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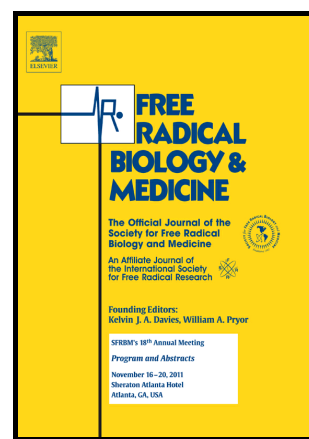
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***N*-acetylcysteine ethyl ester as GSH enhancer in human primary endothelial cells: a comparative study with other drugs**

Daniela Giustarini^{1*}, Federico Galvagni², Isabella Dalle Donne³, Aldo Milzani³, Filiberto Maria Severi⁴, Annalisa Santucci² and Ranieri Rossi¹

¹Department of Medicine, Surgery and Neurosciences, University of Siena, Via A. Moro 2, I-53100, Siena, Italy

²Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Via A. Moro 2, I-53100, Siena, Italy

³Department of Biosciences, Università degli Studi di Milano, via Celoria 26, I-20133 Milan, Italy

⁴Department of Molecular and Developmental Medicine, Via delle Scotte, University of Siena, Siena, Italy.

*Correspondence to: Daniela Giustarini, Department of Life Sciences, Laboratory of Pharmacology and Toxicology, University of Siena, via A. Moro 4, I-53100, Siena, Italy Tel.: +39-0577-234198.

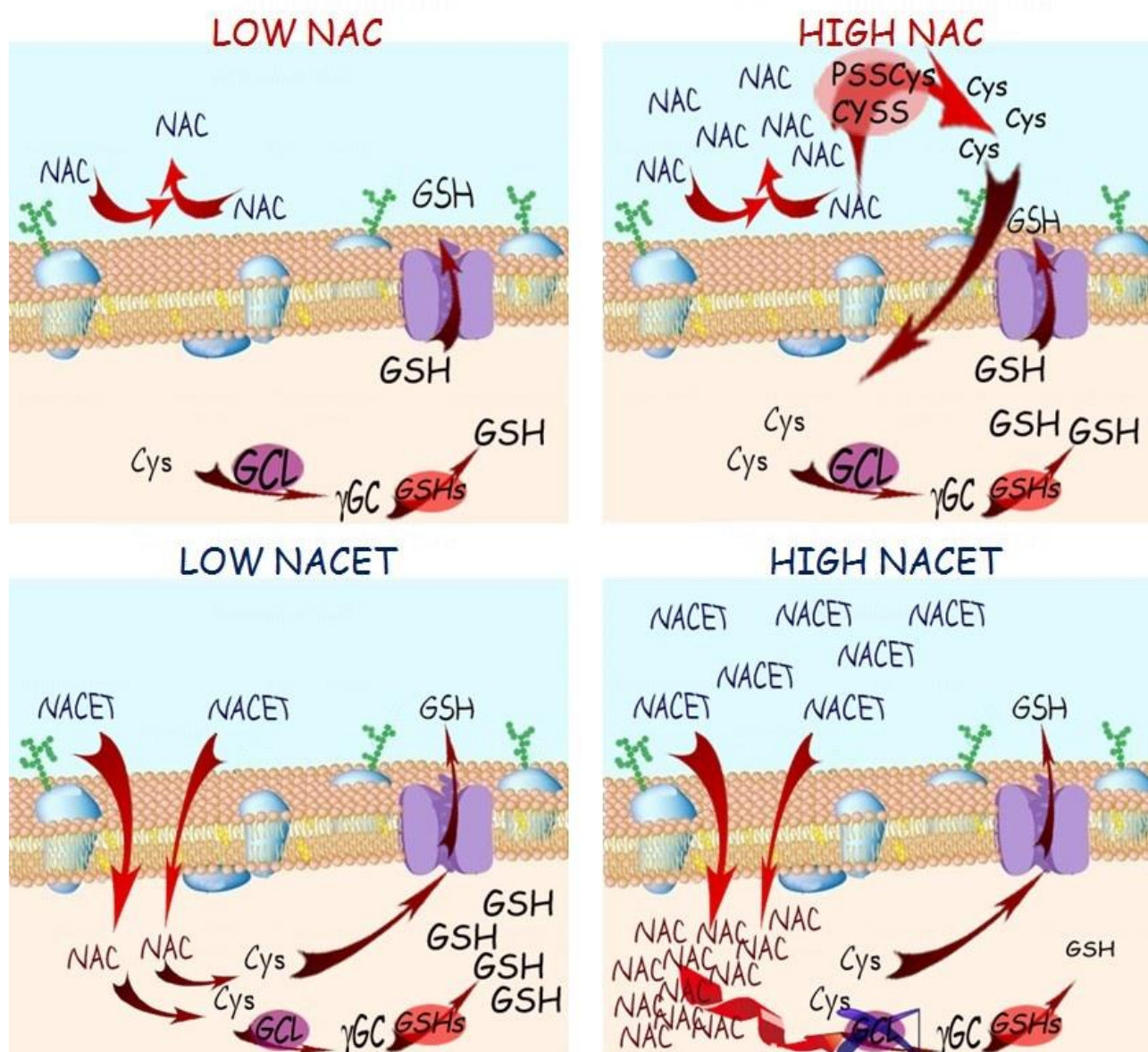
Fax: +39 0577 234476. E-mail: giustarini@unisi.it

Abstract

Several drugs are currently in use as glutathione (GSH) enhancers in clinical, pre-clinical and experimental research. Here we compare the ability of *N*-acetylcysteine (NAC), 2-oxothiazolidine-4-carboxylic acid (OTC), glutathione ethyl ester (GSH-EE) and *N*-acetylcysteine ethyl ester (NACET) to increase the intracellular concentration of GSH using primary human umbilical vein endothelial cells (HUVEC) as in vitro model. Our experiments highlighted that NACET is largely the most efficient molecule in increasing the intracellular levels of GSH, cysteine, and γ -glutamylcysteine. This is because NACET is lipophilic and can freely cross plasma membrane but, inside the cell, it is de-esterified to the more hydrophilic NAC, which, in turn, is trapped into the cell and slowly transformed into cysteine. The higher availability of cysteine is matched by an increase in GSH synthesis, cysteine availability being the rate limiting step for this reaction. Surprisingly, the increase in GSH concentration was not linear but peaked at 0.5 mM NACET and gradually decreased when cells were treated with higher concentrations of NACET. We

demonstrated that this puzzling ceiling effect was due to the fact that NAC released from NACET turned out to be a competitive inhibitor of the enzyme glutamate-cysteine ligase, with a K_i value of 3.2 mM. By using a cell culture medium lacking of cysteine and methionine, we could demonstrate that the slight increase in intracellular levels of cysteine and GSH induced by NAC in HUVEC grown in standard medium was due to the reduction of the cystine present in the medium itself there rather than to the action of NAC as Cys pro-drug. This fact may explain why NAC works well as GSH enhancer at very high concentrations in pre-clinical and in vitro studies, whereas it failed in most clinical trials.

