Published in final edited form as:

Biometals. 2013 December ; 26(6): 1041–1050. doi:10.1007/s10534-013-9681-8.

EGCG inhibit chemical reactivity of iron through forming an Ngal–EGCG–iron complex

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Abstract

Accumulated evidence indicates that the interconversion of iron between ferric (Fe^{3+}) and ferrous (Fe^{2+}) can be realized through interaction with reactive oxygen species in the Fenton and Haber–Weiss reactions and thereby physiologically effects redox cycling. The imbalance of iron and ROS may eventually cause tissue damage such as renal proximal tubule injury and necrosis. Many approaches were exploited to ameliorate the oxidative stress caused by the imbalance. (–)-Epigallocatechin-3-gallate, the most active and most abundant catechin in tea, was found to be involved in the protection of a spectrum of renal injuries caused by oxidative stress. Most of studies suggested that EGCG works as an antioxidant. In this paper, Multivariate analysis of the LC–MS data of tea extracts and binding assays showed that the tea polyphenol EGCG can form stable complex with iron through the protein Ngal, a biomarker of acute kidney injury. UV–Vis and Luminescence spectrum methods showed that Ngal can inhibit the chemical reactivity of iron and EGCG through forming an Ngal–EGCG–iron complex. In thinking of the interaction of iron

and ROS, we proposed that EGCG may work as both antioxidant and Ngal binding siderophore in protection of kidney from injuries.

Keywords

EGCG; Ngal; Siderophore; Antioxidant; Renal injury

Introduction

Iron can interconvert between ferric (Fe^{3+}) and ferrous (Fe^{2+}). These two forms of iron interact with reactive oxygen species (ROS) in two main reactions. Hydrogen peroxide (H_2O_2) can react with (free or poorly liganded) Fe^{2+} in the Fenton reaction and produce Fe^{3+} (Rodríguez et al. 2001). Superoxide ($\text{O}_2^{\bullet-}$) reaction with Fe^{3+} in the Haber–Weiss reaction (Kehrer 2000) to produce Fe^{2+} again, thereby, effects redox cycling. Under normal physiological circumstances, proteins such as transferrin and ferritin sequester iron to reduce the threat of iron–ROS interaction. However, when pathological changes happen, over-production of ROS may free iron from ferritin, producing more ROS. Iron and ROS thus positively interact with each other and produce more serious problem under such imbalance of iron and RO. As a result, it may eventually cause tissue damage such as renal proximal tubule injury and necrosis. These tissues (kidney, heart or liver) injury or dysfunction caused by iron–ROS positively interaction underpins the early stage of related pathological symptom. ROS such as hydroxyl radicals and $\text{O}_2^{\bullet-}$, as well as uremic toxins are mainly responsible for renal failure (Paller et al. 1984; Wills 1985; Yokozawa et al. 1991). In addition, free radicals play a considerable role in the synthesis of the uremic toxins Cr, MG and GSA (Fujitsuka et al. 1994). Loading animals with iron prior to the ROS damage aggravated the injury (Mori et al. 2005) while the injury was decreased when given iron chelators such as deferoxamine (Paller and Hedlund 1994), apotransferrin (de Vries et al. 2004), microbial origin chelators (Horwitz et al. 1998), or antioxidants (Shedlofsky 1998; Yokozawa et al. 2003; Abdel-Raheem et al. 2010) in advance. Consequently, iron chelators or antioxidant agents could be two types of therapeutic approaches for tissue damage caused by iron–ROS-interaction.

