Free Radical Research, August 2005; 39(8): 845–851

Rapid increase in serum lipid peroxide 4-hydroxynonenal (HNE) through monocyte NADPH oxidase in early endo-toxemia

HIROKO KIMURA[1], SHUANG LIU[2], SATOSHI YAMADA[3], KOJI UCHIDA[4], KAZUKO MATSUMOTO[5], MASAHIRO MUKAIDA[6], & KEN-ICHI YOSHIDA[2]

[1]*Department of Forensic Medicine, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan,* [2]*Department of Forensic Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-0033, Japan,* [3]*Life Science Group NOF Corporation Tsukuba Research Laboratory, 5-10, Tokodai, Tsukuba, Ibaraki 300-2635, Japan,* [4]*Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Furocho 1, Chigusa-ku, Nagoya 464-8601, Japan,* [5]*Department of Chemistry, Waseda University, Okubo 3-4-1, Shinjuku-ku, Tokyo 169-8555, Japan, and* [6]*Department of Forensic Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama, Japan*

Accepted by Professor E. Niki

(Received 4 February 2005; in revised form 14 April 2005)

Abstract

We have developed a time-resolved fluoroimmunoassay (TR-FIA) for a lipid peroxide 4-hydroxynonenal (HNE), which is 100-fold more sensitive than conventional enzyme-linked immunosorbent assay (ELISA) and is an easier technique to use for a large number of samples without pre-treatment. By this assay, we found that a low dose of bacterial lipo-polysaccharide (LPS), injected intra-peritoneally (0.5 mg/kg), increased serum HNE level by 28-folds, with a peak at 20 min. LPS also increased HNE *in vitro* to a much higher level in the monocyte-enriched plasma than in the leukocyte-enriched plasma, with a peak at 10 min. The HNE production after LPS treatment was inhibited by apocynin, a specific NADPH oxidase inhibitor *in vivo* and *in vitro*, and to a lesser extent by dimethylsulfoxide a solvent for apocynin and a hydroxyl radical scavenger *in vitro*. These data suggest that monocyte NADPH oxidase is involved in the lipid peroxidation (HNE formation) in the LPS-challenged rat. This is the first clear demonstration of the link between an inflammatory stimulus and lipid peroxidation in the blood.

Keywords: *4-hydroxynonenal (HNE), time-resolved fluoroimmunoassay, lipid peroxide, lipo-polysaccharide, NADPH oxidase*

Introduction

Oxidative stress and lipid/protein oxidation are well known to promote atherosclerosis [1], and ischemic heart disease [2]. Low-density lipoprotein (LDL) oxidation is involved in atherogenesis, whereas the link between inflammation and oxidative stress in association with atherogenesis remains elusive [1,3]. The causality between acute inflammation and oxidative stress is a focus of atherosclerosis research. Additionally, although some anti-hyperlipidemia drugs have anti-oxidative properties [4], there is no substantial method to evaluate their effect in the blood samples.

4-Hydroxynonenal (HNE), a major aldehydic product of lipid peroxidation [5–7], is believed to exert primary pathogenic effects of oxidative stress. HNE is one of the most toxic, biologically active, and stable lipid peroxides under various pathological

Correspondence: K. Yoshidab, Department of Forensic Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-0033, Japan. Tel/Fax: 81 3 5841 3367. E-mail: kyoshida@m.u-tokyo.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10715760500161546

settings [8]. In the patients and animals, measurement of stable lipid peroxides show that oxidative stress is associated with inflammation [9–12]. For example, the lipid peroxides such as malondialdehyde (MDA) have been detected in inflammation by use of HPLC and GC-MS methods [13–15]. There are also reports on enhanced MDA production in the plasma or liver under oxidative stress as detected by 2-thiobarbituric acid (TBA) method [16,17].

Lipo-polysaccharide (LPS) is the most important mediator of bacterial infection and endotoxemia. Administration of a large amount of LPS (20 mg/kg, i.p.) was shown to increase plasma MDA by 2.7-fold, using the colorimetric assay [16]. LPS also increased HNE-modified proteins in the liver after LPS administration, as detected by western blotting [18]. However, there is neither a simple and sensitive assay for HNE nor the findings on the change in HNE under mild inflammatory stimulus as conferred by low dose LPS.

Here, we have developed a highly sensitive time-resolved fluoroimmunoassay (TR-FIA) for a lipid peroxide HNE and show a line of evidence for the causality between early endotoxemia and HNE production through monocyte NADPH oxidase.

