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Induction of cystine transport in bovine pulmonary artery endothelial cells by sodium arsenite

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Sodium arsenite is one of a number of agents reported to induce a 30–34 kDa 'stress' protein in cells. Other agents which induce this stress protein, including diethyl maleate (DEM) and H_2O_2, have also been reported to be inducers of cystine transport in fibroblasts, macrophages, endothelial cells and other cell types. We have determined that micromolar levels of sodium arsenite increase cystine transport in bovine pulmonary artery endothelial cells (BPAEC), resulting in increases in intracellular glutathione (GSH). The increase in cystine transport appears to be due to stimulation of the synthesis of a protein analogous to the x_c^- transport system, a sodium-independent system specific for cystine and glutamate. We have determined that this stimulation is maximal between 8–16 h after addition of sodium arsenite and is inhibited by exogenous GSH. Others have reported that synthesis of the 30–34 kDa stress protein is maximal between 2–4 h and returns to baseline by 6–10 h. We conclude that cystine transport may be considered a 'secondary' stress response and is likely to be modulated by sulfhydryl-reactive agents.

Introduction

Various oxidizing or thiol reactive agents have been reported to induce a family of 'stress' proteins in a number of cell types, including both bacteria and eucaryotic cells [1–6]. Attention has been focused on a group of proteins with molecular weights of 30–34 kDa [5,7–11]. In some cell types, e.g., Chinese hamster ovary (CHO) cells and mouse macrophages, the 30–34 kDa protein induced by electrophilic agents, cadmium, arsenite, H_2O_2 or hypoxia has been identified as a heme oxygenase [12,13]. The stimulation of the synthesis of the typical stress protein begins within 30 min to 2 h after exposure, reaches a maximum between 2–4 h, and declines by 6 h or more after the initial reaction [1,4–12].

Glutathione involvement in stress protein induction has been explored. Glutathione reagents, such as DEM and arsenite, have been implicated in stress protein induction, but the absolute levels of GSH were not considered to be the determining factor, since near total depletion of GSH by the γ-glutamylcysteine synthetase inhibitor BSO resulted in minimal stress protein induction [4,5,7,9,10,12]. However, in at least one case, BSO has been reported to be an inducer of heme oxygenase [14].

In our previous studies of bovine pulmonary artery endothelial cells (BPAEC), we have reported that treatments with various agents, including DEM, BSO, BCNU, H_2O_2 and hyperoxia, induced a protein-synthesis dependent transport system specific for cystine and glutamate [15–17,19]. Induction of cystine transport was accompanied by an increase of total intracellular glutathione. Induction of cystine transport has been reported directly only in cells in culture, albeit in a wide variety of cells from different organs and species [17–22]. It seems likely, however, that increased cystine transport leading to increased intracellular glutathione levels is an important adaptive response to oxidant related stresses in vivo. Under normal conditions, utilization of circulating cysteine might be adequate for glutathione synthesis but, since plasma cystine levels are much higher than cysteine levels, increased access to this additional sulfur amino-acid pool during stress conditions may have physiological importance. Although our treatments which induced cystine transport were identical to those reported by others to increase the 30–34 kDa stress

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protein, the transport function did not increase before 4 h and the rate of increase was most rapid between 6–16 h after onset of exposure.

To examine further the mechanisms of induction of cystine transport and to explore the role of thiol reactions in the regulation of protein synthesis, we have characterized the effect of sodium arsenite on the cystine transport system in endothelial cells.

Materials and Methods

Chemical reagents. L-[2,3-^{3}H]Glutamic acid, [2,3-^{3}H]aspartic acid and [^{3}H]leucine were obtained from DuPont-New England Nuclear (Boston, MA). [^{3}H]Cystine was obtained from Amersham (Arlington Heights, IL). Dulbecco's phosphate buffered saline (PBS), RPMI-1640 and trypsin-EDTA were purchased from Gibco (Chagrin Falls, OH). Fetal calf serum and substrates for the GSH assays were purchased from Sigma (St Louis, MO).

