

# Ergothioneine produces relaxation in isolated rat aorta by inactivating superoxide anion

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**Abstract. – OBJECTIVE:** Ergothioneine (EGT) is a ubiquitous, sulphur-containing derivative of amino acid histidine, acquired by higher order plants and animals solely through dietary means. The antioxidant and cytoprotective effects of ergothioneine have been demonstrated by *in vitro* studies, but its physiological role remains unclear. This study aims to investigate the effects of ergothioneine (EGT) on basal and acetylcholine-stimulated activity of nitric oxide.

**MATERIALS AND METHODS:** Effects of EGT on basal and acetylcholine (ACh)-stimulated activity of nitric oxide (NO) were tested in isolated rings of rat thoracic aorta. In parallel experiments, relaxant responses to ACh were evaluated following incubation with Cu/Zn superoxide dismutase inhibitor diethyldithiocarbamate (DETCA) and superoxide anion generating system hypoxanthine/xanthine oxidase (HX/XO). Generation of reactive oxygen species (ROS) in aortic rings was measured by means of lucigenin- and luminol-enhanced chemiluminescence, in the presence and in the absence of EGT.

**RESULTS:** EGT (1-200  $\mu$ M) produced a concentration-dependent relaxation in endothelium-intact aortic rings which was abolished by endothelial denudation or NO synthase inhibition. Impaired response to ACh in DETCA and HX/XO treated rings was recovered by EGT treatment. This recovery by EGT was characterized by a significant decrease in the production of superoxide anion.

**CONCLUSIONS:** Ergothioneine, at levels normally present in blood, may protect NO from destruction by superoxide anion and play a physiologically important role in preserving NO-dependent endothelial function.

*Key Words:*

Ergothioneine, Nitric oxide, Superoxide anion, Endothelial dysfunction.

## Introduction

Ergothioneine (EGT) is a ubiquitous, water-soluble, sulphur-containing derivative of amino acid histidine, acquired by higher order plants

and animals solely through dietary means<sup>1-4</sup>. In humans, EGT has been shown to accumulate in cells and tissues frequently exposed to oxidative stress with highest levels in the millimolar range occurring in liver, bone marrow, lens of the eye, seminal fluid, and blood<sup>5,6</sup>. Cellular uptake of EGT is mediated through the organic cation transporter OCTN1, an integral membrane protein encoded by SLC22A4<sup>7</sup>. In contrast to glutathione, EGT is resistant to autooxidation which is attributed to its existence in thione form in neutral aqueous solutions and does not form disulphides under physiological conditions<sup>4,8</sup>. Several *in vitro* studies revealed that EGT is a powerful scavenger of reactive oxygen species (ROS) and protects cells against a wide range of stressors<sup>9-15</sup>. Although these findings have not been verified *in vivo*, accumulation, tissue distribution and scavenging properties, all highlight the potential for EGT to function as a physiological antioxidant<sup>1</sup>.

In the cardiovascular system, the signaling molecule nitric oxide (NO) is crucial for maintaining continuous vasodilator tone and for regulating local perfusion and systemic blood pressure<sup>16</sup>. It also modulates proliferation of vascular smooth muscle cells and inhibits platelet aggregation<sup>16</sup>. Oxidative stress, defined as an imbalance between the formation of ROS and the antioxidant response in favour of the former, is a critical parameter which limits vascular bioavailability of NO. Superoxide anion ( $O_2^{\cdot-}$ ), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions<sup>17</sup>. In vascular tissue, a decline in antioxidant defenses leads to excessive production of superoxide anion by NADPH oxidase, xanthine oxidase and mitochondrial superoxide producing enzymes. Consequently, a rapid and spontaneous reaction between superoxide anion and NO results in formation of potent oxidants peroxynitrite and peroxynitrous acid, which in turn has been shown to uncouple endothelial NO

synthase (eNOS). Uncoupling of eNOS has been demonstrated in 1) conditions associated with endothelial dysfunction such as atherosclerosis, diabetes mellitus, ischemia-reperfusion (I/R) injury, hypertension, and chronic flow overload; 2) cardiac hypertrophy with ventricular remodeling; and 3) diastolic heart failure<sup>18-25</sup>. These findings strongly suggest that restoring and conserving adequate NO signaling in the cardiovascular system may be a promising therapeutic approach.

