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ORIGINAL ARTICLE

Comparative effects of a fixed *Polypodium leucotomos*/Pomegranate combination versus *Polypodium leucotomos* alone on skin biophysical parameters

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Abstract

OBJECTIVES: *Polypodium leucotomos* extract is a commonly used systemic photoprotective agent. In an exploratory fashion, the current study aimed to compare the effects of oral supplementation with a fixed *Polypodium leucotomos*/pomegranate combination (PPmix®) versus *Polypodium leucotomos* alone (Fernblock®) on skin biophysical parameters of Caucasian adults.

METHODS: Forty healthy adult volunteers (20 males and 20 females; mean age: 37.2±5.5 years) were randomized in a 1:1 fashion to a fixed *Polypodium leucotomos*/pomegranate combination (480 mg/day; n=20) or *Polypodium leucotomos* alone (480 mg/day; n=20) for 3 months. Six skin biophysical parameters (skin sebum content, hydration, transepidermal water loss [TEWL], erythema index, melanin index, and elasticity) were measured at baseline and after 3 months by personnel blinded to participant allocation.

RESULTS: At the end of the study, hydration and elasticity were significantly improved and TEWL was reduced in both groups, without significant intergroup differences. The erythema index was decreased by both treatments, although the fixed *Polypodium leucotomos*/pomegranate combination was significantly more effective. Finally, melanin index and skin sebum content were reduced by the fixed Polypodium leucotomos/pomegranate combination, whereas *Polypodium leucotomos* alone did not affect them.

CONCLUSIONS: Our results suggest that a fixed *Polypodium leucotomos*/pomegranate combination provides a greater improvement of skin biophysical parameters compared to *Polypodium leucotomos* alone in adult Caucasians. Our findings may have implications for optimizing systemic skin photoprotection and beautification strategies.

INTRODUCTION

Exposure of the skin to ultraviolet radiation (UVR) may directly produce varying levels of free radical formation and also deplete or inhibit to various degrees levels of endogenous antioxidants (Katta & Brown 2015; Emanuele *et al.* 2014). Consequently, supplementation of skin with antioxidants may be useful to compensate for their UVR-induced depletion (Fernández-García 2014), ultimately preventing oxidative damage to DNA and proteins in different skin cell types (Emanuele *et al.* 2014). The extract of *Polypodium leucotomos* – a species of fern in the Polypodiaceae family found in South America – is one of the most common oral supplements used for systemic photoprotection (Gonzalez *et al.* 2007; Choudhry *et al.* 2014; El-Haj & Goldstein 2015; Winkelmann *et al.* 2015; Bhatia 2015; Palomino 2015; Parrado *et al.* 2016). Research in healthy volunteers has shown that oral administration of *P. leucotomos* for two days is sufficient to protect the skin against subsequent exposure to artificial UVR (Middelkamp-Hup *et al.* 2004). Molecular studies demonstrated that *P. leucotomos* can decrease erythema following acute UVR exposure effect mainly by scavenging reactive oxygen species (ROS), ultimately mitigating UVR-induced formation of oxidized DNA bases (Zattra *et al.* 2009). Because long-term administration of *P. leucotomos* does not pose safety concerns (Nestor *et al.* 2015; Murbach *et al.* 2015), the extract has been also tested in a variety of skin conditions, including atopic dermatitis (Ramírez-Bosca *et al.* 2013) and as an adjunct in the treatment of pigmentation disorders – including vitiligo and melasma (Nestor *et al.* 2014). Although results have been generally promising, the mechanisms underlying the potential utility of *P. leucotomos* in these conditions have not yet been completely elucidated (Berman *et al.* 2016).

Apart from *P. leucotomos,* the characteristic biochemical composition of pomegranate fruit (*Punica granatum*) – being rich in antioxidants and anti-inflammatory polyphenols (e.g., punicalagin and other ellagitannins) (Syed *et al.* 2013; Johanningsmeier & Harris 2011) – has drawn attention in skin photoprotection and inhibition of photocarcinogenesis (Baccarin *et al.* 2015). Recent studies found that topical application of *P. granatum* extract can downregulate proinflammatory molecules – including COX-2 – both in an *ex vivo* model of porcine skin (Houston *et al.* 2016) and when administered orally to mice (Khan *et al.* 2012). *In vitro* experiments also demonstrated that *P. granatum* extract inhibits tyrosinase activity and melanin production (Rana *et al.* 2013). In addition, *P. granatum* concentrated solution has been shown to enhance hyaluronan synthesis, as well as suppress elastase, collagenase, and metalloproteinase (MMP)-1 activity, potentially exerting significant cosmetic effects (Kang *et al.* 2015).

In light of these findings, we hypothesized that a combination of *P. leucotomos* and *P. granatum* might exert additive or synergistic effects compared to the widely used *P. leucotomos* alone for systemic skin care applications. The current study was therefore designed to compare in an exploratory fashion the effects of oral supplementation with a fixed *Polypodium leucotomos*/pomegranate combination (PPmix®) *versus Polypodium leucotomos* alone (Fernblock®) on skin biophysical parameters of healthy Caucasian adults.

METHODS

Study participants

The study population comprised 40 volunteers aged >18 years (20 males and 20 females; mean age: 37.2±5.5 years) in apparently good physical health. All participants were of Caucasian descent (Fitzpatrick skin phototype II–III) and were free of any known dermatological conditions. Subjects with a history of significant neurologic, psychiatric, hematological, endocrine, cardiovascular, respiratory, renal, hepatic, or gastrointestinal disease, or coagulation deficits were excluded. Women who were pregnant or breastfeeding were also ineligible. The study was approved by the local ethics committee and complied with the tenets of the Declaration of Helsinki. Before the study, each participant was informed in detail about the purpose of the research, and signed informed consents were obtained.

