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Uptake and protective effects of ergothioneine in human endothelial cells

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Running title: Ergothioneine protects endothelial cells

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Non-standard abbreviation used: COX-1, cyclooxygenase 1; DCFH-DA, dichlorodihydrofluorescin diacetate; eNOS, endothelial nitric oxide synthase; HBMECs, human brain microvascular endothelial cells; H_2O_2, hydrogen peroxide; MTT, 3-(4,5-Dimethylthgiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NO, nitric oxide; NOX-1, NADPH oxidase 1; OCTN-1, organic cation transporter novel type-1; $OH^{\bullet}$, hydroxyl radicals; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; S.E.M., standard error of mean; SOD, superoxide dismutase.

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Abstract

Ergothioneine is a thiourea derivative of histidine found in food, especially mushrooms. Experiments in cell-free systems and chemical assays identified this compound as a powerful antioxidant. Experiments were designed to test the ability of endothelial cells to take up ergothioneine and hence benefit from protection against oxidative stress. RT-PCR and Western blotting demonstrated transcription and translation of an ergothioneine transporter in human brain microvascular endothelial cells (HBMECs). Uptake of [^{3}H]ergothioneine occurred by the organic cation transporter novel type-1 (OCTN-1), was sodium-dependent and was reduced when expression of OCTN-1 was silenced by siRNA. The effect of ergothioneine on the production of reactive oxygen species (ROS) in HBMECs was measured using dichlorodihydrofluorescin and lucigenin, and the effect on cell viability was studied using the 3-(4,5-Dimethylthgiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. ROS production and cell death induced by pyrogallol, xanthine oxidase plus xanthine and high glucose were suppressed by ergothioneine. The antioxidant and cytoprotective effects of ergothionine were abolished when OCTN-1 was silenced using siRNA. The expression of NADPH oxidase 1 was decreased, and those of glutathione reductase, catalase and superoxide dismutase enhanced by the compound. Ergothioneine attenuated the reduction in relaxation of isolated rat basilar arteries to the endothelium-dependent vasodilator acetylcholine caused by pyrogallol, xanthine oxidase plus xanthine or incubation in high glucose. Chronic treatment with the compound improved the response to acetylcholine in arteries of rats with streptozotocin-induced diabetes. In summary, ergothioneine is taken up by endothelial cells via OCTN-1, where the compound then protects against oxidative stress, curtailing endothelial dysfunction.

Introduction

Endothelial cells synthesize and release various factors, in particular nitric oxide (NO), that regulate vascular tone, angiogenesis, inflammatory responses, haemostasis, and permeability. Endothelial dysfunction is associated with a number of cardiovascular disease processes including hypertension, atherosclerosis, heart failure, coronary syndrome and stroke (Fèlètou and Vanhoutte, 2006). Oxidative stress is an important pathogenic element in endothelial dysfunction. Reactive oxygen species (ROS) such as superoxide anions reduce the bioavailability of NO by scavenging it avidly, resulting in the formation of peroxynitrite (Rubanyi and Vanhoutte, 1986; Gryglewski et al., 1986).

Ergothioneine was first discovered in the rye ergot *Claviceps purpurea*. A variety of foods contain ergothioneine in trace quantities, with the highest amounts found in some mushroom species including *Lentinus edodes* (shiitake), *Pleurotus ostreatus and P. eryngii*, while moderate amounts are found in certain meat products such as kidney (Ey et al., 2007). Ergothioneine is a dietary water-soluble sulfur-containing histidine derivative (Fig. 1). Ergothioneine was first identified as an antioxidant in cell-free experiments. It inhibits formation of hydroxyl radical (Motohashi and Mori, 1986), and superoxide anions, (Cargnoni et al., 1995), singlet oxygen production (Obayashi et al., 2005), lipid peroxidation (Deiana et al., 2004) and peroxynitrite oxidative damage (Aruoma et al., 1997). As opposed to other naturally occurring thiols (e.g., glutathione, N-acetylcysteine), ergothioneine is present in aqueous solutions predominantly as a thione rather than a tautomeric thiol structure; as a consequence, ergothioneine is a stable antioxidant that is non-autoxidizable at physiological pH (Hartman, 1990). Ergothioneine is positively charged at physiological pH, and requires the specific carrier protein organic action transporter novel type-1 (OCTN-1) to cross the cell membrane (Nakamura

et al., 2008; Grigat et al., 2007). OCTN-1 is mainly found in kidney, trachea, and bone marrow (Tamai et al., 1997; Koepsell et al., 2007), but its existence in other cell types has not been explored.

Number of compounds possess antioxidant properties *in vitro* but are ineffective *in vivo*. A typical example is flavonoids (Halliwell, 2009). Although *in vitro* studies demonstrate that ergothioneine may act as a cytoprotectant (Aruoma et al., 1999; Rahman et al., 2003; Jang et al., 2004; Markova et al., 2009), few *in vivo* findings with ergothioneine are available. Oral administration of ergothioneine protects fatty acids against ferric-nitrilotriacetate-induced oxidation in rat kidney and liver (Deiana et al. 2004). *In vivo* treatment of rats with ergothioneine also protects intestinal and liver tissues from damage during ischemia/reperfusion injury (Bedirli et al, 2004; Sakrak et al., 2008). In addition, chronic administration of ergothioneine inhibits brain lipid peroxidation, protects against cisplatin-induced neuronal injury and enhances cognition in the mouse (Song et al., 2010). Endothelial cells are exposed to oxidative stress, in particular in diabetic subjects (Guzik et al., 2002). However, the potential protective effects of ergothioneine on endothelial cells and blood vessels have not been investigated. The present experiments were designed to examine ergothioneine uptake by endothelial cells, its effects on ROS production and cytotoxicity, and its ability to preserve vascular function during oxidative stress. The *in vivo* effects of ergothioneine on vascular responsiveness were studied in rats with streptozotocin–induced diabetes.

Materials and Methods

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Materials

SuperScript II first-stand synthesis system, primers, agarose, and TRIzol reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). All primary and secondary antibodies were bought from Abcam (Cambridge, UK). [^{3}H]Ergothioneine was purchased from Moravek (Brea, CA, USA). MTT, hypoxanthine, xanthine oxidase, pyrogallol, Triton X-100, streptozotocin, acetylcholine and ergothioneine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human brain microvascular endothelial cells (HBMECs) were purchased from ScienCell™ Research Laboratories (San Diego, California, USA) and cultured according to the manufacturer's procedures. Cells were incubated in 5% CO_2 / 5% air at 37°C. Specific culture medium for HBMECs was purchased from ScienCell™ and the medium was supplemented with 5% fetal bovine serum, 1% endothelial cell growth supplement (ScienCell™) and 1% penicillin/streptomycin. The medium was changed every three days. Experiments were performed between the second and sixth passage.

RNA isolation and RT-PCR

Total RNA was isolated from HBMECs using TRIzol reagent (Invitrogen). Two µg of total RNA were used for first strand cDNA synthesis using random hexamer primers and Superscript II RNase Reverse Transcriptase (Invitrogen). The resulting first strand cDNA was directly used for PCR amplification. The two primers used for amplifying OCTN-1 (accession number AF079117) were 5'-CTGGATGCTCCTAATTTACATGG-3' (sense) and 5'-

AGGAGACTCTCTAGAAATGGTTGG-3' (antisense), which generated a 785 bp fragment. The program was as follows; 30 cycles of 94°C for 1 minute, 55°C for 1.5 minute, and 72°C for 1.5 minute. Products were analyzed by agarose gel electrophoresis and visualized under ultraviolet light by staining with ethidium bromide.

