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L-ergothioneine reduces nitration of lactoferrin and loss of antibacterial activity associated with nitrosative stress

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ABSTRACT

Lactoferrin (LF) is a multifunctional antimicrobial, anti-inflammatory, and antioxidant protein that occurs naturally in mammals, most notably in exocrine gland tissues and fluids, such as in the eye. Nitrosative stress can promote changes to tyrosine and other amino acid residues of the protein, which also reduces the activity of LF. L-ergothioneine (ET) is a potent anti-inflammatory antioxidant present in the eye and other tissues through nutrition or supplementation and that may play a role in the prevention or treatment of a variety of diseases. Here we investigated the ability of ET to reduce 3-nitrotyrosine (NTyr) formation using two separate substrates, with the goal of determining whether ET can protect the antibacterial function of LF and other proteins when exposed separately to peroxynitrite and tetranitromethane as nitrating reagents. Native human LF was used as a simple protein substrate, and lamb corneal lysate was chosen as one example of mammalian tissue with a more complex mixture of proteins and other biomolecules. Nitration was monitored by absorbance and fluorescence spectroscopy as well as sandwich (nitrated LF) and direct NTyr (corneal lysate) enzyme-linked immunosorbent assays (ELISAs). We found that pretreatment with ET reduced chemical modification of both native LF and corneal lysate samples and loss of antibacterial LF function due to exposure to the nitrating reagents. These initial results suggest that ET, raised to sufficiently elevated levels, could be tailored as a therapeutic agent to reduce effects of nitrosative stress on LF and in turn sustain the protein activity.

1. Introduction

Lactoferrin (LF) is an important, natural mammalian protein formed in secondary granules of neutrophils and mucosal epithelial cells. The protein is released into the blood and tissues in response to inflammation [1,2]. LF provides an array of diverse physiological defensive functions within body tissues and fluids, and the overall immune response is reduced when LF concentrations are lower. For example, Venge and colleagues found LF concentrations of 210 μg L^{-1} in infection-prone and leukemic children compared to 330 μg L^{-1} in the healthy population [3]. LF plays important roles in the immune response, in part, because of its natural antimicrobial properties [4]. LF can act as both a bactericidal agent, by binding to bacterial iron, and as a bacteriostatic agent, by disturbing the bacterial membrane [5,6]. Because of its immune properties, LF levels are frequently used as a biomarker for a variety of diseases [7–9].

Nitrosative stress accompanying the immune response and inflammatory diseases like diabetes, asthma, and dry eye disease, can induce post-translational modification (PTM) of proteins and deleterious effects on protein function [10,11]. One particular PTM efficiently mediated by nitrosative stress is the conversion of accessible tyrosine (Tyr) units within selected proteins to 3-nitrotyrosine (NTyr). Consequently, NTyr is frequently used as a marker for reactive nitrogen species (RNS) and reactive oxygen species (ROS) [12]. Protein nitration that generates NTyr can alter both protein structure and function [13]. LF is a key biomarker for several inflammatory diseases and a target for modification by RNS and ROS [14–17]. For example, modification of LF via reaction with peroxynitrite ($ONOO^-$) alters the physiological function of LF by reducing its iron-binding capability and, accordingly, its antibacterial function [18]. The latter observations suggest that during periods of high nitrosative stress, e.g. during episodes of inflammation, secondary physiological effects from PTM of LF is likely to reduce the

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immune response and antibacterial defense normally mediated by native LF. The latter concept might be particularly important in the eye, where LF is an important endogenous protective agent [19,20], and because the eye is frequently exposed to nitrosative stress from inflammation and air pollution [21–23].

Among possible efforts to reduce effects of protein nitration is the use of the natural product L-ergothioneine (N-α-trimethyl-2-thiohistidine; ET) [24,25]. ET is a histidine derivative synthesized by many species of fungi and bacteria, but not by higher plants or animals [26]. ET predominantly exists in its thione form (carbon double-bonded to sulfur) at physiological pH (Figure S1), which prevents ET from autoxidizing and allows it to accumulate in tissues [21,27,28]. In addition, ET has a higher redox potential than other naturally occurring thiols with an E^0 of −0.06 V [29]. ET acts as an antioxidant by quenching singlet oxygen and scavenging hydroxyl radicals and $ONOO^-$ [28,30,31].

