6-2011

The Role of Protein Kinase C Epsilon in Hydrogen Peroxide and Nitric Oxide Release During Oxidative Stress Caused by Extracorporeal Shockwave Lithotripsy

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THE ROLE OF PROTEIN KINASE C EPSILON IN HYDROGEN PEROXIDE AND NITRIC OXIDE RELEASE DURING OXIDATIVE STRESS CAUSED BY EXTRACORPOREAL SHOCKWAVE LITHOTRIPSY

A Thesis in Endothelial Dysfunction by Edward S. Iames

Submitted in Partial Fulfillment of the Requirements for the Degree of Masters in Biomedical Sciences
June 2011

We the undersigned duly appointed thesis committee have read and examined this manuscript and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

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ABSTRACT

The Role of Protein Kinase C Epsilon on Hydrogen Peroxide and Nitric Oxide Release during Oxidative Stress caused by Extracorporeal Shock Wave Lithotripsy

Clinical extracorporeal shock wave lithotripsy (ESWL) treatment to ablate kidney stones can cause acute damage to the renal microvasculature. Accumulation of continued treatment with shockwave therapy can lead to chronic damage to the kidney, and lead to clinical hypertension. Shockwaves have been shown to stimulate endothelial cells to release superoxide (SO), which is converted to hydrogen peroxide (H_2O_2), and reacts with nitric oxide (NO) to produce peroxynitrite anion ($OONO^-$), creating a powerful oxidant that increases oxidative stress while simultaneously reducing NO bioavailability. Increased oxidative stress during events such as ESWL, also uncouples NO production reaction in endothelial nitric oxide synthase (eNOS), causing eNOS to produce SO instead of NO, exacerbating the oxidative insult. NO is an essential signaling molecule responsible for vasodilation, which also functions in inhibiting platelet adhesion and reducing leukocyte-endothelial interactions. This increased oxidative stress, decreased NO bioavailability, and the direct physical force of the shock wave causes prolonged renal vasodilation and eventually vascular endothelial dysfunction in the renal vasculature. Protein kinase C epsilon (PKC-ε) positively regulates eNOS, increasing its activity regardless of whether eNOS is producing SO or NO. We hypothesized that the PKC-ε peptide inhibitor (N-Myr-EAVSLKPT, MW = 1054) would attenuate ESWL-induced increased H_2O_2 release and increase NO release compared to ESWL-saline control rats, while the PKC-ε peptide activator (N-Myr-HDAPIGYD, MW 1097) would increase ESWL-induced H_2O_2 release and reduce NO release. H_2O_2 and NO was

measured in real-time by inserting a H_2O_2 or NO microsensor (100 um diameter) into the left renal vein in anesthetized male Sprague-Dawley rats. ESWL treatment was administered with 16 kV shock waves for 13 minutes in a period of 500 shocks at 60 beats/min then 500 shocks at 120 beats/min by an Epos Ultra lithotripter. Immediately post-ESWL treatment, saline or drug was infused through the external jugular vein. Infusion of PKC-ε inhibitor in ESWL-treated rats significantly reduced H_2O_2 release ($n = 5$) from 5 minutes ($p < 0.05$) to 30 minutes ($p < 0.01$) compared to ESWL-saline controls ($n = 5$). PKC-ε inhibitor also significantly increased NO release ($n = 5$) from 5 minutes ($p < 0.01$) to 30 minutes ($p < 0.01$) compared to ESWL-saline controls ($n = 5$). Contrary to the hypothesis that PKC-ε activator would increase H_2O_2 release and reduce NO release, results from PKC-ε activator showed a similar but not statistically significant different trend to ESWL-saline controls in H_2O_2 release ($n = 5$) and NO release ($n = 5$). The data shows that inhibition of PKC-ε effectively decreases ESWL-induced increased H_2O_2 release and significantly restores NO release, which suggests that uncoupled eNOS is a significant source of oxidative stress during ESWL treatment. This results in decreased oxidative stress, attenuating endothelial dysfunction and further damage to the renal microvasculature.

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ACKNOWLEDGMENTS

I would like to acknowledge the following for their contributions to my thesis:

Dr. Lindon Young, Ph.D., Professor of Pathology, Microbiology, Immunology and Forensic Medicine, Thesis Advisor

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Kyle Bartol, Dan Eskinazi, Michael Iyamu, Lucy Joo, Maria Kern, Michael Minni, Kerry-Anne Perkins, Brian Rueter, Philadelphia College of Osteopathic Medicine Students

Antony Lavigna, Technical Consultant, Multimed Technical Services, Inc. Beltsville, Maryland

Dr. Marcus Bell, Professor of the Neuroscience, Physiology and Pharmacology.

This study was supported by NHLBI Grant 2R15HL-76235-02 and the Center for the Chronic Disorders of Aging at Philadelphia College of Osteopathic Medicine.

INTRODUCTION

Extracorporeal shock wave lithotripsy, or ESWL, is an effective, non-invasive therapy utilized to fragment stones in the kidney and urinary tract. Lithotripsy provides a less detrimental alternative to invasive treatments, which can lead to parenchymal fibrosis in the kidney[37], permanently damaging the delicate structure of numerous nephrons. A lithotripter generates high-energy acoustic pulses and propagates those shockwaves through a lens that focuses on the location of the stone, breaking up the stone. After treatment, fragmentation of the stone allows the debris to be cleared by the flow of the urinary tract. Although ESWL provides a safer solution to removing harmful stone, the forces fragmenting the stone can also cause underlying damage to vascular endothelium due to increased oxidative stress, acute damage not easily detected. ESWL treatment can not only lead to damage from the physical forces of the shock waves, but oxidative stress indirectly caused by shock waves, which can lead to acute injury post-treatment and may lead to chronic adverse effects. This endothelial dysfunction can be attenuated pharmacotherapeutically by signaling the endothelium to reduce release of reactive oxygen species, such as superoxide (SO) and hydrogen peroxide (H_2O_2).

