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EFFECT OF 1-METHYL-2-NITROSOIMIDAZOLE ON INTRACELLULAR THIOLS AND CALCIUM LEVELS IN CHINESE HAMSTER OVARY CELLS

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Abstract—The cellular reduction of 2-nitroimidazoles under hypoxic conditions can lead to cell killing. One of the postulated toxic intermediates is the two-electron reduction product, the nitrosoimidazole. 1-Methyl-2-nitrosoimidazole (INO) was used as a model to study the reactivity of 2-nitrosoimidazoles with sulfhydryls. INO reacted within minutes with bovine serum albumin (BSA) in a stoichiometric fashion as measured by the loss of its characteristic absorption at 360 nm. It appeared to react specifically with the SH group of BSA as demonstrated by the loss of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) reactive groups and by the loss of INO reactivity if BSA was previously reacted with DTNB. INO also depleted glutathione (GSH) and protein sulfhydryls (Pr-SH) in Chinese hamster ovary (CHO) cells in a concentration-dependent fashion. INO at 25 μM, a non-toxic concentration in terms of cell colony-forming ability, depleted GSH to 10–20% of control levels within 5 min after treatment. Pr-SH were depleted more slowly to 60% of control levels. GSH recovered to near control levels over 3–4 hr but Pr-SH remained depressed. The recovery of GSH was blocked by buthionine sulfoximine (BSO), suggesting that the recovery was due to *de novo* synthesis of GSH. At a toxic concentration of INO (45 μM), GSH was again depleted to 10–20% and Pr-SH to 50% of control levels. No recovery of either was observed up to 4 hr. The effect of this extensive oxidative stress on intracellular calcium (Ca_i^{2+}) levels was monitored using 1-[2-amino-5-(6-carboxyindole-2-yl)-phenoxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxy methylester (INDO-1 AM). At toxic concentrations of INO, Ca_i^{2+} increased in a sustained, non-physiological manner starting at approximately 60 min after the addition of INO. No increase in Ca_i^{2+} was observed when cells were treated with non-toxic concentrations of INO. INO toxicity may be modulated by an uncontrolled influx of Ca^{2+} which can trigger the activation of cellular enzymes and lead to cell death.

2- and 5-Nitroimidazoles have been investigated in clinical trials as radiation sensitizers of hypoxic cells [1–4] with a limited degree of success. They also exhibit selective toxicity towards hypoxic mammalian cells in experimental studies [5, 6], and this aspect of their mechanism of action may have a role to play clinically [1, 4]. This selective toxicity is believed to result from the reductive metabolism of the drug to nitroso, hydroxylamine or amine derivatives via radical intermediates [7–9]. This reduction is blocked or greatly reduced by oxygen acting at the nitro radical anion step. Using 1-methyl-2-nitroimidazole (INO_2§) as a model of 1-substituted 2-nitroimidazoles, it was shown that the corresponding nitrosimidazole, 1-methyl-2-nitrosoimidazole (INO), was very toxic towards both hypoxic and aerobic cells [10, 11] when compared to the corresponding hydroxylamine and amine which showed only limited toxicity. The nitrosoimidazole, INO, is a likely candidate for the proximate reduction product responsible for the hypoxic toxicity of INO_2 although it is not known whether INO is acting directly on a critical cellular target or if further transformation is needed for biological activity.

The nature of the critical targets for the cytotoxic action of nitrosoimidazoles is unclear. One possible target is DNA. INO has been shown to cause extensive DNA strand breaks in human cells in culture as measured by alkaline elution assays.‖ Sulfhydryls also are a possible critical target, and reaction of INO with sulfhydryls could lead to an alteration in the redox state of the cell. INO reacts rapidly with glutathione (GSH) in a cell free system yielding oxidized glutathione (GSSG) and imidazole-GSH adducts [12]. INO also reacts rapidly with GSH in mammalian cells *in vitro* [11, 13].

Alteration in the redox state of the cell via

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§ Abbreviations: INO_2, 1-methyl-2-nitroimidazole; INO, 1-methyl-2-nitrosoimidazole; GSH, glutathione; GSSG, oxidized glutathione; Pr-SH, protein sulfhydryl; CHO, Chinese hamster ovary; BSA, bovine serum albumin; BSO, L-buthionine sulfoximine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DEM, diethyl maleate; PBS, phosphate-buffered saline; INDO-1 AM, 1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxy methylester; GR, glutathione reductase; α-MEM, Minimum Essential Medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; and TCA, trichloroacetic acid.

‖ Mulcahy T, personal communication, 1988 cited with permission.

depletion of GSH and protein sulfhydryls (Pr-SH) has been proposed as a key event leading to the acute toxicity of the quinone menadione in rat hepatocytes [14]. Oxidative stress due to redox cycling of the quinone leads to GSH and Pr-SH oxidation as well as a sustained increase in cytosolic Ca^{2+} concentration in hepatocytes [15]. This non-physiological rise in Ca^{2+} is believed to inactivate non-specifically Ca^{2+}-dependent catabolic enzymes such as phospholipases, proteases and endonucleases that may contribute to cell death [16]. The mechanism of sulfhydryl depletion is probably different for INO compared to menadione. However, they both lead to extensive depletion of sulfhydryl in the range of concentrations where toxicity is observed. In the present work, an investigation has been made of the ability of different concentrations of INO to react with Pr-SH in a cell free system using bovine serum albumin (BSA) and *in vitro* using Chinese hamster ovary (CHO) cells. The time course of these reactions was followed in both systems. In the cell system, the effect of INO on intracellular Ca^{2+} levels also was monitored.