Materials and methods

Animal experiments

The animal study was conducted in accordance with the guidelines of the Committee of the Animal Experiments of Graduate School of Medicine, University of Tokyo. Male Sprague-Dawley rats, aged 4 weeks, were purchased (CLEA Japan Inc., Tokyo, Japan). LPS (L-2630 Sigma, St Louis, MO) in physiological saline (0.5 mg/kg, i. p.) was administered to the rats ($n = 4$ for each group). Sham rats received the equal volume of saline. Cardiac blood was harvested at 10, 20, 30 min, 1, 2, 4, 8, 12, 24 h after LPS administration under anesthesia with sodium pentobarbital (75 mg/kg, i. p.). To examine the contribution of NADPH oxidase, 3 group ($n = 4$ for each group) of rats were pre-treated either with apocynin (Calbiochem, la Jolla, CA, 5 mg/kg), dimethylsulfoxide (DMSO) (0.6%) or saline for 30 min. Then, the rats were injected with LPS and left for 20 min. The samples were stored at -80°C until biochemical analyses.

In vitro experiment

Human peripheral blood was separated by Ficoll (Mono-poly resolving medium, Dainippon pharm., Osaka, Japan) density gradient centrifugation. The fractions of mononuclear leukocyte and polymorphonuclear leucocytes were separated and washed twice with phosphate-buffered saline (PBS, pH 7.3) and each fraction was re-suspended in the half volume of the plasma. LPS (7.2 μg/ml plasma) was added to either of the plasma, which were allowed to stand at 37°C for 10, 20, 30, and 60 min and stored at -80°C until use. After disrupting the cells by freeze and thaw, the HNE levels in the supernatant were determined by TR-FIA. To examine the involvement of NADPH oxidase, monocyte-enriched plasma was pretreated with a NADPH oxidase inhibitor apocynin (Calbiochem, la Jolla, CA) for 30 min, before incubation with LPS for 15 min at 37°C. In preliminary experiments, we obtained optimal concentrations of apocynin and its solvent DMSO as 0.2 mM and 0.2%, respectively, for treatment with LPS (5 μg/ml plasma).

Antibodies and antigens

Mouse monoclonal anti-HNE was purchased (Nikken Seil, Shizuoka, Japan). Polyclonal anti-HNE was obtained by immunizing rabbits with a HNE-keyhole limpet hemocyanin (KLH) conjugate [19]. Anti-HNE purified by affinity-chromatography was used. Biotinylated goat polyclonal anti-rabbit IgG was obtained from Vector Laboratories (Burlingame, CA). HNE-bovine serum albumin (BSA) was prepared by incubating 1 mg of BSA (ICN Pharmaceuticals, Cleveland) and 10 mM of HNE ($MW = 156$, Calbiochem) in 2 mL of 67 mM PBS (pH 7.4) for 24 h at 37°C, and then the mixture was dialyzed twice in PBS. The HNE concentration was determined by competitive enzyme linked immunosorbent assay (HNE-histidine adduct ELISA system, NOF Corporation, Tsukuba) using anti-HNE antibody, and HNE-acetyl-histidin. Since it is known that almost all of HNE binds with BSA, we assumed that the concentration of the HNE-BSA preparation is 5 μmol/ml and used as a standard solution.

Labeling of Streptavidin with 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)-chlorosulfo-o-terphenyl (BHHCT)

Streptavidin (SA) was obtained (Chemicon International Inc., Temecula, CA, USA). The SA and BSA conjugates (SA-BSA) were prepared as reported previously [20]. To intensify the labeling ratio of BHHCT to SA, the SA-BSA was labeled with a new chelate, BHHCT as described previously [21].

Time-resolved fluoroimmunoassay (TR-FIA) for HNE proteins

Sandwich type immunoassay using three antibodies was employed (Figure 1). The wells of microtiter plates (Fluoro Nunc module plate, Nalge Nunc International, Denmark) were coated with monoclonal anti-HNE (0.25 μg/0.1 ml) dissolved in 0.1 M carbonate-bicarbonate buffer (pH 9.3) and washed with 0.05 M Tris–HCl buffer containing 0.05%

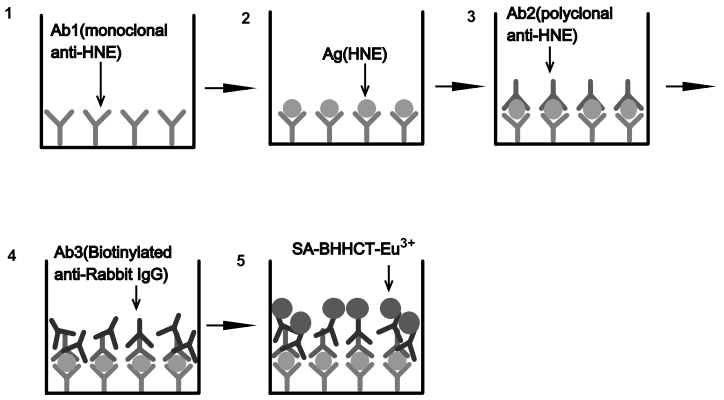

Figure 1. Time-resolved fluoroimmunoassay procedure for HNE modified protein. A large numbers of streptavidin (46 SA/BSA molecule)-conjugated Eu chelate binds to biotinylated anti-rabbit IgG (step 4 and 5). Time-resolved measurement reduces the non-specific fluorescence derived from biological samples. For details, see text.

