Biochemical and Biophysical Research Communications 425 (2012) 842–847

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Lipid peroxidation dysregulation in ischemic stroke: Plasma 4-HNE as a potential biomarker?

Wan-Chi Lee [a,c], Hau-Yang Wong [b,c], Yun-Yong Chai [b], Chan-Wei Shi [a], Naoya Amino [d], Shizuki Kikuchi [d], Shin-Hai Huang [a,c,*]

[a] Department of Neurology, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan
[b] Department of Life Science, National Taipei University, Taipei 112, Taiwan
[c] Department of Neurology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA
[d] Department of Pharmacology, Keio University School of Medicine, Tokyo, Japan

ARTICLE INFO

Article history:
Received 28 July 2012
Available online 7 August 2012

Keywords:
Oxidative stress
4-HNE
ALDH2
Ischemic stroke
Biomarker
Apoptosis
Autophagy

ABSTRACT

4-hydroxynonenal (4-HNE) is a major aldehyde produced during the lipid peroxidation of ω-6 polyunsaturated fatty acids. Recently, 4-HNE has been reported to contribute to the pathogenesis of neuronal diseases such as Alzheimer's disease. However, the role of 4-HNE in ischemic stroke is unclear yet. In this study, we found that plasma 4-HNE concentrations were higher in the genetic stroke-prone rats (stroke-prone spontaneously hypertensive rats) and experimental stroke rats with middle cerebral artery occlusion (MCAO). Moreover, administration of 4-HNE via intravenous injection before MCAO surgery not only enlarged cerebral ischemia-induced infarct area, but also increased oxidative stress in brain tissue, which was evidenced by the enhanced ROS/MPA levels, and the reduced GSH/GSSG ratio and MnSOD levels. Overexpression of aldehyde dehydrogenasesbcl-2 (ALDH2), an enzyme catalyses 4-HNE, rescued neuronal survival against 4-HNE treatment in PC12 cells. The plasma 4-HNE concentrations in patients with ischemic stroke were higher than those in control subjects. In a small sample population ($N = 60$), the plasma 4-HNE concentration was positively correlated with the plasma homocysteine concentration, a risk factor for ischemic stroke. Taken together, our study suggests that the plasma 4-HNE level is a potential biomarker for ischemic stroke.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Cerebral ischemic stroke is a leading cause of mortality and morbidity worldwide [1]. At present, tissue plasminogen activator (tPA) is the only therapy for acute ischemic stroke approved by the Food and Drug Administration of United States [2,3]. However, the strict 3-h time window represents a major obstacle for the use of intravenous injection of tPA [2,3]. Thus, identification of biochemical markers for pathogenic alterations during cerebral ischemia would contribute to the timely medical examination or intervention, which may reduce the risk of cerebral ischemia occurrence.

Previous studies have shown that the toxic reactive oxygen species (ROS) and other chemical active factors could be produced by hypoxic cells and accumulated in the infarct area during cerebral ischemia [4,5]. In fact, oxidative stress is widely accepted as a fundamental mechanism of neuronal damage in ischemic stroke. Furthermore, items with antioxidant activity, including dietary supplements such as grapes [6] and red wine [7], or compounds such as resveratrol [8,9], albumin [10] and dehydroascorbic acid [11], have been shown to be able to reduce stroke-related brain damage in animal models. Lipid peroxidation, the oxidative degradation of lipids, is also implicated in the pathogenesis of ischemia stroke [12,13]. The peroxidation of polyunsaturated fatty acids in membrane phospholipids continues until the substrate is completely consumed or termination occurs due to antioxidants, leading to structural or functional damage. The toxic effects of lipid peroxidation products such as malondialdehyde [14] and thiobarbituric acid-reactive substances [15] in ischemic stroke have been documented.

4-hydroxynonenal (4-HNE), an α,β-unsaturated hydroxyalkenal produced by lipid peroxidation, is widely recognized as a specific marker of oxidative stress [13]. Okun et al. [16] and Nagotani et al. [17] reported that 4-HNE protein level in the ischemic cerebral cortex increased within 2 h of stroke induction. However, the role of plasma 4-HNE level during ischemic stroke has never been studied. Whether the plasma 4-HNE level is changed during cerebral ischemia? If the answer is yes, then the plasma 4-HNE level may be a potential biochemical marker for cerebral ischemia and is very useful for predicting or diagnosing ischemic stroke. In

* Corresponding author. Address: Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. Fax: +886 4 2359 0331.
E-mail address: shinhh69@gmail.com (S.-H. Huang).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.002

the current investigation, we studied this possibility in genetic stroke-prone rats (stroke-prone spontaneously hypertensive rat, SHR-SP), experimental stroke rat with middle cerebral artery occlusion (MCAO), and human patients.