Graphical Abstract:



Abbreviations

BSO, buthionine sulfoximine; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; GCL, glutamate-cysteine ligase; GR, glutathione disulfide reductase; GSH, glutathione; GSH-EE, GSH monoethyl ester; GSSG, glutathione disulfide; HUVEC, human umbilical vein endothelial cells; mBrB, monobromobimane; NAC, *N*-acetylcysteine; NACET, *N*-acetyl cysteine ethyl ester; NEM, *N*-ethylmaleimide; OTC, 2-oxothiazolidine-4-carboxylic acid; PBS, phosphate buffered saline solution; PSSG, *S*-glutathionylated proteins; R-SH, thiols; ROS, reactive oxygen species; TCA, trichloroacetic acid

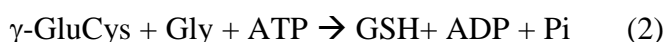
Keywords:

intracellular glutathione, cysteine derivatives, cysteine pro-drugs, glutamate-cysteine ligase

Introduction

The most common way to increase glutathione (GSH) levels in cells is to provide them with cysteine (Cys) or with a source of this molecule, since Cys is usually the limiting substrate in GSH synthesis. Because of its zwitterionic feature, Cys has difficulty to permeate cell membranes and specific transporters for cysteine and cystine (the disulfide form) are needed on the plasma membranes of mammalian cells [1]. Little is known about the structure and activity of these transporters and, in particular, about their kinetic characteristics (e.g. K_M for cysteine). However, it is known that cysteine and cystine enter cells by two different transporters. Cell supply of cysteine is ensured by its uptake from extracellular fluids, where cysteine is derived from the diet or from intracellular protein turnover. An efficient inter-organ exchange of cysteine occurs during starvation and oxidative stress [2-5]. Particularly during prolonged starvation, skeletal muscle can deliver cysteine to the plasma by degradation of proteins. Methionine can also serve as a source of cysteine through the transsulfuration pathway [6-7].

Regulation of GSH synthesis and concentration in different cells is still poorly known. GSH is synthesized from cysteine, glycine (Gly) and glutamic acid (Glu) by a two-step reaction that requires ATP as energy donor:



Glutamate-cysteine ligase (GCL) catalyzes the first step (reaction 1) and this ligation between the amino group of cysteine and the γ -carboxy group of glutamic acid is the rate limiting step in de novo GSH synthesis. The concentration of cysteine is considered rate-limiting in GSH synthesis because it is far below the K_M of GCL [8]. A fundamental in-depth study of the kinetic properties of GCL was carried out by Chen and colleagues [9], who determined that the enzyme is actually a holoenzyme being composed by both catalytic and regulatory subunits.

To enhance cellular GSH levels is a challenge, mainly because of the difficulty of providing enough cysteine for its intracellular synthesis. This is for a couple of reasons: the low concentration of Cys in the extracellular fluids [3] and the low efficiency of the transporter for cystine, whose extracellular concentration is much higher than that of Cys [1]. The use of more lipophilic cysteine derivatives can solve the problem of its uptake, provided that, once inside the cell, the cysteine precursors are transformed into Cys. Several cysteine derivatives have been proposed to this aim. Among these, *N*-acetylcysteine (NAC) is the most widely used to fulfill this requirement. However, recent large clinical trials failed to confirm the supposed beneficial effects of NAC in preventing oxidative stress-related diseases [10,11], whereas *in vitro* experiments and animal models gave controversial results. We reported that these negative or controversial results could be due to the low bioavailability of NAC [12]. As matter of fact, extremely high concentrations of NAC (millimolar range) are in most cases used in *in vitro* experiments, in particular with cell cultures, to increase GSH levels [see 13 and 14 as an example]. Instead, the pharmacological concentrations of NAC reached in human plasma are two-three orders of magnitude lower.

The relatively low oral bioavailability of NAC has stimulated the search for alternative, pharmacologically favorable Cys pro-drugs. Several Cys and NAC derivatives have been prepared and tested for this purpose [15,16]. Interesting Cys pro-drugs are represented by thiazolidines (e.g., 2-oxothiazolidine-4-carboxylic acid, OTC) and the esters of GSH [17]. OTC has shown a moderate effect in increasing GSH, but it was reported to reverse endothelial dysfunction in patients with coronary artery disease [18]. The most common GSH monoethyl ester (GSH-EE) was shown to

protect against ischemia and hypoxia in rat hippocampal slices [19]. However, in a double-blinded placebo-controlled study carried out to evaluate the safety and efficacy of OTC for the treatment of respiratory distress syndrome, it failed in reducing the symptoms [20]. We demonstrated that the esterification of the carboxyl group of NAC to produce *N*-acetyl cysteine ethyl ester (NACET) drastically increases the lipophilicity of NAC, thus greatly improving its bioavailability [12].

In the present study, we compare the ability of NAC, OTC, GSH-EE and NACET to increase the intracellular concentration of GSH in primary human umbilical vein endothelial cells (HUVEC). The peculiar effect and the biochemical characteristics of NACET are characterized. Furthermore, the influence of the intermediate metabolites of NACET on enzyme machinery for GSH synthesis is investigated.

2. Materials and Methods

2.1 Materials

Monobromobimane (mBrB) was obtained from Calbiochem (Milan, Italy). HPLC grade solvents were purchased from Mallinckrodt-Baker (Milan, Italy). NAC and all other reagents were obtained from Sigma-Aldrich (Milan, Italy) unless otherwise indicated.

The synthesis, purification and the mass spectrometry, ¹H NMR, infrared spectrometry, and polarimetry characterization of NACET (C₇H₁₃NO₃S, MW 191.2, mp 44.1–44.5°C) were previously reported [21]. Briefly, NACET was prepared under argon atmosphere by *N*-acetylation of L-cysteine ethyl ester (Merck, Darmstadt, Germany) in dichloromethane with equimolar amounts of acetic anhydride (Merck, Darmstadt, Germany). HPLC analysis with UV (215 nm) absorbance detection of the isolated product revealed a chemical purity of >99% for NACET.

Cell cultures and treatments

Human umbilical vein endothelial cells (HUVEC) (Lonza, C-2517) were grown on 1% (w/v) gelatin (Sigma Aldrich) in EBM-2 medium (Lonza) as previously described [22]. At confluence, the culture medium was removed and cells were washed twice (1 min each) with phosphate buffered saline solution (PBS), pH 7.4, at 4°C, lysed by treatment with 0.5 ml of 4% (w/v) trichloroacetic acid (TCA, Sigma Aldrich, Milan, Italy) containing 1 mM K₃EDTA and collected after scraping. Samples were either immediately analyzed or stored at -80°C until analyses. Measurements were always carried out within 10 days from sample preparation.

Stock solutions (20 mM) of the drugs (NAC, NACET, OTC, GSH-EE) were prepared the day of the experiment in H₂O and then diluted to final concentration in cell medium.

For experiments carried out with culture medium lacking of methionine and cysteine, cells were incubated with Gibco™ DMEM, High Glucose, No Methionine, No Cystine (Gibco/BRL,

Bethesda, MD, cat. #21013024) for 8 hours.