Tween 20, pH 7.8 (Tris–HCl buffer). The serum samples were diluted 2–5-folds with the Tris–HCl buffer containing 0.2% BSA. In the wells, 50 μl of standard HNE-BSA solutions or the serum (plasma) samples were allowed to react overnight at 5°C. After washings 3 times with the Tris–HCl buffer containing 0.05% Tween 20 (pH 7.8), 50 μl of polyclonal anti-HNE rabbit IgG antibody (2 μg/ml) were added and incubated at 5°C overnight. After washing with the Tris–HCl buffer containing 0.05% Tween 20 (pH 7.8), 50 μl of the biotinylated anti rabbit IgG antibody (7.5 μg/ml) was added and incubated at room temperature for 4 h. Then 50 μl of the BHHCT-Eu^{3+} labeled SA-BSA (SA-BSA-BHHCT-Eu^{3+}) was added after washing, and incubated at 37°C for 1 h. Finally, the wells were washed 3 times with Tris–HCl buffer containing 0.05% Tween 20 (pH 9.1), and the plates were subjected to the time-resolved fluorometric measurement, using an Arvo SX multilabel counter (PerkinElmer Life Sciences, Boston, MA, USA), with excitation at 340 nm, delay time of 0.2 ms, and window time of 0.4 ms. Fluorescence intensity was measured at 615 nm.

Rat C-Reactive protein (CRP) assay

Serum CRP was measured by use of rat C-reactive protein assay kit (Alpha Diagnostics, Tex, USA).

Malondialdehyde (MDA) assay

Serum MDA levels were determined by immunoassay performed by SRL (Tokyo, Japan).

Statistical analysis

Results are expressed as means ± SE. Statistical significance ($P < 0.05$) was evaluated by StatView-J5.0 software (Abacus Concepts Inc., CA, USA) using one-factor ANOVA, with post hoc test of Tukey-Kramer.

Results

The calibration curve of HNE

The calibration curve of HNE is shown in Figure 2. In our TR-FIA, the detection limit of HNE was 1.1 p mol/ml, which is 3-folds of the standard deviation (SD) of the background, as previous reported [21]. In the intra-assay, the coefficients of variation were 1.2–6.7% (mean CV = 3.8%), whereas in the inter-assay, those were 9.2–15.8% (mean CV = 13.3%) at 7 different concentrations (Table I). The detection limit is about 100-times lower than that of conventional ELISA, as previously reported [22]. The recoveries, obtained by adding 0.1, 0.01 and 0.001 nmol/ml of standard HNE-BSA to human sera with known intrinsic HNE level (0.157 nmol/ml), were 83.7, 90.4, and 103.2%, respectively.

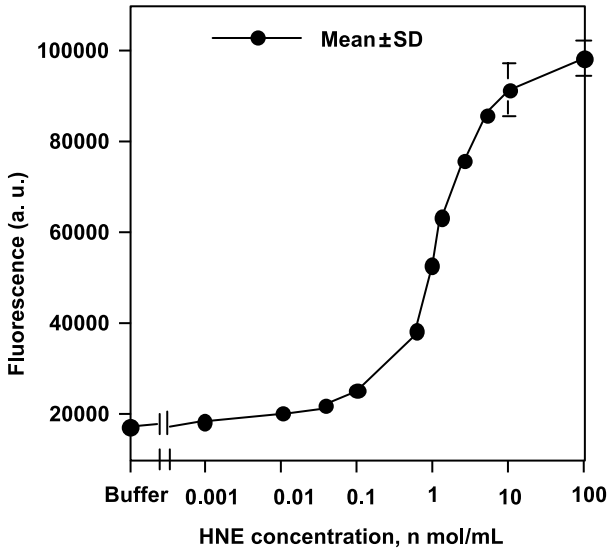

Figure 2. The calibration curve for HNE by time-resolved fluoroimuunoassay. Bars represent mean ± SD. Minimum detection limit (1.1 p mol/ml) is 100-times smaller than ELISA previously reported. For details, see text.

