Oxidation of Sulphydryl Groups of Ribonuclease Inhibitor in Epithelial Cells Is Sufficient for Its Intracellular Degradation*

Montserrat Blázquez, J esús M. Fominaya, and J an Hofsteenge†

From the Friedrich Miescher-Institut, CH-4002 Basel, Switzerland

Ribonuclease inhibitor (RI) is a cytoplasmic protein (50 kDa) that inhibits a variety of pancreatic type RNases. The porcine inhibitor contains 30 cysteine residues, all of which occur in the reduced state. It is well known that in vitro modification of the thiol groups inactivates the protein and greatly increases its susceptibility to proteolysis. Here we show that oxidation of thiol groups in RI can also occur within the cell. Induction of an oxidative insult in cultured LLC-PK₁ cells, either with a general oxidant, H₂O₂, or with a thiol-specific oxidant, diamide, led to the loss of RI activity. By using specific antibodies it was demonstrated that the decrease correlated with a decline in the amount of RI protein in the cells. Furthermore, analysis of RI mRNA levels and half-life of the protein excluded inhibition of the synthesis of RI as the cause of its depletion. The results indicate that oxidation of thiol groups in RI is sufficient to cause its rapid inactivation and disappearance from the cell. Most likely this results from intracellular degradation of the protein.

The cytosol of virtually all cells contains a powerful inhibitor protein for ribonucleases of the pancreatic type (1). The approximately 50-kDa protein forms a 1:1 complex with the enzymes with a dissociation constant in the femtomolar range (2–4). Apart from the interesting primary and tertiary structure (5–8), this protein has a peculiarly high number (9–10) of free thiol groups (4). Comparison of the amino acid sequences of RI from human, pig, and rat shows that 27 of these cysteine residues are conserved (5–7, 9), suggesting that they play an important structural and/or functional role. One possible function is that modification of the thiol groups might be a means to regulate the activity of RI (10, 11). This hypothesis is based on the observations that RI is rapidly inactivated by thiol-blocking reagents like p-hydroxymercuribenzoate and N-ethylmaleimide (12–14) or oxidizing agents like 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (15). In vitro studies using stoichiometric concentrations of DTNB have revealed two interesting features. First, the reaction occurs in an "all-or-none" fashion, i.e. it is highly cooperative, leading to the formation of inactive RI containing 15 disulfide bridges. Second, the disulfide cross-linked RI was very sensitive to proteolysis as compared to the native protein. Thus, the structural prerequisites for regulation of RI activity by means of thiol groups are given.

Intracellular oxidative stress is caused by increased concentrations of reactive oxygen species (reactive oxygen species, e.g. O₂⁻, OH, H₂O₂) (16). This may result from aging or pathogenic conditions, e.g. cataract formation (17, 18), or the presence of chemicals that mediate the production of such oxygen species (19). It has been known that these reactive oxygen species may damage proteins by oxidizing the amino acid side chains of cysteine, methionine, tryptophan, tyrosine, histidine, and even aliphatic amino acid residues. Oxidatively damaged proteins are removed from the cell by proteolysis (20, 21). In addition, oxygen radical production has also been implicated in apoptosis (22, 23).

Apart from these phenomena, oxidation and reduction have been proposed to play a role in a number of proteins (mainly transcription factors) whose activity is regulated by a thiol switch that is supposedly operated by changes in the intracellular redox potential (24–27).

Given the changes induced in RI by thiol oxidation in vitro (15), it would be of interest to examine whether oxidation of thiol groups can also occur within the cell and whether it results in increased sensitivity to proteolysis. To this end we have incubated LLC-PK₁ cells (28) with hydrogen peroxide and with diamide, a thiol-specific oxidant. The results show that oxidation of thiol groups is sufficient to cause removal of RI activity and protein from the cell. Analysis of mRNA levels and protein half-life indicate that most likely a proteolytic mechanism is involved.