Cells and media. All experiments were carried out using 4th to 6th passage bovine pulmonary artery endothelial cells obtained as previously described [15,16,23]. Culture media consisted of RPMI-1640 supplemented with 10% fetal calf serum, penicillin, streptomycin and amphotericin B as previously reported. Cells were plated at densities of $(4–8) \times 10^4$ per 35 mm dish. The medium was changed every 48 h and before the start of any experimental procedure. Exposures to sodium arsenite were begun after cells reached confluence ($> 1.5 \times 10^6$/35 mm dish), usually 4–5 days after plating. Once arsenite was added to the media, it remained on the cells until uptake measurements or GSH measurements were begun.

Uptake experiments. To measure uptake of cystine and other amino acids, cells were washed twice with Dulbecco's PBS supplemented with 14 mM glucose, and preincubated for 60 min with 2 ml PBS + glucose at 37°C. The cells were then washed twice and incubated in PBS + glucose containing 0.06 mM of the radioactive amino acid at the desired specific activity. In experiments to assess the sodium dependence of uptake, LiCl was substituted for NaCl and Tris buffer for sodium phosphate for pre-washes and during uptake. We have previously shown that cystine and glutamate uptake in endothelial cells is linear for 30 min under these conditions [17]. We have also found that aspartate uptake into BPAEC is essentially linear for at least 15 min and that leucine uptake is linear up to 5 min of incubation (unpublished data). Cells were incubated for 1 or 10 min, followed by four washes with ice-cold PBS. The cells were then dissolved in 1% Triton X-100 and an aliquot counted in Ecolite scintillation fluid. Each experiment was carried out with 4–6 replicate plates for each experimental parameter and repeated at least twice on separate cell preparations.

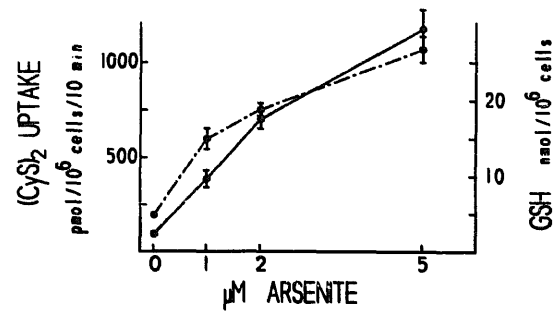

Fig. 1. Concentration dependence of cystine uptake and glutathione increases following sodium arsenite exposure. Solid line is stimulation of [^{3}H]cystine uptake into BPAC measured for 10 min following 24 h pre-exposure to increasing concentrations of sodium arsenite. (- - -), increase in intracellular levels of glutathione in BPAC following 24 h of exposure to increasing concentrations of sodium arsenite. Vertical bars denote S.E. for six separate determinations.

Cell counts and assays. Cell counts were performed on the same dishes used for GSH measurements using a calibrated Coulter Counter as described previously [17,19]. Cell sizes were determined with a Coulter Channelizer. For GSH measurements, an aliquot of the cells was treated with 10% perchloric acid, sonicated, centrifuged and immediately frozen for later GSH assay by the method of Tietze [24], as described by Akerboom and Sies [25]. Alternatively, 2–4 dishes were washed, scraped directly into 1% perchloric acid and derivatized for HPLC assay by the method of Reed and co-workers [26], as previously described [17,19]. Toxicity was determined by counting adherent and non-adherent cells and by Trypan blue exclusion.

Calculations and statistics. All uptakes were expressed as picomoles of cystine or other amino acid per 10^6 cells or expressed as % control value. GSH levels are expressed as nmol/10^6 cells. Statistical significance was determined by analysis of variance with the post-hoc Scheffe test for groups with significant differences.

Results

Exposure of endothelial cells to arsenite resulted in a concentration dependent increase in both cystine uptake and intracellular GSH levels (Fig. 1). The increase in cystine uptake was protein-synthesis dependent (Table I). Cystine uptake was also sodium-independent (Table II). In addition, the increase in amino-acid uptake was specific for cystine and glutamate. No significant increases were seen in uptake of leucine or aspartate (Table III).

