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THE ROLE OF THIOL OXIDATION IN COBALT(II)-INDUCED TOXICITY IN HAMSTER LUNG

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Abstract—Exposure of lung tissue to Co(II) ions both *in vivo* and *in vitro* results in toxicity, a relatively early event of which is the oxidation of cellular glutathione. In this study we have attempted to delineate the relationship between this oxidation of glutathione and the subsequent development of cellular dysfunction. Simultaneous incubation with H_2O_2 potentiated Co(II)-induced increases in both levels of oxidized glutathione (GSSG) and the activity of the pentose phosphate pathway in hamster lung slices. This effect was initially synergistic and, thereafter, both parameters were maintained at significantly greater levels than with either treatment alone throughout the incubation period until the onset of detectable cellular dysfunction. When dysfunction occurred, however, it was not quantitatively increased by the co-treatment over that occurring with $CoCl_2$ alone. Similarly, pretreatment of slices with the glutathione reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) potentiated the Co(II)-induced increase in levels of GSSG. However, this effect was again not associated with an enhancement of cell dysfunction. Since the Co(II)-induced cell damage appeared not to be related directly to the oxidation of glutathione, the quantitative significance of the latter was investigated by comparison with the known oxidant *tert*-butyl hydroperoxide (t-BOOH). At a concentration of 100 μM, t-BOOH caused an increase in the concentration of GSSG in BCNU-pretreated lung slices which was comparable to that after treatment with Co(II)/H_2O_2 or CO(II)/BCNU. None of these treatments resulted in a loss of protein thiols. Furthermore, in contrast to Co(II), t-BOOH/BCNU treatment did not result in impaired cell functions. However, at a t-BOOH concentration of 250 μM, t-BOOH/BCNU treatment caused a significantly greater increase in the level of GSSG than that caused by the previous treatments and was associated with both a loss of protein thiols and increased cell dysfunction. We have concluded from these data that under our experimental conditions, Co(II)-induced cell dysfunction is not a consequence of oxidation of cellular glutathione. The reason for this appears to be that the extent of glutathione oxidation by Co(II) even at a concentration which induces cell dysfunction is not of sufficient magnitude to result in the oxidation of protein thiol groups, an event which is likely to constitute the critical consequence of glutathione oxidation in the toxic process.

Occupational exposure to cobalt-containing dust has been associated with various forms of lung disease, an extreme form being the development of a potentially fatal interstitial fibrosis [1–3]. The biochemical mechanisms underlying this toxicity are not understood [4, 5]. However, we have suggested that the generation of oxidative damage may play a role in this process, having demonstrated previously that the oxidation of glutathione and an increased activity of the pentose phosphate pathway (PPP†)—changes indicative of the generation of oxidative stress—constitute early biochemical events in lung tissue exposed to Co(II) ions [6]. The nature of the reactive species involved in the oxidation of glutathione and the relevance of thiol oxidation in the pulmonary toxicity of Co(II) ions remain, however, unestablished.

Several recent studies have demonstrated that in the test tube, Co(II) ions can react with H_2O_2 to produce a reactive species which appears to be at least in part hydroxyl radical [7–9]. On the basis of these observations, we have put forward the hypothesis that the generation of reactive oxidant species in such a reaction between Co(II) ions and endogenous H_2O_2 may underlie the oxidative changes observed in Co(II)-treated lung tissue. In this study, we demonstrate a synergistic effect of $CoCl_2$ and exogenous H_2O_2 in oxidizing glutathione and stimulating the PPP in hamster lung slices, and examine the significance of these changes in thiol status in terms of cell toxicity. In addition, we have employed the glutathione reductase inhibitor BCNU as a second method of enhancing Co(II)-induced glutathione oxidation and, again, have related this increased oxidation to indices of cellular dysfunction.

MATERIALS AND METHODS

Reagents. $CoCl_2 \cdot 6H_2O$ (99% pure) was obtained from U.C.B. Belgium (Vel SA/NV, Leuven, Belgium). H_2O_2 (30% v/v) was purchased from Merck-Schuchardt (Munich, Germany). [1,4-^{14}C]-Putrescine dihydrochloride (110 mCi/mmol), [1-^{14}C]glucose (55.9 mCi/mmol), [6-^{14}C]glucose (55.7 mCi/mmol) and L-[4,5-^{3}H]leucine (140 Ci/mmol) were purchased from Amersham SA/NV (Brussels, Belgium). Waymouths MB 752/1 medium

