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REVIEW

Post-translational disulfide modifications in cell signaling—role of inter-protein, intra-protein, S-glutathionyl, and S-cysteaminyl disulfide modifications in signal transmission

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Abstract

Cell signaling entails a host of post-translational modifications of effector-proteins. These modifications control signal transmission by regulating the activity, localization or half-life of the effector-protein. Prominent oxidative modifications induced by cell-signaling reactive oxygen species (ROS) are cysteinyl modifications such as S-nitrosylation, sulfenic acid and disulfide formation. Disulfides protect protein sulfhydryls against oxidative destruction and simultaneously influence cell signaling by engaging redox-regulatory sulfhydryls in effector-proteins. The types of disulfides implicated in signaling span (1) protein S-glutathionylation, e.g. as a novel mode of Ras activation through S-glutathionylation at Cys-118 in response to a hydrogen-peroxide burst, (2) intra-protein disulfides, e.g. in the regulation of the stability of the protein phosphatase Cdc25C by hydrogen-peroxide, (3) inter-protein disulfides, e.g. in the hydrogen peroxide-mediated inactivation of receptor protein-tyrosine phosphatase α (RPTPα) by dimerization and (4) protein S-cysteaminylation by cystamine. Cystamine is a byproduct of pantetheinase-catalyzed pantothenic acid recycling from pantetheine for biosynthesis of Coenzyme A (CoA), a ubiquitous and metabolically indispensable cofactor. Cystamine inactivates protein kinase C-ε (PKCε), γ-glutamylcysteine synthetase and tissue transglutaminase by S-cysteaminylation-triggered mechanisms. The importance of protein S-cysteaminylation in signal transmission *in vivo* is evident from the ability of cystamine administration to rescue the intestinal inflammatory-response deficit of pantetheinase knockout mice. These mice lack the predominant epithelial pantetheinase isoform and have sharply reduced levels of cystamine/cysteamine in epithelial tissues. In addition, intraperitoneal administration of cystamine significantly delays neurodegenerative pathogenesis in a Huntington's disease mouse model. Thus, cystamine may serve as a prototype for the development of novel therapeutics that target effector-proteins regulated by S-cysteaminylation.

Keywords: *Cystamine, inter-protein disulfides, intra-protein disulfides, redox signaling, S-cysteaminylation, S-glutathionylation*

Introduction

Cell signaling entails a host of post-translational modifications of effector proteins, some transient and others sustained. The modifications control signal transmission by effector proteins, in some cases serving as on/off switches and in others as rheostats transmitting graded responses to signal input. This entails post-translational regulation of the activity, localization or half-life of the effector protein. Among these post-translational alterations are oxidative protein-sulfhydryl modifications induced by cell-signaling reactive oxygen species (ROS),

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e.g. hydrogen-peroxide and superoxide, and reactive nitrogen species (RNS), e.g. nitric oxide and peroxynitrite. Prominent among these cysteinyl modifications are S-nitrosylation, sulfenic acid formation and disulfide induction, the focus of this review. Post-translational disulfide modifications implicated in cell signaling span intra-protein disulfides in signaling proteins, e.g. the protein phosphatase Cdc25C [1], inter-protein disulfides cross-linking signaling proteins, e.g. receptor protein-tyrosine phosphatase α (RPTPα) dimerization [2], protein S-glutathionylation, e.g. a novel mode of Ras activation [3], and protein S-thiolation by other physiological S-thiolating species, e.g. oxidative inactivation of the effector-protein transglutaminase by cystamine [4]. Disulfide formation may specifically affect the activity, localization or half-life of a signaling component, e.g. cystine-induced S-cysteinylation inactivates PKCε [5], and hydrogen peroxide-induced intramolecular disulfide bond formation in the dual-specificity protein phosphatase Cdc25C both regulates the half-life of the phosphatase and influences its subcellular localization [1]. At the same time, disulfide modifications protect protein sulfhydryls against oxidation to sulfonic acid and other species, a concept advanced by Thomas and now firmly established in the literature [6].