For experiments with the GSH synthesis inhibitor, buthionine sulfoximine (BSO), cells at confluence were treated with 0.2 mM BSO for 24 hours. Where indicated, cells were pre-treated with 0.2 mM NACET for 24 hours before exposure to BSO.

Analysis of intracellular and extracellular thiols

Thiols in cell lysates were measured by HPLC after labelling of the –SH group with mBrB [12]. Analyses of total thiols in culture medium were carried out by the same HPLC method after reduction of disulfides with dithiothreitol (DTT).

Protein analysis

Protein content was measured by the Bradford assay [23] after protein pellet resuspension in 0.1 N NaOH. Bovine serum albumin was used as standard.

GCL enzyme activity

Whole cell extract for enzyme analyses was obtained from HUVEC cells grown under the above described conditions and lysed in PBS by sonication for 5 min at 0°C. Cell extracts were then centrifuged at 13.000×g for 15 min at 4°C. Supernatants obtained were loaded onto PD10 desalting columns and eluted with 0.1 M TRIS-HCl buffer, pH 7.6, containing 1 mM EDTA in order to remove all low molecular weight thiols. Enzyme activity and K_M were measured following the method described by Chen et al [9] with minor modifications. Briefly, the cytosol was 1:10 diluted with standard buffer and incubated at 37°C in the presence of 0.4 mM DTT. After incubation, proteins were removed by addition of 5% (final concentration) TCA and centrifugation. Samples were then incubated with 2 mM mBrB (final concentration) and separated by HPLC as previously described [12]. The concentration of DTT was lower than that reported in the literature (3 mM) and it was selected to reduce the interference with the fluorescent probe, mBrB, we used to label thiols. We used mBrB instead of *o*-phtalaldehyde in that it offers a good selectivity, being able to form high fluorescent complexes by a selective link with thiols. Inhibition with NAC was evaluated under the same conditions in the 0-20 mM range in the presence of 0.1 mM Cys. Results were fit to the classic dose-response “S” shaped curve by the use of SigmaPlot software.

An Agilent series 1100 HPLC (Agilent Technologies, Milan, Italy) equipped with diode array and a fluorescence detector was used for all determinations.

Statistics

Data are the mean \pm SD. Differences between means were evaluated using the unpaired two-tailed Student's *t*-test for comparisons between two groups and the one-way or two-way ANOVA followed by the post hoc Bonferroni's test for comparisons of multiple groups. A value of $p < 0.05$ was considered statistically significant. Data analysis was performed by using GraphPad Prism 6 statistical software (GraphPad Software Inc., San Diego, CA).

Results

Cell treatment with GSH enhancers

The time course of intracellular levels of GSH in HUVEC after treatment with several drugs acting as GSH enhancers is reported in Fig. 1A. After 18 hours only a minimal (if any) increase in GSH level was elicited by the drugs, with the only exception being NACET. Treatment with NACET also increased the levels of both GSH precursors, Cys and γ -glutamylcysteine (γ -GluCys) (Fig. 1B and C). It is worth of attention the nearly tripled increase in γ -GluCys levels (the intermediate that is formed during the GSH synthesis). Conversely, OTC, GSH-EE and NAC did not significantly influence intracellular concentrations of any of the measured low molecular weight thiols, with the exception of Cys, whose intracellular concentration slightly increased after 18 hours of treatment with NAC. Intracellular levels of NAC are reported in Fig. 1D. As clearly shown, treatment with NACET elicited high intracellular concentrations of NAC; paradoxically, it induced a higher concentration of intracellular NAC than treatment with NAC.

We then evaluated the dose-dependence of the observed phenomenon. An 18-h HUVEC incubation with 0.5-5 mM NAC, NACET, GSH-EE or OTC led to dramatic changes of intracellular GSH levels only when NACET was used (Fig. 2A). The maximum increase in GSH concentration was observed at 0.5 mM NACET, whereas GSH levels gradually decreased at NACET concentrations higher than 0.5 mM. OTC and GSH-EE did not affect GSH levels at any of the used concentrations, while NAC induced a minimal increase in intracellular GSH level only at the highest concentration. Measuring intracellular levels of GSH intermediates and NAC in HUVEC treated with GSH enhancers (Fig. 2 B-D), we noted a linear, though minimal, increase in Cys, γ -GluCys and NAC levels in NAC-treated cells, whereas we observed a massive dose-dependent increase in Cys and NAC levels in NACET-treated cells. γ -GluCys levels peaked at 0.5 mM NACET then rapidly dropped in parallel with the increase in Cys and NAC concentration.

We then verified how long can last the effect of NACET-induced increase in cellular GSH levels. For this aim, after a 18 h-incubation with 0.5 mM NACET, cells were washed and maintained in a NACET-free medium (Fig. 3). Once externally deprived of NACET, intracellular GSH concentration returned to near basal levels. The same trend was observed also for the GSH

precursors: NAC, Cys and γ -GluCys. A significant portion of GSH was found in the extracellular medium suggesting that cells actively export it.

Regulation of GSH synthesis

To try to clarify the observed ceiling effect of NACET on the rise of GSH levels, we measured the activity of GCL in the presence of different concentrations of its substrates. In good agreement with what is reported in the literature, we found that K_M for cysteine is limiting for enzyme activity (Table 1) as it is 0.266 mM. Considering that the mean volume of a single HUVEC is about 2000 femtoliters [24] and that the number of cells in our experiments was about 2×10^6 , intracellular Cys concentration under basal conditions turned out to be about 20 μ M. Therefore, the Michaelis-Menten kinetics of GCL ($V/V_{\max} = [\text{Cys}]/(K_M + [\text{Cys}])$) was about 6.3% of V_{\max} and this implies that any increase in intracellular Cys concentration induces a parallel increase in GSH production. However, NAC turned out to be a competitive inhibitor of GCL with a calculated IC_{50} of 4.48 ± 0.23 mM in the presence of 0.1 mM Cys (Fig. 4). According to the Cheng-Prusoff relationship for competitive inhibitors (3) [25], the calculated K_i value for NAC is about 3.2 mM.

$$IC_{50} = K_i (1 + [S]/K_M) \quad (3)$$

Since NACET, once it enters the cells, is transformed into the poorly membrane-permeable NAC, which remains confined inside the cell [12], it can be assumed that, at high concentrations, NACET strongly hampers GSH synthesis. In short, we are in the presence of a complex phenomenon where GSH production is, on the one hand, boosted by increased cysteine concentration and, on the other hand, hampered by enhanced levels of both NAC (inhibition of GCL) and GSH (feedback inhibition).

Modulation of thiol availability

To further depict the influence of NAC and NACET on the complex regulation of GSH synthesis, we incubated HUVEC in medium lacking of any font of thiols, namely cysteine and methionine. Therefore, the cysteine needed to synthesize GSH could only derive from NAC/NACET or protein degradation. This thiol starving procedure pointed even more the differences between NAC and NACET. Indeed, after 8 h of incubation, GSH and cysteine levels dramatically increased upon NACET supplementation, as expected (Fig. 5A and B). NAC had a minimal effect on both GSH and cysteine intracellular concentrations, unlike the results obtained in the experiments shown in Fig 2. In particular, this difference is evident by comparing the intracellular concentrations of Cys

measured in the two experiments (Fig 5A, inset). This suggests that the Cys-increasing effect of NAC previously observed at high concentration (Fig. 2, 2 and 5 mM) was due to the reduction of cystine present in the culture medium (both as cystine and albumin-cystine mixed disulfide) rather than to the action of NAC as Cys prodrug.