2. Materials and methods

2.1. Animals and reagents

Male SHR-SP and age-matched control rats (Wistar-Kyoto rats, WKY), which were originated from the Charles River Breeding Laboratories (Tokyo, Japan), were purchased from the National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan. Procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University.

The 4-HNE was purchased from Cayman Chemical Company (USA). The 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma–Aldrich. OxiSelect™ 4-HNE ELISA kit was purchased from Cell Biolabs Inc. (San Diego, CA). Homocysteine ELISA kit was purchased from IBL-America (Immuno-Biological Laboratories, Minneapolis, MN). Assay kits for mycophenolic acid (MPA), ROS, reduced glutathione (GSH), oxidized glutathione (GSSG) and manganese superoxide dismutase (MnSOD) were purchased from Cayman Chemicals (Ann Arbor, MI). Antibodies against aldehyde dehydrogenasesbcl-2 (ALDH2), bax, bcl-X/L and bad were purchased from Millipore Chemicon International (Temecula, CA, USA). Antibodies against p62, LC3 and beclin-1 were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Antibody against tubulin was from Sigma. Enhanced chemiluminescence and protease/phosphatase inhibitors were purchased from Pierce (Rockford, IL, USA).

2.2. MCAO surgery and infarct area quantification

MCAO was performed as described previously [18] with some modification. During all surgical procedures, the rats were anesthetized via inhalation of 2% isoflurane in O_2 delivered via face mask during surgery. Body temperature was maintained by a heating system coupled to a rectal thermistor probe. The left jugular vein was cannulated for drug administration. Rats were placed under an operating microscope and the right MCA was exposed. A monofilament nylon (3–0) was introduced retrogradely into the MCA and occluded, a distance of 20–22 mm from the carotid bifurcation. After surgery, rats were maintained in a warm environment using a 100-W lamp. After 2 h of MCAO, rats were re-anesthetized and the intraluminal suture was carefully removed. In control animals, surgery was also carried out but the MCA was left intact (Control group). For 4-HNE administration, rats were injected with 0.1 ml of saline or 4-HNE (10 mg/ml) via the left jugular vein at 20 min before MCAO surgery. At 24 h after MCAO, rats were killed for various examinations after neurological deficits scoring. The brain was isolated and cut into 6 sections. The sections were put in a glass Petri dish containing 2% TTC solution to determine the infarct area (the infarct area was white). The ratio of infarct area was measured using ImageJ software (National Institutes of Health, Bethesda, MD) [19].

2.3. Samples and biochemical assays

For evaluating ROS, MPA, GSH/GSSG and MnSOD levels in infarct brain tissue, another set of rats were subjected to 4-HNE injection and followed by MCAO surgery. At 24 h after MCAO, the fresh infarct brain tissues were harvested and homogenated with RIPA buffer plus protease inhibitors [20]. After centrifuging (3000 rpm, 10 min), the supernatant was harvested and stored at −80 °C until assayed. The assays of ROS, MPA, GSH/GSSG and MnSOD were performed according to the manufacturer's instructions. The color intensity was measured by TECAN microplate fluorometer (TECAN, Research Triangle Park, NC) [21].

The plasma 4-HNE levels were measured using OxiSelect™ 4-HNE ELISA kit. (Cell Biolabs, San Diego, CA, USA). Briefly, 50 μl plasma was diluted with PBS to 100 μl. The samples were then added into the 96-well protein binding plate overnight at 4 °C. The absorbance was detected at 450 nm after incubation with a mouse monoclonal anti-HNE–His antibody followed by HRP-conjugated secondary anti-mouse antibody. The plasma 4-HNE concentrations were calculated according to the standard curve.

2.4. Cell culture, vector construction and transfection

PC12 cells were obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in RPMI-1640 medium (Sigma), which contains 2 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 incubator at 37 °C [22]. For vector construction, the full-length ALDH2 gene was amplified by polymerase chain reaction (PCR) from mouse liver tissue and subcloned into pIRES2-EGFP vector (Invitrogen). The control vectors or vectors carrying ALDH2 gene were transfected into PC12 cell using Lipofectamine™ LTX with Plus™ (Invitrogen) according to the manufacturer's instruction [23].

2.5. Immunoblotting

Immunoblotting analyses on tissue extracts were performed as described previously [24]. Samples were subjected to 10% SDS–PAGE, and transferred onto PVDF membranes at 100 V for 1∼2 h. After being blocked in blocking buffer with 5% (w/v) nonfat milk and 0.1% (v/v) Tween 20 in phosphate-buffered saline for 4 h, the membrane was incubated with specific primary antibody and then followed by HRP-labeled secondary antibody. The membranes were then detected using the enhanced chemiluminescence system as described previously [25].

