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PROTEIN-SPECIFIC S-THIOLATION IN HUMAN ENDOTHELIAL CELLS DURING OXIDATIVE STRESS

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Abstract—Confluent human umbilical vein endothelial cells were treated with diamide, *t*-butyl hydroperoxide (*t*-BH) or the hydrogen peroxide generating system glucose/glucose oxidase and the effects on glutathione oxidation and protein S-thiolation were examined. In the presence of all three oxidants glutathione was rapidly oxidized to a similar extent and S-thiolation of a limited number of proteins occurred. Diamide caused considerable S-thiolation of proteins with molecular masses of 44, 34, 24 and 14 kDa, of which the protein with molecular mass of 44 kDa was most extensively modified. *t*-BH caused extensive modification of proteins with molecular masses of 24 and 14 kDa whilst hydrogen peroxide caused S-thiolation of proteins of 39, 24 and 14 kDa. This study shows that S-thiolation of proteins is an important metabolic response to oxidant insult in human endothelial cells and that the specificity of the response depends on the chemical nature of the oxidant.

Endothelial cells, particularly within the capillary bed of the lungs, have been shown to be sensitive to oxidant insult both *in vivo* and *in vitro* [1]. The source of the oxidant insult in the lung may vary from exposure to oxidant gases such as NO_x and O_3 [2, 3], exposure to high partial pressure of oxygen [4], or exposure to reactive metabolites, such as $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$, generated during the reperfusion of ischemic tissue [5] or by activated polymorphonuclear leucocytes [6]. Several studies have concurred that neutrophil-mediated endothelial toxicity may be caused by hydrogen peroxide [7, 8].

Endothelial cells possess an extensive network of both soluble and membrane-associated antioxidants [9], including vitamin E [10] and glutathione, along with its associated peroxidase and reductase enzymes [9, 11]. However, cells derived from human sources appear to be deficient in catalase activity [11, 12], indicating that the human endothelium relies rather heavily on the glutathione system for the detoxication of reactive oxygen metabolites [9, 11]. Exposure of cells to oxidants results in the formation of GSSG (glutathione, oxidized form†), which is generally not accumulated within the cell. Both rapid rereduction, catalysed by a NADPH-dependent GSSG reductase [13], and extrusion from the cell by the activity of ATP-dependent translocases located on the cell plasma membrane [14] account for this phenomenon. In addition, GSSG has been shown to partake in protein S-thiolation with protein thiols to form mixed disulfides [15]. It is generally assumed that the absolute intracellular GSSG level may be critical to the amount of protein mixed disulfides formed [16]. Glutathione–protein mixed disulfide formation has been suggested to occur with a certain degree of specificity (selectivity) to cellular proteins, since protein thiol groups exhibit a considerable heterogeneity in terms of their individual pK_a values and their location in protein structures. Indeed, work in myocytes has shown that *t*-butyl hydroperoxide (*t*-BH)- and diamide-stimulated protein S-thiolation is protein-specific [17].

All three of the above processes involving GSSG, i.e. rereduction, extrusion and protein mixed disulfide formation, may be involved in oxidant-induced cell damage in human endothelial cells. We have therefore studied the fate of GSH (glutathione, reduced form) and GSSG during short-term oxidative stress in cultured human umbilical vein endothelial (HUVE) cells. The data indicate rapid oxidation of GSH to GSSG induced by *t*-BH, H_2O_2 and diamide, with subsequent oxidant- and protein-specific S-thiolation of HUVE cell proteins and the extrusion of cysteine equivalents from the cells.

MATERIALS AND METHODS

Chemicals and media. Diamide, *t*-BH, cycloheximide and cystine were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Glucose oxidase was from Boehringer-Mannheim (Mannheim, Germany). L-[^{35}S]Cystine (955.7 Ci/mmol) was obtained from DUPONT, NEN Research products (Dreieich, Germany). Acrylamide, *N*,*N*-ethylene-bisacrylamide and SDS were obtained from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of the highest purity commercially available. All materials for the isolation and culture of HUVE cells were obtained from Nordvacc (Stockholm, Sweden), except for human serum

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† Abbreviations: GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; HUVE cells, human umbilical vein endothelial cells; *t*-BH, *t*-butyl hydroperoxide; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; PCA, perchloric acid.