ET in humans and other mammals originates exclusively through nutritional intake and exhibits a stable concentrations of 0.1–2 mM in cells and tissues, including the eye, liver, heart, and lungs, and in fluids such as tears and serum [26,27,32–34]. It has been used widely as a dietary supplement, but peer-reviewed evidence supporting its activity is relatively lacking [35–37]. The organic cation transporter novel-type 1 (OCTN1) is responsible for ET uptake, accumulation, and selective distribution in tissues [21,38]. L-ergothioneine has been shown to accumulate with high concentrations in various cells and tissues, including cornea of the eye [32]. High expression of OCTN1, along with corresponding elevated concentrations of ET, occurs at sites of inflammation in tissues [21,32]. ET has many roles, including scavenging of free radical precursors such as $ONOO^-$ and providing antioxidant activity by inhibiting the redox process [27,39]. ET can also regulate microbial pathogenesis of several microbes through its metal chelating properties that confer antimicrobial activity [40]. ET is more effective at inhibiting protein nitration than other antioxidant molecules, including glutathione [24].

Nitration of proteins in the body can also occur via reaction with RNS and ROS in the atmosphere [41]. Outer surfaces of the eye, such as the cornea, may be particularly susceptible due to their constant interaction with atmospheric pollutants and other gases. Studies have also shown ET levels of 9.5 μmol g^{-1} in ocular tissues and 1.2 μmol mL^{-1} (mM range) in the aqueous humor of bovine and porcine animals [29,32]. Few studies have investigated the protective roles of ET in ocular diseases, however [21]. In addition, despite the rich literature on the properties of ET and its antioxidant efficacy in humans, no studies have demonstrated the capacity of ET to protect proteins in eye tissue and, more specifically, from a loss of antibacterial activity due to exposure to $ONOO^-$ or tetranitromethane (TNM).

We report that pre-treatment with ET protects LF from nitration, whether LF is present in pure form or in a complex mixture of proteins normally present in corneal tissue. We also found that ET may help preserve the antibacterial function of LF from dysfunction mediated by reaction with either nitrating reagent. This is the first indication that ET can prevent chemical modification of LF upon exposure to nitrating reagents $ONOO^-$ or TNM and may also help preserve antibacterial activity of LF reduced during nitrosative stress. Nitration of corneal proteins could offer a biochemical explanation for decreases in LF levels and activity in patients with inflammatory diseases such as dry eye disease [42]. The possibility emerges that ET, elevated through therapeutic treatment, could reduce the consequences of LF nitration in all parts of the body and in turn, for example, reduce both infectious and non-infectious ocular diseases involving inflammation and nitrosative or oxidative stress.

2. Materials and methods

2.1. Materials

Lactoferrin purified from human milk (LF; L0520), bovine serum albumin (BSA; A7030), tetranitromethane (T25003), and sodium peroxynitrite (NaONOO; 516620) were purchased from Sigma Aldrich (St. Louis, MO, USA). Details and commercial sources associated with all other materials are listed in the online supplementary material.

2.2. Lamb cornea tissue dissection

Lamb eyes were harvested from lamb heads acquired from a local butcher shop. Corneas were extracted from the whole lamb eye using a surgical razor blade, cut into four pieces, weighed using an analytical scale and flash-frozen using liquid nitrogen. The cornea was placed in a cryogenic microtube and stored at −80 °C until extraction [43].

2.3. Protein extraction from lamb cornea

A quarter piece of the cornea (1084.6 mg) was washed using Tris-HCl buffer and placed into the extraction buffer at a concentration of 10% m/v (corneal tissue mass per Tris-HCl buffer volume; 10 mg mL^{-1}). The Eppendorf tube containing the mixture was placed on ice and the reaction mixture was stirred continuously for 25 min and sonicated (level 8) for 2 min. The mixture was centrifuged at 1400 g for 30 min, the supernatant was collected, and an aliquot was placed into a cryogenic microtube and stored at −20 °C (from days to three months) until further analysis. The total protein concentration of the lysate was measured as 1.34 mg mL^{-1}, using the bicinchoninic acid (BCA) assay following manufacturer protocol. The presence of protein in the corneal lysate was detected using UV–Vis analysis at wavelength 280 nm. LF sandwich ELISA and the concentration evaluated the total protein concentration of LF in the corneal lysate after the LF calibration curve calculated 1:10 dilution factor was 4.6 ng/mL.

