4-Hydroxy-2-nonenal Induces Calcium Overload via the Generation of Reactive Oxygen Species in Isolated Rat Cardiac Myocytes

KAZUFUMI NAKAMURA, MD, PhD,[1] DAIJI MIURA, PhD,[1,2] KENGO FUKUSHIMA KUSANO, MD, PhD,[1] YOSHIHISA FUJIMOTO, MD, PhD,[3] WAKAKO SUMITA-YOSHIKAWA, MD,[1] SOICHIRO FUKE, MD,[1] NOBUHIRO NISHII, MD, PhD,[1] SATOSHI NAGASE, MD, PhD,[1] YOSHIKI HATA, MD, PhD,[1] HIROSHI MORITA, MD, PhD,[1] HIROMI MATSUBARA, MD, PhD,[3] TOHRU OHE, MD, PhD, FACC,[1,2] AND HIROSHI ITO, MD, PhD[1]

Okayama, Japan

ABSTRACT

Background: It has been reported that that the amount of 4-hydroxy-2-nonenal (HNE), which is a major lipid peroxidation product and a cytotoxic aldehyde, is increased in the human failing myocardium. This study was designed to determine whether HNE has a pro-oxidant effect in cardiac myocytes and whether HNE causes Ca^{2+} overload.

Methods and Results: Exposure to HNE for 10 minutes in the presence of ferric nitrilotriacetate induced the production of hydroxyl radical (·OH) in the rat myocardium as assessed by electron spin resonance spectroscopy, and HNE induced the generation of reactive oxygen species (ROS) in a dose-dependent manner as assessed by 2′, 7′-dichlorofluorescein diacetate fluorescence. HNE increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as assessed by fura-2 ratio in a dose- and time-dependent manner. After 20 minutes of HNE (400 μmol/L) exposure, hypercontracture was induced in 67% of the cells. Catalase, an antioxidative enzyme that can decompose hydrogen peroxide (H_2O_2), significantly attenuated the increase in $[Ca^{2+}]_i$ and completely inhibited hypercontracture. Carvedilol, a β-blocker with potent antioxidant activity, also significantly attenuated the increase in $[Ca^{2+}]_i$ and completely inhibited hypercontracture, but propranolol had no effect on either $[Ca^{2+}]_i$ increase or hypercontracture.

Conclusions: HNE induces the formation of ROS, especially H_2O_2 and ·OH, in cardiomyocytes and subsequently ROS cause intracellular Ca^{2+} overload. HNE formation may play an important role as a mediator of oxidative stress in heart failure. (*J Cardiac Fail 2009;15:709−716*)

Key Words: Reactive oxygen species, heart failure, calcium overload.

Oxidative stress has been implicated in the pathogenesis of heart failure. Reactive oxygen species (ROS) are produced in the failing myocardium[1−4] and cause hypertrophy, apoptosis/cell death, and intracellular Ca^{2+} overload in cardiac myocytes.[5−9] Furthermore, ROS cause damage to lipid cell membranes in the process of lipid peroxidation. In this process, several aldehydes, including 4-hydroxy-2-nonenal (HNE), are generated. HNE is recognized as the most reliable marker of lipid peroxidation,[10−14] Mak et al have reported that the amount of HNE is increased in the plasma of patients with heart failure.[15] Moreover, we have demonstrated that the amount of HNE is increased in the human failing myocardium.[2,3] HNE is recognized not only as a reliable marker of oxidative stress, but also as a toxic aldehyde to many types of cells.[10−13] HNE exhibits cytopathological effects, such as enzyme inhibition and inhibition

From the [1]Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [2]Department of Cardiology, Cardiovascular Center, Sakakibara Hospital, Okayama, Japan; and [3]Division of Cardiology, National Hospital Organization Okayama Medical Center, Okayama, Japan.

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Reprint requests: Kazufumi Nakamura, MD, PhD, Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Tel: +81-86-235-7351; Fax: +81-86-235-7353. *E-mail addresses:* ichibun@cc.okayama-u.ac.jp or ichibun@yahoo.ac.jp

The first 2 authors contributed equally to this work.

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of DNA, RNA, and protein synthesis, and then HNE has pro-oxidant properties. HNE can markedly induce intracellular production of ROS in cultured rat hepatocytes and human neuroblastoma cells.[13,16,17] It is speculated that HNE also exacerbates heart failure by increasing oxidative stress in myocardium. Therefore, we hypothesized that HNE induces Ca^{2+} overload via the generation of ROS in cardiac myocytes. To test this hypothesis, we examined whether HNE induces the generation of ROS, whether HNE increases intracellular calcium concentration ($[Ca^{2+}]_i$), and whether antioxidants (catalase, an enzyme which can decompose hydrogen peroxide (H_2O_2) and carvedilol (a β-blocker with potent antioxidant activity) inhibit the increase of $[Ca^{2+}]_i$ in isolated rat cardiac myocytes.