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† Abbreviations: GSSG, oxidized glutathione; GSH, reduced glutathione; PPP, pentose phosphate pathway; KRP, Krebs–Ringer phosphate medium; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; t-BOOH, *tert*-butyl hydroperoxide.

was purchased from Gibco BRL (Paisley, U.K.). BCNU (Nitrumon) was a gift from Sintessa SA/NV (Brussels, Belgium). Soluene 350 tissue solubilizer, and Ultima Gold and Emulsifier Safe scintillation cocktails were obtained from Packard Instruments (Brussels, Belgium). 4-Vinylpyridine was obtained from Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were obtained from either U.C.B. Belgium (Vel SA/NV, Leuven, Belgium) or Sigma Chemie GmbH (Deisenhofen, Germany).

Animals. Six- to ten-week-old male or female Syrian Gold hamsters bred locally in conventional animal facilities and of body weight approximately 85 g were used throughout. No differences were apparent between males and females either in basal results or in response to treatments.

Incubation of lung slices with $CoCl_2/H_2O_2$. Animals were lightly anesthetized with ether and killed by an overdose of pentobarbital sodium (Sanofi, Brussels, Belgium) and (on cessation of reflex) severance of the abdominal vessels. The lungs were washed free of blood *in situ*, by the injection of 10 mL 0.9% saline via the pulmonary artery, a small cut being made in the left atrium to allow the efflux of the perfusate. The lungs were removed and slices 0.7 mm thick were prepared using a McIlwain tissue chopper (Mickle Laboratories, Guildford, U.K.). The slices were incubated in 3 mL modified KRP [NaCl (130 mM), KCl (5.2 mM), $CaCl_2$ (1.9 mM), $MgSO_4$ (1.29 mM), Na_2HPO_4 (10 mM), glucose (11 mM), pH 7.4] at 37° in a shaking water bath (120 cycles/min). $CoCl_2$, H_2O_2 and/or t-BOOH were added to the incubation medium immediately after the addition of the tissue, so that any generation of oxidant species would take place entirely in the presence of the tissue.

Analytical procedures. To determine levels of protein thiols, lung slices (30–40 mg) were homogenized in 2 mL 6% (w/v) trichloroacetic acid at 4° and centrifuged (20 min, ~1500 *g*, 4°). The resultant pellet was washed again with 2 mL trichloroacetic acid (6% w/v) and suspended in 2 mL Tris–HCl (0.5 M, pH 7.6) to which was added 100 μL 10 mM 5,5′-dithiobis(2-nitrobenzoic acid). After standing at room temperature for 20 min, the insoluble material was removed by centrifugation (10 min, 1500 *g*, 4°) and the absorbance of the resulting supernatant fraction was measured at 412 nm against a standard curve of GSH, using a Beckman Du-65 spectrophotometer.

GSSG was determined as described previously [6] by a modification of the glutathione reductase recycling assay of Griffith [10], adapted from that of Tietze [11]; 4-vinylpyridine being used for the removal of GSH.

The metabolism of glucose through the PPP or the glycolytic/tricarboxylic acid pathway was determined as the NaOH-trapped $^{14}CO_2$ derived from [1-^{14}C]- or [6-^{14}C]glucose respectively, and protein synthesis was measured as the stable incorporation of L-[4,5-^{3}H]leucine into acid insoluble cellular material, as described previously [6].

Preincubation with BCNU. The effect of BCNU on glutathione oxidation was determined using an adaptation of the lung slice system developed by Hardwick *et al.* [12]. Lung slices were incubated with BCNU (100 μM) for 45 min at 37° in 3 mL Waymouths MB 752/1 medium supplemented with serine (0.2 mM). BCNU was added in ethanol (final concentration 0.3%). Control slices were treated in the same way except that BCNU was omitted. (BCNU-pretreated slices retained normal levels of GSH throughout the subsequent incubation period, in agreement with the findings of Hardwick *et al.* [12]). After preincubation, slices were removed from the medium, washed with 2 × 1 mL KRP and incubated in 3 mL KRP as described above.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test for paired data. Significance was determined at $P < 0.05$. Usually, experimental groups contained four animals. In most instances, experimental treatments were repeated on separate days, allowing control data to be pooled and giving a group size of eight.

Synergy was said to occur when the effect of a combined treatment was significantly ($P < 0.05$) greater than the sum of the effects of the individual treatments. An "effect" was defined as the measured absolute difference between control and treated samples.