Present study aims at investigating the effects of EGT on basal and agonist-stimulated activity of NO in isolated rings of rat thoracic aorta, in a cross-talk between its physiological concentrations and ROS-scavenging capacity.

## Materials and Methods

### Animals

Three-month old male Wistar rats (150-175 g, n = 30) were used for the study (Lemali Ltd., Ankara, Turkey). Protocol for animal studies was approved by Ethics Committee of Dokuz Eylül University, Izmir, Turkey (B.30.2/DEU/0.01.00.00/9402 - 08 May 2009). The rats were maintained under identical temperature conditions ( $22 \pm 2^\circ\text{C}$ ) with day and night cycles of 12 h and were given pelleted food and water *ad libitum*.

### Drugs

Ergothioneine, acetylcholine hydrochloride, catalase (bovine liver), diethyldithiocarbamate (DETCA), hypoxanthine, NG-nitro-L-arginine methyl ester (L-NAME), phenylephrine hydrochloride and xanthine oxidase (butter-milk) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in saline (0.9% NaCl) except for hypoxanthine which was dissolved in 0.1% sodium hydroxide.

### Preparation of Tissues

Rats were euthanized by decapitation under ketamine/xylazine anesthesia. The thoracic aorta was removed, cleaned of fat and loose connective tissue, and cut into 2 mm wide transverse rings. In some experiments, the endothelium was removed by gentle abrasion of the intimal surface using a blunt forceps. Rings were then mounted under 1.5 g resting tension on stainless steel hooks within 25 ml organ chambers and maintained at  $37^\circ\text{C}$  in physiological salt solution (Krebs-Henseleit), continuously gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The composition of the solution

was (in mM): NaCl, 118; KCl, 4.7;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2.5;  $\text{KH}_2\text{PO}_4$ , 1.20;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.17; Glucose, 11.1;  $\text{NaHCO}_3$ , 25. Tension was measured isometrically with MLT0201/RAD force transducers (AD Instruments, Inc., Colorado Springs, CO, USA) and recorded on LabChart Pro (Version 7.1, AD Instruments, Inc., Colorado Springs, CO, USA). Tissues were allowed to equilibrate for 45 min before experimental procedures were started, during which time the resting tension was re-adjusted to 1.5 g. In this period, tissues were washed out with Krebs-Henseleit solution for every 15 minutes.

### Experimental Protocol for Vascular Reactivity Studies

Since experimental procedures employed have been shown to affect vascular responses to contractile agents, all experiments involving relaxation on control, endothelium-intact aortic rings were performed following induction of 40-60% of maximal phenylephrine (PE)-induced tone ( $1.78 \pm 0.04$  g, n = 6)<sup>26,27</sup>. This tone was achieved with PE at 0.3-1  $\mu\text{M}$ . Thereafter, cumulative concentration-response curves for relaxation to ergothioneine (EGT; 1  $\mu\text{M}$ -200  $\mu\text{M}$ ) were constructed on endothelium-intact rings. The chambers were repeatedly washed out, allowing the tissues to re-equilibrate for at least 30 min before further experimentation.

