S-Nitrosoglutathione Inactivation of the Mitochondrial and Cytosolic BCAT Proteins: S-Nitrosation and S-Thiolation[†]

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ABSTRACT: Specific proteins with reactive thiol(ate) groups are susceptible to nitric oxide (NO) modification, which can result in S-nitrosation, S-thiolation, or disulfide bond formation. In the present study the effect of NO modification on the functionality of human mitochondrial and cytosolic branched-chain aminotransferases (hBCATm and hBCATc, respectively) was investigated. Here, the NO reactive agents, *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetyl-DL-penacillamine, and sodium nitroprusside, inactivated both isoforms in a dose-dependent manner. Furthermore, low concentrations of GSNO caused a time-dependent loss in BCAT activity (50 ± 3% and 77 ± 2% for hBCATc and hBCATm, respectively) correlating with the loss of four and one to two thiol groups, respectively, confirming the thiols as targets for NO modification. Analysis of GSNO-modified hBCATc by quadrupole time-of-flight mass spectrometry identified a major peak containing three NO adducts and a minor peak equivalent to two NO adducts and one glutathione (GSH) molecule, the latter confirmed by Western blot analysis. Moreover, prolonged exposure or increased levels of GSNO caused increased S-glutathionylation and partial dimerization of hBCATc, suggesting a possible shift from regulation by NO to one of adaptation during nitrosated stress. Although GSNO inactivated hBCATm, neither S-nitrosation, S-glutathionylation, nor dimerization could be detected, suggesting differential mechanisms of regulation through NO between isoforms in the mitochondria and cytosol. Reversal of GSNO-modified hBCAT using GSH alone was only partial, and complete reactivation was only possible using the glutaredoxin/GSH system (97 ± 4% and 91 ± 3% for hBCATc and hBCATm, respectively), implicating the importance of a full physiological redox system for activation/inactivation. To conclude, these results clearly demonstrate distinct functional/mechanistic responses to GSNO modification between BCAT isoforms and offer intriguing comparisons between the BCAT proteins and the respective cytosolic and mitochondrial hTrx and hGrx proteins.

Nitric oxide (NO)[1] is reported to exert diverse effects on cell function, playing important roles in physiological and pathophysiological conditions such as inflammation (*1, 2*) and cell signaling cascades (*3, 4*). Although NO homeostasis is paramount for normal cell function, prolonged elevated intracellular NO levels have been implicated in the pathogenesis of many diseases including diabetes, malignancy, atherosclerosis, and neurodegeneration (*5*−*8*). Intracellular targets for NO include transition metal containing moieties such as heme groups, tyrosine residues, and reactive thiol groups of cysteine residues (*9*). Critical solvent-accessible cysteine thiol(ate)s (Cys-SH/S$^-$) of proteins offer potential sites for redox modification and protein regulation by NO through the formation of posttranslational oxidized intermediates such as *S*-nitrosothiols (SNO) (*9, 10*). Although *S*-nitrosothiols have been detected in vivo, the mechanism of formation is unclear (*11, 12*). The biologically important NO nitrosating agent *S*-nitrosoglutathione (GSNO) is involved in the modification of protein thiols that can result in protein activation and/or inactivation through transnitrosation, disulfide bond formation, and/or S-glutathionylation (*13*−*16*). Proteins directly regulated by NO (PSNO) include enzymes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and creatine kinase (CK)), cytoskeletal proteins (tubulin, S-actin), receptors (ryanodine receptor), and transcription factors (TF cJun) (*16*−*19*). Furthermore, both mitochondrial and cytosolic isoforms of human thioredoxin (hTrx) and glutaredoxin (hGrx), which have

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[1] Abbreviations: NO, nitric oxide; SNO, *S*-nitrosothiols; GSNO, *S*-nitrosoglutathione; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CK, creatine kinase; hTrx, human thioredoxin; hGrx, human glutaredoxin; hBCAT, human branched-chain aminotransferase; hBCATm, human mitochondrial branched-chain aminotransferase; hBCATc, human cytosolic branched-chain aminotransferase; WT, wild type; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; PLP, pyridoxal 5′-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; H_2O_2, hydrogen peroxide; PVDF, polyvinylidene difluoride; TNB, 2-nitro-5-thiobenzoate; Q-TOF MS, quadrupole time of flight mass spectrometry; GSH, glutathione; GSSG, glutathione disulfide; Grx, glutaredoxin.

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redox-active CXXC motifs, are differentially regulated through NO (*20, 21*).

The human branched-chain aminotransferases (hBCATs) are key metabolic enzymes that catalyze the reversible transamination of the branched-chain amino acids (BCAAs) to their respective α-keto acids (*22*). The BCAAs, particularly leucine, readily cross the blood−brain barrier, where it is thought that the BCAAs participate in an intracellular shuttle between neurons and astroglia, regulating the levels of the major excitatory neurotransmitter glutamate (*23, 24*). In humans there are two BCAT isoforms: a ubiquitous mitochondrial isoform (hBCATm) and a largely neuronal specific cytosolic isoform (hBCATc) (*23, 24*). In the central nervous system it is postulated that these proteins operate in series to provide nitrogen for optimal rates of de novo glutamate synthesis (*25*). Only mammalian BCATs have redox-active CXXC motifs, and the cytosolic isoform has two to three additional reactive thiols (*26*−*28*). X-ray crystallography and biochemical studies have demonstrated that the thiols of the CXXC motif can exist in both reduced and oxidized forms, representing the active and inactive protein, respectively (*28*−*31*). Moreover, our recent work has shown redox associations of the brain-specific isoform with several neuronal proteins suggesting potential roles for these thiol(ate)s in G protein cell signaling and/or S-thiolation (*30*).

Intriguingly, the BCAT proteins share several common features with the redox repair enzymes hTrx and hGrx. Like BCAT, both mammalian reducing proteins have two isoforms (cytosolic and mitochondrial), with redox-active CXXC motifs, which differ in size and catalytic properties, and like hBCATc, the cytosolic isoforms hTrx1 and hGrx1 have two to three additional solvent-accessible thiols (*20, 21, 32, 33*). Moreover, as described for the BCAT isoforms (*29, 30*) the hTrx and hGrx isoforms show differential susceptibility to oxidation and S-thiolation (*20, 21, 34, 35*). Recent studies illustrated that NO modification of hTrx1 and hGrx1 results in protein inactivation through S-nitrosation with intradisulfide bond formation or complex dimer/multimer structural rearrangements, suggesting similar modes of inactivation by NO (*20, 21*). In contrast, hGrx2 (the mitochondrial isoform) was insensitive to S-nitrosation (*21*). This differential reactivity to S-nitrosation potentially reflects different cellular mechanisms of regulation mediated by NO. Therefore, investigation into NO regulation of the redox-active BCAT isoforms was warranted to detect any commonalities between the mammalian BCATs and the reducing proteins of the cell or diversity between isoforms.

Our current study reports for the first time that inhibition of the BCAT proteins can occur by S-nitrosation and is mechanistically different between isoforms, and NO modification of hBCATc is similar to that reported for hTrx1 and hGrx1. NO modification is targeted preferentially through the N-terminal cysteine and mediates its inactivation primarily through S-nitrosation for both isoforms. Transition from S-nitrosation to S-glutathionylation is discussed with respect to its potential role during oxidative stress, where using the Grx system deglutathionylation facilitates complete reactivation of the BCAT proteins. These findings strongly suggest that the BCAT proteins are differentially inactivated through NO modification, suggesting alternative mechanisms of regulation in response to levels of NO in the mitochondria and the cytosol.