The principle that a given cysteinyl redox center in a signaling protein may undergo alternative oxidative modifications *in vivo* in response to different ROS/RNS and thereby mediate divergent functional responses has been demonstrated in an elegant study of the prokaryotic transcription-factor OxyR. In that study, Stamler and colleagues discovered that OxyR contains a single accessible and reactive sulfhydryl (Cys 199) which is modified from the native reduced form (SH) to stable S-nitrosyl (SNO), sulfenic acid (SOH) or S-glutathionyl (SSG) species in response to different inducers of nitrosative or oxidative stress *in vivo*. Differential effects of the various Cys 199 modifications on the OxyR protein structure were revealed by circular-dichroism analysis of the isolated, modified OxyR species. This allowed the investigators to compare the OxyR regulatory responses elicited by alternative Cys 199 modifications (DNA binding and transcriptional activity). Native OxyR (SH) bound an oxyS binding-site DNA-fragment non-cooperatively with high affinity, the SNO and SOH species bound the fragment cooperatively (Hill coefficients of 1.7–1.8) with distinct and somewhat lower affinities than native OxyR, and the SSG species bound the fragment with the highest affinity of all in a non-cooperative manner. Furthermore, the transcriptional activity of these OxyR species at the oxyS promoter spanned a ~30-fold range, with a rank order of SH < SNO < SOH < SSG [7]. The implication of these findings is to multiply the number of potential signaling events that may be elicited through reversible oxidative modifications of cysteinyl redox centers in signaling proteins, by factoring in the largely unexplored possibility that alternative oxidative modifications at a given sulfhydryl redox-center modulate the degree or transform the qualitative nature of the functional response elicited. This additional layer of complexity provides further impetus to identify and sort out protein-sulfhydryl modifications at play in cell signaling and their functional consequence.

RNS/ROS signaling regulates effector proteins by S-glutathionylation

Ras activation by S-glutathionylation

A recent study of angiotensin II (AII) signaling in vascular smooth muscle cells (VSMC) established that a burst of hydrogen-peroxide production triggered by AII activated the small guanosine triphosphatase (GTPase) Ras by S-glutathionylation at Cys-118 [3]. AII stimulates hydrogen-peroxide production in VSMC by an NADPH oxidase-dependent mechanism. Hydrogen-peroxide induction of Ras activation by S-glutathionylation was demonstrated in VSMC by showing that Ras activation and S-glutathionylation were induced by AII simultaneously, and were simultaneously attenuated by (1) treatment of the cells with a chemical inhibitor of NADPH oxidase, (2) forced expression of a dominant-negative NADPH-oxidase subunit (DN $p47^{phox}$), (3) forced expression of the peroxide-scavenger catalase and (4) forced expression of glutaredoxin, a dethiolase that specifically recognizes S-glutathionyl disulfides in proteins. Three Cys residues in Ras were identified as S-glutathionylated by mass spectrometry after isolation of the GTPase from AII-treated VSMC. Forced expression of a Ras construct with a site-specific mutation at one of the S-glutathionylated Cys (C118S Ras) revealed that the construct functioned in a dominant-negative mode, abrogating the activation of p38 and Akt in response to AII as well as AII-induced protein synthesis in VSMC. Taken together, these results strongly implicate Ras activation by Cys 118 S-glutathionylation in the induction of vascular hypertrophy by AII [3].

Ras is a pivotal mediator of myriad cell signaling pathways triggered by growth factors, integrins and other extracellular signals. Ras is influential in key cellular functions, e.g. cell proliferation, survival and migration as well as commonly occurring, insidious pathological conditions, e.g. neoplastic transformation [8]. Therefore, the finding that Cys-118 (Ras) is a redox-sensitive switch that mediates Ras activation upon S-glutathionylation has broad and far-reaching implications to redox regulation of cell signaling networks. Signaling by platelet-derived growth factor (PDGF) in VSMC entails hydrogen-peroxide production and is abrogated by catalase

and other scavengers of this ROS species [9]. This raises the intriguing question of whether Ras activation by S-glutathionylation is also a critical event in the PDGF pathway. By the same token, might Ras activation by S-glutathionylation occur in hydrogen peroxide-mediated signaling induced by EGF/EGFR in epithelial cells? [10] Whether S-glutathionylation of oncogenic forms of Ras influences the constitutive activity of the GTPase in Ras-transformed cells is also worth exploring. Purified Ras is subject to diverse oxidative modifications at Cys 118 and other Cys residues, including S-nitrosylation, S-glutathionylation and S-cysteaminylation [11]. This suggests potential for complexity akin to OxyR redox regulation [7] through alternative oxidative Ras modifications at Cys 118 induced by RNS/ROS in various *in vivo* environments.

SERCA activation by S-glutathionylation

Nitric oxide (NO) signaling transduces relaxation of cardiac, skeletal and vascular smooth muscle by stimulating the sarco/endoplasmic reticulum calcium ATPase (SERCA) to pump intracellular Ca^{2+} into the sarcoplasmic reticulum. SERCA activation by S-glutathionylation at Cys 674 has been strongly implicated in the muscle relaxation response. The requirement for both NO and superoxide to induce SERCA S-glutathionylation and elicit muscle relaxation points to peroxynitrite, which is formed by reaction of NO with superoxide, as the RNS mediating the cellular response [12].