Supplements

P. leucotomos extract (Fernblock®) was from IFC Group (Madrid, Spain). The fixed *P. leucotomos*/pomegranate combination (PPmix®) was purchased from Bioenx (Florence, Italy). The preparation – in form of a red-brownish hygroscopic powder – was obtained through a patented proprietary technology by spraying *P. leucotomos* powder with pomegranate juice containing polyphenols (punicalagin and other hydrolyzable tannins, gallic acid, anthocyanins, flavones). The relative percentages of *P. leucotomos* and pomegranate in the fixed *P. leucotomos*/pomegranate combination are covered by industrial intellectual protection.

Allocation

All participants were asked to withdraw any topical skin product 14 days before the beginning of the study. In addition, they were not allowed to use any topical skin intervention throughout the entire study period. With the use of a computer-generated random-allocation sequence, patients were assigned in a 1:1 fashion to receive tablets containing a proprietary fixed *P. leucotomos*/pomegranate combination (480 mg/day, n=20) or *P. leucotomos* alone (480 mg/day, n=20) for 3 months. Both the investigators and the study participants were blinded to supplement allocation.

Outcome measures

There were six skin biophysical parameters targeted as outcome measures in the study: skin sebum content,

Tab. 1. Changes from baseline to 3 months in skin biophysical parameters in two study groups.

	Fixed *Polypodium leucotomos*/pomegranate combination (n=20)		*Polypodium leucotomos* alone (n=20)	
	Baseline	**3 months**	**Baseline**	**3 months**
Skin sebum content	53.6±15.2	44.1±18.4*,†	55.0±13.6	57.1±14.5
Hydration	50.1±11.8	59.3±14.6*	51.7±12.9	60.1±18.3*
TEWL	14.4±5.3	10.3±4.2*	13.6±5.9	9.9±4.8*
Erythema index	359±101	287±93*,†	351±98	311±103*
Melanin index	175±48	154±60*,†	180±53	174±58
Elasticity	0.30±0.11	0.37±0.16*	0.33±0.13	0.41±0.19*

TEWL, transepidermal water loss. *p<0.001 versus baseline; †p<0.001 versus *P. leucotomos* alone.

hydration, transepidermal water loss (TEWL), erythema index, melanin index, and elasticity. All measurements were performed at baseline and repeated after 3 months of supplementation.

Assessment of skin biophysical parameters

All skin biophysical parameters were measured in the same anatomical location (right cheek) by personnel blinded to the allocated supplement. All assessments were performed at room temperature (20–25 °C) with a relative humidity of 40–50% according to previously described protocols (Firooz *et al.* 2012). Skin sebum content was measured with a Sebumeter SM 815 (Courage & Khazaka electronic GmbH, Cologne, Germany) and expressed as μg/cm^2. Hydration was assessed using a Corneometer CM 825 and the results were expressed using system-specific arbitrary units. TEWL was quantified with a TEWAmeter TM 300 and was expressed as g/m^2/h. The erythema index was calculated with Mexameter MX 18 from the strength of the absorbed and the reflected light at 568 and 660 nm, respectively. The melanin index was determined in a similar manner at 660 and 880 nm, respectively. Finally, skin elasticity was measured with a cutometer MPA 580 and expressed in arbitrary units.

Statistical analysis

Data are given as means ± standard deviations or counts, as appropriate. Categorical data were analyzed with the χ^2 test. Unpaired Student's t-tests were performed to compare the continuous variables at baseline. One-sample paired Student's t-tests were used for within-group comparisons between baseline and post-treatment skin biophysical parameters. Linear mixed models were utilized to detect potential interactions which might influence the relation between treatment and change in the study variables (including age and sex). All calculations were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Two-tailed p values <0.05 were considered statistically significant.

RESULTS

The mean age of subjects in the fixed *P. leucotomos*/pomegranate combination group was 37.5±5.9 years (age range: 29–50 years) and was not significantly different from that observed in the *P. leucotomos* alone group (37.1±5.3 years; age range: 29–48 years; p=0.80). The fixed *P. leucotomos*/pomegranate combination group comprised 9 males and 11 females, whereas the *P. leucotomos* alone group consisted of 11 males and 9 females (χ^2=0.10, p=0.75). No participant withdrew from the study and no adverse events were reported.

There were no statistically significant differences in baseline skin biophysical parameters between the two study groups (all p=ns). Table 1 shows the changes in skin biophysical parameters in the two study arms after 3 months of supplementation. At the end of the study, hydration and elasticity were significantly improved and TEWL was reduced in both groups, without significant differences between the two arms. The erythema index was decreased by both treatments, although the fixed *P. leucotomos*/pomegranate combination was significantly more effective (p<0.001 *versus P. leucotomos* alone). Finally, melanin index and skin sebum content were reduced by the fixed *P. leucotomos*/pomegranate combination (both p<0.001 *versus* baseline), whereas *P. leucotomos* alone did not affect them. Linear mixed models showed no interactions between age, sex, and any of the observed changes in skin biochemical parameters.

DISCUSSION

The central question addressed in this exploratory pilot study was whether a fixed *P. leucotomos*/pomegranate combination and *P. leucotomos* alone could display different effects on skin biochemical parameters when orally administered to adult healthy Caucasian individuals. Our main results were as follows: 1) the fixed *P. leucotomos*/pomegranate combination and *P. leucoto-*

mos were equally effective in improving hydration and elasticity, as well as in reducing TEWL; 2) the erythema index was significantly decreased in both arms, albeit the fixed *P. leucotomos*/pomegranate combination was significantly more effective; and 3) the melanin index and skin sebum content were significantly reduced by the fixed *P. leucotomos*/pomegranate combination, whereas *P. leucotomos* alone did not change these parameters.