EXPERIMENTAL PROCEDURES

Reagents. Glycine, HEPES, isopropyl β-D-thiogalactopyranoside (IPTG), and hydrogen peroxide (H_2O_2) (30%) were obtained from Fisher Scientific (Loughborough, U.K.). 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2,4-dithiothreitol (DTT), reduced glutathione (GSH), α-keto isocaproate (KIC), α-keto isovalerate (KIV), *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), and TEMED (99%) were obtained from Sigma-Aldrich Co. (Gillingham, U.K.). Amido black was obtained from Bio-Rad Laboratories Ltd. (Hemel Hempstead, U.K.). Hyperfilm ECL and nitrocellulose hybond ECL were obtained from GE Healthcare (Buckinghamshire, U.K.). ECL Western blotting substrate, fluorescein 5′-malemide (F5M), and GelCode blue Coomassie reagent were obtained from Pierce Biotechnology (Rockford, IL). PD10 columns were obtained from GE Healthcare (Buckinghamshire, U.K.). ^{14}C-Labeled valine was obtained from Tocris (Bristol, U.K.). Mouse monoclonal anti-glutathione (anti-GSH) was obtained from Virogen (Watertown, MA). Rabbit polyclonal mouse IgG HRP conjugate was obtained from Dako (Ely, U.K.).

Site-Directed Mutagenesis of the CXXC Motif Cysteine Residues of hBCATc and hBCATm. The cysteine residues in the CXXC motif of hBCATc and hBCATm were mutated to serine and alanine, respectively, as described in Conway et al. (*29, 30*). Using the QuikChange kit (Stratagene, La Jolla, CA), synthetic oligonucleotides and their exact complements with the chosen mutations (C335S, C338S, and C335/338S for hBCATc and C315A and C318A for hBCATm) were prepared as previously described (*29, 30*). All mutations and fidelity of PCR amplification were confirmed by DNA sequence analysis using the ABI 377 DNA sequenator in the DNA Sequencing Core Facility at the University of the West of England, Bristol. Both WT and mutant proteins were overexpressed using the pET-28a expression vector and transformed into *Escherichia coli* BL21(DE3) cells as described in Davoodi et al. (*36*).

Overexpression and Purification of WT and Mutant hBCAT Proteins. The overexpression and purification of WT and mutant hBCAT proteins were performed as described in Conway et al. (*26*). Briefly, the proteins were purified using nickel affinity chromatography followed by anion-exchange chromatography using a HiTrap Q HP strong anion column. The purified proteins were eluted using a concentration gradient between 0 to 500 mM sodium chloride in 100 mM potassium phosphate buffer (pH 8.0) at a flow rate of 1 mL min^{-1} for 20 min. The concentration of purified protein was determined using the Schaffner and Weissmann (*37*) method or calculated from the absorbance at 280 nm using the extinction coefficients of 86300 and 67000 M^{-1} cm^{-1} per monomer for hBCATc and hBCATm, respectively (*36*). The purified hBCAT proteins were then dialyzed at 4 °C overnight into storage buffer (50 mM Tris (pH 7.4) with 150 mM NaCl, 5 mM glucose, 1 mM EDTA, 1 mM KIC, and 5 mM DTT). Electrospray ionization mass spectrometry showed that the observed molecular mass of the respective mutant enzymes corresponded to the predicted molecular masses,

and the purity of each protein was determined to be >98%. Proteins were stored for 1 month at −20 °C in 30% (w/v) glycerol.

Spectrophotometric Measurement of Reduced Cysteine Sulfhydryls. The thiol content of the hBCAT proteins and their mutants was assessed using a photometric assay described in Conway et al. (*31*). The hBCAT proteins were exchanged into 50 mM HEPES (pH 7.2) and 1 mM EDTA using a PD10 column. An absorbance reading at λ 280 nm was used to calculate the hBCAT concentration using the molar extinction coefficients $\epsilon = 86300$ and 67000 M^{-1} cm^{-1} per monomer for hBCATc and hBCATm, respectively, as reported by Davoodi et al. (*36*). A final concentration of 2 μM hBCAT was incubated with a 100-fold molar excess of DTNB (total protein to reagent) for 20 min at room temperature. The absorbance change at λ 412 nm due to free 2-nitro-5-thiobenzoate (TNB) liberation was monitored over the course of the titration. The final reduced cysteine thiol count was calculated using $\epsilon = 13600$ M^{-1} cm^{-1} for TNB (*38*).

Measurement of hBCAT Activity. BCAT activity measurements for each experiment were performed using the transaminase assay (*36*). Briefly, BCAT activity was measured at 37 °C in buffer containing 25 mM potassium phosphate (pH 7.8), 5 mM DTT, 1 mM [1-^{14}C]KIV, and 0.25 mM PLP. A unit of hBCAT activity was expressed as 1 μmol of ^{14}C-valine formed per minute at 37 °C. All assays were performed in triplicate.

Screening Nitrosating Agents for BCAT Inhibition. To assess the ability of nitrosating agents to modify the functionality of the BCAT proteins, WT and mutant proteins were exchanged into 50 mM HEPES (pH 7.2) and 1 mM EDTA using PD10 chromatography. The protein (4 μM) was incubated at 37 °C for 30 min with constant shaking using the concentration ranges 10, 30, 100, 300, and 1000 μM SNP, SNAP, and GSNO, respectively. Specific hBCAT activity and reduced cysteine sulfhydryls were subsequently assessed as described above.

Time-Dependent S-Nitrosation of hBCAT. Wild-type hBCAT and their mutant proteins were exchanged into 50 mM HEPES (pH 7.2) and 1 mM EDTA by desalting, where each respective BCAT (4 μM) was incubated at 37 °C for 30 min with constant shaking with 300 μM GSNO (for hBCATc) or 150 μM GSNO (for hBCATm). Aliquots were removed at specific time points (ranging from 0 to 60 min) for the assessment of residual specific activity relative to control and the number of reduced cysteine thiol groups remaining. At each time point additional aliquots were taken, and each respective hBCAT (3 μM) was incubated for a further 30 min at 37 °C with 10 mM DTT or 10 mM GSH, respectively, and subsequently assessed for the reactivation of BCAT. Furthermore, aliquots of these samples were separated on a nonreducing 12% SDS−PAGE system to assess the effect of these reductants on protein disulfide bond formation.

Quadrupole Time of Flight Mass Spectrometry of GSNO-Modified hBCAT Proteins. Quadrupole time of flight (Q-TOF) MS was used to determine the type of modification induced by GSNO on the BCAT proteins. The hBCAT proteins were exchanged into a buffer containing 10 mM ammonium bicarbonate (pH 7.5). A final concentration of 4 nmol of each respective hBCAT was incubated in the presence of 300 μM GNSO for 30 min at 37 °C prior to Q-TOF MS analysis. In brief, samples (after addition of acetonitrile to a final concentration of 50% and 1% formic acid) were separated by Q-TOF MS (Waters), where each single analysis required 20−30 scans (1 s scans for 1−2 min), and the data were processed using MassLynx version 4.0 and the Maximum Entropy software supplied with the program to generate spectra on the absolute molecular weight scale. Sample analysis was carried out using the Q-TOF MS from the Bristol Genomics Research Institute, University of the West of England.

Detection of GSNO-Induced S-Glutathionylated Adducts by Western Blotting. In brief, 4 μM hBCATc proteins treated with GSNO over a concentration range (0−1 mM) were denatured in a solution containing 60 mM Tris (pH 6.8), 2% SDS, 10% glycerol, and 0.025% bromophenol blue for 5 min at 95 °C. Modified proteins (1 μg) were resolved using a 12% SDS−PAGE system under nonreducing conditions. The hBCAT proteins were transferred to hybond nitrocellulose ECL and blocked overnight (15−17 h) at 4 °C in TBST (2 mM Tris, 200 mM NaCl, and 0.1% Tween-20), adjusted to contain 5% BSA. The membranes were incubated with a 1:2000 dilution of mouse monoclonal anti-GSH (Virogen) in TBST plus 5% BSA for 1 h at room temperature. To observe S-glutathionylated WT hBCATc, membranes were incubated with rabbit anti-mouse IgG HRP for 1 h at room temperature prior to addition of ECL substrate and exposure to ECL hyperfilm. The membranes were stained with amido black to confirm equal transfer.