Relatively low concentrations of peroxynitrite (10–50 μM) were shown to induce SERCA S-glutathionylation, when administered with glutathione (GSH) to purified SERCA, aorta homogenates or lysates of HEK293 SERCA-transfectants. S-glutathionylation was associated with significant enhancement of the Ca^{2+} uptake activity of SERCA. Mass spectrometry revealed that Cys 674, which is a particularly reactive Cys in SERCA by virtue of a low pK_a, was S-glutathionylated in these experiments. Furthermore, the C674S SERCA mutant was resistant to stimulation by peroxynitrite/GSH. Superoxide dismutase (SOD) overexpression and treatment with the superoxide-scavenger Tiron each attenuated the relaxation response and SERCA S-glutathionylation in NO-treated smooth muscle cells, implicating peroxynitrite as the mediator of C674 S-glutathionylation in SERCA. Intriguingly, mass spectrometry analysis of atherosclerotic aorta tissue, which is defective in the NO-induced relaxation response, revealed oxidation of Cys 674 (SERCA) to sulfonic acid (RSO_3H), an irreversible modification [12]. Thus, SERCA S-glutathionylation at Cys 674 fits the paradigm of dual functionality as a physiological redox-switch in an effector-protein and a protective mechanism against oxidative destruction leading to a disease state.

The C674S and *wt* SERCA constructs in [12] are human SERCA2b, which is expressed in most tissues and viewed as a housekeeping gene [13]. Inhibition of SERCA2b by the natural-product thapsigargin potently induces apoptosis of a variety of cell types through perturbation of endoplasmic reticulum (ER) Ca^{2+} regulation, i.e. "ER stress" [13,14]. This suggests that SERCA2b activation by C674 S-glutathionylation may play a protective role against apoptosis in diverse tissues, provided that the ROS/RNS produced intracellularly induce the modification.

TNFα induces ROS-dependent apoptosis and protein S-glutathionylation

Tumor necrosis factor-α (TNFα) is a pro-inflammatory cytokine that activates multiple pathways, including pro-apoptotic and survival signaling, by interacting with its cognate receptors (TNF-R1 and TNF-R2) [15]. Co-administration of protein-synthesis inhibitors, such as cycloheximide (chx) or emetine, suppresses TNFα survival signals in HeLa cells and thereby elicits apoptosis, allowing focus on the death-inducing arm of physiological TNFα signaling. Using this paradigm, TNFα apoptosis signaling was shown to involve a burst of mitochondrial ROS production. This was demonstrated by antioxidant suppression of TNFα/emetin-induced HeLa cell apoptosis and by the attenuated apoptotic responses of HeLa cell variants that were defective in mitochondrial respiratory-chain components [16] These observations suggested potential involvement of protein-sulfhydryl oxidation in apoptosis signaling by TNFα.

Analysis of proteins S-glutathionylated in HeLa cells in association with TNFα/chx-induced mitochondrial ROS production and apoptosis revealed that S-glutathionylation of numerous protein species was upregulated by the stimulus. Furthermore, forced expression of the anti-apoptotic protein Bcl-2 abrogated both the surge in mitochondrial ROS and the induction of protein S-glutathionylation elicited by TNFα/chx. Two of the S-glutathionylated proteins were identified by HPLC, mass spectrometry and Western analysis as thioredoxin peroxidase II and annexin II. Cys 9 was pinpointed as the sole site of annexin II S-glutathionylation in TNFα/chx-treated HeLa cells by the resistance of the mutant C9G annexin II to S-glutathionylation [17].

Annexins are characterized by Ca^{2+}-dependent binding interactions with cellular membranes as well as with filamentous actin (F-actin). Through these interactions, annexins exert broad influence over membrane trafficking and cytoskeletal dynamics. A subsequent study of the regulatory effects of

diamide-induced S-glutathionylation on purified annexin II (also termed annexin A2) found that annexin II was S-glutathionylated at two sites, the Cys residue modified in TNFα/chx-treated HeLa cells (Cys 9) and Cys 132. S-glutathionylation potentiated diamide-induced inactivation of the phospholipid and F-actin binding-activities of annexin II, and substantial reversal of this effect by glutaredoxin verified that S-glutathionylation mediated inactivation [18].