Limitations of ESWL

Lithotripsy remains the only non-invasive technique available in the removal of stone, however; the treatment does not come without its limitation. Over the past couple decades, urologists have scrutinized the ESWL treatment and its usage has been restricted over concerns of efficacy of stone fragmentation and safety of the

procedure. A major limitation to lithotripsy that has dictated procedure is the stone burden, which includes the stone size and the stone type[32]. Because of the limited capacity of the kidney and ureters to clear debris, shock wave lithotripsy is generally regarded as the primary method for treatment if the stone is 2.5cm or smaller and there are no abnormalities in the patient's renal anatomy[32]. Certain stone types may be resistant to shock wave lithotripsy, among them brushite, calcium oxalate monohydrate, and cysteine stones or may be easily fragmented but not completely comminuted, leading to re-treatment [32]. Stones treated with lithotripsy are ultimately broken up by two forces: a positive pressure phase, in which the direct force of the shock wave compresses the stone and a negative pressure phase, which causes cavitation bubbles to form and collapse near the stone. Low-energy shock waves are sufficient enough to break up the stone, however, higher-energy shock waves are needed to create an energy scattering effect caused by the accumulation of stone fragments from the initial shock waves[35]. Stress waves and cavitation have been shown to work in a synergistic manner in ESWL[35]. Unfortunately, the stress forces not only break up the stone, but also attribute to ESWL-induced injury. Intraluminal bubbles formed from cavitation have been linked to damage and rupturing of blood vessels[35].

The greatest concern of the safety of lithotripsy grew from animal and clinical studies showing lesions from shock waves involving ruptured blood vessels that would cause intraparenchymal hemorrhage and renal[35]**.** Damage from shock waves were found to be dose-dependent, according to the number and the amplitude of ESWL, and localized, but not limited to the focal zone of the lithotripter[32]. Research

has shown that these injuries can rupture primarily in blood vessels, which in turn causes bleeding leading to inflammatory responses that damage functional renal tissue[32]. These injuries from ESWL, although not directly proven to be the cause of chronic disease, do promote concern and has stimulated the investigation that has generated much support that patients, especially though who have had multiple lithotripsy treatments, have an increased risk to developing hypertension and diabetes mellitus[32].

Improvements to ESWL Technique

Ongoing research has intensely studied the implications of ESWL-treatment protocols in order to determine the safety parameters as well as the highest efficacy within those parameters. To determine safer strategies, researchers have investigated factors such as number of shock waves delivered, frequency of shock wave administration, energy output of shock waves, step-wise change and constant energy output, and pretreatment protocols[32]. Ultimately, the primary goal was to complete stone comminution with the least amount of shock waves at the lowest possible power setting and slowest rate of delivery[32]. The results of research have shown many beneficial changes to the treatment protocol, which is a non-standardized procedure. Researchers used 2000 shock waves at above 20kV energy output and at a constant rate of 120 shock waves per minute (sw/min) to simulate a general lithotripsy procedure. Utilizing this procedure, the quick, powerful, and numerous shock waves would yield easily quantifiable trauma to kidney tissue[32]. Adjusting the rate to slower frequencies proved to significantly reduce the damage from ESWL, even at the same

energy output, showing significantly better treatment at 60 sw/min compared to the typical 120 sw/min[32]. In a separate study, increasing the energy output from lower levels in a step-wise fashion increased stone comminution without changing any other parameters, while a decreasing or constant energy output yielded poorer clearance[35].

A protective effect was observed in the kidney when a low frequency, lower energy output pretreatment of 100 shocks was given before the treatment phase. Results from this study showed that the initial exposure to shock waves resulted in a vasoconstrictive response during the shock wave delivery, rather than a vasoconstrictive response post-ESWL treatment seen in the standard procedure[32]. This observation has led to the conclusion that the protective effect from pretreatment lies in the vasoconstriction of renal vasculature during incoming shock waves. Although inconclusive, there has been some speculation to the mechanism behind protective vasoconstriction. Low pressure from shock waves at low output voltages may cause vessels to vasoconstrict and reduce intraluminal volume of blood, thus lowering the number of potential cavitation bubble nuclei, protecting against the high-pressure phase of the shock waves[35]. Another theory suggests that the vasoconstriction itself strengthens the integrity of the vasculature to prevent vessel rupture. With these developments, researchers were able to devise a new, current standard that provides better treatment with less injury and effective stone comminution, recommending a slow rate at increasing energy output from lower –to moderate levels, using as few shock waves as necessary[32].

Despite improvements to ESWL technique that shows dramatically reduced injury, recent research by this laboratory has shown that oxidative stress is increased

post-ESWL treatment even under the recommended changes to ESWL procedure. In that study, ESWL was shown to induce increases in H_2O_2 and decreases in nitric oxide (NO), characteristics of endothelial nitric oxide synthase (eNOS) dysfunction found in ischemia-reperfusion injury. This has led our laboratory to further investigate the role of eNOS during oxidative stress caused by ESWL, explore mechanisms involved in its regulation, and potentially attenuate the oxidative stress through manipulation of eNOS regulation. Treatment of oxidative stress in the vascular endothelium may be pivotal in preventing underlying acute injury that leads long-term damage to kidney tissue.

Endothelium and Nitric Oxide

Endothelium is the pivotal barrier between the cardiovascular system and the entire body. Continued maintenance of proper endothelium function is essential for not only the physical barrier the endothelium provides, but also for vascular homeostasis. Endothelial cells (ECs) effectively form a semipermeable barrier throughout the entire vasculature and control molecular transfer between the blood and tissue. This layer of cells, capable of paracrine and endocrine function, can sense the dynamic changes of blood and regulate vascular tone. ECs can release vasoconstrictors, such as endothelin-1 and prostaglandins H2, or vasodilators, prostacyclin, endothelium-derived hyperpolarizing factor, and most importantly, nitric oxide (NO). NO serves as a primary vasodilator, relaxing smooth muscle cells, and also provides anti-thrombotic, anti-atherosclerotic, anti-inflammatory functions for the endothelium[29].

