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Preventive Effect of Curcumin and Quercetin against Nitric Oxide Mediated Modification of Goat Lung Cystatin

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Cysteine proteinase inhibitors are of prime physiologic importance inside the cells, controlling the activities of lysosomal cysteine proteases. The present work aimed to realize the effects of nitric oxide on the structure and function of goat lung cystatin (GLC) and to evaluate antinitrostative efficacy of curcumin and quercetin. Nitric oxide induced structural modifications were followed by fluorescence spectroscopy and PAGE and functional inactivation by monitoring the inhibition of caseinolytic activity of papain. Ten millimolar sodium nitroprusside (SNP) caused time dependent inactivation of GLC-I with complete functional loss precipitating at 180 min. Curcumin (50 μM) and quercetin (250 μM) opposed such loss in papain inhibitory activity of GLC-I. Loss in tertiary structure of GLC-I (fluorescence quenching and 15 nm red shift) was observed on SNP treatment. Inhibition of functional and structural SNP mediated damage of GLC-I by curcumin (50 μM) and quercetin (250 μM) reaffirms their NO scavenging potency.

KEYWORDS: NO; protease inhibitor; asthma; curcumin; quercetin

INTRODUCTION

Nitric oxide (NO) is an omnipresent intercellular messenger in all vertebrates, modulating blood flow (*1*), thrombosis (*2*) and neural activity (*3*). The production of nitric oxide is also important for nonspecific host defense, helping to kill tumors and intracellular pathogens. However, under pathophysiological conditions, NO has damaging effects. It is found to be involved in neural disorders involving oxidative stress such as Parkinson's disease (*4*), Alzheimer's disease (*5*), ischemia (*6*), Huntington's disease (*7*), amyotrophic lateral sclerosis (*8*) and multiple sclerosis (*9*). Excessive production of NO (a known mediator of inflammation) is correlated with nitrostative stress in tissues. Although $NO^{\bullet}$ is a free radical, it has selective reactivity and reacts predominantly with other paramagnetic species, including ferrous or ferric iron in heme proteins or iron−sulfur centers, and other radical species such as molecular oxygen (O_2), superoxide anion ($^{\bullet}O_2^-$) and lipid or protein radicals (*10*−*14*). Reaction of NO with $^{\bullet}O_2^-$ at a near diffusion limited rate yields peroxynitrite ($ONOO^-$), a powerful oxidizing species, to which many of the cytotoxic properties of $NO^{\bullet}$ have been attributed (*15*).

Lung diseases like cancer, emphysema and idiopathic pulmonary fibrosis are known to be caused by an imbalance between the activities of endogenous inhibitors and cysteine proteinases (cathepsins) (*16*). Cystatins, noncovalent competitive inhibitors of cysteine proteinases (cathepsins B, H, L and S) (*17*), are ubiquitously present in mammalian system. These inhibitors protect the cells from unwanted proteolysis which may otherwise cause a number of pathologies like purulent bronchitis (*18*), Rheumatoid arthritis (*19*), osteoporosis (*20*), Alzheimer's disease (*21*), metastasizing cancer (*22*) and microbial invasion (*23*).

NO is a thermodynamically unstable molecule and tends to react with other bio molecules especially proteins resulting in their oxidation, nitration and nitrosylation, with the concomitant effects on many cellular mechanisms. Increased ROS/RNS ($NO^{\bullet}$) levels are consequential to chronic/acute lung inflammation. A common factor in inflammation is that the equilibrium between lysosomal enzymes released by macrophages or neutrophilic granulocytes and their endogenous inhibitors in the extra cellular space is disturbed. This imbalance may originate from many reasons, one of which may be inactivation by ROS/RNS released by neutrophilic granulocytes during the process of inflammation.

Various researches have been undertaken to evaluate the potential benefits of complementary and alternatives medicines (botanicals, flavonoids, herbs, etc.) to oppose the toxicity associated with reactive species generated during various pathogenic states.