2.4. Protein nitration reaction

The protocol to nitrate LF and corneal lysate using both TNM and $ONOO^-$ was outlined by Alhalwani et al. [18]. For the TNM reaction, native LF was buffered in Tris-HCl buffer to a final concentration of 1 mg mL^{-1}. TNM was added to yield a TNM/Tyr molar ratio of 10/1 ([TNM] 2.7 mM) and stirred at room temperature (RT) for 2 h to yield nitrated LF (NLF). For the $ONOO^-$ reaction, native LF was buffered in 1x phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 1 mg mL^{-1}. The $ONOO^-$ reaction occurred by adding NaONOO (160–200 mM) after being thawed on ice to yield a $ONOO^-$/Tyr molar ratio of 10/1 ([$ONOO^-$] 2.7 mM) and stirred on ice for 2 h. Both the TNM and $ONOO^-$ reactions were quenched via centrifugation with Amicon centrifugal filters with 10 kDa cutoff membranes. The nitration reactions for the corneal lysate were performed using the same protocol as for LF, but using different volumes to account for the fact that the corneal lysate contained a complex and uncharacterized mixture of proteins with unknown amounts of Tyr residues. The corneal lysate was buffered to a final protein concentration of 1.0 mg mL^{-1}. Additionally, the TNM stock was prepared at higher concentration (8.35 M) than the $ONOO^-$ (160–200 μM), so a smaller volume of TNM (1.16 μL; [TNM] 9.7 mM) than $ONOO^-$ (17.05 μL; [$ONOO^-$] 3.4 mM) was used to prepare the nitrated corneal lysate (N-lysate). The $ONOO^-$ concentration is nominally set to 200 μM and immediately flash-frozen by the manufacture. However, minor differences in freezing and thawing conditions lead to a slight degradation in concentration as a function of time, and so the range of concentration here is shown as an approximate range, as calculated by the manufacture.

For the ET-treated samples, ET was suspended in 250 μL nanopure water and added to produce solutions with final concentrations of 0.1 mM, 1.0 mM, and 5.0 mM prior to the addition of nitrating reagents (NR). A wide range of ET concentrations was used for this first investigation of its effect of within the given reaction types. An upper limit of 5.0 mM ET was used to match the concentration used by Aruoma et al. Sample conditions are summarized in Table 1 [25].

Table 1
Summary of sample preparation conditions. Protein nitration m/m ratio; nitrating reagent (NR) mass to protein mass. Note that Tyr nitration ratio of nitrated corneal lysate (N-lysate) is unknown (*n/a*) because of unknown types and concentrations of proteins and tyrosine subunits in the sample.

Sample Number	Sample Name	Protein Concentration (mg mL^{-1})	Nitrating Reagent (NR)	NR mass: Protein mass	NR moles: Tyr moles	ET Concentration (mM)
1	LF	1.0	–	–	–	–
2	NLF	1.0	TNM	2.3	10	–
3	NLF	1.0	TNM	2.3	10	0.1
4	NLF	1.0	TNM	2.3	10	1.0
5	NLF	1.0	$ONOO^-$	71	10	–
6	NLF	1.0	$ONOO^-$	71	10	0.1
7	NLF	1.0	$ONOO^-$	71	10	1.0
8	Lysate	1.0	–	–	–	–
9	N-lysate	1.0	TNM	9.3	*n/a*	–
10	N-lysate	1.0	TNM	9.3	*n/a*	5.0
11	N-lysate	1.0	$ONOO^-$	2.1	*n/a*	–
12	N-lysate	1.0	$ONOO^-$	2.1	*n/a*	5.0

2.5. Absorbance analysis

The spectrophotometric absorption of light at 350 nm is a common way to detect NTyr, due to the addition of the NO_2 group [44]. Absorbance measurements were performed on a UV–vis spectrophotometer (Cary 100 BIO) at RT in the wavelength range of 250–500 nm in 2 nm increments. Each sample was diluted in its respective reaction buffer, PBS (for $ONOO^-$) or Tris-HCl (for TNM), for a final protein concentration of 1.0 mg mL^{-1}. It should be noted, however, that spectroscopic monitoring of the nitration process is not unambiguous, because of spectral changes due to nitration of other aromatic amino acids and other post-translational modifications, as discussed below. For this reason and due to the qualitative nature of the spectroscopic analysis, exemplary results are shown for only one replicate.