Long-term levels of arterial pressure can be regulated by the medullary blood flow of the kidney. Reductions of 15-30% to renal medulla blood flow have been shown to lead to hypertension[13]. Essentially, NO has an important role in the regulation of medullary blood flow, which regulates arterial blood pressure and sodium homeostasis. NO acts in low renal vascular resistances and stimulates natriuresis and dieresis regardless of the renal perfusion pressure[13]. Studies show that NO can reduce sodium reabsorption in most tubular segments, notably the proximal and distal tubules, thick ascending limb, and cortical collecting ducts[13]. NO associates tubular metabolic needs to blood flow and protects the medulla from underperfusion and macula densa NO acts as tubuloglomerular feedback control of afferent arteriolar resistance in the regulation of renal blood flow[13].

Studies have shown that NO can limit platelet adhesion and leukocyte-endothelial interactions by inhibiting exocytosis of inflammation-inducing granules within endothelial cells via targeting *N*-ethylmaleimide sensitive factor through *S*-nitrosylation of exocytic machinery[33]. By decreasing granule trafficking to the cell membrane and accelerating endocytosis, NO regulates the transport of leukocyte-endothelial interacting proteins and inhibits leukocyte interaction with the EC membrane. In this effect, NO decreases expression of P-selectin on the EC surface, the transmembrane protein responsible for leukocyte rolling, when ECs loosely attach to leukocytes upon P-selectin glycoprotein ligand-1 on their surfaces interaction with P-selectins[33]. Additionally, integrins and intercellular adhesion molecule expression is decreased by NO[33]. NO works antagonistically to inflammatory signals that activate

the exocytosis of resting EC granules to translocate to the cell membrane that lead to inflammatory responses[33].

The Structure and Function of eNOS

eNOS is the primary producer of nitric oxide along the endothelium of the cardiovascular system. eNOS, also known as NOS3, is one of three nitric oxide synthases, and different palmitoylation and myristoylation sites for subcellular targeting from other NOS isoforms. Although functionally primarily localized within caveolae distributed through the EC surface, eNOS is a dynamic protein, which is also found inside the Golgi apparatus and the plasma membrane[20]. The enzyme consists of two identical monomer units with a dual domain, a flavin-reductase domain and heme-oxygenase domain, connected together by a calmodulin-binding peptide[40]. The flavin-reductase domain consists of a NADPH oxygenase, FAD, and FMN subunits, while the heme-oxygenase domain contains a prosthetic heme group and a binding site for pterin molecules[45]. The two monomers are held together by a zinc-tetrasulfate bond, contributed by two cysteine residues from each monomer[30]. This bond is critical to maintaining the homodimer structure and can be subject auto-inhibition by oxidation or *S*-nitrosylation that will dissociate the structure into monomers when levels of NO or H_2O_2 become too high[17].

In order for eNOS to undergo its catalytic activity, the two monomers must form a homodimer. This functional configuration is maintained by the presence of pterin molecules located adjacent to the heme group. eNOS also requires the Ca^{2+}-dependent binding of calmodulin to the calmodulin-binding peptide. Upon binding of calmodulin, eNOS can conduct its catalytic activity. Physiologically, BH_4 is the

necessary pterin cofactor required by eNOS not only for the production of NO and but also the stability of the homodimer through numerous hydrogen bonds. eNOS produces NO the catalysis of a two-step reaction sequences that requires BH_4 cofactor for both steps as an electron donor. The first of the two reactions is the oxidation of the substrate, L-arginine, to the stable intermediate *N*-hydroxy-L-arginine followed by the oxidation of the intermediate to yield NO and L-citrulline. These two reactions depend on an electron flow from the oxidation of NADPH to FAD, then FMN, ultimately to O_2, which is bound to the iron-heme group, forming a ferrous-dioxygen complex[53,65].

eNOS Coupling and Uncoupling

Not only is eNOS a primary producer of NO, eNOS can also change its enzymatic profile to produce SO (Figure 1). BH_4 competes with another physiological pterin, BH_2, for the cofactor binding site. When BH_2 replaces BH_4 in the cofactor binding site, the transfer of electrons from the reductase domain to the oxygenase domain is uncoupled[64].Without BH_4 to facilitate the electron transfer, molecular oxygen sequesters an electron from the heme group it is bound to, dissociating from the heme-dioxygen complex as SO. With a similar structure to BH_4 without its catalytic capabilities, BH_2 may stabilize the catalytic homodimer to produce SO. SO has a relatively short half-life, but has an extremely large reactivity constant with NO. SO produced from eNOS is thus capable of quenching NO also produced from eNOS, which leads to the formation of the highly reactive peroxynitrite anion ($ONOO^-$). The scavenging of NO by SO to produce $ONOO^-$ decreases NO bioavailability and causes further oxidative stress, leading to more eNOS uncoupling, as well as damaging the

cell. $ONOO^-$ contributes greatly to oxidative stress by targeting its limited number of biological targets. Because of a high in vivo concentration of CO_2 reacting with $ONOO^-$ at a fast rate, many molecules do not compete with $ONOO^-$ before its conversion to bicarbonate[14]. However, $ONOO^-$ is capable of oxidizing BH_4, leading directly to eNOS uncoupling[2]. $ONOO^-$ oxidizes BH_4 to BH_2, with a reaction order much higher than $ONOO^-$ with ascorbate, glutathione, or thiol groups[28]. Although SO can also react with BH_4, the rate for this reaction is much slower than its scavenging of NO[63]. Therefore, as the product of SO scavenging of NO, $ONOO^-$ is the likely oxidant for BH_4 oxidation and the direct contributor to the oxidative stress that leads to eNOS uncoupling, further exacerbating oxidative stress caused by eNOS[21, 58]. $ONOO^-$ can also target heme-containing proteins and proteins containing zinc-thiolate centers, oxidizing bonds and destabilizing their protein structure. eNOS falls into both these categories, showing that it is a readily available target for destabilization, which may in turn allow for oxidation of bound BH_4 or replacement of BH_4 with BH_2. BH_2 competes for the pterin-binding site in eNOS with similar affinity, allowing a free exchange of the two pterin molecules which may be dependent upon the concentration ratio of the two molecules within the cell.