Reduced skin hydration and elasticity are well-known markers of skin aging and are strongly related to decreased dermal collagen content (Choi *et al.* 2013). Besides loss of cutaneous hydration and elasticity, a higher TEWL – which reflects an increased cutaneous evaporation rate – is a common age-related skin problem that could ultimately lead to xerosis (Luebberding *et al.* 2013). Improvements in skin elasticity and moisture content – as observed in our study – suggest that long-term supplementation with either a *P. leucotomos*/pomegranate combination or *P. leucotomos* alone could contribute to maintain a youthful skin appearance. Although further investigations are needed, it could be speculated that such positive effects could be related to an increase of dermal matrix macromolecule biosynthesis and/or inhibition of MMPs. Although such effects were demonstrated for both *P. leucotomos* (Berman *et al.* 2016) and pomegranate extract (Kang *et al.* 2015) separately, no synergistic or additive effects of their combination were evident in terms of skin hydration, elasticity, and TEWL in the current study. In contrast, our results indicated that *P. leucotomos*/pomegranate combination or *P. leucotomos* alone displayed significant differences in terms of erythema and melanin indexes. As far as the erythema index is concerned, the study groups showed both a significant reduction, with improvements being significantly superior in subjects who received the fixed *P. leucotomos*/pomegranate combination. A more marked decrease in the skin erythema index in these participants may also be explained by additive effects exerted by pomegranate (as compared with *P. leucotomos* alone) on skin hyperemia and/or inflammation. Interestingly, the melanin index – which reflects the extent of skin pigmentation – was unaffected by supplementation with *P. leucotomos* alone but decreased significantly in subjects who received *P. leucotomos*/pomegranate combination. These observations suggest that active compounds present in the pomegranate component of the combination were responsible for the reduction of skin pigmentation, with punicalagin being the most plausible candidate (Rana *et al.* 2013). These results may pave the way for the use of the *P. leucotomos*/pomegranate combination in systemic skin lightening products. We are nonetheless aware that baseline skin pigmentation levels may be a confounding factor when pigmentary skin changes are assessed (Firooz *et al.* 2012). Because the present study included Caucasian subjects with limited range of skin phototype (II–III), further investigations will be needed to establish whether the same effect could be detected in different skin types. Finally, we observed that skin sebum content was significantly decreased by the *P. leucotomos*/pomegranate combination but not by *P. leucotomos* alone. An increased sebum production is associated with hyperplasia of the sebaceous glands and represents one of the major concurrent events associated with the development of acne (Zouboulis 2004). Interestingly, the biological function of sebocytes is stimulated by several factors – including activation of peroxisome proliferator-activated receptors (Trivedi *et al.* 2006) – which are known to be downregulated by pomegranate extract (Hontecillas *et al.* 2009). Additional research is therefore needed to disentangle the exact molecular mechanisms by which sebum production is decreased by the *P. leucotomos*/pomegranate combination.

Our findings need to be interpreted in the context of some limitations. First, it is known that skin biophysical parameters vary with age, sex, body site, and ethnicity (Firooz *et al.* 2012). In our study, age and sex were well-matched in the two study arms and their confounding impact on our results is likely to be minor, if any. To minimize the effect of body site, all measurements of skin biophysical parameters were performed in the right cheek. Second, analysis of human skin explants was not performed in the current report, and the histological changes that might be responsible for the observed biophysical effects of supplementation deserve further scrutiny. Finally, our study was limited to Caucasian individuals and might not be generalizable to other ethnic groups.

In summary, our results suggest that a fixed *P. leucotomos*/pomegranate combination provides a greater improvement of skin biophysical parameters compared to *P. leucotomos* alone in adult Caucasians. Our findings may have implications for optimizing systemic skin photoprotection and beautification strategies.

Conflicts of interest

Enzo Emanuele is a shareholder of Bioenx srl. All other authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this manuscript.

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ORIGINAL ARTICLE

Tyrosine hydroxylase gene expression is facilitated by alcohol followed by the degradation of the protein by ubiquitin proteasome system

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Abstract

OBJECTIVES: Alcohol intake induces brief periods of euphoria; however, its continuous consumption can lead the development of alcohol tolerance. The euphoria, an intense feeling of wellbeing, is deeply associated with dopamine. Dopamine biosynthesis is strictly regulated by tyrosine hydroxylase (TH), a rate-limiting enzyme of dopamine. The aim of this study was to examine the transient or chronic effects of ethanol treatment on TH protein level in vitro.

METHODS: Cultured primary mesencephalic neurons were prepared and exposed to 100 mM ethanol for 48 hours or 168 hours. TH and cAMP-responsive element (CRE)-mediated transcriptional activity was measured by reporter gene assay using pTH9.0kb-Luc and pCRE-Luc reporter plasmid. TH protein expression and TH phosphorylation was analyzed by Western blot analysis. Dopamine content was measured by high-performance liquid chromatography (HPLC).

RESULTS: Ethanol treatment for 48 hours facilitates TH transcriptional activity and TH protein expression in a cAMP-dependent protein kinase A (PKA) and MAPK/Erk kinase (MEK)-dependent manner in cultured mesencephalic neurons. Ethanol also facilitated TH phosphorylation, which resulted in the elevation of dopamine content. On the other hand, treatment with ethanol for 168 hours did not show significant elevation of TH gene expression and dopamine biosynthesis. Intriguingly, simultaneous treatment with MG-132, a 26S proteasomal inhibitor, recovered the ethanol-induced increase of TH protein expression and dopamine biosynthesis.

CONCLUSION: Transient ethanol-treatment facilitates TH gene expression and its phosphorylation in a PKA- and MEK-dependent manner to elevate dopamine biosynthesis, whereas continuous exposure to ethanol abolishes its potent effects on the dopaminergic function to reduce dopamine content. This reduction seems to originate from the decrease of TH protein level by degradation of the protein. Our current data may contribute to the better understanding of alcohol tolerance associated with degradation of TH protein to reduce total-TH level and dopamine biosynthesis.

