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Acute exposure to an electric field induces changes in human plasma 9-HODE, 13-HODE, and immunoreactive substance P levels: Insight into the molecular mechanisms of electric field therapy

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Abstract

Medical treatment using high-voltage electric potential (HELP) devices to generate an electric field (EF) is an alternative therapy commonly used in Japan. However, the underlying mechanisms of the potential health benefits are not fully understood. To address this issue, we performed plasma lipidomics using liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS). 9-Hydroxyoctadecadienoic acid (HODE), 13-HODE, and 13-hydroperoxy-octadecadienoic acid (HpODE) levels were significantly upregulated after HELP (18 kV, 30 min) exposure. However, there was no effect on HODE-related diol- metabolites, epoxide-metabolites, ketone- metabolites, or prostaglandins (PGs). We further examined the effect of HELP exposure on plasma concentrations of mediators using enzyme-linked immunosorbent assay (ELISA)/enzyme immunoassay (EIA). Immunoreactive substance P (SP) and brain-derived neurotrophic factor (BDNF) levels were significantly upregulated after HELP exposure. Under these conditions, HELP exposure had no effect on immunoreactive levels of vasoactive intestinal peptide (VIP), bradykinin, calcitonin gene-related peptide (CGRP), or motilin. Our findings provide insight into the possible relationship between the pharmacological modulation of neuromediators and that of HODEs by EF exposure. They may also be important in the development of electroceuticals.

Abbreviations: α-MSH: *alpha*-melanocyte-stimulating hormone; AA: arachidonic acid; BDNF: brain-derived neurotrophic factor; CGRP: calcitonin gene-related peptide; COX: cycloxygenase; CYP4A: cytochrome P450 oxidase 4A; DiHOME: dihydroxy-octadecenoic acid; EF: electric field; EIA: enzyme immunoassay; ELISA: enzyme-linked immunosorbent assay; EpOME: epoxy-octadecenoic acid; GPR: G protein-coupled receptor; HELP: high-voltage electric potential; HETE: hydroxyeicosatetraenoic acid; HODE: hydroxyoctadecadienoic acid; HOTrE: hydroxyoctadecatrienoic acid; HpODE: hydroperoxy-octadecadienoic acid; LA: linoleic acid; LC-MS/MS: liquid chromatography in combination with tandem mass spectrometry; LOX: lipoxygenase; NGF: nerve growth factor; OEA: oleoylethanolamide; OxoODE: oxo-octadecadienoic acid; PG: prostaglamdin; 15-PGDH: 15-hydroxy prostaglandin dehydrogenase; PLA_2: phospholipase A_2; PLA_2G_2D: group IID secretory phospholipase A_2; PPAR-γ: peroxisome proliferator-activated receptor-*gamma*: SP: substance P; TRPV1: transient receptor potential vanilloid 1; and VIP: vasoactive intestinal peptide

Introduction

A therapeutic device designed to expose the human body to high-voltage electric potential (HELP) has been approved by the Ministry of Health, Labour and Welfare in Japan [1-9]. High-voltage electric field (EF) therapy is reported to be an effective treatment for stiff shoulders, constipation, insomnia, and headaches [1-9]. However, the mechanisms by which EF exposure induces its variety of health benefits are poorly understood. Key mediators, such as neuropeptide and endogenous metabolites, have been suggested as candidate molecules that represent the interface between symptoms and electroceutical target proteins [10-16]. Our previous attempts to find an EF exposure-induced biomarker using non-targeted plasma metabolomics led to the detection of changes in an endogenous lipid-derived signaling molecule oleoylethanolamide (OEA), and unsaturated fatty acids such as oleic acid, linoleic acid (LA), *cis*-11-eicosenoic acid, *cis*-11,14-eicosadienoic acid, *cis*-8,11,14-eicosatrienoic acid, *cis*-5,8,11,14,17-eicosapentaenoic acid, *cis*-4,7,10,13,16,19-docosahexaenoic acid, and arachidonic acid (AA) [15]. In particular, OEA activates the transient receptor potential vanilloid 1 (TRPV1) on perivascular sensory nerves [17]. In our previous study, we found that OEA induces marked upregulation in group IID secretory phospholipase A_2 (PLA_2G_2D) expression in human subcutaneous cultured adipocytes [15]. Liberation of LA or AA in the PLA_2 reaction is believed to represent the rate-limiting step of the cascade leading to the formation of bioactive lipid mediators. Thuren *et al*. reported that activation of PLA_2-catalyzed hydrolysis was induced