Western blotting

HBMECs were grown to confluence in 10 cm culture dishes. The cells were washed three times with ice cold phosphate buffered saline (PBS), and suspended in 2 ml of 5 mM sodium phosphate (pH 8) and protease inhibitor cocktail (Sigma-Aldrich) was added to a 1:100 (v/v) ratio. Cells were sonicated briefly and centrifuged at 3,000 *g* for ten minutes to remove nuclei and unbroken cells. The resulting supernatant was resolved on 9% (w/v) SDS-PAGE and electrotransferred onto nitrocellulose membranes. After blocking with 5% (w/v) nonfat dry milk in PBS overnight at 4°C, nitrocellulose membranes were incubated with the primary antibodies (1:100 [v/v] dilution in blocking solution) at room temperature for 2 hours. The membranes were then washed extensively with 0.02% (v/v) Triton X-100 in PBS. After washing, they were incubated with horseradish-conjugated secondary antibody (1:5000 [v/v] dilution in blocking solution) at room temperature for 2 hours. Excess secondary antibody was removed by washing, and the bound secondary antibody was detected by enhanced chemiluminescence using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA, USA). The expected molecular weights of OCTN-1, NADPH oxidase 1 (NOX-1), xanthine oxidase, endothelial nitric oxide synthase (eNOS), cyclooxygenase 1 (COX-1), glutathione reductase, catalase, Zu/Cu-superoxide dismutase (SOD) and Mn-SOD were calculated as 62, 65, 150, 140, 69, 58, 60, 17 and 25 kDa, respectively.

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Silencing of OCTN-1

HBMECs were transiently transfected with siRNA specific for OCTN-1 (Abcam, Cambridge, UK) for 10 hours with RNAifect Transfection Reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. HBMECs were then further cultured for 24 to 48 hours before being used for RT-PCR, Western blotting and [^{3}H]ergothioneine uptake studies. Cells transfected with a scrambled sequence served as control.

Uptake of [^{3}H]ergothioneine

HBMECs were cultured in 24-well plates until confluence. The culture medium was discarded and the cells were washed twice with PBS. The wash buffer was removed and Ringer solution (or sodium-free Ringer solution) containing [^{3}H]ergothioneine (2 µCi/ml, 1 nM) was added to the cells. The Ringer solution was composed of 135 mM NaCl, 5 mM KCl, 3.33 mM NaH_2PO_4, 0.83 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 5 mM HEPES. The sodium-free Ringer solution was composed of 140 mM NMOG, 5 mM HEPES, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM glucose. Sodium-containing and sodium-free Ringer solutions were adjusted to pH 7.4. The cells were incubated at room temperature for 1 to 15 minutes. To terminate the uptake, the [^{3}H]ergothioneine was removed and cells were quickly washed five times with ice cold PBS. The 24-well plates were air dried at room temperature. Complete cell lysis was achieved by overnight incubation with Triton-X100 (10%; 500 µl per well). Radioactivity was measured with a β-scintillation counter (Model LS6500; Beckman Coulter; Brea, CA, USA).

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ROS production

Intracellular concentrations of reactive oxygen species (ROS) in HBMECs were measured using OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence; Cell Biolabs Inc, San Diego, CA, USA). Cells were first cultured in 24-well plates. Prior to ROS measurement, the culture medium was discarded and the cells were washed gently three times with PBS. 2'-7-Dichlorodihydrofluorescin diacetate (DCFH-DA) was added to each well and incubation allowed in 5% CO_2 / 95% air at 37°C for sixty minutes. Cells were then washed three times with PBS. Lysis buffer was added to each well and further incubation allowed in 5% CO_2 / 95% air at 37°C for 5 minutes. Lysates were transferred to a black 96-well fluorometric plate and fluorescence was measured using a FLUOstar OPTIMA (BMG Labtechnologies, Offenburg, Germany) with excitation and emission wavelengths of 485 and 520 nm, respectively.

The release of ROS from HBMECs was also measured using the lucigenin chemiluminescence assay. Briefly, confluent monolayers of HBMECs were detached with trypsin (0.05% and 0.02% EDTA) and re-suspended in Ringer solution. After 20 minutes, lucigenin (5 µM) was added and the emitted chemiluminescence was immediately recorded for 1 minute with a luminometer (Model MLX; Dynex Technologies; Chantilly, VA).

Cell viability

HBMECs were cultured in 24-well plates and the cell viability was measured using the 3-(4,5-Dimethylthgiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. After washing twice with PBS, MTT (5 mg/ml) was added to each well. The cells were incubated in 5% CO_2 / 95% air at 37°C for 4 hours. Following formation of MTT formazan crystals, 10% SDS in 0.01 M HCl was added to each well and incubation overnight at 37°C allowed to lyse the cells and

solubilize the MTT formazan crystals. Lysates were transferred to a 96-well plate and the absorbance at 580 nm was measured.

Animals and tissue preparations

All animal experiments performed in this study were approved by the Committee on the Use of Live animals in Teaching and Research of The University of Hong Kong. Sprague–Dawley rats (450–600 g, 16 weeks old, male) were used. Diabetes was induced by a single administration of streptozotocin (55 mg/kg, injected in the tail vein) (Shi et al., 2006). Following 72 hours of food deprivation, tail blood samples were obtained and blood glucose concentrations were measured using a one-touch glucometer (LifeScan Inc, USA). Induction of diabetes was considered successful when the blood glucose level was higher than 16.6 mM (day 0). Non-diabetic control rats were injected with vehicle solution alone and kept under identical conditions. In certain experiments, chronic treatment with ergothioneine (10-1000 μg/kg/day) was started on day 0. This dosage range of ergothioneine has been used in other studies and no toxicity was observed (Bedirli et al., 2004; Moncaster et al., 2002). In the rat, a steady blood concentration of 20 μg/ml (~87 μM) is achieved when a single intravenous dose of 100 mg/kg ergothioneine is given (Kaneko et al., 1980). Ergothioneine was dissolved in drinking water and given to both control and streptozotocin-treated rats by gavage. Six to 12 weeks later, the rats were sacrificed. Non-fasting blood glucose levels were measured on the day of the experiment. Control rats with glucose higher than 11.1 mM were excluded from the study.

Isometric tension recording

The basilar arteries were carefully isolated from the rat brain under a dissecting microscope and

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placed in Krebs–Henseleit solution [118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose (control solution)]. The arteries were cut into rings of 2 mm length. The rings were suspended in the 5 ml organ chambers of a Mulvany-Halpern wire myograph (Model 610M, Danish Myo Technology A/S, Aarhus, Denmark) using 25 μm steel wires to record tension. The organ chambers were filled with control solution at 37°C and aerated with 95% O_2/5% CO_2 to maintain pH at 7.4. Changes in isometric tension were relayed to a MacLab 4 amplifier (ADInstruments; Bella Vista, Australia) and saved to a computer system (sampling rate 100/s).

During the initial equilibration period of 45 minutes, the rings were stretched until the resting tension held steady at approximately 0.6 g. U46619 (100 nM) was added to establish stable contractile tone. In order to study endothelium-dependent relaxations, a cumulative concentration–response curve to acetylcholine was obtained during the sustained contraction to U46619.

Statistical analysis

Data are expressed as means ± standard error of mean (S.E.M.) of three experiments performed in triplicate. Student's *t*-test and analysis of variance were used for paired and multiple variants, respectively. *P* values less than 0.05 were considered to indicate statistically significant differences.

Results

Ergothioneine uptake

Reverse transcriptase-PCR amplified a DNA fragment of the apparent size of the OCTN-1 gene

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in RNA isolated from HBMECs (Fig. 2A), and sequencing confirmed the expression of the transporter in these cells. Western blotting demonstrated the presence of the OCTN-1 protein in HBMECs (Fig. 2A).

To investigate whether or not ergothioneine can enter endothelial cells, [^{3}H]ergothioneine uptake was measured. The radioactive compound was taken up by HBMECs in a time-dependent manner (Fig. 2B), and this uptake could be abolished by 50 mM of non-radiolabeled ergothioneine, or when sodium was removed in the bathing solution, showing specificity and sodium-dependency, respectively, of the transport. To verify the contribution of OCTN-1 to ergothioneine transport in HBMECs, OCTN-1 expression was knocked down using siRNA. In OCTN-1 silenced cell lines, protein levels were lowered by 90.2% (Fig. 3A). Silencing of OCTN-1 caused a 74.3% reduction in [^{3}H]ergothioneine uptake (Fig. 3B).