RESULTS

Effect of hydrogen peroxide on Co(II)*-induced glutathione oxidation*

Incubation of lung slices with $CoCl_2$ (1 mM) resulted in significantly increased levels of GSSG which were maintained throughout the 4-hr incubation period (Fig. 1a). Incubation with H_2O_2 (250 μM) had a similar effect, although statistical significance was not achieved until 2 hr, after which levels remained relatively constant throughout the period of incubation. Incubation of the slices with $CoCl_2$ and H_2O_2 together resulted, after 1 hr, in an increase in levels of GSSG which was significantly greater than the sum of the effects of the individual treatments (Fig. 1a). This synergistic effect was evident only after the first hour of incubation. Nevertheless, levels of GSSG remained significantly higher than with either treatment alone throughout the first 3 hr of incubation, falling at 4 hr to levels which were not significantly different from those seen after either individual treatment (Fig. 1a).

Similarly, the activity of the PPP was significantly increased throughout the period of incubation with $CoCl_2$ or H_2O_2 alone (Fig. 1b). Again, incubation with the two together resulted in a stimulation which, during the first hour, was greater than the sum of the effects of the individual treatments. Again, although this synergism was not evident at subsequent time points, the activity of the pathway remained significantly greater after co-treatment than after either treatment alone, for 3 hr. During the 4th hr, the stimulation after co-treatment was not significantly different from that after either individual treatment (Fig. 1b).

During the first 3 hr of incubation, no significant changes were observed with any of the treatments in the ability of the lung slices to metabolize [6-^{14}C]glucose to $^{14}CO_2$ (Fig. 2a) or to incorporate [^{3}H]leucine into protein (Fig. 2b), parameters which we considered to be indicative of cell viability.

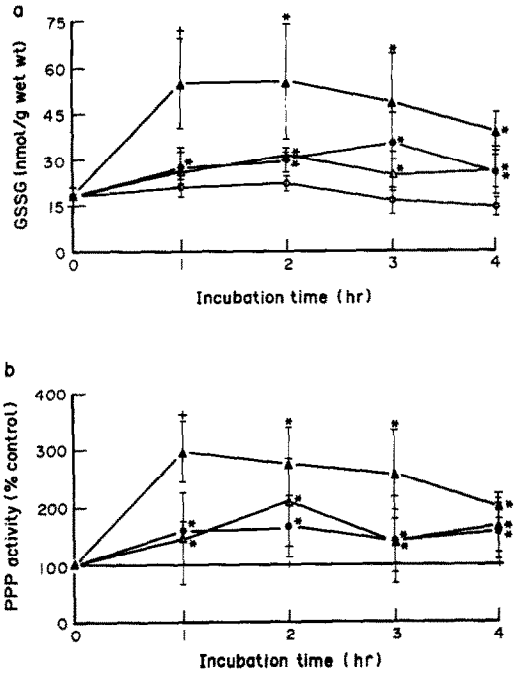

Fig. 1. (a) Levels of GSSG and (b) activity of the PPP in hamster lung slices incubated with $CoCl_2$ (1 mM) and/or H_2O_2 (250 μM). GSSG was determined by the glutathione reductase recycling assay, after the removal of GSH with 4-vinylpyridine. The activity of the PPP was determined as the trapped $^{14}CO_2$ radioactivity derived from [1-^{14}C]-glucose minus that from [6-^{14}C]glucose. Control values were 4217 ± 1120, 5143 ± 674, 9171 ± 2122 and 12170 ± 3317 dpm/100 mg wet weight during the 1st, 2nd 3rd and 4th hr of incubation, respectively. Results are expressed as the means ± SD of eight observations. *$P < 0.05$ compared to control; + $P < 0.05$ compared to the sum of the effects of the individual treatments. (○) Control, (●) 1 mM $CoCl_2$, (△) 250 μM H_2O_2, (▲) 1 mM $CoCl_2$ + 250 μM H_2O_2.