2.6. Fluorescence analysis

Fluorescence measurements were performed at RT on a Cary Eclipse Fluorescence Spectrometer using an excitation wavelength of 280 nm and measuring emission as a total value integrated over the range 200–500 nm, recorded in 2 nm increments. The degree of nitration from native Tyr and tryptophan can be monitored by the reduction in fluorescence signal intensity upon conversion to the nitrated forms. Sample concentrations of 0.2 mg mL^{-1} were diluted in the respective reaction buffer, PBS or Tris-HCl. Exemplary replicates are shown for only one replicate, for reasons discussed above with respect to the absorbance analysis.

2.7. ELISA for quantifying NLF and NTyr

Direct ELISA was used to quantify NTyr in the lysate samples (Table 1). BSA was used as a calibrant, because it is a standard protein and has 21 Tyr residues, which is similar to the 20 Tyr residues in LF. Samples were diluted into a carbonate buffer at concentration 5 μg mL^{-1}. The samples were coated in triplicate with each sample solution and added separately to three wells as a blank measurement. The plate was covered and incubated at RT for 2 h. Washing steps to each well were applied after the incubation using 200 μL of PBST (PBS with 0.05% Tween). After washing, the plate was blocked by the non-specific binding areas using 200 μL of blocking buffer (5% BSA in PBS). This was added to each well and incubated at RT for 2 h. Following the washing step, mouse monoclonal to NTyr biotinylated antibody (α-NTyr) was diluted in blocking buffer in 1:500 dilution ratio, and 50 μL of the dilution was added to each well and incubated at RT for 1 h. Next, streptavidin-HRP, diluted 1:10,000 in blocking buffer, was added in 50 μL aliquots to each well and incubated at RT for 1 h. The tetramethylbenzidine (TMB) substrate (100 μL) was added to each well and incubated at RT until the color developed from colorless to blue (~10 min). Aqueous sulfuric acid (0.5 N, 100 μL) was added, and the color instantly changed to yellow. The plate was measured using the optical absorbance of each well using a microwell plate reader, following a similar protocol [45]. The final absorbance value used is the background-subtracted absorbance at 450 nm (blue) minus the background-subtracted absorbance at 620 nm (yellow).

Sandwich ELISA was used for detection of NLF in the pure protein samples (Table 1), following published procedures [46]. The approach involves using a goat polyclonal antibody to LF non-conjugated antibody (α-LF) for capture and a mouse monoclonal to NTyr biotinylated antibody (α-NTyr) for detection (Table S1). The microplate was coated with 50 μL of 1:1000 capture antibody in carbonate buffer at 4 °C overnight. The plate was washed and blocked as described above. Samples were prepared in 10/1 ratios of either TNM or $ONOO^-$ to Tyr, both with and without ET (Table 1), producing NLF solutions at concentrations of 5 μg mL^{-1}. NLF samples were added in triplicate to the plate (50 μL each) and incubated at RT for 1 h. Blocking buffer (5% BSA in PBS) was used for blank measurements. Detection antibody (50 μL), diluted 1:100 in blocking buffer, was added and the plates were incubated with shaking (UltraRocker, BIO-RAD) at RT for 1 h. Next, streptavidin-HRP (50 μL), diluted 1:10,000 in blocking buffer, was added in to each well and incubated at RT for 1 h. TMB substrate (100 μL) was then added to each well and incubated at RT until the color developed from colorless to blue after about 10 min. Aqueous sulfuric acid (0.5 N, 100 μL) was added the substrate and the color quickly changed to yellow. The absorption of each well was measured using a microwell plate reader (Tecan Infinite M1000 PRO) following a standard protocol (Abcam). The final absorbance value is calculated in identical fashion to the direct ELISA (above).

2.8. Broth microplate assay for detecting antibacterial activity

The microplate assay used for detection of antibacterial activity followed procedures outlined previously [18,47,48]. Briefly, *E. coli* prepared from a 1 μL aliquot of glycerol stock was inoculated over the surface of the TSA plates at 37 °C for 24 h. Several (3–5) separate bacterial colonies were then selected, transferred into 4 mL Tryptic Soya broth (TSB), and incubated at 37 °C for 24 h. Subsequently, the 4 mL of *E. coli* in TSB was diluted 1:10 by adding 36 mL of TSB to make *E. coli* broth stock. TSB (200 μL) was then added to each well. All samples were prepared in TSB and are outlined in Table S2. *E. coli* broth stock (5 μL) was added to each well and plates were allowed to incubate overnight at 37 °C. The absorbance of each sample in the microwell plates was then measured at 600 nm using a plate reader to monitor bacterial growth. Using the absorbance wavelength at 600 nm is common for estimating the number of live cells in a liquid suspension [49,50]. All samples were analyzed in triplicate.