Abbreviations:

TH	- tyrosine hydroxylase
pSer40-TH	- tyrosine hydroxylase phosphorylated at 40Ser
PKA	- cyclic AMP-dependent protein kinase
ERK	- extracellular signal-regulated kinase
MEK	- mitogen-activated protein kinase kinase/ERK kinase
CRE	- cAMP-responsive element

INTRODUCTION

Alcohol consumption is known to induce brief periods of euphoria. These feelings of wellbeing are deeply associated with monoamines and other hormones (Dfarhud *et al.* 2014). Dopamine is one of the representative "happiness" hormones and its biosynthesis is strictly regulated by the rate-limiting enzyme of dopamine, tyrosine hydroxylase (TH). As TH catalyzes the rate limiting step in catecholamine biosynthesis, it has proven to be the focus of many recent studies regarding the direct effect of alcohol consumption, due to its proven susceptibility over its expression and or function. As the adaptive response to ethanol exposure, there is an increase of TH expression in cell culture (Gayer *et al.* 1991) and in rat brain (Ortiz *et al.* 1995), indicating alcohol positively regulates TH gene expression.

The effects of either increased or decreased dopamine are thoroughly studied, such as the fact that increased blood-ethanol levels (Jones *et al.* 2006; Jones *et al.* 2004; Lewis & June 1990) exhibit stimulating effects, thought to be caused by a rise in dopamine content and the use of dopamine receptors antagonists, resulting in a loss of motor activity, the mechanisms by which this increase takes place is still not altogether understood. It is a sound belief that TH expression is enhanced as a result of ethanol exposure, as several studies have used TH inhibitors resulting in the suppression of the excitatory effects of ethanol. In this context, understanding how ethanol modifies the activity of the mesolimbic dopamine system, through the study of changes in TH gene expression, would identify a number of neurochemical and molecular markers associated with brain addiction, abstinence and reward responses (Gerlai *et al.* 2009). Recent studies suggest that continued exposure to ethanol results in the accumulation of TH via the cAMP / PKA pathway in association with other functional proteins, suggesting as to the cause of its stabilization and functionality within 24 hours of exposure (He & Ron 2008). However, the relation between the continuous consumption of alcohol and the acquisition of alcohol tolerance is still not fully understood.

Alcohol consumption is affected mainly by two major variables, the consumed amount and the length of consumption. Either caused by elevated TH gene expression or as a direct product of dopamine positive feedback loops, few studies have weighed the possibility of TH phosphorylation as the underlying cause for the effects of alcohol consumption (Nowicki *et al.* 2015; Yao *et al.* 2010), even though it might prove to be one of the primary mechanisms responsible for the differential effect of ethanol on different catecholaminergic systems that contribute to the observed differences in sensitivity and resultant expression within *in vivo* studies (French & Weiner 1984), such as significant gene expression variations following acute ethanol administration in the nigrostriatum (Oliva *et al.* 2008; Pellegrino *et al.* 1993). In addition, we previously showed that persisting TH phosphorylation results in the accumulation of phosphorylated TH to be degraded to reduce total-TH protein (Kawahata *et al.* 2009; Kawahata *et al.* 2015). We therefore focus in this study on the effect of ethanol not only on TH gene expression but also on TH phosphorylation and the degradation mechanism of TH.

When we get a tolerance for alcohol in the chronic drinking stage of alcoholism, the amount of alcohol consumption is elevated as its effects become progressively weaker. With intermittent alcohol administration, for instance, once daily for 7 days, elicited functional and structural plasticity in the dorsomedial striatum has been reported (Wang *et al.* 2015), however, the effects of persistent treatment with alcohol on dopaminergic function is still unclear. In this study, we examined the effects of ethanol treatment for 48 hours (transient model) or 168 hours (chronic model) on TH gene expression, phosphorylation and its degradation in cultured dopaminergic neurons. Our reporter gene assay and Western blot analysis revealed that 48 hours-treatment with ethanol facilitated TH gene expression and phosphorylation to increase dopamine content, but persistent treatment for 7 days promoted degradation of TH and attenuated TH's total protein level to reduce dopamine biosynthesis. These data suggest a possible mechanism for the alcohol tolerance why continuous consumption of alcohol decreases sensitivity to feel euphoria.

MATERIALS AND METHODS

Culture of primary mesencephalic neurons

The culture of primary mesencephalic neurons was prepared as described previously (Wakita *et al.* 2010). Briefly, two-thirds of the ventral mesencephalon was dissected from Wistar rat embryos (Japan SLC) on the 16th day of gestation. Dissected tissues were then chemically and mechanically dissociated into single cell suspensions. Cells were plated onto poly-L-lysine-coated 48-well multi-well plates or 35 mm plastic culture dishes at a density of 1.3×10^5 cells/cm^2. Cultures were maintained in Eagle's minimum essential medium (Nissui) supplemented with 10% fetal calf serum. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 100% relative humidity.

Ethanol and inhibitors

Ethanol (boiling point, 78.37 °C) was purchased from Wako (Japan). H-89, an inhibitor of cAMP-depen-

dent protein kinase A (PKA) or U0126, an inhibitor of MAPK/Erk kinase (MEK), were purchased from Calbiochem, Merck Millipore (Germany). Cultured mesencephalic neurons were treated with ethanol at a concentration indicated in the results. H-89 and U0126 were used at a concentration of 10 μM and simultaneously treated with ethanol. MG-132, a 26S proteasome was also purchased from Calbiochem, Merck Millipore, and were used at a concentration of 250 nM (Kawahata *et al.* 2009), For 1 week ethanol treatment, with or without MG-132, medium was changed every 48 hours to fresh one containing 100 mM ethanol and 250 nM MG-132.