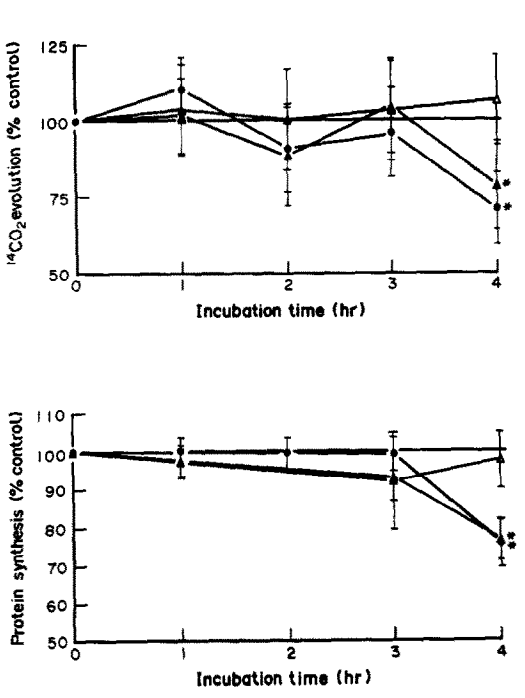

Fig. 2. (a) Metabolism of [6-^{14}C]glucose and (b) protein synthesis by hamster lung slices during incubation with $CoCl_2$ (1 mM) and/or H_2O_2 (250 μM). [6-^{14}C]Glucose metabolism was defined as the $^{14}CO_2$ radioactivity trapped during incubation. Control values were 2127 ± 412, 6651 ± 720, 11,132 ± 1339 and 15,261 ± 3355 dpm/100 mg wet weight during the 1st, 2nd, 3rd and 4th hr of incubation respectively. Protein synthesis was determined as the [^{3}H]-leucine-derived radioactivity associated with acid-insoluble cellular material after washing with inorganic and organic solvents. Control values were 10,038 ± 1721, 9471 ± 2170, 11,321 ± 2003 and 9432 ± 1132 dpm/100 mg weight weight during the 1st, 2nd, 3rd and 4th hr of incubation, respectively. Results are expressed as the means ± SD of eight observations. *$P < 0.05$ compared to control; (●) 1 mM $CoCl_2$, (△) 250 μM H_2O_2, (▲) 1 mM $CoCl_2$ + 250 μM H_2O_2.

During the 4th hr, slices incubated in control medium or with H_2O_2 alone again exhibited no changes in either of these functions; however, significant decreases in both parameters were observed at the 4th hr in those slices incubated with $CoCl_2$ alone or with $CoCl_2$ and H_2O_2 together. In each case, these changes did not significantly differ, quantitatively, between the two treatments (Fig. 2a and b).

Effect of BCNU on Co(II)*-induced glutathione oxidation*

Preincubation of lung slices with BCNU (100 μM) alone had no significant effect on GSSG levels or the activity of the PPP (Fig. 3a and b). However, when BCNU-treated slices were subsequently incubated with $CoCl_2$ (1 mM), levels of GSSG became significantly higher than those in non-pretreated slices. This effect was maintained throughout the incubation period (Fig. 3a). BCNU pretreatment also resulted in complete abolition of the Co(II)-dependent stimulation of the PPP observed in non-pretreated slices (Fig. 3b).

Preincubation with BCNU had no effect on the evolution of $^{14}CO_2$ from [6-^{14}C]glucose or the incorporation of [^{3}H]leucine into proteins. These parameters were decreased by $CoCl_2$ (1 mM) in both non-preteated and BCNU-pretreated slices but without a significant difference between the two groups (Fig. 4a and b).

Comparison of Co(II)*-induced glutathione oxidation with that of t-BOOH*

Since the previous experiments suggested that there was no direct relationship between Co(II)-induced glutathione oxidation and cellular dysfunction, the glutathione status of Co(II)-treated lung slices was compared with that of slices treated with the known oxidant, t-BOOH.

Incubation of BCNU (100 μM)-pretreated slices for 1 hr with t-BOOH (100 μM) resulted in an increased level of cellular GSSG which was comparable to that observed after a 2-hr incubation