The effect of an inhibitor of GSH synthesis, i.e. BSO, was also evaluated (Table 2). HUVEC pre-treated with NACET lost almost all GSH after 24 h of incubation with BSO in analogy to control (NACET untreated) cells, although GSH concentration was more than doubled without BSO (137 ± 11 vs 62.5 ± 9.8 nmol/mg prot). This suggests that cells export GSH (GSH lost from cells was found extracellularly, not shown) at the same rate regardless of its intracellular concentration, suggesting the presence of a first order kinetics for GSH transporter. Interestingly, when cells were challenged simultaneously with both BSO and NACET, the intracellular concentration of GSH was even lower, thus suggesting that, under these experimental conditions, NAC may reinforce the BSO-induced inhibition of GCL.

4. Discussion

The widespread concept on GSH is that this molecule plays a key role in cell resistance to oxidative and nitrosative damage as well in detoxification reactions of xenobiotics by its addition to their electrophilic centers. Both roles are fundamental for health maintenance. Under normal conditions, GSH is oxidized to glutathione disulfide (GSSG), which, in turn, is reduced back to GSH by glutathione reductase using NADPH as a cofactor. Generally, this system maintains very low GSSG levels and rarely the GSH/GSSG ratio is found to be >100 in mammalian cells/tissues [26,27].

The loss of intracellular GSH can be due to the export of GSSG, GS-X (i.e., GSH thioethers, which are further metabolized *via* the mercapturic acid pathway) and the GSH itself, a phenomenon whose significance is still poorly understood. Extracellularly, GSH undergoes a rapid hydrolysis and its constituent amino acids can be reused for either GSH *de novo* synthesis or other functions [28]. Differently, GSH-thioethers result in loss of Cys, which is transformed into mercapturic acid and excreted with urine, while GSH-derived glycine and glutamate are recycled. GSH is synthesized intracellularly by the action of two enzymes, i.e., GCL and glutathione synthase (GS). GSH biosynthesis is regulated by GSH feedback inhibition of GCL and Cys availability [8].

Dozens of molecules have been proposed and investigated with the aim of increasing intracellular GSH concentrations, but only a few gave promising results. It is obvious that the easiest way to increase intracellular GSH concentration is to provide cells with higher levels of its constituent amino acids, but it's not easy to do that. First of all, amino acids have specific saturable

transporters. Moreover, cellular levels of Gly and Glu are sufficient to saturate GCL; thus, it would be vain to raise their levels. The only way to increase intracellular GSH concentrations is to add cysteine or its precursors, as the K_M of GCL for this amino acid is much higher than intracellular Cys concentrations. For this purpose, synthetic compounds including NAC, GSH esters, thiazolidines, have been used largely in animal or cell culture models [15,17,29]. The efficacy of these supplements remains questionable in many topics. NAC is routinely used as an antidote in paracetamol intoxication at extremely high doses (i.e. 10-20 g), through endovascular route. For other purposes, for example as mucolytic agent, the dose ranges from 400 to 1200 mg/die per os [10]. Systematic reviews and meta-analyses suggested that prolonged treatment with NAC is inefficacious in chronic obstructive pulmonary disease and in preventing the occurrence of cancer [30,31]. NAC infusion has been widely used in acute hepatic failure but convincing evidence for benefit of NAC in this pathology is lacking [32]. Initially, NAC was suggested to be effective in preventing radio contrast-induced nephropathy [33], but subsequently highly inconsistent results were reported [11].

We and others have previously suggested that some of the inconsistent or null results related to the use of NAC could be due to its low oral bioavailability, which is between 5% and 10% [12,34]. Contrary to what has been observed for treatment with NAC in humans, a large number of important reports suggest that NAC works well both in cell cultures and animal models [10]. Our results presented here can offer some explanation to these apparently contradictory findings. It is evident that NACET (the ethyl ester derivative of NAC) is much more efficient than the other studied molecules in boosting intracellular GSH concentrations (Figs. 1 and 2). The biochemical explanation of this is in the enhanced activity of GCL as the result of increased cellular Cys levels (Figs. 1 and 2, Table 1). We calculated that, under basal conditions, intracellular cysteine concentration is about 20 μ M in HUVEC cells, a value similar to those measured in most cell cultures analyzed, red blood cells included [our laboratory, unpublished results, 35]. It is noteworthy that the reported concentration of cysteine in tissues is considerably higher [6,27], but this can be reasonably due both to the contribution of extracellular compartment and to sample handling (e.g. the activity of γ -glutamyltranspeptidase); in any case, it is much less than the K_M of GCL. Treatments with NACET raised cysteine levels up to five-fold the basal levels and, evidently, led to an increase in *de novo* synthesis of GSH, as the parallel dramatic augmentation of its immediate precursor, namely γ -GluCys, shows (Figs. 1 and 2). However, it was surprising that NACET did not show a dose-dependent effect. As a matter of fact, concentrations of NACET above 0.5 mM gradually decreased intracellular GSH levels to a concentration similar, if not lower, to that of untreated cells. The possible explanation for this result is that NAC, the membrane-impermeable

metabolite of NACET that is trapped inside cells, acts as a competitive inhibitor of GCL, probably due to the structural similarity with the enzyme substrate, i.e., cysteine. The measured K_i was about 3 mM, well in concordance with the observed effect. In fact, treatments with 1-5 mM NACET increased intracellular NAC levels until about 10 mM and inhibited GCL activity (92% inhibition as calculated from equation: $V = (V_{max} \cdot [S]) / (K_M (1 + [I]/K_i) + [S])$). The fact that NACET-derived NAC accumulates inside cells whereas cysteine does not is probably due to the saturation of the enzyme(s) that deacetylates NAC and to the lack of specific transporters for NAC. The overall situation can be described by two overlapping phenomena: i) export of cysteine and saturation of cellular de-acetylases (Fig. 6), which lead to much greater increase in NAC levels than cysteine ones. As shown by data reported in Fig. 2, once NACET enters cells, it is rapidly de-esterified, forming large quantities of NAC, but very slowly de-acetylated, forming low Cys levels, with the lower Cys/NAC ratio the higher NACET treatment (Fig. 2). This leads to NAC accumulation and, in parallel, to the inhibition of GCL activity by NAC itself. It was surprising that GSH-EE and OTC were not able to increase GSH, even at the highest concentration used. We have not a logical explanation for this result, which is in contrast to some reports of the literature [36,37]. Instead, NAC was able to enhance GSH level but only at the highest concentrations, which are out of the pharmacological range. This is in good agreement with both some previously published papers that describe an effect of NAC only when it is used at very high concentrations [see citations in refs 10 and 38] and the inefficacy of most clinical trials with NAC [10,30,31]. The explanation is rather straightforward: NAC is not effective at low concentrations because it is poorly membrane-permeable, having a low partition coefficient and lacking of specific transporters. Instead, at high concentrations, only a minimal part of NAC enters the cells, whereas the extracellular amount of NAC is able to reduce the cysteine present in cell culture media both as free cystine and as mixed disulfide with albumin. This latter effect is shown by experiments reported in Fig. 5, where HUVEC were incubated with NAC or NACET in culture medium without Cys and Met. In this case, it can be deduced that the majority of NAC effect is due to the presence of disulfide forms of cysteine that, once reduced, can cross cell membrane *via* specific transporters and increase GSH synthesis. In any case, it must be considered that NAC could work as a GSH enhancer at different concentrations depending on the cell type, mostly because the efficiency of both the transporters and de-acetylases can vary. Last but not least, we must take into consideration that when experiments with cell cultures are carried out at high non-pharmacological NAC concentrations to demonstrate the efficacy of this treatment in counteracting the detrimental actions of oxidants, the antioxidant effect can simply be due to the direct reaction of NAC with the oxidant itself in the extracellular medium, without any implication of intracellular GSH.