Luciferase reporter gene assay

For reporter gene assays, a firefly luciferase reporter plasmid, pTH9.0kb-Luc (Iwawaki *et al.* 2000) or firefly luciferase reporter plasmid containing CRE (Clontech) was employed as a reporter plasmid. Transfection and reporter gene assays were conducted as reported previously (Kawahata *et al.* 2013). Primary mesencephalic neurons were plated at the density described above. After co-transfection with reporter plasmids (1.6 μg/ml/well) and phRG-TK, a Renilla luciferase control plasmid (Promega, WI, USA) (0.32 μg/ml/well), using Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA), cells were further cultured for 48 hours with 100 mM ethanol and then assayed for reporter activity. phRG-TK was used as an internal control to normalize for differences in transfection efficiency. All determinations were carried out in quadruplicate, and five independent experiments were performed.

Western blot analysis

For Western blotting, cells were plated on 35 mm plastic dishes at a density of 5×10^5 cells/dish and later washed twice with ice-cold PBS, and lysed as described previously (Kawahata *et al.* 2009). Proteins from cell lysates were subjected to sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) then transferred to PVDF membranes, and blots were blocked with 5% skim milk or 5% BSA/TBS-T at room temperature for 1 h, followed by overnight incubation with primary antibodies at 4°C. The following antibodies were used for immunoblotting: mouse anti-TH (1:1000) (Hatanaka & Arimatsu 1984), rabbit anti-phosphorylated TH at serine 40 residue (1:1000) (CST), anti-ubiquitin monoclonal antibody (1:1000) (LifeSensors) and mouse anti-β-actin (1:5000) (Sigma). Immunoreactive bands were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000; CST) and Immobilon Western Chemiluminescent HRP Substrate (Millipore). Images were obtained with LAS3000 (Fujifilm) or FUSION SOLO (VILBER). Multi Gauge software (Fujifilm) was used for quantification of immunoreactive bands, and five independent experiments were evaluated.

Dopamine assay

PC12D cells or cultured mesencephalic neurons were homogenized in 0.4 N perchloric acid in 1.5 ml tubes and centrifuged at 20,000 × *g* for 15 min. The dopamine levels in the supernatant were analyzed by high-performance liquid chromatography (HPLC) (Shimazu, Japan) with an SC5-ODS column (EICOM, Japan) and a mobile phase buffer containing 84 mM acetic acid-citrate (pH 3.5), 5 μg/ml EDTA, 190 mg/ml sodium 1-octane sulfonate, and 16% methanol. Monoamines were detected by electrochemical detection (ECD-700; EICOM). Five independent assays were carried out.

Data normalization

In our work, data normalization was employed only in the cases described below:

Reporter gene assay. To eliminate the difference of transfection efficiency in the control and chemotherapeutic agent-treated group, we divide the value of reporter gene transcription (TH) by the internal control, RG-TK. The value indicates the raw data ratio of reporter gene to internal control.

Statistical analysis

Statistical analysis was performed by two-way ANOVA analysis of variance with a *post-hoc* Tukey's multiple comparison test using Prism 5 software (GraphPad Software).

RESULTS

48 hour-ethanol treatment facilitates TH transcriptional activity in PKA and MEK-dependently in cultured mesencephalic neurons

To analyze the effects of alcohol treatment on the dopaminergic system, we first tested whether ethanol effects on the transcriptional activity of TH. Cultured mesencephalic neurons were exposed to ethanol at a concentration of 100 mM for 48 hours (He & Ron 2008; Gayer *et al.* 1991; Crews *et al.* 1999). Our reporter gene assay using pTH9.0kb-Luc TH reporter gene revealed that ethanol facilitated TH transcriptional activity (Figure 1 left, *** $p<0.001$ vs. control). When we treated the cells concomitantly with H-89, an inhibitor of cAMP-dependent protein kinase A (PKA), or U0126, an inhibitor of MAPK/Erk kinase (MEK), ethanol-induced facilitation of TH transcriptional activity was abolished (Figure 1 left, $^{\#\#\#}p<0.001$ *vs.* vehicle). These data suggest the participation of cAMP response elements (CRE) in the ethanol-induced facilitate of TH transcription. Expectedly, our CRE reporter gene assay revealed that ethanol facilitated CRE-mediated transcriptional activity (Figure 1 right, $^{***}p<0.001$ vs. control), which was abolished by the simultaneous treatment with H-89 or U0126 (Figure 1 right, $^{\#\#\#}p<0.001$ vs. vehicle). These results indicate that facilitation of TH transcription inducted by 48 hour-ethanol treatment is regulated by a PKA and MEK-dependent CRE-mediated pathway.

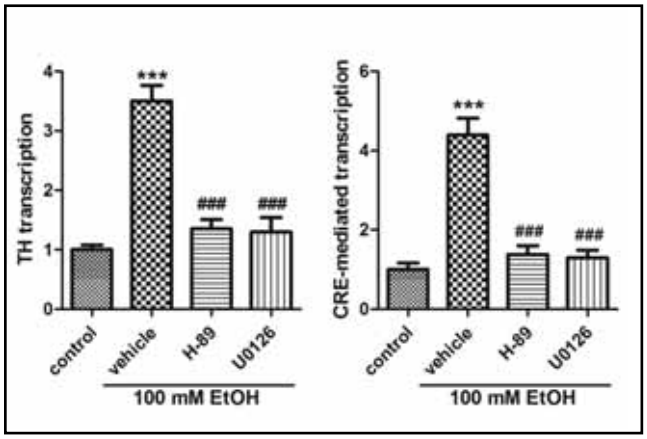

Fig. 1. Effects of 48 hour-treatment with ethanol on TH and CRE-mediated transcriptional activity in cultured mesencephalic dopaminergic neurons. Cultured neurons were transfected with pTH9.0kb-Luc reporter gene (left) or firefly luciferase reporter plasmid containing CRE (right) at 5 days *in vitro* (DIV). 24 hours after the transfection, neurons were exposed to 100 mM ethanol for 48 hours. 10 μM H-89 or U0126 was simultaneously treated with 100 mM ethanol. Values are expressed as the means ± SEM (n=5). ***p<0.001 *vs*. control; ### p<0.001 *vs*. vehicle (chequered column).