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by EF [18]. Thus, we hypothesized that the increase in unsaturated fatty acids, such as LA and AA, after EF exposure may be affected to change in lipoxygenase (LOX)-derived metabolites in plasma. In this study, we investigated the levels of LA- and AA-derived lipid metabolites using LC－MS/MS in plasma samples obtained from healthy subjects before and after exposure to a single HELP stimulation. We report that the LA-derived lipid mediator 9-HODE and 13-HODE can be upregulated by HELP (18 kV, 30 min) exposure. Because 9-HODE treatment to cultured trigeminal ganglia neurons induces the release of sensory neuropeptide by the activation of TRPV1 [19], we also investigated the effect of HELP (18 kV, 30 min) exposure on several mediators.

Materials and methods

EF exposure

The system used for EF exposure has been previously described [8,9,15,16]. The EF system was equipped with a transformer, a seat, and two insulator-covered electrodes. One electrode was placed on a floor plate on which the subject's feet were located, and the other was placed above the subject's head. EF generated by the HELP apparatus (Healthtron PRO-18T, H9000, or HES-A30; Hakuju Institute for Health Science Co., Ltd., Tokyo, Japan) was uniformly created by transforming a 50-Hz alternating current at 18 kV. The safety of this system for human use was established by the Japanese government in 1963.

Subjects

Thirty-five healthy adults (12 males and 23 females; mean age, 46.3 ± 1.1 years; mean body mass index (BMI), 22.3 ± 0.5 kg/m^2) participated in experiment 1 (exposure condition: 18 kV, 30 min). Ten healthy adults (3 males and 7 females; mean age, 46.8 ± 2.9 years; mean BMI, 22.5 ± 1.0 kg/m^2) participated in experiment 2 (exposure condition: 18 kV, 30 min). Ten healthy adults (5 males and 5 females; mean age, 42.4 ± 2.8 years; mean BMI, 23.6 ± 1.0 kg/m^2) participated in experiment 3 (exposure condition: 18 kV, 15 min). Ten healthy adults (5 males and 5 females; mean age, 42.5 ± 2.9 years; mean BMI, 21.4 ± 1.0 kg/m^2) participated in experiment 4 (exposure condition: 9 kV, 15 min). Ten healthy adults (6 males and 4 females; mean age, 45.9 ± 2.9 years; mean BMI, 22.9 ± 1.0 kg/m^2) participated in experiment 5 (exposure condition: 30 kV, 15 min). All experiments were performed in the morning and all participants signed an informed consent form after receiving verbal and written information about the study. All experiments were conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the human ethics committee of Hakuju Institute for Health Science Co., Ltd. (Tokyo, Japan).

Plasma preparation

Blood samples were collected in vacutainer tubes coated with ethylenediaminetetraacetic acid (VP-NA070K; Terumo Corporation, Tokyo, Japan) and immediately centrifuged at 800 x *g* for 5 min to separate plasma from other cellular materials. Plasma was then transferred to a fresh Eppendorf tube and stored at -80°C until processing.

LC－MS/MS analysis

Lipid metabolites were measured as described previously [20]. LC－MS/MS analysis of lipid metabolites was performed using a API 4000 mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with the ACQUITY UPLC separation module (Waters Corporation, Milford, MA, USA). Chromotographic separation was performed at 40°C on a HSS T3 column (1.8 μm, 2.1 x 150 mm, Waters Corporation, Milford, MA, USA) under gradient conditions at a flow rate of 0.153 mL/min. The moblile phases consisted of 0.1% acetic acid/acetonitrile (7:3, v/v) and acetonitrile/2-propanol (1:1, v/v). The turbo ion spray interface was operated in the negative ion mode. Quantification was performed using Analyst 1.5.2 (AB SCIEX, Foster City, CA, USA). Multiple reaction monitoring (MRM) m/z transitions were 9-HODE=295 /171; 13-HODE=295/195; 9-HOTrE=293/171;13-HOTrE=293/195; 9,10-DiHOME=313/201; 12,13-DiHOME=313/183; 9(10)-EpOME=295/277;12(13)-EpOME=295/171; 9-HpODE=293/185; 13-HpODE=293/113; 9-OxoODE=293/185; 13-OxoODE=293/113; 5-HETE=319/301; 12-HETE=319/301; 15-HETE=319/219; 20-HETE=319/301; PGD_2=351/315; PGE_2=351/271; $PGF_{2\alpha}$=353/309; 13,14-dihydro-15-keto-PGD_2=351/333; 13,14-dihydro-15-keto-PGE_2=351/333; and 13,14-dihydro-15-keto-$PGF_{2\alpha}$=353/182.