Antioxidant and cytoprotective effects

DCFH is more specific for the detection of hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and peroxynitrite ($ONOO^-$), while lucigenin is more specific for detection of superoxide anions (O_2^-) (Myhre et al., 2003; Tarpey and Fridovich, 2001). Pyrogallol (100 μM) increased DCHF-induced fluorescence 7.6-fold (Fig. 4A) and increased lucigenin-induced chemiluminescence 2.9-fold (Fig. 4B). The pyrogallol-induced ROS generation was reduced by ergothioneine in a concentration-dependent manner. The MTT assay was used to investigate if ergothioneine was able to protect endothelial cells against oxidative stress-induced cell death. Ergothioneine itself (up to 10 mM) was not toxic to the cells (data not shown). The compound reduced the cytotoxic effect of pyrogallol and this cytoprotective effect was concentration-dependent; 1 mM of ergothioneine lowered the cytotoxic effect of 100 μM pyrogallol from 77% to 22% (Fig. 4C).

A combination of 1 mM hypoxanthine plus 10 mU of xanthine oxidase was also used to induce oxidative stress in HBMECs. Hypoxanthine/xanthine oxidase caused a 2.3-fold and 1.5-fold increase in DCHF-induced fluorescence and lucigenin-induced chemiluminescence, respectively (Figs. 5A and 5B). Hypoxanthine/xanthine oxidase-induced ROS decreased cell viability, and this cytotoxicity was alleviated by ergothioneine in a concentration-dependent manner; 1 mM ergothioneine lowered the cytotoxic effect from 33% to 6% (Fig. 5C).

DCHF-induced fluorescence and lucigenin-induced chemilluminescence increased by 80% and 46%, respectively, when the concentration of glucose in the culture medium was augmented from 5.5 mM to 25 mM for three days (Figs 6A and 6B). In addition, the viability of HBMECs was reduced by 29% when they were incubated in 25 mM glucose (Fig. 6C). The high glucose-induced oxidative stress and cytotoxicity were abolished by ergothioneine (100 μM and 1 mM). The antioxidant and cytoprotective effects of ergothioneine (1 mM) were comparable to those of tiron (10 mM) and stronger than those of vitamin C (100 μM) (Figs. 4-6).

To investigate whether or not the protective effects of ergothioneine depend on its uptake into the endothelial cells, experiments were repeated in HBMECs with OCTN-1 knocked down using siRNA. In OCTN-1 silenced cells, the antioxidant and cytoprotective effects of ergothioneine were abolished (Fig. 7).

Oxidative and antioxidant enzymes

To evaluate the involvement(s) of different oxidative and antioxidant enzymes in the action of ergothioneine, expression levels of NOX-1, xanthine oxidase, eNOS, COX-1, glutathione reductase, catalase, Cu/Zn-SOD and Mn-SOD were measured in HBMECs. Ergothioneine (1 mM) decreased the expression level of NOX-1, and increased the expression levels of

glutathione reductase, catalase, Cu/Zn-SOD and Mn-SOD (Fig. 8). There were no apparent changes in the expression of xanthine oxidase, eNOS and COX-1 (Fig. 8).

Endothelium-dependent relaxations

To study endothelium-dependent relaxations, rat basilar arteries were contracted with the TP receptor agonist U46619 (100 nM) and exposed to increasing concentrations of acetylcholine. Ergothioneine itself had no effect on acetylcholine-induced relaxations. Pre-incubation with pyrogallol (100 μM), hypoxanthine (1 mM) plus xanthine oxidase (10 mU) or glucose (25 mM) impaired the acetylcholine-induced relaxations (Fig. 9). This impairment in relaxation was inhibited by ergothioneine in a concentration-dependent manner, and this effect was comparable to that of tiron (10 mM).

Streptozotocin-induced diabetes resulted in a blunting of the maximal relaxations of the basilar artery to acetylcholine to 33% (Fig. 10). Chronic treatment with ergothioneine (100 μg/kg/day or 1000 μg/kg/day) did not affect the response to acetylcholine of basilar arteries of control rats. Ten and 100 μg/kg/day of ergothioneine also had no significant effect on acetylcholine-induced relaxations in preparations of streptozotocin-treated rats. However, when the streptozotocin-treated rats were fed with a higher dosage of ergothioneine (1000 μg/kg/day) for 6 weeks, the maximal relaxation to acetylcholine averaged 73% (Fig. 10). This effect was significantly greater than that of vitamin C (1000 mg/kg/day) and comparable to that of apocynin (16 mg/kg/day) (Fig. 10).

Discussion

The investigations of the antioxidant effects of ergothioneine in cells have yielded controversial

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results. In the concentration range of 10 to100 μM, the compound failed to protect rabbit hearts from oxidative injury or prevent glutathione oxidation (Cargnoni et al., 1995). However, in toxicity assays, millimolar concentrations of ergothioneine protect against cell damage (Aruoma et al., 1999; Rahman et al., 2003; Jang et al., 2004). In addition, at concentrations above 100 μM, ergothioneine reduces the cellular level of ROS, and protects DNA, proteins, and lipids from damage in keratinocytes subjected to solar-simulating ultraviolet light (Markova et al., 2009). These studies imply that the antioxidant effect of ergothioneine is concentration-dependent and may be highly dependent on the amount taken up by the cells. Ergothioneine carries a positively charged ammonium group. Theoretically, this chemical property makes it membrane impermeable (Gründemann et al., 2005). The cellular uptake of ergothioneine relies mainly on a specific transporter known as OCTN-1 (Gründemann et al., 2005). This dependency is illustrated in knockdown experiments demonstrating that OCTN-1 gene expression is directly proportional to the decrease in ergothioneine uptake in HeLa cells (Paul and Snyder 2010). Furthermore, transfection of the OCTN-1 gene into HEK 293 cells allows uptake of ergothioneine and reduces Cu^{2+}-induced cytotoxicity (Nakamura et al., 2008).

Endothelial cells are subjected to oxidative stress (Félétou and Vanhoutte, 2006). The present experiments aimed to determine whether or not OCTN-1 is expressed in this cell type. The RT-PCR and Western blotting data clearly identified the presence of OCTN-1 mRNA and protein in HBMECs, while the [^{3}H]ergothioneine uptake experiments demonstrated that the compound is transported actively into these endothelial cells. The fact that non-radiolabeled ergothioneine could compete with [^{3}H]ergothioneine in these experiments, strongly indicates that uptake occurs via a specific transporter rather than by passive diffusion. OCTN-1 transports ergothioneine more efficiently when sodium is present (Gründemann et al., 2005; Nakamura et

al., 2008). This earlier finding was corroborated by the present study which demonstrated that [^{3}H]ergothioneine uptake in endothelial cells is also sodium-dependent. A specific inhibitor of the OCTN-1 transporter is unavailable at present, hence a pharmacological approach could not be adopted to verify its involvement in the uptake of ergothioneine. Instead, a siRNA approach was utilized, which demonstrated that [^{3}H]ergothioneine uptake was greatly diminished when OCNT-1 expression was silenced, further demonstrating the role of OCTN-1 in transporting ergothioneine into endothelial cells.

Several studies are relevant for a vascular protective effect of ergothioneine. The compound reduces hydrogen peroxide-induced cell death in rat pheocromocytoma cells (Colognato et al., 2006), HeLa cells (Paul and Synder, 2010) and human neuronal hybridoma cells (Aruoma et al., 1999). Ergothioneine attenuates ischemia/reperfusion injury in rat intestine and liver by increasing heat shock proteins-70 and decreasing tumor necrosis factor-α, interleukin-1β, malondialdehide and myleoperoxidase levels in the tissues (Sakrak et al., 2008; Bedirli et al., 2004). Ergothioneine is obtained from diet and its concentration in human blood ranges from 11.9 to 13.5 mg/L (i.e. 51.9 to 58.9 μM) for normal subjects, 11.6 to 13.6 mg/L (i.e. 50.6 to 60.1 μM) for type I diabetic subjects and 11.9 to 21.9 (i.e. 51.9 to 95.5 μM) for type II diabetic subjects (Epand et al., 1988). A more recent study reported that the median serum ergothioneine level is as low as 1.01 μM in middle-aged and older adults (Sotgia et al., 2013). This discrepancy in the blood levels of ergothioneine may be due to the different sensitivities of chemical analysis and the age of study population (36-55 versus 55-85 years old). In the present study, pharmacological concentrations of ergothioneine (i.e. > 100 μM, and as high as 10 mM) were not toxic to HBMECs. To study the cytoprotective effect of ergothioneine in endothelial cells, oxidative stress was induced. Pyrogallol generates ROS and induces apoptosis in human

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lymphoma (Saeki et al., 2000) and glioma cells (Sawada et al., 2001), and in rat mesangial cells (Moreno-Manzano et al., 2000). The present experiments also showed that ergothioneine reduces ROS production in HBMECs and protects them against pyrogallol-induced cell death. Likewise, when oxidative stress was induced by hypoxanthine plus xanthine oxidase [known to generate O_2^-, H_2O_2 and uric acid (Radi et al., 1990)], ergothioneine reduced ROS generation and cell death in HBMECs. These findings are in line with the observations in porcine coronary arteries that administration of purine and xanthine oxidase caused endothelial dysfunction and sustained contraction which could be prevented by ROS scavengers such as mannitol and catalase (Ishihara et al., 2008).