2.6. Human patients

Twenty-four human subjects were included in this study: 24 men with ischemic stroke (mean age, 67 years; median National Institutes of Health Stroke Scale 18) and 36 age-matched normal men. Informed consent was obtained according to an established protocol approved by the Ethic Committee of Kaohsiung Medical University. In ischemic patients, plasma was obtained by fingerstick using a commercial dry plasma collection device (Chemcard Plasma Collection Device) within 3 h from symptom onset and frozen at −80 °C until assayed. The ELISA assays of plasma 4-HNE and homocysteine were performed according to the manufacturer's instructions and laboratory validated protocols. The absorbance of each well on a microplate reader was read at 450 nm [26].

2.7. Statistical analysis

Data are expressed as mean ± SD. Differences were evaluated by two-tailed Student's *t* test. Linear regression analysis was performed to evaluate the correlation between plasma homocysteine and 4-HNE concentrations. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Higher plasma 4-HNE concentrations in SHR-SP and MCAO rats

As shown in Fig. 1A, the plasma 4-HNE concentrations in 6-month old SHR-SP were significantly higher than those in age-matched WKY rats. Next, we tested whether plasma 4-HNE concentrations can be induced in experimental ischemic stroke model. As expected, at 24 h after MCAO surgery, the plasma 4-HNE concentrations in MCAO rats were higher than those in control rats (Fig. 1B). These results suggest that the plasma 4-HNE levels were increased by cerebral ischemia and might be an important feature of ischemic stroke.

3.2. Administration of 4-HNE aggravated ischemic brain damage, increased oxidant stress and enhanced apoptosis/autophagy in MCAO model

We administrated saline (control) or 4-HNE in rats, and performed MCAO surgery on these rats twenty minutes later. As shown in Fig. 2A, 4-HNE treatment enlarged the cerebral infarct area in MCAO rats by approximately 50%. It did not induce cerebral infarct in sham-operated rats. We also measured several parameters of oxidative stress. In sham-operated rats, 4-HNE slightly changed the ROS levels (Fig. 2D) and GSH/GSSG ratio (Fig. 2E). In MCAO rats, 4-HNE induced intensive changes of oxidative stress parameters. It not only increased MPA (Fig. 2B) and ROS (Fig. 2C) levels, but also decreased GSH/GSSG ratio (Fig. 2D) and MnSOD (Fig. 2E) levels, indicating that 4-HNE treatment further increased oxidative stress in the cerebral ischemia condition.

Neuronal damage caused by ischemia involved apoptosis and excessive autophagy. As shown in Fig. 2F, 4-HNE administration further increased levels of apoptotic proteins, evidenced by the decreased bcl-2/bax and bcl-X/L/bad ratios in MCAO rat brain tissue. Moreover, the 4-HNE administration increased autophagic proteins levels, evidenced by the enhanced p62, beclin-1 and LC3-II expressions (Fig. 2G).

3.3. Overexpression of ALDH2 rescued neuronal survival against 4-HNE treatment in PC12 cells

ALDH2 can metabolize 4-HNE and is responsible for the detoxification of 4-HNE. Thus, we overexpressed ALDH2 in PC12 neuronal cells (Fig. 3A) to test whether overexpression of ALDH2 can protect against 4-HNE-induced neuronal toxicity. As shown in Fig. 3B, 4-HNE-induced decrease of cell viability in PC12 cells was partly blocked by ALDH overexpression. The LDH leakage induced by 4-HNE treatment was also partly blocked by overexpression of ALDH2 (Fig. 3C).

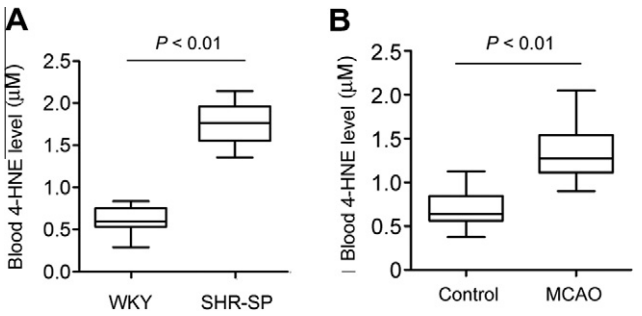

Fig. 1. Plasma 4-HNE concentrations are higher in SHR-SP and MCAO rats. (A) The plasma 4-HNE concentrations in SHR-SP and normal control WKY rats ($N = 10$). (B) The plasma 4-HNE concentrations in control rats and MCAO rats at 24 h after MCAO surgery ($N = 10$). SHR-SP, stroke-prone spontaneously hypertensive rat; MCAO, middle cerebral artery occlusion.

