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Original Article

Ergothioneine oxidation in the protection against high-glucose induced endothelial senescence: Involvement of SIRT1 and SIRT6

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ABSTRACT

Ergothioneine (Egt), the betaine of 2-mercapto-L-histidine, is a dietary antioxidant protecting against many diseases, including cardiovascular disease (CVD), through a redox mechanism different from alkylthiols. Here, experiments were designed to evaluate the mechanisms underlying the beneficial effect of Egt against hyperglycaemia-induced senescence in endothelial cells. To this end, cells were incubated with increasing concentrations of Egt (0.01–1.00 mM) for 12 h followed by incubation for 48 h with high-glucose (25 mM). Cell evaluation indicated that viability was not affected by mM concentrations of Egt and that the high-glucose cytotoxicity was prevented with the highest efficacy at 0.5 mM Egt. The cytoprotective effect of Egt was paralleled by reduced ROS production, cell senescence, and, interestingly, the formation of hercynine (EH), a betaine we recently found to be produced during the Egt oxidation pathway. Notably, the Egt beneficial effect was exerted through the upregulation of sirtuin 1 (SIRT1) and sirtuin 6 (SIRT6) expression and the downregulation of p66Shc and NF-κB. SIRT1 activity inhibition and SIRT6 gene silencing by small interfering RNA abolished the protective effect of Egt against the high-glucose-induced endothelial senescence. These data provide the first evidence of the Egt ability to interfere with endothelial senescence linked to hyperglycaemia through the regulation of SIRT1 and SIRT6 signaling, thus further strengthening the already assessed role of these two histone deacetylases in type 2 diabetes.

1. Introduction

Endothelial dysfunction is a multifaceted disorder associated with a number of pathophysiological processes strictly linked to risk factors of cardiovascular disease (CVD), such as hypertension, atherosclerosis, or diabetes [1,2]. Oxidative stress, the common denominator underlying endothelial dysfunction in CVD, results in a high accumulation of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$), and a reduced nitric oxide (NO) bioavailability, thus driving endothelial cells (EC) towards senescence and apoptotic processes which interfere with the mechanisms of endothelial repair and regeneration [1,3]. In particular, the enhanced generation of reactive oxygen species (ROS) and the inflammatory activation characterize the functional alteration of the endothelium during hyperglycaemia, a key feature of diabetes and its vascular complications [4–6].

The hyperglycaemia-induced endothelial dysfunction is mediated by the downregulation of sirtuin 1 (SIRT1) and sirtuin 6 (SIRT6) [4,7–9]. SIRT1 protects blood vessels from hyperglycaemia-induced endothelial dysfunction through the downregulation of p66Shc expression [10]. In EC, SIRT1 prevents hydrogen peroxide-induced premature senescence by deacetylating the tumor suppressor p53 and protects from hyperglycaemia-induced endothelial dysfunction through the transcriptional regulation of endothelial nitric oxide synthase (eNOS) and the downregulation of p66Shc expression, a unique protein isoform of SHC (Src homology 2 domain containing) transforming protein 1 [11]. In EC,

Abbreviations: CVD, cardiovascular disease; EC, endothelial cells; Egt, ergothioneine; EH, hercynine; eNOS, endothelial nitric oxide synthase; ESH, 2-mercaptohistidine betaine; ESO_2H, ergothioneine sulfinic acid; ESOH, ergothioneine sulfenic acid; ESSE, ergothioneine disulfide; GSH, glutathione; hGluc, high-glucose; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein-1; NADH, nicotinamide adenine dinucleotide; NF-κB, nuclear factor-kappaB; NO, nitric oxide; OCTN1, organic cationic transporter 1; PKCβII, protein kinase c βII; ROS, reactive oxygen species; SHC, Src homology 2 domain containing; SIRT1, sirtuin 1; SIRT6, sirtuin 6; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; EIC, extracted ion chromatogram; ICC, ion charge control; ESI, electro spray ionization; FIA, flow injection analysis; HPLC, high-performance liquid chromatography; MS/MS, tandem mass; MRM, multiple reaction monitoring

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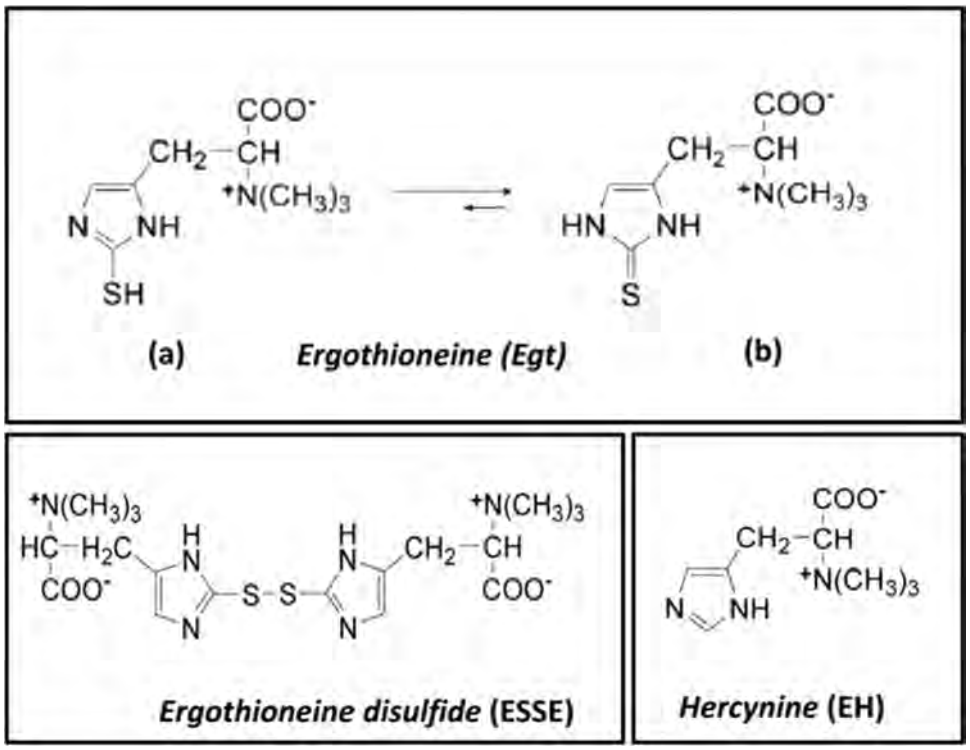