ELISA/EIA assay

Plasma immunoreactive levels of SP, VIP, bradykinin, CGRP, motilin, BDNF, and NGF were measured using a human SP EIA kit from Cayman Chemical (Ann Arbor, MI), human VIP and NGF ELISA kit from LifeSpan BioSciences (Seattle, WA), human bradykinin ELISA kit from Cloud-Clone (Houston, TX), human CGRP ELISA kit from Bertin Pharma (Montigny le Bretonneux, France), human motilin ELISA kit from Cusabio Biotech (Wuhan, Hubei, China), or human BDNF ELISA kit from Phoenix Pharmaceuticals (Belmont, CA).

Statistical analysis

Data were analyzed using Welch's *t*-test. A probability (p) value < 0.05 was considered statistically significant.

Results

Effect of HELP exposure on lipid metabolites in plasma from healthy humans

We assessed LA-derived lipid metabolites in the plasma obtained from 35 healthy participants using LC－MS/MS. HELP (18 kV, 30 min) exposure showed significantly higher plasma concentrations of 9-HODE (2307 ± 142 vs. 1956 ± 136 pg/mL, p=0.004), 13-HODE (4419 ± 264 vs. 3762 ± 272 pg/mL, p=0.001), and 13-HpODE (486 ± 50 vs. 427 ± 47 pg/mL, p=0.016) than pre-exposure levels (Figure 1 and Table 1). Under these conditions, HELP exposure had no effect on 9-hydroxyoctadecatrienoic acid (HOTrE); 13-HOTrE; 9,10-dihydroxy-octadecenoic acid (DiHOME); 12,13-DiHOME; 9(10)-epoxy-octadecenoic acid (EpOME); 12(13)-EpOME; 9-HpODE; 9-oxo-octadecadienoic acid (OxoODE); or 13-OxoODE (Figure 1 and Table 1).

To investigate whether other hydroxyl- metabolites were affected by HELP exposure, we tested the effect of HELP (18 kV, 30 min) exposure on AA-derived HETEs and PGs. As shown in Table 1, HELP exposure had no effect on 5-HETE; 12-HETE; 15-HETE; 20-HETE; 13,14-dihydro-15-keto-PGD_2; 13,14-dihydro-15-keto-PGE_2; 13,14-dihydro-15-keto-$PGF_{2\alpha}$;PGD_2; PGE_2; or $PGF_{2\alpha}$.

Effect of HELP exposure on immunoreactive SP levels in plasma from healthy humans at time points following EF

The results of EIA analysis of immunoreactive SP are shown in Figure 2a. Plasma imunoreactive SP concentrations significantly at the 30-min time point (A30) after HELP exposure when compared