The observed phenomenon of GSH export (Fig. 5) cannot be neglected. All cells export GSH, red blood cells included [39] and it is well known the occurrence of an inter-organ flux of GSH. The systems that extrude GSH from cells are probably the same involved in the multidrug resistance mechanisms and have the function of exporting thioethers of GSH [40]. These systems probably act independently from intracellular GSH concentration, as demonstrated in Table 2 where, in the presence of an inhibitor of GSH synthesis (BSO), cells do not spare their stocks of GSH. The fact that GSH levels in NACET-treated cells return to the initial values within 24 hours (Fig. 3) let us hypothesize that these systems are finely tuned. GSH cannot enter cells directly and must be made inside the cell, from its three constituent amino acids: glycine, glutamate and cysteine. Therefore, once it is exported from cells, GSH must be divided into its constituent amino acids, which enter the cells through specific transporters. This was interpreted as a safer form of transport for cysteine, in that high plasmatic Cys concentrations can be detrimental (the reason for this is far from being clarified). The fact that cells export high amounts of GSH and must resynthesize it continuously notwithstanding its synthesis requires energy (ATP), let us speculate that this output is framed in the need to maintain a proper concentration of glutathione inside cells. To complete the picture, there is also some experimental evidence that the catabolism of GSH by γ -glutamyltranspeptidase can have a signaling function and cannot be considered a mere "futile cycle". In fact, it has been demonstrated that the metabolites of GSH generate ROS by reaction with free iron. The ultimate effect is the oxidative modification of several molecular targets in the cell surface, such as the tumor necrosis factor receptor-1 in melanoma cells that plays a central role in the signaling of apoptotic and anti-apoptotic stimuli [41,42].

In conclusion, here we demonstrated that, among the most used drugs to increase GSH levels, only NACET is able to influence intracellular GSH levels at pharmacological concentrations; OTC, NAC and GSH-EE were ineffective at the same concentrations. We also showed that treatment of HUVEC with NACET has an evident ceiling effect. The levels reached by intracellular GSH after treatments with NACET turned out to be a balance between the induction of its synthesis (by increased cysteine) and enzyme inhibition (by increased intracellular NAC). This must be considered in experimental preclinical studies with NACET, since it is possible that massive doses can have the paradox effect of decreasing GSH rather than increasing it. Finally, we pointed out that cellular stores of GSH are fine-tuned, probably to avoid GSH overload, which can represent an unnatural condition with unforeseeable consequences. Our data can give useful information in order to select the best way to increase intracellular levels of GSH. It should be stressed that the rise of a single antioxidant can help to clarify the causality relationship between redox

homeostasis/perturbation and physiological phenomena, an important goal that needs to be investigated, as recently discussed [43].

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Figure 1. HUVEC treatment with GSH enhancers for different times. HUVEC at confluence were treated with 0.2 mM NAC, NACET, GSH-EE, or OTC. At the indicated times, cells were washed with PBS and lysed by TCA. Low molecular mass thiols were measured in cell lysates by HPLC. Data are the mean \pm SD. Number of replicates = 3. *** $p < 0.001$ NACET vs. control; ## $p < 0.01$ NAC vs. control ; two-way ANOVA followed by Bonferroni post hoc analysis.

Figure 2. HUVEC treatment with GSH enhancers at different concentrations. HUVEC at confluence were treated with 0.5-5 mM NAC, NACET, GSH-EE or OTC for 18 h. At the end of treatment, cells were washed with PBS and lysed by TCA. Low molecular mass thiols were measured in cell lysates by HPLC. Data are the mean \pm SD. Number of replicates = 3. *** $p < 0.001$ NACET vs. NAC, GSH-EE and OTC; ## $p < 0.01$ NAC vs. NACET, GSH-EE and OTC; °°° $p < 0.001$ NAC vs. GSH-EE and OTC; one-way ANOVA followed by Bonferroni post hoc analysis.

Figure 3. Study of the long-lasting effect of NACET as GSH enhancer in HUVEC. HUVEC at confluence were treated with 0.5 mM NACET (time point 0) for 18 hours. Cells were then washed (the time is indicated by the arrow) and maintained in a NACET-free medium. At the indicated time points, low molecular mass thiols were measured both in cell lysates (black symbols) and in the culture medium (white symbols) by HPLC. Data are the mean \pm SD. Number of replicates = 3. ** $p < 0.01$ vs. 0 time cell lysates; *** $p < 0.001$ vs. 0 time cell lysates; # $p < 0.05$ vs. 24h culture medium; ### $p < 0.001$ vs. 24h culture medium; one-way ANOVA followed by Bonferroni post hoc analysis.

Figure 4. Determination of IC₅₀ of NAC for glutamylcysteine lyase. GCL was extracted from cytosolic fraction of HUVEC. The enzyme activity was measured by HPLC in the presence of 1 mM Cys and 0-20 mM NAC. The results were fitted according to the classic dose-response « S » shaped curve. A representative experiment of 4 replicates is shown.

Figure 5. The effect of NAC and NACET in HUVEC incubated in Cys- and Met-free medium. HUVEC at confluence were incubated in Cys- and Met-free medium and treated with NAC (triangles) or NACET (squares) for 8 hours. At the end of the treatments, cells were washed with PBS and lysed by TCA. Cys (panel A) and GSH (panel B) were measured in cell lysates by HPLC. In the inset the levels of Cys in NAC-treated cells either in Cys/ Met-free medium (closed triangles) or in complete medium (data of Fig. 2C, open triangles) are reported. Data are the mean \pm SD. Number of replicates = 3. *** $p < 0.001$ NACET vs. NAC; one-way ANOVA followed by

Bonferroni post hoc analysis; °p<0.05 vs. baseline; °°°p<0.001 vs. baseline ; one-way ANOVA followed by Bonferroni post hoc analysis.