In order to test the effects of EGT on agonist-stimulated activity of NO relaxation to acetylcholine (ACh; 1 nM-100  $\mu\text{M}$ ) was evaluated in the presence and in the absence of EGT. In these experiments, EGT (200  $\mu\text{M}$ ) was given as a 30 min pretreatment before induction of tone with PE. In parallel experiments, effects of EGT on relaxation to ACh were evaluated under conditions of oxidative stress. For this purpose, aortic rings were incubated with Cu/Zn superoxide dismutase (SOD) inhibitor DETCA (0.1 mM for 90 min). After washout, rings were treated with xanthine oxidase (XO; 16  $\mu\text{M}$ ) for 30 min. Following precontraction with PE, hypoxanthine (HX; 0.1 mM) was added and the effects of EGT on ACh-induced relaxation were observed within 5 min. Experiments involving the use of DETCA and HX/XO were conducted in the presence of catalase (1000 u/ml) to guard against accumulation of hydrogen peroxide<sup>26</sup>.

The effects of inhibition of nitric oxide synthase with L-NAME (100  $\mu\text{M}$ , 30 min) and of endothelial denudation were both examined on relaxation to EGT and ACh.

### Detection of Reactive Oxygen Species

Levels of superoxide anion and other reactive species in aortic rings were determined according to the method described by Wang et al<sup>28</sup>, with slight modifications. Briefly, aortic rings were taken into organ chambers filled with Krebs-Henseleit solution maintained at 37°C and gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Afterward, experimental procedures which were used to induce oxidative stress in vascular reactivity studies were repeated in the presence and in the absence of EGT. Aortic rings were then transferred to solid white well-plates containing 200 ml of HEPES-buffered Krebs-Henseleit solution (pH 7.4). After addition of chemiluminescence enhancers, lucigenin or luminol (final concentration of 5 μmol/L for either), ROS were quantified using a multi-plate reader (Victor III-1420, Perkin Elmer, Turku, Finland). Counts were obtained at 10-second intervals and corrected for wet tissue weight. Results were expressed as the area under curve (AUC) for a counting period of 5 min AUC of relative light units (rlu)/mg wet tissue<sup>29</sup>.

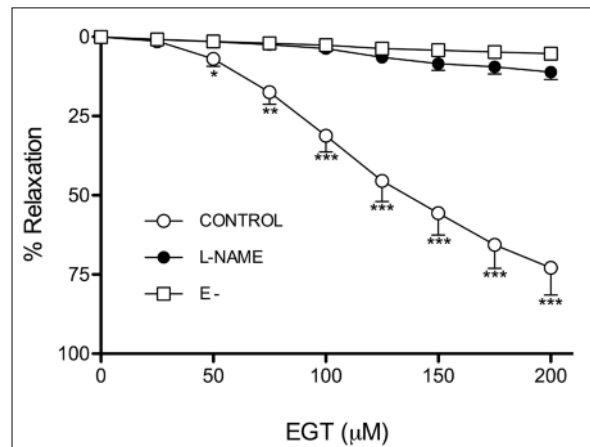
### Statistical Analysis

All data are expressed as means ± SEM. Relaxant responses are expressed as percentage (%) relaxation of PE-induced tone. ANOVA followed by Tukey's Multiple Comparison test was performed using GraphPad Prism (GraphPad Software, Version 5.0 for Mac OS X, San Diego, CA, USA). A value of  $p < 0.05$  was considered significant.

## Results

### Effects of Ergothioneine on Rat Aorta

Following precontraction with phenylephrine in endothelium-intact rings of rat thoracic aorta,