(B+ve), which was obtained from a local blood donation center.

Cell culture. Umbilical cords were obtained less than 6 hr *post-parturitum* from a local delivery center. The cells were isolated by perfusion of the umbilical vein with collagenase according to the method of Jaffe *et al.* [18]. Isolated cells were applied to gelatinized culture plates (94 mm) and allowed to adhere overnight in medium M 199 supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), fungizone (2.5 μg/mL) and human serum (10% v/v). Non-adherent cells (erythrocytes and smooth muscle cells) were removed by washing the endothelial cell monolayer with phosphate-buffered saline (PBS) (KCl 0.2 g/L, NaCl 8 g/L, KH_2PO_4 0.2 g/L, $Na_2HPO_4 \times 7\ H_2O$ 2.16 g/L, pH 7.4). Cells were grown to confluency (4–5 days) in the culture medium.

For the experiments, cells of the second and third passage were depleted of their glutathione [19] by a 24 hr incubation in medium devoid of any low molecular mass sulfur-containing components, e.g. cystine, cysteine, GSH and methionine, [M 199(−)] supplemented with penicillin (100 U/mL)/streptomycin (100 μg/mL) and human serum (10% v/v). As shown previously [19], the human bank serum does not provide any reduced low molecular mass thiols due to their oxidation and subsequent mixed disulfide formation with serum proteins during storage. The cells were then incubated for 6 hr in fresh medium M 199(−) containing cycloheximide (10 μM) and L-[^{35}S]cystine (50 μM, 15 μCi/plate for gel samples and 5 μCi/plate for the determination of free glutathione and cellular [^{35}S]cysteine equivalent efflux) in order to label intracellular GSH without introducing radioactivity into the cell protein [20]. Hydrogen peroxide produced by glucose (1 g/L in medium) and glucose oxidase (10 U/mL) in M 199(−)-supplemented with 10% (v/v) human serum and 50 μM cystine was determined by the method of Boutin *et al.* [21]. The viability of the cells was measured by a lactate dehydrogenase (LDH) leakage assay [22].

Gel electrophoresis. Cell extracts were prepared from rapidly washed cultures (4 × 5 mL PBS) by perchloric acid (PCA) precipitation of the protein (7% PCA, containing 50 mM *N*-ethylmaleimide). The protein precipitate was scraped from the plate, centrifuged (10,000 *g* for 5 min) and washed three times with PCA (7% PCA containing 50 mM *N*-ethylmaleimide) and once with ice-cold ether. Cell protein was solubilized in SDS (1% w/v), sonicated for 30 sec and resolved electrophoretically on 1.5 mm thick, 12% SDS–polyacrylamide gels according to the method of Laemmli [23]. Gels were stained with Coomassie Brilliant Blue R-250, photographed and the dried gels were exposed to Kodak SB-5 film for autoradiography.

Analysis of cellular thiols and glutathione reductase. Glutathione was analysed by the method of Reed *et al.* [24] with the modifications described [25]. For the determination of the specific activity of [^{35}S]-glutathione GSH was analysed using monobromobimane [26] because of the higher sensitivity and the much shorter processing time required. Protein-bound GSH was quantitated by determination of the radioactivity of the washed (3 × 5 mL 5% PCA) and dissolved protein (in 2% SDS w/v) using a Beckman Model LS 1801 Liquid Scintillation Counter and correlation with the specific activity values of the cellular GSH. Glutathione reductase activity was measured by the method of Racker [27] and protein was determined using the method of Peterson [28].

Efflux of [^{35}S]*cysteine equivalents.* Preliminary experiments showed that it was not possible to detect the possible efflux of GSSG into the medium using the method of Reed *et al.* [24] because of the high dilution in the medium. Similarly, GSH could not be detected in the medium by the monobromobimane method [26]. Thus, we decided to measure the efflux of ^{35}S-equivalents from labeled cells. Cells were depleted of their GSH and repleted with a [^{35}S]-cystine-containing medium for 6 hr as described above, and then washed with PBS supplemented with 1% bovine serum albumin (4 × 5 mL at 37°) in order to remove the unbound radioactivity from the plate. Fresh M 199(−) was added and the cells were treated with the oxidants. At various time points triplicate medium samples (200 μL) were taken and the radioactivity determined. At the end of the incubation time cells were lysed (1% Triton v/v, containing 1% SDS w/v) and the residual cell-associated radioactivity was determined.