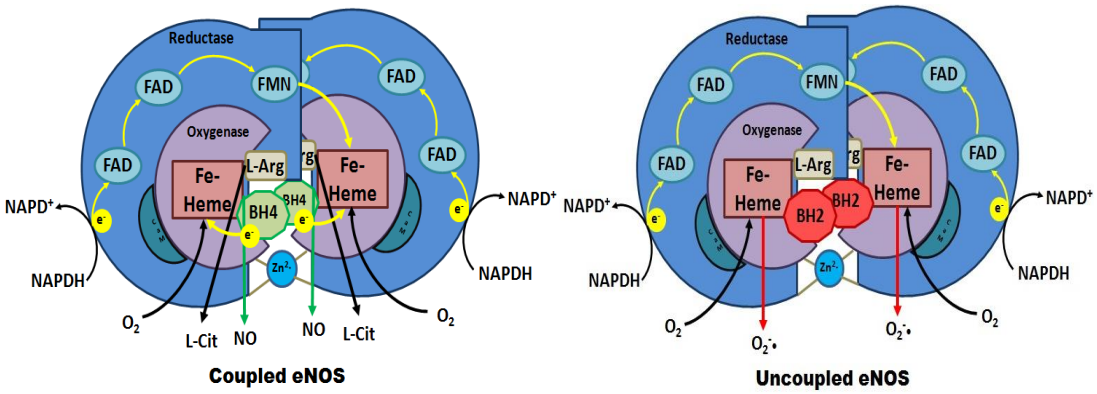

Figure 1. eNOS Enzymatic Profiles: Coupled State and Uncoupled State. eNOS is a homodimer with three domains: an oxygenase domain, reductase domain, and calmodulin-binding domain. In eNOS, electrons from NAPDH are shuttled to the ferrous-dioxygen complex. The BH_4 cofactor donates an electron to couple oxygen reduction with L-arginine oxidation in the ferrous-dioxygen complex to produce NO. During oxidative stress, BH_4 is oxidized to BH_2, thereby increasing BH_2 to BH_4 ratio. In the presence of BH_2, eNOS is unable to utilize electrons from BH_2, uncoupling oxidation of L-arginine from oxygen reduction, causing the ferrous-dioxygen complex to produce SO instead of NO.

Protein Kinase C and the Epsilon Isoform

One of the discovered regulatory mechanisms of eNOS is through the signal transduction cascade of protein kinase C. Protein kinase C consists of serine/threonine protein kinase family complete with three classes of 11 different isozymes, which are expressed in many cell types, which include polymorphonuclear leukocytes (PMNs) and vascular endothelial cells[5,73]. Each isoform has the ability to directly regulation eNOS through phosphorylation at specific residues. The effects of each isoform vary; some may phosphorylate to enhance eNOS activity, or inhibit its function[41].Protein kinase C epsilon (PKC-ε) in particular is constitutively expressed in vascular endothelial cells and not in PMNs and increases eNOS activity via phosphorylation at serine residue 1177[74].

PKC enzymes consist of two domains, the N-terminal regulatory domain with C1 and C2 subdomains, and the conserved C-terminal catalytic domain. C1 and C2 subdomains act as mediators between PKC and secondary messengers and interactions with other cellular molecules[5]. Cell permeable peptides due to myristoylation can be used as activators or inhibitors to eNOS activity. For PKC-ε, these peptides are derived from the C2/V1 domain of the regulatory region of PKC-ε and are capable of directly modulating PKC-ε activity when within the cell, thus indirectly regulating eNOS[5]. PKC-ε activator peptide activates PKC-ε by targeting its receptor for activated C kinase (RACK) region, which facilitates the translocation of PKC-ε in the cytosol to the cell membrane where the enzyme will phosphorylate eNOS. Activator peptide interferes with the PKC autoinhibitory intramolecular interactions, allowing for its activation[5]. On the other hand when PKC-ε inhibitor peptide binds to RACK, it interferes with the protein and PKC-ε translocation to the cell membrane is inhibited, preventing phosphorylation of eNOS, thus decreasing its activity. In ischemia-reperfusion models involving the heart, PKC-ε activator has shown to be cardioprotective through preconditioning, but damaging to heart tissue during reperfusion, which may be due to enhanced activity of uncoupled eNOS, leading to an increase in SO release[61]. On the contrary, PKC-ε inhibitor has resulted in cardioprotective effects during reperfusion, decreasing uncoupled eNOS activity, decreasing SO release and improving NO bioavailability[61].

