The expression of ET-1 has been observed in a variety of organs including the heart, vessels, lung, brain and kidney [6]. The activation of ET_A and ET_B receptors mediates many functions, including vasoconstriction, cardiovascular remodeling, cell proliferation, cell differentiation, extracellular matrix production, and control of water and sodium secretion, and ROS generation [7]. ET-1 may cause an increase of $O_2^{\bullet -}$ by the NADPH oxidase-dependent mechanism [8,9], by the mitogen-activated protein kinase (MAPK) pathway [9], the protein kinase C (PKC) [10], the nuclear factor-kappa B (NF-κB) pathway [11] or the c-Jun-terminal kinase (JNK) [12].

PKCs comprise a family of serine/threonine kinases characterized by at least eleven different isotypes, which are classified into conventional, novel and atypical groups. The conventional PKCα, PKCβ1/II and PKCγ isotypes are Ca^{2+} and diacylglycerol dependent. PKCs act as regulatory enzymes in many cellular responses in the lung, including permeability, migration, hypertrophy, proliferation, apoptosis and secretion, and play a critical role in mediating inflammation and respiratory diseases [13]. PKCs induce ET-1 synthesis by numerous agents, including mechanical strain [14,15], angiotensin and vasopressin [16], and ET-1 [17]. Cosentino-Gomez et al. [18] report that PKC family members are highly sensitive to oxidative stress. These enzymes containing cysteine residues and free sulfhydryls at the regulatory and catalytic sites are themselves susceptible to oxidative modification [18].

A review of PubMed reveals a paucity of data concerning the influence of a PKC αβ1 inhibitor and its activator (PMA) on the development of lung edema. Moreover, the role of PKCs in the regulation of ROS generation induced by ET-1 administration is yet unclear. Therefore, the objective of this study was to determine whether ET-1-induced generation of ROS and TNF-α in lung tissue is mediated by protein kinase C αβ1, and whether activation of PKC αβ1 by PMA could affect ROS and TNF-α generation in lung tissues.

Materials and methods

Chemicals

Endothelin-1 (powder), 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole (Gő6976), PMA, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), sodium acetate trihydrate (TEA), triethanoloamine hydrochloride, 5-sulfosalicylic acid hydrate (5-SSA), 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), β-nicotinamide adenine dinucleotide phosphate (β-NADPH), glutathione reductase (GR), 2-vinylpyridine, horseradish peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were obtained from POCH (Gliwice, Poland) and were of analytical grade. Endothelin-1 was dissolved in 0.9% NaCl. Gö6976 and PMA were dissolved in dimethyl sulfoxide (DMSO), the stock solution was freshly diluted before each experiment. The final concentration of DMSO was 0.1%.

Animals

The experiments were performed on male Wistar rats weighing 260–280 g, aged 2–3 months. The animals were acquired from the Medical University of Lodz animal quarters and were housed in individual cages under standard laboratory conditions: 12/12 h light–dark cycle (light on at 7.00 a.m.) at 20 ± 2 °C ambient temperature and 55 ± 5% air humidity. All rats received a standard laboratory diet and water *ad libitum* and they were maintained for 1 week in the laboratory for adaptation. The experimental procedures followed the guidelines for the care and use of laboratory animals and were approved by the Medical University of Lodz Ethics Committee No. 28/LB520/2010.

Experimental design

The experimental animals were divided into six groups, each group comprising six rats:

Group I (control rats): received two doses of 0.2 ml saline, 0.5 h apart. Group II (ET-1 group): received one dose of 0.2 ml saline and 0.5 h later ET-1 at a dose 3 μg/kg b.w. Group III (Gö6976): received one dose of 0.2 ml saline and 0.5 h later inhibitor PKCαβ1 Gö6976 at a dose 2 μg/kg b.w. Group IV (Gö6976 + ET-1): received one dose of Gö6976 (2 μg/kg b.w.) and 0.5 h later ET-1 at a dose 3 μg/kg b.w. Group V (PMA): received 0.2 ml saline and 0.5 h later PMA at a dose 50 μg/kg b.w. Group VI (PMA + Gö6976): received 0.2 ml saline and 0.5 later Gö6976 at a dose 2 μg/k b.w. All compounds were administered into the femoral vein.

Animal preparations

The animals were anaesthetized by an intraperitoneal injection of 10% urethane (2 ml/100 g b.w.). When a sufficient level of anesthesia was achieved, the skin and subcutaneous tissues on the neck were cut and a 2 cm-long polyethylene tube (2.00 mm O.D.) was inserted into the trachea. The right femoral vein was catheterized for drug infusion. At the end of the experiment, after 5 h, the rats were killed by cervical decapitation. Lungs from control and experimental groups of rats were excised, rinsed with ice-cold saline, dried by blotting between two pieces of filter paper, weighed on an electronic scale then stored at −76 °C for measurement of oxidative parameters and levels of TNF-α.

Lung edema assay

The lungs were rinsed with ice-cold saline, weighed (wet weight) and placed in a drying oven at +80 °C for 8 h, before being weighed again (dry weight). Finally, the lung wet-to-dry weight ratios were determined as an indicator of lung edema.

Preparation of lung tissue homogenate

An accurately weighed portion of lungs (50 mg) was homogenized in either 0.15 M KCl for the estimation of lipid peroxidation and H_2O_2 level, or in 5% SSA for the estimation of glutathione. Homogenates were centrifuged at 7.450 rpm for 10 min at +4 °C for glutathione measurement or at 3.500 rpm for 15 min at +4 °C, for lipid peroxidation assay. The resulting supernatant was used for biochemical analysis.

Determination of lipid peroxidation

Lipid peroxidation is an important process in the establishment of lung injury. The formation of thiobarbituric acid-reactive substances (TBARS) was used to quantify the degree of lipid peroxidation in tissues and was assayed according to Yagi [19]. The peroxidation product content in lung homogenates was assayed as the concentration of TBARS in the butanol layer, measured spectrofluorometrically using an LS-50 Perkin Elmer Luminescence Spectrometer (Norwalk, CT, USA). Excitation was set at 515 nm and emission was measured at 546 nm. The TBARS concentration in the sample was calculated by the use of a regression equation prepared from triplicate assays of six increasing concentrations of tetramethoxypropane (range 0.01–50 μM) as a standard for TBARS. Finally, the results were calculated for 50 mg of the lung tissue.