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INACTIVATION OF LACTATE DEHYDROGENASE BY SEVERAL CHEMICALS: IMPLICATIONS FOR IN VITRO TOXICOLOGY STUDIES

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Abstract

Lactate dehydrogenase (LDH) release is frequently used as an end-point for cytotoxicity studies. We have been unable to measure LDH release during studies using *para*-aminophenol (PAP) in LLC-PK₁ cells. When LLC-PK₁ cells were incubated with either PAP (0–10 mM) or menadione (0–1000 μM), viability was markedly reduced when assessed by alamar Blue or total LDH activity but not by release of LDH into the incubation medium. In addition, we incubated cells with PAP or menadione and compared LDH activity using two different assays. Both assays confirmed our observation of decreased LDH activity in cell lysates without corresponding increases in LDH activity in incubation media. Using purified LDH and 10 mM PAP, we found that PAP produced loss of LDH activity that was inversely proportional to the amount of LDH initially added. In additional experiments, we incubated 0.5 units of LDH for 1 h with varying concentrations of PAP, menadione, hydrogen peroxide (H₂O₂) or cisplatin. All four chemicals produced concentration-dependent decreases in LDH activity. In previous experiments, inclusion of antioxidants such as reduced glutathione (GSH) and ascorbate protected cells from PAP toxicity. GSH (1 mM) preserved LDH activity in the presence of toxicants while ascorbate (1 mM) only prevented LDH loss induced by PAP. These studies suggest that LDH that is released into the incubation medium is susceptible to degradation when reactive chemicals are present.

INTRODUCTION

Lactate dehydrogenase (LDH) release is commonly used as a marker for necrotic/oncotic cell death (Valentovic and Ball, 1998; Lash *et al.*, 1995). Most cells contain LDH and when these cells are lethally injured, loss of membrane integrity can be assessed by monitoring activity of LDH in the incubation medium. Recently, we attempted to measure LDH release in LLC-PK₁ cells (a spontaneously immortalized cell line derived from pig kidney) following incubation with concentrations of *para*-aminophenol (PAP) that produce apoptotic or oncotic responses. With LLC-PK₁ cells, there were visibly fewer cells in wells treated with oncotic concentrations of PAP and alamar Blue fluorescence was decreased, indicating loss of viability. However, we were unable to detect LDH activity in the medium although we could easily determine that intracellular (lysate) LDH activity was decreased following PAP treatment.

CONFLICT OF INTEREST

Neither author has any affiliation or association with companies or organizations that could influence the scope or interpretation of the work presented in this manuscript.

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Therefore, we speculated that PAP interfered with the determination of LDH activity. The present study was conducted to more thoroughly investigate LDH activity in the presence of toxic chemicals.

METHODS AND MATERIALS

Chemicals

Lactate dehydrogenase (LDH) from rabbit muscle, *para*-aminophenol (PAP), menadione (2-methyl-1,4-naphthoquinone), ascorbic acid, cisplatin, reduced glutathione (GSH), NADH and pyruvate were obtained from Sigma Chemical Company. Hydrogen peroxide (H₂O₂) was obtained from Fisher Scientific. CytoTox-ONE™ LDH assay kit was obtained from Promega Corporation. All other reagents were obtained from Fischer Scientific.

Cell viability using Alamar Blue, LDH release or total LDH

LLC-PK₁ cells from the American Type Tissue Collection (Manassas, Virginia) were cultured in Dulbecco's modified essential medium/Ham's F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were seeded at 5 x 10⁴ cells/well in 24-well plates and allowed to attach overnight.