MATERIALS AND METHODS

Chemicals and reagents. INO was synthesized chemically following the procedure reported by Noss *et al.* [10]. For all experiments concentrated stock solutions of INO were prepared in deionized and distilled water at the time of the experiment. The concentration of INO was determined spectrophotometrically using ε_{360} = 20,000. L-Buthione sulfoximine (BSO), glutathione reductase (GR), NADPH, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), BSA fraction IV and RPMI 1640 medium were purchased from the Sigma Chemical Co. (St. Louis, MO). Diethyl maleate (DEM) was purchased from the Aldrich Chemical Co. (Milwaukee, WI) and concentrated solutions in phosphate-buffered saline (PBS) were prepared by sonication [17]. The fluorescent dye, 1-[2-amino-5-(6-carboxyindole-2-yl)-phenoxy]-2-(2'-amino-5'-methylphenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid pentaacetoxy methylester (INDO-1 AM) used for Ca^{2+} measurements was purchased from Molecular Probes (Eugene, OR). Ionomycin was from Calbiochem (San Diego, CA).

Reaction of INO with BSA. INO was mixed with different concentrations of BSA at room temperature in a spectrophotometer cell and the reaction was monitored by the loss of INO absorption at 360 nm as a function of time. The earliest time point that could be measured routinely was 30 sec after mixing.

BSA-SH assay. Stock solutions of 1 mM BSA were prepared in PBS. At the time of the experiment DTNB was added (1 mM final concentration) to aliquots of BSA in PBS and absorbance at 412 nm was followed for 20 min at room temperature, using a recording spectrophotometer. In experiments with INO, samples with different ratios of INO to BSA were allowed to react for 20 min. DTNB (1 mM final concentration) was then added to the mixture and absorbance at 412 nm was followed over time.

Cells. Cells used in the experiments were the Chinese hamster ovary cell subclone, AA8-4, obtained originally from Dr. L. H. Thompson of Lawrance Livermore Laboratories, CA, and grown routinely in suspension cultured at 37° in growth medium consisting of Minimum Essential Medium (α-MEM) plus 10% fetal bovine serum (FBS). Exposure of cells to INO under aerobic or hypoxic conditions, sampling, dilutions and assaying for colony-forming ability were carried out as previously described [10].

PrSH assay. Protein sulfhydryls in the cell were assayed according to the procedure of Di Monte *et al.* [18]. Briefly, cells were washed and resuspended in 9.9 mL of ascorbic acid free α-MEM plus 10% FBS at 10^6 cells/mL and were equilibrated in vials with a prehumidified gas mixture of 95% air/5% CO_2 (aerobic conditions) or 95% N_2/5% CO_2 (hypoxic conditions), while being continuously stirred and maintained at 37°. An 0.1-mL aliquot of INO stock solution was added to the vials to give the desired final concentration. Cell samples were removed at different time points, pelleted, washed in cold PBS, and resuspended in a 6% trichloroacetic acid solution at 10^6 cells/mL. Cells were centrifuged again and the pellet was resuspended in 1.8 mL of 10 mM Tris–HCl buffer, pH 7.6. An 0.2-mL aliquot of 1 mM DTNB was then added to the sample and the absorbance of 412 nm was read after 20 min. A second assay was also used in some cases [17]. Briefly, cells were lysed in a 0.1 M Tris buffer, pH 8.0, containing 0.25% sodium doedecyl sulfate (SDS) and 2 mM EDTA. An aliquot equivalent to 10^6 cells in 1 mL was then layered on a Sephadex G-25 column (10 mL bed volume) (Pharmacia, Uppsala, Sweden). The sample was eluted with 10 mM phosphate buffer, pH 7.5, containing 0.25% SDS and 2 mM EDTA to separate small non-protein sulfhydryls, such as GSH, from Pr-SH. Pr-SH were assayed by adding 0.1 mL of 100 μM DTNB to 0.9 mL of the eluate.

GSH assay. Intracellular glutathione was measured using the procedure of Tietze [19] as modified by Bump *et al.* [17].