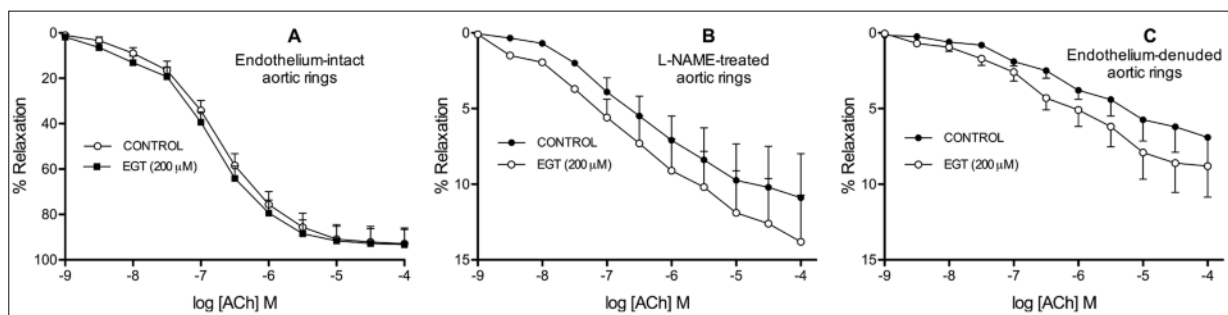


**Figure 1.** Concentration-response curves showing relaxation to ergothioneine (EGT) on phenylephrine-contracted, endothelium-intact rings of rat thoracic aorta (Control) and the effects of endothelial denudation (E-) or treatment with L-NAME (100 μM) on these relaxations. Each point is the mean ± standard error of mean of 6 observations. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  indicate a significant difference in maximal relaxation from L-NAME and E-.

EGT produced a concentration-dependent relaxation (Figure 1). Maximal relaxation achieved by EGT was  $72.9\% \pm 8.60$  ( $n = 6$ ). EGT-induced relaxation was almost completely abolished by endothelial denudation or pretreatment with L-NAME (Figure 1).

### Effects of Ergothioneine on Acetylcholine-Induced Relaxation in Rat Aorta

Pretreatment for 30 min with EGT up to 200 μM did not affect acetylcholine (ACh)-induced (1 nM-100 μM) relaxation in endothelium-intact rings (Figure 2). Also, EGT did not affect relaxation to ACh in L-NAME-treated or endothelial-

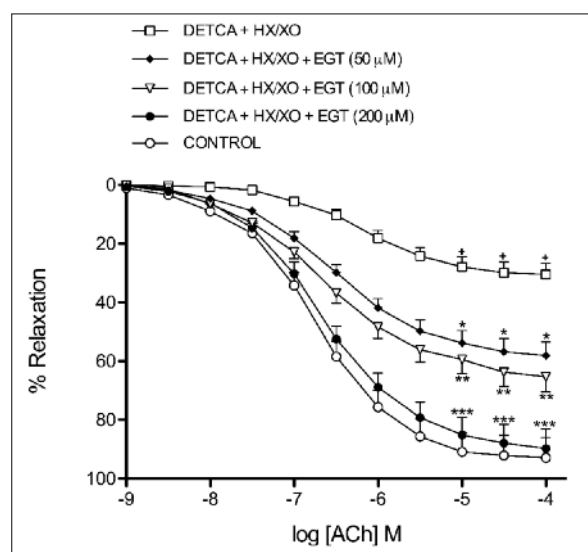


**Figure 2.** Effects of pretreatment for 30 min with ergothioneine (EGT; 200 μM) on relaxation to acetylcholine in phenylephrine-contracted rings of rat thoracic aorta. **A**, Endothelium-intact aortic rings. **B**, L-NAME-treated aortic rings. **C**, Endothelium-denuded aortic rings). Each point is the mean ± standard error of mean of 6 observations.

denuded aortic rings (Figure 2). Inhibition of endogenous Cu/Zn SOD with diethyldithiocarbamate (DETCA, 0.1 mM, 90 min) followed by treatment with hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 u/ml) led to significantly decreased relaxations to acetylcholine and shifted concentration-response curve to the right (Figure 3). Maximal relaxation ( $E_{max}$ ) fell from  $92.9 \pm 6.9\%$  to  $30.5 \pm 3.7\%$  (Table I,  $^+p < 0.001$ ,  $n = 6$ ). Pretreatment with EGT (50  $\mu\text{M}$ ) improved ACh-induced relaxation (Figure 3,  $^*p < 0.05$ ,  $n = 6$ ) without changing sensitivity (Table I). EGT at higher concentrations (100 and 200  $\mu\text{M}$ ) produced a more significant recovery in ACh-induced relaxation (Figure 3) while increasing sensitivity to ACh (Table I).