S-Glutathionylation induced by changes to the redox environment of the BCAT proteins was assessed using Western blot analysis and measuring the effect on BCAT functionality. Here, 4 μM protein was incubated in buffer containing 50 mM HEPES (pH 7.2) and 1 mM EDTA with varying ratios of 2GSH:GSSG including 10 mM:0 mM, 7.5 mM:1.25 mM, 5.0 mM:2.5 mM, 2.5 mM:3.75 mM, and 0 mM:5.0 mM, respectively, for 30 min at 37 °C. Aliquots were removed and assayed for percent residual BCAT activity and also analyzed for S-glutathionylation by Western blot analysis as described above.

Fluoresein 5′-Malemide Assessment of hBCAT Modified with GSNO. Fluoresein 5′-malemide (F5M) was used to investigate the role of the reactive thiols in NO-modified BCAT and to determine if disulfide bond formation contributed to the inhibition observed with GSNO. Wild-type hBCAT proteins (4 μM) were treated with GSNO (concentration range: 10−300 μM) and incubated with a 20-fold molar excess of F5M (total protein to reagent) in 50 mM HEPES (pH 7.2) and 1 mM EDTA for 2 h at room temperature in the dark. Proteins labeled with F5M were resolved using 12% SDS−PAGE in the dark. The gel was fixed for 30 min at room temperature in 7% (v/v) glacial acetic acid/40% (v/v) methanol and scanned at λ 526 nm using a Typhoon 9400 variable mode imager (GE Healthcare). Image Quant version 5.2 (Molecular Dynamics) was used to determine the pixel density of hBCAT protein bands.

Reduction of S-Glutathionylated hBCAT by the Glutaredoxin (Grx) System. Aliquots of GSNO treated WT hBCAT (2−8 nmol) were incubated in 100 mM potassium phosphate (pH 7.0) with 1 mM EDTA, 1 mM GSH, 100 μg/mL BSA, and 100 μM NADPH containing 0.14 unit/mL glutathione reductase and 50 nM Grx. The reaction was monitored at λ 340 nm for 5 min, and Grx activity was calculated using the

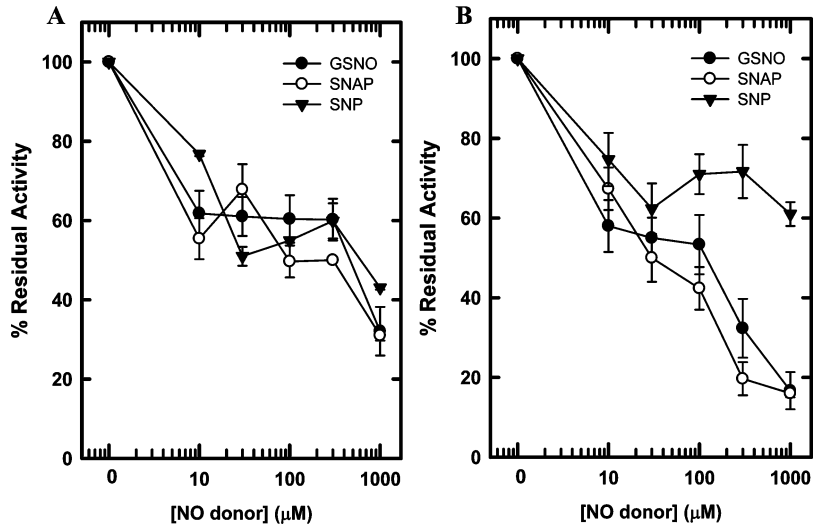


FIGURE 1: Concentration-dependent inactivation of the BCAT proteins using a panel of nitrosating agents. Three nitric oxide agents were investigated (GSNO (●), SNAP (○), and SNP (▼)) for their potential to inhibit hBCATc (panel A) and hBCATm (panel B) activity. Four nanomoles of each isoform, respectively, was incubated with increasing concentrations (10−1000 μM) of each NO agent in 50 mM HEPES, pH 7.2, and 1 mM EDTA at 37 °C for 30 min. BCAT activity was measured as described in Experimental Procedures. The data are represented as a mean ± SEM ($n = 4$).

molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADPH. Samples were monitored before and after GSNO treatment for hBCAT activity.

RESULTS

Screening of Several Nitrosating Agents and Their Interaction with the BCAT Proteins. The sensitivity of hBCAT isoenzymes to Cys-SH/S^- modification by NO was examined using a panel of three NO reagents (GSNO, SNAP, and SNP) at various concentrations (10−1000 μM) for 30 min. Here, a dose-dependent loss of hBCAT activity was mediated through all nitrosating agents for both isoforms, although the degree of inhibition was lower with SNP for the mitochondrial isoform (Figure 1A (cytosolic) and 1B (mitochondrial)). Loss in hBCAT activity for both proteins (approximately 42 ± 10%) occurred over the initial low concentration of 10 μM using GSNO and SNAP, with 23 ± 7% inhibition recorded for SNP (Figure 1A). At higher concentrations up to 67 ± 9% loss in hBCATc activity occurred for all three donors. In contrast, GSNO or SNAP resulted in a rapid loss of 88 ± 2% hBCATm activity, but only partial sensitivity to SNP (loss of 38 ± 6%) was observed (Figure 1B). Thus, the extent of inhibition for hBCATm is greater than observed for hBCATc by SNAP or GSNO, and SNP is not as potent an inhibitor for hBCATm relative to the cytosolic protein.

Effect of GSNO-Mediated Inactivation of the BCAT Proteins. To evaluate the relationship between the physiological NO agent, GSNO, and its inactivation of the BCAT proteins, hBCATc and hBCATm were incubated with 300 and 150 μM GSNO over 60 min, concentrations that are equivalent or lower than used in similar studies (*17, 20, 21*). A lower dose of GSNO was used for hBCATm since rapid inactivation occurred with 300 μM donor (data not shown). Inactivation of hBCATc resulted in a time-dependent loss of 50 ± 2% activity compared with an overall loss of 77 ± 3% for hBCATm (panels A and B of Figure 2, respectively). The decrease in activity correlated with a loss of 3.9 ± 0.4 and 1.3 ± 0.2 thiol groups for hBCATc and hBCATm, respectively (panels A and B of Figure 2, respectively). Thus, it is evident that the reactive thiols are targets for GSNO modification, and this modification correlates with inhibition of BCAT activity.

The Role of the CXXC Motif in GSNO-Mediated hBCAT Inhibition. Using the CXXC mutant proteins for hBCATc (C335S, C338S, C335/338S) and hBCATm (C315A, C318A), the effect of GSNO on the functional role of the thiols of the CXXC motif was investigated. Mutation alone of the N-terminal thiols C335S (hBCATc) and C315A (hBCATm) resulted in a 32 ± 5% and 18 ± 2% loss, respectively, in BCAT activity (Figure 3A (cytosolic) and 3B (mitochondrial)). Inhibition of these mutants with GSNO resulted in a total overall loss in BCAT activity for C335S of 52 ± 3%, comparable to inactivation of WT hBCATc with the oxidation of ~4 Cys-SH/S^- (Table 1). However, the overall loss in activity for the C315A mutant (hBCATm) reached only 47 ± 1%, significantly less compared to WT hBCATm (77 ± 3%), which correlated with a loss of 0.4 ± 0.3 Cys-SH/S^- at 60 min (Table 1).