3. Results and discussion

3.1. Inhibition of LF nitration

Spectroscopic and ELISA analyses were used to investigate the extent

to which the addition of ET in the nitration reaction of LF, driven separately by $ONOO^-$ and TNM, could protect the native protein against NTyr formation (Fig. 1). For absorbance (Fig. 1a) and ELISA (Fig. 1c) nitration results in a larger measurement response (higher bar), whereas nitration produces a lower response for fluorescence (Fig. 1b).

The addition of both concentrations of ET to the reaction mixtures reduced the normalized absorbance at 350 nm from 1.0 to between 0.34 and 0.53 (Fig. 1a). The results were similar following sandwich ELISA analysis (Fig. 1c), though the resulting absorbance signal due to NLF remained higher for the TNM-mediated reactions (0.55 and 0.46 for 0.1 mM and 1.0 mM ET, respectively) than for the $ONOO^-$-mediated reactions (0.13 and 0.01, respectively). In all cases, the fluorescence results (Fig. 1b) showed substantial reduction in fluorescence following all reactions. Protection provided by the 0.1 mM and 1.0 mM ET concentrations show only minor differences, with statistical significance from the sandwich ELISA results (Fig. 1c) provided by *p*-values of 0.038 and 0.0035 for TNM and $ONOO^-$, respectively (Fig. 1c, Table S3). The main message here is that that ET shows a reduction in nitration compared to the unmitigated NLF case (no ET). Further work will be needed to separate effects due to increasing ET amount and to establish thresholds of ET concentration required to provide consistent protection against nitration.

It is important to note that using spectroscopic methods alone is insufficient to monitor changes in nitration of tyrosine within proteins. This is because other polyaromatic ring-based amino acids (tryptophan, phenylalanine) can also be nitrated and will show confounding spectroscopic change [51]. Many other post-translational modifications are also possible upon reaction with TNM or $ONOO^-$, and so the spectroscopic results are used only as a first indication of nitration. The sandwich ELISA method used (Fig. 1c), however, is specific to NTyr within LF, and the direct ELISA (Fig. 2c) is specific to NTyr more broadly. So, while the absorbance, fluorescence, and ELISA measurements are not equivalent, they all show the same qualitative trend of reduced nitration-like effects when ET is administered.

3.2. Inhibition of corneal tissue lysate nitration

A matching set of experiments was performed on the corneal lysate (Fig. 2) as an exemplary system with physiological complexity, including a wide variety of proteins and other biomolecules. The only other difference with the experiments performed on the pure protein system (Fig. 1) was that an ET concentration of 5 mM was applied to the lysate compared to 0.1 mM or 1.0 mM for previous experiments. A higher ET concentration (5 mM) was chosen, to match the procedure used by Aruoma et al., who showed that 5 mM of ET during oxidative exposure by $ONOO^-$ was well-tolerated by and increased viability of the cells and protected from oxidative damages [25].

The results of the corneal lysate experiments are qualitatively similar to the pure protein experiments, in that the ET-treated samples showed intermediate nitration response between pure lysate and lysate samples treated with either TNM or $ONOO^-$. As measured by either absorbance or fluorescence, however, the 5.0 mM ET provided less protection against nitration to these samples, as compared with the lower concentration of ET added to reaction mixtures of pure protein. This relatively weaker response compared to the pure protein is likely due to complexity of the lysate samples having a wide and unknown mixture of protein types, and so the relative ratio of nitrating reagent or ET to protein active surface is also unknown. Additional investigation will be needed to further probe differences of effect resulting from varying ratios of both nitrating reagent and ET to protein or lysate mass.