Table I. Intra- and Inter-experimental variations in TR-FIA of HNE.

HNE concentration (n mol/ml)	Intra assay CV(%) $n = 4$	Inter assay CV(%) $n = 7$
0 (buffer)	1.2	15.0
0.001	4.0	13.3
0.01	2.6	9.8
0.1	6.7	15.9
1	1.6	14.9
10	6.5	11.1
100	4.0	13.0
Mean CV	(3.8)	(13.3)

Temporal change in HNE after LPS injection

The levels of rat serum HNE after i.p. injection of LPS were determined by TR-FIA. As shown in Fig 3, HNE was increased rapidly and greatly (28-folds) with a sharp peak at 20 min. The basal level of HNE in plasma of human and experimental animals (0.1–10 nmol/ml) [23] was below the detection limit of the ELISA (10 nmol/ml), as previously reported [22]. The rapid return of the HNE level at 30 min suggests the rapid metabolism [23,24]. HNE also showed small peaks at 4 and 12 h (Figure 3).

An inflammation marker CRP was increased at 1 h and later (Figure 4). The two peaks around 12 h gap suggest different mechanisms of CRP production in the early and late phases.

Source of HNE

As for the source of HNE, LPS increased HNE production in the monocyte-enriched plasma (7.4-folds vs. control) by 3.4-folds higher than in the polymorphonuclear leukocyte-enriched plasma (2.1-folds vs. control), with a peak at 10 min (Figure 5). The peak *in vitro* was earlier than the peak (at 20 min) in sera of the LPS-treated rats (Figure 3).

To identify the enzyme for the HNE production, a NADPH oxidase inhibitor [25], apocynin was employed. The increase in serum HNE in LPS-treated rat was inhibited by apocynin as compared with control, and the solvent dimethylsulfoxide (DMSO) (Figure 6). Apocynin also inhibited HNE production in the monocyte-enriched plasma after LPS-treatment, with much faster peak at 10 min (Figure 7). DMSO a hydroxyradical scavenger also reduced the *in vitro* increase in HNE by LPS to a lesser extent (Figure 7).

Comparison with malondialdehyde (MDA) assay

ELISA for MDA could not detect any increase in the monocyte-enriched plasma after LPS treatment (data not shown).

Discussion

The most important finding of this study is the very rapid and outstanding production of a lipid peroxide HNE, through monocyte NADPH oxidase in the rat after injection of a small amount of LPS. We could get these results by the TR-FIA with much higher sensitivity than ELISA. CRP is known to be a sensitive marker for atherosclerosis or ischemic heart disease. However, HNE was increased much faster and higher than CRP under the settings of mild inflammation.

To our knowledge, there are only two reports on basal plasma HNE as detected by HPLC and by GC-MS [26,27], but none for the change in HNE by any stimulus. The TR-FIA for HNE is superior to HPLC, GC-MS, silica gel chromatography, or fluorometric assay for MDA or other lipid peroxides [13–15,28] since many samples can be measured simultaneously without pre-treatment. The new assay method is convenient and applicable to evaluate oxidative status and effect of therapies under various clinical settings.

The result clearly shows that early mild inflammation enhances oxidative stress and lipid peroxidation and also suggests the causality between inflammation and atherosclerosis through the lipid

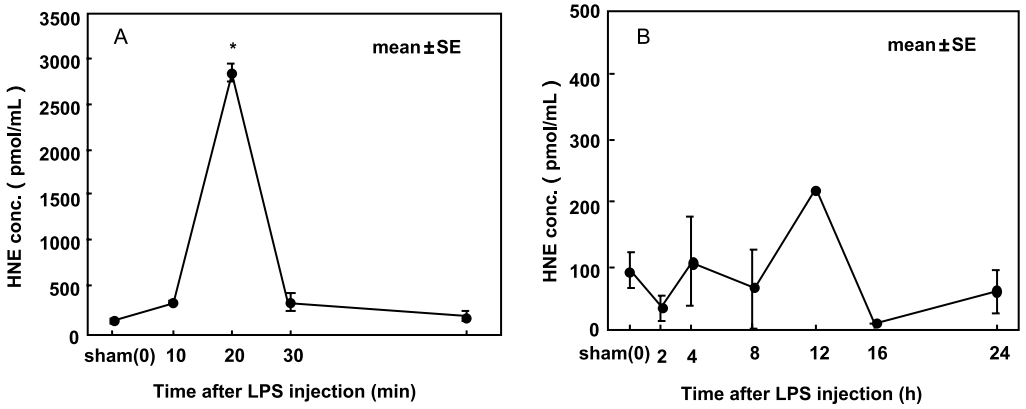

Figure 3. Temporal change in serum HNE after LPS injection. After i.p. injection of LPS in the rat, serum HNE increased with a peak at 20 min (panel A, $*P < 0.05$ vs. sham) and a small peak at 12 h (panel B, not significant). Note the difference in dimension of the *y*-axis.