RESULTS

Cultured endothelial cells at the second and third passage contained 16–20 nmol GSH/mg protein. When cells were incubated in a sulfur-deficient medium [M 199(−)] for 24 hr cellular GSH levels

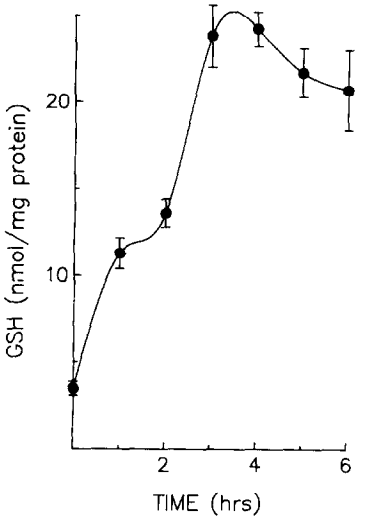

Fig. 1. The kinetics of resynthesis of glutathione in medium-depleted cells in the presence of cycloheximide. HUVE cell cultures were depleted of GSH as described in Materials and Methods and repleted with 50 μM cystine in fresh medium [M 199(−)], supplemented with 10 μM cycloheximide for the times indicated. Cellular glutathione was assayed as in Materials and Methods. Data are means ± SD (N = 3).

Table 1. The effects of diamide, *t*-BH and hydrogen peroxide on the reduced, oxidized and protein-bound glutathione contents of HUVE cells

Treatment	Time (min)	GSH (nmol/mg)	GSSG* (nmol/mg)	Protein-bound GSH† (nmol/mg)
None	0	16.2 ± 0.5	ND	1.0 ± 0.4
Diamide (0.2 mM)	1	3.3 ± 0.6	ND	6.0 ± 2.0
	3	2.7 ± 0.4	0.9 ± 0.3	8.0 ± 3.2
	7	3.3 ± 0.2	0.4 ± 0.1	14.7 ± 7.4
	15	6.9 ± 0.6	0.2 ± 0.1	8.5 ± 3.6
t-BH (0.2 mM)	1	5.6 ± 1.7	0.5 ± 0.1	3.0 ± 1.2
	3	5.8 ± 1.9	0.6 ± 0.2	3.7 ± 0.6
	7	6.7 ± 0.2	0.7 ± 0.4	3.1 ± 1.3
	15	6.3 ± 0.4	0.2 ± 0.1	4.6 ± 2.3
Glucose/glucose oxidase (10 U/mL)	1	3.4 ± 1.2	0.9 ± 0.4	1.9 ± 1.3
	3	4.6 ± 2.1	0.6 ± 0.1	3.2 ± 2.2
	7	3.8 ± 1.5	1.2 ± 0.6	3.8 ± 2.9
	15	3.7 ± 0.7	1.0 ± 0.1	3.9 ± 2.3

* The cell cultures were treated as indicated, washed, extracted and GSSG was analysed. ND, not detected (< 0.1 nmol/mg).

† The cells were treated as indicated, washed and precipitated. Radioactivity was counted in the washed cell protein. Protein-bound GSH was calculated on the basis of the specific activity of GSH (2–4 × 10^3 dpm/nmol).

The values in control cells did not change during the 15 min of incubation. Data are means ± SD (N = 3).

were decreased to *ca.* 15% of control values (Fig. 1). When the cells were then reincubated with medium containing only cystine (50 μM) as low molecular mass sulfur source, in the presence of cycloheximide (10 μM), cellular GSH levels were rapidly repleted to control levels by 6 hr of incubation. When these repleted cells were treated with diamide, *t*-BH or H_2O_2 cellular GSH levels declined rapidly with the concurrent formation of GSSG. Table 1 shows that the incubation of HUVE cells with 0.2 mM diamide resulted in an 80% depletion of GSH within the first minute. This level remained stable for the subsequent 6 min of the incubation but rose to 45% of controls by 15 min. Accumulation of GSSG was first detected by the third minute of the incubation at a peak of 0.9 ± 0.3 nmol/mg protein, declining steadily thereafter. Similar kinetics of depletion and repletion of