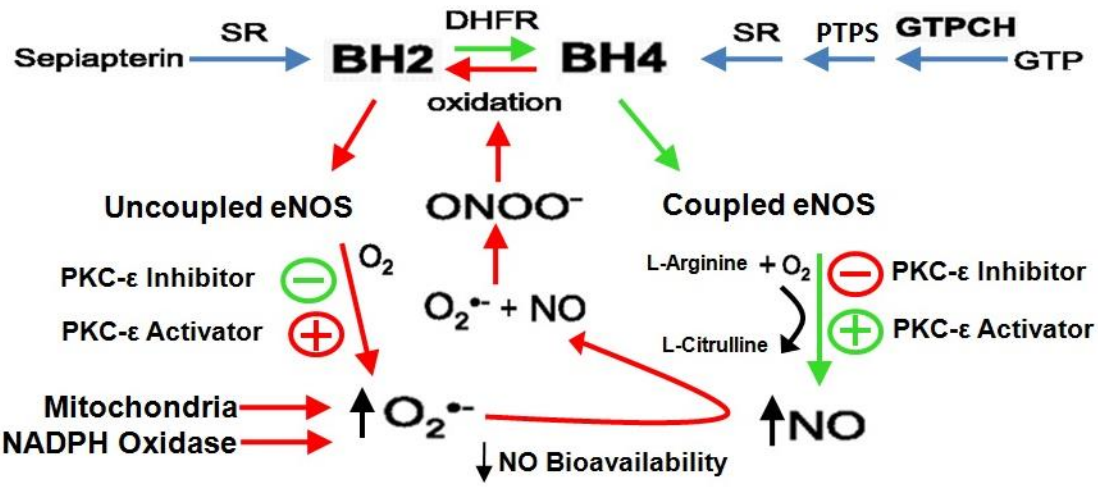

Figure 2. Sources of Oxidative Stress and Effects of PKC-ε Activator and Inhibitor. (1) NADPH Oxidase releases SO ($O_2^{-}\cdot$) from leukocytes and endothelial cells; (2) Mitochondria releases SO via incomplete oxidative phosphorylation; (3) Uncoupled eNOS releases SO in the presence of BH_2 . Increased SO quenches NO produced from coupled eNOS, reducing NO bioavailability and further increases oxidative stress through peroxynitrite ($ONOO^-$). PKC-ε activator/inhibitor peptides increase/decrease coupled and uncoupled eNOS activity, respectively.

The previous study by this laboratory using the ESWL model investigated the effects of ESWL on NO and H_2O_2 levels and experimented with regulation from another PKC isoform, PKC-βII. Their studies showed that ESWL results in an increase in H_2O_2 release as well as a decrease in NO, confirming ESWL-induced oxidative stress after lithotripsy treatment. Using PKC-βII inhibitor, PKC-βII activity was attenuated, which preserved NO bioavailability by preventing the activation of SO producer, NADPH oxidase by PKC-βII[26]. In this study, we will again test the effects of ESWL on NO and H_2O_2 release, and target PKC-ε in an effort to identify if eNOS is the primary source of oxidative stress in the renal vasculature and explore the effects of PKC-ε regulation through PKC-ε activator and inhibitor peptides on ESWL-induced oxidative stress.

HYPOTHESIS

This project will study the implications of ESWL on the release of H_2O_2 and NO in rat renal veins and the changes of this release by the effects of PKC-ε activator (N-Myr-HDAPIGYD, MW=1097, Genemed Synthesis, San Antonio, TX) and inhibitor (N-Myr-EAVSLKPT, MW=1054, Genemed Synthesis) peptides on the enzyme and its role on eNOS.

Effects of ESWL on NO and H_2O_2 Release Compared to No-ESWL Controls

Introduction of ESWL treatment will increase H_2O_2 release and decrease NO release compared to controls not treated by ESWL. The stress from the shear forces of ESWL will damage endothelium, stimulating H_2O_2 release while dropping NO bioavailability. The non-ESWL treatment group, or sham group, will not experience any deviation from baseline release levels.

PKC-ε Activator Effects on NO and H_2O_2 Release

Infusion of PKC-ε activator will trigger further eNOS uncoupling, increasing H_2O_2 release while simultaneously decreasing NO release. PKC-ε activator will indiscriminately activate both coupled eNOS and uncoupled eNOS, increasing both NO release and H_2O_2 release. However, oxidative stress induced by ESWL oxidize BH_4 to BH_2, and will set up increased uncoupling of eNOS thereafter leading to increases levels of H_2O_2 release and decreased levels NO release.

PKC-ε Inhibitor Effects on NO and H_2O_2 Release

PKC-ε inhibitor infusion after ESWL will attenuate H_2O_2 release and lessen the decrease of NO release. PKC-ε inhibitor, as with activator, will inhibit both coupled eNOS and uncoupled eNOS, attenuating both H_2O_2 release and NO release. Since more eNOS are uncoupled after ESWL treatment, inhibition of eNOS will cause a greater effect in attenuating H_2O_2 while allowing NO bioavailability to remain less affected by ESWL.

METHODS

Measurement of NO and H_2O_2 Release from Rat Renal Veins post-ESWL Treatment

Each rat was anesthetized with an induction dose of sodium pentobarbital (60mg/kg) via intraperitoneal injection. A maintenance dose (30mg/kg) was delivered at intervals of 45 minutes unless otherwise necessary. The rat was then placed on the operating table with the left kidney positioned within the range of the lithotripter's focal point and injected intraperitoneally with 1 milliliter (mL) sodium heparin (1000 USP units/mL). A 24-gauge catheter was inserted into the external jugular for drug infusion after ESWL treatment. The animal was then subjected to a mid-line laparotomy and the left renal vein was isolated.

Upon catheterization of the left renal vein with a 22-gauge angiocatheter, the microsensor was inserted through the catheter and connected to the free radical analyzer. The free radical trace was recorded until a decrease of one picoamp (pA) per second, indicating a stable baseline. After the establishment of a baseline, ESWL treatment was induced by a total of 1000 shocks in two periods of 500 shocks. The first low-frequency period included 500 shocks at 60 beats per minute followed by the high-frequency second period of 500 shocks at 120 beats per minute, resulting in approximately 13 minutes of shockwave treatment[67]. Immediately post-ESWL treatment, 0.5mL saline or drug bolus was infused through the jugular vein followed by 0.5mL of saline as a flush. Recordings were taken at the beginning and end of treatment, then every five minutes until 30 minutes post-treatment.

Animal Model

The Institutional Animal Care and Use Committee of the Philadelphia College of Osteopathic Medicine approved the animal protocol performed in this study. Male Sprague-Dawley rats (275-325 grams, Ace Animals, Boyertown, PA) were used for all experiments.