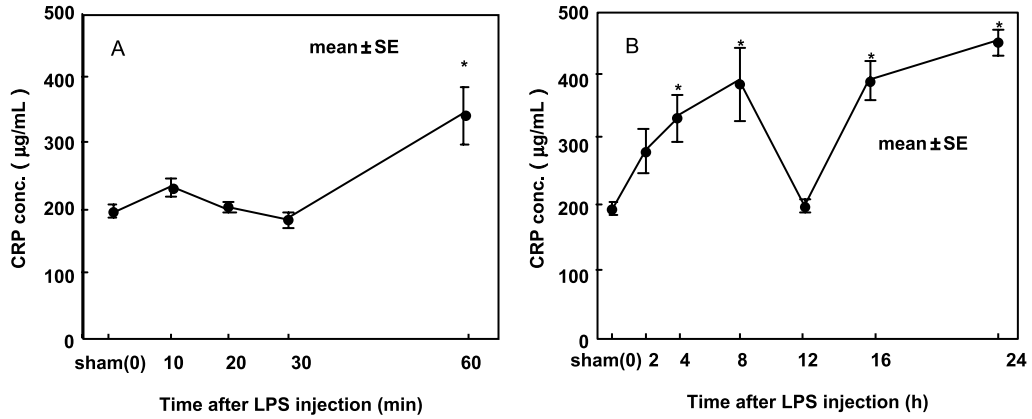

Figure 4. Temporal change in CRP after LPS injection. After LPS injection, serum CRP level increased at 1 (panel A), 4,8,16 and 24 h (panel B) (*$P < 0.05$ vs. sham). For details, see text.

peroxidation (Figure 3 and Figure 4). HNE is a multi-potential lipid mediator in various biochemical reactions [29–31]. For example, HNE exerts transcriptional activation of cyclo-oxygenase-2 [29], enzyme inactivation [30], and cellular response [31]. Additionally, HNE is not only generated during oxidation of LDL but also contributes to the oxidative modification of LDL [32,33]. The HNE-modification of LDL is supposed to enhance atherosclerosis [32,33]. We have undertaken to identify HNE-modified proteins by western blotting to find the clue to the patho-physiological role of HNE in endotoxemia.

The monocyte is the most plausible source for HNE formation after LPS treatment. LPS increased the HNE predominantly in the monocyte-enriched plasma, though there was a small increase in the leukocyte-enriched plasma (Figure 7). The kinetics of HNE production post-LPS was faster *in vitro (*peak at 10 min) (Figure 5) than *in vivo* (peak at 20 min) (Figure 3). This delay *in vivo* would reflect the time for adsorption of intraperitoneal LPS.

NADPH oxidase is the most potential source for HNE formation post-LPS. A specific NADPH oxidase inhibitor apocynin inhibited the HNE generation in the serum of the rat (*in vivo*, Figure 6) and in the monocyte-enriched plasma (*in vitro*, Figure 7) after LPS treatment. A hydroxyl radical scavenger DMSO reduced the *in vitro* increase in HNE by LPS (Figure 7), suggesting the contribution of Fenton reaction. The quick return of plasma HNE to the near-basal level shows the contribution of a blood metabolizing enzyme (Figure 6), such as aldose reductase [24].

There is growing body of evidence that monocyte NADPH oxidase plays important roles in the pathogenesis of atherosclerosis. Chlamydia-primed monocyte generates reactive oxygens [34], while LDL is oxidized by monocyte NADPH oxidase [35]. In atherosclerotic plaques, monocytes are activated upon

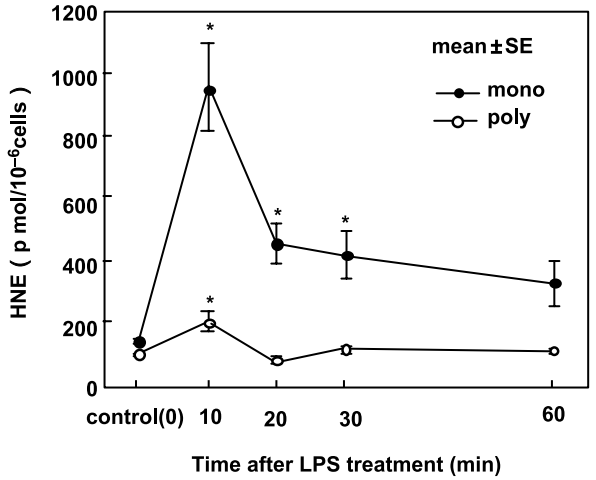

Figure 5. Temporal changes in HNE in monocyte or leukyocyte-enriched plasma after LPS treatment. HNE increased higher (5-folds) in the monocyte-enriched plasma (close circles) than in the leukocyte-enriched plasma (open circles *$p < 0.05$ vs. sham), with a peak at 10 min.