The importance of protein sulfhydryl oxidation in TNFα/chx-induced HeLa cell apoptosis is strongly suggested by the robust potentiation of apoptosis achieved by knockdown of either thioredoxin-1 (Trx-1) or Trx-related protein-14 (TRP14) [19], which function as protein-disulfide reductases in cells [20,21]. However, thioredoxin family members also function by regulating signaling proteins by non-redox interactions, e.g. Trx-1 (but not TRP14) thus inhibits the apoptosis signal regulating kinase-1 (ASK1) [19,22]. This raises the possibility that loss of Trx-1/TRP14 actions other than protein disulfide reduction may contribute to apoptosis potentiation by Trx-1/TRP14 knockdown [19].

TNFα-induced disulfide formation in the LC8 dynein light chain has been directly demonstrated in HeLa cells through forced expression of a TRP14 mutant. In the mutant, the last Cys in the disulfide-reductase motif CXXC was changed to Ser. Because the last Cys of the motif cleaves the disulfide that links the first Cys with substrate in the reaction-intermediate to produce oxidized TRP14 and release of reduced substrate (RSH), the CXXS TRP14 mutant traps substrates in disulfide-linked complexes. Through this approach, LC8 was found trapped in a complex with mutant (but not *wt*) TRP14 in TNFα-treated HeLa cells, indicating TNFα induction of disulfide bond(s) in LC8 [19]. Interestingly, LC8 is also implicated in redox signaling by its inhibitory activity against neuronal NO synthase and its upregulation following cerebral ischemia [23].

While the specific actions of TNFα-induced mitochondrial ROS that are required for signal transmission of HeLa cell apoptosis remain elusive, the discovery of attendant disulfide modifications in proteins involved in membrane trafficking and cytoskeletal dynamics offers tantalizing clues for further investigation. This may help to answer the broad question of whether TNFα and other physiological agents, such as β_2-microglobulin, that trigger ROS-dependent apoptosis signaling by binding at the cell surface [16,24] accomplish this by convergent or divergent mechanisms at the levels of ROS species and ROS effector-proteins.

Stress responses and protein S-glutathionylation

Protein S-glutathionylation has also been linked to the mammalian stress response. Exposure of eukaryotic cells to heat, known as heat shock, or various chemical stressors induces transcriptional upregulation of heat shock proteins (HSP's). For the most part, HSP's have a chaperone function that protects the structural integrity of cell proteins and deters stress-induced protein aggregation. In mammals, stress-induced upregulation of HSP's is orchestrated by the transcription-factor heat shock factor 1 (HSF-1). HSF-1 is constitutively expressed as a monomer in the cytoplasm, where it is on call for activation in response to environmental stress. HSF-1 activation entails trimerization, nuclear translocation and transcriptional activation of HSP promoters by binding at resident heat shock elements (HSE's) [25,26].

Protein disulfide modifications are strongly implicated in cell signaling events that trigger the mammalian stress response. This is because the disulfide-forming agent diamide potently induces the stress response in both primary cultures and cells in long-term culture [primary cultures of guinea pig gastric mucosal cells and Chinese hamster ovary (CHO) cells] [26,27]. Diamide, [molecular formula, $(CH_3)_2NCON{=}NCON(CH_3)_2$], is a potent inducer of disulfides in biological systems. Disulfide induction entails addition/displacement of reactive cysteinyl-sulfhydryls at the diazene bond of diamide, which yields the products protein disulfide (or glutathione disulfide, etc.) and reduced diamide (hydrazine) [28]. Based on its highly selective oxidant activity, diamide can be used as a chemical probe of the consequences of disulfide formation in cells [28].

The analysis of diamide-treated CHO cells established that the thiol-specific oxidant upregulated HSP's and produced thermal tolerance, indicative of an authentic stress response [27]. Mechanistic analysis of the stress response in primary gastric mucosal cells revealed that induction of HSP60, HSP70 and HSP90 expression by diamide treatment of the cells was accompanied by HSF-1 activation. HSF-1 activation was evident from a marked increase in HSE binding-activity, with supershifting by HSF-1 Ab, in gel mobility shift assays of whole cell extracts prepared from diamide-treated cells [26]. The possibility that the diamide-induced stress response entails direct regulatory effects on HSF-1 through induction of intra-protein or inter-protein disulfides within or between HSF-1 monomers can be ruled out. First, diamide treatment of a cytosolic fraction from HeLa cells stabilized inactive human HSF-1 in a disulfide cross-linked monomer that was resistant to heat-induced trimerization and activation (HSE binding-activity), but fully recovered functionality after disulfide reduction by dithiothreitol (DTT) [29]. Second, diamide also inhibited the activated HSF-1 species in nuclear extracts. The addition of diamide to nuclear extracts prepared from stressed human endothelial cells markedly inhibited the HSE

binding-activity of HSF-1, and subsequent addition of DTT restored the activity [30].

