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Thiol supplementation inhibits metalloproteinase activity independent of glutathione status

Paola Bogani [a,b], Monica Canavesi [a], Tory M. Hagen [c], Francesco Visioli [d], Stefano Bellosta [a,*]

[a] Department of Pharmacological Sciences, University of Milan, via Balzaretti 9, 20133 Milan, Italy
[b] Centro Cardiologico Monzino IRCCS, Milano, Italy
[c] Linus Pauling Institute, Oregon State University, Corvallis, OR, USA
[d] UMR7079, Université Pierre et Marie Curie—Paris6, Paris, France

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Abstract

Matrix metalloproteinases (MMPs) are proteolytic enzymes that regulate both integrity and composition of the extracellular matrix (ECM). Excessive ECM breakdown by MMPs is implicated in many physiological and pathological conditions, such as atherosclerosis. Activated macrophages, especially in the atherosclerotic lesion, are a major source of reactive oxygen species (ROS). Antioxidants protect against ROS-induced MMPs activation and inhibit gelatinolytic activity. We sought to determine whether the antioxidants glutathione (GSH), *N*-acetylcysteine (NAC), or lipoic acid (LA) affect gelatinase production and secretion. The results show that thiol compounds affect MMPs expression and activity in different ways. MMP-2 activity is directly inhibited by NAC and GSH, while LA is ineffective. On the contrary, MMP-9 expression is inhibited by LA at a pretrascriptional level, and MMP-9 activity is stimulated by GSH through a direct interaction with the gelatinase itself. Although all thiols, these compounds have different properties and different cellular uptakes and metabolic characteristics, and this could explain, at least in part, their differential effects on MMPs.
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Matrix metalloproteinases (MMPs) are a multigene family of enzymes that regulate both integrity and composition of the extracellular matrix (ECM). These enzymatic systems play a pivotal role in the control of signals elicited by matrix molecules, which regulate cell proliferation, differentiation, and apoptosis [1]. Such proteases are widely distributed according to cell types and to pathological status [2]. MMPs are mainly secreted as inactive zymogens, and once activated can completely degrade all ECM components, playing an important role in ECM remodeling [1]. Excessive ECM breakdown by MMPs is implicated in many physiological and pathological conditions, particularly in inflammatory status such as atherosclerotic lesion development, cell infiltration and proliferation [2]. In particular, gelatinases, such as the 72-kDa gelatinase A (MMP-2) and the 92-kDa gelatinase B (MMP-9), are abundantly expressed in various malignant tumors, play an active role in angiogenesis, and may also influence the process of atherosclerotic lesion formation and destabilization [2]. The atherosclerotic plaque is characterized by an intense infiltration of inflammatory cells, constituted predominantly by monocyte/macrophages [3]. Macrophages are able of degrading ECM by phagocytosis or by secreting MMPs, weakening the fibrous cap of the atherosclerotic plaques, and predisposing its rupture [4,5]. Previous studies showed that MMP-9 is present in atheroma and abdominal aortic aneurysm [6] and elevated levels are associated with stroke or cardiovascular death [7].

Activated macrophages, especially in the atherosclerotic lesion, are a major source of reactive oxygen species (ROS)

* Corresponding author. Fax: +39 02 5031 8284.
E-mail address: Stefano.Bellosta@unimi.it (S. Bellosta).

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and treatment with antioxidants protects against ROS-induced MMPs activity [8,9]. Recent data suggest that antioxidant treatment inhibits gelatinolytic activity [10–12]. However, how oxidative stress regulates MMPs expression is not yet fully understood.

In this study, we sought to determine whether treatment with the endogenous antioxidant glutathione (GSH), its thiol precursor *N*-acetylcysteine (NAC), or lipoic acid (LA, which indirectly can enhance GSH synthetic capacity) could affect gelatinases production and secretion. To this end, we tested the effects of the above mentioned compounds on MMP-2 and MMP-9 expression in different cell culture systems.