3.4. Plasma 4-HNE concentrations are higher in patients with ischemic stroke and positively correlated with plasma homocysteine concentrations

Next, we studied the plasma 4-HNE concentrations in human patients. Interestingly, the plasma 4-HNE concentrations in patients with ischemic stroke were higher than those in control people ($P = 0.029$, Fig. 4A). Moreover, we studied the correlation between the plasma concentrations of 4-HNE and homocysteine, a well-established risk factor for ischemic stroke [27]. As shown in Fig. 4B, a positive correlation was found between plasma concentrations of 4-HNE and homocysteine ($r^2 = 0.36$, $P = 0.005$).

4. Discussion

In the present study, using a genetic stroke model (SHR-SP) and an experimental stroke model (MCAO), we found that plasma 4-HNE concentrations could be induced by ischemic stroke. This result prompted us to further evaluate the influence of 4-HNE on neuronal damage during ischemic stroke *in vivo*. As expected, the administration of 4-HNE before MCAO surgery markedly enlarged the cerebral ischemia-induced infarct area, and increased oxidative stress in brain tissue. Overexpression of ALDH2 rescued neuronal survival against 4-HNE treatment in PC12 cells. Furthermore, the plasma 4-HNE concentrations of patients with ischemic stroke were higher than those in control subjects. At last, in a small sample population, we found that the plasma 4-HNE levels were positively correlated with plasma homocysteine levels. Based on these results, we proposed that the plasma 4-HNE level may be a biomarker for ischemic stroke.

This is the first report describing the enhanced plasma 4-HNE concentration during ischemic stroke in rat model and humans. Previously, 4-HNE concentrations were found to be elevated in the ventricular fluid of subjects with Alzheimer's disease [28] and Parkinson's disease [29]. McKracken et al. reported that 4-HNE immunoreactivity was increased in human hippocampus tissue after global ischemia [30], which was confirmed by later reports [16,17], indicating that local oxidant stress was induced by cerebral ischemia. Our data provide the first evidence that the plasma 4-HNE levels were also enhanced in ischemia stroke, suggesting the increased systemic oxidative stress.

4-HNE has neurotrophic effect at low concentrations (0.8–2.8 μM), activating signaling pathways through increasing basal and GTP-stimulated phospholipase C, adenylate cyclase, and decreasing ornithine decarboxylase activities [31]. However, 4-HNE is cytotoxic in high concentration (>10 μM). It can disturb calcium homeostasis, inhibit DNA and RNA synthesis, block mitochondrial respiration and induce membrane disintegration [32]. Because the plasma 4-HNE levels were increased by ~3-fold during ischemia, we considered that the increase of plasma 4-HNE concentration might impact on the neuronal injury caused by cerebral ischemia. As expected, we found that the administration of 4-HNE markedly enlarged the infarct area, as well as increased apoptotic and autophagic protein levels in infarct brain tissues. While apoptosis is a well-described event contributing to neuronal damage in cerebral ischemia [33], autophagy is a new concept and focus in recent studies on ischemic stroke [34]. As a double-edged sword, autophagy is a process for turnover of intracellular organelles and molecules, and protects cells during stress responses [35]. Mild autophagy is neuroprotective [36–38], whereas excessive autophagy is vital and neurotoxic [39–41]. Although some previous studies have described the relationship between neuronal oxidative stress and autophagy [42,43], the effect of