The mechanism of the diamide-induced stress response remains an open question, but investigations manipulating cellular GSH levels and monitoring protein S-glutathionylation provide clues that implicate S-glutathionylation of cellular proteins in the response. Pretreatment of the primary gastric mucosal cells with the specific γ-glutamylcysteine synthetase inhibitor buthionine-[S,R]-sulfoximine (BSO) markedly lowered the cellular GSH level and abrogated diamide-induced HSF-1 activation (HSE binding-activity) and HSP upregulation [26]. Interestingly, a strong correlation was observed between HSP upregulation and a rise in cellular protein S-glutathionylation in diamide-induced stress responses in human endothelial ECV304 cells and human lung epithelial A549 cells; (the stress responses were authenticated by demonstrating resistance to thermal and oxidative stress.) [31] Might HSF-1 be S-glutathionylated and activated by the modification? An answer to this question seems likely to be part of the next installment of this interesting story.

Protein S-glutathionylation is also implicated in the oxidative stress response produced during cardiac ischemia/reperfusion. By probing isolated rat hearts with biotinylated GSH, Eaton et al., established that numerous cardiac proteins, including 20 dominant species, are S-glutathionylated in the initial 10 min of the reperfusion phase, amounting to a 15-fold increase in bulk protein S-glutathionylation; one of the S-glutathionylated proteins was identified by N-terminal sequence-analysis and HPLC peptide-mapping as a critical metabolic enzyme, glyceraldehyde phosphate dehydrogenase (GAPDH) [32]. These observations link protein S-glutathionylation to mammalian stress responses.

Regulation of cell signaling transmission by thiol/disulfide exchange reactions between protein sulfhydryls and cystamine

FBPase activation by S-cysteaminylation

Horecker's laboratory was the first to definitively demonstrate protein regulation by thiol-disulfide exchange with a physiologically-occurring disulfide in a classic paper published in 1967 [33]. In that report, the disulfide was cystamine, and its action was to stimulate the activity of fructose-1,6-bisphosphatase (FBPase), a key enzyme in the metabolic-pathway gluconeogenesis, three- to four-fold with concomitant [^{35}S] cystamine labeling of the enzyme. Reducing agents removed the [^{35}S] label and reversed cystamine-induced FBPase activation, indicative of FBPase regulation by thiol-disulfide exchange with cystamine. Several other physiological disulfides were tested, including GSH disulfide (GSSG), cystine and oxidized coenzyme A, but none affected FBPase activity. This provided a measure of the selectivity of cystamine, and inspired the authors to speculate that cystamine might be a physiological regulator of protein function by S-thiolation of select protein targets [33].

Cystamine targeting of Caspase-3 and Tgase—implications to HD therapy

Huntington's disease (HD) is an inherited neurodegenerative disorder that inexorably leads to chorea, dementia, severe weight loss and death. Pathogenesis of HD and several other neurodegenerative disorders is due to the expression of a protein mutated by expansion of a polyglutamine domain (polyGln) encoded by a CAG trinucleotide-repeat [34]. Proteolytic cleavage of polyGln mutants of the Huntington protein (htt) by caspase-3 produces N-terminal polyGln-containing fragments that aggregate with self and other proteins. One important mechanism at play in aggregate formation is transglutaminase (Tgase) catalysis of inter-protein Gln-Lys cross-links through ε-(γ-glutamyl)lysine isopeptide bond formation. Tgase and caspase-3 are currently viewed as rational targets for HD therapy, based on their involvement in mutant htt processing, [4,35,36]. Cystamine is a potent inhibitor of both Tgase [37] and caspase-3 [35]. Remarkably, intraperitoneal administration of cystamine to transgenic mice expressing mutant htt extended survival and ameliorated neurodegeneration-associated symptoms in two independently conducted studies [4,36].

Caspase-3 is an effector caspase common to mitochondrial- and death receptor- initiated apoptosis-signaling pathways. Caspases contain a critical catalytic Cys residue and are inactivated by Cys modification. For example, thiol-disulfide exchange with the thiuram-disulfide disulfiram inactivates caspases [38]. The mechanism of caspase-3 inhibition by cystamine also appears to entail thiol-disulfide exchange. This is because when human neuroblastoma SH-SY5Y cells were treated with cystamine prior to an apoptosis-inducing agent, the cystamine concentrations that inhibited caspase-3 activity measured in the cell lysates were only several-fold higher than concentrations that achieved equivalent inhibition when added directly to recombinant active human caspase-3 assays ($IC_{50} = 25\,\mu M$) [35]. Inhibition entailing reversible binding of cystamine or cysteamine to active caspase-3 in the cells would presumably be lost upon dilution during cell lysate preparation and therefore undetectable. It is necessary to note, however, that the authors did not design their study to address the question of reversible vs. S-cysteaminylation-triggered caspase-3 inhibitory mechanisms and did not make any claims based on their results with regard to this issue. The above interpretation