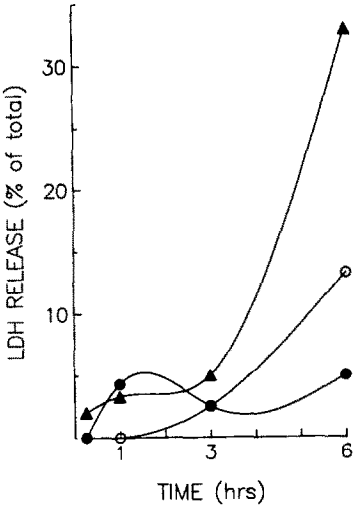

Fig. 2. The acute cytotoxicity of diamide, *t*-BH and glucose/glucose oxidase in HUVE cells. Cells were depleted of their GSH and repleted again as described in Fig. 1. Cellular viability was assessed by the leakage of LDH from the cells. Data are means ± SD (N = 3). Cells treated with 0.2 mM *t*-BH (▲), 1 g/L glucose/10 U/mL glucose oxidase (○) or 0.2 mM diamide (●).

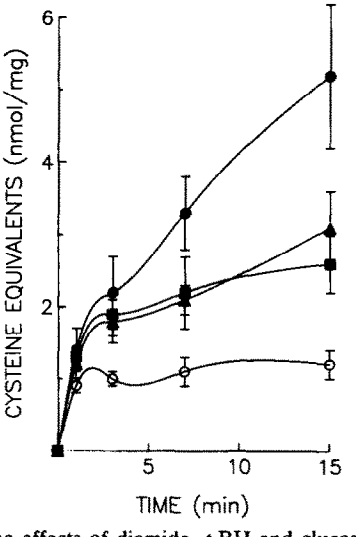

Fig. 3. The effects of diamide, *t*-BH and glucose/glucose oxidase on the efflux of [^{35}S]cystine equivalents from HUVE cells. Cell cultures were depleted of glutathione and repleted as in Fig. 1 but with the addition of L-[^{35}S]-cystine during resynthesis. Cultures were then treated with either 0.2 mM diamide (●), 0.2 mM *t*-BH (△) or 1 g/L glucose/10 U/mL glucose oxidase (■) [control cells (○)]. Radioactivity was measured in the medium and the data were converted to nmoles cysteine equivalents based on the specific activity of cellular GSH (4.3 × 10^3 dpm/nmol). Data are expressed as means ± SD of four experiments with different cell preparations and analysed using Student's unpaired *t*-test. After 1 min of incubation $P \leq 0.05$ on all test points. All other values differed significantly ($P \leq 0.005$) from controls.

HUVE cell GSH were noted when the cells were treated with 0.2 mM *t*-BH. However, GSSG accumulation was detected after the first minute, rising to a peak of 0.7 ± 0.4 nmol/mg protein by the seventh minute and declining by the end of the incubation (15 min). Treatment of the cells with 10 U/mL glucose oxidase produced 0.52 ± 0.05 mM H_2O_2 (N = 4) after 1 min of incubation, increasing to 0.99 ± 0.08 mM H_2O_2 (N = 4) after 15 min of incubation and caused a sustained depletion of GSH of between 70% and 80% throughout the test period and accumulation of GSSG of between 0.6 ± 0.1 and 1.2 ± 0.6 nmol/mg protein. GSH was never completely oxidized and the cells always contained at least three times as much GSH as GSSG. HUVE cells at the second and third passage contained a glutathione reductase activity of 11.5 ± 2.5 nmol/mg/min (N = 3). There was considerable inter-batch variability in response of the cells to the oxidants but effects on the cellular GSH/GSSG ratios displayed similar tendencies in the three experiments performed. During these treatments no loss of viability occurred (as assessed by LDH leakage, Fig. 2) and the cells did not show any morphological changes. However, extended exposure of the cells to the oxidants at the doses employed resulted in release of LDH which amounted to 33, 13 and 5% of control cellular levels for *t*-BH, glucose oxidase and diamide, respectively, by 6 hr of incubation (Fig. 2).