Ca^{2+} assay. Intracellular Ca^{2+} concentration was measured using the fluorescent dye INDO-1 AM following the method of Nasmith and Grinstein [20] with slight modifications. Briefly, CHO cells at 5×10^6 cells/mL were incubated with 3 μM INDO-1 AM at 37° for 30 min in RPMI 1640 medium without FBS. After incubation with INDO-1 AM, cells were centrifuged and resuspended in 1 mL of RPMI 1640 medium at 5×10^6 cells/mL. An aliquot of 1×10^6 cells/mL was microfuged (Eppendorf centrifuge, at 5000 rpm for 5 sec) and resuspended in 2 mL of the following buffer: $CaCl_2$, 1 mM; NaCl, 140 mM; KCl, 5 mM; sodium-Hepes (free acid), 10 mM; glucose, 10 mM; pH 7.35. Fluorescence measurements were performed using a Perkin–Elmer model LS-3 spectrofluorometer with excitation at 331 nm and emission at 410 nm. The solution was maintained at 37° and stirred continuously. Ionomycin and $MnCl_2$ were used in order to calibrate the assay [21], and an aliquot of a concentrated solution of INO (in distilled H_2O) was added to the solution to give the appropriate drug concentration.

RESULTS

Interaction of INO with Pr-SH. It is known that

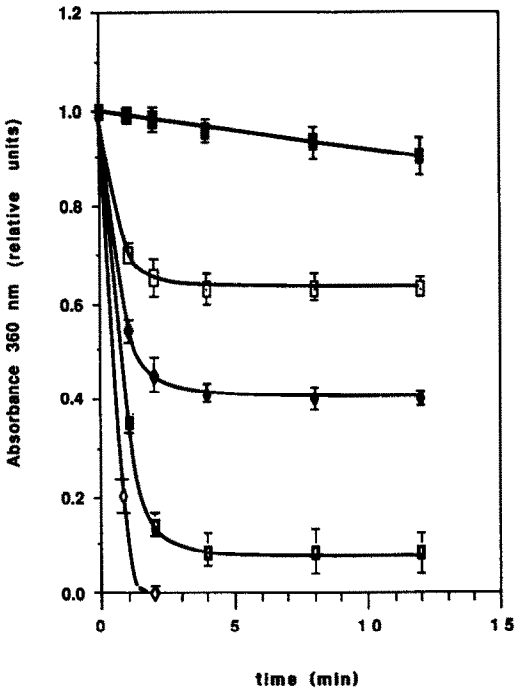

Fig. 1 Reaction of BSA with INO monitored by absorbance at 360 nm. Different concentrations of BSA were mixed with 50–80 μM INO and allowed to react at room temperature in PBS and the absorbance of the solutions was monitored as a function of time. Different curves represent different molar ratios of BSA to INO. Key: (■) INO control, (□) 0.74, (◆) 1.30, (⊡) 2.40, and (◇) 5.17. Each point is the mean ± SD of three independent experiments.

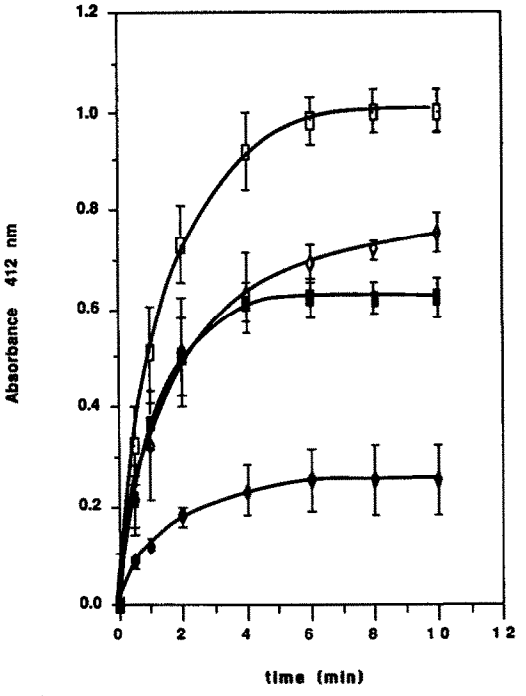

Fig. 2. Reaction of DTNB with BSA sulfhydryl groups after reaction with INO monitored by absorbance at 412 nm. Key: (□) BSA, 200 μM; (◇) BSA, 150 μM; (■) BSA, 200 μM, + INO, 21 μM; and (◆) BSA, 150 μM, + INO, 32 μM. Each point is the mean ± SD of three independent experiments.

INO rapidly reacts with GSH in solution to give GSSG and an adduct of GSH and INO [12]. Therefore, the possibility that INO would be able to oxidize or react with Pr-SH was investigated. INO was added to BSA in PBS at room temperature. BSA contains one free SH group per molecule [22]. The absorbance of INO at 360 nm was monitored as a function of time at different INO to BSA ratios. The results in Fig. 1 clearly indicate that INO can react with BSA as seen by the rapid loss of the characteristic absorbance of INO at 360 nm. The reaction appears to be stoichiometric as reflected by the ratio-dependent levels of the plateaus of absorbance with approximately two BSA molecules required to cause the rapid loss in absorption of one INO molecule. For example, at a molar ratio of 0.74 BSA/INO, Fig. 1 indicates a loss of approximately 0.38 mol of INO (0.74/0.38 = 1.9). This can be seen quantitatively by plotting the ratio of the concentration of INO lost at the plateau to its initial concentration versus the initial molar ratio of BSA to INO. However, when the present sample of BSA was titrated for free SH groups, a value of approximately 0.5 SH/BSA was obtained, suggesting that in the BSA samples about half of the free SH groups were already oxidized. If this is so, the stoichiometry becomes one INO per one DTNB reactive BSA-SH group. The small decay of INO in the control is in agreement with previous work [10]. The lack of decay of unreacted INO, seen as a constant absorbance value at times greater than 4 min in Fig. 1, reflects an apparent stabilizing effect of the sulfhydryl-depleted BSA molecules. A prolongation of INO lifetime in PBS has also been seen in the presence of DNA (50 μg/mL) which is not reactive with INO (data not shown).