that the results reflect inhibition by thiol-disulfide exchange requires an assumption that neither cystamine nor cysteamine inhibits the conversion of the zymogen to active caspase-3. On the other hand, the definitive demonstration that disulfiram inactivates caspases by S-thiolation [38] lends support to the notions that cystamine inhibits active caspase-3 by S-cysteaminylation, and that the protective effects of cystamine against neurodegenerative pathogenesis involve this mechanism.

Tgase is an effector protein that is activated by signals which elevate cellular Ca^{2+} levels. As in the case of caspase-3, the contribution of S-cysteaminylation in the inhibition of Tgase by cystamine has not been precisely defined [39,40]. Tgase contains an active-site Cys that is essential to its Gln-Lys cross-linking activity and implicated as the redox switch mediating oxidative inactivation by cystamine [41]. Tgase is rendered inactive by either site-directed mutagenesis of the active-site Cys or treatment with sulfhydryl alkylating reagents [42]. Teasing out the contribution of the S-cysteaminylation-triggered mechanism to Tgase inhibition by cystamine has been hindered by the complication that cystamine has in common with other small primary amines, e.g. cysteamine and cadaverine, the capacity to inhibit Tgase by serving as a substrate that competes with the Lys-bearing protein substrate [37,39,40].

In support of oxidative Tgase inactivation by cystamine *in vivo*, treatment of human lung WI-38 cells with cystamine was found to potently inhibit Tgase activity measured in the cell lysates, whereas several other primary amines that serve as competitive substrates failed to do so, despite being taken up by the cells [40]. The implication of this finding was that cystamine inhibited cellular Tgase by covalent modification, i.e. S-cysteaminylation and potentially rearrangement to intra-protein or inter-protein disulfides. However, as noted by the authors, it is also plausible that inhibition of Tgase occurred after intracellular reduction of cystamine to cysteamine. Unfortunately, cysteamine was not among the primary amines analyzed [40]. Another line of evidence for oxidative Tgase inactivation by cystamine was provided in a report showing that preincubation with cystamine abrogated Tgase-catalyzed formation of insoluble brain-neurofilament polymers *in vitro* [43], although here too cysteamine was not investigated, so that the possibility that cystamine abrogated the reaction by serving as a competing substrate cannot be ruled out.

The breakthrough finding that cystamine therapy slows HD pathogenesis in mouse models has heightened interest in the unresolved mechanistic questions regarding cystamine inhibition of Tgase and caspase-3. Answers to these questions may allow rational design of polyGln-disorder therapeutics that are based on the action of cystamine.

Cystamine availability in mammalian cells and tissues

Horecker's prediction in 1967 that cystamine may physiologically regulate protein function through thiol-disulfide exchange still awaits an answer. However, recent investigations in mouse models with attenuated cysteamine/cystamine tissue-levels have shed new light on this question that suggests the prediction will turn out to be correct. It is now recognized that cysteamine/cystamine are produced in a broad spectrum of mammalian tissues, indicating a potential physiological role for S-cysteaminylation in protein regulation.

Coenzyme A (CoA) is an indispensable cofactor that plays obligate roles in carbohydrate, lipid and amino-acid metabolism, and its biosynthesis requires pantothenic acid, a structural component of the coenzyme [44]. Pantothenic acid recycling entails hydrolysis of an isopeptide bond in the CoA degradation-product pantetheine to yield pantothenic acid and cysteamine. This reaction is catalyzed by pantetheinases, which are ubiquitously expressed in mammalian tissues [45–47]. Hence, cysteamine is ubiquitously produced in mammalian tissues to various extents [46].

Evidence of the propensity of cysteamine to form cystamine or other disulfides in cells has been provided by observations that cysteamine undergoes thiol-disulfide exchange with cystine in fibroblasts from cystinosis patients. These cells are characterized by highly elevated lysosomal cystine stores due to a defect in lysosomal membrane transport. Cysteamine depletes the cells of cystine by forming cysteine-cysteamine mixed disulfides that rapidly efflux from the cells [48]. Likewise, the elevation of plasma cysteamine levels after oral administration of cysteamine to cystinosis patients correlates with cystine depletion in circulating leukocytes [49]. The reactivity of cysteamine with cystine in cystinosis cells is concordant with the low pK_a of cysteamine, which is attributed to the proximal amine [50]. This suggests that cysteamine-containing disulfides, such as cystamine, may normally be present along with cysteamine in mammalian tissues under physiological conditions. In fact, increased metabolic activity typically shifts the intracellular redox set-point to a more pro-oxidant character and can be expected to elevate cysteamine levels by accelerating pantothenic acid recycling. Therefore, intracellular redox conditions are likely to become more favorable for cysteamine oxidation to cystamine in tandem with accelerated production of cysteamine.