Materials and methods

Chemicals and reagents. Methanol, chloroform, acetic acid (all of HPLC grade), GSH, GSSG, dansyl chloride, cresol purple, lipoic acid, and *N*-acetyl L-cysteine were purched from Sigma (Milan, Italy) and gamma-L-glutamyl-L-glutamic acid was from Acros (Rodano, Italy).

Cell culture. Bovine Aortic Endothelial Cells (BAEC; Cambrex Bio Science, Caravaggio, Italy) were plated at a density of 2.5×10^5 cells in a 35 mm dish and grown in DMEM supplemented with 15% fetal calf serum (FCS). BAEC between passages 5 and 8 were used in all experiments. Chinese Hamster Ovary (CHO) were maintained in medium containing 10% FCS, at 37 °C in a humidified atmosphere of 5% CO_2.

The effects of thiol compounds were examined in cells that were incubated for 24 h at 37 °C in serum free medium containing 0.2% BSA (Sigma) and NAC 5 mmol/L, LA 1 mmol/L, GSH 1 mmol/L, or vehicle. At the end of the treatment period, the conditioned medium was collected. The gelatinolytic activity was measured by gelatin zymography and cell protein content was estimated with the Bradford method [13].

SDS–PAGE zymography. The gelatinolytic activity was measured in aliquots of conditioned medium by BAEC (for MMP-2) or CHO (for MMP-9) by gelatin zymography, as previously described [14].

To evaluate the effect of thiol compounds on the activity of secreted MMP-2 and -9, aliquots of gelatinase-containing medium underwent electrophoresis on 7.5% polyacrylamide gels containing 10% SDS and gelatin (1 mg/ml). The gels were then washed in 2.5% Triton X-100 (Sigma) at room temperature, then cut in strips and incubated overnight at 37 °C in the activation buffer (Tris 50 mM, pH 7.5, containing NaCl 150 mM, CaCl2 10 mM, ZnCl2 1 μM) containing the tested compounds. At the end of the incubation, the gels were stained with Coomassie briliant blue R-250 (Sigma–Aldrich, Italy).

Determination of intracellular MMP-9 activity. Intracellular MMPs activity was measured by gelatin zymography in CHO cell homogenates prepared in STEN buffer (Tris 1 M pH 7.6, NaCl 5 M, and 1% nonidet P40). Total cellular protein content was determined with the Bradford method.

Transient transfection assays. CHO cells were transfected using the calcium phosphate co-precipitation technique [15]. Cells were plated at a density of 6×10^4 cells/well in DMEM containing 10% FCS, for 3 days at 37 °C. A unique co-precipitate containing each reporter plasmid/luciferase plus pCMVβ-gal was prepared and aliquoted in different wells to assure that all samples were transfected with the same amount of plasmid DNA (1.2 μg of luciferase plasmid plus 0.3 μg of β-galactosidase plasmid DNA/well). After 16 h at 37 °C, cells were washed with PBS and incubated for 24 h in medium containing the compounds to be tested or the vehicle (control).

Enzyme assay. Luciferase and β-galactosidase assays were performed using a luminometer (Lumat 9501, Berthold, Germany) and a microtiter plate reader (Bio-Rad, Hercules, CA), respectively, as previously described [16]. Luciferase activities were normalized versus galactosidase activities. Results are expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD values of triplicate samples.

Glutathione status. After derivatization with dansyl chloride, intracellular glutathione status was measured by HPLC following the method of Jones et al. [17].

Statistical analysis. Each experiment was performed at least three times with different preparations of cells.

Gelatinase activity was quantified by scanning densitometry. We used a video camera and a computer analysis package (NIH Image 1.52 image analysis software) to perform a densitometric scanning for quantization of zymograms.

Data are presented as means of % vs control ± SD. Statistical analysis was done by two-tailed Student's *t*-test. A $P < 0.05$ was considered as statistically significant.

Results

Effects of the antioxidants on MMP-2 activity

To examine the effect of thiol agents on MMP-2 activity, we incubated BAEC for 24 h with NAC 5 mmol/L, LA 1 mmol/L, or GSH 1 mmol/L. Then media were collected and analyzed by gelatin zymography. Supplementation with NAC (5 mmol/L) significantly inhibited MMP-2 activity by almost 60%; GSH (1 mmol/L) inhibited gelatinase A activity by approximately 50%, whereas LA (1 mmol/L) did not significantly affect MMP-2 gelatinolytic activity (Fig. 1).