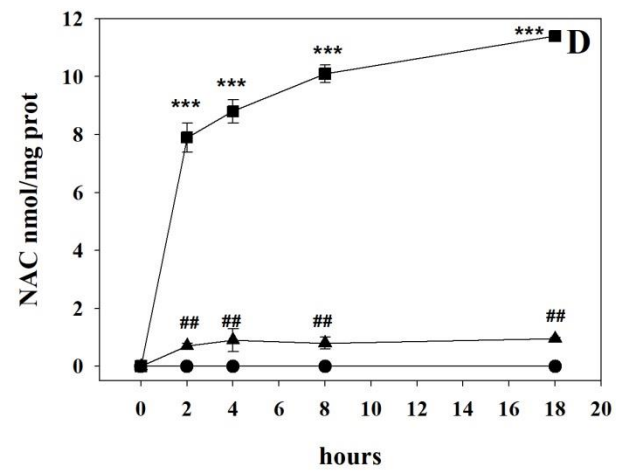
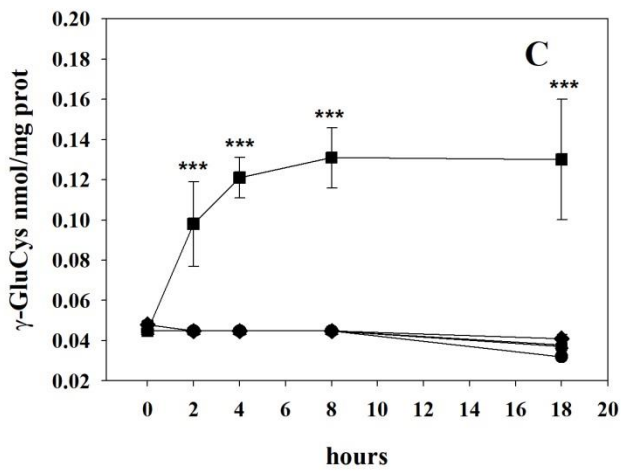
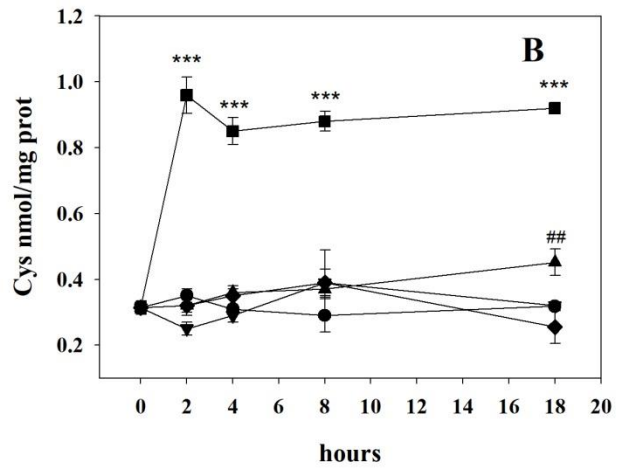
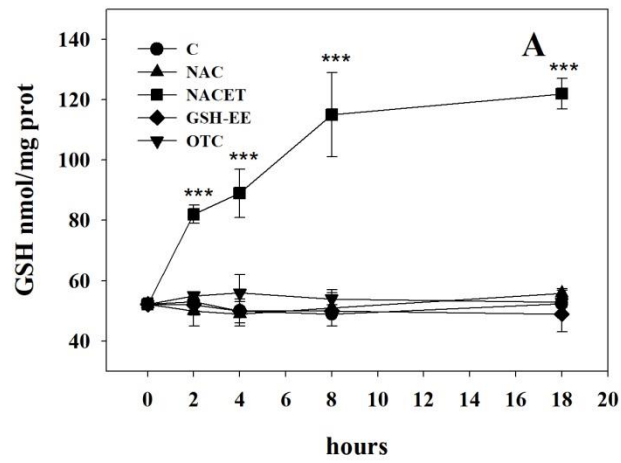
Figure 6. Concentration dependent effects of NACET on intracellular GSH. At low concentrations (0.1-0.5 mM), NACET enters HUVEC, where it is de-esterified to NAC that, in turn, is de-acetylated to cysteine. Cys has two main fates: it participates to the synthesis of GSH and, in part, is exported from cells. At concentrations higher than 0.5 mM, NACET produces a large amount of intracellular NAC, which does not cross the plasma membrane and, therefore, cannot be exported, thus saturating intracellular de-acetylases. The high levels of NAC inhibit the activity of glutamate-cysteine ligase leading to an unexpected decrease in GSH concentration.

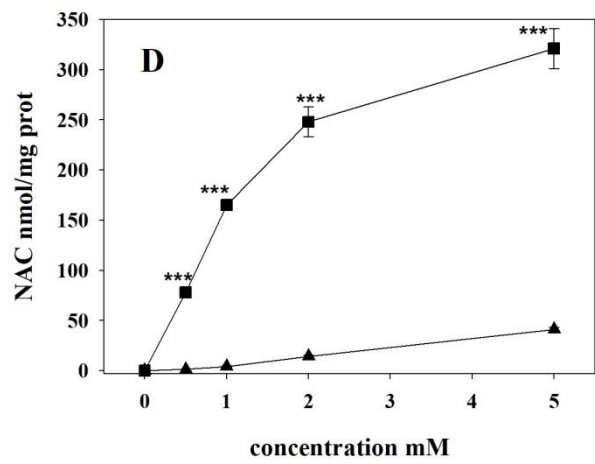
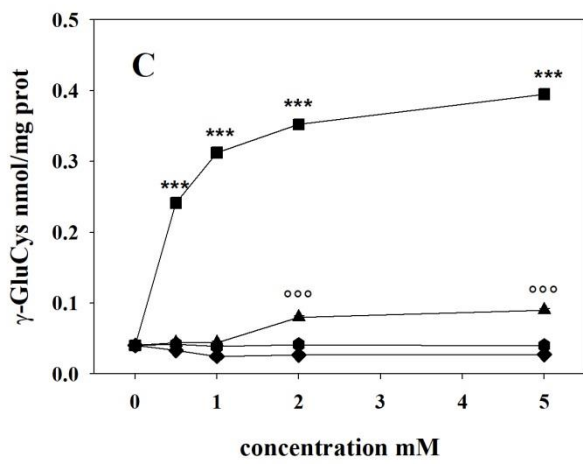
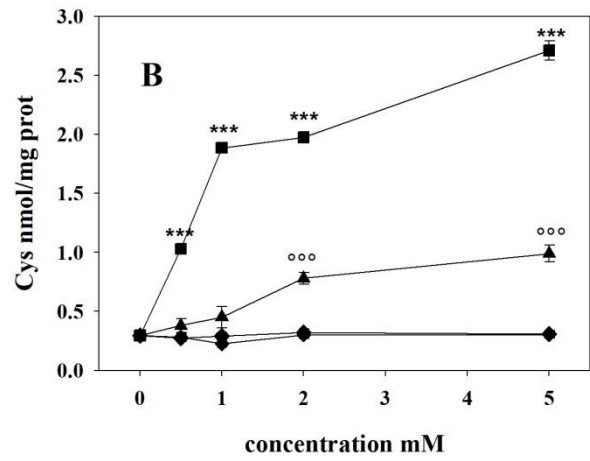
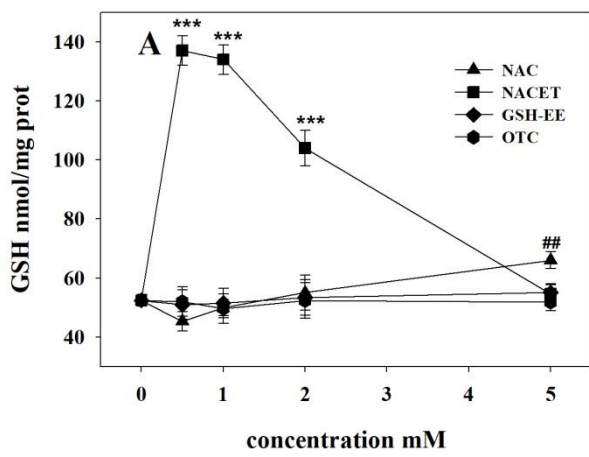
Table 1. Vmax of GCL in HUVEC extracts depending on substrate concentration. GCL activity was measured in HUVEC extracts at varying concentration of substrate (cysteine). The activity was measured monitoring the formation of γ GC by HPLC after labelling of thiols with mBrB. Data are the mean \pm SD, n = 3.