An intriguing hypothesis pertaining to why physiological roles for cysteamine/cystamine may have evolved was introduced by Miller. He noted that the structure of CoA seems inordinately elaborate for its function of activating carbonyl groups. He then reasoned that pantetheine might have been formed on the prebiotic earth from the hypothesized available supplies of

β-alanine, pantoyl lactone and cysteamine. Miller's research team substantiated this hypothetical scenario by demonstrating the synthesis of pantetheine from these precursor reactants after moderate heating of the dried mixture, to recapitulate prebiotic conditions of a dried-out lagoon baking in the sun [51]. The probable availability of cysteamine on the pre-biotic earth not only suggests that this sulfhydryl species may have been a key component in the still unfathomable origins of life, but also that physiological roles of various sorts may have evolved for cysteamine/cystamine in prokaryotes and eukaryotes. This notion is congruous with the presence of cysteamine/cystamine in diverse mammalian cells and tissues.

Cystamine rescue of a pantetheinase knockout phenotype

Pantetheinases are encoded by the vanin gene family; the mouse genome contains two and the human genome three vanin genes. Physiological roles for cysteamine/cystamine have been revealed by investigations of vanin-1 knockout mice. The vanin-1 gene-product is a glycosylphosphatidylinositol (GPI)-anchored ecto-pantetheinase that is expressed at variable levels in most or all mouse tissues, with particularly high expression in renal, intestinal and hepatic tissues [46]. The lack of detectable cysteamine in renal and hepatic tissues of vanin-1 knockout mice contrasts with the cysteamine levels measured in the tissues of *wt* mice and supports the view that the primary source of endogenous cysteamine is pantetheine catabolism [46].

Vanin-1 knockout mice develop normally and thus provide an appropriate model to explore the effects of cysteamine deficiency on physiological responses in adult mice. To evaluate the potential involvement of cysteamine/cystamine in inflammatory responses, Martin et al., focused on intestinal inflammation in vanin-1 knockout vs. *wt* mice, because the vanin-1 gene product is the predominant pantetheinase in intestinal tissue. Indomethacin is a nonsteroidal antiinflammatory drug (NSAID) that at high doses produces the toxic side-effect of intestinal injury. Using indomethacin at toxic doses as a model of acute intestinal injury and inflammation, they found that the intestinal villi of *wt* mice exhibited morphological changes indicative of injury whereas no such injury was evident in vanin-1 −/− mice. Furthermore, the indomethacin treatment upregulated expression of inflammatory genes in *wt* mice and only to a much lesser extent in vanin-1 knockout mice. Qualitatively similar results were obtained in a *Schistosoma mansoni* infection model of chronic intestinal inflammation. Remarkably, oral cystamine administration to vanin-1 −/− mice reversed protection against the inflammatory responses, pinpointing cysteamine deficiency as the causative factor for the inflammatory-response deficit.

γ-Glutamylcysteine synthetase (γGCS) catalyzes the rate-limiting step of GSH synthesis. The results also provided evidence for physiological inhibition of γGCS and attenuation of GSH stores by cysteamine/cystamine, based on the analysis of vanin-1 knockout vs. *wt* mice under control conditions, i.e. without toxic insult. Thus, hepatic γGCS activity and GSH levels were elevated in vanin-1 −/− mice compared to *wt* mice and returned to baseline upon oral cystamine administration to the knockout mice [47]. These *in vivo* findings are congruous with early observations by Meister that cystamine inactivates purified γGCS by thiol-disulfide exchange [52]. This suggests that cystamine and not cysteamine mediates γGCS inhibition *in vivo*. Furthermore, the results clearly define a role for cysteamine/cystamine in inflammation and also in the *in vivo* regulation of the redox set-point of hepatocytes.