Since only a small percentage of the GSH lost was recovered as intracellular GSSG, the release of [^{35}S]cystine equivalents into the medium was measured from cells repleted as above in the presence of [^{35}S]cystine (Fig. 3). Control cells released 0.9 ± 0.1 nmol cysteine equivalents/mg protein into the medium after 1 min, with little additional accumulation of radioactivity noted during the 15 min of incubation. All of the oxidants tested caused elevated efflux of ^{35}S from the cells. Similar kinetics of release caused by *t*-BH and H_2O_2 were noted with peak levels of 3.1 ± 0.5 and 2.6 ± 0.4 nmol/mg noted after 15 min, respectively. Diamide stimulated a considerably greater release of radioactivity with 5.2 ± 1.0 nmol cysteine equivalents/mg protein noted in the medium by 15 min of incubation.

The oxidant-stimulated S-thiolation of HUVE cell bulk protein is shown in Table 1. By separation of these S-thiolated proteins on gels the specificity of mixed disulfide formation could be determined (Figs 4–6). Diamide caused rapid and extensive S-thiolation of HUVE cell proteins with 6.0 ± 2.0 bound nmol GSH/mg protein noted after 1 min,

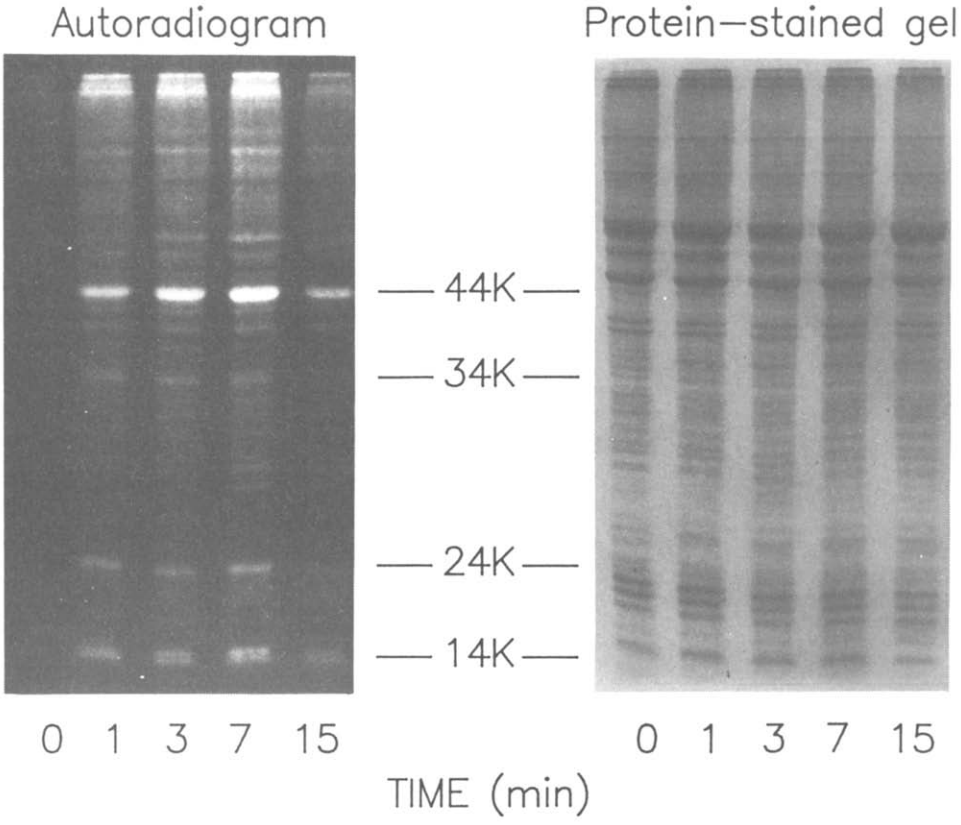

Fig. 4. The protein specificity of diamide-induced S-thiolation of HUVE cell protein. HUVE cell glutathione was labeled with L-[^{35}S]cystine as in Fig. 3. Radioactive cultures were treated with 0.2 mM diamide for the times indicated. Cells were then extracted and proteins were separated by electrophoresis and autoradiographed as described in Materials and Methods.

