Journal of the American College of Cardiology
© 2007 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 49, No. 4, 2007
ISSN 0735-1097/07/$32.00
doi:10.1016/j.jacc.2006.10.043

PRECLINICAL RESEARCH

Lipopolysaccharide Activates Calcineurin in Ventricular Myocytes

Jun Suzuki, MD, PHD,*† Evelyn Bayna, PHD,* Hai Ling Li, PHD,* Erminia Dalle Molle, BS,*† Wilbur Y. W. Lew, MD, FACC*†

San Diego, California

Objectives We investigated whether lipopolysaccharide (LPS), a proximate cause of inflammation, activates calcineurin in cardiac myocytes and if calcineurin regulates apoptosis in this setting.

Background Calcineurin regulates myocardial growth and hypertrophy, but its role in inflammation is unknown. Calcineurin has proapoptotic or antiapoptotic effects depending on the stimuli.

Methods Calcineurin activity was measured in left ventricular myocytes from adult Sprague Dawley rats. Cardiac apoptosis was measured by terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling staining and caspase-3 activity after in vitro and in vivo exposure to LPS.

Results Lipopolysaccharide increased calcineurin activity in myocytes over 1 to 24 h (t 1/2 = 4.8 h) with an EC_{50} of 0.80 ng/ml LPS ($p < 0.05$, $n = 4$). The LPS (10 ng/ml) effects were mimicked by angiotensin II (Ang II) (100 nmol/l); both increased calcineurin activity and induced apoptosis without additive effects ($p < 0.05$, $n = 5$ to 9). Lipopolysaccharide and/or Ang II effects were prevented by 1 h pre-treatment with an Ang II type 1 receptor blocker (losartan, 1 μmol/l), calcineurin inhibitor (cyclosporin A, 0.5 μmol/l), calcium chelator (1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl) ester, 0.1 μmol/l), or by inhibiting sarcoplasmic reticulum (SR) calcium (Ca)-ATPase (thapsigargin, 1 μmol/l) or SR calcium release channel (ryanodine, 1 μmol/l). Left ventricular apoptosis increased from 4 to 24 h after LPS (1 mg/kg intravenously) in vivo, but not in rats pre-treated with cyclosporin A (20 mg/kg/day subcutaneously) for 3 days ($p < 0.05$, $n = 5$).

Conclusions In cardiac myocytes, LPS activates calcineurin in association with apoptosis by Ang II and SR calcium-dependent mechanisms. This expands the paradigm for cardiac calcineurin to be activated by low levels of LPS in inflammation and chronic conditions (e.g., infections, smoking, and heart failure). (J Am Coll Cardiol 2007;49:491–9)

Calcineurin is an important mediator of cardiac growth and hypertrophy (1,2). The stress signals that activate cardiac calcineurin in physiological and pathophysiological conditions, such as pressure overload and ischemia, have been studied extensively. However, little is known about the role of calcineurin in inflammation. Inflammation contributes to the pathogenesis of atherosclerosis and vascular events (3) and may contribute to the progression of heart failure (4). It is unknown if inflammation activates calcineurin in cardiac myocytes.

See page 500

Lipopolysaccharide (LPS) from gram negative bacteria is one of the most common causes of inflammation and the best characterized activator of innate immunity (5,6). Low levels of LPS may mediate vascular inflammation in atherosclerosis (7). Cells sense minute amounts of LPS through Toll-like receptor-4 (TLR-4), an LPS receptor that recognizes pathogen-associated molecular patterns and is required for cell signaling. Cardiac myocytes express TLR-4 (8), although they are not professional cells involved in innate immunity. Studies from this laboratory demonstrated that LPS directly activates cardiac myocytes to depress contractility (9) and induce apoptosis (10), independently of secondary mediators released by non-myocytes. This occurs with low levels of LPS comparable to those found circulating in subacute

From the *Cardiology Section, Department of Medicine, V.A. San Diego Healthcare System, San Diego, California; and the †University of California, San Diego, San Diego, California. This research was supported by the Medical Research Service, Department of Veterans Affairs, and by Tobacco-Related Disease Research Program Grant TRDRP 9RT-0166 from the University of California, Office of the President. Joel S. Karliner acted as guest editor for this article. Dr. Suzuki is currently affiliated with the Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan; and Dr. Li is currently affiliated with Discovery Research, Schering Plough Biopharma, Palo Alto, California.

Manuscript received November 1, 2005; revised manuscript received August 31, 2006, accepted September 1, 2006.

JACC Vol. 49, No. 4, 2007
January 30, 2007:491–9

Abbreviations and Acronyms

Ang II = angiotensin II

AT_1 = angiotensin II type 1 receptor

BAPTA-AM = 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester

LPS = lipopolysaccharide

MPT = mitochondrial permeability transition pore

RyR = ryanodine receptor

SR = sarcoplasmic reticulum

TLR-4 = Toll-like receptor-4

TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

and chronic conditions, such as chronic infections, smoking, and heart failure (11–13).