Tea is the second most highly consumed beverage worldwide other than water. However, unlike water, tea contains thousands of chemical components including polyphenols (mainly catechins, flavonoids and its glycosides, proanthocyanidins, phenolic acids and their derivatives), purine (xanthine) alkaloids, terpenoids and its glycosides, fatty acids, amino acids, carbohydrates, etc. (Ulrich 2010), which showed a wide spectrum of bioactivities. The catechins (Flavan-3-ols), accounting for about 30 % dry weight of tea (Balentine et al. 1997), are known to be excellent antioxidants that directly scavenge free radicals and protect against pathological damage such as hypertension, tumorigenesis, and renal diseases (Yokozawa et al. 2003). (–)-Epigallocatechin-3-gallate (EGCG, Fig. 1a), the most active and most abundant catechin in tea (Ulrich 2010), is a potential anti-inflammatory and antioxidant agent having been exploited to be involved in quenching free radicals, chelating transition metals, and interrupting lipid peroxidation chain reaction (Peng et al. 2011). Accordingly, tea polyphenols may play some roles in protecting kidney through strong antioxidant

activity or as iron chelators. There were several reports on the protection by tea polyphenols or EGCG against nephrotoxicity (Yokozawa et al. 2003; Abdel-Raheem et al. 2010; Peng et al. 2011; El-Mowafy et al. 2010; Ryu et al. 2011; Jang et al. 2006; Rah et al. 2007; Hisamura et al. 2006; Shi et al. 2003). Nonetheless, in addition to the full concerns on its antioxidant activity as for the mechanistic basis for it, there is much more to be worked out.

Neutrophil gelatinase associated lipocalin (Ngal) is expressed only at low levels in normal tissues of rodents and humans, but it is markedly expressed in response to injuries caused by ischemia–reperfusion or cytotoxic agents (Mori et al. 2005; Mishra et al. 2005; Nickolas et al. 2008; Bao et al. 2010). While it is plausible that in these aseptic diseases Ngal plays a prophylactic antimicrobial role, it is also possible that Ngal binds novel ligands from other resources such as endogenous or food origin. Here we report findings that Ngal inhibit the chemical reactivity of iron and may interrupt the positive feedback loop of iron and oxidative stress, thus protecting kidney from related factors caused injury through forming an Ngal–EGCG–iron complex.

Materials and methods

Materials

Loose tea and Jingwei Fu tea were kindly provided by Shaanxi Cangshan Tea Industry Co., Ltd. Standard EGCG was obtained commercially from Shanghai Tauto Biotech (USA, purity >98 %). HPLC-grade acetonitrile was purchased from Oceanpak Company, Sweden. Formic acid was of HPLC grade, and was purchased from Agilent (USA). Deionized water was prepared using a water purification system (Millipore, USA). $^{55}\text{FeCl}_3$ (45 mCi/mg) was from PerkinElmer. Enterobactin and ferric Enterobactin (FeEnt) were from EMC Microcollections, Germany. Ngal was expressed in BL-21 bacteria (Mori et al. 2005; Bao et al. 2010).

Preparation of tea extracts

Tea (2.5 g) was reflux extracted by EtOAc (3×200 mL, 1 h each), MeOH (3×200 mL, 1 h each), and water (3×200 mL, 1 h each), separately, and thus the EtOAc, MeOH and H_2O extracts of tea were prepared. Each extract was frozen dry for assay use. A final concentration 0.5 mg/mL of tea extracts was used in the binding assay.

Binding assays (Mori et al. 2005; Bao et al. 2010)

To test whether EGCG or tea extracts bind iron, we spotted the EGCG (1 nmol, in $\text{MeOH:H}_2\text{O}$, 1:1) or tea extracts (10 μg) with $^{55}\text{Fe}^{3+}$ (1 pmol, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in water) on Whatman chromatography grade paper (3 mm, 8 inches high) and separated from free $^{55}\text{Fe}^{3+}$ by developing the paper chromatogram with water. All binding assay data were normalized to Ent (100 % value tested at 10 μM , pH 7.5, RT).

To test whether Ngal binds iron through the tea extracts or EGCG, Ngal (Unless otherwise specified, concentration of Ngal used in all binding assays is 10 μM), $^{55}\text{Fe}^{3+}$ (1 μM + cold FeCl_3 9 μM) and EGCG (for EGCG, 0.5 mg/mL for tea extracts) or the positive control Enterobactin (Ent, 10 μM) were incubated in 150 mM NaCl, 20 mM Tris (pH 7.4), room

temperature (RT, 25 °C) for 60 min. Different pH buffers utilized Tris Base mediated by HCl and NaOH. The mixture was then washed three times with the Tris buffer on an Ultracel-10 K Amicon Ultra (Millipore, Ireland Ltd) and the retained ⁵⁵Fe was measured with a scintillation counter.