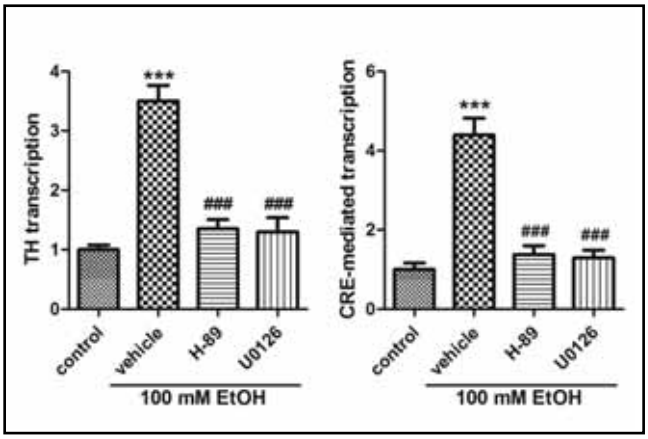

Fig. 2. Effects of ethanol on the protein expression and phosphorylation of TH in cultured mesencephalic dopaminergic neurons. (**A**) Cultured neurons were treated with 100 mM ethanol for 48 hours under the same condition as the reporter gene assay in Fig. 1. Neurons were lysed and subjected ito Western blot analysis. 10 μM H-89 or U0126was simultaneously treated with 100 mM ethanol. (**B**) Quantitative analysis of the result in **A**. Values are expressed as the means ± SEM (n=5). ***p<0.001 *vs*. control; ### p<0.001 *vs*. vehicle (chequered column).

48 hour-ethanol treatment facilitates TH protein expression and TH phosphorylation in cultured mesencephalic neuron

We next analyzed the effects of alcohol treatment on TH protein expression using cultured mesencephalic neurons. Cultured neurons were exposed to ethanol in the same manner as in the reporter gene assay shown in result above. Consistent with the facilitation of TH transcription (Figure 1), our Western blot analysis showed that TH protein expression was augmented by 100 mM ethanol treatment (Figure 2A and Figure 2B, ***p<0.001 *vs*. control). Ethanol-induced facilitation of TH protein expression was also abolished by simultaneous treatment with H-89, indicating that ethanol treatment-induced TH level elevation is mediated by a PKA-dependent pathway (Figure 2A and Figure 2B, ###p<0.001 *vs*. ethanol-treatment). Simultaneous treatment with ethanol and U0126 also resulted in the inhibition of ethanol-induced facilitation of TH expression (Figure 2A and Figure 2B, ***p<0.001 *vs*. control; ###p<0.001 *vs*. ethanol-treatment), suggesting that ethanol promote TH expression via MEK-dependent pathway.

TH phosphorylation plays an important role in regulating its dopamine biosynthesizing activity (Ramsey & Fitzpatrick 1998; Haycock 1990; Dunkley *et al.* 2004). Thus, we also tested the effect of ethanol on TH phosphorylation, by measuring the level of TH phosphorylation at Ser40 (pSer40-TH). As shown in Figure 2, 100 mM ethanol treatment up-regulated TH phosphorylation, which was mediated in a PKA-dependent manner. Simultaneous treatment with ethanol and U0126 resulted in the partial inhibition of the ethanol-induced facilitation of TH phosphorylation (Figure 2A and Figure 2B, ***p<0.001 *vs*. control; ###p<0.001 *vs*. ethanol-treatment), suggesting that ethanol partially promotes TH phosphorylation in a MEK-dependent cascade. These data indicates that ethanol facilitated TH gene expression and up-regulated the phosphorylation level in a PKA- and MEK-dependent manner.

Dopamine biosynthesis is facilitated by a 48 hour-ethanol treatment in cultured mesencephalic neurons

Promotion of TH phosphorylation results in the elevation of dopamine biosynthesizing activity (Dunkley *et al.* 2004). Therefore, we next analyzed the effect of ethanol treatment on dopamine biosynthesis in cultured mesencephalic neurons. Our HPLC analysis revealed that 100 mM methanol increases dopamine content in dopaminergic neurons (Figure 3, ***p<0.001 *vs*. control). This elevation in dopamine biosynthesis was suppressed by simultaneous treatment with H-89 (Figure 3, ###p<0.001 *vs*. ethanol-treatment). U0126 partially inhibited the ethanol-induced elevation of dopamine content (Figure 3, #p<0.001 *vs*. ethanol-treatment). These data suggests that 48 hour-ethanol treatment facilitates dopamine biosynthesis in a PKA- and in part MEK-dependent manner.