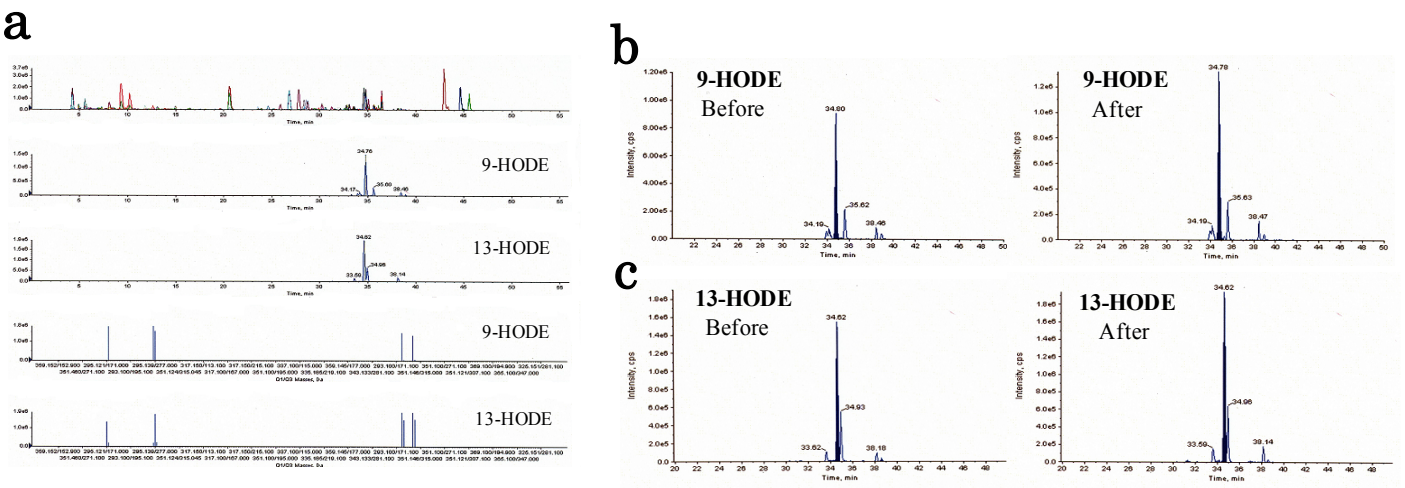

Figure 1. Lipidomic analysis of human healthy plasma between before and after HELP (18 kV) exposure for 30 min.

(a) Typical MS/MS spectrum for 9-HODE and 13-HODE from healthy human plasma sample. (b) Typical 9-HODE peak in healthy human plasma. (c) Typical 13-HODE peak in healthy human plasma.

Table 1. Lipid metabolite profiles in human healthy plasma between before and after HELP (18 kV; 30 min) exposure.

Metabolites	Before (pg/mL)	After (pg/mL)	Ratio	
	Mean ± SE	Mean ± SE	After/Before	*p* value
LA-derived (n = 35)				
Alcohols				
9-HODE	1956 ± 136	2307 ± 142	1.18	0.004 **
13-HODE	3762 ± 272	4419 ± 264	1.17	0.001 **
9-HOTrE	80.4 ± 12.7	89.7 ± 14.3	1.12	0.221
13-HOTrE	432 ± 66	428 ± 52	0.99	0.929
Diols				
9,10-DiHOME	293 ± 30	326 ± 32	1.11	0.163
12,13-DiHOME	1136 ± 140	1150 ± 76	1.01	0.898
Epoxides				
9(10)-EpOME	514 ± 127	560 ± 69	1.09	0.627
12(13)-EpOME	3829 ± 421	4338 ± 297	1.13	0.171
Hydroperoxides				
9-HpODE	15.3 ± 2.0	16.9 ± 2.0	1.10	0.182
13-HpODE	427 ± 47	486 ± 50	1.14	0.016 *
Ketones				
9-OxoODE	1131 ± 91	1080 ± 76	0.96	0.554
13-OxoODE	756 ± 47	791 ± 55	1.05	0.351
AA-derived (n = 10)				
5-HETE	530 ± 58	491 ± 25	0.93	0.351
12-HETE	568 ± 139	433 ± 86	0.76	0.391
15-HETE	268 ± 35	245 ± 16	0.91	0.445
20-HETE	250 ± 54	259 ± 14	1.04	0.882
13,14-dihydro-15-keto-PGD_2	4.60 ± 0.55	4.16 ± 0.39	0.90	0.272
13,14-dihydro-15-keto-PGE_2	13.8 ± 2.4	12.8 ± 1.9	0.93	0.689
13,14-dihydro-15-keto-$PGF_{2\alpha}$	13.0 ± 2.5	11.3 ± 1.9	0.87	0.521
PGD_2	4.26 ± 1.32	4.26 ± 1.19	1.00	0.998
PGE_2	6.18 ± 1.12	8.33 ± 1.11	1.35	0.220
$PGF_{2\alpha}$	13.1 ± 2.4	10.2 ± 1.8	0.78	0.384

* Indicates a significant difference (*p<0.05, ** p<0.01, t-test).

with pre-exposure levels (0 min, 1.83-fold, p=0.111; 30 min, 2.37-fold, p=0.032). The effect of HELP on immunoreactive SP levels was investigated using treatment for 15 min at 18 kV. The ratios of after/before were 1.43 (p=0.136), 1.72 (p=0.007), and 2.37 (p=0.003) for the 0-min time point (A0), 15-min time point (A15), and 45-min time point (A45), respectively (Figure 2b).