LC–MS data acquisition

HPLC-photodiode array detector (PAD)—electrospray ionization (ESI)—high resolution mass spectrometry (HRMS) analysis was carried out on an Agilent 6210 time-of-flight (TOF) LC/MS system with a binary high-pressure mixing pump, auto sampler, column oven, PAD, TOF MS with an ESI source and an Agilent workstation. Analytical separations were carried out on an Agilent Poroshell 120 column (C-18, 2.7 µm, 100 × 3.0 mm i.d.). Liquid samples were concentrated with a Senko R 206 rotary evaporator and frozen dried with a Laboconco freeze dryer.

LC parameters were as follows: injection volume, 5 µL; column temperature, 40 °C; flow rate, 0.3 mL/min; and the eluates were monitored with a PAD at full length scan from 200 to 600 nm. The mobile phase A = 0.1 % formic acid in water, B = 0.1 % formic acid in acetonitrile, and gradient elution was carried out: 10–30 % B for 0–5 min; 30–70 % B for 5–40 min; 70–100 % B for 40–41 min; 100 % B for 41–51 min. The column was reconditioned with initial gradient for 12 min. The mass spectrometer parameter settings used for the measurement were as follows: ionization mode, positive and negative; gas temperature, 350 °C; drying gas, 12 L/min; nebulizer pressure, 45 psi; capillary voltage, 4,000 V in positive mode and 3,500 V in negative mode; fragmentor voltage, 215 V in positive mode and 170 V in negative; skimmer voltage, 60 V; OCT 1 RF, 250 V. Data acquisition was performed in the *m/z* range 50–1,100 Da.

Data processing and statistical analysis

The raw data from LC-TOF chromatograms were preprocessed by MassHunter software (Agilent Technologies, Santa Clara, CA, USA) using the molecular feature extraction (MFE) algorithm for automated peak detection and chromatographic deconvolution. Peaks with signal-to-noise (S/N) ratios lower than 5 were rejected. The mass/retention time/peak height data array for each sample were generated and exported as .csv file. Then all the data were uploaded to MetaboAnalyst for subsequent data process and statistical analysis (Xia and Wishart 2011; Xia et al. 2009). Peaks were aligned across all samples using the parameters of 0.01 Da and 0.5 min tolerance. Finally, the processed data were downloaded for multivariate analysis. Orthogonal projection on latent structures discriminant analysis (OPLS-DA) were performed by SIMCA-P+ (version 12.0, Umetrics, Umea, Sweden). Pareto scaling was applied for OPLS-DA derived from LC–MS data sets.

Metabolites identification

Metabolite identification was performed in the following approaches: (1) The molecular formula calculated by the MassHunter software based on the accurate mass and isotopic pattern recognitions, was used for confirming putative identities by searching against web databases. (2) The UV–Vis spectra were used in the identification whenever possible. (3)

The ambiguous metabolites were identified by comparison with authentic compounds available and/or by referring to the published data about tea compounds.

Dose response curve, and pH response curve of the binding assay

Use the same procedure for binding assay except that different concentration of EGCG (Concentration: 1.0, 5.0, 10, 30, 50, 100 μM) was added to the solution for dose response curve test or different pH washing solution (pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5) was prepared for pH response curve test. Each was tested three to seven times.

UV spectrum of Ngal–EGCG–iron complex

UV was detected with U-5100 UV/Visible Spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan). Ngal, EGCG (or catechol, Enterobactin), iron ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) solution was mixed together to make final concentration of Ngal (170 μM), EGCG (or catechol, Enterobactin, 1,700 μM), iron (1,700 μM). The mixture was cultured at RT for 60 min and then washed three times with the Tris buffer on a Ultracel-10 K (Millipore). The retained mixture was tested by the UV-spectrophotometer (Bao et al. 2010).