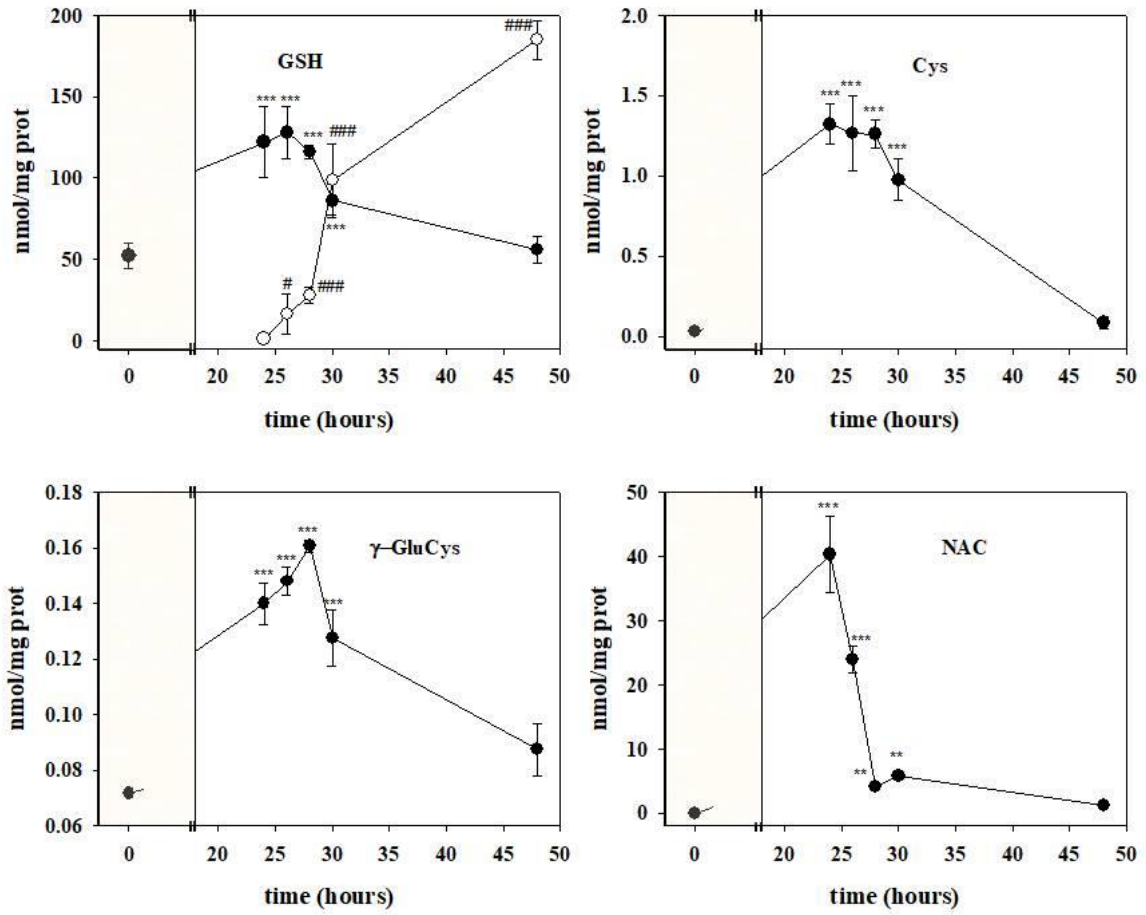
Cysteine mM	Vmax (mU/mg prot)
0.05	0.139 \pm 0.005
0.1	0.239 \pm 0.008
0.25	0.413 \pm 0.021
0.5	0.572 \pm 0.051
1	0.692 \pm 0.033
2.5	0.792 \pm 0.064
5	0.832 \pm 0.055