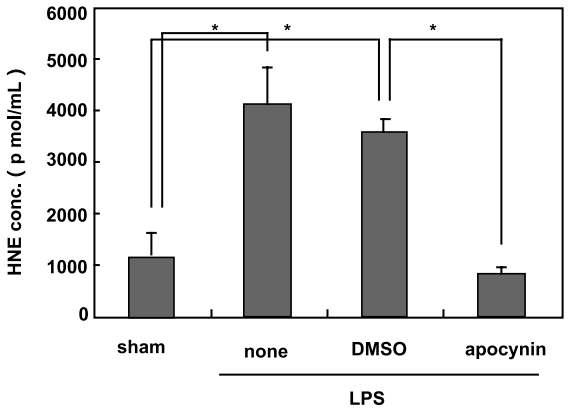

Figure 6. Effect of apocynin on HNE production after LPS injection in rat. Either of apocynin (5 mg/kg), 0.6% DMSO or saline was injected (i.p.) in the rats ($n = 4$ for each) before i.p. administration of LPS. LPS increased serum HNE in the rat after 20 min, in the presence of 0.6%DMSO, the solvent for apocynin. Apocynin blocked the increase in HNE (*$p < 0.05$).

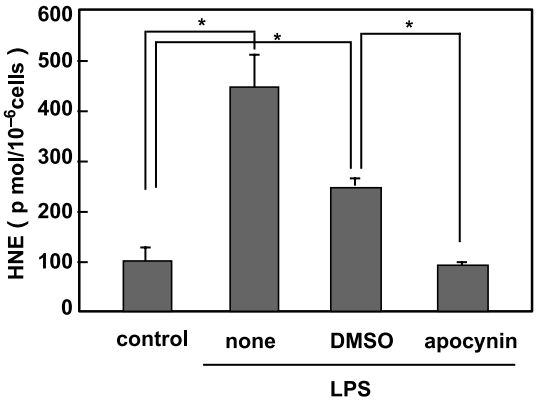

Figure 7. Effect of apocynin on HNE increase after LPS treatment *in vitro*. Apocynin reduced the increase in HNE after LPS treatment for 15 min in monocyte-enriched plasma as compared with control, and DMSO. DMSO also reduced the increment in HNE after LPS treatment ($*p < 0.05$).

phagocytosis of oxidized LDL, and transformed into foamy macrophage. Expression of scavenger receptors and adhesion molecules, regulated by oxidative stresses, are involved in these processes [1]. In hypertension, diabetes, and hyperlipidemia, reactive oxygens derived from monocyte NADPH oxidase [36,37,38] were shown to promote these processes that lead to atherosclerosis. Reactive oxygens produce lipid peroxides such as HNE in the atherosclerosis plaques, and HNE promotes atherosclerosis through activation of cyclo-oxygenase-2 that generates inflammatory mediators such as prostanoids [39].

In conclusion, this study shows the rapid and great increase in serum lipid peroxide HNE in the LPS-treated rat by highly sensitive TR-FIA. It is likely that monocyte NADPH oxidase is a predominant source for HNE. The assay of serum HNE level will be useful in the clinical studies on pathogenesis and pharmacological evaluation of oxidative stress.

Acknowledgements

This work has been supported by Grant-in-Aid for Scientific Research No. 15590588 from the Ministry of Education, Science, Sports and Culture, and also by Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST).

References

[1] Stocker R, Keaney Jr., JF. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004;84:1381–1478.

[2] Chen J, Henderson GI, Freeman GL. Role of 4-hydroxynonenal in modification of cytochrome c oxidase in ischemia/reperfused rat heart. J Mol Cell Cardiol 2001;33: 1919–1927.

[3] Toyokuni S, Yamada S, Kashima M, Ihara Y, Yamada Y, Tanaka T, Hiai H, Seino Y, Uchida K. Serum 4-hydroxy-2-nonenal-modified albumin is elevated in patients with type 2 diabetes mellitus. Antioxid Redox Signal 2000;2:681–685.