To determine whether the inhibitory effect of NAC and GSH was due to a direct interference with the activity of secreted MMP-2, we used serum-free medium containing MMP-2. Aliquots of this media was run on gelatin containing gels. Then the gels were cut in strips and incubated for 24 h at 37 °C in the activation buffer containing the antioxidants. GSH and NAC reduced MMP-2 by 40% and 45%, respectively, while LA was ineffective (Table 1). These data imply a direct inhibitory effect of GSH and NAC on MMP-2 activity.

Effects of the antioxidants on MMP-9 activity

Then we evaluated the effect of the compounds on MMP-9 secretion. The data show that the compounds have different effects. LA addition significantly decreased MMP-9 levels by approximately 50% as compared with controls

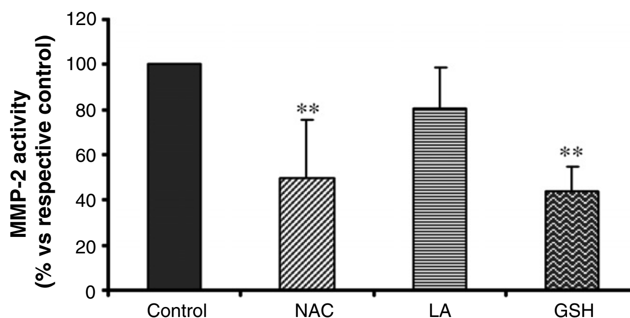

Fig. 1. Effect of NAC (5 mmol/L), LA (1 mmol/L), and GSH (1 mmol/L) on MMP-2 gelatinolytic activity in BAEC. Data were quantified by densitometric scanning and expressed as mean ± SD. $^{**}P < 0.01$ vs. controls.

($P < 0.01$, Fig. 2). This effect was most probably due to an inhibition of MMP-9 gene transcription since LA addition reduced it by 45% (Fig. 3). The inhibition of MMP-9 gene transcription resulted also in reduced intracellular MMP-9 levels up to 25% (Fig. 4).

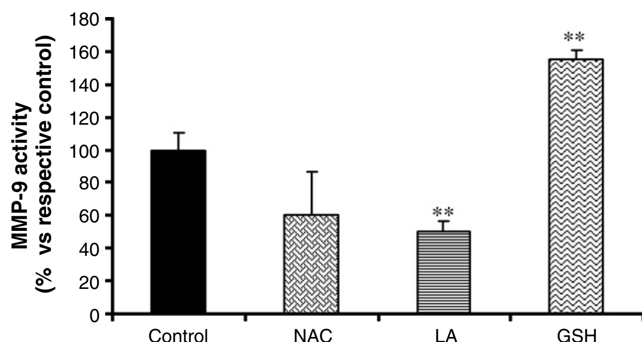

Fig. 2. Effect of thiols on MMP-9 secretion by CHO cells. The graph represents data from three independent experiments in which conditioned medium was incubated in the presence of NAC 5 mmol/L, GSH 1 mmol/L, or LA 1 mmol/L for 24 h. **P < 0.01 vs. control.

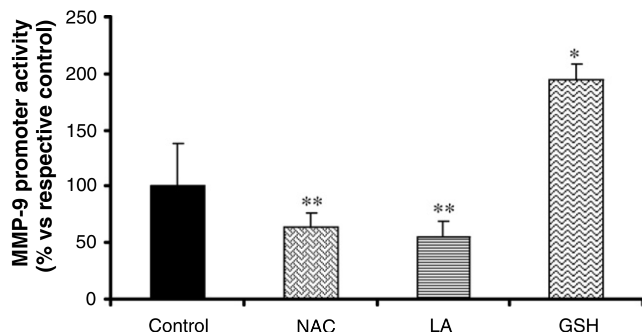

Fig. 3. Effect of thiols on MMP-9 promoter-driven transcription. CHO cells were transfected and incubated for 24 h with the indicated concentrations of compounds. A luciferase assay was then performed as described in Materials and methods. Luciferase activities were normalized to β-galattosidase activities. Values are expressed relative to controls. $^{*}P < 0.05$, $^{**}P < 0.01$.