rising to 14.7 ± 7.4 nmol bound GSH/mg protein by 7 min and declining thereafter (Table 1). These kinetics of S-thiolation of the bulk protein caused by diamide were almost exactly paralleled by the labelling of individual protein bands with the molecular masses of 14, 24, 34 and 44 kDa (major S-thiolated species). Labeled proteins were also noted with molecular masses > 200 kDa (Fig. 4). All radioactive bands were completely removed by pretreatment of the protein with 10 mM dithiothreitol before electrophoresis (data not shown). In contrast to diamide, both hydroperoxides induced lower and generally stable levels of S-thiolation of HUVE cell bulk protein (Table 1). Again in contrast to diamide, neither *t*-BH nor H_2O_2 induced labeling of the 44 kDa protein band (Figs 5 and 6), but *t*-BH did stimulate the labeling of a band at 24 kDa, which was stable throughout the experimental period, and a band at 14 kDa whose labeling increased steadily with time (Fig. 5). H_2O_2 also stimulated the labeling of the 14 kDa and 24 kDa bands, but the 14 kDa band was more heavily labeled than in the cells treated with the organic hydroperoxide. A highly labeled band at 39 kDa was also noted with H_2O_2, which reached a maximum after 7 min and declined thereafter (Fig. 6).

DISCUSSION

It is well known that oxidants such as H_2O_2 deplete endothelial cells of their GSH [29]. In the present paper we have studied the fate of glutathione in human endothelial cells undergoing oxidant insult. Diamide and the two hydroperoxides caused rapid depletion of GSH but the kinetics of GSSG accumulation and extrusion from the cell, as well as the extent of protein S-thiolation, were all clearly oxidant-specific. For instance, although GSSG was detected in the hydroperoxide-treated cells after only 1 min of incubation, an equimolar concentration of diamide initially caused measurable GSSG accumulation at 3 min. The reason for the delay in GSSG accumulation in the presence of diamide may be due to the mechanism of GSH oxidation by diamide which involves an initial adduct formation. Furthermore, GSSG levels declined after peak values in diamide- and *t*-BH-treated cells. When the cells were incubated with continuously generated H_2O_2, GSSG levels remained stable. In most cells the activity of GSSG reductase is central to the maintenance of the GSH/GSSG ratio within the cells. HUVE cells of the second and third passage contain only about 20% of the glutathione reductase