The antioxidant effect of ergothioneine can be accounted for by its ability to form stable complexes with copper, zinc and iron ions (Motohashi et al., 1986; Hartman, 1990). Ergothioneine also interacts with ROS, most probably through the quenching of singlet oxygen (Hartman et al., 1988). In addition, the compound reacts with hydroxyl radicals (Rougee et al., 1988; Akanmu et al., 1991), and scavenges hypochlorous acid (Akanmu et al., 1991), peroxyl radical (Asmus et al., 1996) and peroxynitrite (Aruoma et al., 1997). The effects of ergothioneine on oxidative and/or antioxidant enzymes are largely unexplored. It inhibits tyrosinase but the reported IC_{50} value of 4.47 mM is relatively high, suggesting a weak correlation between tyrosinase inhibition and antioxidant activity (Liao et al., 2012). The present study documents that, besides being a direct ROS scavenger, ergothioneine reduces the expression NOX1, a major source of oxygen-derived free radicals in the vascular wall (Guzis et al., 2002) but increases that of antioxidant enzymes such as glutathione reductase, catalase, and superoxide dismutase (SOD). These findings add a new dimension to the potential role of ergothioneine in protecting against oxidative stress.

The antioxidant and cytoprotective effects of ergothioneine on HBMECs suggested that ergothioneine may preserve endothelial integrity and hence prevent vascular dysfunction, particularly under conditions related to oxidative stress such as diabetes. Acute administration of scavengers of superoxide anions such as SOD improves the abnormal endothelium-dependent responses in different models of diabetes (Pieper et al., 1996; Bohlen and Lash 1993; Pieper et al., 1997). Similarly, treatment with antioxidants such as probucol (Tesfamariam and Cohen, 1992), N-acetylcysteine (Pieper and Siebeneich, 1998), vitamin E (Keegan et al., 1995) prevents the development of endothelial dysfunction in experimental diabetes. Unfortunately, chronic treatment with antioxidants such as vitamins C and E usually fails to improve endothelial function in human diabetics (Pellegrini et al., 2004; Gazis et al., 1999). The reason for this failure is not known, but disease duration in relation to antioxidant treatment may be important. In studies of animal models of diabetes, antioxidant supplements usually are given from the onset on of the disease. By contrast, humans may have had impaired glucose tolerance and/or undiagnosed diabetes for years before initiation of treatment. Another possible reason for the divergent effects of antioxidants in animal and human studies is related to the dose used. For example, the effects of vitamin C have been assessed in humans with Type II diabetes, but up to 1 mM administered directly into the brachial artery was required to counteract the reduction in methacholine-induced vasodilatation (Ting et al., 1996). Such high local concentrations of vitamin C cannot be achieved by oral administration. Consistent with earlier work on endothelial dysfunction, the present study shows that relaxations to the endothelium-dependent vasodilator acetylcholine (Furchgott and Zawadzki, 1980) were dampened by ROS producers (pyrogallol; hypoxanthine/xanthine oxidase) (Abrahamsson et al., 1992; Kamata et al., 2006) and hyperglycemic conditions (25 mM glucose *in vitro*; streptozotocin-induced diabetes *in vivo*)

(Guo et al,, 2000; Pieper et al., 1995; Leo et al., 2011). This oxidative stress-associated impairment of relaxations was prevented by ergothioneine. Importantly, ergothioneine was more potent than vitamin C in protecting the endothelial integrity and vascular function, reflecting the potential beneficial effects of the substance as regards to vascular health.

Ergothioneine has long been recognized as an antioxidant amino acid present in food (especially in mushrooms) (Kawano et al., 1982). The compound is rapidly cleared from the circulatory system and retained in the body with minimal metabolism (Mayumi et al., 1978). The absence of toxicity, high potency, and broad spectrum antioxidant properties, together with the high water solubility and stability at physiological pH, make ergothioneine, especially when compared with existing conventional antioxidants, a very attractive candidate for protecting endothelial cells against oxidative stress and thus for the treatment and/or prevention of oxidative stress-associated cardiovascular diseases.

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Authorship Contributions

Participated in research design: Li, Yang, Sit, Kwan, Lee, Hoi, Chan, Hausman, Vanhoutte, Leung

Conducted experiments: Li, Yang, Sit, Leung

Contributed new reagents or analytic tools: Kwan, Lee, Hoi, Chan

Performed data analysis: Li, Yang, Sit, Leung

Wrote or contributed to the writing of the manuscript: Hausman, Vanhoutte, Leung

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Footnotes

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Figure Legends

Fig. 1 Structure of ergothioneine.

Fig. 2. (A) Detection and measurement of OCTN-1 (A) mRNA (top panel) using RT-PCR (positive control with human kidney cDNA indicates the expected size of 785 bp), and protein (bottom panel) using Western blotting (positive control with human kidney protein indicates the expected molecular size of 62 kDa). (B) Time-course of ergothioneine uptake in HBMECs. [^{3}H]Ergothioneine uptake (2 μCi/ml, 1 nM) was measured in the presence of non-radioactive ergothioneine (50 mM) or in the absence of Na^+ as indicated. Values are means ± S.E.M. of three experiments carried out in triplicate. *P < 0.05 versus with Na^+.

Fig. 3. Effects of siRNA knockdown of OCTN-1 on ergothioneine uptake in HBMECs. (A) Protein expression in cells transfected with OCTN-1 siRNA and a scrambled control sequence. Protein levels were normalized against β-actin. (B) [^{3}H]Ergothioneine uptake in cells transfected with OCTN-1 siRNA and a scrambled control sequence. Values are means ± S.E.M. of three separate experiments. *P <0.05 versus control.

Fig. 4. Effects of ergothioneine on pyrogallol-induced ROS generation and cytotoxicity. Cells were treated with 100 μM pyrogallol in the absence (control) or presence of different concentrations of ergothioneine (1 μM to 1 mM) for 48 hours. The generation of ROS is indicated by the amount of (A) DCF-induced fluorescence and (B) lucigenin-induced chemilluminescence. Relative fluorescence units and relative light units were normalized to total cellular protein. (C) Cell viability was measured by the MTT assay. Tiron (1 mM) and vitamin C

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(100 μM) were used as positive controls and for comparison. Values are means ± S.E.M. of three experiments. *P <0.05 versus group with pyrogallol but without ergothioneine.

Fig. 5. Effects of ergothioneine on hypoxanthine/xanthine oxidase-induced ROS generation and cytotoxicity. Cells were treated with 1 mM hypoxanthine and 10 mU xanthine oxidase in the absence (control) or presence of different concentrations of ergothioneine (1 μM to 1 mM) for 48 hours. The generation of ROS is indicated by the amount of (A) DCF-induced fluorescence and (B) lucigenin-induced chemilluminescence. Relative fluorescence units and relative light units were normalized to total cellular protein. (C) Cell viability was measured using the MTT assay. Tiron (1 mM) and Vitamin C (100 μM) were used as positive controls and for comparison. Values are means ± S.E.M. of three experiments. *P <0.05 versus group with hypoxanthine and xanthine oxidase but without ergothioneine.

Fig. 6. Effects of ergothioneine on high glucose-induced ROS generation and cytotoxicity. Cells were treated with 25 mM glucose in the absence (control) or presence of different concentrations of ergothioneine (1 μM to 1 mM) for 48 hours. The generation of ROS is indicated by the amount of (A) DCF-induced fluorescence and (B) lucigenin-induced chemiluminescence. Relative fluorescence units and relative light units were normalized to total cellular protein. (C) Cell viability was measured by the MTT assay. Tiron (1 mM) and vitamin C (100 μM) were used as positive controls and for comparison. Values are means ± S.E.M. of three experiments. *P <0.05 versus 25 mM glucose without ergothioneine.