Though the data in Fig. 1 indicate that INO reacts with BSA, it does not indicate whether this reaction involves the SH group in BSA or not. To test for this, 200 or 150 μM BSA was reacted with INO at approximately 10 to 1 and 5 to 1 ratios for 10 min. The BSA was then assayed for free SH with DTNB (Fig. 2). Clearly, previous reaction of BSA with INO reduced the number of DTNB reactable sulfhydryls but did not alter the kinetics of the reaction of the remaining sulfhydryls appreciably. If one INO reacts per DTNB reactive SH group (see above), then at 10 to 1 and 5 to 1 BSA/INO ratios one might expect a 20 and 40% loss of sulfhydryls based on 0.5 free SH per BSA molecule in the present BSA stock. What was observed was a 40 and a 75% loss. Thus, the calculated stoichiometry may not represent the complete reaction if INO-BSA adducts reacted with other SH groups on other BSA molecules. Reversing the experiment and reacting BSA with DTNB and then looking for the reactivity of INO by monitoring the rapid loss of its

Table 1. Pr-SH in CHO cells after INO or DEM treatment as assayed by two different methods*

	Pr-SH† (nmol/10^6 cells)		
	Control	60 μM INO	1 mM DEM
Method I	27.3 ± 3.0	15.0 ± 2.5	9.5 ± 2.7
Method II	25.7 ± 3.4	11.9 ± 1.8	—

* Cells were removed after 30 min and were assayed for Pr-SH according to the method of Bump *et al.* [17] with slight modifications (method I) or the method of Di Monte *et al.* [18] (method II).

† Values are the means ± SD of 4 independent experiments. The results for the two methods were not significantly different from one another using the unpaired Student's *t*-test with $P < 0.05$.

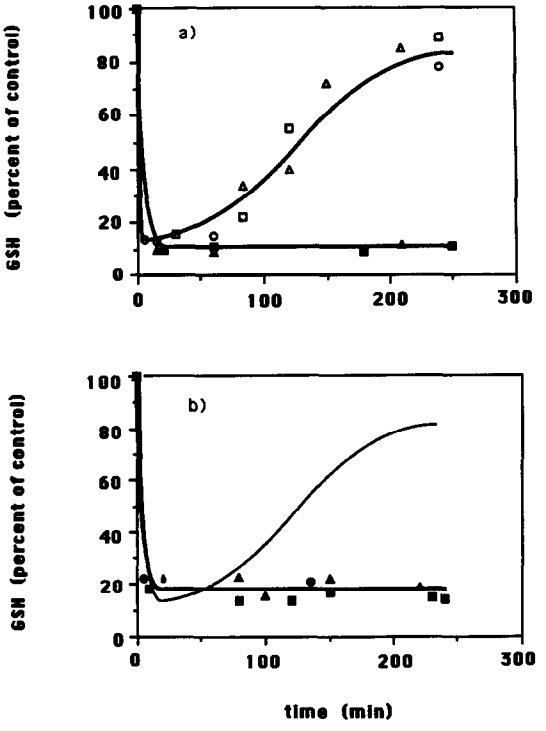

Fig. 3. GSH levels following treatment with INO. (a) CHO cells (10^6 cells/mL) were incubated with INO under aerobic conditions and GSH was assayed as a function of time. Open symbols: INO, 25 μM. Closed symbols: INO, 45 μM. Different symbols represent different experiments. (b) CHO cells (10^6 cells/mL) were incubated in the presence of 25 μM INO with or without BSO at 0.1 mM. BSO was added either 15 min before (■, ●) or after (▲) addition of INO. Different symbols represent different experiments. Solid line without symbols is 25 μM INO, no BSO from 3a.

360 nm absorbance as in Fig. 1 indicated that DTNB prevented INO from reacting with BSA, presumably by its prior reaction with the sulfhydryl group in BSA (data not shown).

Intracellular interaction of INO with Pr-SH and GSH. Initial studies were carried out to see if INO could reduce Pr-SH in CHO cells. A single concentration of 60 μM, expected to reduce cell survival to 0.1% [10], was added to 10^6 cells/mL in α-MEM plus 10% FBS. The cells were assayed for Pr-SH 10 min later. As can be seen in Table 1, control cells had Pr-SH levels of 27 nmol/10^6 cells which was reduced by 45% after INO treatment using the column technique of Bump *et al.* [17]. DEM was used as a positive control for Pr-SH depletion. This assay was compared with the procedure of Di Monte *et al.* [18] which makes use of trichloroacetic acid precipitation to separate GSH from Pr-SH. As can be seen in Table 1, there was good agreement between the two assays. Because of the greater ease of the TCA technique, it was used in subsequent experiments.