In contrast, the C-terminal mutants showed distinctive modes of inhibition by GSNO. Like WT hBCATc no further loss in C338S activity was observed beyond 50% (Figure 3A), which correlated to a final oxidation of ~4 Cys-SH/S^- (Table 1). In contrast, inhibition of C318A relative to WT hBCATm occurred at a slower rate of inactivation, resulting in a final overall inhibition of 58 ± 1% and oxidation of 0.9 ± 0.2 Cys-SH/S^- (Table 1), thus not reaching full inactivation (77% ± 2%) seen with WT protein. Finally, no further inhibition of the hBCATc double CXXC mutant protein occurred following exposure to NO (Figure 3A). These results show that the N-terminal cysteine residues are the primary targets for modification and that overall modification relating to inactivation is mechanistically different for each isoform.

Modification of Cys-SH/S^- Mediated through GSNO. Inhibition induced by GSNO can potentially occur through several modifications including S-nitrosation, disulfide bond formation, or S-thiolation. Quadrupole TOF MS, Western blot analysis, and the thiol-specific fluorescent probe fluo-

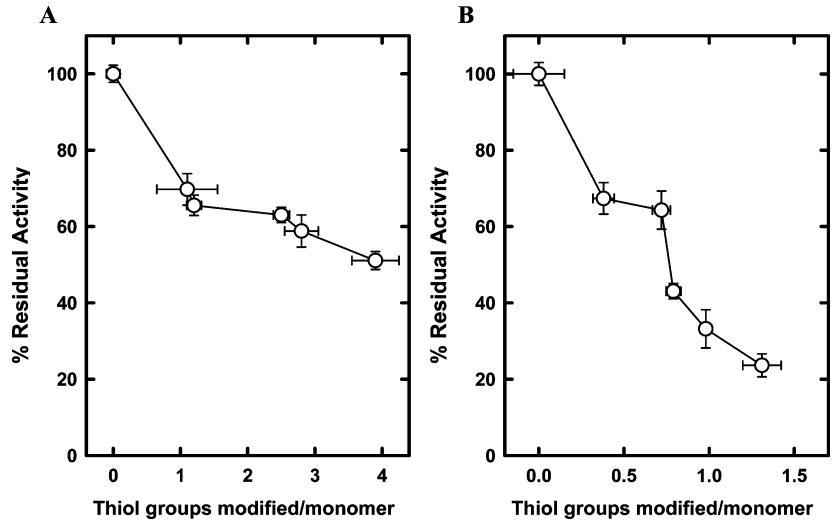


FIGURE 2: Effect of GSNO on both the activity and thiol content of the BCAT proteins. Both hBCATc and hBCATm (4 nmol) were incubated with 300 and 150 μM GSNO, respectively, for 60 min at 37 °C. At each time point 2 nmol of labeled BCAT was removed for titration with DTNB, and the percent residual activity was measured as described in Experimental Procedures. Panels A (hBCATc) and B (hBCATm): the number of thiol groups per monomer modified after GSNO modification of hBCATc and hBCATm, respectively, correlated with the percent loss in residual activity. The data are represented as a mean ± SEM ($n = 4$).

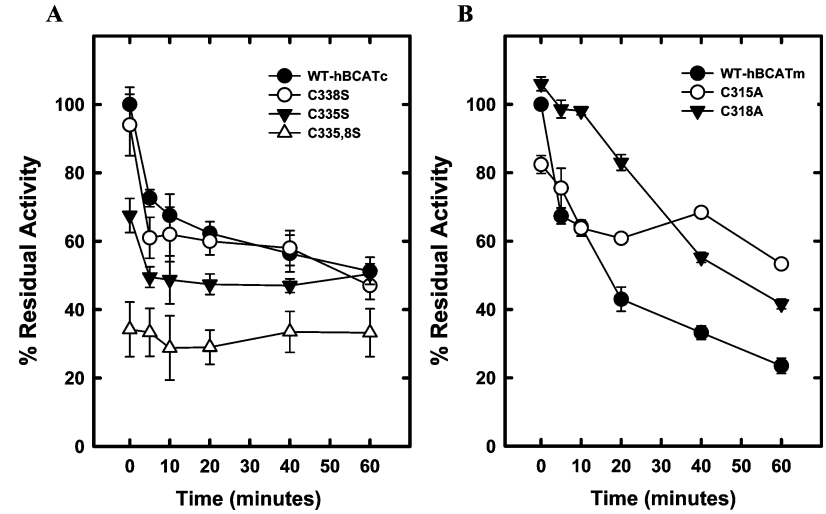


FIGURE 3: Time-dependent GSNO inhibition of WT hBCAT and the CXXC mutant proteins. WT hBCATc and WT hBCATm and their CXXC mutant proteins (hBCATc, C335S, C338S, and C335/338S; hBCATm, C315S and C318S) were incubated with 300 μM (cytosolic) and 150 μM (mitochondrial) GSNO for 60 min at 37 °C. BCAT activity was measured at various time intervals up to 60 min, where the percent residual activity was calculated. Panel A: WT hBCATc (●), C338S (○), C335S (▼), and C335/338S (△). Panel B: WT hBCATm (●), C315S (○), and C338S (▼). Data are represented as a mean ± SEM ($n = 3$).

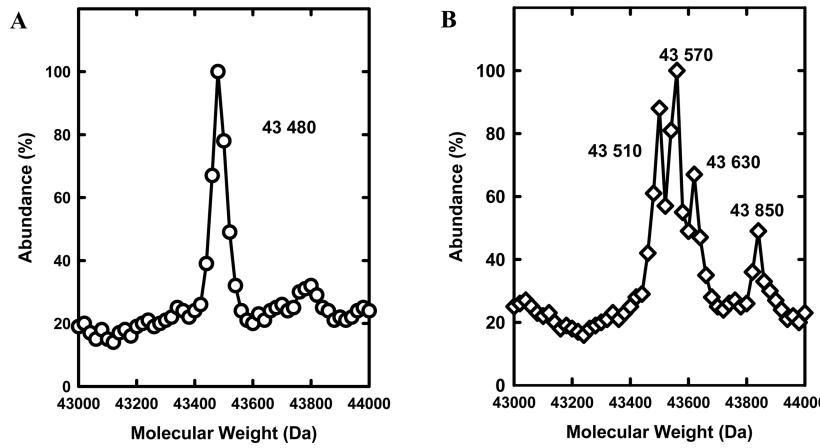
Table 1: Modification of the Reactive Cys-SH/S$^-$ of WT BCAT and the CXXC Mutant Proteins with GSNO[a]

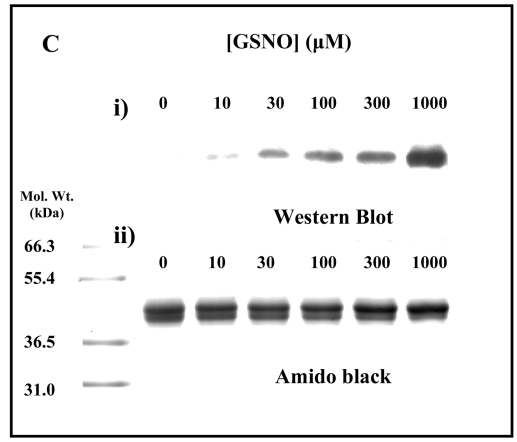
enzyme	0 min	60 min
hBCATc	5.7 ± 0.2	1.8 ± 0.4
C335S	5.1 ± 0.7	0.6 ± 0.1
C338S	4.8 ± 0.2	0.7 ± 0.2
C335/338S	3.6 ± 0.3	0.8 ± 0.1
hBCATm	2.2 ± 0.7	0.8 ± 0.7
C315A	1.1 ± 0.2	0.7 ± 0.3
C318A	1.2 ± 0.5	0.4 ± 0.2

[a] The moles of thiol groups per mole of protein are reported as the mean ± SEM from four determinations. This was estimated by titrating 2 nmol of protein with a 100-fold excess of DTNB and measuring the increase in the absorbance at 412 nm over the course of 10 min. The moles of thiol group per mole of protein were determined using the molar extinction coefficient 13600 M^{-1} cm^{-1} for TNB (*36*).

rescein 5-maleimide (F5M) were used to ascertain the type of modification responsible for the inactivation of the BCAT proteins (Figure 4). Compared to WT hBCATc a spectrum of four peaks corresponding to masses of 43510, 43570, 43630, and 43850 Da was reported for hBCATc incubated with 300 μM GSNO for 30 min (Figure 4A,B). In comparison to WT hBCATc this related to 1 NO, 3 NO, 5 NO, and 2 NO plus 1 GSH adduct, respectively. Labeling of these thiol(ate)s was confirmed through DTNB titration studies, which showed a loss of four to five thiol groups after 30 min (data not shown). Although treatment of hBCATm resulted in almost complete inactivation of the protein, Q-TOF MS analysis did not detect the NO adduct.