Direct ELISA was used to detect nitrated proteins in the corneal lysate samples, because the direct assay detects all proteins with the NTyr substitutions, rather than as specific modifications to LF alone. As a result, the results of the ELISA experiments (Fig. 2c) are conceptually different from the matching experiments with pure LF (Fig. 1c). In this way, the direct ELISA experiments are analogous to the absorbance and

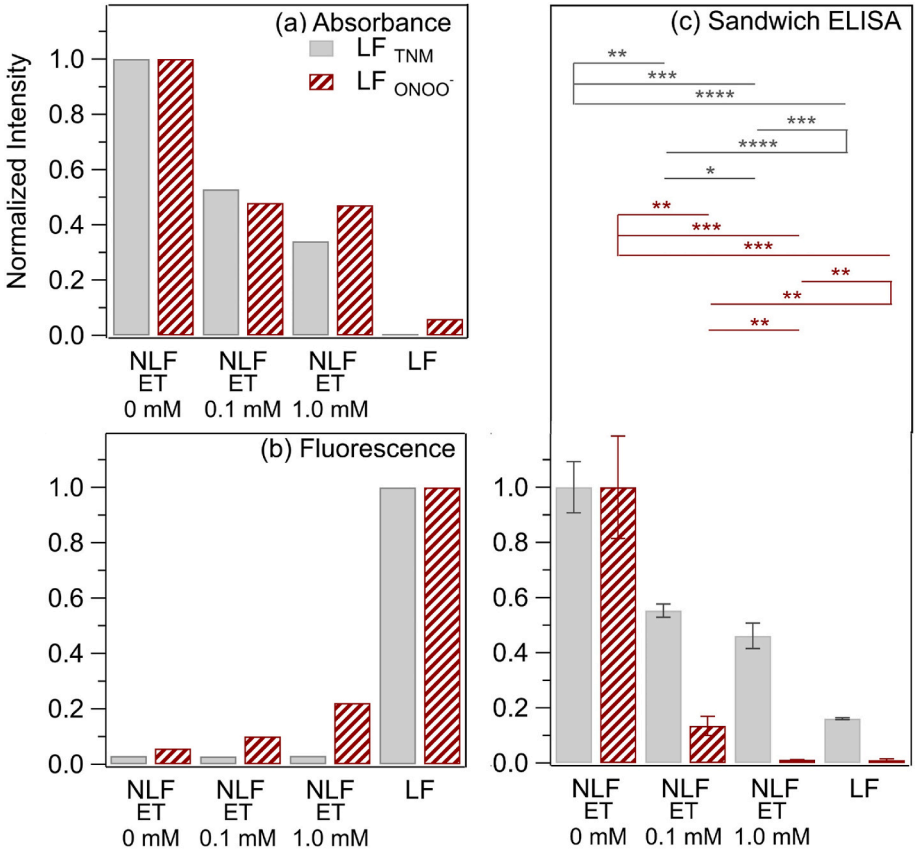

Fig. 1. Relative nitration degree of LF, with and without protective ET. Results shown via: (a) absorbance at 350 nm, (b) fluorescence emission (280 nm excitation), and (c) sandwich ELISA. NLF produced via TNM (solid gray) or $ONOO^-$ (hatched red). ELISA uncertainty bars show mean values ± standard deviations (n = 3). Spectroscopic measurements each represent single samples. Data normalized to a maximum of unity. (d) Statistical significance of differences between separate bars (indicated by horizontal lines) in (c), where increasing significance shown with additional * symbols (see Table S6 for definitions). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