Inhibition assay of 50 fold red enterobactin (FeEnt) or mutant Ngal

The assay was tested same procedure as the binding assay other than that 50 fold FeEnt was added to the assay or Mutant Ngal (the specific binding sites for catecholate siderophore, lysines K125 and K134, was mutated by Alanine) was used instead of the normal Ngal.

Reduction of iron (FeCl_3) by EGCG and inhibition of the reaction by Ngal

The reaction mixture contained 0.24 M potassium phosphate buffer (pH 7.4), 30 mM sodium citrate, 15 μM FeCl_3 , 50 μM EGCG (in 50 mM potassium phosphate buffer, pH 6.5) and 5 mM *O*-phenanthroline (in ethanol). For inhibition test, Ngal (60 μg , 15 μM) was added. A time course Fe^{3+} reduction was monitored by absorbance (512 nm) 5 min after mixing. All spectrophotometric measurements were carried out with a 10 mm light path, with a UV–Vis spectrophotometer at RT (Iwahashi et al. 1989).

Generation of hydroxyl radicals and inhibition of the reaction by Ngal

Hydroxyl radicals were detected by the conversion of HPF to fluorescein based on the method (Setsukinai et al. 2003) with modification. The reaction mixture contained 0.10 M sodium phosphate buffer (pH 7.4), 3 mM EDTA, 100 μM FeCl_3 , 300 μM EGCG, 2 mM H_2O_2 , and 10 μM 3'-(*p*-hydroxyphenyl) fluorescein (HPF; Invitrogen) or 20 μM F1300 (Invitrogen). For inhibition test, Ngal (100 μM) was added. After 1 h at RT, the fluorescence of the reaction mixture was measured with a Luminescence Spectrometer, Ex 490 nm, Em 515 nm.

Results

Association of extracts of two different teas with iron through binding Ngal

Loose tea is a dark green tea, the starting material of Jingwei Fu Tea. Jingwei Fu tea is a kind of post-fermented tea which is fermented by the dominant yellow fungi *Eurotium* spp.

(also called “golden flora”, growing within the tea leaves under controlled temperature and moisture because of a “fungal fermentation” stage in its manufacturing process) (Ling et al. 2010). According to our binding assay (Fig. 1b), there is no significant difference between the two kinds of teas in general ($p = 0.396$, two tailed t test); however, the big difference can be found among different extract methods. The water extract is the most active fraction (compared with MeOH extract, $p < 10^{-6}$, two tailed t test) followed by the EtOAc extract (compared with MeOH extract, $p < 10^{-5}$, two tailed t test), while the MeOH extract has very little binding activity. These results possibly are caused by different chemical constituents in different extracts. In that idea, an HPLC-HR-ESI MS analysis of the components of different extracts was conducted as follows.

LC–MS and multivariate analysis

The binding assay showed that H₂O extracts of both teas had the strongest binding activity, which should be caused by the different chemical constituents in the extracts (Fig. 1b). Several studies used multivariate analysis to correlated variance in chemical constituents of extracts and related bioactivities by different models (Gao et al. 2010; Nguyen et al. 2009; Shyur and Yang 2008). Here, the OPLS-DA models of the tea extracts were established using one predictive and three orthogonal components ($R^2X = 0.709$, $R^2Y = 0.527$, $Q^2 = 0.617$). A paired comparison of OPLS-DA can clearly discriminate the different extracts (Fig. 2a, from right to left: EtOAc, black rectangles; MeOH, red circles; H₂O, blue diamonds) and different teas as well (Fig. 2a, Jingwei Fu tea: top red dashed oval; Loose tea: bottom dashed black oval). Subsequently, the S-plots from the OPLS-DA models were constructed to understand the chemical constituents responsible for the differentiation (Fig. 2b). S-plots showed the most relevant variable affecting differentiation is at the peak 458.08/6.51. And the contribution score of H₂O fractions normalized to average showed that the peak 458.08/6.51 provided the biggest contribution for its difference as well (Fig. 2c). According to the HRESIMS calculation and also comparison with the LC–MS of standard compounds, 458.08/6.51 was identified as the peak of EGCG, which gave a hint that EGCG may contribute to the binding activity most in thinking of its high concentration in tea. Therefore, we conducted more detailed binding assay for EGCG next. At the same time, concentration of EGCG in the different extracts (Fig. 2d) showed that significant difference exists between different extraction, with the highest concentration in H₂O extraction while the smallest amount in the EtOAc extraction ($p < 0.0001$). Generally, Loose tea has more EGCG than Jingwei Fu tea which is logically reasonable, because the EGCG of Loose tea (the starting material as a green tea) will endure big loss during the following processing and microbial fermentation period of Jingwei Fu tea production (Ulrich 2010).