Cells were incubated with serum- and phenol red-free DMEM/F-12 containing PAP (0–10 mM) or menadione (0–1000 μM) for 4 h at 37°C in an atmosphere of 95% O₂ / 5% CO₂. After 4 h of incubation, wells in two of four rows were washed with chemical-free Hank's Balanced Salt Solution (HBSS), then incubated with 5% alamar Blue (Trek Diagnostics) in HBSS. Alamar blue was used as an indicator of cell viability (Hallman et al., 2000). Incubation with PAP or menadione was continued for an additional 2 h in the wells not incubated with alamar Blue. Since both assays, alamar blue reduction and LDH activity, use conversion of blue resazurin to pink resorufin, it was not possible to measure viability by both methods in a single well. Therefore, we processed two of four rows of wells (6 treatment groups) for alamar Blue and the remaining two rows of wells (6 treatment groups) for LDH activity at the end of the alamar Blue incubation period (6 h after initial exposure to PAP or menadione). To determine alamar Blue reduction, fluorescence (530 nm excitation, 590 nm emission) was determined initially and at the end of a 2 h incubation period using a Perkin-Elmer HTS3000 Plus plate reader (Perkin-Elmer Corporation, Wellesley, MA).

Following the final fluorescence reading for alamar Blue, the remaining wells in each plate were processed for LDH determinations. Media was collected and cells were lysed according to the manufacturer's protocol. LDH activity was measured using a commercial assay (CytoTox-ONE, Promega) and LDH activity in media and lysate is reported in arbitrary fluorescence units. In the CytoTox-One assay, LDH enzymatically converts lactate to pyruvate in a reaction coupled to diaphorase-mediated conversion of resazurin to resorufin (Promega Technical Bulletin). Resorufin is a highly fluorescent chemical (excitation 560 nm, emission 590 nm) and was measured using a microplate reader. Total LDH activity was determined by adding fluorescence values in lysate and media, adjusted for background fluorescence. For background fluorescence, we used 100 μl aliquots of distilled water or triton X-100 lysis solution instead of media or lysate samples. Fluorescence intensity was not different between samples of distilled water or triton X-100 lysis solution so values were pooled and used as background correction. LDH release was calculated as the ratio of media fluorescence compared with total fluorescence in media plus lysate and the results were expressed as a percentage. Viability using LDH release was calculated by subtracting the percentage of media LDH activity from total LDH activity of untreated samples. Viability using total LDH was calculated by dividing total LDH activity from treated wells by total LDH activity of untreated wells, expressed as a percentage.

In a separate series of experiments, we seeded cells at a density of 1×10^5 cells/well in 24-well plates. Cells were allowed to attach overnight, then incubated with HBSS containing PAP (0–10 mM) or menadione (0–1000 μ M) for 4 h. We determined LDH release using the CytoTox-One assay kit and calculated LDH release and viability as described above. These experiments allowed us to determine LDH activity in the absence of pyruvate in the media, since DMEM/F-12 contains pyruvate whereas HBSS does not. Pyruvate in the incubation medium may inhibit LDH activity and reduce the fluorescence signal (Promega Technical Bulletin; Stambaugh and Post, 1966).

LDH release measured by NADH oxidation

To confirm our observations using the CytoTox-One assay, we determined LDH release by measuring changes in absorbance at 340 nm due to NADH oxidation (Bergmeyer and Bernt, 1963). For these experiments, we seeded LLC-PK₁ cells at 5×10^5 cells/dish in 60 mm dishes. Cells were allowed to attach overnight, then incubated with 1 ml/dish of HBSS containing PAP (0–10 mM) or menadione (0–1000 μ M). At the end of 4 h, the media was recovered and 1 ml of a lysis solution containing 0.1% triton X-100 in 100 mM sodium phosphate buffer (pH 7.4) was added to each dish. Dishes were incubated for 5 minutes at room temperature and cell lysis was confirmed microscopically. To assay LDH, we mixed 2.85 ml 0.3 mM pyruvic acid in 50 mM phosphate buffer (pH 7.5) with 50 μ l of 8 mM NADH, also in 0.3 mM pyruvic acid solution. We determined absorption at 340 nm and then initiated reactions using 100 μ l aliquots of media or lysate (Bergmeyer and Bernt, 1963). Background or spontaneous NADH oxidation was measured by substituting 100 μ l aliquots of distilled water or triton X-100 lysis solution instead of media or lysate samples into the reaction mixture. Samples were mixed and immediately loaded into a Genesys 6 spectrophotometer (Thermo Electron Corporation, Waltham, MA). Absorbance was recorded every minute for 5 minutes and the change in absorbance (ΔA_{340}) was determined. In the absence of media or lysate samples, $\Delta A_{340}/\text{min}$ was negligible over 5 minutes. Total LDH activity was determined by adding $\Delta A_{340}/\text{min}$ in lysate and media samples. LDH release was determined by dividing $\Delta A_{340}/\text{min}$ in the media sample by total $\Delta A_{340}/\text{min}$. Viability was determined by dividing total $\Delta A_{340}/\text{min}$ in treated samples by total $\Delta A_{340}/\text{min}$ for control samples, expressed as a percentage.