Curcumin (diferuloylmethane), the major coloring pigment present in the rhizomes of *Curcuma longa* (turmeric), is a spice widely used in Indian cooking and for medicinal purposes (*24*). Curcumin has been shown to possess many therapeutic properties including antioxidant (*25*), anti-inflammatory (*26*) and anticancer properties (*27*). It also protects DNA against singlet-oxygen-induced strand break (*28*), lipids from peroxidation (*29*) and oxyhemoglobin from nitrite induced oxidation (*30*).

Quercetin is the main flavonoid in the diet and is consumed as quercetin-3-rutinoside (in black tea about 40%), quercetin-4′-glucoside (45% in onions) and quercetin-3-glucoside (mainly in tea, apples and tomatoes) (*31*, *32*). Flavonoids exhibit several positive health aspects; they possess anticarcinogenic, antimutagenic, antioxidant, immune-stimulating and estrogen-active

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properties. They inhibit lipid peroxidation, LDL oxidation and chelate transition metals (*33*−*35*).

Combating the damage inflicted by reactive species is the need of the hour. The main objective of present work was to access the effects of NO on structure and function of GLC and to evaluate their suppression by dietary antioxidants, curcumin and quercetin.

MATERIALS AND METHODS

Materials. Curcumin (1, 7-bis [4-hydroxy-3-methoxyphenyl]-1, 6-heptadiene-3, 5-Dione) was obtained from Sigma (St. Louis, MO) and quercetin from Qualigens (India). Sodium nitroprusside, sulphanilamide and naphthylenediamine dihydrochloride were of analytical grade.

Methods. *Purification of GLC*. Goat lungs were homogenized and fractionated with ammonium sulfate and the fraction, precipitated by 40−60%, was dialyzed against 50 mM sodium phosphate buffer pH 7.4 containing 0.1 M NaCl. This was chromatographed on DEAE-cellulose ion exchange column. Two separate protein peaks with papain inhibitory activity were obtained. First inhibitory peak (GLC-I), corresponding to 66 kDa, was used in the following study (*36*).

Nitric Oxide Production from Sodium Nitroprusside. Nitric oxide was generated from sodium nitroprusside and measured by the Griess reagent. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by use of the Greiss reagent (*37*). SNP (100 mM) was prepared by dissolving the powder in phoshphate buffer saline (PBS) pH 7.4. The reaction mixture (2 mL) containing 100 mM SNP (0.2 mL, final concentration 10 mM) and PBS (1.8 mL) was incubated at 25 °C for 180 min. At 30 min intervals, samples (1 mL) of the incubation were removed and diluted with 1 mL of Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine dihydrochloride in 2% H_3PO_4). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylene-diamine was read at 540 nm and referred to the absorbance of standard solution of sodium nitrite treated in the same way with Greiss reagent. The plot between the concentration of nitrite and time of incubation exhibited the best incubation time for nitrite production from SNP.

Functional Inactivation of GLC-I by Nitric Oxide and Its Impediment by Curcumin and Quercetin. The purified thiol protease inhibitor (GLC-I) was incubated with 10 mM SNP for varying time intervals in the absence and presence of varying concentration of (10−50 μM) curcumin and (10−250 μM) quercetin. Inhibitory activity was measured by ability of GLC-I to inhibit caseinolytic activity of papain by the method of Kunitz (1974) (*38*). Papain inhibitory activity signifies the activity of GLC-I. Papain has been used as model cysteine protease in the experiments which is inhibited by GLC-I (Cysteine protease inhibitor). Percent remaining activity is the activity of GLC-I remaining after various treatments.

Nitric Oxide Induced Conformational Changes in Goat Lung Cystatin (GLC-I) and Protection by Curcumin and Quercetin. The conformational changes in goat lung cystatin induced by NO generated from SNP were detected by fluorescence measurements which were carried out on a Shimadzu spectrofluorimeter model RF-540 equipped with a data recorder DR-3. GLC-I (1 μM) was incubated with varying concentration of SNP (0.05−10 mM) for 30 min in a final reaction volume of 1000 μL. In a separate set of experiments, curcumin (50 μM) and quercetin (250 μM) were also added to the reaction mixture. Appropriate controls were run for each set and corrections were made wherever necessary. The fluorescence was recorded in the wavelength range of 300−400 nm after exciting the protein solution at 280 nm.