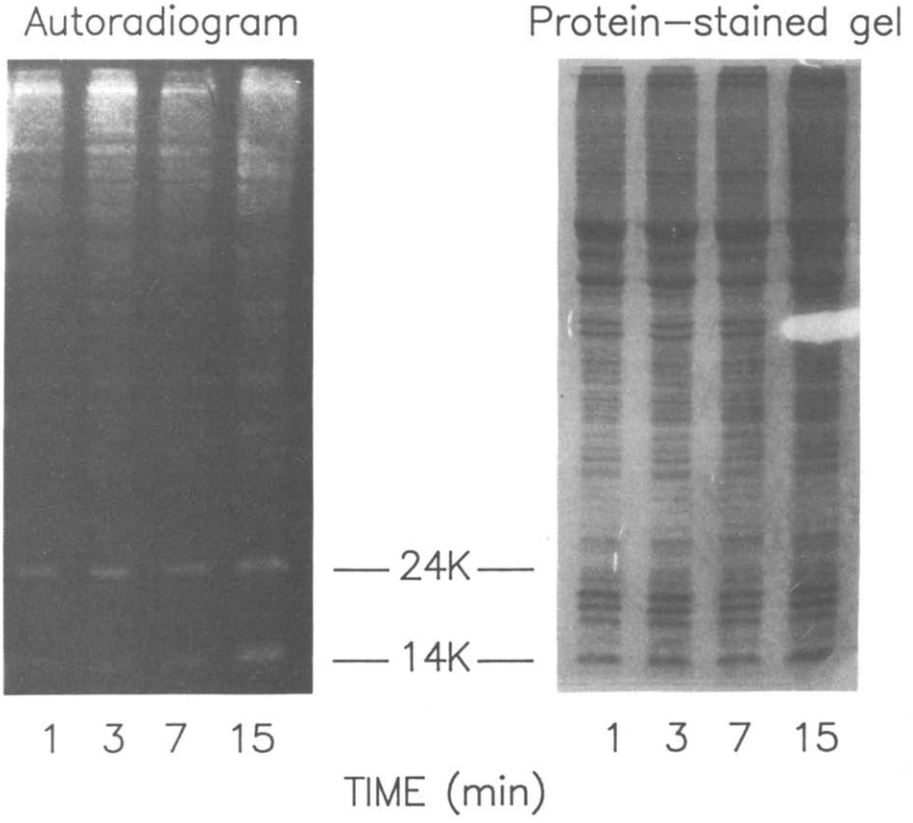

Fig. 5. The protein specificity of *t*-BH-induced S-thiolation of HUVE cell protein. HUVE cell GSH was labeled with L-[^{35}S]cystine as described in Fig. 3. Radioactive cultures were treated with 0.2 mM *t*-BH for the times indicated. Cells were extracted and proteins were separated by electrophoresis and autoradiographed as described in Materials and Methods.

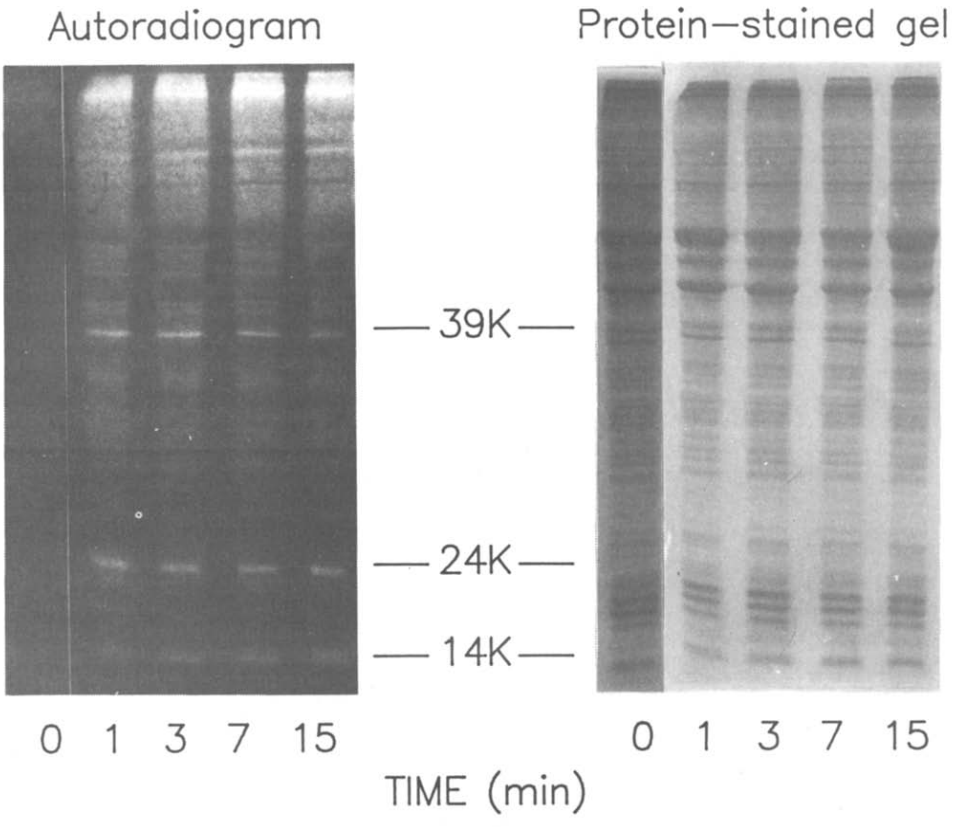

Fig. 6. The protein specificity of hydrogen peroxide-induced S-thiolation of HUVE cell protein. HUVE cell GSH has labeled with L-[^{35}S]cystine as described in Fig. 3. Radioactive cultures were treated with 10 U/mL glucose oxidase for the times indicated. Cells were extracted and proteins were separated by electrophoresis and autoradiographed as described in Materials and Methods.

activity of hepatocytes [13]. This much lower activity in cultured HUVE cells may not be sufficient to ensure full rereduction of GSSG during oxidant insult and allow other GSSG-specific processes to dominate the disposition of the disulfide.