[4] Hussein O, Shneider J, Rosenblat M, Aviram M. Valsartan therapy has additive anti-oxidative effect to that of fluvastatin therapy against low-density lipoprotein oxidation: Studies in hypercholesterolemic and hypertensive patients. J Cardiovasc Pharmacol 2002;40:28–34.

[5] Esterbauer H, Cheeseman KH, Dianzani MU, Poli G, Slater TF. Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe^{2+} in rat liver microsomes. Biochem J 1982;208:129–140.

[6] Benedetti A, Comporti M, Esterbauer H. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. Biochim Biophys Acta 1980;620:281–296.

[7] Benedetti A, Pompella A, Fulceri R, Romani A, Comporti M. Detection of 4-hydroxynonenal and other lipid peroxidation products in the liver of bromobenzene-poisoned mice. Biochim Biophys Acta 1986;876:658–666.

[8] Esterbauer H, Zollner H, Schaur RJ. Aldehydes forms by lipid peroxidation: Mechanisms formation, occurrence and determination. In: Vigo-Pelferey C, editor. Membrane Lipid Oxidation. Vol. 1. Boca Raton: CRC Press; 1990. p 239–268.

[9] Childs A, Jacobs C, Kaminski T, Halliwell B, Leeuwenburgh C. Supplementation with vitamin C and N-acetyl-cysteine increases oxidative stress in humans after an acute muscle injury induced by eccentric exercise. Free Radic Biol Med 2001;31:745–753.

[10] Fabris C, Pirisi M, Panozzo MP, Soardo G, Toniutto P, Hocza V, Bartoli E. Intensity of inflammatory damage and serum lipid peroxide concentrations in liver disease. J Clin Pathol 1993; 46:364–367.

[11] Zuyderhoudt FM, Jorning GG, De Haan JG, Samson G, Van Gool J. Rat liver storage iron and plasma ferritin during D-galactosamine-HCl-induced hepatitis. Clin Sci (Lond) 1980; 58:321–325.

[12] Sulpice T, Boucher F, Pucheu S, de Leiris J. Contribution of leukocyte infiltration to lipoperoxidation occurring in the non-ischemic region of the rat heart submitted to permanent left coronary artery occlusion. J Mol Cell Cardiol 1994;26:831–840.

[13] Liu J, Yeo HC, Doniger SJ, Ames BN. Assay of aldehydes from lipid peroxidation: Gas chromatography-mass spectrometry compared to thiobarbituric acid. Anal Biochem 1997;245:161–166.

[14] Karatas F, Karatepe M, Baysar A. Determination of free malondialdehyde in human serum by high-performance liquid chromatography. Anal Biochem 2002;311:76–79.

[15] Rosiers CD, Rivest MJ, Boily MJ, Jette M, Carrobecohen A, Kumar A. Gas-chromatographic-mass spectrometric assay of tissue malondialdehyde, 4-hydroxynonenal, and othe aldehydes after their reduction to stable alcohols. Anal Biochem 1993;208:161–170.

[16] Ben-Shaul V, Lomnitski L, Nyska A, Zurovsky Y, Bergman M, Grossman S. The effect of natural antioxidants, NAO and apocynin, on oxidative stress in the rat heart following LPS challenge. Toxicol Lett 2001;123:1–10.

[17] Aniya Y, Koyama T, Miyagi C, Miyahira M, Inomata C, Kinoshita S, Ichiba T. Free Radical Scavenging and Hepatoprotective Actions of the Medicinal Herb, Crassocephalum crepidioides from the Okinawa Islands. Biol Pharm Bull 2005;28:19–23.

[18] Minamiyama Y, Takemura S, Toyokuni S, Imaoka S, Funae Y, Hirohashi K, Yoshikawa T, Okada S. CYP3A induction aggravates endotoxemic liver injury via reactive oxygen species in male rats. Free Radic Biol Med 2004;37:703–712.

[19] Uchida K, Szweda LI, Chae H-Z, Stadman ER. Immunohistchemical detection of 4-hydoroxynonenal protein adducts in oxidized hepatocytes. Proc Natl Acad Sci 1993;90: 8742–8746.