### Effects of Ergothioneine on Generation of Reactive Oxygen Species

Incubation of aortic rings with DETCA followed by HX/XO treatment significantly elevated ROS levels. Lucigenin-enhanced chemiluminescence was approximately 7 times higher than



**Figure 3.** Relaxation to acetylcholine (ACh) in phenylephrine-contracted, endothelium-intact rings of rat thoracic aorta (Control), and the blockade of these relaxations following treatment with diethyldithiocarbamate (DETCA) and hypoxanthine/xanthine oxidase (HX/XO). The effects of pretreatment for 30 min with ergothioneine (EGT) at 50-200  $\mu\text{M}$  on the blockade of relaxation are also depicted. Each point is the mean  $\pm$  standard error of mean of 6 observations.  $^+p < 0.001$  indicates a significant difference in maximal ACh-induced relaxation from Control.  $^*p < 0.05$ ,  $^{**}p < 0.01$  and  $^{***}p < 0.001$  indicate a significant reversal of the decline in ACh-induced relaxation in aortic rings treated with DETCA and HX/XO.

**Table I.** Effects of ergothioneine (EGT; 50, 100 and 200  $\mu\text{M}$ ) on maximum relaxation ( $E_{max}$ ) to acetylcholine (ACh) and  $pD^2$  obtained from diethyldithiocarbamate (DETCA) and hypoxanthine/xanthine oxidase (HX/XO)-treated rat aortic rings. Data are expressed as mean  $\pm$  standard error of mean of 6 observations.

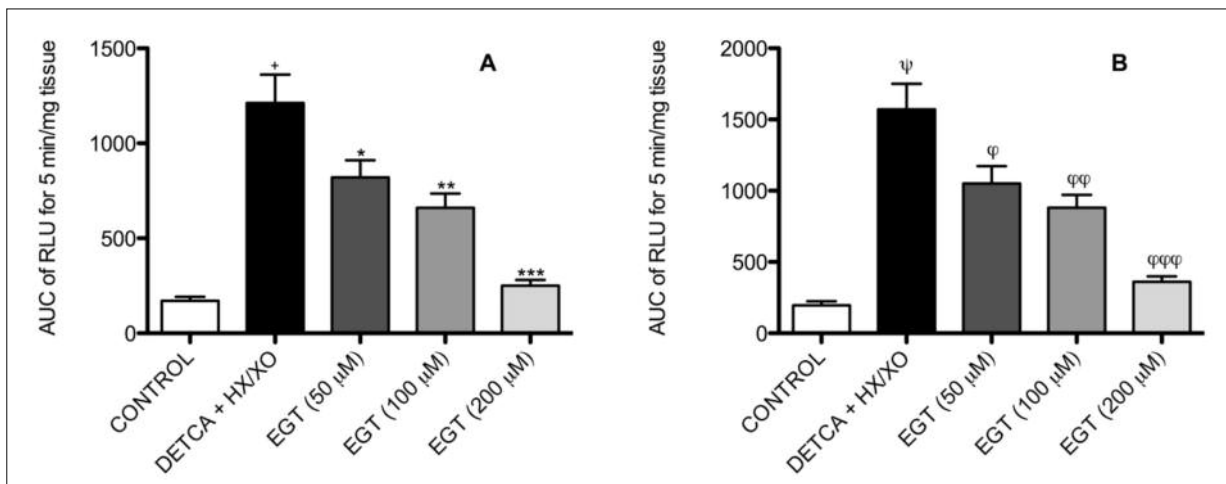
Group	$pD^2$	$E_{max}$
DETCA +HX/XO	$6.18 \pm 0.12^\psi$	$30.5 \pm 3.7^+$
EGT (50 $\mu\text{M}$ )	$6.53 \pm 0.08$	$89.7 \pm 6.5^*$
EGT (100 $\mu\text{M}$ )	$6.65 \pm 0.09^\phi$	$65.3 \pm 5.2^{**}$
EGT (200 $\mu\text{M}$ )	$6.66 \pm 0.08^{\phi\phi}$	$58.1 \pm 5.6^{***}$