Binding assays of EGCG

Since the results of LC–MS and Multivariate analysis suggested that EGCG may be contribute most to the Ngal binding activity of tea infusion, EGCG was tested through both paper chromatography and 10 K filter cutoff experiments. The paper chromatography together with Ent suggested EGCG can mobilize Fe³⁺ along the paper by water (Fig. 3a), which means EGCG is an effective iron chelator. To determine whether EGCG can form a complex with Ngal and iron, Ngal (10 μ M), EGCG (1–100 μ M) and iron (1 μ M ⁵⁵Fe + cold FeCl₃ 9 μ M) were incubated for 1 h at RT. The mixture solution was then washed

repetitively on a 10 K cutoff filter ($n = 3-7$ independent experiments, Fig. 3c). The dose responsive curve (Normalized to Ent) showed EGCG has the strongest binding activity at 30 μM , suggested that Ngal: EGCG: $\text{Fe}^{3+} = 1:3:1$ is the best stoichiometric condition for their binding. Then a concentrated Ngal-EGCG- Fe^{3+} complex solution was prepared and detected by UV, which showed a shoulder peak at around 400–700 nm just as those of Cat or Ent (Fig. 3b, apoNgal, black line; Ent, green line; Cat, blue line; EGCG, red line), suggesting that EGCG was associated with Ngal and iron through forming a stable Ngal–EGCG–iron complex.

The pH response experiments suggested acidification of Ngal–EGCG–iron complex can release the iron. The best pH condition for the Ngal–EGCG–iron complex is at pH 6.5 (Fig. 3c). Interestingly, a second high peak at pH 5.5 suggested that its dissociation mechanism is different from Catechol whose dissociation started as soon as the pH decreased from its highest peak at pH 7 (Bao et al. 2010). From pH 5.5, the Ngal–EGCG–iron complex decomposed quickly as well. Its different appearance of acidification releasing iron from catechol may be caused by the fact that two pyrogallol groups exist in the structure of EGCG (Fig. 1a). Transferrin and iron complex can be released by acidified endosomes, therefore, acidification release of the iron from iron complex is very important for iron transportation. The pH response experiment suggested that Ngal–EGCG–iron complex may endure the same procedure as transferrin for releasing iron intracellularly.

The binding sites of EGCG at Ngal

Structural studies showed Ngal recognition of siderophores mechanism depends on electrostatic and cation– π interactions between the two lysine side chains (K125 and K134) of Ngal and catechol groups of siderophores (such as Ent or Cat) (Bao et al. 2010; Hoette et al. 2008; Holmes et al. 2005). To determine whether Ngal recognized EGCG by a similar type of interaction, a 50 fold of red ferric enterobactin (FeEnt) was added to the normal binding assay (Fig. 4, gray bar). The inhibition suggested that EGCG may bind the same sites as Ent. Since lysines K125 and K134 interact with the catechol groups of Ent (Hoette et al. 2008; Holmes et al. 2005) through donating cation– π bonds (Holmes et al. 2005), we evaluated a mutant form of Ngal, both of the two lysines were mutated to alanine (Fig. 4, white bar). Ngal became ineffective in the capture of Ent:Fe, Cat:Fe and EGCG:Fe in this case. So, EGCG should interact with Ngal at the same positions K125 and K134 as Ent or Cat does.