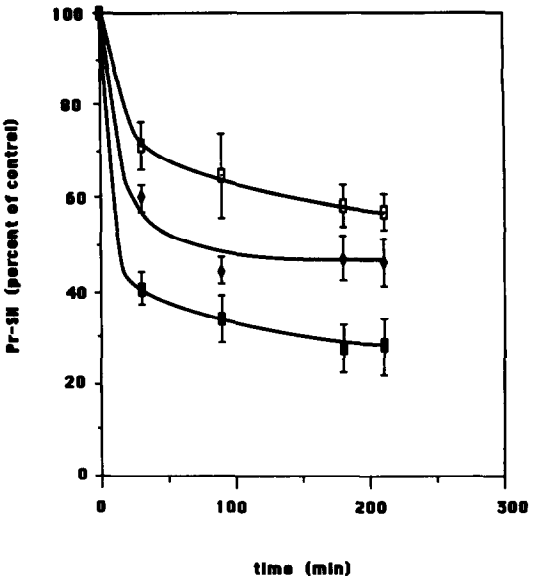

Fig. 4. Pr-SH levels following treatment with INO. CHO cells (10^6 cells/mL) were incubated with INO under aerobic conditions and Pr-SH were assayed as a function of time. Key: (□) 25 μM INO, (◆) 45 μM INO, and (■) 65 μM INO. Each point is the mean ± SD of three independent experiments.

Previous studies have indicated that INO depletes GSH in the concentration range where it is toxic in terms of cell colony-forming ability [10, 11, 13]. Table 1 indicates that a 10-min exposure to INO also depletes Pr-SH in the range where it is toxic. To study the concentration and time dependency of INO depletion of GSH and Pr-SH levels, toxic and non-toxic concentrations were studied. CHO cells at 10^6/mL were exposed to 25, 45 or 65 μM INO at 37°, and samples were removed as a function of time and assayed for their levels of GSH and Pr-SH. These concentrations correspond to cell survival levels of 90, 5 and 0.1%, respectively (see Fig. 7). Addition of INO at all these concentrations led to a rapid depletion of GSH to 10–20% of the normal level at the earliest time tested (Fig. 3a). Results at 65 μM (data not shown) were similar to those at

45 μM. INO concentrations of 25, 45 and 65 μM led to depletion of Pr-SH to 70, 60 and 40%, respectively, of the normal level when measured 30 min after INO addition (Fig. 4). With time, 1–4 hr after the addition of INO, GSH levels returned to normal following treatment with the non-toxic drug concentration (25 μM) but remained at the initial depressed level after treatment with toxic concentrations (Fig. 3a). In contrast, Pr-SH levels did not return to normal even at the non-toxic concentration of INO up to 3 hr (Fig. 4). The measurement of GSH and Pr-SH was repeated under hypoxic INO exposure conditions. The samples were gassed for 45 min before adding INO. Cells were held under hypoxia throughout the experiment (up to 4 hr). GSH and Pr-SH levels in control cells under hypoxia increased marginally (10–15% at 4 hr). However, the results with INO were not significantly different from those obtained in air (data not shown).

The loss of GSH could be due to its oxidation to GSSG and/or to its binding to INO or its reduction/oxidation products. Previous studies had not detected any GSSG formation under these exposure conditions after 30 min [13]. Thus, the recovery might be due to instability of possible GSH-INO adducts or resynthesis of new GSH. To test for the latter possibility CHO cells were preincubated with BSO, a specific inhibitor of GSH synthesis, 15 min before INO was added and the level of GSH measured as a function of time. As can be seen in Fig. 3b, there was no recovery of GSH in cells treated with 25 μM INO. However, even this short treatment with BSO was found to deplete as much as 30% of the cell's GSH (data not shown). Therefore, it is possible that the sensitivity of the cells to INO was modified in such a way as to prevent recovery. To investigate this possibility, BSO was added 15 min after treatment with 25 μM INO at a time when GSH is already depleted. Even under these conditions, no recovery was observed. It is concluded that GSH recovery after addition of a non-toxic dose of INO is due to *de novo* synthesis.

Previous results had indicated a plateau in the ability of high INO concentrations to deplete GSH, suggesting the presence of GSH in a non-accessible (resistant) compartment such as mitochondria [11, 13]. To determine if Pr-SH could be reduced beyond the 40% level, higher concentrations of INO were used. The results shown in Fig. 5 indicate that 30 min after exposure to INO, total Pr-SH can be reduced to as low as 10%. There appears to be a compartment of 60% of the cellular Pr-SH which is sensitive to depletion by low INO concentrations. The remaining 40% component requires much higher concentrations.