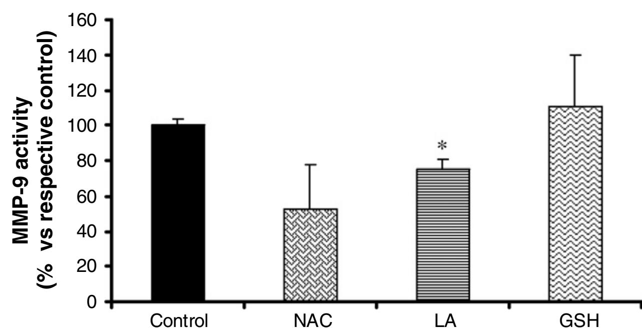

Fig. 4. Effect of thiols on intracellular MMP-9 levels. CHO cells were incubated for 24 h with thiols (NAC 5 mmol/L, GSH 1 mmol/L, or LA 1 mmol/L) and intracellular MMP-9 measured by zymography. Data are mean ± SD of 3 experiments. Student's *t*-test $^{*}P < 0.05$.

NAC reduced MMP-9 promoter activity by 25% ($P < 0.01$, Fig. 3), this lead to a reduced MMP-9 release and intracellular levels, although these results did not reach a statistically significant values (Figs. 2 and 4, respectively).

Conversely, GSH addition stimulated MMP-9 promoter driven transcription (Fig. 3). This caused an increased secretion of MMP-9 (Fig. 2), while intracellular MMP-9 levels were unaffected (Fig. 4).

We than evaluated if the compounds had a direct effect on the activity of MMP-9 already secreted. As shown in Table 1, NAC and LA did not show any effect, while GSH increased directly the activity of secreted MMP-9 by 97%.

Finally, we evaluated if the effects of the compounds on MMPs expression were possibly correlated with intracellular glutathione levels. To this end, we measured if the addition of the compounds changed the amounts of cellular total glutathione (GSH + GSSG; μg total glutathione/mg protein). While LA supplementation significantly increased glutathione content by 52%, the other thiol compounds had no effect (data not shown).

Discussion

It has been shown that ROS activate gelatinase B (MMP-9) and that antioxidants are able to limit ROS-induced MMPs activity [10]. We tested thiol-related compounds because of their efficacy in modulating cellular redox status by increasing intracellular glutathione level [18–20] (or in modulating thiol status directly) and because of their role as GSH precursors. We evaluated the effects of a thiol compound, *N*-acetylcysteine (NAC, which is also a GSH precursor), a dithiol, lipoic acid (LA), and a monothiol such as glutathione (GSH), on MMPs secretions and activity and whether an increased cellular glutathione content might be directly related to these effects. We focused on gelatinases since they are ubiquitous in normal and atherosclerotic human vessel; MMP-2 is mainly produced *in vitro* by endothelial cells and SMC; and MMP-9 is chiefly expressed by macrophages [21]. Our results show that thiol compounds may affect MMPs expression and activity in different ways. NAC reduced both MMP-2

Table 1
Effect of thiol compounds on the activity of secreted MMPs

Treatment (mmol/L)	MMP-2 activity (% of control ± SD)	MMP-9 activity (% of control ± SD)
Control	100.00 ± 11.09	100.00 ± 9.77
NAC (5)	60.25 ± 12.15	96.50 ± 16.11
LA (1)	109.47 ± 6.51	98.29 ± 4.43
GSH (1)	55.88 ± 0.71*	197.44 ± 39.66*

To evaluate if the compounds have a direct effect on the activity of MMP-2 or MMP-9 already secreted, aliquots of gelatinase-containing medium were run on gelatin gels, and the indicated compounds added into the activation buffer, as described in Materials and methods. Student's *t*-test, two tail.