**EXPERIMENTAL PROCEDURES**

Materials

Bovine pancreatic RNase A, diamide, and l-buthionine-(S,R)-sulfoximine (BSO), were obtained from Sigma. Perhydrol 30% (H₂O₂) was from Merck. DTNB, GSH, Triton X-100, leupeptin, pepstatin A, benzamidine HCl, and phenylmethylsulfonyl fluoride were from Fluka Chemie AG. Tween-20, nitrocellulose, and peroxidoase-conjugated goat anti-rabbit secondary antibody were purchased from Bio-Rad. Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), phenol, methionine, and cysteine were from Life Technologies, Inc. Trypan blue solution 0.5% was from Serva. ECL Western blotting detection reagents and Hybond-N⁺ membranes were obtained from Amersham Corp. Protein A-Sepharose 4 Fast Flow was from Pharmacia Biotech Inc. DIG RNA labeling (SP6/T7) and DIG nucleic acid detection kits were from Boehringer Mannheim. Trans²⁺S-labeled was purchased from ICN Pharmaceuticals. All other chemicals were of the highest purity available.

Methods

In Vitro Assay with Diamide and Thermolysin Digestion—RI (1 µM) was incubated with various concentrations of diamide (0.125–1 mM) in 50 mM Tris-HCl, pH 7.5, containing 0.6 mM EDTA at 37 °C for 30 min. Aliquots were diluted 500-fold in 0.1% bovine serum albumin in H₂O and used directly in the RNase assay.

Cleavage of diamide-treated RI with thermolysin and analysis by SDS-polyacrylamide gel electrophoresis were performed as described.
previously (15).

Cell Culture and Treatments—LLC-PK1 cells (28) were obtained from Dr. Y. Nagamine (Friedrich Miescher-Institut, Basel) and maintained in DMEM supplemented with 10% v/v heat-inactivated FCS in a 95% air, 5% CO2 humidified incubator at 37 °C.

LLC-PK1 cells in logarithmic phase (2 × 105 cells/10 cm dish) were treated with 5 mM H2O2 for 30 min either with or without pretreatment with 250 μM BSO for 24 h in order to deplete GSH levels. Cells at the same density were also treated with 250 μM diamide for various lengths of time. In this case, glucose was omitted from the medium. Unless specified otherwise, incubations with the various oxidants were carried out in serum-free medium.

Preparation of Cytoplasmic Extracts—At the end of the incubations, cells were washed three times with phosphate-buffered saline, frozen in liquid nitrogen, and kept at −80 °C until used. Before extraction, the cells were thawed on ice and removed from the plate with a rubber policeman in 0.3 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2 mM diithiothreitol, 0.25 mM ascorbate, 0.1% Triton X-100, 0.1 μg/ml leupeptin, 0.1 μg/ml pepstatin A, 1 μg/ml benzamidine HCl, 0.1 mM phenylmethylsulfonyl fluoride). Unless specified otherwise, all steps were performed at 4 °C.

Cells were lysed by vortexing and incubation on ice for 10 min. The extracts were centrifuged at 3,000 × g for 10 min, and the supernatants were centrifuged at 270,000 × g for 30 min. The final supernatants were ether frozen in dry ice and stored at −80 °C or processed immediately.

RI Activity Assay—RI activity was determined by titrating the inhibitor with a known amount of RNase A. The concentration of RNase A was determined from its absorbance at 280 nm, using A280 = 0.73 mg ⁻¹·ml·cm⁻¹ (29, 30). The remaining RNase activity was determined using total yeast RNA as a substrate as described previously by Fornay et al. (31). A Shimadzu UV-240 spectrophotometer was used to measure the absorbance at 260 nm, and the readings were corrected for the appropriate blanks. RI activity was assayed as units of RI/mg of protein, defining 1 unit as the amount of RI required to inhibit the activity of 5 ng of RNase A by 50% (13).

Protein Determination—The total protein concentration in cell extracts was calculated using the method of Bradford (32) as adapted by BioRad.

Lactate Dehydrogenase Assay—Enzyme activity was assayed following the conversion of pyruvate and NADH into lactate and NAD⁺ according to the procedure described by Storrie et al. (33). The decrease in absorbance at 340 nm was registered and lactate dehydrogenase activity was assayed as nmol of NAD⁺ min⁻¹·mg⁻¹ protein.

Cell Viability—To determine the percentage of viable cells after each treatment, the cells were trypsinized, resuspended in medium, mixed with trypan blue solution (1:1 v/v), and counted. Cell viability was calculated as the percentage of trypan blue-excluding cells with respect to the total cell count.