Fig. 7. Effects of ergothioneine on pyrogallol-induced ROS generation and cytotoxicity in OCTN-1-silenced HBMECs. HBMECs were transfected with OCTN-1 siRNA or a scrambled control sequence. Cells were treated with 100 μM pyrogallol in the absence or presence of ergothioneine (1 mM) for 48 hours. The generation of ROS is indicated by the amount of (A) DCF-induced fluorescence and (B) lucigenin-induced chemilluminescence. Relative fluorescence units and relative light units were normalized to total cellular protein. (C) Cell viability was measured by the MTT assay. Values are means ± S.E.M. of three experiments. *P <0.05 versus group with pyrogallol but without ergothioneine.

Fig. 8. Effects of ergothioneine on the enzyme expression. Western blot analysis was carried out to measure changes in expression of (A) NOX1, (B) xanthine oxidase, (C) eNOS, (D) COX-1, (E) glutathione reductase, (F) catalase, (G) Zn/Cu-superoxide dismutase and (H) Mn-superoxide dismutase after HBMECs were incubated without (control) or with ergothioneine (1 mM). The amount of protein was normalized against β-actin. Values are means ± S.E.M. of three separate experiments. *P <0.05 versus control.

Fig. 9. Effects of ergothioneine on endothelium-dependent relaxations of rat basilar arteries. Rat basilar arteries were contracted with U46619 (100 nM) and acetylcholine-induced relaxations were measured after incubation of the arteries with (A) pyrogallol (100 μM) for 30 minutes, (B) 1 mM hypoxanthine and 10 mU of xanthine oxidase for 30 minutes, and (C) 25 mM glucose for 6 hours in the absence (control) or presence of different concentrations of ergothioneine (10 μM to 1 mM). Tiron (1 mM) served as a positive control and for comparison. Changes in tension are expressed as a percentage decrease of the contraction to U46619. Values are means ± S.E.M. of

five experiments. *P <0.05 versus control.

Fig. 10. Effects of ergothioneine on endothelium-dependent relaxations of basilar arteries in streptozotocin-induced diabetic rats. Rats were injected without (control) or with intravenous administration of streptozotocin (55 mg/kg) and fed with or without ergothioneine (10 to 1000 μg/kg/day) for 6 weeks. Basilar arteries were contracted with U46619 (100 nM) and acetylcholine-induced relaxations were measured. Vitamin C (1000 mg/kg/day) and apocynin (16 mg/kg/day) served as a positive control and for comparison. Changes in tension are expressed as a percentage decrease of the contraction to U46619. Values are means ± S.E.M. of five experiments. *P <0.05 versus control. †P < 0.05 versus streptozotocin.