Western blot analysis using an anti-GSH monoclonal antibody was used to validate S-glutathionylation over the different concentrations of GSNO utilized. Here, it was demonstrated that as the concentration of GSNO increased, the extent of S-glutathionylation increased (Figure 4C (i)). Using amido black staining of the membrane, migration of these modified proteins on a 12% SDS−PAGE system demonstrated equal loading and evidence of disulfide bond





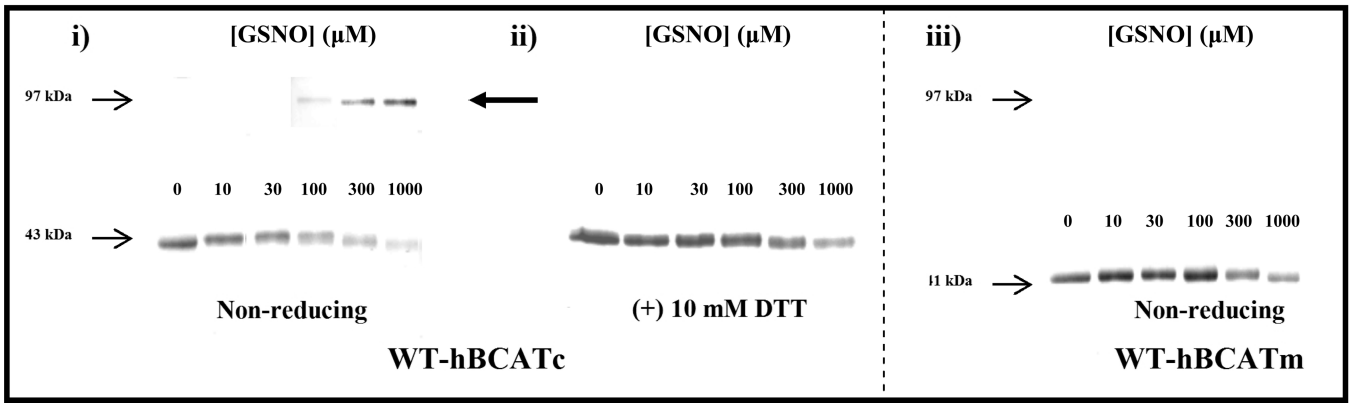


FIGURE 4: GSNO-mediated S-nitrosation and S-glutathionylation of hBCATc. Twenty nanomoles of hBCATc was exchanged into 10 mM ammonium hydrogen carbonate (pH 7.5) and incubated with 300 μM GSNO for 30 min at 37 °C prior to Q-TOF MS analysis as described in Experimental Procedures. Panel A: The principal peak has a mass of 43480 corresponding to control hBCATc. Panel B: A total of four peaks were identified with GSNO-modified protein, with masses of 43510, 43570, 43630, and 43850, corresponding to hBCATc with 1 NO, 3 NO, 5 NO, and 2 NO plus 1 GSH adducts, respectively. In addition, 4 nmol of hBCATc in 50 mM HEPES (pH 7.2) and 1 mM EDTA was incubated for 30 min at 37 °C with increasing concentrations of GSNO. Aliquots were removed for Western blot analysis using the anti-GSH antibody (Virogen). Panel C, lanes 1−6: hBCATc was incubated with 0, 10, 30, 100, 300, and 1000 μM GSNO, respectively. (i) Western blot analysis of these NO modified proteins using anti-GSH. (ii) The membrane was subsequently stained with amido black. The extent of thiol modification was monitored using F5M as described in Experimental Procedures. Panel D, lanes 1−6: (i) hBCATc incubated with 0−1 mM GSNO; (ii) GSNO (0−1 mM) modified hBCATc + DTT; (iii) hBCATm incubated with 0−1 mM GSNO.

formation (Figure 4C (ii)). Lane 5 of the Western blot represents 300 μM GSNO, corresponding to an aliquot of the same sample used for the Q-TOF MS, relating to the peak of 43840 Da, which correlates to WT hBCATc plus 2 NO and 1 GSH molecule. However, S-glutathionylation of hBCATm was not detected under these experimental conditions. These results suggest that at lower GSNO concentrations WT hBCATc is modified largely through S-nitrosation, whereas as the dose of GSNO is raised, the extent of S-glutathionylation increased.

In addition to S-nitrosation and S-thiolation both intra- and inter-disulfide bond formation was observed as a product for hBCATc inhibition with GSNO but not for hBCATm (Figure 4C (ii) and 4D (i−iii)). Under nonreducing conditions