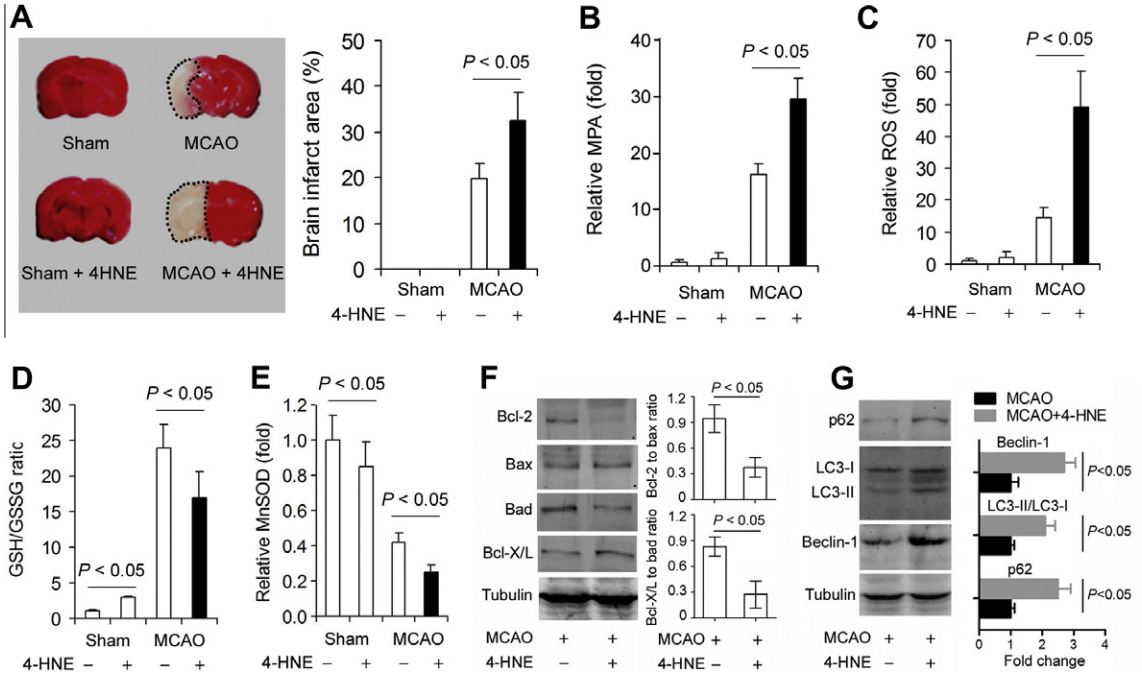

Fig. 2. 4-HNE treatment augmented cerebral infarct area, increased oxidative damage and aggravated apoptosis/autophagy in MCAO model. (A) Representative image and quantitative analysis of infarct area in sham-operated rats and MCAO rats with or without 4-HNE administration. The rat brain was harvested at 24 h after MCAO surgery and stained with 2% TTC solution. The white area was infarct brain tissue ($N = 10$). (B–E) Levels of MPA, ROS, GSH/GSSG ratio and MnSOD in brain tissue ($N = 10$). (E) Representative image and quantitative analysis of immunoblotting of apoptotic proteins (bax, bcl-2, bad and bcl-X/L) in brain tissues from MCAO rats and MCAO rats with 4-HNE. Tubulin was used as a loading control ($N = 4$). (F) Representative image and quantitative analysis of immunoblotting of autophagic proteins (p62, LC3 and beclin-1) in brain tissues from MCAO rats and MCAO rats with 4-HNE. Tubulin was used as a loading control ($N = 4$).

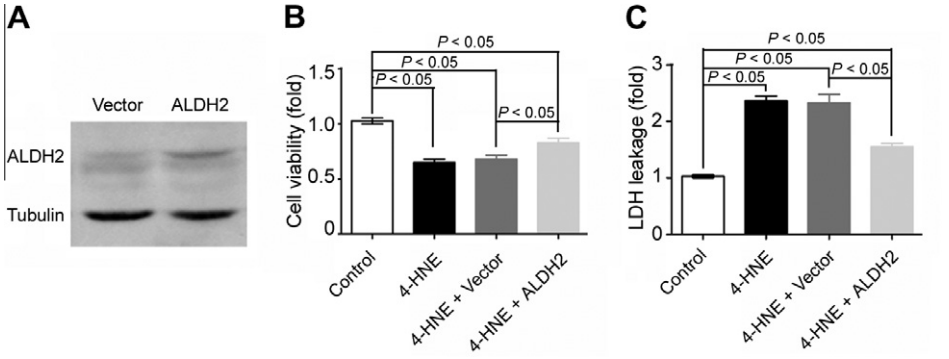

Fig. 3. Overexpression of ALDH2 rescued neuronal survival against 4-HNE treatment in PC12 cells. (A) Representative immunoblotting image of overexpression of ALDH2 in PC12 cells. (B) Overexpression of ALDH2 partly blocked the decrease of cell viability induced by 4-HNE treatment (30 μM) ($N = 8$). (C) Overexpression of ALDH2 partly prevented the LDH leakage induced by 4-HNE treatment (30 μM) ($N = 8$).