Experimental Apparatus

During the experiment, free radical concentration data was recorded and analyzed by a TBR 4100 Free Radical Analyzer (World Precision Instruments, Sarasota, FL). Only one microsensor, H_2O_2 or NO, was connected to the free radical analyzer via a via an interface inside the microsensor cable handle and the free radical analyzer linked with a computer to an analytical program, DataTrax2 (World Precision Instruments, Sarasota, FL), which read and recorded the data. A Dornier Epos Ultra HE (high-energy) lithotripter transmitted the ESWL treatment with shock waves at intensity level 13 (16kv), the maximum for this model of lithotripter.

Microsensor Calibration

Each microsensor was calibrated before each experiment to calculate a standard curve, which provided an accurate and consistent response to translate data across different animals. Traces were recorded from electrical responses in picoamps and translated into molar concentration in vivo. The standard curve was generated by a stepwise dose-response of the microsensor to the appropriate standard solution.

Since there are no sensors to directly measure SO, H_2O_2 microsensors were used instead. Superoxide dismutase present in cells catalyzes the conversion of SO and hydrogen ions to H_2O_2, thus H_2O_2 was measured to gauge SO release. The H_2O_2 standard solution, a 1.0mM H_2O_2 solution in deionized water was prepared while the reference tip was submerged in a 10mL 0.01M PBS (phosphate buffered saline) solution until the registered trace reached a stable baseline. Doses of 2.5µL, 5µL, 10µL, and 20µL of 1mM H_2O_2 were added stepwise to record the H_2O_2 standard curve.

For the NO microsensors, 50mL aqueous solution with solutes of 1mg ethylene diamine tetraacetic acid and 1.06mg of S-nitroso-*N*-acetyl-1,1-penicillamine (SNAP) was prepared as the NO standard solution. The NO reference tips were soaked in 10mL of 0.2M $CuSO_4 \cdot 5H_2O$ until baseline, then doses of 1µL, 5µL, 10µL, and 20µL the SNAP solution were used to record the NO standard curve.

Treatment and Control Groups

Each group was exposed to the same number and frequency of shockwaves. Only the left renal vein can be sufficiently isolated to insert the catheter, limiting one microsensor per experiment and thus only NO or H_2O_2 could be measured at one time.

Table A. Treatment and Control Groups

Group	Nitric Oxide (NO)	Hydrogen Peroxide (H_2O_2)
No-ESWL Control (No drug)	**n = 5**	**n = 5**
ESWL Control (1mL 0.9% Saline)	**n = 6**	**n = 6**
PKC-ε Activator (.9mg/kg, 10μM)	**n = 5**	**n = 5**
PKC-ε Inhibitor (.8mg/kg, 10μM)	**n = 5**	**n = 5**

Statistical Analysis

All data in the text and figures were presented as means ±SEM. The data for each time-point in the recordings were analyzed by ANOVA using post hoc analysis with Fischer's PLSD test for data. Probability values of less than 0.05 were considered to be statistically significant.

RESULTS

H_2O_2 and NO release in Rat Left Renal Vein

All graphs in this section are measured as relative changes. A relative change graph arbitrarily sets the baseline at 0. When a drug treated group exhibits decreased NO or H_2O_2 release it is represented as a change from 0 to a negative number on the graph.

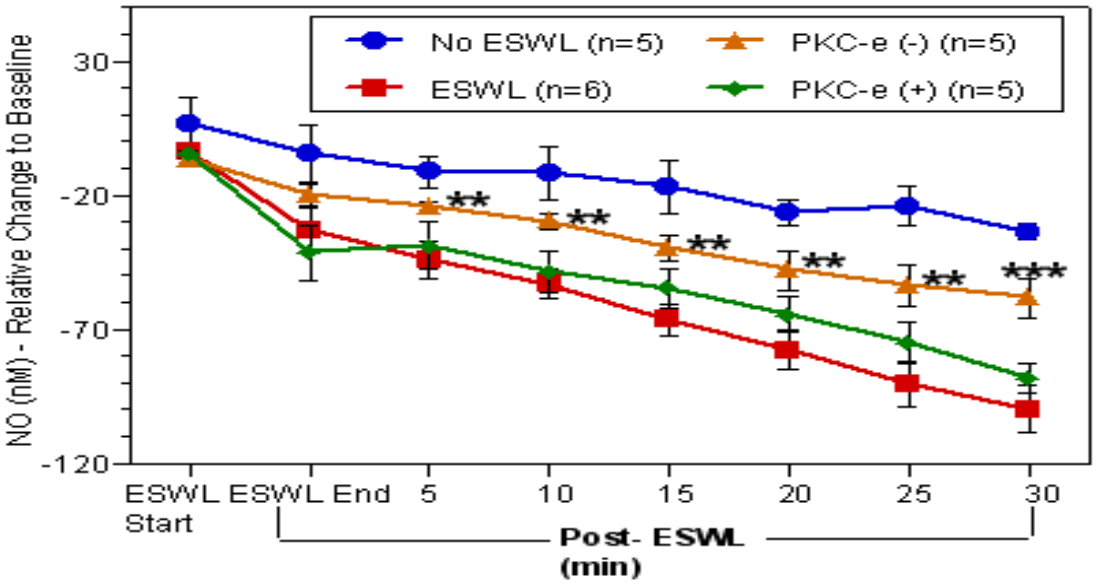

Figure 3. Real-time Blood NO Changes During Experiment. This graph shows change in NO renal vein blood values post-ESWL. ESWL significantly induced a 70nm decrease in NO release compared to no-ESWL and this effect was significantly attenuated by PKC-ε inhibitor. Saline and PKC-ε activator significantly reduced NO levels compared to no-ESWL controls ($^{\#}p\leq0.05$, $^{\#\#}p\leq0.01$). PKC-ε inhibitor significantly attenuated the ESWL-induced decrease in NO level (*$p\leq0.05$, **$p\leq0.01$, compared to ESWL).