Ca^{2+} *homeostasis*. Oxidative stress caused by the quinone menadione results in loss of cellular GSH and Pr-SH and perturbs Ca^{2+} levels in rat hepatocytes [14, 15]. Since INO treatment also results in GSH and Pr-SH loss, albeit by a different mechanism, the effect of INO on the intracellular concentration of Ca^{2+} was investigated. CHO cells were preloaded with the fluorescent dye INDO-1 AM as described in Materials and Methods. The fluorescence of control cells remained constant over 2 hr indicating little leakage of INDO-1 from the cells (data not shown). Toxicity of INDO-1 after 2 hr was not more than 15% as measured by a colony assay. A non-toxic (25 μM) or a toxic (45 μM) concentration of INO was added to 10^6 cells/mL and fluorescence of the cells was followed continuously at 37° as a function of time. As seen in Fig. 6, there was an immediate drop in fluorescence of the cells after INO addition which was concentration dependent but recovered within approximately 20 min. This has proven to be due to the absorbance of the INO solution at 330 nm, the excitation wavelength used for the measurements (data not shown). At 60–70 min after the addition of INO there was a gradual rise in fluorescence of the cells treated with 45 μM INO, indicating an increase in intracellular calcium levels. The sustained increase was very significant in relation to the maximum signal seen when cells were treated with the calcium ionophore ionomycin. The actual concentration of calcium calculated according to Grynkiewicz *et al.* [21] was 0.3 μM for control cells and 0.9 μM (at 100 min) for cells treated with 45 μM INO. Several other INO concentrations were assayed in this fluorescence assay to investigate their effect on intracellular calcium levels. These results are shown in Fig. 7 where relative fluorescence of INDO-1 at 2 hr is plotted as a function of the INO concentration added and compared to INO-mediated cell killing measured by a colony-forming assay. There is a very good inverse correlation between increased intracellular calcium levels and cell survival.

DISCUSSION

INO has been shown to react with GSH both in a chemical [12] system and intracellularly [11, 13]. The present work indicates that INO can also interact with Pr-SH in a chemical system as well as intracellularly. The rapid loss of the characteristic absorbance of INO at 360 nm in the presence of BSA in a stoichiometric fashion was the initial indication of this reaction (Fig. 1). Using DTNB to measure Pr-SH in BSA, it was found that the INO-BSA reaction could involve all the sulfhydryl groups detectable by DTNB. At present, it is not known if the loss of Pr-SH was due to oxidation to a disulfide form or by conjugation with INO or a reduction product of INO such as the hydroxylamine. Studies by Raleigh and Koch [23] have stressed the important role for Pr-SH in the reaction of reduced 2-nitroimidazoles to form a stable bound adduct. The work of Varghese and Whitmore [24] demonstrated the binding of a reduction product of misonidazole to BSA using a chemical reduction system. Thus, there are strong precedents for covalent binding of 2-nitroimidazole reduction products to protein sulfyhydryls.

The 2-nitroimidazole misonidazole is able to deplete GSH and Pr-SH over a period of minutes to hours in hypoxic mammalian cells [25]. Absence of oxygen is required for the efficient reduction of misonidazole which gives rise to the reduction products believed to be responsible for sulfhydryl depletion. It was found that INO was capable of depleting Pr-SH in both hypoxic and aerobic mammalian cells to a similar extent, consistent with

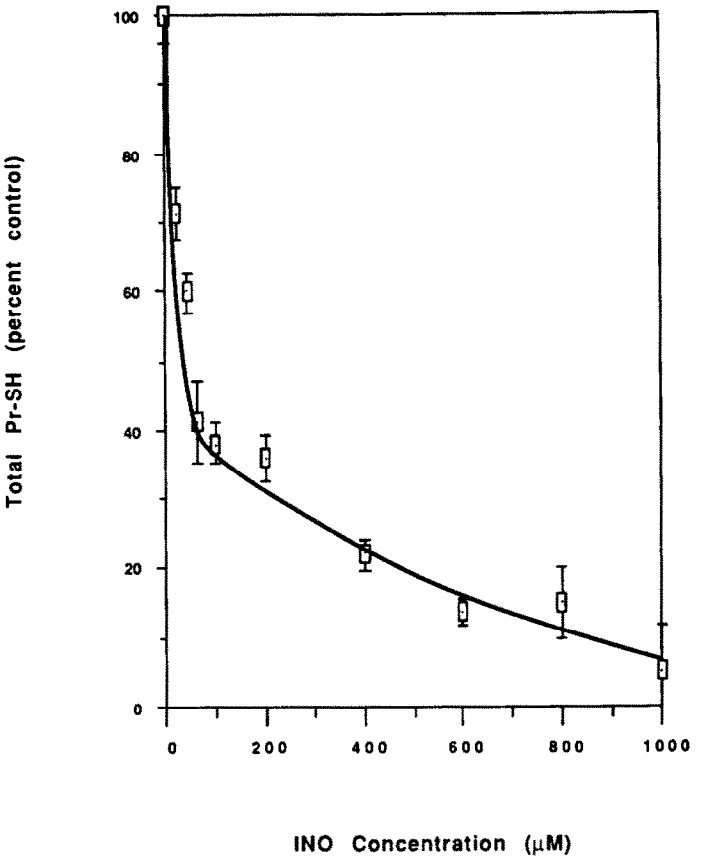

Fig. 5. Pr-SH levels as a function of INO concentration. CHO cells (10^6 cells/mL) were incubated with INO at the indicated concentration for 30 min under aerobic conditions before being assayed for Pr-SH. Each point is the mean ± SD of three independent experiments.