Structural modification of GLC-I by NO. Structural changes of GLC-I by NO were investigated using Polyacrylamide gel electrophoresis. GLC-I (50 μg) was incubated with different concentration of SNP (0.05 mM, 5 mM and 10 mM) for 2 h and then was run on PAGE. In a separate set of experiments, GLC-I (50 μg) was incubated with SNP (10 mM) in presence of curcumin (50 μM) and quercetin (250 μM) and subjected to analysis by PAGE. The gels were stained with 0.01% CBB-R250.

Statistical Analysis. All the experiments were conducted in replicates of 4. All data are expressed as Mean ± SEM. Significance of difference in mean values were evaluated using one way ANOVA. A probability level of $p < 0.001$ was selected as indicating statistical significance. Activity of GLC-I treated with 10 mM SNP was taken as control for all calculations.

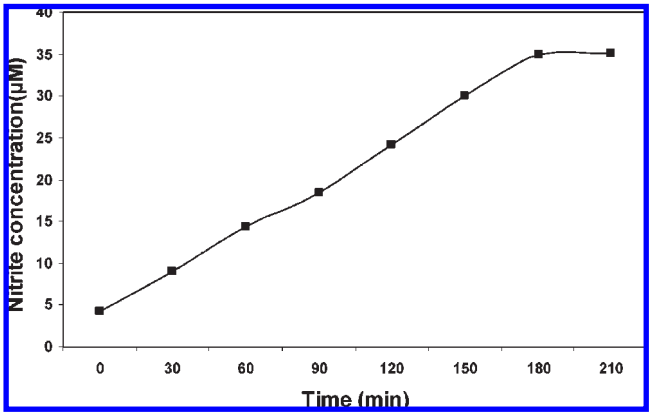

Figure 1. Time-dependent nitric oxide production from a solution of 10 mM sodium nitroprusside. At 30 min intervals, 1 mL of incubation solution was removed and reacted with 1 mL of Griess reagent. The absorbance was read at 540 nm and referred to the absorbance of standard solution of sodium nitrite treated in the same way with Griess reagent.

Table 1. Effect of Varying Time Intervals of Incubation with 10 mM SNP on GLC- I Antiproteolytic Activity[a]

S.No.	treatment and time of incubation	% remaining activity	% loss of nhibitory activity
1.	Native GLC-I	88 ± 2	0
2.	GLC-I+SNP (0 min)	86.5 ± 1	1.5
3.	GLC-I+SNP (15 min)	78.5 ± 1.5[b]	9.5
4.	GLC-I+SNP (30 min)	73.5 ± 2.1[b]	14.5
5.	GLC-I+SNP (45 min)	53.5 ± 2.2[b]	34.7
6.	GLC-I+SNP (60 min)	46.61 ± 1.3[b]	41.4
7.	GLC-I+SNP (90 min)	3.9 ± 1.5[b]	48.2
8.	GLC-I+SNP (120 min)	25.7 ± 2.1[b]	62.3
9.	**GLC-1+SNP (150 min)**	**15.9 ± 1.1[b]**	**72.1**
10.	GLC-I+SNP (180 min)	0	100

[a] control used for calculations was GLC-I treated with 10 mM SNP. [b] $p < 0.001$ by one way ANOVA.

RESULTS

Nitric Oxide Production from Sodium Nitroprusside. SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce a nitrite ion which can be estimated using Griess reagent. Incubation of solutions of SNP in PBS at 25 °C resulted in linear time-dependent nitrite production (**Figure 1**).

Functional Inactivation of GLC-I by Nitric Oxide and Its Impediment by Curcumin and Quercetin. GLC-I on incubation with SNP lost its inhibitory activity (**Table 1**). More than 50% lost in activity was observed within 60 min of incubation with SNP (NO donor). Within 180 min, inhibitory activity trailed off to zero. Curcumin (50 μM) caused 95% retrieval of inhibitory activity of GLC-I (**Table 2**) while much higher concentration of quercetin (250 μM) was needed to gain back 93.8% of activity (**Table 3**).