Facilitation of TH gene expression by ethanol is abolished by the chronic treatment caused by the proteasomal degradation of TH protein

Chronic drinking of alcohol results in the development of alcohol tolerance. Thus, to test the effect of persistent treatment with ethanol as a possible model of the continuous consumption of alcohol, we finally analyzed the

effect of a longer ethanol treatment on TH gene expression. 168 hour-treatment with 100 mM ethanol showed a tendency to facilitate TH gene expression, however, it did not elevate the TH protein amount significantly (Figure 4A and 4B, no significance *vs.* control). Notably, the elevation of the TH protein level by a 168-hour treatment with ethanol is much weaker than that observed in the 48 hour-treatment. We previously reported that accumulated pSer40-TH by facilitation of TH phosphorylation results in the degradation of TH protein by the ubiquitin proteasome system (Kawahata *et al.* 2009; Kawahata *et al.* 2015). These data raise the possibility that a part of accumulated pSer40-TH by ethanol treatment is degraded by proteasomes. Thus, we treated cultured neurons simultaneously with MG-132, a 26S proteasomal inhibitor, as well as ethanol, and then subjected to Western blot analysis. As expected, abolished elevation of TH protein level by 168 hour-ethanol treatment was recovered by simultaneous treatment with 250 nM MG-132 (Figure 4A and 4B, $^{***}p<0.001$ *vs.* control; $^{###}p<0.001$ *vs.* vehicle treatment). Additionally, 168 hour-treatment with 100 mM ethanol did not significantly elevate the pSer40-TH level, which was successfully recovered by MG-132 co-treatment (Figure 4A and 4B, $^{***}p<0.001$ *vs.* control; $^{###}p<0.001$ *vs.* vehicle treatment). The effect of MG-132 on the proteasomal inhibition was confirmed by the Western blot analysis using anti-ubiquitin monoclonal antibody (Figure 4C, EtOH+MG-132). It is noteworthy that, consistent with the result in Figure 4, dopamine content was not elevated by a 168-hour treatment with ethanol alone, whereas co-treatment with MG-132 recovered the ethanol-induced facilitation of dopamine biosynthesis (Figure 5, $^{***}p<0.001$ *vs.* control; $^{##}p<0.001$ *vs.* vehicle treatment). These data indicate that ethanol-induced facilitation of TH gene expression and accumulated TH phosphorylation is followed by the degradation of TH protein by 26S proteasome, possibly by the degradation of pSer40-TH.

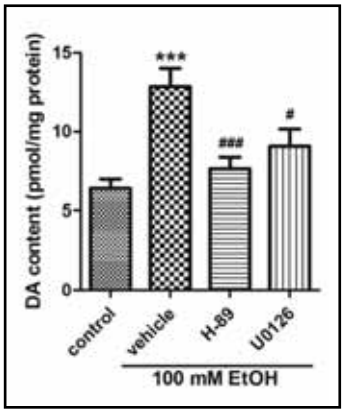

Fig. 3. Effects of ethanol on dopamine biosynthesis in cultured mesencephalic dopaminergic neurons. Cultured neurons were treated with 100 mM ethanol for 48 hours under the same condition as reporter gene assay in Fig. 1. Neurons were deproteinized in 0.4 N perchloric acid and centrifuged clear supernatants were subjected into HPLC analysis. Values are expressed as the means ± SEM (n=5). $^{***}p<0.001$ *vs.* control; $^{###}p<0.001$, $^{#}p<0.05$ *vs.* vehicle (chequered column).

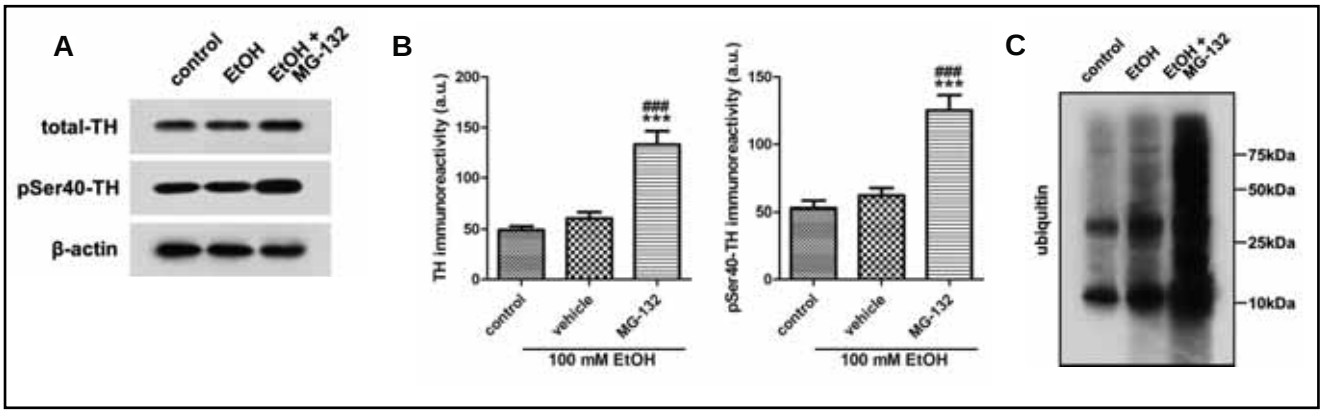

Fig. 4. Effects of 168 hour-treatment with ethanol and proteasome inhibition on the protein expression, phosphorylation of TH and ubiquitination in cultured mesencephalic dopaminergic neurons. (**A**) Cultured neurons were exposed to 100 mM ethanol with or without 250 nM MG-132 for 168 hours from 5 DIV, medium was changed every 48 hours to fresh one containing ethanol with/without 250 nM MG-132. At 12 DIV after 168 hours treatment with reagents, neurons were lysed and subjected to Western blot analysis. (**B**) Quantitative analysis of the result in **A**. Values are expressed as the means ± SEM (n=5). $^{***}p<0.001$ *vs.* control; $^{###}p<0.001$ *vs.* vehicle (chequered column). (**C**) Cultured neurons were simultaneously treated with 100 mM ethanol and 250 nM proteasomal inhibitor MG-132 in the same condition described in (A). Ubiquitinated proteins were visualized with anti-ubiquitin antibody.