In hepatocytes a plasma membrane ATPase is known to transport GSSG out of the cell [14]. HUVE cells may have such an extrusion mechanism as indicated by the release of labelled cysteine equivalents, presumably mainly due to GSSG, in the presence of all three oxidants. The rate of extrusion was highest following diamide treatment and may be due to some direct oxidative alteration of the extrusion mechanism by diamide. Indeed, the ATP-dependent GSSG translocase in hepatocytes has been shown to be activated by agents which modify protein thiols [14]. Determining the nature of this glutathione extrusion mechanism will be an aim for further studies.

Much of the GSSG in oxidant-treated HUVE cells resides in DTT-reducible mixed disulfides with proteins. Work in isolated hepatocytes [16] and in perfused liver [30] has suggested that the formation of mixed disulfides is strictly correlated with the GSSG concentration measured in the cells. This may not be the case in HUVE cells. Although the oxidants used in the present study result in similar levels of GSSG accumulation in the cells, there is oxidant-specificity in the extent of protein S-thiolation. The origins of these differences may lie in factors such as the rate of and cellular localization of the reaction between the oxidant and GSH, as well as the proximity and reactivity of recipient protein thiols.

In comparison with another well studied cell type, the hepatocyte, the extent to which oxidant-stimulated S-thiolation of HUVE cell protein can occur appears to be greater. Table 1 shows that about 80% of the diamide-oxidized GSH is located on the cellular protein after 7 min of incubation. This is in contrast to hepatocytes undergoing redox cycling-dependent oxidative stress stimulated with either paraquat [16] or diquat [31] which sustain a maximum of $<10\%$ and 5%, respectively, of their GSH bound to hepatocellular protein. Diamide stimulated more rapid and extensive protein S-thiolation than the hydroperoxides. It is a chemical oxidant and may directly oxidize protein and GSH causing S-thiolation without passing through GSSG as an intermediate. Hydrogen peroxide and *t*-BH generate GSSG essentially by glutathione peroxidase activities in the cytosol. However, Park and Thomas [32] have suggested an oxiradical- and H_2O_2-initiated mechanism of protein S-thiolation during oxidative stress without extensive oxidation of glutathione to GSSG.

HUVE cell proteins with a molecular mass of 14 and 24 kDa appear to be common substrates for S-thiolation caused by all of the oxidants tested. Additionally, oxidant- and protein-specific reversibility of S-thiolation was evident during the experiments. Thus, diamide-induced S-thiolation of bulk cellular protein was to some extent reversible between 7 and 15 min of the incubation (Table 1), and was reversible on the gels, especially for the 44 kDa protein (Fig. 4).

Reversibility of S-thiolation of HUVE cell protein caused by the hydroperoxides was not, however, evident from the bulk protein measurements. On the gels, the labeling of the 39 kDa band stimulated by H_2O_2 was reversible to some extent between 7 and 15 min of incubation, although the cells were continuously exposed to H_2O_2 produced by glucose/glucose oxidase. The mechanism of the rereduction of protein mixed disulfides is uncertain and may involve enzymes such as thiol transferases [33], protein disulfide isomerase [34] or GSSG reductase itself. The role of such enzymes in the control of redox events on HUVE cell proteins is the subject of further investigations.

The activity of a number of receptors, enzymes and structural proteins may be modulated by alterations to their thiol–disulfide redox balance [35]. Such alterations may compromise the delicate integration of such activities into complex physiological processes and predispose the cell to cytotoxicity. However, that the rapid stimulation of protein S-thiolation seen in these experiments, with the doses of the oxidants used, did not lead to immediate cytotoxicity. Indeed, stimulated S-thiolation alone may not be sufficient to initiate toxicity as the viability of the diamide-treated cells was largely unaffected by 6 hr of incubation, whereas *t*-BH elicited 30% toxicity at this time. In addition to stimulating glutathione oxidation and protein S-thiolation, the hydroperoxide is also able to elicit other oxidative changes within the cells such as lipid peroxidation and DNA damage.

Further studies are required in order to determine the effects of higher doses of oxidants on protein S-thiolation and cell viability, as well as the effect of extended periods of exposure on steady state levels of oxidants and the kinetics of rereduction of mixed disulfides following removal of the stimulus. The possibility of enzyme-catalysed thiol-disulfide exchange reactions being involved in these observations is also the subject of continuing study.

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