Conformation Stability of GLC-I in the Presence of NO and Its Prevention by Curcumin and Quercetin. Intrinsic fluorescence is an excellent spectroscopic probe to investigate conformational changes in the tertiary structure of the protein. The modification of microenvironment of aromatic residues of GLC-I in the presence of NO has been studied by monitoring the changes in

Table 2. Effect of Varying Concentration of Curcumin on Antiproteolytic Activity SNP Treated GLC-I[a]

S.No.	treatment of curcumin with GLC-I	% remaining activity
1.	Native GLC-I	88 ± 2
2.	GLC-I + SNP	15.9 ± 1.1
3.	GLC-I + SNP + Curcumin (10 μM)	24.3 ± 1.5[b]
4.	GLC-I + SNP + Curcumin (20 μM)	42.1 ± 2.2[b]
5.	GLC-I + SNP + Curcumin (30 μM)	65.4 ± 2[b]
6.	GLC-I + SNP + Curcumin (40 μM)	79.5 ± 2.4[b]
7.	**GLC-I + SNP + Curcumin (50 μM)**	**84.4 ± 2**[b]

[a] Concentration of SNP used was 10 mM. Time of incubation of the reaction mixture was 2 1/2 h. Control used for calculations was GLC-I treated with 10 mM SNP. [b] $p < 0.001$ by one way ANOVA.

Table 3. Effect of Varying Concentration of Quercetin on Antiproteolytic Activity SNP Treated GLC-I[a]

S.No.	treatment of quercetin with GLC-I	% remaining activity
1	Native GLC-I	88 ± 2.0
2	GLC-I+SNP	15.9 ± 1.1
3	GLC-I+SNP+Quercetin(10 μM)	16.5 ± 1.5
4	GLC-I+SNP+Quercetin(20 μM)	19.8 ± 2.0
5	GLC-I+SNP+Quercetin(30 μM)	25.4 ± 2.5[b]
6	GLC-I+SNP+Quercetin(40 μM)	29.3 ± 1.5[b]
7	GLC-I+SNP+Quercetin(50 μM)	31.5 ± 2.2[b]
8	GLC-I+SNP+Quercetin(100 μM)	42.6 ± 1.5[b]
9	GLC-I+SNP+Quercetin(150 μM)	57.2 ± 2.5[b]
10	GLC-I+SNP+Quercetin(200 μM)	69.5 ± 2.3[b]
11	**GLC-I+SNP+Quercetin(250 μM)**	**82.6 ± 1.9**[b]

[a] Concentration of SNP used was 10 mM. Time of incubation of the reaction mixture was 2 1/2 h. Control used for calculations was GLC-I treated with 10 mM SNP. [b]: $p < 0.001$ by one way ANOVA.

intensity and wavelength of emission maxima as a function of SNP concentration. Fluorescence spectra of GLC-I in the presence of NO shows quenching of fluorescence intensity coupled with 15 nm red shift (**Figure 2**a and b). However, this loss in tertiary structure of GLC-I by NO is clearly prevented by curcumin (50 μM) and quercetin (250 μM) (Figure **4**).

Structural Modification of GLC-I by NO. Structural changes in GLC-I by NO were investigated using polyacrylamide gel electrophoresis. As the concentration of SNP increased from 0.05 to 10 mM, there is formation of aggregates as indicated by the shift of protein band to a upper position in the gel. However, this change in conformation of GLC-I in the presence of 50 μM curcumin and 250 μM quercetin is visibly repressed (**Figure 3**a and b).