Fig. 1

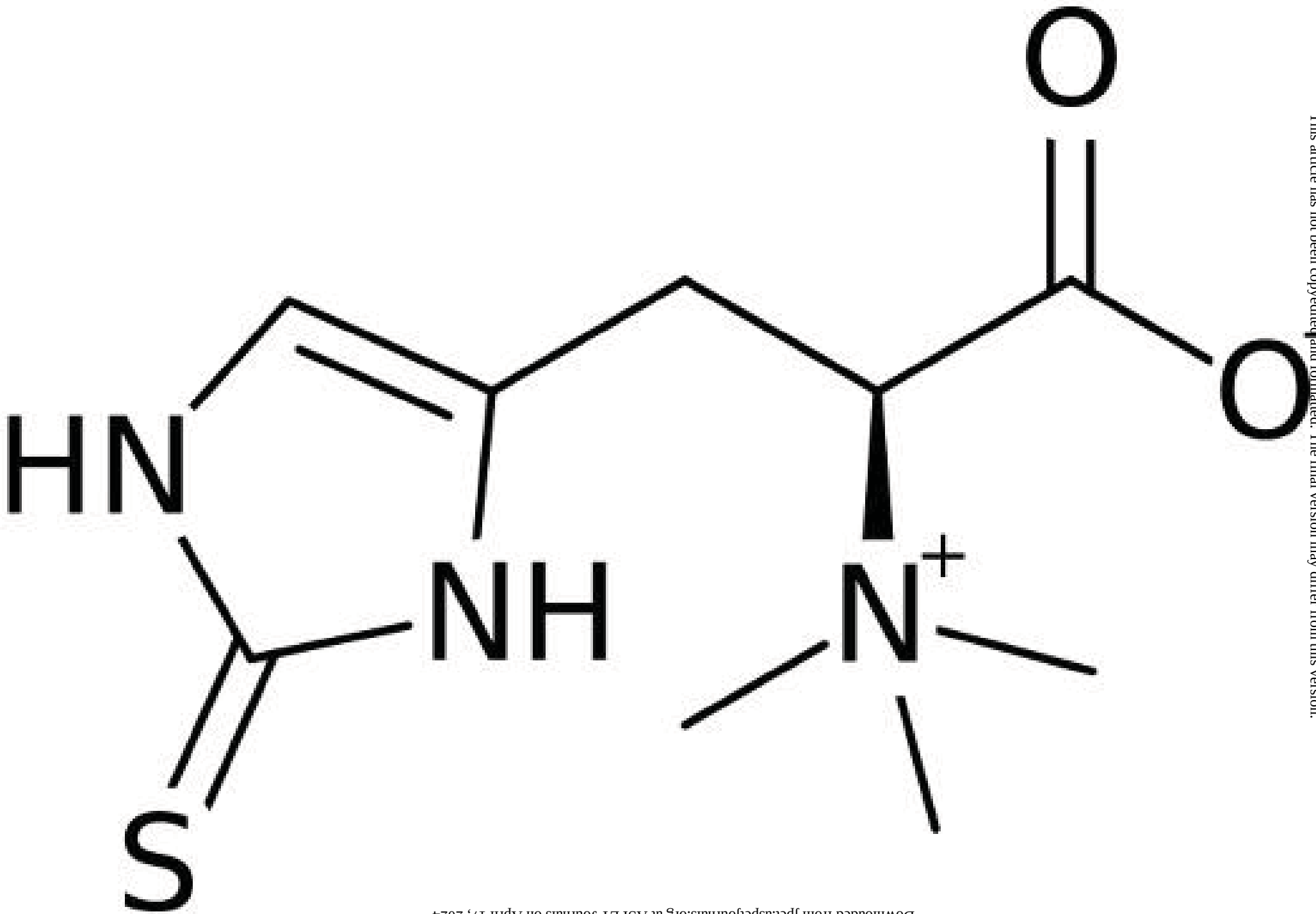

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Fig. 2

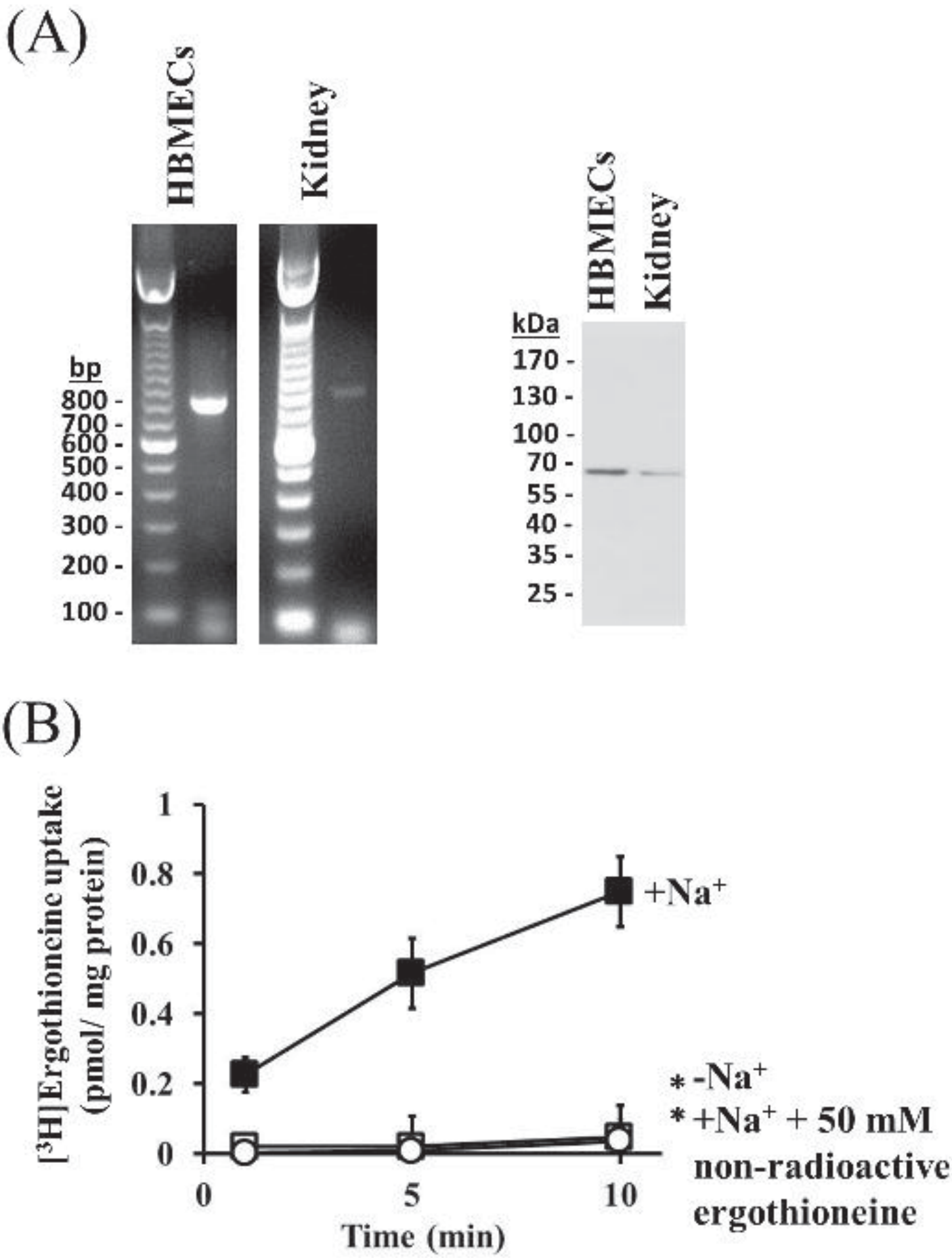

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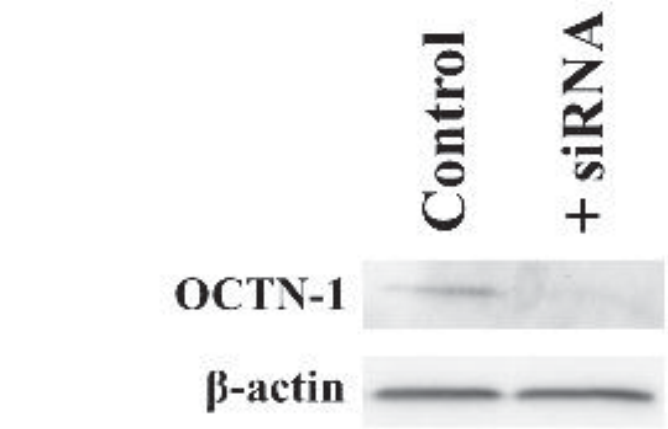
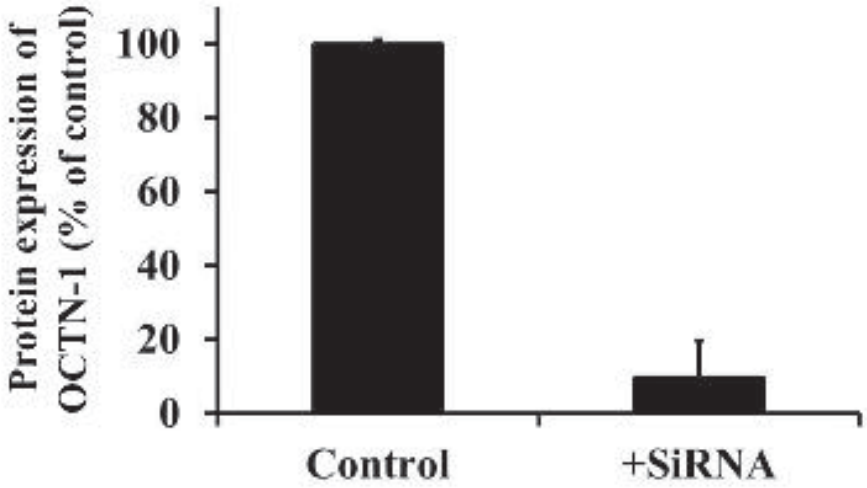
Fig. 3

(A)





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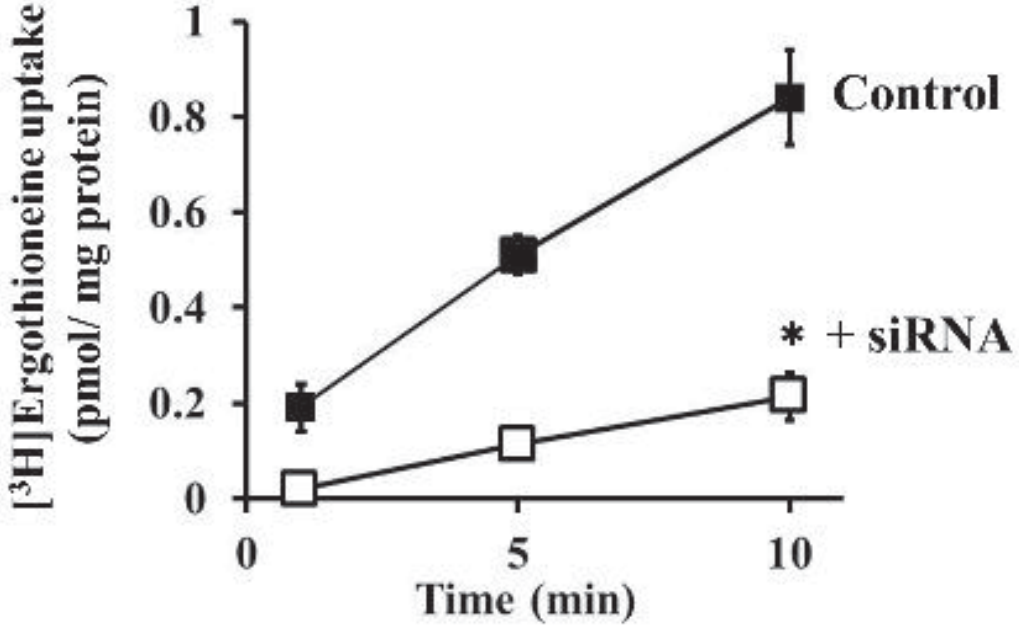