$^\psi p < 0.01$  indicates a significant difference in ACh sensitivity from Control.  $^\phi p < 0.05$  and  $^{\phi\phi} p < 0.01$  indicate a significant recovery in ACh sensitivity in aortic rings treated with DETCA and HX/XO.  $^+ p < 0.001$  indicates a significant difference in maximal ACh-induced relaxation from Control.  $^* p < 0.05$ ,  $^{**} p < 0.01$  and  $^{***} p < 0.001$  indicate a significant reversal of the decline in  $E_{max}$  in aortic rings treated with DETCA and HX/XO.

in that of control tissues (Figure 4). Similarly, luminol-enhanced chemiluminescence was increased up to 8 fold (Figure 4). EGT, significantly reduced the increased production of superoxide anion and other reactive species (Figure 4).

## Discussion

Ubiquitous presence of ergothioneine (EGT) and its extensive uptake and accumulation in tissues predisposed to high levels of oxidative stress and inflammation highlight the possibility that EGT may function as a physiological antioxidant. This supposition is further supported by *in vitro* studies demonstrating that EGT is a powerful scavenger of hydroxyl radicals, hypochlorous acid and peroxynitrite<sup>9-15</sup>. EGT was also shown to deactivate singlet oxygen at a higher rate than GSH<sup>30</sup>. Moreover, in cell culture studies EGT was identified as a scavenger of superoxide anion which is regarded as one of the most harmful derivative of molecular oxygen for vascular endothelium<sup>31</sup>. These findings may imply a role for EGT in preserving NO-dependent endothelial function, especially in conditions of oxidative stress. In order to test this hypothesis, we firstly determined whether EGT could produce endothelium-dependent relaxation. Agents which remove superoxide anion, such as authentic superoxide dismutase (SOD), SOD-mimetics, and ascorbate have previously been reported to produce relaxation by inhibiting the reaction be-



**Figure 4.** Lucigenin (**A**) and luminol (**B**)-enhanced chemiluminescence (CL) in endothelium-intact rings of rat thoracic aorta (CONTROL) and increased superoxide anion / ROS levels following treatment with diethyldithiocarbamate (DETCA) and hypoxanthine/xanthine oxidase (HX/XO). The effects of pretreatment for 30 min with ergothioneine (EGT) at 50–200  $\mu\text{M}$  on the increased levels of superoxide anion (A) and other ROS (B) are also depicted. Results are expressed as mean  $\pm$  standard error of mean of area under curve (AUC) for a counting period of 5 min  $n = 6$ , AUC of relative light units (rlu)/mg wet tissue. \* $p < 0.001$  indicates a significant difference in the production of superoxide anion from CONTROL. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  indicate a significant reversal of the increased superoxide anion production by EGT in aortic rings treated with DETCA and HX/XO. <sup>ψ</sup> $p < 0.001$  indicates a significant difference in the production of ROS from CONTROL. <sup>φ</sup> $p < 0.05$ , <sup>φφ</sup> $p < 0.01$  and <sup>φφφ</sup> $p < 0.001$  indicate a significant reversal of the increased ROS production by EGT in aortic rings treated with DETCA and HX/XO.