The goal of this study was to determine if LPS activates calcineurin in cardiac myocytes. This would expand the list of known activators of cardiac calcineurin to include inflammation, along with previously established conditions of cardiac growth, hypertrophy, and ischemia-reperfusion. Calcineurin may be activated by LPS in macrophages (14), but it is unknown if LPS activates calcineurin in cardiac myocytes.

Calcineurin is a serine/threonine protein phosphatase that is activated by a sustained increase in calcium. Lipopolysaccharide increases intracellular calcium in cardiac myocytes (15,16). It is unknown if this is sufficient to activate calcineurin, which may depend on specialized pools of calcium at specific intracellular sites (17,18). Several sites for activating calcium have been proposed, but none have been proven experimentally (17). We hypothesized that LPS activates calcineurin with calcium cycling through the sarcoplasmic reticulum (SR). The rationale is that the SR plays a central role for calcium handling and there is a physical and functional association between calcineurin and the SR calcium release channel or ryanodine receptor (RyR) (19,20). The calcineurin inhibitor FK506 targets FK506 binding proteins, which regulate RyR function (21).

A secondary goal was to determine if calcineurin plays a regulatory role to enhance or inhibit cardiac apoptosis induced by LPS. Calcineurin enhances cardiac apoptosis induced by beta-adrenergic stimulation (22), but inhibits apoptosis induced by oxidative stress or ischemia-reperfusion (23,24). Calcineurin can induce or inhibit apoptosis in the same cell depending on the concurrent activation of downstream signaling pathways (25,26). Thus, the effects of calcineurin on apoptosis are stimuli and context-dependent. As a subsidiary goal, we evaluated if calcineurin is activated by the same cell signaling pathway as LPS-induced apoptosis, which we found to be mediated by activating cardiac renin-angiotensin to stimulate angiotensin II type 1 (AT_1) receptors (10,27). Angiotensin II (Ang II) increases calcineurin in cardiac myocytes (28,29).

This study demonstrates that LPS activates calcineurin in association with apoptosis in cardiac myocytes. This occurs with low levels of LPS found in chronic conditions. This provides a unique link between inflammation activated by LPS, and calcineurin, an important signaling pathway for myocardial growth and hypertrophy.

Methods

Experiments were performed in accordance with institutional guidelines and the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cardiac myocyte preparation and protocols. Cardiac myocytes were isolated from adult Sprague Dawley rats (250 to 400 g, either gender), as previously described (10). In brief, rats were anesthetized with 40 mg/kg sodium pentobarbital intraperitoneally. The heart was excised and perfused with 15 to 30 mg/kg of depyrogenated collagenase B and protease containing <0.3 to 0.5 ng/ml LPS (*Limulus* amobocyte lysate test QCL-1000, BioWhittaker, Walkersville, Maryland) (30). Freshly isolated myocytes were plated on dishes pre-coated with laminin in a Dulbeccos modified Eagle's media and stored at 37°C in 5% CO_2 (10).

Myocytes were incubated with LPS (*Escherichia coli* 055, LPS no. B5, lot 2039F, List Biological Laboratories, Campbell, California) and/or Ang II, preceded by 1 h exposure to inhibitors including losartan (AT_1 receptor inhibitor, a kind gift from Merck and DuPont, Rahway, New Jersey), 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM) (calcium chelator), thapsigargin (SR calcium ATPase inhibitor), ryanodine (SR calcium release channel inhibitor, Calbiochem, San Diego, California), cyclosporin A (calcineurin inhibitor), or nicotine (an inhibitor of LPS-induced cardiac apoptosis) (27) (unless otherwise noted, all reagents from Sigma Chemical, St. Louis, Missouri).

Calcineurin and apoptosis assays in cardiac myocytes and left ventricle. Calcineurin assays were performed on cardiac myocytes harvested after 16 h of incubation. After cell lysis, cellular calcineurin (PP2B) phosphatase activity was measured using a BIOMOL GREEN Calcineurin Assay Kit (BIOMOL Research Laboratories, Plymouth Meeting, Pennsylvania). Ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid (EGTA) was used as the calcineurin inhibitor, and results were expressed as Total − EGTA phosphate nmol/μg total protein. Protein content was determined using a standard colorimetric assay (BCA, Pierce Chemical, Rockford, Illinois). The calcineurin activity data were fit to curves using GraphPad Prism, version 4.0 from GraphPad Software, Inc. (San Diego, California).

Apoptosis was assessed in cardiac myocytes fixed with 4% formalin phosphate-buffered saline after 24 h of incubation. Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed using a CardioTACS In Situ Apoptosis Detection kit (R&D Systems). At least 2,000 cells were scored from each group with the observer blinded to the treatment condition.

In vivo studies were performed in rats injected with LPS (1 mg/kg) or saline into a tail vein (10). After 24 h, the heart was excised and fixed in 3.7% formaldehyde solution for