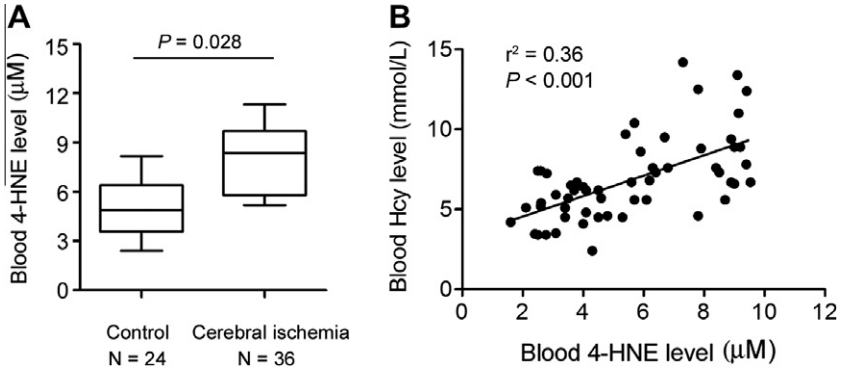

Fig. 4. Plasma 4-HNE concentrations in human patients with ischemic stroke and the correlation between plasma 4-HNE and homocysteine concentrations. (A) ELISA assay showing the plasma 4-HNE concentrations in human patients with ischemic stroke and control people. Plasma of ischemic patients was harvested with 3 h from symptom onset. (B) Correlation between plasma 4-HNE and homocysteine concentrations in a small sample population ($N = 60$). Hcy, homocysteine.

4-HNE on autophagy has never been studied. In our study, 4-HNE further increased the expressions of autophagic proteins in MCAO rat brain tissue, suggesting 4-HNE may induce excessive autophagy.

A large number of results from clinical studies have confirmed that the elevated plasma homocysteine concentration is a potent risk factor for cardiovascular disease, including ischemic stroke [27,44]. In humans, we found that plasma 4-HNE concentrations were positively correlated with plasma homocysteine levels. This result indicates that the people with higher plasma homocysteine level, who are at high risk for stroke, also have a high plasma 4-HNE level. However, the sample size of our study is relatively small ($N = 60$). Consequently, future large-scale prospective controlled clinical investigations are warranted to assess the exact role of plasma 4-HNE concentrations in ischemic stroke.

In summary, we demonstrated that the plasma-4HNE levels were higher in genetic stroke rats, experimental stroke rats and patients with ischemic stroke. 4-HNE administration augmented cerebral infarct area, increased oxidative stress, and enhanced apoptosis/autophagy in MCAO rats. Overexpression of ALDH2, an enzyme can detoxify 4-HNE, rescued neuronal survival against 4-HNE treatment in PC12 cells. Moreover, in a small-size population, we found that the plasma 4-HNE levels were positively correlated with homocysteine levels, a well-known risk factor for ischemic stroke. All these results indicate that the plasma 4-HNE level may be a potential biochemical marker for ischemic stroke.

5. Disclosure statement

All the authors declared no conflict of interests.

Acknowledgment

This work was supported by grants from the Young Science Foundation of Kaohsiung Medical University (NO. 20109023).