the model for nitrocompound action in which the nitroso is beyond the oxygen-sensitive step. Whether INO acts directly or needs further reduction in cells in order to deplete sulfhydryls is not known. Chemical experiments have shown that the hydroxylamine can rearrange to give a nitrenium ion which can react with sulfhydryl-containing molecules [26]. Therefore, it is possible that INO and/or some of its reduction products react directly with Pr-SH *in vitro*. An indirect depletion of Pr-SH is also possible. It is known that GSH and GSSG can undergo extensive mixed disulfide exchange with Pr-SH when cells are subjected to oxidative stress; thus, depletion of GSH by INO could lead to formation of mixed disulfides. A non-toxic concentration of INO (25 μM) caused depletion of Pr-SH and GSH to 70 and 15% of control level, respectively, under aerobic conditions. GSH but not Pr-SH, was able to recover over the next 3–4 hr (Figs. 3 and 4). The behavior of GSH and Pr-SH after treatment with INO may have important implications for the efficient use of nitroimidazoles both as radiosensitizers and chemosensitizers of hypoxic cells since GSH and Pr-SH are both believed to be involved [27]. It has been shown that glutathione-S-transferases may be involved in the reaction of various drugs with GSH [28]. It is possible that the reaction of INO with GSH is catalysed by glutathione-*S*-transferases. However, considering the fast rate of the chemical reaction between INO and GSH both in chemical [12] and cellular systems, this appears unlikely. No qualitative differences between hypoxic and aerobic depletion of GSH and Pr-SH and the recovery of GSH at low INO concentrations were observed. This result was not surprising since INO did not show any differential toxicity towards hypoxic versus aerobic cells [10].

Figure 3b shows that there was no recovery of GSH after treatment with a non-toxic concentration of INO if BSO was added either 15 min before or after INO, which implies recovery is due to *de novo* synthesis. The rate of GSH resynthesis after INO treatment was 3–4 times faster than resynthesis after similar levels of depletion, 80–90%, by non-toxic BSO treatment, but is quantitatively similar to recovery after DEM treatment [29]. BSO slowly depleted GSH to 5–10% (12–24 hr), whereas DEM and INO depleted GSH to a similar extent much more rapidly suggesting that the mechanism by which GSH is depleted may be a factor influencing the kinetics of its recovery. At least one report suggests that the rate of GSH synthesis as well as GSH levels may be important factors governing bacterial cell sensitivity to extended radiation exposure or drug [30]. The finding that GSH recovery

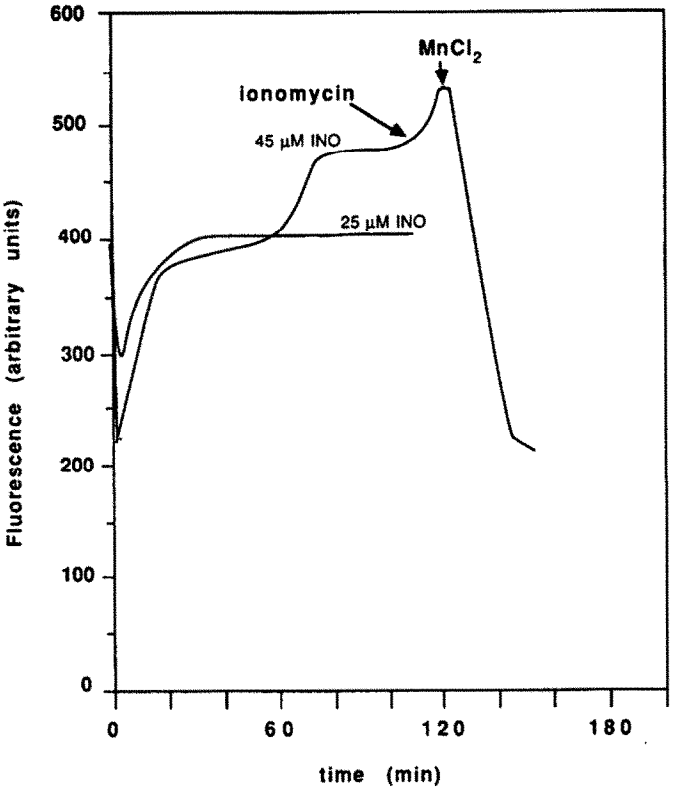

Fig. 6. Calcium levels in CHO cells (10^6 cells/mL) incubated with INO. Cells were loaded with INDO-1 AM and relative cellular fluorescence was monitored continuously after addition of 25 or 45 μM INO. After 120 min, ionomycin (3 μM) followed by $MnCl_2$ (1.5 mM) were added to calibrate the assay. The concentration of intracellular calcium was approximately 0.3 μM in control cells and increased to approximately 0.9 μM in cells treated with 45 μM INO. The traces shown are representative of six independent experiments.

is due to resynthesis is consistent with the fact that GSH can form adducts with reduced misonidazole [3]) and that reaction of INO and GSH in chemical systems also produces similar adducts [12]. Such adducts in cells would be stable since they would not be expected to be a substrate for glutathione reductase. The presence of such adducts in cells is currently being sought.