DISCUSSION

In this study, we showed the effect of ethanol treatment on TH gene expression and its phosphorylation level in cultured mesencephalic neurons. 48 hour-ethanol treatment facilitated TH transcription and CRE-mediated transcription (Figure 1), which were H-89 and U0126 sensitive. 48 hour-ethanol treatment also elevated TH protein level and pSer40-TH level (Figure 2), resulting in the increase of dopamine content (Figure 3). These data indicate that facilitation of TH gene expression and dopamine biosynthesis by ethanol treatment is regulated by PKA and MEK-dependent CRE-mediated pathway. On the other hand, 168 hour-treatment did not augment the TH protein level significantly. The abolished ethanol-induced facilitation of TH protein

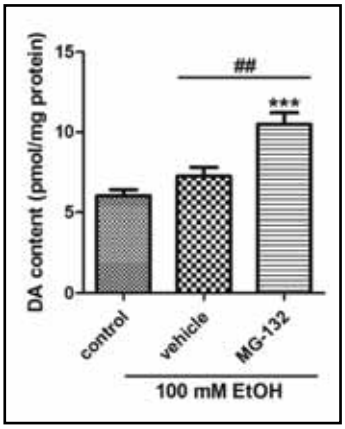

Fig. 5. Effects of 168 hour-treatment with ethanol and proteasome inhibition on dopamine biosynthesis in cultured mesencephalic dopaminergic neurons. Cultured neurons were treated with 100 mM ethanol for 168 hours with or without 250 nM MG-132. Neurons were deproteinized in 0.4 N perchloric acid and centrifuged clear supernatants were subjected into HPLC analysis. Values are expressed as the means ± SEM (n=5). ***$p<0.001$ *vs.* control; ##$p<0.01$ vehicle *vs.* MG-132.

expression and dopamine biosynthesis was successfully recovered by simultaneous treatment with MG-132, an inhibitor of the 26S proteasome (Figure 4). These data suggest that increase of TH protein level by ethanol treatment is abolished by the degradation of TH protein. Our data provide evidence that short-term ethanol treatment facilitates TH gene expression to increase dopamine content but long-term treatment does not have such potent effects on elevating TH protein level.

Previously, we found that a part of the accumulated TH protein is degraded by the 26S proteasome (Kawahata *et al.* 2009). We also reported that facilitation of TH phosphorylation in the dopamine and/or biopterin-deficient state, which resulted in the ubiquitination and degradation of pSer40-TH to reduce total-TH protein level (Kawahata *et al.* 2015). Therefore, our data in this study suggest the possibility that long-lasting TH phosphorylation induced by the ethanol treatment for 168 hours accelerates the degradation of accumulated pSer40-TH in cultured dopaminergic neurons to reduce dopamine biosynthesis. Consistently, 168 hour-ethanol treatment apparently increased ubiquitin-immunoreactive bands (Figure 4C, lane 2 EtOH). These data raise the possibility that continuous consumption of alcohol without intervals causes an attenuation of the facilitative effect of alcohol on TH gene expression by the degradation of TH protein via ubiquitin proteasome pathway.

In this study, we treated the cultured dopaminergic neurons with ethanol at concentration of 100 mM. This concentration is consistent with the ones used in previously reported studies that analyzed the effect of ethanol on dopamine producing cell lines and biogenic amine neurons (He & Ron 2008; Gayer *et al.* 1991; Crews *et al.* 1999). It is noteworthy that the blood concentration of alcohol shifts from approximately 40 mM to 60 mM in human after the consumption of alcohol (Jones *et al.* 2006). These data suggest that the ethanol concentration used in our study is somewhat similar to the one in human blood, therefor raise the possibility that consumed alcohol effects on the TH protein in the similar pharmacological mechanism shown in this study to be degraded by proteasome to decrease the alcohol sensitivity.

We cannot exclude the possibility of the participation of other pharmacological mechanisms in developing alcohol tolerance. For instance, long-term alcohol exposure may lead to development of alcohol tolerance in consequence of altered neurotransmitter functions. Alterations in the function of N-methyl-D-aspartate (NMDA) receptors are supposed to contribute to the development of the tolerance to ethanol (Nagy 2008). This is in part due to the increase of alcohol-dependent NMDA receptor phosphorylation. Also, Chronic ingestion of ethanol up-regulates NMDA receptor 1 subunit expression in rat hippocampus (Trevisan *et al.* 1994), indicating that increased NMDA receptor subunit levels in the hippocampus after chronic ethanol exposure may represent an important neurochemical substrate for some of the features associated with ethanol dependence and withdrawal. In addition, chronic ethanol-induced decreases in the a-subunit of the γ-Aminobutyric acid $(GABA)_A$ receptor may contribute to modify the tolerance to alcohol (Montpied *et al.* 1991; Mhatre *et al.* 1993; Sanna *et al.* 2003; Liang *et al.* 2007; Keir & Morrow 1994; Hirouchi *et al.* 1993; Buck *et al.* 1991). These data suggest the involvement of not only the dopaminergic system but also NMDA and GABA receptors in the acquisition of alcohol tolerance and dependence.

In conclusion, this study provides crucial evidence that ethanol potently regulates TH gene expression in a PKA- and MEK-dependent CRE-mediated pathway. It also facilitates TH phosphorylation PKA-dependently, which is accompanied by the elevation of dopamine biosynthesis. However, continuous exposure to ethanol brings an attenuation of its facilitative effects on the dopaminergic system, as accumulated pSer40-TH can be targeted to the 26S proteasome for degradation. Our data raised the possibility that intermittent alcohol consumption causes a generalized sense of euphoria brought upon by the elevation of dopamine level, but continuous consumption aids in the development of alcohol tolerance, presenting a diminished level of dopamine biosynthesis by decreased TH protein and its phosphorylation. Combined with the non-dopaminergic mechanisms described above, our finding may contribute to understand the mechanism of acquisition of alcohol tolerance and dependence.

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