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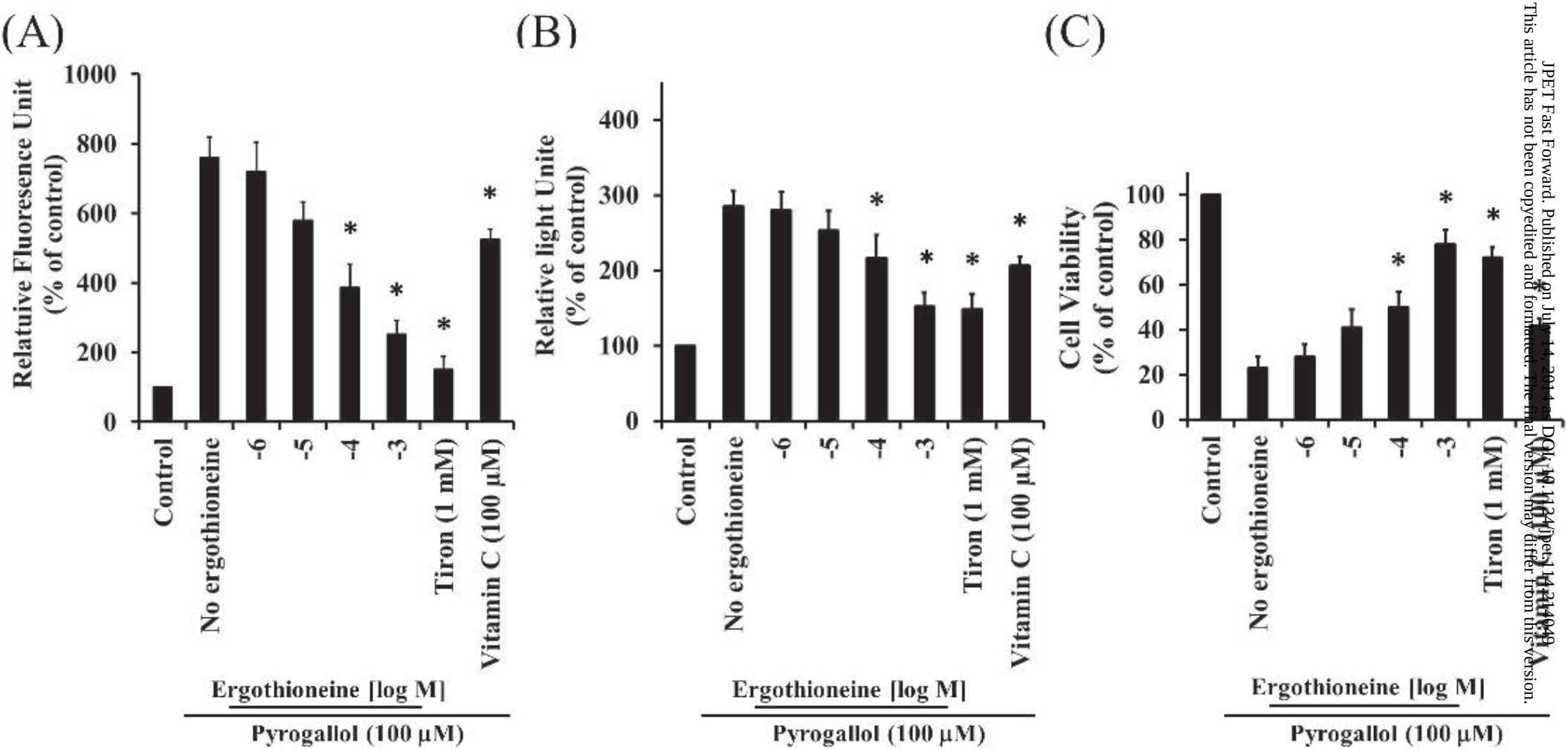

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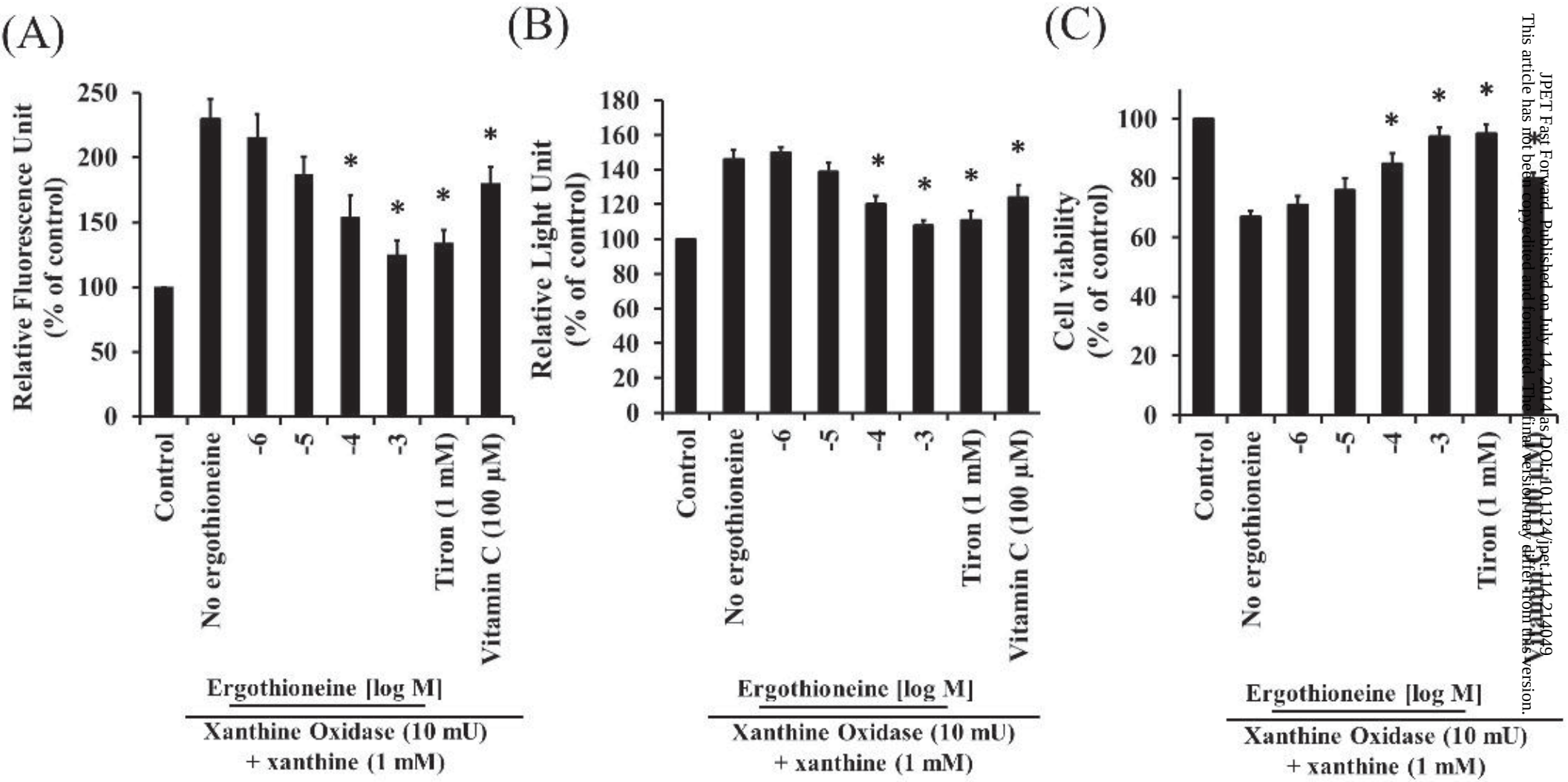

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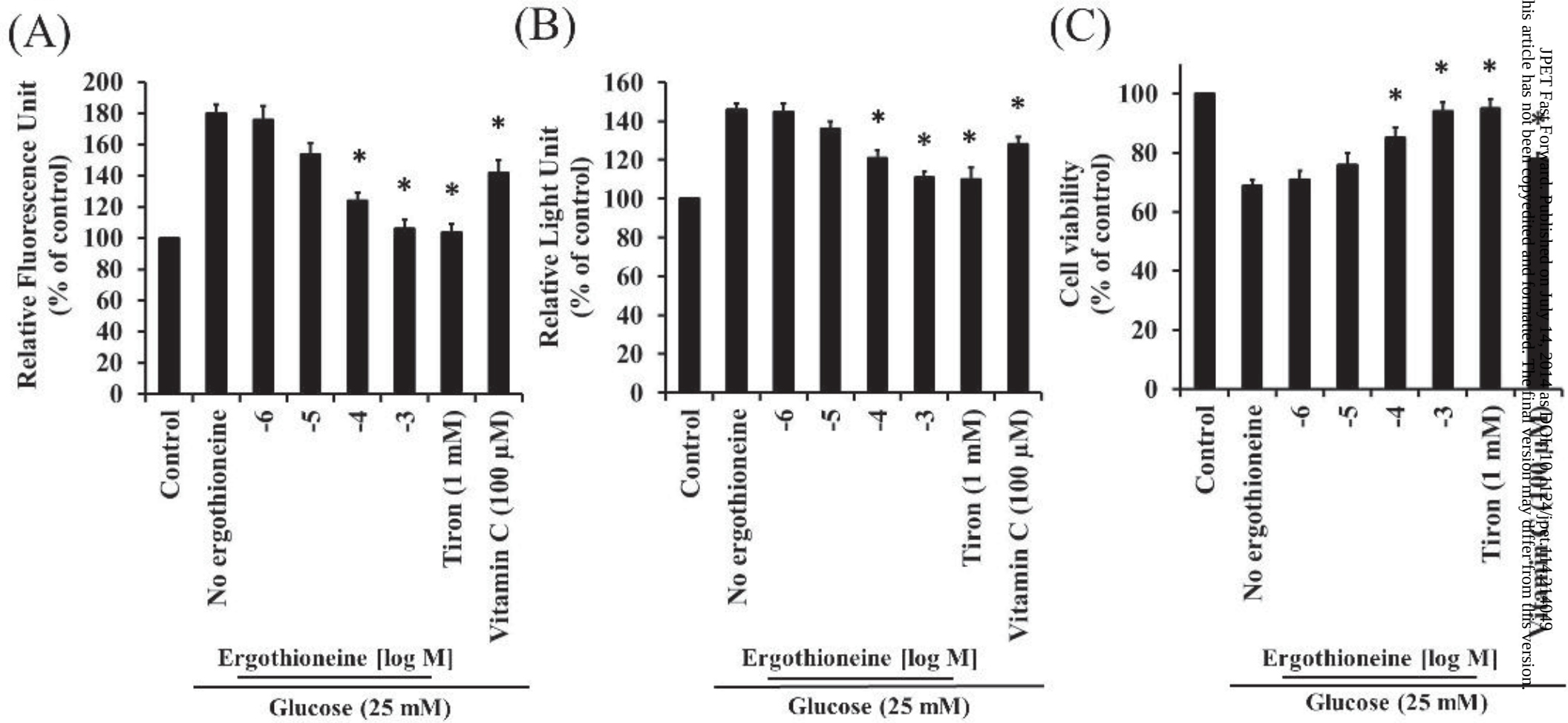