Table 2. Effect of HELP (18 kV, 30 min) exposure on mediators in plasma from healthy humans at multiple time points.

Mediators	Before Mean ± SE	0 min	30 min
		After HELP Mean ± SE	**After HELP** Mean ± SE
	n = 10	n = 10	n = 10
Peptide hormones			
VIP (pg/mL)	221 ± 27	197 ± 27	227 ± 18
Bradykinin (pg/mL)	11.0 ± 3.0	8.7 ± 2.1	7.1 ± 1.5
CGRP (pg/mL)	51.2 ± 13.8	46.6 ± 10.4	38.4 ± 7.5
Motilin (pg/mL)	28.3 ± 3.6	25.4 ± 1.7	27.4 ± 2.6
Neurotrophins			
BDNF (ng/mL)	21.3 ± 1.8	23.9 ± 3.0	29.7 ± 4.4 *
NGF (pg/mL)	19.9 ± 3.6	17.5 ± 3.9 *	20.1 ± 4.1

*p < 0.05 compared with before.

The nature of the immunoreactive SP responsible for EF strength was then investigated using treatment for 15 min. The ratios of immunoreactive SP levels after the 45-min time point (A45) / before were 1.04 (p=0.792) and 1.29 (p=0.011) for 9 and 30 kV, respectively. .

Effect of HELP exposure on mediators in plasma from healthy humans at different time points following EF

Given that the release of SP is thought to contribute to the modulation of mediators [21,22], we evaluated the effect of 30 min of HELP exposure to 18 kV on neurotrophins and peptide hormones in plasma. As shown in Table 2, plasma immunoreactive BDNF concentrations significantly increased at the 30-min time point after HELP exposure compared with pre-exposure levels (1.39-fold, p=0.041). In contrast, plasma immunoreactive NGF concentrations significantly decreased immediately after HELP exposure compared with pre-exposure levels (0.88-fold, p=0.017). Under these conditions, HELP exposure did not affect the immunoreactive levels of VIP, bradykinin, CGRP, or motilin (Table 2).

The nature of the immunoreactive BDNF responsible for EF strength was then investigated using treatment for 15 min. The relative

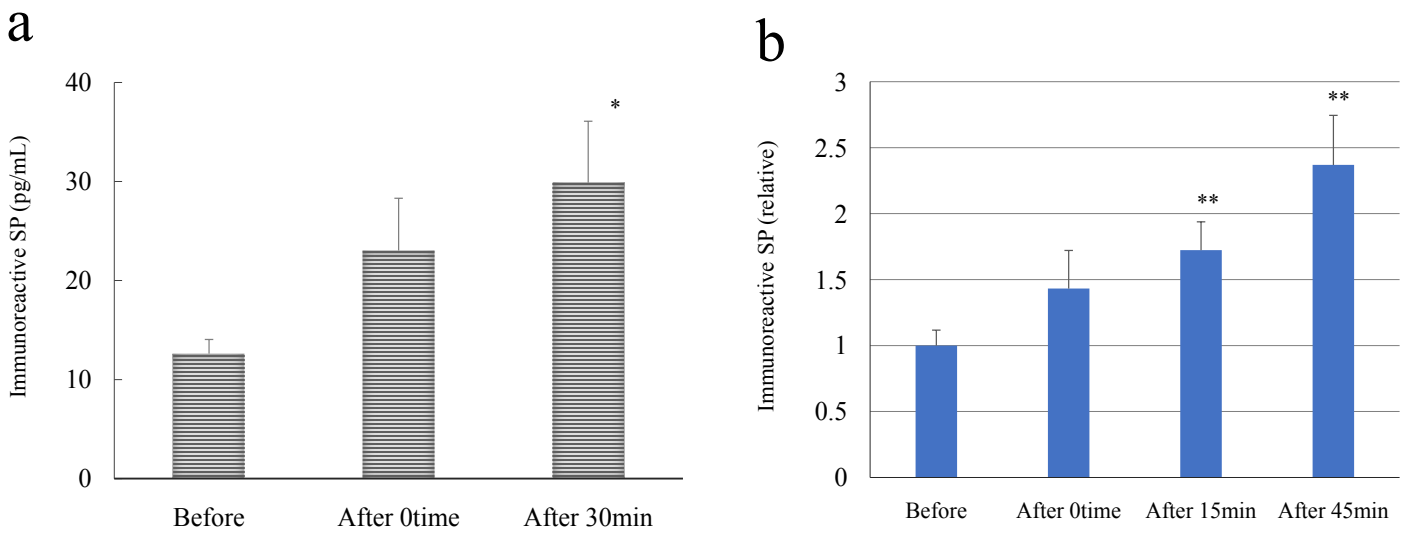

Figure 2. Effect of HELP exposure on immunoreactive SP levels in plasma from healthy humans at multiple time points.