DISCUSSION

The generation of reactive oxygen species (ROS) and other free radicals (R•) during metabolism is a necessary and normal process that ideally is compensated by an elaborate endogenous antioxidant system. However, due to many environmental, lifestyle, and pathological situations, there can be an excess of radicals resulting in a situation of oxidative stress, which has been implicated in cardiovascular disorders, cancer, and other chronic diseases accounting for a major portion of deaths today. Proteins are major targets for damage by free radicals because of their abundance in biological system. They are modified at the backbone or at the side chain groups. As many proteins are catalytic in nature, modifications by free radicals can have an amplified effect on their activity, that is, susceptibility to proteolysis, inactivation and aggregation. Davies (2005) reported that proteins, which have been exposed to certain radicals, exhibit altered primary, secondary and tertiary structures and can undergo spontaneous fragmentation or increased proteolytic susceptibility.

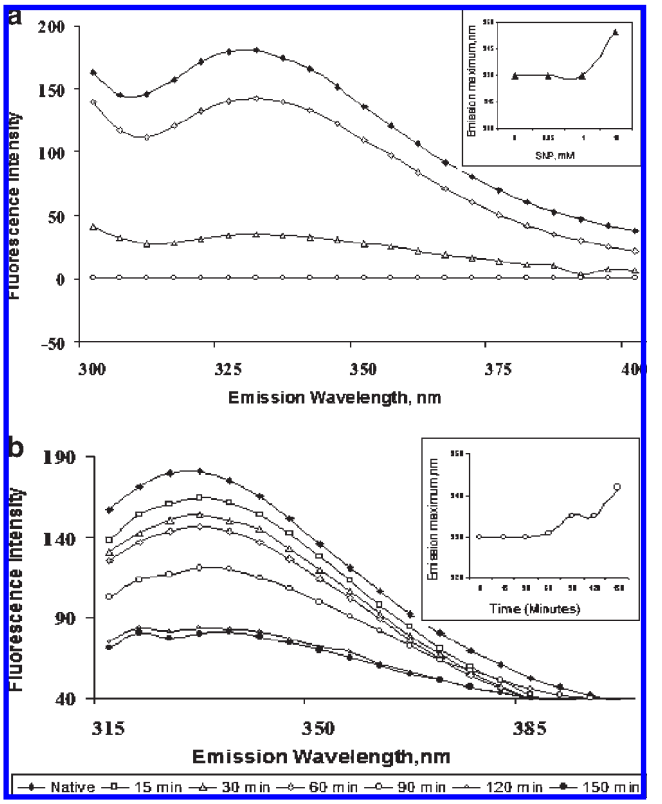

Figure 2. (a) Effect of varying concentration of NO on secondary structure of GLC-I. GLC-I was incubated with different concentrations of SNP (0.05 mM, 1 mM and 10 mM). The protein concentration used was 1 μM. The excitation wavelength was 280 nm and their emissions were recorded in the range of 300−400 nm. (b) Effect of varying time intervals of incubation of GLC-I with SNP (50 μM). The protein concentration used was 1 μM. The excitation wavelength was 280 nm and their emissions were recorded in the range of 300−400 nm.

The treatment of cystatin from goat lungs with the NO-generating compound SNP causes concentration- and time-dependent loss of enzyme activity (**Table 1** and **Figure 2**a and b). This functional inactivation may arise due to modification of active site amino acids or may also be due to oxidation of critical tryptophan residues as shown by the quenching of fluorescence coupled with 15 nm red shift suggesting the complete unfolding of protein (GLC-I) in the presence of NO. This result is in accordance with previous pronouncement that NO may reversibly inhibit enzymes with transition metals or with free radical intermediates in their catalytic cycle. NO in micromolar concentrations reversibly inhibited catalase and cytochrome *P*-450 (*40*, *41*). GLC-I treated with SNP, exhibits mobility less than the untreated sample due to aggregation visible in nondenaturing PAGE. When the same sample was analyzed by SDS-PAGE, higher mobility material than the native GLC-I (untreated) was observed. This implies that the aggregation product visible in native PAGE of SNP treated GLC-I might be a random conglomerate of protein fragments (gel not shown).