Fig. 7

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Fig. 8

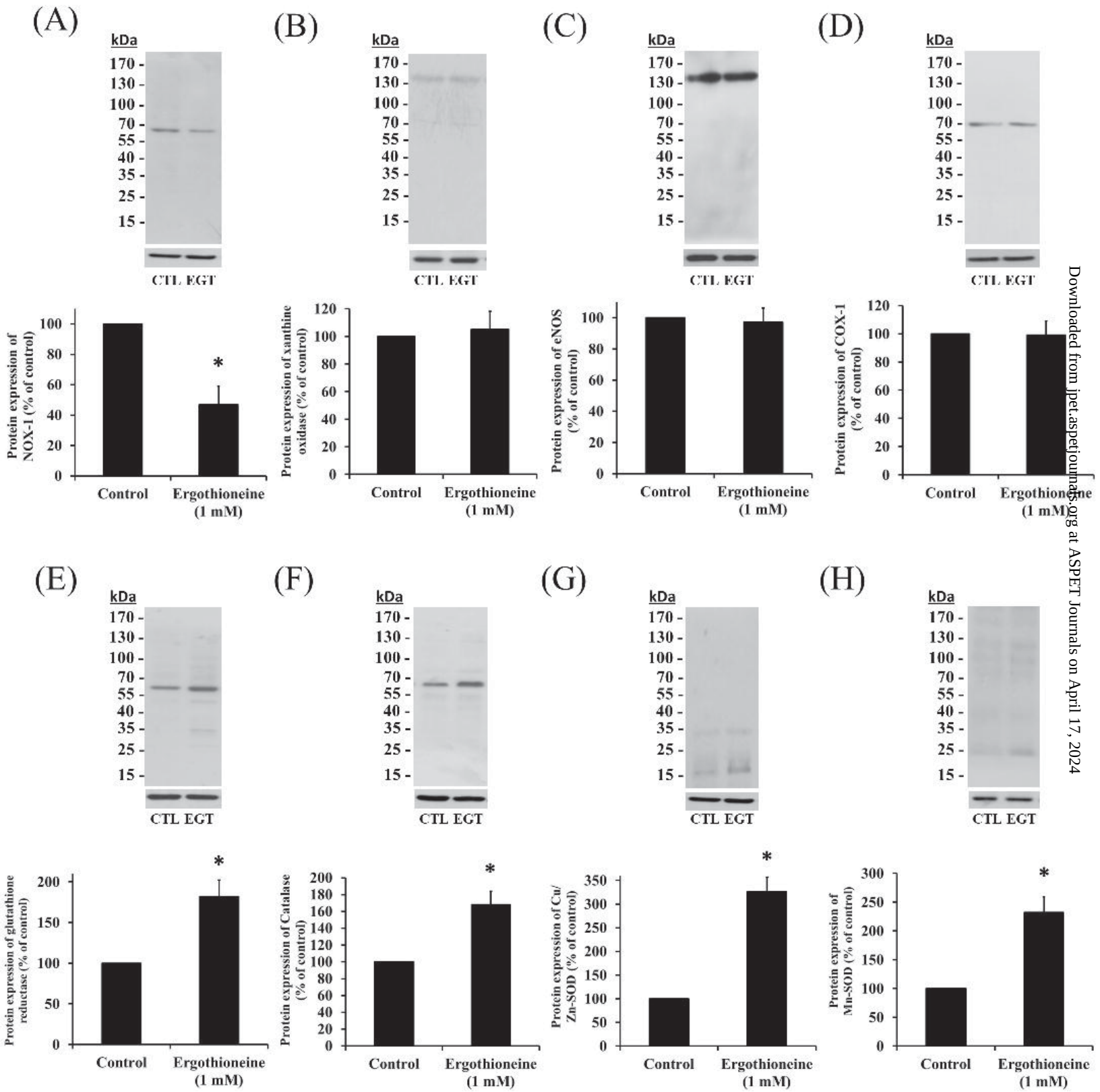

Fig. 9

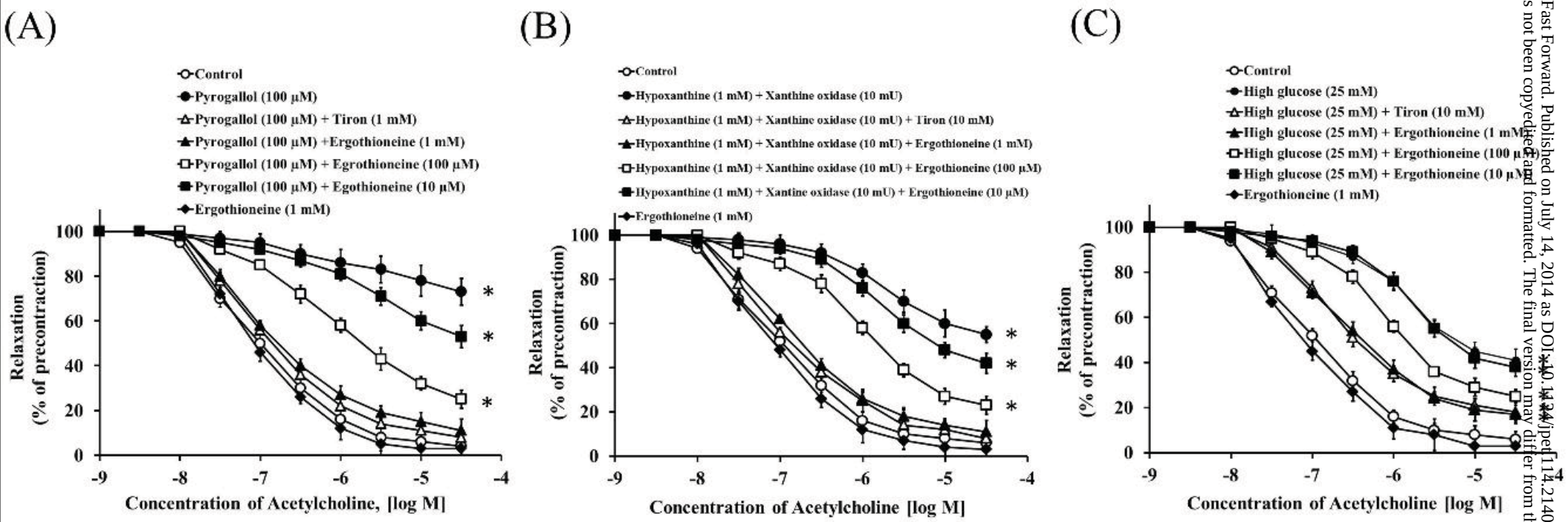

Fig. 10