(a) Effect of HELP (18 kV; 30 min) exposure on plasma immunoreactive SP levels at multiple time points. (b) Effect of HELP (18 kV, 15 min) exposure on plasma immunoreactive SP levels at multiple time points. Results are presented as mean ± SEM (n = 10). * $p < 0.05$ compared with before, ** $p < 0.01$ compared with before.

ratios of after 45-min time point (A45) / before were 1.39 (p=0.031) and 1.26 (p=0.006) for 9 and 30 kV, respectively.

Discussion

In this study, we showed that LA (18:2n-6)-derived hydroxy- (9-HODE and 13-HODE) and hydroperoxy- (13-HpODE) fatty acids in healthy human subjects are sensitive to acute EF exposure. However, AA (20:4n-6)-derived hydroxy-fatty acids (20-HETE, 15-HETE, 12-HETE, and 5-HETE) and AA-derived 13,14-dihydro-15-keto-PGs were not affected by EF exposure. 9-HODE and 13-HODE can be generated *via* 15-LOX in macrophages and vascular cells [23]. However, Upston *et al.* have shown that non-enzymatic oxidation of LA produces an approximately equal mixture of 9-HODE and 13-HODE [24]. Thus, EF exposure may generate, at least in part, 9-HODE and 13-HODE by non-enzymatic oxidation but not 5-LOX, 12-LOX, cytochrome P450 oxidase 4A (CYP4A), and cyclooxygenase (COX)/15-hydroxy prostaglandin dehydrogenase (15-PGDH). However, the detailed mechanisms of EF-induced changes in 9-HODE, 13-HODE, and 13-HpODE remain to be elucidated.

Our findings show that immunoreactive SP is upregulated by acute EF exposure. EF exposure did not appear to adversely alter physiological peptide hormone levels, at least those of VIP, bradykinin, CGRP, or motilin. The molecular mechanisms of changes in immunoreactive SP concentrations following EF exposure are complex and can be interpreted in several ways. Recently, a new member of the tachykinin family that displays high sequence identity with SP has been discovered [25]. To better understand the properties of immunoreactive SP, it is important to clarify the specificity of SP against the novel tachykinin peptide family including hemokinin 1. Further studies may elucidate the identity of immunoreactive SP. TRPV1 is a nonselective cation channel present on sensory neurons that is activated by heat (> 43°C), protons, capsaicin, and endovanilloids [26-28]. OEA, an endovanilloid, has been suggested to function as an endogenous agonist of TRPV1 [17,29]. Kendall *et al.* recently reported that OEA and LA-derived hydroxy fatty acids, such as 9- and 13-HODE, were present in human skin at high concentrations [30]. Neuropeptides, such as SP and CGRP, are present in human skin [31]. Interestingly, Patwardhan *et al.* reported that 9-HODE and 13-HODE as endogenous ligands for TRPV1 were formed in animal skin biopsies after exposure to noxious heat at a temperature range of 40-55°C [19]. Of note is that application of 9-HODE to cultured trigeminal ganglia neurons stimulates the release of neuropeptide such as CGRP [19]. Nathan *et al.* reported that TRPV1-mediated SP release from primary sensory neurons [32]. Moreover, Miranda-Morales *et al.* have recently reported that axon reflexes evoked by TRPV1 activation are mediated by tetrodotoxin-resistant voltage-gated Na^+ channels in intestinal afferent nerves [33]. There is also evidence that electrical stimulation has a stimulating effect on release of SP from peripheral nerve terminals in the skin [34]. Thus, several endogenous ligands for TRPV1 may exert effects *via* SP release induced by the axon reflex at nerve terminals of peripheral sensory neurons in human intestine or skin [35]. Considerable evidence on the prevention of swallowing disorders has been obtained from studies of improvement of the swallowing reflex by capsaicin administration [36]. Ebihara *et al.* reported that administration of capsaicin improved the swallowing reflex by increasing SP levels [37,38]. In future, it will be important to assess the alleviative effect on dysphagia in clinical trial using EF exposure.