The reactivity of EGCG and iron can be inhibited through forming Ngal–EGCG–Fe complex

EGCG, like Cat, can activate the Fenton reaction by reducing iron ($\text{Fe}^{3+} \Rightarrow \text{Fe}^{2+}$) (Rodríguez et al. 2001) and thereby accelerating hydroxyl-radical formation, which was confirmed by detecting phenanthroline reactive Fe^{2+} after incubating Fe^{3+} with EGCG (red line). However, the addition of stoichiometric quantities of Ngal (green line, Ngal:EGCG 1:3, respectively) inhibited the reaction ($\text{EGCG} + \text{FeCl}_3 \pm \text{Ngal}$: $p = 0.039$ through 10 min, two-tailed t test, $n = 4$; Fig. 5a), while Ngal itself cannot induce the Fenton reaction (blue line) and not inhibit Fe^{2+} by itself either (black to purple line, $p = 0.29$, two-tailed t test, $n = 4$; Fig. 5a). This experiment thus suggested Ngal limit the reactivity of iron and EGCG through the formation of Ngal-EGCG- Fe^{3+} complex.

Similarly, while EGCG:Fe induced the conversion of 3'-(*p*-hydroxyphenyl) fluorescein (HPF) to fluorescein in the presence of H₂O₂ (red line, Fig. 5b), the addition of Ngal (green line, Fig. 5b) blocked the reaction (HPF + EGCG + FeCl₃ ± Ngal: $p < 0.001$, $n = 3$; Fig. 5b) (Setsukinai et al. 2003). These data demonstrate that through complexation with EGCG: iron, Ngal maintained Fe³⁺ in the oxidation state, and thereby limited its reactivity in Fenton reaction. Consequently, it could stop the continuing aggravation of cellular and organismal deterioration caused by ROS.

Discussion and conclusions

Kidney is one of the most sensitive tissues to ROS. When acute injury caused by ROS happens, the level of Ngal will be increased by orders of magnitude in the renal tissues for its own protection (Mori et al. 2005). Numbers of studies suggested that EGCG can protect the kidney from acute injury caused by ischemia, antibiotics and cytotoxic agents and they emphasized the role of EGCG as an antioxidant. This paper suggested in addition to its antioxidant role, EGCG could work as a stable siderophore through binding Ngal when massive iron released by the injured kidney. EGCG may thus serve both roles as antioxidant and effective siderophore for the reduction of redox stress and as the result protecting the kidney from injury.

Although several studies on tea polyphenols particularly EGCG protection effect on kidney cells, renal tissue injury or failure, the mechanism about its effect is scarcely touched upon. More recently, a hypothesis was suggested that green tea polyphenols attenuate CsA-induced kidney injury, at least in part, through stimulation of mitochondrial biogenesis (MB) (Rehman et al. 2013). However, whether this stimulation of MB requires binding of polyphenols to a specific receptor remain to be investigated. In this study, we tested that the major tea polyphenol EGCG can bind the protein, Ngal, which is a 21 kDa protein highly expressed in injured renal tissues. Although the exact mechanism is not clear how the Ngal originated in such a speedy way with 100–1,000 increment as soon as the injury started, one paper suggested that oxidative stress can upregulate Ngal gene expression and this express can be inhibited by antioxidants (Roudkenar et al. 2007). Through our previous and present study, a possible pathway could be provided for the effect of Ngal on kidney injury. When kidney injury initiated by ROS, the proximal tube is easily affected. For its recovery, Ngal is highly upregulated (Roudkenar et al. 2007) and bound to some endogenous small molecular siderophores together with iron. Through forming the Ngal–siderophore–iron complex, on the one hand, Ngal can inhibit the chemical reactivity of the largely released iron from injured tissues, and thus suppress free iron promoted ROS generation and the following progressive tissue damage. On the other hand, Ngal–siderophore–iron complex can provide certain chemicals especially antioxidants for stimulation of mitochondrial biogenesis, and thereby produce energy and provide iron and nutritionals for converting renal progenitors into epithelial tubules and thus protecting adult kidney epithelial cells or accelerating their recovery from damage. Additionally, the complex can inhibit microbial growth through sequestering iron the microbial growth needed. However, limited endogenous small molecular siderophores cannot satisfy the needs if overdue injury happens, in which case exogenous siderophores are highly needed. EGCG is an active antioxidant and can form a stable EGCG–iron complex through binding Ngal, and thus can provide siderophores and