At levels of arsenite of 2.5 μM or less, there was no apparent toxic effect of the arsenite on the endothelial cells, as measured by inhibition of cell growth and Trypan blue exclusion or observation by light microscopy. Levels of 5 μM did typically produce some

TABLE I

Effect of cycloheximide on induction of cell GSH levels and cystine uptake

CH, cycloheximide; As, sodium arsenite. Cells were exposed to sodium arsenite for 24 h. Numbers are means ± S.E., $n = 6$ dishes.

	Cystine uptake (pmol/10^6 cells per 10 min)	GSH level (nmol/10^6 cells)
Control	89.7 ± 6.96	3.38 ± 0.58
+ CH	89.0 ± 10.7	5.62 ± 0.68 [a]
2.5 μM As	618.9 ± 77.9 [a]	16.11 ± 1.20 [a]
+ CH	92.9 ± 7.5 [b]	6.08 ± 1.07 [a,b]

[a] Significantly above control levels without arsenite, $P < 0.01$.
[b] Significantly less than treated cells without cycloheximide.

TABLE II

Sodium independence of stimulation of cystine uptake by arsenite

+ Na, uptakes were measured in PBS. − Na, uptakes were measured in a Tris-LiCl balanced salts solution. As, sodium arsenite. Cells in RPMI + 10% FCS were exposed to sodium arsenite for 24 h before measurement of cystine uptake. Values are means ± S.E. for $n = 6$ dishes.

	Cystine uptake (pmol/10^6 cells per 10 min)
Control + Na	111.2 ± 5.75
Control − Na	114.8 ± 14.8
+ 2.5 μM As + Na	491.9 ± 55.1 *
+ 2.5 μM As − Na	585 ± 37.1 *

* Significantly above control levels without arsenite, $P < 0.01$.

decrease in cell numbers (10–20%) after 24 h. No inhibition of protein synthesis was seen in arsenite exposed cells, as determined by incorporation of [^{3}H]leucine into protein.

Since arsenite can form complexes with GSH, we measured the effect of arsenite on GSH levels at 1, 2, and 4 h after addition of 2.5 μM sodium arsenite to the BPAEC. Cystine uptake was also measured at these time points. No significant differences were seen between intracellular GSH levels in control and arsenite

TABLE III

Amino-acid uptakes

Cells were exposed to 2.5 μM sodium arsenite for 24 h. Uptakes are values for 1 min incubations with the labeled amino acids at 0.06 mM.

Amino acid	Amino-acid uptake (pmol/10^6) cells per min)	
	control	2.5 μM As
Cystine	7.24 ± 0.37	26.3 ± 3.60 *
Glutamate	9.51 ± 1.49	32.39 ± 6.46 *
Aspartate	3.71 ± 0.23	4.23 ± 0.67
Leucine	44.57 ± 1.81	39.50 ± 2.15

* Significantly above control, $P < 0.01$.

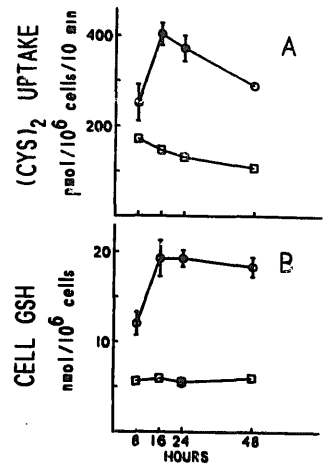

Fig. 2. (A) Time dependence of stimulation of cystine uptake in BPAEC following addition of sodium arsenite. Uptake of cystine in cells exposed to 2.5 μM sodium arsenite (○) is compared to cells maintained in RPMI + 10% FCS without added arsenite (□). Vertical bars denote S.E. for measurements on six separate plates. (B) Time dependence of changes in intracellular GSH levels in cells following addition of sodium arsenite. Total GSH equivalents in cells exposed to 2.5 μM sodium arsenite (○) is compared to cells maintained in RPMI + 10% FCS without added arsenite (□). Vertical bars denote S.E. for measurements on six separate plates.