SP, LA, 13-HODE, and 13-HpODE induces endothelial-mediated vasorelaxation in the coronary artery of the pig [39,40]. Interestingly, OEA also causes endothelium-dependent vasorelaxation in the rat small mesenteric artery [41]. OEA activates on perivascular sensory nerves and induces neuropeptide release [41]. A similar vasodilation was reported by Tochio *et al.* in a study examining the effect of EF exposure on the rat small mesenteric artery [42]. Thus, it is conceivable that the increase of 9-HODE, 13-HODE, 13-HpODE, OEA, and immunoreactive SP levels in plasma is, at least in part, responsible for the improvement observed in patients with stiff shoulders who undergo EF treatment [7].

Capsaicin is used for chronic pain relief as a defunctionalization inducer of nociceptor [27,28,43]. For example, a capsaicin dermal patch is available for the treatment of peripheral neuropathic pain [44]. Our previous study has shown that acute EF (18 kV, 30 min) exposure

induces an increase in plasma OEA levels [15]. An experimental pretest—posttest design study by Shinba *et al.* showed that repetitive stimulation with EF exposure reduced the visual analog scale for pain in chronic pain with no obvious underlying disease [8]. Although repetitive EF treatment was not performed in this study, EF exposure may alleviate pain, at least in part, *via* desensitization of TRPV1 by 9-HODE, 13-HODE, or OEA. However, 9-HODE and 13-HODE also activates peroxisome proliferator-activated receptor-*gamma* (PPAR-γ) [45], raising the possibility that these receptors also serve as targets for 9-HODE/13-HODE during EF exposure. Further studies are in progress.

The results of this study also indicate that acute EF exposure affects plasma BDNF levels in healthy human subjects. Considerable evidence for the regulation of BDNF by *alpha*-melanocyte-stimulating hormone (α-MSH) has been obtained from the study of BDNF expression [46]. Nicholson *et al.* reported that BDNF release was induced by stimulation of the melanocortin-4 receptor [47]. We have reported that EF exposure induces an increase of the nonselective melanocortin receptor agonist α-MSH levels in plasma [16]. It is thus reasonable to speculate that EF exposure activates BDNF release/secretion or production through the upregulation of α-MSH. However, it is unclear at present whether changes in BDNF levels can be attributed to neurons, astrocytes, microglia, mast cells, fibroblasts, leukocytes, platelets, or keratinocytes. Further studies are needed to identify the BDNF signaling pathways induced by EF exposure. Notably, intact BDNF in peripheral circulation can cross the blood—brain barrier *via* a high-capacity, saturable transport system [48]. Positive correlations between blood BDNF and hippocampal BDNF levels have been observed in rats and pigs [49]. Neurotrophic activities in the hippocampus have been suggested to play a key role in spatial learning and memory function [50,51]. Interestingly, Yanamoto *et al.* have reported that EF exposure (5 h/day for 3 weeks) induces an increase of hippocampal BDNF levels in mice and an improvement of Morris water maze tasks in infarct lesions of mice [52]. In contrast, Campolongo *et al.* reported that post-training administration of OEA in rats enhances memory consolidation in a Morris water maze performance [53]. Thus, it is reasonable to speculate that EF exposure facilitates spatial learning and memory function *via* upregulation of OEA and BDNF. A recent study has shown negative correlations between plasma BDNF levels and objective evaluation of tinnitus severity [54]. In future, it will be of interest to evaluate the possible effect of EF exposure on tinnitus.

In conclusion, acute EF exposure exerted marked effects on plasma 9-HODE, 13-HODE, SP, and BDNF levels in healthy subjects. Our findings provide insight into the molecular mechanisms of health benefits induced by the HELP device (PRO-18T) and may also be important in the development of therapies for dysphagia, chronic pain, mild cognitive impairment, and tinnitus.

Competing interests

YN-Y, HH and AH are employed by Hakuju Institute for Health Science Co., Ltd., FN is employed by CMIC Pharma Science Co., Ltd., and MS is employed by Acel Inc.. All other authors have no competing interests.

Authors' contributions

YN-Y designed and supervised the research, and wrote the manuscript. FN performed LC—MS/MS. YN-Y, MS, HH, and AH performed the EF exposure and biochemical experiments. All authors have read and approved the final version of the manuscript.

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