LDH Stability

In preliminary experiments, we incubated LDH ranging from 0.1 to 1 unit/ml at 37°C in an atmosphere of 95% O₂ / 5% CO₂ for 1 h. LDH was dissolved in phosphate-buffered saline (PBS, pH 7.4). At the end of the incubation, LDH was assayed using the CytoTox-One protocol as described above. The fluorescence signal was linear over the range of LDH concentrations used and we elected to use 0.5 units of LDH for additional experiments.

LDH (0.5 units/ml in PBS) was incubated with PAP, menadione, H₂O₂ or cisplatin (all at concentrations of 1–10 mM) for 1 h at 37°C in an atmosphere of 95% O₂ / 5% CO₂. An equivalent amount of solvent (PBS for PAP, menadione and H₂O₂; DMSO for cisplatin) was added to control (0 mM) incubations. At the end of the incubation period, LDH activity was determined. In separate experiments, we tested the ability of L-ascorbic acid or reduced GSH (both at 1 mM) to preserve LDH activity. LDH was incubated with 5 mM concentrations of PAP, menadione, H₂O₂ or cisplatin in the presence or absence of antioxidants. Mixtures were incubated for 1 h at 37 °C in an atmosphere of 95% O₂ / 5% CO₂ and LDH activity was determined. All incubations were run in triplicate and LDH determinations were done in duplicate.

We measured NADH absorbance at 340 nm in the presence or absence of 1 mM PAP, menadione, cisplatin or H₂O₂. There were no significant changes in absorption over a 2 h period with any of the chemicals tested, suggesting no interactions with NADH or interference with

its detection spectrophotometrically. We also measured resorufin fluorescence in the presence or absence of 5 mM PAP, menadione, cisplatin and H_2O_2 and found no interference either to enhance or quench resorufin fluorescence. None of these chemicals produced detectable fluorescence at the wavelengths used for the CytoTox-One assay (560 nm excitation, 590 nm emission).

Data and Statistical Analyses

For experiments using purified LDH, comparisons were made using raw fluorescence data. Data were analyzed using ANOVA followed by Student-Newman-Keuls analysis to determine significant differences. The criterion for significance was $p < 0.05$. For experiments using LLC-PK₁ cells, data were expressed as a percentage of cells not exposed to toxic chemicals. In addition, we determined total LDH by adding the fluorescence signals of media and lysate. The same statistical analysis and criterion for significance was used.

RESULTS

We compared viability as determined by LDH release into media, total LDH (media plus lysate) and alamar Blue fluorescence for LLC-PK₁ cells incubated with either PAP or menadione. With PAP, viability (as measured by alamar Blue) was significantly reduced at concentrations greater than 0.1 mM following 6 h of incubation. PAP at 5 and 10 mM reduced viability to less than 10% of control values (Figure 1, squares). Viability as measured by LDH release was significantly reduced to $79.1 \pm 3.3\%$ of control viability with 10 mM PAP (Figure 1, open circles). LDH activity in the media was not increased and was, in fact, significantly decreased at all concentrations of PAP (Figure 2 and inset). The decrease in viability observed in Figure 1 using total LDH activity was due to a dramatic decrease in lysate fluorescence, as shown in Figure 2. For PAP, the concentrations required to decrease viability by 50% (LC₅₀) were approximately 0.75 mM for alamar Blue and 2.5 mM for total LDH activity. LC₅₀ for PAP could not be estimated from LDH release (Figure 1).