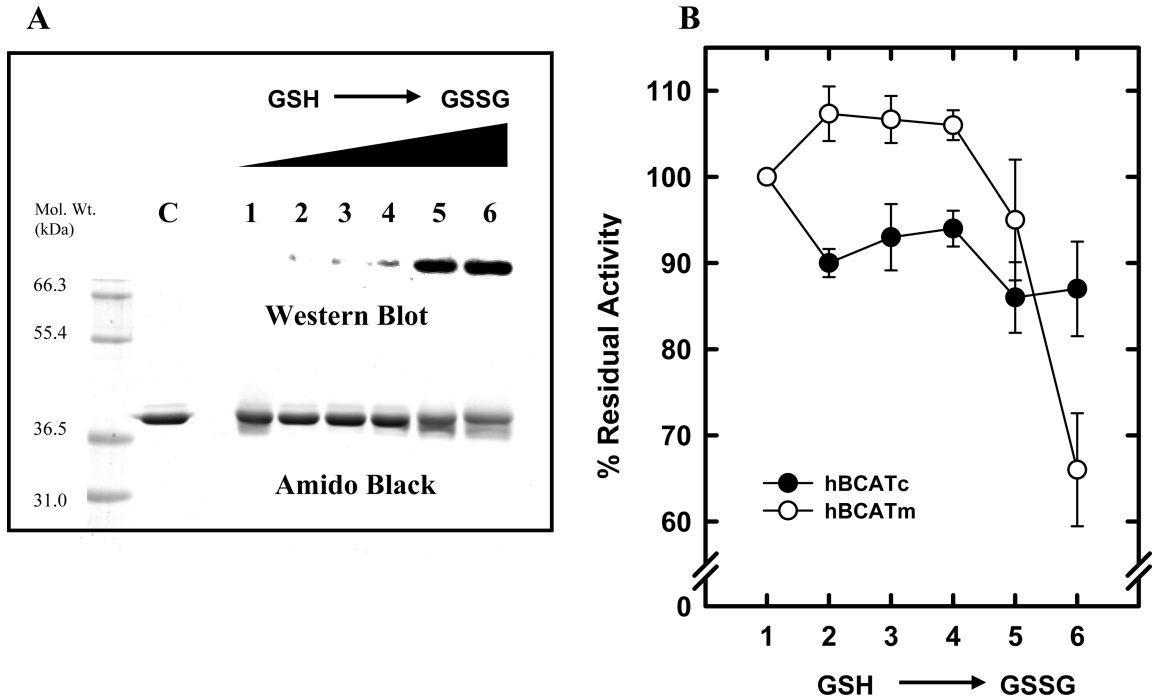


FIGURE 5: S-Glutathionylation of hBCATm. A final concentration of 4 nmol of WT hBCATc (●) or WT hBCATm (○) was incubated with increasing ratios of GSSG:GSH. Aliquots of modified hBCATm were removed for Western blot analysis using an anti-GSH monoclonal antibody (Virogen) to detect the S-glutathionylated adduct. Panel A (i, Western blot analysis) and (ii, amido black), lanes 1−6 (GSH:GSSG): buffer alone, 10 mM:0 mM, 7.5 mM:1.25 mM, 5.0 mM:2.5 mM, 2.5 mM:3.75 mM, and 0 mM:5.0 mM, respectively, Also, the percent residual activity remaining on treatment with these ratios was monitored as described in Experimental Procedures. Panel B: S-Glutathionylation of hBCATc (●) compared with hBCATm (○) correlated with the percent residual BCAT activity. Data are represented as a mean ± SEM ($n = 3$).

partial oxidation was observed for hBCATc prior to treatment with GSNO (Figure 4C (ii, lane 1)). However, this band was less evident as the concentration of GSNO increased (Figure 4C (ii, lanes 2−7)). Using the fluorescent thiol-specific probe, F5M, a structural shift resulting in dimer formation at concentrations as low as 100 μM was observed (Figure 4D (i, lane 4)). The disulfide bonds were reduced when the samples were treated with DTT, where migration of the protein is observed as a tight focused band (Figure 4D (ii)) compared with the oxidized protein which is more diffuse (Figure 4C (ii)). Thus, at concentrations >100 μM GSNO there is a reversible structural induced dimerization of the cytosolic isoform. Although fluorescence is significantly reduced for hBCATm labeled with GSNO, there is no evidence of either intersubunit disulfide bond formation or dimerization (Figure 4D (iii)). Hence, in addition to evidence for functional differences between both isoforms there are also structural alterations observed for hBCATc but not for hBCATm in response to GSNO inhibition.

Assessment of S-Glutathionylation of hBCATm. Previously we demonstrated that incubation of WT hBCATc with varying ratios of GSH:GSSG resulted in S-glutathionylation (*30*). Here, S-glutathionylation of hBCATm was observed, which increased as the ratio of GSSG:GSH increased (Figure 5A Western blot). This correlated with a decrease in activity (40 ± 5%) (Figure 5B) and the formation of mixed disulfide bonds evident from the migration on 12% SDS−PAGE (Figure 5A, amido black). Thus, both proteins are differentially S-glutathionylated where hBCATm is only S-glutathionylated under predominantly an oxidizing environment.

Reversibility of GSNO-Mediated BCAT Inhibition. To investigate whether GSNO-mediated BCAT inactivation could be restored, proteins were subsequently treated with DTT or GSH. Glutathione and DTT restored hBCATc activity to 80−96 ± 5% of control after 20 min GSNO exposure, with only 76−78 ± 4% recovery after 60 min (Figure 6A). Similar to hBCATc, DTT recovered hBCATm activity to 92 ± 3% and 68 ± 5% after 20 and 60 min, respectively (Figure 6B). However, GSH only restored hBCATm activity to 56 ± 1% at 60 min (Figure 6B). Migration of the modified proteins with GSH and DTT confirms that the protein was reduced, where the modified protein moves from the diffuse characteristic double band of an intrasubunit disulfide bond to the tighter single reduced monomeric form (Figure 6C (ii) and (iii), respectively). Therefore, both reducing agents can reverse GSNO-mediated inactivation but differ in their overall reducing capacity between isoforms.

The Glutaredoxin/Glutathione Reductase System and GSNO-Modified BCAT. As BCAT activity was not completely recovered with GSH at 60 min (Figure 6), we investigated whether the physiological glutaredoxin/glutathione reductase (Grx/Gr) system could fully recover BCAT activity. Full BCAT activity is not recovered when GSNO-mediated inhibition of hBCATc or hBCATm is incubated with GSH alone (Figure 7). Whereas after incubation with Grx/Gr, BCAT activity was recovered to 97 ± 4% and 91 ± 3% (Figure 7) for hBCATc and hBCATm, respectively. Thus, Grx is required to fully reverse GSNO-mediated inhibition of the BCAT isoforms.

DISCUSSION

Proteins with reactive thiol(ate)s are primary targets for NO modification (*39*). Products of NO modification are both transitory and reversible and have been implicated in NO