iron for the injured kidney. Besides, the Ngai:siderophore:Fe complex upregulates heme oxygenase-1 (Mori et al. 2005; Wu et al. 2006), a protective enzyme, preserves proximal tubule N-cadherin, and inhibits cell death, through protecting Nuclear factor-erythroid-2-related factor 2 (Nrf2) activated by oxidative stress signals from various sources (Ruiz et al. 2013). According to one recent study, EGCG may also play as iron chelator (siderophore) as deferoxamine did in inhibiting chelatable iron released by lysosomes and taken up by mitochondria and thus suppressed iron promoted ROS generation, the mitochondrial permeability transition and cell death (Zhang and Lemasters 2013). Collectively, this study provides a possible new mechanism in which EGCG protects kidney from injury through complexing with Ngai and iron.

Acknowledgments

Financial assistance was received with appreciation from China National Science Foundation 81170654/H0507, Anhui Agricultural University Talents Foundation (YJ2011-06), Anhui Outstanding Youth Science Foundation 1108085J04, and Program for Changjiang Scholars and Innovative Research Team in University IRT1101.

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Fig. 1.
a the structure of EGCG. **b** Binding assay of tea extracts of Loose tea and Jingwei Fu tea (data normalized to Ent)

Fig. 2. Multivariate analysis of LC-MS data. **a** A paired comparison of OPLS-DA analysis of samples according to various extracts of two teas: OPLS-DA score plots derived from HPLC-MS spectra ($R^2X = 0.709$, $R^2Y = 0.527$, $Q^2 = 0.617$). **b** S-plot generated from OPLS-DA models of the six extracts of two types of tea shows covariance w and correlation p (corr), $w[1] \geq 0.1$, p (corr) ≥ 0.5 , the variables ($p < 0.01$, t test) was identified as the metabolites related to grouping. **c** The contribution score of H2O fractions and esp. EGCG (normalized to average). **d** EGCG relative concentration in the six extracts (normalized to average). (Color figure online)

Fig. 3. Association of EGCG with Ngal and iron and releasing iron in acid condition. **a** Paper chromatography of free Fe^{3+} , Fe^{3+} + Ent, Fe^{3+} + EGCG and Fe^{3+} + tea water infusion (tea). **b** UV spectrum of apoNgal (black line), Ngal–Ent–iron (green line), Ngal–Cat–iron (blue line), and Ngal–EGCG–iron (red line) complex. **c** Dose responsive curve of EGCG binding iron through Ngal (Ngal 10 μM , EGCG 1.0, 5.0, 10, 30, 50, 100 μM , normalized to Ent). **d** Dissociation of iron from the complex in acid condition: pH responsive curve of EGCG binding iron through Ngal (washing buffer pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, normalized to Ent). (Color figure online)

Fig. 4.
The binding sites at the protein Ngal: The formation of Ngal–siderophore–iron complex (Ent, Cat, EGCG, *black bar*) can be inhibited by 50 fold red Enterobactin (FeEnt, *grey bar*), and the site specific mutant (K125 and K134 to Alanine, *white bar*) of Ngal cannot form a complex with EGCG, Ent, Cat and iron. Data was normalized to Ent

Fig. 5. EGCG reducing power and prooxidant capacity can be inhibited by Ngal. **a** UV detected the conversion of Fe^{3+} to Fe^{2+} by EGCG (red line) can be inhibited by adding Ngal (green line), $p = 0.039$ through 10 min, two-tailed t test, $n = 4$. **b** Conversion of HPF to fluorescein (Ex 490 nm, Em 515 nm) was detected in the presence of EGCG, Fe^{3+} and H_2O_2 (red line), but the addition of Ngal blocked the reaction (green line; HPF + EGCG + $\text{FeCl}_3 \pm$ Ngal: $p < 10^{-5}$, $n = 3$, two-tailed t test) (each experiment contained Fe^{3+} , which was not shown on the figure legends). (Color figure online)