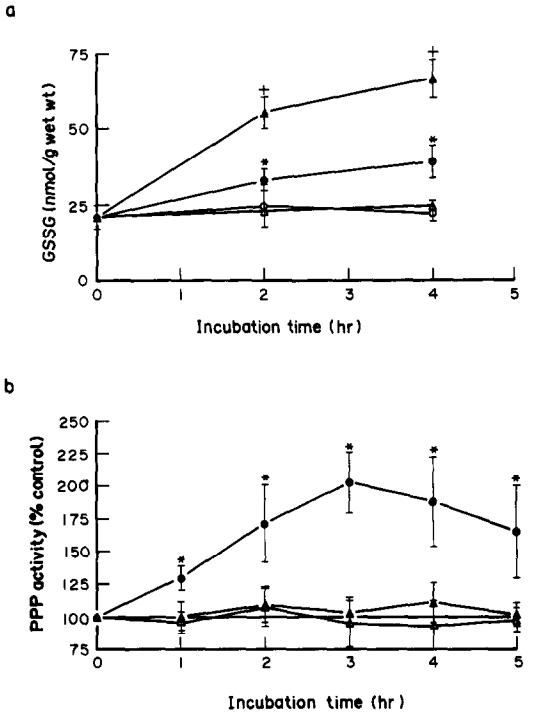

Fig. 3. Effect of preincubation with BCNU on Co(II)-induced increases in (a) levels of GSSG and (b) activity of the PPP. Slices were incubated for 45 min with BCNU (100 μM) prior to incubation with $CoCl_2$ (1 mM). GSSG was determined by the glutathione reductase recycling assay after removal of GSH with 4-vinylpyridine. The activity of the PPP was determined as the trapped $^{14}CO_2$ radioactivity derived from [1-^{14}C]glucose minus that from [6-^{14}C]glucose. Control values were 4258 ± 1147, 5847 ± 1555, 8989 ± 2245, 12,196 ± 2669 and 14,596 ± 3001 dpm/100 mg wet weight during the 1st, 2nd, 3rd, 4th and 5th hr of incubation, respectively. Results are expressed as the means ± SD of eight observations. *$P < 0.05$ compared to control; †$P < 0.05$ compared to Co(II) treatment alone. (○) Control, (△) 100 μM BCNU, (●) 1 mM $CoCl_2$, (▲) 100 μM BCNU + 1 mM $CoCl_2$.

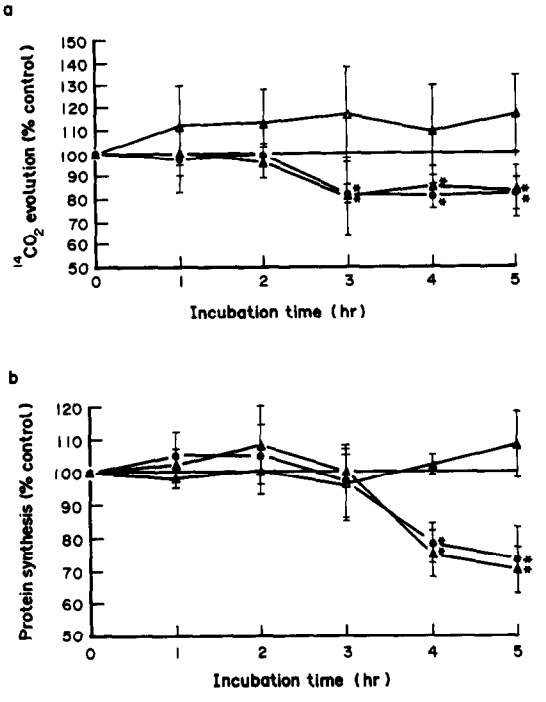

Fig. 4. Effect of preincubation with BCNU on Co(II)-induced decrease in (a) glucose oxidation and (b) protein synthesis. Glucose oxidation was defined as the $^{14}CO_2$ radioactivity trapped during incubation with [6-^{14}C]glucose. Control values were 2336 ± 801, 6965 ± 693, 11,245 ± 2562, 14,998 ± 2336 and 16,986 ± 3658 dpm/100 mg wet weight during the 1st, 2nd, 3rd, 4th and 5th hr of incubation, respectively. Protein synthesis was determined as the stable incorporation of radioactivity from L-[^{3}H]leucine into acid-insoluble cellular material. Control values were 11,245 ± 1458, 10,236 ± 1785, 11,698 ± 2004 and 12,889 ± 1269 dpm/100 mg wet weight during the 1st, 2nd, 3rd and 4th hr of incubation respectively. Results are expressed as the means ± SD of eight observations. *$P < 0.05$ compared to control; (△) 100 μM BCNU, (●) 1 mM $CoCl_2$, (▲) 100 μM BCNU + 1 mM $CoCl_2$.

with Co(II) (1 mM), either after BCNU pretreatment or in the presence of H_2O_2 (250 μM) (Fig. 5, cf. Figs 1a and 3a). None of these treatments resulted in detectable decreases in the levels of protein thiols (Fig. 6) or increased metabolic dysfunction (Fig. 7a and b, cf. Figs 2 and 4). However, incubation for 1 hr with t-BOOH (250 μM), again after pretreatment with BCNU (100 μM), resulted in significantly greater increases in GSSG levels than the aforementioned treatments and was associated with both a loss of protein thiols (Fig. 6) and increased cell dysfunction (Fig. 7a and b).