Materials and Methods

Isolation of Adult Rat Cardiac Myocytes

Cardiac myocytes were isolated from the left ventricular free wall of the adult rat heart essentially as described elsewhere.[18] In brief, hearts were quickly excised from 11- to 13-week-old male Wistar rats (CLEA Japan, Inc., Tokyo, Japan), the ascending aorta were cannulated, and the heart were retrogradely perfused with a collagenase (0.8 mg/mL, Nitta Zeratin, Osaka, Japan)-containing buffer of the following composition (Tyrode's solution) (mmol/L): NaCl, 135; KCl, 5; HEPES, 10; MgCl2 1.0; EGTA, 1.25; albumin, 0.5 mg/mL; pH 7.4. When the heart had become soft, the left ventricle was dissected free and gently minced. Dissociated myocytes were seeded into 35-mm glass base dishes (IWAKI, Funabashi, Chiba, Japan) coated with poly L-lysine (SIGMA-ALDRICH, St. Louis, MO), fed with medium M199 (PAA Laboratories, Pasching, Austria) containing kanamycin (100 μg/mL), and kept in a humidified environment of 5% CO_2 at 37°C. All animal protocols were approved and conducted according to the recommendations from Okayama University on Animal Care and Use. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Quantification of ROS by Electron Spin Resonance Spectroscopy

Electron spin resonance (ESR) spectroscopy was used for the assessment of intracellular ROS formation in rat heart essentially as previously described.[19] Hearts were quickly excised from 11- to 13-week-old male Wistar rats (CLEA Japan, Inc., Tokyo, Japan), weighed, and retrogradely perfused with HNE (100 μmol/L) (Calbiochem, San Diego, CA) or a diluent (control) and ferric nitrilotriacetate (Fe-NTA) (100 μmol/L)-containing buffer at 37°C for 10 minutes. Bhatnagar reported that 400 μmol/L HNE induces rigor shortening of myocytes within 60 minutes.[20] Therefore, we used less than 400 μmol/L HNE (10 or 100 μmol/L) to investigate the generation of ROS in the rat myocardium. Fe-NTA facilitates formation of the hydroxyl radical (·OH) in the presence of H_2O_2 via the Fenton reaction.[6] The hearts were homogenized at 4° for 1 minute in 10 volumes of 50 mM Tris-HCl buffer. The homogenate was centrifuged at 12,000*g* for 10 minutes at 4°. The resultant supernatant was reacted with a spin trapping agent, alpha-phenyl-N-tert butylnitrone (250 mM final concentration, LABOTEC, Ltd, Tokyo, Japan). The mixture was incubated at 37° for 90 minutes. The hydroxyl radical (·OH) adduct of alpha-phenyl-N-tert butylnitrone was measured at room temperature using ESR spectroscopy (JES-FR30, JEOL Ltd, Tokyo, Japan). Quantification of the alpha-phenyl-N-tert butylnitrone signal intensity was performed by comparing the amplitude of the standard Mn^{2+} marker. The ESR settings were as follows: power, 4 mW; magnetic field, 335.6 ± 5 mT; modulation frequency, 9.41 GHz; modulation amplitude, 1 × 0.1 mT; response time, 0.1 seconds; sweep time, 2 minutes.

Analysis of Dichlorofluorescein (DCF) Fluorescence

A fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes Inc., Eugene, OR), was used for the assessment of intracellular ROS formation in rat cardiac myocytes as previously described.[5,21] This is a nonspecific detector of the production of intracellular ROS such as H_2O_2, ·OH, and hydroperoxides.[22,23] DCFH-DA was dissolved in absolute ethanol at a concentration of 5 mmol/L. Isolated rat cardiac myocytes were seeded into 35-mm glass-base dishes coated with poly L-lysine. HNE (10 or 100 μmol/L) was administered simultaneously with DCFH-DA (5 μmol/L) in medium M199. After incubation at 37°C for 10 minutes, cardiac myocytes were washed with M199. Fluorescence images were acquired with a fluorescence microscope (IX71, Olympus, Tokyo, Japan). The fluorescence intensity per cardiac myocyte was measured using the software Lumina Vision Ver2.4 (Mitani, Osaka, Japan).

Assay for Cell Viability

Cell viability was examined by the trypan blue assay as previously described.[24] Cardiac myocytes were exposed to HNE (400 μmol/L) at room temperature for 20 minutes and then stained with trypan blue (Nacalai Tesque, Kyoto, Japan). We used a high level of HNE (400 μmol/L) by reference to a previous report.[20] Viable cells with an intact membrane excluded trypan blue and were not stained.

Measurement of Relative Intracellular Calcium Concentration ($[Ca^{2+}]_i$) of Isolated Cardiac Myocytes under Resting Condition

Isolated cardiac myocytes in 35-mm glass-base dishes coated with poly L-lysine were loaded in a dark room at room temperature in M199 containing 2 μmol/L of the acetoxymethyl ester of Fura-2 (Fura-2 AM, Molecular Probes, Eugene, OR) as described elsewhere.[25] After 20 minutes, cells in the dishes were washed, and maintained in M199 under resting condition. The dishes were placed on an inverted fluorescence microscope (Axiovert 200, Carl Zeiss, Jena, Germany) and fluorescence images were acquired.

$[Ca^{2+}]_i$ of isolated cardiac myocytes under resting condition was obtained as a relative value by calculating the ratio of fluorescence intensity at 510 nm emitted from dye activated at 2 wavelengths (340 and 380 nm), using the fluorescence ratio imaging system AQUACOSMOS (Hamamatsu Photonics, Hamamatsu, Japan). The myocytes were treated with HNE (final concentration: 50 to 400 μmol/L, dissolved in absolute ethanol at a concentration of 64 mmol/L, Calbiochem, San Diego, CA), catalase (final concentration: 100 U/mL, dissolved in M199 at a concentration of 10,000 U/mL, SIGMA-ALDRICH, St. Louis, MO), carvedilol (final concentration: 0.1 μmol/L, dissolved in absolute ethanol at a concentration of 10 mmol/L, a gift from Daiichi Pharmaceutical Co., Tokyo, Japan), or propranolol (final concentration: 0.2 μmol/L, dissolved in absolute ethanol at a concentration of 10 mmol/L, SIGMA-ALDRICH, St. Louis, MO). We used 50 to 400 μmol/L HNE by reference to a previous report.[20]