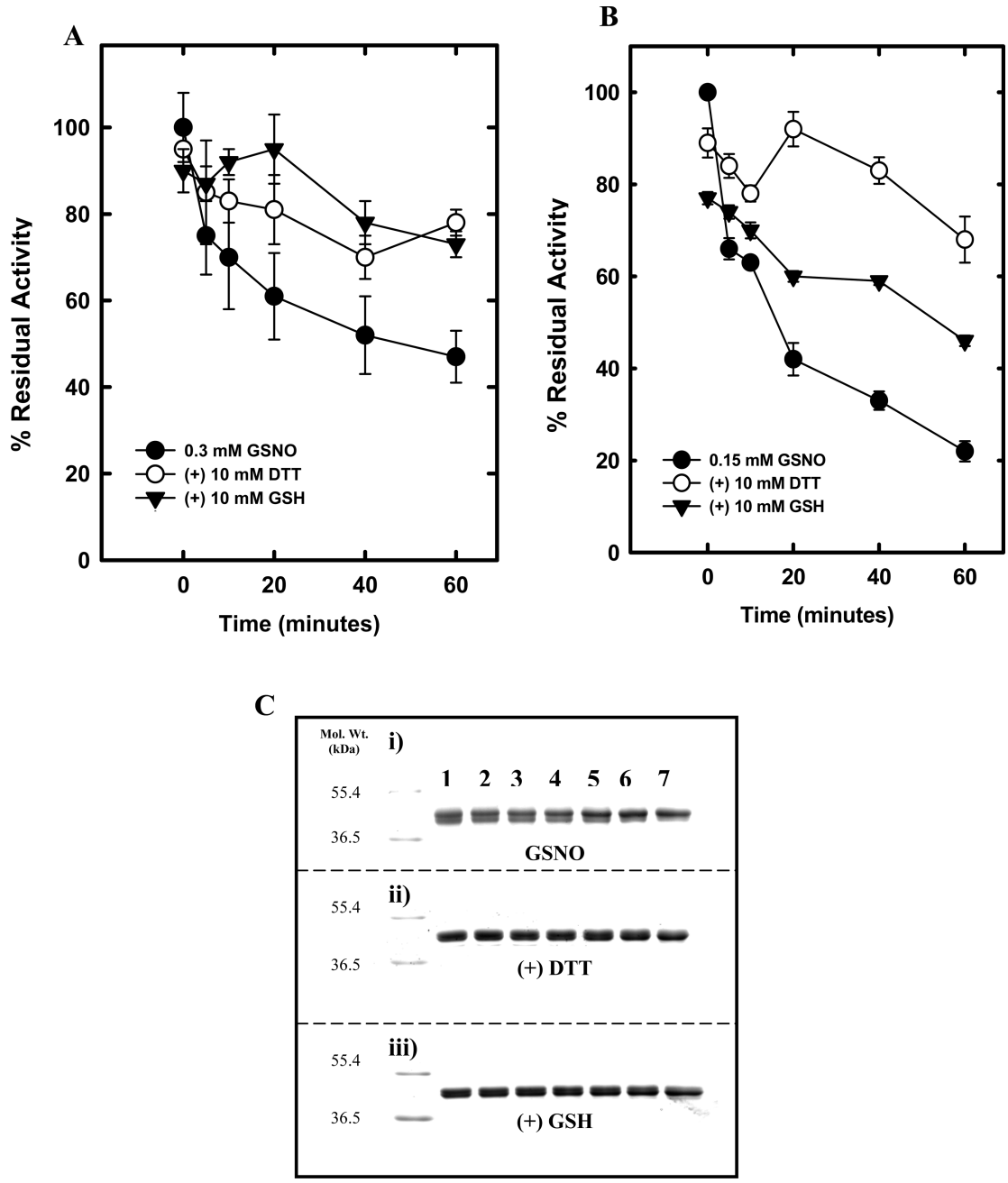


FIGURE 6: Denitrosation/reduction of the BCAT proteins using DTT and GSH. Both hBCATc and hBCATm (4 nmol) were incubated with 300 and 150 μM GSNO, respectively, in 50 mM HEPES (pH 7.2) and 1 mM EDTA for 60 min at 37 °C. Aliquots were taken at various time points for BCAT activity measurements (as described in Experimental Procedures) and incubated with 10 mM DTT or 10 mM GSH, respectively, at 37 °C for 30 min. Panel A: WT hBCATc + 300 μM GSNO control (●), + 10 mM DTT (○), and + 10 mM GSH (▼). Panel B: WT hBCATm + 150 μM GSNO (●), + 10 mM DTT (○), and + 10 mM GSH (▼). Data are represented as a mean ± SEM ($n = 3$). In addition, aliquots at each time point were taken and subjected to SDS−PAGE on 12% resolving gel to compare migrations. Panel C, lanes 1−7: 0, 1, 5, 10, 20, 40, and 60 min, respectively, corresponding to (i) GSNO, (ii) GSNO + DTT, and (iii) GSNO + GSH.

signaling, thiol exchange, and protein regulation, including apoptosis (*40*). However, periods of high levels of NO and/or prolonged NO exposure can potentially lead to altered protein function, ultimately resulting in cellular damage (*41*). Several mechanisms of NO modification have been proposed including S-nitrosation and/or S-glutathionylation, where the outcome largely depends upon the reactivity, charge distribution, and solvent accessibility of the thiol(ate) residue (*15, 16*). Here, the mechanisms of NO inhibition of the BCAT isoforms were determined using specific nitrosating agents, Q-TOF MS, and S-glutathionylation studies. Our findings demonstrate distinct mechanisms of GSNO modification between isoforms, where hBCATc is both S-nitrosated and S-glutathionylated, dependent on the level or duration of NO exposure, whereas hBCATm, although inactivated, neither NO nor S-glutathionylated products were detected. Moreover, full reactivation for both isoforms requires a complete physiological GSH/Grx reducing system, implying that these proteins are possible key targets for reversible NO regulation.

Not all nitrosating reagents result in similar products or equivalent levels of inhibition (*14, 17, 42*); for example, GSNO mediates S-glutathionylation of CK, whereas SNAP favors S-nitrosation (*16*). Here, with the exception to SNP inhibition of hBCATm, a similar response to NO modification for each isoform occurred, suggesting that inactivation is largely donor independent. However, these donors were

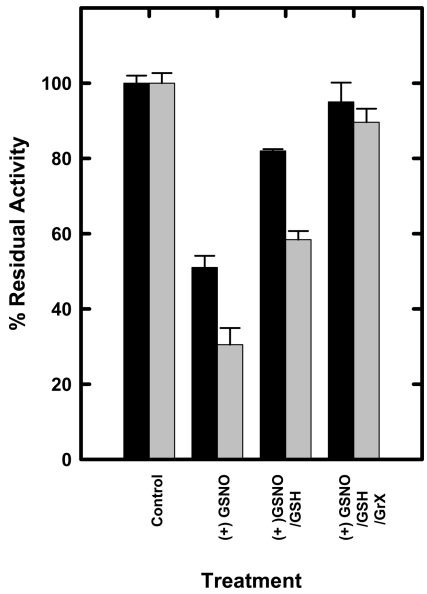


FIGURE 7: The impact of the glutaredoxin repair system on the recovery of BCAT activity from GSNO inhibition. At 60 min BCAT activity was measured ± GSNO, with 10 mM GSH and in the presence of 50 nM Grx/NADPH for 5 min at room temperature. hBCATc (black) and hBCATm (gray): column 1, control; column 2, + GSNO; column 3, + GSNO + GSH; column 4, + GSNO + GSH + Grx. Data are presented as a mean ± SEM ($n = 3$), and full methods are outlined in Experimental Procedures.

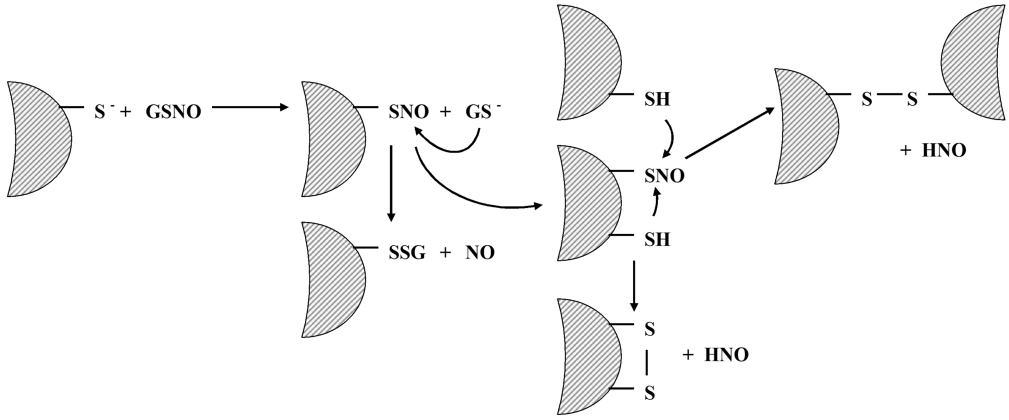
more potent inhibitors of hBCATm than hBCATc, implying different mechanisms of NO inhibition may occur between isoforms (Figure 1). The source of NO from both SNAP and GSNO is through a nitrosated Cys, and NO donation to the BCAT proteins potentially occurs through a rapid transnitrosation reaction with a Cys-SH/S^- without the overall release of NO (*4, 43, 44*). SNP modification differs as it depends on photodegradation for NO liberation, which may occur in either $NO^\bullet$ or NO^+ forms (*44*). Although the chemical structure of SNAP can impose steric hindrance thus affecting SNO reactivity with protein thiols (*17*), here evidently the reactivity and solvent accessibility of the thiol(ate)s permit inhibition by transnitrosation irrespective of the donor, where inhibition by this mechanism is favored over the NO free radical. In support of these results recent studies reported that mitochondrial membrane proteins were insensitive to NO or peroxynitrite but reversibly nitrosated with SNAP or GSNO (*45*).

Our findings demonstrate for the first time that the BCATs are susceptible to NO modification. Inactivation of the BCAT proteins by GSNO reflects a complex reaction, whereby a mixture of species contributes to BCAT inhibition dictated by nitrosating agent concentration and/or duration of exposure (Figures 1 and 2). For hBCATc, inactivation directly correlated to a loss of thiols primarily through S-nitrosation mediated via C335, reflecting the rapid reaction "expected" between reactive thiols and GSNO, i.e., transnitrosation (Scheme 1), where partial intrasubunit disulfide bond formation was also observed. This mechanism is supported by mutation studies where GSNO inhibition of the cytosolic mutant C338S was equivalent to WT protein, suggesting that inactivation is largely dependent on the thiol at position C335. Moreover, when C335 is mutated, further inactivation mediated by NO at C338 is secondary. Similarly, S-nitrosation and disulfide bond formation were also shown to inactivate cytosolic hGrx1 and hTrx1, where both S-nitrosation of C69 and C73 with intrasubunit disulfide bond formation of the active site CXXC motif contributed to hTrx1 inactivation (*20, 46*), indicating that hBCATc shares common mechanisms of GSNO inhibition with the cytosolic reducing proteins. Previously, we showed that under conditions of oxidative stress several key neuronal proteins that are controlled by G-protein cell signaling had redox associations with hBCATc (*30*). These results, taken together, imply that the hBCATc is not only a target for NO modification but more importantly may have a potential role in redox cell signaling mediated through either NO or peroxide.