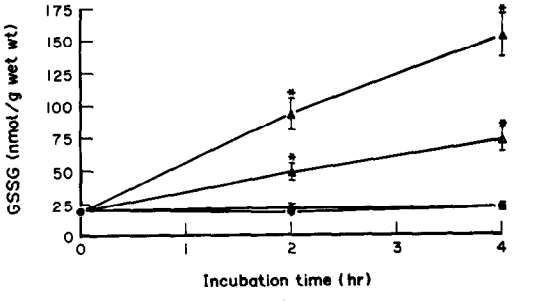

Fig. 5. Effect of t-BOOH on levels of GSSG in hamster lung slices pretreated with BCNU. Slices were incubated for 45 min with BCNU (100 μM) prior to incubation with t-BOOH. GSSG was determined by the glutathione reductase recycling assay, using 4-vinylpyridine to remove GSH. Results are expressed as the means ± SD of four observations. *$P < 0.05$ compared to control. (○) Control, (●) 100 μM BCNU, (△) 100 μM BCNU + 100 μM t-BOOH, (▲) 100 μM BCNU + 250 μM t-BOOH.

DISCUSSION

The role of GSH as a major cellular defence against oxidative insult is well recognized. Its antioxidant mechanism involves the chemical reduction (and, thereby, detoxification) of reactive oxidants, either through a direct reaction with radical

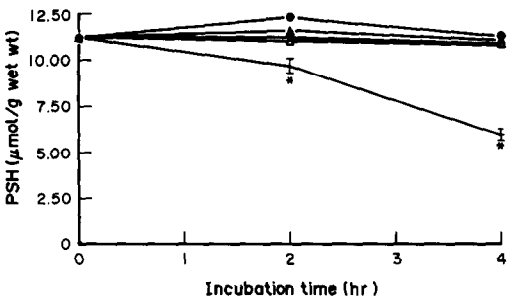

Fig. 6. Levels of protein sulphydryl groups in hamster lung slices. Protein sulphydryls were determined as the absorbance at 412 nm after incubation of acid precipitable cellular material with DTNB. Results are expressed as the means ± SD of four observations. *$P < 0.05$ compared to control. (○) Control, (●) 1 mM $CoCl_2$ + 250 μM H_2O_2, (△) 1 mM $CoCl_2$ after preincubation with 100 μM BCNU, (▲) 100 μM t-BOOH + 100 μM BCNU, (+) 250 μM t-BOOH + 100 μM BCNU.

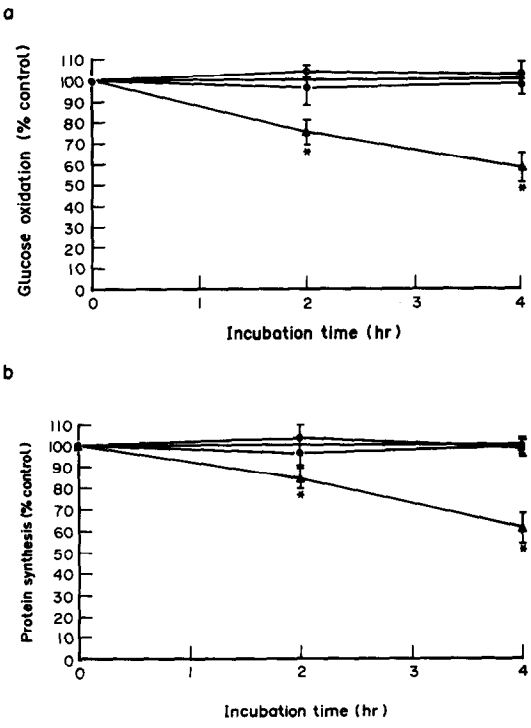

Fig. 7. Effect of t-BOOH on (a) glucose oxidation and (b) protein synthesis in BCNU-pretreated hamster lung slices. Glucose oxidation was defined as the $^{14}CO_2$ radioactivity trapped during incubation with [6-^{14}C]glucose. Control values were 2126 ± 635, 7009 ± 419, 10,016 ± 2111, and 16,229 ± 1933 dpm/100 mg wet weight during the 1st, 2nd, 3rd and 4th hr of incubation, respectively. Protein synthesis was determined as the stable incorporation of radioactivity from L-[^{3}H]leucine into acid-insoluble cellular material. Control values were 14,498 ± 1319 and 13,168 ± 1487 dpm/100 mg wet weight during the 2nd and 4th hr of incubation, respectively. Results are expressed as the means ± SD of four observations. *$P < 0.05$ compared to control; (○) 100 μM BCNU, (●) 100 μM BCNU + 100 μM t-BOOH, (△) 100 μM BCNU + 250 μM t-BOOH.