Over 90% of depletion of GSH by BSO for periods of time up to 24 hr is not sufficient to cause extensive cell death [17, 30]. Similarly, Pr-SH depletion by DEM is well tolerated by cells [17]. Therefore, it is unlikely that GSH and Pr-SH depletion alone are sufficient to explain the toxicity of INO. Mixtures of INO and GSH have been shown to give rise to DNA strand breaks in the plasmid assay.* It has also been shown that the 1-methyl-4-phenyl-5-nitrosoimidazole is bound to DNA and that this incorporation is thiol mediated [32]. Such damage to DNA could produce cellular toxicity. Interestingly, exposure of aerobic hepatocytes to menadione produces depletion of GSH and Pr-SH similar to that observed for INO in CHO cells. This reduction of sulfhydryls in hepatocytes is thought to be mediated by the generation of active oxygen species by the redox cycling of menadione.

As a result of sulfhydryl depletion, hepatocytes were found to have a sustained increase in Ca_i^{2+} upon chronic exposure to toxic doses of menadione [33], and it was suggested that damage to critical SH groups of proteins involved in Ca^{2+} transport was involved. The finding that toxic exposures of cells to 45 μM INO produced a sustained increase in Ca_i^{2+} after 60 min confirms the possibility that menadione and INO may cause toxicity by a similar mechanism. More explicitly, INO rapidly enters the cells and reacts with GSH and Pr-SH. If sulfhydryls are depressed low enough, intracellular calcium levels rise, perhaps due to damage to membrane proteins involved in calcium transport. Increases in Ca_i^{2+} of the level observed could, in turn, activate phospholipases, endonucleases and proteases and induce cell death [33]. The results with menadione also showed extensive cell membrane blebbing and the production of fragmented DNA in the form of nucleosome ladders on gel electrophoresis. These results were interpreted as an apoptotic form of cell death [33]. Though toxic doses of INO give rise to membrane blebbing in CHO cells (data not shown), nucleosome ladders have not

* Milligan JR, Pannicucci R, Zamperoni R, McClelland RA and Rauth AM, manuscript submitted for publication.

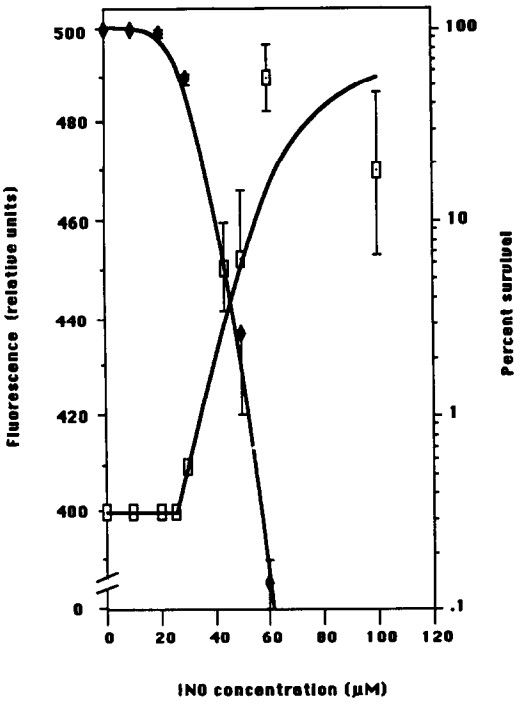

Fig. 7. Calcium levels and survival of CHO cells (10^6 cells/mL) incubated with INO. Values of relative fluorescence at 120 min, left-hand scales, were taken for the concentrations of INO indicated (open symbols). Each point is the mean ± range of two independent experiments. This is compared with the survival of CHO cells, right-hand scale, assayed by colony-forming ability (closed symbols). Each point is the mean ± SD of five independent experiments.

been observed as yet. Thus, whether there is a direct cause–effect relationship between the early increases in intracellular calcium and cell death remains to be established.

It is not clear at present to what degree the rapid molecular and cellular effects of the acute exposure to cells of high external concentrations of INO model the slower effects of 2-nitroimidazoles reduced intracellularly under hypoxic conditions. Qualitatively, the present results for INO seem similar to the previously observed cellular effects of 2-nitroimidazoles, e.g. the depletion of GSH and Pr-SH [25] at toxic drug levels. The results of Mulcahy *et al.* [34] show that INO can act as a chemosensitizer like 2-nitroimidazoles. Further work will be necessary to determine to what degree these acute effects of INO can account for the hypoxic cell toxicity of 2-nitroimidazoles.

To the degree INO effects are predictive of 2-nitroimidazole effects, the present results suggest a number of novel approaches to modulating the cellular toxicity of 2-nitroimidazoles. For example, the importance of calcium levels and/or calcium transport is currently under investigation. Understanding such mechanisms of cell death may be important not only for tumor therapy but also in modulating the dose-related peripheral neuropathy [35], which is the limiting normal tissue toxicity of misonidazole and etanidazole clinically.

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