References

[1] G.A. Donnan, M. Fisher, M. Macleod, S.M. Davis, Stroke, Lancet 371 (2008) 1612–1623.
[2] M. Yepes, B.D. Roussel, C. Ali, D. Vivien, Tissue-type plasminogen activator in the ischemic brain: more than a thrombolytic, Trends Neurosci. 32 (2009) 48–55.
[3] B.A. Liang, R. Lew, J.A. Zivin, Review of tissue plasminogen activator, ischemic stroke, and potential legal issues, Arch. Neurol. 65 (2008) 1429–1433.
[4] S.G. Imre, I. Fekete, T. Farkas, Increased proportion of docosahexanoic acid and high lipid peroxidation capacity in erythrocytes of stroke patients, Stroke 25 (1994) 2416–2420.
[5] A. Cherubini, M.C. Polidori, M. Bregnocchi, S. Pezzuto, R. Cecchetti, T. Ingegni, A. di Iorio, U. Senin, P. Mecocci, Antioxidant profile and early outcome in stroke patients, Stroke 31 (2000) 2295–2300.
[6] Q. Wang, A.Y. Sun, A. Simonyi, D.K. Miller, R.E. Smith, R.G. Luchtefeld, R.J. Korthuis, G.Y. Sun, Oral administration of grape polyphenol extract ameliorates cerebral ischemia/reperfusion-induced neuronal damage and behavioral deficits in gerbils: comparison of pre- and post-ischemic administration, J. Nutr. Biochem. 20 (2009) 369–377.
[7] M.F. Ritz, P. Ratajczak, Y. Curin, E. Cam, A. Mendelowitsch, F. Pinet, R. Andriantsitohaina, Chronic treatment with red wine polyphenol compounds mediates neuroprotection in a rat model of ischemic cerebral stroke, J. Nutr. 138 (2008) 519–525.
[8] Y. Sakata, H. Zhuang, H. Kwansa, R.C. Koehler, S. Dore, Resveratrol protects against experimental stroke: putative neuroprotective role of heme oxygenase 1, Exp. Neurol. 224 (2010) 325–329.
[9] M. Mauthe, A. Jacob, S. Freiberger, K. Hentschel, Y.D. Stierhof, P. Codogno, T. Proikas-Cezanne, Resveratrol-mediated autophagy requires WIPI-1-regulated LC3 lipidation in the absence of induced phagophore formation, Autophagy 7 (2011) 1448–1461.
[10] L. Belayev, Y. Liu, W. Zhao, R. Busto, M.D. Ginsberg, Human albumin therapy of acute ischemic stroke: marked neuroprotective efficacy at moderate doses and with a broad therapeutic window, Stroke 32 (2001) 553–560.
[11] J. Huang, D.B. Agus, C.J. Winfree, S. Kiss, W.J. Mack, R.A. McTaggart, T.F. Choudhri, L.J. Kim, J. Mocco, D.J. Pinsky, W.D. Fox, R.J. Israel, T.A. Boyd, D.W. Golde, E.S. Connolly Jr., Dehydroascorbic acid, a blood-brain barrier transportable form of vitamin C, mediates potent cerebroprotection in experimental stroke, Proc. Natl. Acad. Sci. USA 98 (2001) 11720–11724.
[12] A. Cherubini, C. Ruggiero, M.C. Polidori, P. Mecocci, Potential markers of oxidative stress in stroke, Free Radical Biol. Med. 39 (2005) 841–852.
[13] E. Niki, Lipid peroxidation: physiological levels and dual biological effects, Free Radical Biol. Med. 47 (2009) 469–484.
[14] P.C. Sharpe, C. Mulholland, T. Trinick, Ascorbate and malondialdehyde in stroke patients, Ir. J. Med. Sci. 163 (1994) 488–491.
[15] C.Y. Chang, Y.C. Lai, T.J. Cheng, M.T. Lau, M.L. Hu, Plasma levels of antioxidant vitamins, selenium, total sulfhydryl groups and oxidative products in ischemic-stroke patients as compared to matched controls in Taiwan, Free Radical Res. 28 (1998) 15–24.
[16] E. Okun, T.V. Arumugam, S.C. Tang, M. Gleichmann, M. Albeck, B. Sredni, M.P. Mattson, The organotellurium compound ammonium trichloro(dioxoethylene-0,0′) tellurate enhances neuronal survival and improves functional outcome in an ischemic stroke model in mice, J. Neurochem. 102 (2007) 1232–1241.
[17] S. Nagotani, T. Hayashi, K. Sato, W. Zhang, K. Deguchi, I. Nagano, M. Shoji, K. Abe, Reduction of cerebral infarction in stroke-prone spontaneously hypertensive rats by statins associated with amelioration of oxidative stress, Stroke 36 (2005) 670–672.
[18] L. Belayev, O.F. Alonso, R. Busto, W. Zhao, M.D. Ginsberg, Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model, Stroke 27 (1996) 1616–1622 (discussion 23).
[19] I.L. Hsin, C.C. Ou, T.C. Wu, M.S. Jan, M.F. Wu, L.Y. Chiu, K.H. Lue, J.L. Ko, GMI, an immunomodulatory protein from *Ganoderma microsporum*, induces autophagy in non-small cell lung cancer cells, Autophagy 7 (2011) 873–882.
[20] S.Y. Chen, L.Y. Chiu, M.C. Maa, J.S. Wang, C.L. Chien, W.W. Lin, ZVAD-induced autophagic cell death requires c-Src-dependent ERK and JNK activation and reactive oxygen species generation, Autophagy 7 (2011) 217–228.
[21] S.K. Bhutia, S.K. Das, B. Azab, R. Dash, Z.Z. Su, S.G. Lee, P. Dent, D.T. Curiel, D. Sarkar, P.B. Fisher, Autophagy switches to apoptosis in prostate cancer cells infected with melanoma differentiation associated gene-7/interleukin-24 (Mda-7/IL-24), Autophagy 7 (2011) 1076–1077.
[22] M. Germain, R.S. Slack, MCL-1 regulates the balance between autophagy and apoptosis, Autophagy 7 (2011) 549–551.
[23] J. Lim, H.W. Kim, M.B. Youdim, I.J. Rhyu, K.M. Choe, Y.J. Oh, Binding preference of p62 towards LC3-ll during dopaminergic neurotoxin-induced impairment of autophagic flux, Autophagy 7 (2011) 51–60.
[24] Y.L. Liu, P.M. Yang, C.T. Shun, M.S. Wu, J.R. Weng, C.C. Chen, Autophagy potentiates the anti-cancer effects of the histone deacetylase inhibitors in hepatocellular carcinoma, Autophagy 6 (2010) 1057–1065.
[25] H.H. Lu, S.Y. Kao, T.Y. Liu, S.T. Liu, W.P. Huang, K.W. Chang, S.C. Lin, Areca nut extract induced oxidative stress and upregulated hypoxia inducing factor leading to autophagy in oral cancer cells, Autophagy 6 (2010) 725–737.
[26] G. Petrovski, G. Ayna, G. Majai, J. Hodrea, S. Benko, A. Madi, L. Fesus, Phagocytosis of cells dying through autophagy induces inflammasome activation and IL-1beta release in human macrophages, Autophagy 7 (2010) 321–330.
[27] Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis, JAMA 288 (2002) 2015–22.
[28] M.A. Lovell, W.D. Ehmann, M.P. Mattson, W.R. Markesbery, Elevated 4-hydroxynonenal in ventricular fluid in Alzheimer's disease, Neurobiol. Aging 18 (1997) 457–461.
[29] K. Zarkovic, 4-hydroxynonenal and neurodegenerative diseases, Mol. Aspects Med. 24 (2003) 293–303.
[30] E. McKracken, D.I. Graham, M. Nilsen, J. Stewart, J.A. Nicoll, K. Horsburgh, 4-Hydroxynonenal immunoreactivity is increased in human hippocampus after global ischemia, Brain Pathol. 11 (2001) 414–421.
[31] A.A. Farooqui, L.A. Horrocks, Phospholipase A2-generated lipid mediators in the brain: the good, the bad, and the ugly, Neuroscientist 12 (2006) 245–260.
[32] X.R. Lu, W.Y. Ong, B. Halliwell, L.A. Horrocks, A.A. Farooqui, Differential effects of calcium-dependent and calcium-independent phospholipase A(2) inhibitors on kainate-induced neuronal injury in rat hippocampal slices, Free Radical Biol. Med. 30 (2001) 1263–1273.
[33] F. Zhang, W. Yin, J. Chen, Apoptosis in cerebral ischemia: executional and regulatory signaling mechanisms, Neurol. Res. 26 (2004) 835–845.
[34] F. Tian, K. Deguchi, T. Yamashita, Y. Ohta, N. Morimoto, J. Shang, X. Zhang, N. Liu, Y. Ikeda, T. Matsuura, K. Abe, *In vivo* imaging of autophagy in a mouse stroke model, Autophagy 6 (2011) 1107–1114.
[35] H.M. Shen, P. Codogno, Autophagic cell death: loch ness monster or endangered species?, Autophagy 7 (2011) 457–465
[36] S. Carloni, S. Girelli, C. Scopa, G. Buonocore, M. Longini, W. Balduini, Activation of autophagy and Akt/CREB signaling play an equivalent role in the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia, Autophagy 6 (2010) 366–377.
[37] R. Sheng, L.S. Zhang, R. Han, X.Q. Liu, B. Gao, Z.H. Qin, Autophagy activation is associated with neuroprotection in a rat model of focal cerebral ischemic preconditioning, Autophagy 6 (2010).
[38] N. Rodriguez-Muela, P. Boya, Axonal damage, autophagy and neuronal survival, Autophagy 8 (2011) 286–288.
[39] Y. Grishchuk, V. Ginet, A.C. Truttmann, P.G. Clarke, J. Puyal, Beclin 1-independent autophagy contributes to apoptosis in cortical neurons, Autophagy 7 (2011) 1115–1131.
[40] B.J. Bartlett, P. Isakson, J. Lewerenz, H. Sanchez, R.W. Kotzebue, R.C. Cumming, G.L. Harris, I.P. Nezis, D.R. Schubert, A. Simonsen, K.D. Finley, P62, Ref(2)P and