species in a radical transfer process or through its role as reductant in the glutathione peroxidase-mediated reduction of H_2O_2 and lipid hydroperoxides. In both cases, GSH is oxidized to the disulphide, GSSG [13]. This can be reduced back to the sulphydryl by NADPH-dependent glutathione reductase. A consequent decrease in levels of NADPH is associated with an increased activity of the PPP which in the lung constitutes the major source of reducing equivalents (i.e. NADPH) [14]. Thus, an increase in cellular levels of GSSG and an increased activity of the PPP may represent early cellular changes in response to oxidative stress [15]. We have found previously that these events occur in lung tissue upon exposure to Co(II) ions [6]. In the present study the concentrations of GSH were not measured specifically. However, we have good evidence from other experiments [6] and as yet unpublished work, that GSH levels decrease upon incubation of lung slices with Co(II) ions, such that the GSSG/GSH ratio is certainly increased when GSSG concentrations increase. It is fair to assume that this is also the case with the other oxidant treatments used here, certainly within the time frame of our *in vitro* experiments.

In this study, we have observed that the initial increases in levels of GSSG and in the activity of the PPP in hamster lung slices after simultaneous incubation with $CoCl_2$ and H_2O_2 were significantly greater than the sum of the effects of the individual treatments (Fig. 1b). This indicates a cooperative interaction between the two treatments in the generation of an oxidizing activity.

A possible explanation for this effect is the occurrence of a Co(II)-mediated Fenton-type reaction with the generation of the highly reactive oxidant, hydroxyl radical.

$$Co^{2+} + H_2O_2 \rightarrow Co^{3+} + OH^- + OH^{\cdot}. \quad (1)$$

This hypothesis is based on observations that Co(II) ions are able to generate a hydroxyl radical-like activity, in the presence of H_2O_2, in the test tube [7–9]. An Fe-dependent generation of hydroxyl radicals by a similar mechanism has been proposed to underlie H_2O_2-induced DNA damage in a macrophage-derived cell line [16] and in human fibroblasts [17]. However, in reaction mixtures containing Co(II) and H_2O_2, electron spin resonance studies have identified the presence of singlet oxygen and superoxide anion in addition to that of hydroxyl radical [8, 9]. The present data do not allow us to eliminate a possible role for singlet oxygen or superoxide in the observed oxidation of glutathione; so far our use of various radical scavengers to provide an answer to this has not given conclusive results (unpublished data).

Effect of BCNU on Co(II)*-induced changes in glutathione status*

We have shown that Co(II)-induced oxidation of glutathione was potentiated by the preincubation of lung slices with BCNU (Fig. 3a). Presumably this is a consequence of the inhibition of glutathione reductase by BCNU leading to an impaired ability to reduce GSSG formed as a result of oxidative stress. We also observed that under these conditions,

the Co(II)-induced stimulation of the PPP was abolished. The activity of glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of the PPP, is known to be inhibited by NADPH with this inhibition being lost upon oxidation to $NADP^+$. This has been proposed to represent a means by which the pathway may be regulated (i.e. the removal of inhibition by NADPH during oxidative stress increases the activity of the pathway and thereby promotes the regeneration of NADPH) [18]. However, it has also been suggested that the inhibition of glucose-6-phosphate dehydrogenase by NADPH can be overcome by GSSG and that this represents a further degree of "fine control" of the pathway [19]. The data in the present study indicate that the prevention of NADPH utilization by glutathione reductase through the inhibition of the enzyme results in the abolition of the Co(II)-induced stimulation of the PPP. Moreover, this occurs despite increased levels of GSSG (due to the prevention of its reduction by the inhibition of glutathione reductase). This would suggest that, under these conditions, it is a decrease in levels of NADPH which underlies the stimulation of the PPP by Co(II) and not the increase in levels of GSSG.

Effect of glutathione oxidation on cell function

In our study we have used impairments in glucose oxidation and protein synthesis as indices of cellular dysfunction rather than, for example, the more conventional lactate dehydrogenase release which we feel might be a late and insensitive index of cell injury. Although changes in glucose oxidation and protein synthesis might be brought about by physiological adaptive mechanisms, we have evidence (submitted for publication) that, in the case of Co(II) exposure, impaired glucose oxidation is irreversible and therefore presumably not a simple biochemical response to oxidant stress.