GSH Measurement—The DTNB colorimetric method (34) was used for determination of GSH. Cells were washed three times with phosphate-buffered saline and scraped off with a rubber policeman in 0.3 ml of 0.67% (w/v) freshly prepared metaphosphoric acid. This treatment resulted in cell lysis and precipitation of the proteins. Under these conditions, GSH represents the only measurable soluble thiol within the cells. Samples were centrifuged and aliquots of 0.2 ml were mixed with 0.7 ml of 0.3 mM sodium phosphate buffer, pH 8.7, and 0.1 ml of 0.04% DTNB in 1% sodium dodecyl sulfate buffer, pH 6.8. After 5 min, the absorbance at 412 nm was measured and the amount of soluble thiol was determined by comparison with GSH standards.

Western Blotting—Protein samples (2.5 μg) were analyzed by SDS-polyacrylamide gel electrophoresis on 10 or 12.5% slab gels according to the method of Laemmli (35). The separated proteins were transferred to nitrocellulose and the blots were blocked overnight at 4 °C with a nonfat dry milk in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl (TBS), containing 0.1% Tween 20 (TBS-T). RI was detected with an affinity-purified rabbit anti-ribonuclease inhibitor antibody (20 ng/ml) against recombinant porcine RI and a horseradish peroxidase-coupled goat anti-rabbit secondary antibody (1:10,000 dilution). The blots were developed using the enhanced chemiluminescence (ECL) detection system according to the manufacturer's protocol (Amersham Corp.). Exposure time on x-ray film ranged from 20 to 60 s.

RNA isolation and Northern Blot Analysis—Cells were grown until the late exponential phase on 6-cm tissue culture dishes were washed with methionine- and cysteine-free medium (FCS was also omitted), followed by metabolic labeling with 100 μCi/ml Trans[35S]label in the above medium for 50 min. Cells were washed three times with DMEM and chased in the same medium containing 2 mM Met and 1 mM Cys and supplemented with 10% FCS for the indicated times. Cells extractions and preparation for Northern analysis were immunoprecipitated by incubation for 1 h at 4 °C with the purified antibodies (0.125 μg/μg of total protein), followed by an overnight incubation with 50 μl of a 50% solution of protein A-Sepharose beads (Pharmacia). The immunocomplexes collected with the beads were washed three times with 10 mM sodium phosphate, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, and 0.1% Tween 20, and the antigens were eluted from the beads by boiling for 3 min in gel sample buffer as described (37). The samples were resolved using a 10% SDS-polyacrylamide gel, and the radiolabeled bands containing RI were quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

RESULTS

Effect of Hydrogen Peroxide—The incubation of cultured LLC-PK1 cells with 5 mM H2O2 for 30 min decreased the level of RI activity by 40% compared to untreated control cells (Fig. 1A). Reduced glutathione (GSH) can act as a scavenger for H2O2 and radicals generated from it (38). Therefore, it was of interest to examine the effect of buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis (39). Pretreatment of LLC-PK1 cells with 250 μM BSO for 24 h reduced the concentration of GSH to 20% of control levels, but the RI activity remained essentially the same (Fig. 1A). After further incubation with 5 mM H2O2 for 30 min, the RI activity had decreased to approximately 20% of that of untreated cells. Under the experimental conditions used, total cell number did not decrease, and cell viability as determined by trypan blue exclusion was always higher than 90% (data not shown), indicating that cell death was not the cause of the decrease in RI.

While morphology of the cells incubated either with 5 mM H2O2 or 250 μM BSO was like that of the control, the cytoplasts of the BSO-preincubated cells treated with H2O2 became granular and vacuolated after 15–20 min of incubation (data not shown). The reductions in RI activity correlated with a decrease in the amount of RI protein in the cells as demonstrated by Western blot analysis (Fig. 1B).

These results indicated that an oxidizing environment lead to the loss of RI, but the specific involvement of cysteine residues cannot be concluded given the general nature of hydrogen peroxide oxidation.