ubiquitinated proteins are conserved markers of neuronal aging, aggregate formation and progressive autophagic defects, Autophagy 7 (2011) 572–583.

[41] X. Zhang, L. Li, S. Chen, D. Yang, Y. Wang, Z. Wang, W. Le, Rapamycin treatment augments motor neuron degeneration in SOD1(G93A) mouse model of amyotrophic lateral sclerosis, Autophagy 7 (2011) 412–425.

[42] R.J. West, S.T. Sweeney, Oxidative stress and autophagy: mediators of synapse growth?, Autophagy 8 (2011) 284–285

[43] L. Zheng, A. Terman, M. Hallbeck, N. Dehvari, R.F. Cowburn, E. Benedikz, K. Kagedal, A. Cedazo-Minguez, J. Marcusson, Macroautophagy-generated increase of lysosomal amyloid beta-protein mediates oxidant-induced apoptosis of cultured neuroblastoma cells, Autophagy 7 (2011) 1528–1545.

[44] D. Tanne, M. Haim, U. Goldbourt, V. Boyko, R. Doolman, Y. Adler, D. Brunner, S. Behar, B.A. Sela, Prospective study of serum homocysteine and risk of ischemic stroke among patients with preexisting coronary heart disease, Stroke 34 (2003) 632–636.