* $P < 0.01$, vs. control.

and MMP-9 gelatinolytic capacity, LA affected only MMP-9, while GSH reduced MMP-2 but increased MMP-9 activity.

Gelatinases show similar biochemical characteristics; i.e., they display three repeated modules in their catalytic domain that allow their binding to and denaturation of collagen and elastin [22]. Although all thiols, the compounds we tested have different properties (e.g., NAC and GSH are monothiols whereas LA is a dithiol) and different cellular uptakes and metabolic characteristics, and this could explain, at least in part, their differential effects on MMP-2 and MMP-9. In fact, the reduced activity of MMP-2 caused by NAC and GSH that we observed was most probably consequent to a direct inhibitory action of these two thiols on MMP-2 gelatinolytic capacity, while LA did not show any effect on MMP-2 activity. On the contrary, thiol effects on MMP-9 were more diverse. Our data are consistent with previous report on the capacity of NAC to inhibit gelatinolysis [10,23,24]. In fact, we show that NAC reduces expression of the MMP-9 promoter and its subsequent production, as previously reported by Galis et al. [10]. We used a NAC concentration that approximates the dose that was demonstrated to be effective in reducing MMPs activity *in vivo* [10]. Also, this is the lowest concentration used in *in vitro* studies [10,24].

The glutathione concentration we used was 1 mM, which approximates the intracellular concentration of its reduced form (between 1 and 10 mM, depending on the cell type) [25,26]. We used a similar concentration for LA, which significantly inhibited MMP-9 gelatinolytic capacity acting at a pre-trascriptional level by reducing MMP-9 promoter-driven trascription that resulted in a reduced secretion and accumulation of intracellular levels of MMP-9.

In vivo studies reported that NAC slows the progression of cardiac injury in the hypertensive rat model; this suggests that part of the beneficial effect of NAC is due to its potential ability to restore glutathione status in cardiac tissue, hence preserving cardiac function [27]. However, in our experimental conditions NAC was unable to significantly modify the intracellular total glutathione content. Thus, we hypothesize that, in our cellular model, these thiol-containing antioxidants act by working directly as free radical scavengers.

We did not observe an increase of total GSH concentration after the addition of NAC or GSH, whereas LA increased total intracellular glutathione concentration by 52% and this was paralleled by a reduction of MMP-9 promoter-driven transcription and MMP-9 production by a similar extent. This result is in agreement with previous findings in HeLa cell cultures and in hepatoma cell cultures, where LA addition increased intra- and extra-cellular glutathione levels [28,29]. Maintenance of high intracellular GSH/GSSG ratio is critical because it influences the accumulation of disulfides and provides a reducing environment within the cells [30]. Oxidative stress alters this ratio and modifies a variety of cellular processes [25,31,32]. In turn, our data suggest that the inhibitory effect on MMPs we observed is due to GSH rather than thiols per se. In addition, no effects of GSH supplementation on total glutathione or on the GSH/GSSG ratio were observed. This suggests an extracellular effect, as GSH was not taken up into the cells but still was able to directly enhance MMP-9 gelatinolytic activity.

Previous studies have shown the capacity of LA and NAC to inhibit nuclear factor-kB (NF-kB) activation and the ability of LA to bind DNA [33]. An NF-kB binding site is present inside the promoter of MMP-9, acting as regulatory element of transcription factors [34]. Furthermore, the effects of LA are multi-faceted; some data suggest that part of the effects are also due to its dithiolic form (dihydrolipoate, DHLA), which is synthesized intracellularly and then exported [20,35]. We propose that these compounds (NAC and LA) regulate the redox environment in the cell by acting as antioxidant, supporting the hypothesis that MMP-9 gene expression is redox-regulated. Cantin et al. [36] previously demonstrated, that LA and DHLA are selective inhibitors of gelatinase B, whereas no effects on gelatinase A was shown. Also, GSH was one hundred times less potent than DHLA [36]. On the contrary, our results suggest an up-regulation of MMP-9 transcription and activity after incubation with GSH.

In conclusion, thiols may affect in different ways gelatinase expression and activity, and further studies are ongoing to better clarify this matter.

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