Fig. 1. Chemical structure of ergothioneine (Egt), its disulfide (ESSE), and hercynine (EH). Tautomeric equilibrium between thiol (a) and thione (b) form of ergothioneine.

the atheroprotective effect of SIRT1 is also exerted through the activation of eNOS and the inhibition of NF-κB activity [11]. Several SIRT1 modulators, including resveratrol, resveratrol derivative, and stachydrine showed a beneficial effect on EC dysfunction, through positive regulation of SIRT1 pathway and reduction of superoxide production [12–15]. Indeed, to date, the pharmacological modulation of SIRT1 for the treatment of endothelial dysfunction and vascular-aging disorders is under intensive pre-clinical and clinical research [11].

Ergothioneine (Egt), 2-mercaptohistidine betaine (ESH), is an unusual compound containing the 2-thioimidazole moiety, widely distributed both in the plant and animal kingdoms (Fig. 1). Plants absorb Egt via symbiotic associations between their roots and soil fungi, whereas mammals acquire Egt solely through the diet. Indeed, this naturally occurring water soluble compound, firstly isolated from the ergot fungus (*Claviceps purpurea*) [16], is synthesized only by Actinomycetales and non-yeast like fungi [17–19].

At physiological pH, Egt is mainly in the thione form (Fig. 1). Although many reports describe the Egt chemical properties and synthesis [19], the Egt biosynthetic pathway from *Mycobacterium smegmatis* has been only recently fully reconstituted *in vitro* showing the involvement of Egt sulfenic acid (ESOH) as an intermediate [20]. This biosynthetic profile is consistent with the behavior, identified by Servillo and co-workers [21], of Egt under oxidative conditions, in which ESOH acts as a key intermediate. The Egt disulfide (ESSE) (Fig. 1), formed by Egt in the presence of oxidants, is unstable at physiological pH and undergoes a progressive decomposition. Specifically, ESSE is first hydrolyzed into Egt (ESH) and the highly reactive ESOH that disproportionates producing one molecule of ESH and one of the Egt sulfinic acid (ESO_2H). Successively, the highly unstable ESO_2H irreversibly hydrolyzes into sulfurous acid and hercynine (EH), the betaine of histidine [21].

The fact that Egt can be partially reformed from its disulfide ESSE, without the need of reducing agents, and then oxidized again in a new redox cycle, explains the absolute difference from alkylthiols, such as cysteine and GSH [21].

The endothelial uptake of Egt occurs through the organic cation transporter novel type-1 (OCTN-1) and silencing this specific cell membrane carrier with small interfering RNA abolished the antioxidant and cytoprotective effects of Egt [22]. Owing to the specific carrier, Egt introduced by diet mainly accumulates up to millimolar range also in cells and tissues predisposed to inflammation and oxidative stress [19,23]. Notably, Egt itself is not toxic to EC up to 10 mM [22,24]. A reduced expression of adhesion molecule VCAM-1, ICAM-1 and E-selectin has been observed following treatment of EC with Egt at concentrations of 0.1–0.3 mM [24]. A higher concentration of Egt (1 mM) was effective in reducing monocyte binding to EC [24] and in suppressing reactive oxygen species (ROS) production and cell death in EC exposed to pyrogallol, xanthine oxidase plus xanthine, and high-glucose [22].

The protective action of Egt against CVD during ischemia/reperfusion is achieved through the reduction of ferrylmyoglobin, the modulation of proinflammatory cytokines, and iron chelation [25–27]. Controversial are the findings about the role of Egt in diabetes, since diabetic patients have been reported to have elevated levels of Egt which affects insulin and glucagon storage through chelation of zinc [28,29]. On the other hand, no significant elevation of Egt has been observed in rat model of alloxan-induced diabetes [30]. Moreover, Egt chronic treatment of rats with streptozocin-induced diabetes ameliorated the response to acetylcholine in arteries [22] and Egt supplementation of diabetic pregnant rats reduced diabetic embryopathy, probably through the modulation of hyperglycemia-mediated oxidative stress [31].

While Egt effects on ROS-induced cytotoxicity and vascular responsiveness have been elucidated both *in vitro* and *in vivo* models, our understanding of the molecular mechanism (s) underlying these actions is still relatively limited. Moreover, the recent findings on the peculiar redox behavior of Egt, which widen the scenario of its antioxidant cellular properties, prompted us to investigate the molecular mechanism(s) through which Egt acts in the prevention of CVD, specifically, in the protection of EC against