[20] Yuan J, Matsumoto K, Kimura H. A new tetradentate β-diketonate-europium chelate that can be covalently bound to proteins for time-resolved fluoroimmunoassay. Anal Chem 1998;70:596–601.
[21] Yuan J, Wang G, Kimura H, Matsumoto K. Highly sensitive time-resolved fluoroimmunoassay of human immunoglobulin E by using a new europium fluorescent chelate as a label. Anal Biochem 1997;254:283–287.
[22] Toyokuni S, Uchida K. Publication of unexamined patent applications 1996., No. Hei8-168394
[23] Srivastava S, Dixit BL, Cai J, Sharma S, Hurst HE, Bhatnagar A, Srivastava SK. Metabolism of lipid peroxidation product, 4-hydroxynonenal (HNE) in rat erythrocytes: Role of aldose reductase. Free Radic Biol Med 2000;29:642651.
[24] Srivastava S, Conklin DJ, Liu SQ, Prakash N, Boor PJ, Srivastava SK, Bhatnagar A. Identification of biochemical pathways for the metabolism of oxidized low-density lipoprotein derived aldehyde-4-hydroxy trans-2-nonenal in vascular smooth muscle cells. Atherosclerosis 2001;158: 339–350.
[25] Ben-Shaul V, Lomnitski L, Nyska A, Zurovsky Y, Bergman M, Grossman S. The effect of natural antioxidants, NAO and apocynin, on oxidative stress in the rat heart following LPS challenge. Toxicol Lett 2001;123(1):1–10.
[26] Strohmaier H, Hinghofer-Szalkay H, Schaur RJ. Detection of 4-hydroxynonenal (HNE) as a physiological component in human plasma. J Lipid Mediat Cell Signal 1995;11:51–61.
[27] Spies-Martin D, Sommerburg O, Langhans CD, Leichsenring M. Measurement of 4-hydroxynonenal in small volume blood plasma samples: Modification of a gas chromatographic-mass spectrometric method for clinical settings. J Chromatogr B Analyt Technol Biomed Life Sci 2002;774:231–239.
[28] Yoshino K, Sano M, Fujita M, Tomita I. Studies on the formation of aliphatic aldehydes in the plasma and liver of vitamin E-deficient rats. Chem Pharm Bull (Tokyo) 1990;38:2212–2215.
[29] Kumagai T, Matsukawa N, Kaneko Y, Kusumi Y, Mitsumata M, Uchida K. A lipid peroxidation-derived inflammatory mediator: Identification of 4-hydroxy-2-nonenal as a potential inducer of cyclooxygenase-2 in macrophages. J Biol Chem 2004;279:48389–48396.
[30] Ishii T, Tatsuda E, Kumazawa S, Nakayama T, Uchida K. Molecular basis of enzyme inactivation by an endogenous electrophile 4-hydroxy-2-nonenal: Identification of modification sites in glyceraldehyde-3-phosphate dehydrogenase. Biochemistry 2003;42:3474–3480.
[31] Minekura H, Kumagai T, Kawamoto Y, Nara F, Uchida K. 4-Hydroxy-2-nonenal is a powerful endogenous inhibitor of endothelial response. Biochem Biophys Res Commun 2001; 282:557–561.
[32] Waeg G, Dimsity G, Esterbauer H. Monoclonal antibodies for detection of 4-hydroxynonenal modified proteins. Free Radic Res 1996;25:149–159.
[33] Uchida K, Toyokuni S, Nishikawa K, Kawakishi S, Oda H, Hiai H, Stadtman ER. Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: Markers for atherosclerosis. Biochemistry 1994;33:12487–12494.
[34] Mouithys-Mickalad A, Deby-Dupont G, Mathy-Hartert M, Habraken Y, Nys M, Henrotin Y, Lamy M, Deby C. Effects of glucocorticoids on the respiratory burst of Chlamydia-primed THP-1 cells. Biochem Biophys Res Commun 2004;11: 941–948.
[35] Aviram M, Rosenblat M, Etzioni A, Levy R. Activation of NADPH oxidase required for macrophage-mediated oxidation of low-density lipoprotein. Metabolism 1996;45:1069–1079.
[36] Fortuno A, Olivan S, Beloqui O, San Jose G, MU, Diez J, Zalba G. Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension. J Hypertens 2004;22:2169–2175.
[37] Venugopal SK, Devaraj S, Yang T, Jialal I. Alpha-tocopherol decreases superoxide anion release in human monocytes under hyperglycemic conditions via inhibition of protein kinase C-alpha. Diabetes 2002;5:3049–3054.
[38] Li W, Hellsten A, Jacobsson LS, Blomqvist HM, Olsson AG, Yuan XM. Alpha-tocopherol and astaxanthin decrease macrophage infiltration, apoptosis and vulnerability in atheroma of hyperlipidaemic rabbits. J Mol Cell Cardiol 2004;37:969–978.
[39] Kumagai T, Matsukawa N, Kaneko Y, Kusumi Y, Mitsumata M, Uchida K. A lipid peroxidation-derived inflammatory mediator: Identification of 4-hydroxy-2-nonenal as a potential inducer of cyclooxygenase-2 in macrophages. J Biol Chem 2004;279:48389–48396.