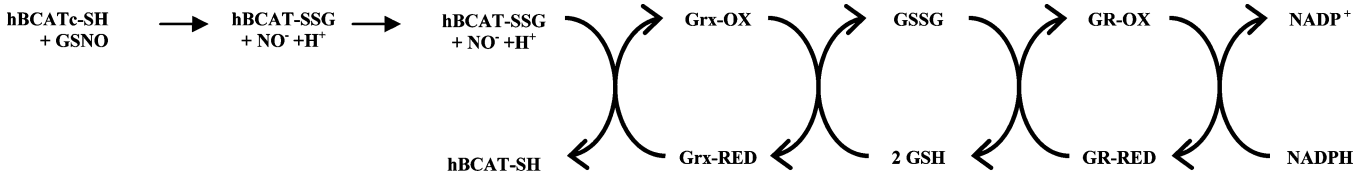
While proteins such as alcohol dehydrogenase, BSA, and S-Actin are only S-nitrosated, GSNO-mediated S-glutathionylation has been reported for a number of enzymes and suggested to occur through several mechanisms (*47*). Our study showed that, in addition to S-nitrosation, both S-glutathionylation and dimerization were evident and became more prominent with increased NO availability or prolonged exposure (Figure 4). In vivo reports of S-glutathionylation occurring in parallel with S-nitrosation, such as that reported for GAPDH in cells exposed to NO, indicate the potential physiological importance of these modifications (*14, 47*−*49*). Here, the hBCATc mixed disulfide can be attributed to the reaction of hBCATc-SNO with GSH released from hBCATc + GSNO leading to hBCATc-SSG (Scheme 1). This reaction is supported from our S-glutathionylation studies, where S-glutathionylation of hBCATc was evident over increasing ratios of GSSG:GSH, where the reactive thiols in addition to those in the CXXC motif provide a buffering effect to peroxide-mediated S-glutathionylation (*30*). Thus, in support of these studies GSNO-mediated S-glutathionylation of hBCATc is clearly a feature of oxidative/nitrosative stress and not the primary mechanism of inhibition, which was shown to be S-nitrosation. The role of proteins as redox buffers has also been suggested for tubulin, where S-glutathionylation of tubulin has been associated with the repair of damaged cytoskeletal proteins (*17*).

Interestingly, dimerization of hBCATc occurred concurrently with GSNO-mediated S-glutathionylation. Peroxide-induced dimerization of the mutant protein C338S was shown to occur; however, this structural rearrangement was not observed with the double mutant C335/8S, which suggests that C335S is integrally linked with intersubunit disulfide bond formation induced under oxidative/nitrosative stress (unpublished data). Further studies are required to investigate this possibility. Dimerization as a feature of oxidative stress has also been reported for hTrx1 where oxidation with diamide resulted in the formation of a second disulfide bond between C62 and C69 for hTrx1 (*33*). Furthermore, hydrogen peroxide induced both dimer and oligomer formation in hGrx1 (*21*). Equally intriguing, like mitochondrial hGrx2 (*21*), we observed that hBCATm does not form GSNO-induced intra-disulfide bonds, dimers, or oligomers. Although NO-mediated dimerization and the formation of multimers have been detailed for other proteins such as tubulin, which has functional implications resulting in the inhibition of tubulin polymerization (*17*), the physiological relevance of dimerization of hBCATc and indeed the reducing proteins hTrx1 and hGrx1 is unknown. However, we can speculate that this reversible structural imposed dimerization for these

Scheme 1



Scheme 2



proteins may have a similar role to S-glutathionylation in protecting the reactive cysteines from irreversible oxidation.

Conversely, although it is evident from GSNO inactivation of C318A that C315 of hBCATm is the primary target for NO modification, the overall reactivity of C315 in the mutant C318A is slower than in WT protein. This implies that C318 is functionally important, possibly affecting the charge distribution and influencing the reactivity of C315, facilitating faster rates of inhibition and overall inactivation seen with WT protein. Although hBCATm is clearly modified by GSNO largely leading to complete inactivation, in contrast to hBCATc, neither S-nitrosation nor S-glutathionylation by GSNO was detected by Q-TOF MS or using the anti-GSH antibody, respectively. This can in part be explained by the insensitive response of hBCATm to S-glutathionylation compared with hBCATc (*30*), where S-glutathionylation of the mitochondrial isoform was not observed until the redox environment switched predominantly to a more oxidizing environment (Figure 5A,B), implying different thiol reactivities between isoforms. Thus, although hBCATm may be S-glutathionylated, the extent may be undetectable under these conditions. In agreement with our findings, GSNO-mediated S-glutathionylation is not reported for the mitochondrial isoform hGrx2 (*28*). Thus, the distinct differential response of BCAT and the reducing protein isoforms to GSNO and peroxide indicate how compartmentalized redox environments and thiol reactivities have evolved to reflect different mechanisms of cell signaling or responses to cellular stress.

The reverse reactions of denitrosation with DTT further validated that NO modification was thiol specific and not linked with modification of tyrosine residues (Figure 6). Here, GSH alone denitrosated hBCATc more efficiently than hBCATm, but complete reactivation was only achieved by the physiological GSH/Grx reducing system (Figure 7 and Scheme 2). With respect to GSNO-modified hBCATc this may be explained by the increased level of GSNO-mediated S-glutathionylation, shown to be concentration and time dependent, contributing to inactivation resulting from S-nitrosylation (Figures 5 and 6). Thus, under these conditions, GSH can reverse S-nitrosation whereas the glutaredoxin reducing/repair system, which operates in a series of thiol exchange reactions, was required to deglutathionylate the protein in order to completely recover the activity of hBCATc. While this finding is consistent with previous S-thiolation studies of hBCATc, which identified oxidized hBCATc as a target for the GSH/Grx reducing system (*30*), these studies are the first to show reversibility of hBCATm. Although S-glutathionylation was not detected in hBCATm treated with GSNO, the ability of the GSH/Grx reducing system to reverse activity relative to GSH alone implies that, over prolonged periods of GSNO inactivation, this inactivation must in part be due to S-glutathionylation. Although denitrosation has been reported in other studies, little is known about its mechanism/importance in cells as some proteins are constitutively S-nitrosylated. However, recent studies have presented evidence that denitrosation of caspase 3 could potentially dictate whether cells undergo apoptosis or necrosis (*50*). Here, the role of denitrosation can be seen as regulatory at physiological levels or one of repair during events of cellular stress. These studies, together with our understanding of the mechanisms of GSNO inhibition of the BCAT isoforms, imply that BCAT proteins are likely oxidant targets which can undergo reversible modification and regulation.

In conclusion, this study has clearly demonstrated that the response of the BCAT proteins to physiological levels of GSNO is through reversible inhibition mediated by transnitrosation via the N-terminal cysteine residues but the association of NO with hBCATm is transitory/less stable. The role of GSNO-mediated S-glutathionylation appears to be one of protection pointing to an adaptive role during periods of oxidative stress. Furthermore, both isoforms require Grx/GSH for complete reactivation, supporting their role in redox regulation. Finally, these studies point to distinct differences between isoforms but also similar mechanisms

of inactivation in terms of oxidation and S-nitrosation to the hTrx and Grx isoforms.

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