Chronic inflammation produces tissue injury and increases the risk of neoplastic transformation. The pro-inflammatory action of cysteamine/cystamine demonstrated in the vanin-1 −/− mouse model [47] is consonant with the inhibitory effects of cystamine against Tgase (↑ cell proliferation), caspase-3 (↑ cell survival), and γGCS (↑ ROS), suggesting that these proteins might function as cystamine-effectors that contribute to the pro-inflammatory outcome. In a hypothetical paradigm where cysteamine/cystamine act as a physiological mediator of inflammatory or stress responses, it is conceivable that cysteamine/cystamine could operate as a rheostat, engaging effectors to activate proliferative, survival and pro-oxidant responses to low or moderate stress and, as the degree of stress further increases, orchestrating a contrary set of effectors to serve as a fail-safe mechanism that attenuates the initial stress response, thus avoiding catastrophic consequences such as tissue injury or neoplastic transformation.

PKCε inactivation by S-cysteaminylation

A signaling protein that in principle meets the requirements for a cystamine-effector in the hypothetical fail-safe mechanism is protein kinase C-ε (PKCε). PKCε is an *sn*-1,2-diacylglycerol/phorbol ester-responsive Ser/Thr protein kinase [53]. Cystamine treatment of cells inactivates PKCε by thiol/disulfide exchange [5,54]. PKCε is oncogenic [55,56], and it is involved in cell proliferative, survival and stress-response signaling. Thus, PKCε inactivation could attenuate these types of responses in some tissue environments. For example, induction of cyclooxygenase-2 (COX-2), a rate-limiting enzyme in prostaglandin synthesis, is associated with increased risk of neoplastic transformation [57]. Cystamine induced COX-2 expression in the intestinal inflammation model in vanin-1 −/− mice [47]. It is conceivable that PKCε inactivation by cystamine

might serve as a fail-safe mechanism that counteracts this, because PKCε-dependent COX-2 induction has been observed in other models. For example, bradykinin-induced COX-2 induction is PKCε-dependent in human airway smooth-muscle cells [58]. In addition, in organ explants of Barrett's esophagus, which is a hyperproliferative epithelial disorder that develops in response to acid reflux, COX-2 induction by acid and bile acid combinations was PKC-dependent and correlated with PKCε activity [59].

PKCε signaling plays a role in heat shock and other stress responses that are associated with pro-oxidant conditions which may foster cysteamine oxidation to cystamine and could lead to a fail-safe mechanism of cystamine-induced PKCε inactivation. For example, heat-induced HSP90β promoter activation and message upregulation are PKCε-dependent in Jurkat cells [60]. It should be noted that phorbol esters, which are non-specific activators of diacylglycerol-responsive proteins, upregulate HSP90 by message stabilization, not promoter activation [61].

PKCε is also implicated in anti-apoptotic signaling. Thus, TRAIL-induced apoptosis is attenuated by forced PKCε expression in human glioma cells [62], and PKCε also attenuates cytotoxic anticancer-drug induction of the mitochondria-initiated apoptosis pathway in human lung cancer cells [63]. In addition, PKCε inhibits pro-apoptotic JNK signaling in HEK293 cells [64]. In the DMBA-TPA mouse skin carcinogenesis model, forced PKCε expression in transgenic mice dramatically enhanced carcinoma formation in response to the pro-oxidant and toxic chemical insult [65,66]. In principle, pro-oxidant apoptosis-inducing agents and/or pro-oxidant apoptosis signaling could reach a critical point of oxidative stress where PKCε-dependent survival signaling is dampened by the fail-safe mechanism of cystamine-induced PKCε inactivation. This could help to foster apoptosis instead of neoplastic transformation of DNA-damaged cells.

Whether or not PKCε turns out to be a cystamine-effector, the $>90\%$ inactivation of PKCε achieved by cystamine treatment of PKCε transfectants [5] and the potency of cystamine against purified human recombinant PKCε ($IC_{50} = 100\,\mu M$) [54] support the notion that a redox-regulatory PKCε-sulfhydryl modified by cystamine may be amenable to development as a target for antineoplastic therapeutics that selectively inactivate PKCε by alkylating the sulfhydryl switch.

Conclusions

The role of cysteinyl redox centers in signaling proteins as transmitters of signal input through reversible oxidative modifications that include intra-protein and inter-protein disulfides, S-glutathionylation and S-cysteaminylation is now coming to light. A continuing challenge in elucidating protein thiol/disulfide regulatory mechanisms is the susceptibility of protein disulfides to rearrangement, which impedes identification of the precise modifications influencing signaling in *in vivo* environments. As this technical hurdle is overcome, participation of other types of protein S-thiolation modifications in cell signaling, e.g. modification by the disulfide pantethine, may be revealed. In addition, cysteinyl redox centers that regulate signal transmission by S-thiolation offer molecular targets for development of drugs that irreversibly alter the function of the target protein by alkylation of the sulfhydryl switch. For example, Tgase inactivators of this type may be of value as therapeutics to delay progression of Huntington's disease.

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