tween NO and endogenously-produced superoxide anion<sup>26,32–34</sup>. In the present study, EGT at concentrations ranging from 1 to 200  $\mu\text{M}$  produced a significant relaxation in endothelium-intact rings of rat thoracic aorta. This effect was abolished by endothelial denudation or inhibition of NO synthase by L-NAME, indicating that it was mediated by NO. It is important to emphasize that concentration range over which EGT exerted its relaxant effects lies within normal plasma limits for humans ( $\sim 40$ –200  $\mu\text{M}$ )<sup>14</sup>. Blood EGT levels increase to 1.5–2.0 mg/100 ml from 1 to 10 years of age (y/o) peaking at around 3.7 mg/100 ml by 18 y/o and gradually declining to 2.3–3.0 mg/100 ml between 19 and 50 y/o and plateauing beyond 51+y/o with an average of 2.8 mg/100 ml<sup>35</sup>. Similar age-related declines have also been reported in NO-mediated vasodilator function and in levels of antioxidant enzymes such as glutathione peroxidase (GPx)<sup>36–38</sup>. Therefore, in this paper, we discuss a possible physiological role for EGT in the modulation of arterial tone, through protection of NO in the vasculature.

Key finding of the present work that EGT produced a NO-dependent relaxation in rat thoracic aorta, prompted us to test the effects of this antioxidant on agonist-stimulated activity of NO. In these studies, pretreatment with EGT at concen-

trations up to 200  $\mu\text{M}$  had no effect on the relaxation to acetylcholine (ACh) on endothelium-intact rings. Additionally, ACh responses both in L-NAME-treated and endothelial-denuded aortic rings remained unchanged. These findings suggested the possibility that EGT may not interfere with basal or agonist-stimulated production of NO and yet may protect NO from destruction by superoxide anion. To test this, we employed a model of oxidative stress which is based on inhibition of endogenous Cu/Zn SOD followed by induction of superoxide anion generation<sup>26</sup>. Using this protocol, we established a 67% reduction in maximal ACh-induced relaxation. In previous studies, authentic SOD and ascorbate have been shown to reverse this blockade<sup>26,33</sup>. In our study, EGT almost completely recovered the attenuated responses observed in ACh-induced relaxation. This finding provides indirect evidence suggesting that scavenging of superoxide anion may underlie NO-mediated relaxant effect of EGT.

Afterward, we measured the levels of superoxide anion and other reactive species in aortic rings, by means of luminol- and lucigenin-enhanced chemiluminescence (CL). Lucigenin specifically emits light upon reaction with superoxide anion and detects only extracellular ROS because it cannot penetrate the cell mem-

brane<sup>39</sup>. Luminol, as distinct from lucigenin, is used for measuring the sum of extra- and intracellular ROS including hydroxyl radicals, hydrogen peroxide and peroxyxynitrite<sup>39</sup>. In our study, an approximately 7-fold increase in lucigenin-enhanced CL was observed in aortic rings treated with Cu-Zn SOD inhibitor (DETCA) and hypoxanthine/xanthine oxidase (HX/XO), while luminol-enhanced CL was increased up to 8 fold. The low difference between lucigenin- and luminol-enhanced CL reflects the specificity of the oxidative stress model to induce superoxide anion production. Since these experiments were performed in the presence of catalase, as a consequence of Fenton chemistry, hydrogen peroxide and hydroxyl radicals are least likely to contribute to luminol-enhanced CL. Additionally, peroxyxynitrite may have a limited effect on luminol-enhanced CL, because luminol also detects superoxide anion. Therefore, for this model, we may state that significantly increased superoxide anion concentration is the major factor leading to impaired ACh responses. Since similar magnitudes of reduction in lucigenin- and luminol-enhanced CL were evident in EGT-pretreated aortic rings, it is conceivable that recovery by EGT of impaired NO-dependent relaxation resulting from oxidant stress in rat aorta, is due to a selective scavenging of superoxide anion.

### Conclusions

We report for the first time that EGT produces NO-dependent relaxation and protects NO from destruction by superoxide anion in rat aorta. This action is observed with EGT at concentrations normally present in plasma. Therefore, maintaining optimal blood levels of EGT may be of critical importance in preserving NO-dependent endothelial function. Our results are in line with previous findings which suggest a potential role for EGT in protection of endothelial cells<sup>40,41</sup>. *In vivo* studies elucidating the role of this endogenous antioxidant within vascular biology may provide new insights into cardiovascular diseases.

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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