Effect of Diamide—To learn more specifically about the role of thiol oxidation in RI inactivation, the effect of diamide, a thiol-specific oxidant (40, 41), was examined in detail. In vitro pilot experiments showed that diamide completely oxidized the 30 thiol groups of RI with concomitant loss of activity (data not shown). Treatment of the fully oxidized protein with thermolysin (1%) w/w for 30 min at 37 °C resulted in complete degradation, as demonstrated by SDS-polyacrylamide gel electrophoresis (data not shown). In contrast, the native protein was completely resistant to proteolysis under the same conditions. Increased susceptibility of RI oxidized to proteolysis has previously been observed using DTNB as the oxidizing reagent (15).

Incubation of LLC-PK1 cells with 250 μM diamide rapidly decreased RI activity in a time-dependent manner, reaching approximately 12% of the control after 50 min (Fig. 2A). This process was preceded by an even faster decline of the concentration of GSH. Western blot analysis showed that the decrease
in RI activity was not simply due to inactivation, as the protein also disappeared from the cells (Fig. 2B).

It was important to ascertain that these changes were not caused by cell death or irreversible cell damage. For this purpose, cells treated with diamide for 40 min were allowed to recover from the oxidative insult for 24 h, during which RI activity was followed. The results in Fig. 3 demonstrate that after this period RI activity was restored to near control levels. This increase was due to reappearance of the RI protein as found by Western blot analysis (data not shown). Furthermore, the increase in the number of cells during this 24-h period was normal, indicating that no irreversible damage had been invoked. Moreover, during the incubation with diamide, total cell number did not decrease and cell viability was always higher than 90%. The morphology of the cells remained normal, at least for the initial 20 min. After this, vacuoles appeared in the cytoplasm (data not shown).

The results presented in Fig. 4 show that diamide did not have an effect on cytoplasmic proteins in general. Neither the activity of a cytoplasmic enzyme, lactate dehydrogenase, nor the recovery of the amount of total protein changed during incubation with diamide.

Given the increased susceptibility of thiol-oxidized RI to proteolysis in vitro, it seemed likely that also intracellularly such a mechanism was responsible for the diamide-induced decrease in the level of RI protein. If, however, RI had a very short half-life, an inhibitory effect of diamide on its synthesis could also explain the observations. Since the half-life of RI has not been determined previously, a pulse-chase experiment was performed. From the results summarized in Fig. 5, a value of $8.5 \pm 0.62$ h was obtained for untreated LLC-PK$_1$ cells, which is much longer than the 50 min period over which the decrease
in RI was observed. Furthermore, diamide did not affect the steady-state level of the mRNA encoding RI, as was demonstrated by Northern blot analysis (Fig. 2C).

To prove that degradation of RI took place in the cell and not during manipulation of the samples, the following experiment was performed. Fully denatured and carboxymethylated RI (cmRI) is easily degraded by proteases (5, 15) and can easily be distinguished from endogenous RI due to its lower electrophoretic mobility in SDS-polyacrylamide gels. Incubation of cmRI for 2 h at 4°C with extracts obtained from cells treated with diamide for 0, 30, or 50 min showed no degradation (Fig. 6). The same result was obtained when the experiment was performed at 37°C (data not shown).

**DISCUSSION**

In vitro studies on RI have shown a clear dependence of its inhibitory activity on the preservation of its cysteines in the reduced state (4, 6). The presence of reducing agents like di-thiothreitol or β-mercaptoethanol (13, 42, 43) as well as antioxidants (metal chelators like EDTA) (44) have been shown to be essential in maintaining RI stable and active. Here we have investigated in cultured cells the effect of oxidants on intracellular RI. Oxidative damage to intracellular proteins is accepted as a "marker" for proteolytic degradation (20, 21). Studies on these aspects of protein turnover usually employ compounds that create reactive oxygen species which can react with a number of amino acids side chains (20, 21).