Providing that reactive species may act as toxins, mediators and modulators of inflammatory gene activation and potential in damaging membranes and cellular workhorses, proteins, efforts have been directed to investigate antioxidant molecules as potential therapeutic agents (*42*). In this context, polyphenols, flavonoids and other natural products are becoming increasingly important (*43*−*45*). The present study thus investigated the protective role of well-known antioxidants curcumin and quercetin against this damage. It was found that curcumin (50 μM)

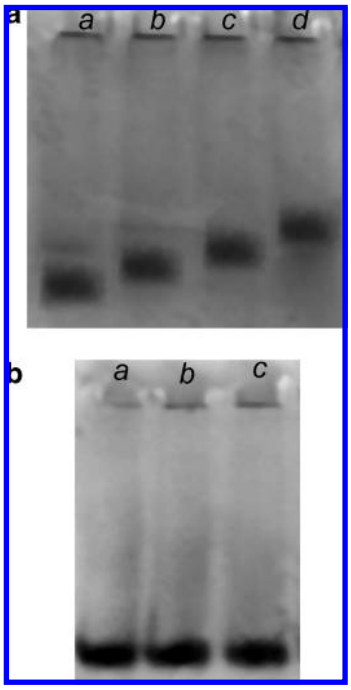

Figure 3. (a) Polyacrylamide gel electrophoresis of GLC-I in presence of nitric oxide generating chemical (SNP). Fifty micrograms of GLC-I were incubated with different concentration of SNP. Lane a is native, lanes b, c and d contain GLC-I incubated with 0.05 mM, 5 mM and 10 mM SNP, respectively, for 2 h. (b) Polyacrylamide gel electrophoresis of GLC-I treated with NO and its scavenger curcumin and quercetin. Lane a is native whereas lanes b and c contain NO treated GLC-I in the presence of 50 μM curcumin and 250 μM quercetin, respectively.

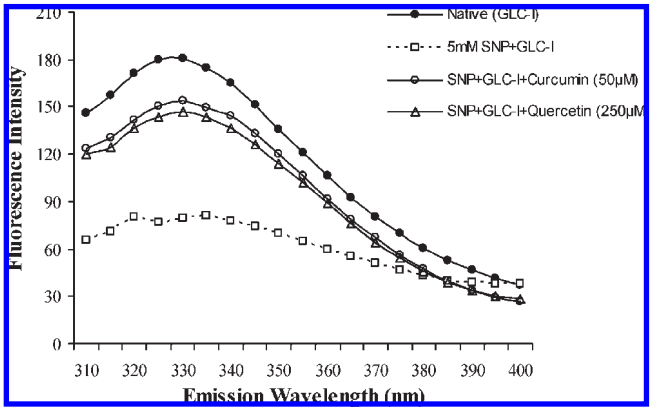

Figure 4. Scavenging role of curcumin (50 μM) and quercetin (250 μM) on SNP (5 mM) treated goat lung cystatin. The protein concentration used was 1 μM. The excitation wavelength was 280 nm and their emissions were recorded in the range of 300−400 nm.

prevented NO induced functional and structural damage to GLC-I whereas for reclamation to a similar extent, quercetin at a concentration 5 times more than curcumin was required. This observation could be explained in part by the fact that curcumin is a specific NO quencher. NO scavenging effects of curcumin have been reported earlier also by various groups (*46*−*48*). Our results obtain support from above findings.

The results of the study are of great significance. Proteins are the major targets of reactive species. As has been emphasized earlier, modification of proteins by reactive species (ROS, NO·, RNS, etc) render them more susceptible to enhanced proteolysis, inactivation and denaturation. The present study documents the suppression of NO· induced inactivation of GLC-I by curcumin and quercetin. Also, structural changes in SNP treated GLC-I are minimized by curcumin and quercetin. The widespread dietary consumption of curcumin and quercetin (and many other polyphenols) in varying proportions and amounts by the human population provides a rather inexpensive therapeutic option against oxidative injury. However, the mechanism by which curcumin and quercetin prevent protein damage in vitro, evaluation of their efficiency in this context in vivo, and elucidation of molecular basis of their action summon future research.

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