Effects of ESWL, PKC-ε Activator, PKC-ε Inhibitor on NO Release

The results shown in Figure 3 display the relative changes of NO release within the renal vein after 30 minutes of ESWL treatment during the no-ESWL control saline control, the ESWL-treated saline control, infusion of PKC-ε activator post-ESWL and infusion of PKC-ε inhibitor. In this graph, the ESWL-saline control

shows a steeper and prolonged decrease of NO release post-ESWL. ESWL reduced NO release levels approximately 70nm compared to the no-ESWL controls. Administration of PKC-ε activator resulted in a similar trend to ESWL-saline controls, decreasing much more than no-ESWL controls. PKC-ε inhibitor caused a counteractive effect against NO decrease from ESWL and attenuated the decrease of NO release from ESWL treatment throughout the 30 minutes of the experiment. PKC-ε inhibitor appeared to have maintained around 70% of NO release as opposed to the saline control.

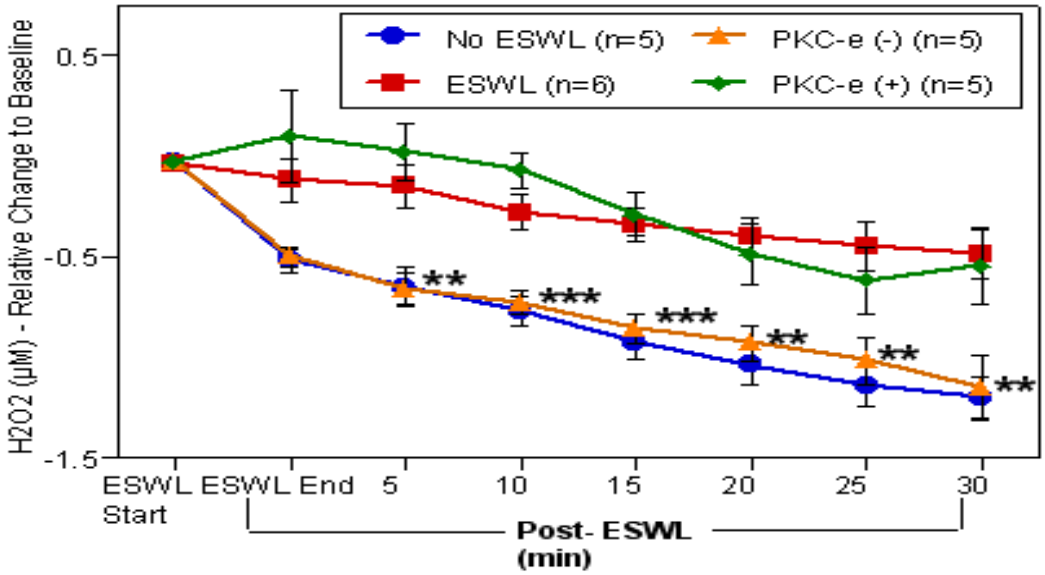

Figure 4. Real-time Blood H_2O_2 Changes Relative to Baseline. This graph shows change in H_2O_2 renal vein blood values post-ESWL. ESWL significantly induced a 750nm increase in H_2O_2 release compared to no-ESWL and this effect was significantly attenuated by PKC-ε inhibitor. Saline and PKC-ε activator H_2O_2 levels significantly rise compared to no-ESWL controls ($^{\#}p\leq0.05$, $^{\#\#}p\leq0.01$). PKC-ε inhibitor significantly attenuated ESWL-induced H_2O_2 release, lowering levels similar to no-ESWL (*$p\leq0.05$, **$p\leq0.01$, compared to ESWL).

Effects of ESWL, PKC-ε Activator, PKC-ε Inhibitor on H_2O_2 Release

The results shown in Figure 4 display the relative changes of H_2O_2 release also within the renal vein after 30 minutes of ESWL treatment during the no-ESWL control saline control, the ESWL-treated saline control, infusion of PKC-ε activator post-ESWL and infusion of PKC-ε inhibitor. In this graph, ESWL-saline controls maintain a higher level of H_2O_2 levels post-treatment. ESWL effectively increased H_2O_2 levels approximately 750nm compared to the no-ESWL controls, which showed a steady decline over the 30 minute time-course. PKC-ε activator infusion again showed similar trend to ESWL-saline controls fluctuating near the levels of the ESWL-saline controls. PKC-ε inhibitor significantly reduced H_2O_2 after ESWL treatment through the rest of the experiment. H_2O_2 levels returned release levels similar to no-ESWL controls, negating the effect of ESWL.

DISCUSSION

Summary of major findings

The major findings conclude the results found in the comparison of no-ESWL treated rats to ESWL-treated rats and between ESWL-treated saline controls to PKC-ε activator and inhibitor groups. In ESWL-treated rats, blood NO decreased while blood H2O2 increased compared to no-ESWL controls. This supports our hypothesis that oxidative stress and reduced NO bioavailability are induced by ESWL. PKC-ε activator was similar to ESWL-saline controls, which may be principally due to increased uncoupled eNOS activity. PKC-ε inhibitor significantly attenuated ESWL-induced effects on H_2O_2 increase and NO decrease, which may be due to inhibition of uncoupled eNOS.

The effect of ESWL on normal levels H_2O_2/NO release

The results from ESWL treatment on levels of H_2O_2 and NO release further confirmed increased oxidative stress post-ESWL treatment. Levels of H_2O_2 release were increased throughout the 30-minute post-ESWL time-course, while NO levels continually decreased after treatment. Even given the current procedure for shock wave treatment, oxidative stress still occurs after ESWL delivery. This oxidative stress can result in endothelial dysfunction by promoting leukocyte-endothelial interactions, thus causing an inflammatory response after ESWL. The decrease in NO bioavailability can cause the upregulation of leukocyte adhesion molecules as seen in other studies by other research done in this lab[9,10]. The invasion of PMNs into renal paremchymal tissue can lead to underlying tissue damage that may cause clinical hypertension, especially in patients that undergo multiple lithotripsies[27].