Incubation of LLC-PK1 cells with hydrogen peroxide caused a decrease in RI activity. Using specific antibodies, we could demonstrate that this decrease was due to disappearance of the protein (Fig. 1B) and not simply to inactivation or complex formation with endogenous RNases. Under normal conditions reduced glutathione maintains a reducing environment in the cytoplasm which protects proteins containing sulfhydryl groups like RI from inactivation (38, 45). In agreement with this it was found that the inactivating effect of H$_2$O$_2$ on RI was augmented by inhibiting GSH synthesis by BSO (Fig. 1). H$_2$O$_2$ is not a specific oxidant for cysteines. It can also oxidize the side chains of Met, Trp, Tyr, and His and generate protein-protein cross-linkage (20, 21). In order to examine the role of thiol oxidation in intracellular RI inactivation, a thiol-specific oxidant was used.
Diamide can react with both protein and nonprotein thiols like GSH. Incubation of LLC-PK₁ cells with diamide rapidly decreased the level of GSH and was followed by a decline in RI activity and protein (Fig. 2). RI is not inactivated due to the low level of GSH. Using BSO to reduce the concentration of GSH to the same level as in the diamide experiment did not result in a decrease in RI (Fig. 1A). The same result was obtained using ethacrynic acid, an inhibitor of glutathione-S-transferase (data not shown). Furthermore, inhibition of the reduction of GSSG with 1,3-bis(2-chloroethyl)-1-nitrosourea had no effect (data not shown). Most likely, the diamide that remains after oxidizing GSH acts directly on intracellular RI, in agreement with the in vitro and complexed state of the protein to this reagent. The rapid decrease in the level of GSH upon addition of diamide demonstrated that the effect of the reagent involved a direct reaction with thiol groups in the cytosolic fraction. In agreement with this, we found that treatment of the cells with DTNB, which is impermeable to cell membranes, did not alter the GSH or RI level (data not shown).

The decrease in RI protein could be due to either an increased rate of degradation or an inhibition of RI synthesis in combination with a normal high turnover. The former seems to be the case. RI has a half-life of 8.5 h in untreated LLC-PK₁ cells. If diamide would only inhibit the synthesis of RI and not affect its degradation, a 6.6% decrease over the experimental period would be expected. Since nearly 90% of RI disappeared, an “active” mechanism must be operating. Given the sensitivity of thiol-oxidized RI to proteolysis in vitro (15) and the role of oxidation in “marking” proteins (20, 21), an intracellular proteolytic event seems a reasonable explanation. The possibility that, in addition to this, diamide has an effect on the translation of the mRNA for RI cannot be excluded. However, the steady-state level of RI mRNA was unaltered, showing that transcription was not influenced by diamide (Fig. 2C). These results show that oxidation of thiol groups in intracellular RI is sufficient for its inactivation and further degradation in the cell. It is important to note that the conditions used in these experiments did not produce a general cell damage and that the effects of diamide were reversible (Fig. 3).

At this point it may be useful to discuss briefly the methods used to determine RI. In the past, numerous studies on the effect of physiological stimuli and chemical compounds on RI have used activity measurements only (1). Since artificial complexes with endogenous RNases may form upon tissue disruption (1, 46) and specific activity and relative amounts of such RNases are not known, the amount of free or complexed RI cannot be determined accurately. In LLC-PK₁ cells the level of endogenous RNases is too low to be detected by the methods used. Thus the RI activity measurements are not affected by fortuitous complex formation. Furthermore, specific probes, antibodies against RI and CDNA encoding RI, were used to qualitatively assess changes in protein and mRNA level. The quantitative application of such analyses to some of the systems in which changes in RI activity have been observed, e.g. growth and development, disease, hormones, compounds affecting cellular functions, or physical stimuli (see Ref. 1 for an overview), will be needed to unravel the biological function of RI.

Whether thiol modification is a physiological mechanism of modulating RI activity as suggested previously (10, 11), as has been found for other proteins (24–27, 47, 48), remains to be examined. It is not known whether thiol oxidation plays any role in the normal turnover (tₜ₀ = 8.5 h) of RI, but it is conceivable that accumulation of oxidized thiols in RI alters its conformation and makes it more susceptible to proteolysis (15). In certain pathological conditions like cataract formation, an increase in the production of oxidants has been observed (17, 18), and under these circumstances RI activity decreases (49–51). However, changes in the levels of RI protein have not been examined in this case. Our results using H₂O₂ and LLC-PK₁ cells directly correlate oxidation, RI instability, and degradation of the protein and extend the studies on cataract formation. It will be of interest to examine the level of RI protein using specific antibodies in this and other pathological situations in which an increase in oxidative stress has been observed.

In summary, we have shown that oxidation of thiol groups in intracellular RI is sufficient for its removal from the cell. In agreement with in vitro experiments (15), increased proteolysis seems to be the most likely mechanism.

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