Although the synergistic effects of the Co(II)/H_2O_2 co-treatment were observed only throughout the first hour of incubation, levels of GSSG and the activity of the PPP after this treatment remained significantly increased, relative to treatment with Co(II) alone, throughout the incubation period until the onset of detectable cellular dysfunction. However, the detection of indices of dysfunction occurred at the same time after both treatments and, moreover, these indices did not differ quantitatively between the two treatments (Fig. 2a and b). Similarly, the potentiation of Co(II)-dependent glutathione oxidation by pretreatment of the slices with BCNU (Fig. 3a) did not result in an enhancement of cell dysfunction (Fig. 4a and b). From these observations, therefore, we must conclude that although glutathione oxidation represents an early event in Co(II)-induced toxicity, it does not appear to be critical to the toxic process.

Studies have demonstrated that increased levels of cellular GSSG result in the oxidation of protein thiols through the formation of mixed disulphides [20, 21] (reaction a of Eqn 2). The oxidation of protein thiols has been suggested to be a significant event in the toxic mechanisms of several oxidants [18, 22, 23]. However, the formation of protein mixed disulphides is opposed by the reduction of GSSG back to GSH by glutathione reductase (reaction b) and by thiol transferase-mediated reduction of protein mixed disulphides to regenerate the protein thiol and GSSG (reaction c).

$$GSH \xleftarrow{b} GSSG + PSH \underset{c}{\overset{a}{\rightleftarrows}} PSSG + GSH \qquad (2)$$

where PSH is reduced protein thiol and PSSG is protein mixed disulphide. Tribble and Jones [24] have demonstrated that the loss of protein thiols in hepatocytes treated with the oxidant diamide occurred only when the ability of the cells to maintain sufficient levels of GSH was overcome, suggesting that the reducing power of GSH is utilized by the cell in the maintenance of protein thiol groups in the reduced form. Thus, the equilibrium of Eqn 2 lies to the left until forced to the right by a sufficient change in the relative concentrations of GSH and GSSG due to oxidation.

In lung slices treated with a concentration of Co(II) which results in cellular dysfunction (assessed by the ability to derive CO_2 from glucose and to synthesize proteins) no loss of protein thiol groups could be detected, even under conditions in which the oxidation of glutathione was potentiated by pretreatment with BCNU or co-incubation with H_2O_2. BCNU-pretreated slices incubated with the known oxidant t-BOOH at a concentration of 100 μM resulted in an increase in GSSG concentration comparable with that in the Co(II)-incubated slices co-treated with BCNU or H_2O_2. Again, no loss of protein thiols could be detected. However, in contrast to the Co(II)-treatment, no decrease in cellular function was apparent. On the other hand, incubation with t-BOOH (250 μM), which resulted in a significantly greater increase in the level of GSSG, was associated with a detectable loss of protein thiols and an increase in cellular dysfunction.

We showed previously that exposure of lung slices to Co(II) ions was associated with an increased level of protein mixed disulphides [6]. However, this represents a loss of only approximately 5% of the total protein thiols and in this study no significant loss of protein thiols could be detected. It appears, therefore, that whilst under our experimental conditions Co(II) ions were able to generate an oxidative stress which was able to perturb the cellular glutathione status; this oxidation of glutathione was not related directly to the observed toxicity. The reason for this may be that the loss of protein thiol groups associated with the oxidation of glutathione was (a) of insufficient magnitude and/or (b) did not occur at the critical cellular proteins to result in toxicity. The cellular dysfunction which occurred in Co(II)-treated tissue under these conditions is likely, therefore, to have been the consequence of an event other than the oxidation of GSH.

We are aware that our slice system is fraught with the problem of the cellular heterogeneity of the lung tissue and that events occurring in a small susceptible fraction of the total cellular population may not be detected against a large background of "no change". Studies with purified cell preparations or focusing on changes in selected enzymes should help to solve this.

Our conclusion does not necessarily contradict the hypothesis that cobalt toxicity in the lung is due to the generation of oxidative damage. Clearly, the increased levels of GSSG observed in this study indicate that Co(II) ions are associated with an oxidizing activity. Although the loss of protein thiols has been put forward as a potentially significant event in the development of oxidative toxicity, other intracellular loci such as DNA or membrane lipids have also been proposed as critical sites of acute oxidative damage [25–27]. Our data do not rule out the possibility that the toxicity of cobalt is mediated by oxidative effects but they suggest that the critical event in the toxic process occurs at a site other than the glutathione system.

In a broader perspective, our findings also suggest that alterations in the glutathione equilibrium need not necessarily be considered as critical events in the process of cell injury.

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