The effect of PKC ε+ on H_2O_2/NO release following ESWL treatment

This study showed that activation of PKC-ε supports and maintains oxidative stress caused by ESWL. Although the levels of H_2O_2 and NO fluctuate during the post-ESWL recordings, these fluctuations are statistically insignificant and generally follow the trend of ESWL-saline controls. The early rise in H_2O_2 may indicate uncoupled eNOS activity is increasing, while the later drop in H_2O_2 levels may show coupled eNOS returning the balance through increased activity of coupled eNOS. In summary, the findings indicate that PKC-ε activator is not protective in oxidative stress in ESWL.

The effect of PKC ε- on H_2O_2/NO release following ESWL treatment

PKC-ε inhibitor caused a remarkable effect on H_2O_2 and NO levels post-ESWL treatment. Through the inhibition of PKC-ε, H_2O_2 levels dropped to the no-ESWL treatment controls, and NO levels increased 60% to 70% compared to ESWL-saline controls. This indicates an opposing role towards PKC-ε activator, and that PKC-ε inhibitor is protective against oxidative stress brought upon by shock waves. Regardless of the enzymatic profile, all eNOS activity is inhibited. Because ESWL leads to an increase in oxidative stress, the oxidative stress is suggested to increase oxidation of BH_4 to BH_2, leading to increased ratio of BH_2-to-BH_4 cofactor that results in an increased ratio of uncoupled eNOS to coupled eNOS. In this situation, uncoupled eNOS outnumber coupled eNOS, producing more SO to quench NO bioavailability. Even though when PKC-ε inhibitor is applied all eNOS is inhibited, the activity of more uncoupled eNOS is decreased, protecting NO production from

coupled eNOS, and the sources, such as hemoglobin and myoglobin reductase, which converts plasma nitrites to NO[76]. This allows NO bioavailability to be increased, but PKC-ε inhibitor did not completely retain no-ESWL levels of NO bioavailability due to the nature of the inhibition. PKC-ε inhibition indiscriminately leads to a reduction of eNOS activity and does not selectively inhibit uncoupled or coupled eNOS. Even with the decrease in coupled eNOS activity, NO bioavailability remains 700nM higher than ESWL-saline control levels after the 30-minute recording, preserving critical NO levels even after shock wave treatment is finished. These results suggest that PKC-ε inhibition may inhibit other sources of reactive oxygen species activity to attenuate the quenching of constitutive blood NO levels.

The role of PKC ε on regulation of eNOS activity on oxidative stress in ESWL

SO production in the blood which leads to increased levels of H_2O_2 can be produced by multiple sources, such as PMNs, endothelial NADPH oxidase, incomplete oxidative phosphorylation in mitochondria and uncoupled eNOS. Since the results in the study indicate that PKC-ε inhibitor, which is constitutively expressed only in ECs, decreased H_2O_2 levels to no-ESWL control levels, increased SO production in PMNs from NADPH oxidase can be ruled out. Since in the ischemia-reperfusion model, transmigrated PMNs did not release cytoxic substances until 30 minutes, PMNs were ruled out as a contributing factor to H_2O_2 release in ESWL-treated kidneys, and SO production was primarily attributed to EC enzymes such as endothelial NADPH oxidase and eNOS[61,73]. In the previous study, PKC-βII was inhibited, thus attenuating its activation of NADPH oxidase. However, in this study, since H_2O_2 level dropped to no-ESWL control levels, PKC-ε inhibitor

completely abolished SO production, suggesting that uncoupled eNOS is the primary producer of SO in the kidney rather than NADPH oxidase[73]. Because PKC-ε inhibition causes a dramatic difference in H_2O_2 and NO release, PKC-ε acts as a major operator in regulation of eNOS during oxidative stress post-ESWL.

Future Studies

In order to assess whether there are increased leukocyte-endothelial interactions and the possibility of a subsequent inflammatory response, western blot and immunohistochemistry staining studies must be performed to determine an upregulation of proteins involved in leukocyte-endothelial reactions, such as P-selectin, ICAM-1, and PECAM-1. The results from those studies will determine if prolonged vasoconstriction resultant of ESWL is an inflammatory response that can lead to damage of renal tissue. Future research will be directed towards investigating the effects of BH_4, BH_2, as well as BH_4-to-BH_2 ratio, to determine whether adjusting the cofactor status of eNOS would provide the same supportive effects towards NO bioavailability. PKC-ε activator and inhibitor will also be tested for pre-conditioning in the lithotripsy model, to gauge its effects before ESWL treatment and compare them to this study's post-ESWL effects. Finally, high-performance liquid chromatography will be utilized to record the levels of BH_2 and BH_4, and the concentration ratio between the two pterins from both treated and non-treated kidneys to investigate their levels under conditions of oxidative stress, provide further evidence for eNOS uncoupling during oxidative stress, and the importance of a maintained BH_4-to-BH_2 ratio.

Significance of Findings

Until the technology to improve imaging and targeting of kidney stones can be developed, treatment for damage from ESWL must rely upon refining the technique of ESWL delivery or pharmaceutical intervention. Although much research has been conducted on fine-tuning the ESWL delivery procedure, not much has been directed toward oxidative stress that may lead to underlying inflammatory response and cell damage. This study further confirms that even under the safer procedure guidelines, levels of H_2O_2 and NO indicate a decreased NO bioavailability and increased oxidative stress post-ESWL treatment. Our research has also found that eNOS is the principle target responsible for oxidative stress after lithotripsy. The data from this study suggests beneficial effects of PKC-ε inhibitor may be due to inhibition of uncoupled eNOS activity and that uncoupled eNOS activity is the primary source of oxidative stress post-ESWL since PKC-ε inhibitor reduces H_2O_2 levels to near no-ESWL levels. By using PKC-ε inhibitor after ESWL, the decreased oxidative stress can attenuate vascular endothelial dysfunction to the renal vasculature. This study suggests that PKC-ε inhibitor may be applied clinically post-ESWL to reduce vascular complications that can lead to development of hypertension.

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