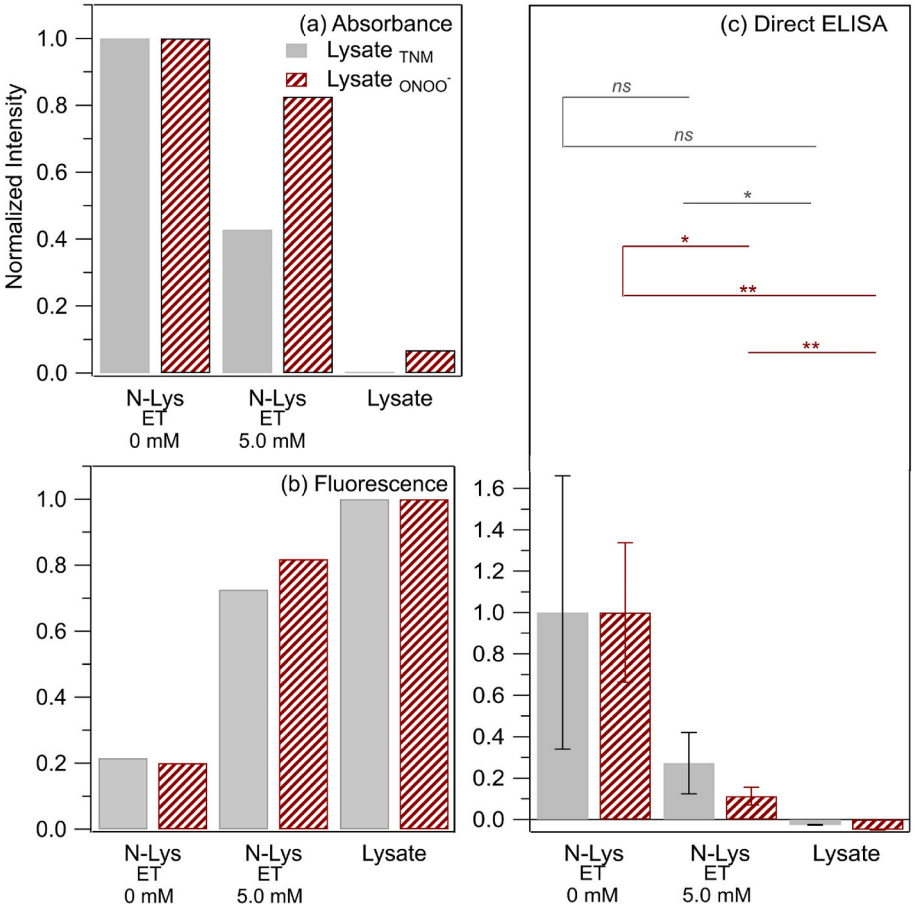

Fig. 2. Relative nitration degree of corneal lysate samples, with and without protective ET. Results shown via: (a) absorbance at 350 nm, (b) fluorescence emission (280 nm excitation), and (c) direct ELISA. NLF produced via TNM (solid gray) or $ONOO^-$ (hatched red). ELISA uncertainty bars show mean values ± standard deviations (n = 3). Spectroscopic measurements each represent single samples. Data normalized to a maximum of unity. (d) Statistical significance of differences between separate bars (indicated by horizontal lines) in (c), where increasing significance shown with additional * symbols (see Table S6 for definitions). *ns* indicates no statistical significance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fluorescence measurements also presented in Fig. 2, with no ability to specifically probe LF. It is worth noting that the ELISA protocol uses anti-NTyr antibodies, which may not specifically bind to NTyr, but also to hydroxy-Tyr [52]. Nitrated lysate with and without ET pre-treatment were statistically inseparable by direct ELISA (Fig. 2c) in the TNM case (*p*-value 0.14) and only weakly separable in the $ONOO^-$ case (0.011, Table S4).

3.3. Protection of LF antibacterial activity

Following investigation of the extent to which ET can reduce nitration in both pure LF and corneal lysate samples, further experiments (Table S2) were conducted to investigate the ability of ET to reduce the antibacterial effects caused by nitration of LF. One of the primary roles LF plays within the immune system is as an antibacterial reagent. As shown previously, LF nitrated with $ONOO^-$ or TNM shows reduced antibacterial activity against *E. coli* compare to native LF, likely due to a combination of the reduction in its iron-binding ability and changes in LF structure upon nitration [18]. ET concentration of 5 mM was chosen to match lysate experiment and the Aruoma et al. study [25], but an additional experiment at 10 mM was conducted to see if doubling ET provided any observable benefit in these preliminary experiments.

Broadly similar to previous results, LF was shown here to reduce *E. coli* growth by 41.4%, compared to untreated samples (blank-corrected comparison), and NLF without ET treatment reduced growth by only 13.7% (Fig. 3). ET was added to the reaction mixtures at 5.0 mM and 10.0 mM concentrations in order to test the hypothesis that ET would provide qualitatively similar protection of antibacterial activity

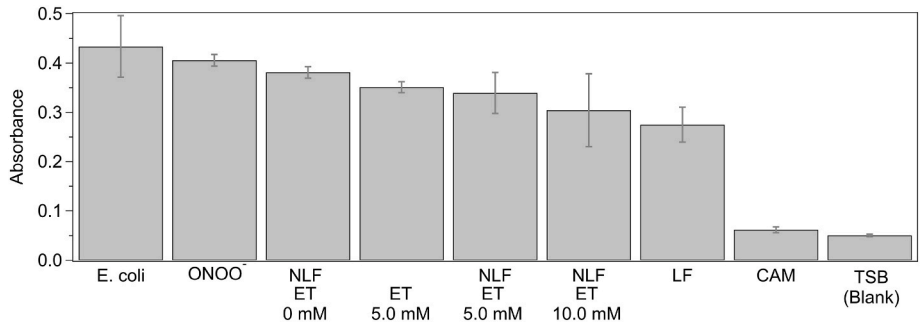

Fig. 3. Antibacterial activity of NLF, with and without protective ET. Absorbance at 600 nm shown for TSB blank-corrected absorbance values for *E. coli* grown against $ONOO^-$, NLF (control), 5.0 mM ET (control), NLF with 5.0 mM and 10.0 mM ET, LF (control), CAM (control), and TSB (blank). Bars show mean values ± standard deviations (n = 3). TSB shown before correction to show magnitude of subtraction.