exposed cells at 1, 2, or 4 h ($P < 0.01$). Maximum increases in both cystine uptake and cell GSH levels were seen between 16–24 h after addition of arsenite

TABLE IV

Effect of extracellular cysteine and glutathione on cystine uptake in arsenite treated endothelial cells

Values shown are means ± S.E. ($n = 4$) of a representative experiment. Cells were exposed to 2.5 μM sodium arsenite for 24 h in the presence of 1 mM cysteine or 1 mM GSH.

Sodium arsenite	Cystine uptake (pmol/10^6 cells per 10 min)		
	control	1 mM CysH	1 mM GSH
0	163 ± 13	197 ± 6.5 *	142 ± 5.7
2.5 μM	644 ± 46 **	772 ± 47 **	425 ± 24 **

* Significantly above controls, $P < 0.01$.
** Significantly above groups without sodium arsenite, $P < 0.01$.

TABLE V

Effect of extracellular cysteine and glutathione on intracellular glutathione in arsenite treated endothelial cells

Values shown are means ± S.E. ($n = 4$) of a representative experiment. Cells were exposed to 2.5 μM for 24 h in the presence of 1 mM cysteine or 1 mM GSH.

Sodium arsenite	Glutathione (nmol/10^6 cells)		
	control	+ 1 mM CysH	+ 1mM GSH
0	2.01 ± 0.28	6.75 ± 0.94 *	6.06 ± 1.12 *
2.5 μM	13.82 ± 0.66 *	19.85 ± 2.4 *	13.05 ± 1.51 *

* Significantly above controls, $P < 0.01$.

TABLE VI

Effect of agents on induction of cystine uptake and cell GSH

Cells were exposed to the above agents for 24 h prior to measurement of cystine uptake. Values are means ± S.E. for six replicate dishes from a representative experiment.

	Cystine uptake * (pmol/10^6 per 10 min)	GSH (nmol/10^6 cells)
Control	53.9 ± 10.3	1.68 ± 0.17
0.025 mM DEM	167 ± 33.3 *	5.49 ± 0.43 *
0.0125 mM disulfiram	87.2 ± 9.8 *	3.80 ± 0.30 *
0.005 mM arsenite	241 ± 83.5 *	6.61 ± 0.54 *
0.005 mM menadione	61 ± 15.0	3.07 ± 0.20 *
0.0125 mM paraquat	71 ± 9.4	2.71 ± 0.43

* Significantly above control, $P < 0.01$.

(Figs. 2a,b). No significant differences were seen between control and arsenite-treated cells at 1, 2, or 4 h after addition ($P < 0.01$).

GSH co-incubated with arsenite partially blocked the stimulation of cystine uptake, but cysteine did not. Incubation with either GSH or cysteine resulted in increased GSH in the control cells. The GSH levels in the arsenite + GSH-treated cells increased less than either the arsenite + cysteine-treated cells or the cells exposed to arsenite alone (Tables IV,V).

We have also compared arsenite to a number of compounds which had previously been investigated for their ability to induce stress proteins in a variety of cell types. Concentrations of the compounds were chosen so that no toxic effects were seen after 24 h of exposure. 5 μM Arsenite was more effective than 25 μM solutions of DEM or 12.5 μM solutions of disulfiram (another sulfhydryl reagent). Paraquat, which has been reported not to induce stress proteins, only slightly stimulated GSH levels and cystine uptake. Menadione had a similarly modest effect on both parameters (Table VI).