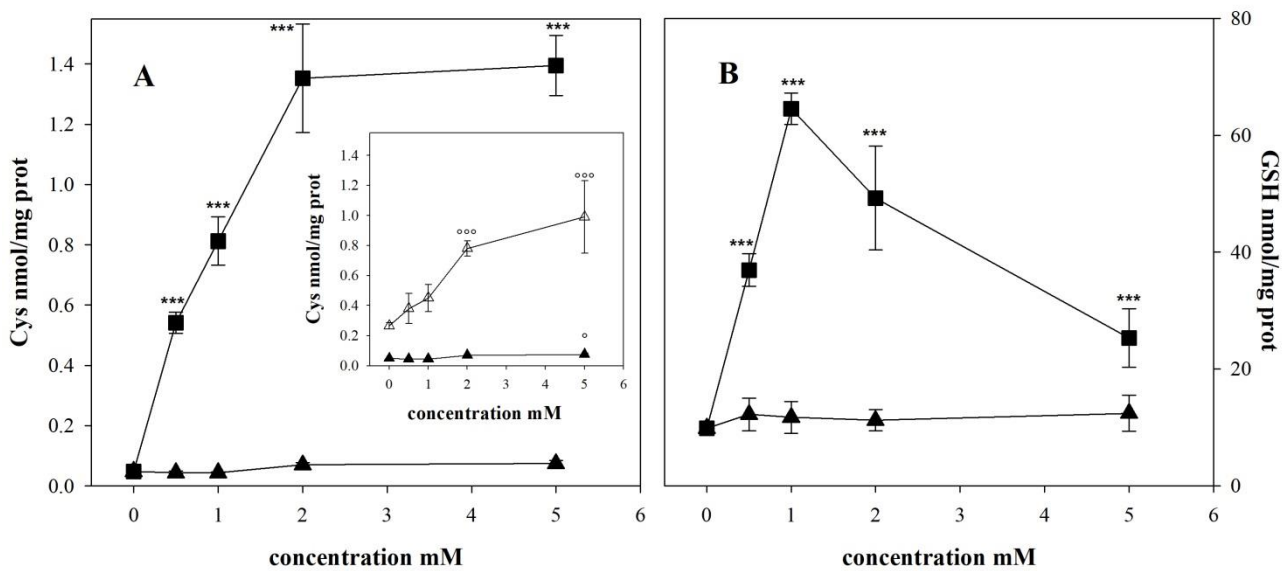
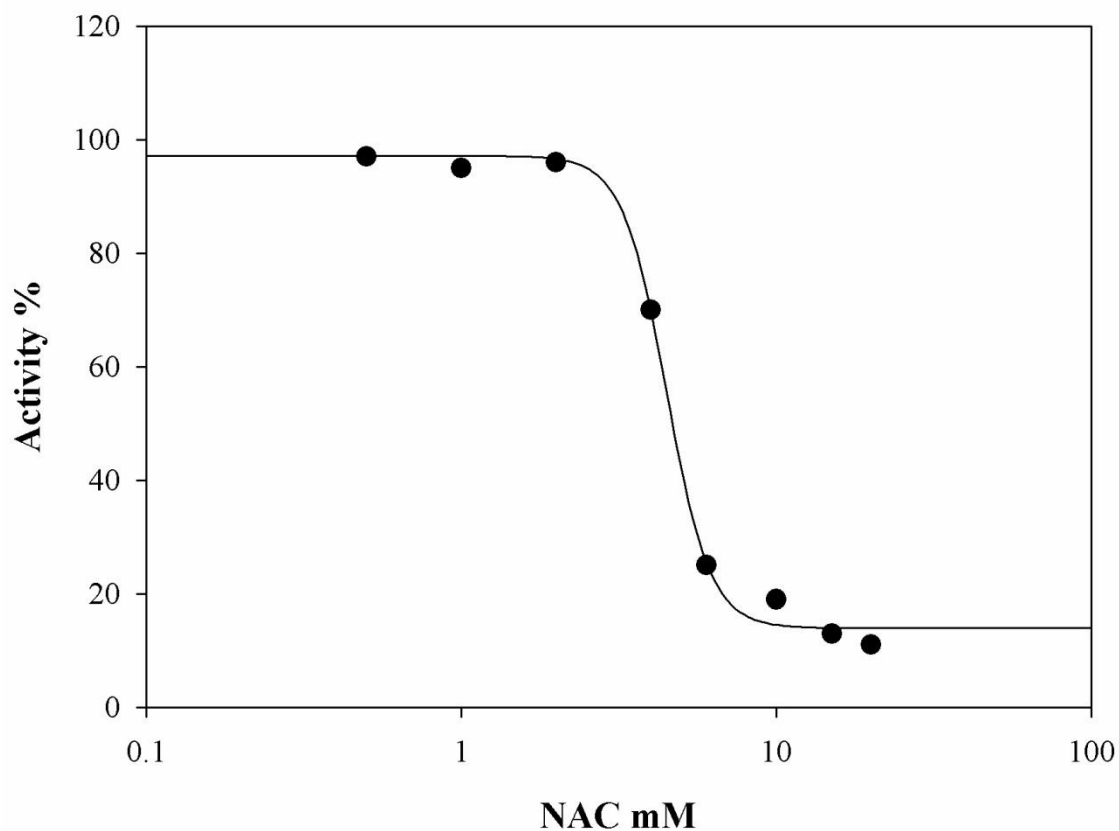
Table 2. Treatment of HUVEC with buthionine sulfoximine. HUVEC were maintained in growing medium for 24+24 hours and treated with vehicle, NACET (0.2 mM) and/or BSO (0.2 mM). Intracellular thiols were measured by HPLC. Data are the mean \pm SD, n = 3, **p<0.01 vs vehicle-vehicle; §§p<0.01 vs NACET-NACET; unpaired two-tailed Student's *t*-test.

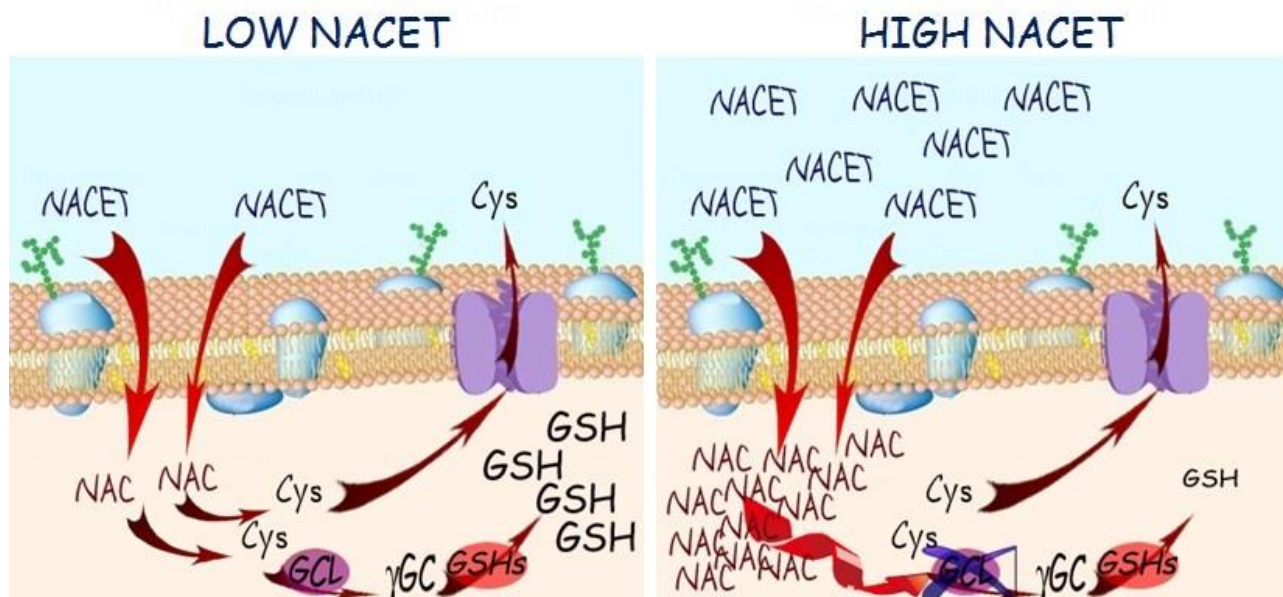
Treatments		Thiols(nmol/mg prot)			
0-24h	24h-48h	Cys	γ -GluCys	GSH	NAC
Vehicle	Vehicle	0.269 \pm 0.058	0.0651 \pm 0.005	62.5 \pm 9.8	n.d.
BSO	Vehicle	0.378 \pm 0.106	0.0210 \pm 0.007**	6.38 \pm 0.71**	n.d.
NACET	BSO	0.787 \pm 0.052**	0.0352 \pm 0.005**	8.90 \pm 2.8**	0.0607 \pm 0.011
NACET	NACET	1.020 \pm 0.040	0.160 \pm 0.012	137 \pm 11	15.9 \pm 0.87
NACET	NACET + BSO	2.22 \pm 0.074§§	0.0123 \pm 0.006§§	2.02 \pm 2.8§§	12.7 \pm 2.6











Highlights

- NACET is by far the most efficient molecule in increasing the intracellular levels of GSH
- NACET is converted to NAC and then to cysteine within cells
- The increase in GSH concentration was not linear in HUVEC but peaked at 0.5 mM NACET
- NAC released from NACET turned out to be a competitive inhibitor of the enzyme glutamate-cysteine ligase
- Treatments of HUVEC with NAC caused a slight increase in GSH mainly by reduction of cystine present in the medium