as it provided against nitration. ET at 5.0 mM showed blank-corrected absorbance from bacterial concentration of 0.289, thus recovering 39.5% of the difference between the LF and NLF samples. The 10.0 mM ET treatment showed 0.304 absorbance, a 72.6% recovery. The effects of various controls on the *E. coli* growth are also shown in Fig. 3 for comparison, including $ONOO^-$ and ET each alone, as well as chloramphenicol antibiotic (CAM) as a strong antibacterial agent and a buffer blank. As noted previously, the minimum inhibitory concentration assay by Wiegand et al. was modified to show semi-quantitative antibacterial activity results [18,53]. Due to the nature of the assay, the results shown in Fig. 3 show preliminary evidence that ET protects the antibacterial activity against nitration. The statistics associated with the analysis are show in SI Table S5, however additional experiments will need to be performed after further constraining procedural variables in order to acquire quantitative results.

4. Discussion and impact

Our results show that ET provides potent protection against nitration via $ONOO^-$ or TNM that may help preserve the anti-bacterial function of LF. A previous study by Aruoma et al. showed that ET can protect Tyr from nitration via $ONOO^-$ and can also protect the α_1-antiproteinase protein from nitration [24]. Another study shows ET protects human neuronal hybridoma cell from $ONOO^-$ and H_2O_2, thus improving cell viability [25]. The present study focused specifically on the effect ET provides against nitration of LF and the subsequent loss of the antibacterial activity offered by LF. The effects were tested at ET concentrations of 5 and 10 mM, which is higher than the maximum concentration known to accumulate naturally in human cells and tissues (μm to 2 mM range) [25,26,32,54]. Higher concentrations of ET may be possible temporarily when supplemented with ET as a therapeutic agent, especially in certain tissues exposed to greater degrees of oxidative stress, e.g. the eye [55]. The purpose here was thus to conceptually test whether higher ET concentrations could provide some benefit by inhibiting nitration or preserving antibacterial activity. Further analyses will be required to determine the threshold at which this protective ability of ET begins and if this is physiologically feasible through dietary supplementation. One mechanism by which LF provides bacteriostatic effects is through its ability to bind iron. We previously showed that the ability of LF to bind iron is reduced upon nitration, likely due to changes at iron binding sites [18]. In the present study we focused on examining the effect of ET to protect LF against nitration following oxidative stress (represented *in vitro* using $ONOO^-$ or TNM), as well as the role ET may have in protecting the antibacterial activity of LF against nitration. Our findings suggest that antibacterial activity is increased by protecting Tyr from nitration [18]. These observations are consistent with the results shown by Brock et al. that LF acts as a bacteriostatic agent that inhibits the growth of bacteria through iron sequestration [19].

The cornea is frequently exposed to high levels of oxidative and nitrosative stress, because it is constantly exposed to atmospheric ROS and RNS. LF is present in the cornea of healthy subjects at an average concentration of 1.42 mg mL^{-1}, representing 25% of total tear proteins [56]. The cornea and other ocular tissues contain high concentrations of ET, as well as OCTN1 mRNA expression [21]. To date, however, there have been no previous studies evaluating the impact of ET on nitration of LF or corneal tissue, nor on the effect of ET on LF antibacterial activity. Further, there have been few studies aiming to understand the interaction of ET with ocular tissues and proteins in reducing the effects of oxidative stress. Thus, our observations fill an important knowledge gap by showing ET efficacy against LF nitration and toward protecting protein defense functionality.

ET decreases nitration of LF in pure protein and corneal samples and ET reduces nitration mediated decreases in LF antibacterial activity. These observations suggest that ET could convey a clinically relevant benefit against nitrosative stress and inflammation in biological systems. More specifically, the results support the possible use of ET as a therapeutic agent for eyes subjected to inflammation, nitrosative stress, and/or infection.

Declaration of competing interest

The authors report no conflict of interest. Amani Alhalwani and John Repine declare U.S. Patent Application No. 16/761,215.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101447.

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