Discussion

We have shown that sodium arsenite is a powerful inducer of the protein-synthesis dependent transport system for cystine and glutamate in bovine pulmonary artery endothelial cells. Previously, we have shown that hyperoxia, DEM (an electrophilic agent which conjugates enzymatically with GSH) and BCNU (an inhibitor of glutathione reductase) also induce synthesis of this protein [15–18]. Bannai and co-workers [21] have recently reported similar effects of sodium arsenite on cystine transport and intracellular GSH levels in mouse peritoneal macrophages, although the stimulation we observed in endothelial cells was even larger than that reported for the macrophages [21]. The transport system induced is sodium-independent and specific for cystine and glutamate, characteristic of the x_c^- system defined by Bannai and coworkers for fibroblasts [20]. No significant depletion of GSH preceded induction of this transport system. We saw no toxic effects of arsenite on our cells at levels of 2.5 μM or lower, as measured by decrease in cell number, increases in non-adherent cells or Trypan blue uptake. Other investigators have similarly reported no significant toxicity of these levels of arsenite in bovine carotid artery endothelial cells measured by Cr^{2+} release [27] or in CHO cells, as measured by decreased plating efficiency [28]. Thus, induction of cystine transport and concomitant increases in intracellular GSH levels occur after exposure to arsenite at subtoxic levels, as well as levels which have no measurable depleting effect on GSH.

As seen in Table IV, both extracellular glutathione and cysteine increase intracellular GSH levels in control cells by 24 h of exposure. This may be due to a combination of effects. Cysteine is more efficiently transported than cystine and initially provides a rapid increase in intracellular glutathione [29]. As it oxidizes, the extracellular concentration of cystine would be increased, providing another source of substrate for the x_c^- transport system, thus, further increasing intracellular glutathione in the arsenite treated cells where the x_c^- system is induced. Glutathione probably increases intracellular glutathione levels by initially reducing cystine in the medium to cysteine which is then transported by the ASC system [30]. Eventually, breakdown of glutathione by γ-glutamyl transpeptidase could also contribute to the cysteine pool available for intracellular synthesis of GSH. The lack of increase in intracellular GSH in arsenite treated glutathione supplemented cells compared to arsenite treated controls suggests that no additional cystine is supplied to the induced x_c^- system by extracellular GSH.

Indeed, extracellular GSH partially inhibits the induction of cystine transport by arsenite (Table VI). This may be due either to formation of a GSH-arsenite complex or because extracellular GSH can protect cell proteins from reactions of sulfhydryls with arsenite. Cysteine, apparently, is ineffective in this regard, perhaps because it is rapidly taken up by the cells.

The stress response of endothelial cells to oxidizing agents or sulfhydryl modifiers thus appears to result in induction of different proteins at different times. The first group, including the 32–35 kDa protein identified as heme oxygenase typically appears within 0.5–2 h following stresses with oxidants or sulfhydryl agents [9,10,12,13]. Synthesis of the cystine-glutamate transport protein induced by hyperoxia, electrophilic agents, H_2O_2 or sulfhydryl-reactive agents begins after a lag of 4–6 h and levels reach a maximum by 16–24 h. Longer term exposure to hyperoxia results in induction of catalase and superoxide dismutase, beginning 2–3 days after onset of exposure [31,32]. Of the agents which we

have shown to induce cystine transport, e.g., hyperoxia, H_2O_2, BSO, DEM, BCNU and arsenite, the common factor may be their ability to directly or indirectly affect the SH levels in cells. Trivalent arsenicals are regarded as sulfhydryl reagents based on their ability to inhibit a variety of thiol-dependent enzyme systems. Direct metabolic regulation by thiol reagents has been demonstrated in a variety of systems, including xanthine oxidase, pyruvate oxidase, protein kinases and cytochrome *b* [33].

The mechanism of regulation of cystine-glutamate transport by arsenite could involve direct reaction with a cysteine residue of a nucleic-acid binding protein or be mediated indirectly. For example, protein kinases may be activated which could then affect nucleic-acid binding phosphoproteins. Alternately, expression of early stress proteins, including heme oxygenase, might be a prerequisite for subsequent induction of cystine transport by mechanisms as yet unknown. Determining the molecular mechanisms for the control of cystine transport and GSH regulation and isolation of the cystine transport protein are targets for future endeavor.

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