Cells incubated in the presence of menadione showed a dramatic drop in viability as assessed by alamar Blue fluorescence (Figure 3, squares). Viability as measured by LDH release dropped significantly at concentrations of menadione $\geq 10 \mu\text{M}$ and remained relatively constant at higher concentrations (Figure 3, open circles). Viability measured using total LDH activity dropped precipitously, virtually paralleling results with alamar Blue. However, concentrations of menadione $\geq 50 \mu\text{M}$ did not show additional decreases in total LDH activity (Figure 3, closed circles). The decrease in viability observed in Figure 3 using total LDH activity was due to a decrease in lysate fluorescence (Figure 4) with relatively constant media fluorescence (Figure 4, inset). For menadione, LC₅₀s for alamar Blue and total LDH activity were similar, estimated at 25 and 45 μM , respectively, while LC₅₀ using LDH release could not be calculated (Figure 3).

To confirm our observations, we compared LDH activity using NADH oxidation or the CytoTox-One assay protocol. These experiments were done using HBSS as an incubation vehicle to circumvent the presence of excess pyruvate that might inhibit LDH activity (Stambaugh and Post, 1966). As seen in Table 1, concentrations of PAP up to 10 mM produced no significant LDH release whether using NADH oxidation or resorufin fluorescence as the endpoint. In contrast, concentrations of PAP as low as 0.5 mM caused statistically significant losses of LDH activity as compared with untreated cells. At 10 mM PAP, total LDH activity was decreased to about 65% of control using both endpoints, NADH oxidation and resorufin formation. Results for total LDH were not significantly different between the two assays. Data for menadione are presented in Table 2. For menadione, the CytoTox-One assay was somewhat more sensitive for LDH loss than was NADH oxidation. Concentrations of menadione equal to or greater than 100 μM produced significant loss of LDH activity in cell lysates using the

CytoTox-One assay whereas using NADH oxidation, significant loss of LDH activity was detectable only with 500 and 1000 μ M menadione. LDH in incubation media was not significantly increased at any concentration of menadione by either assay. However, LDH activity of all media samples, control and menadione-treated, was significantly greater using the CytoTox-One assay as compared with NADH oxidation (Table 2).

We incubated purified LDH from rabbit muscle for 1 h in the presence or absence of 0.5 mM PAP. Fluorescence was dependent on the amount of LDH in the incubation mixture (Figure 5). In addition, 0.5 mM PAP significantly reduced fluorescence at each amount of LDH (Figure 5). When expressed as a percentage of control fluorescence, 0.5 mM PAP reduced fluorescence to $49.4 \pm 2.3\%$, $35.0 \pm 2.0\%$, $54.6 \pm 2.1\%$, and $78.9 \pm 4.0\%$ of the fluorescence due to 0.1, 0.25, 0.5 and 1 units of LDH, respectively. We elected to use 0.5 units of LDH for subsequent experiments since the fluorescence signal was easily differentiated from background fluorescence and within the range of linearity (Figure 5).

To determine if LDH was stable in the presence of chemicals, we incubated 0.5 units of rabbit muscle LDH with 0–10 mM concentrations of four toxicants: PAP, menadione, H_2O_2 , and cisplatin. LDH activity was determined following 1 hour of incubation in the presence or absence of toxicant. For all three concentrations of each toxic agent, LDH activity was significantly reduced compared with activity in the absence of chemical (Figure 6). Cisplatin was the most effective and PAP the least effective in reducing LDH activity. Experiments were performed on different days and control LDH activity was variable when expressed as fluorescence units. Therefore, data are expressed as a percentage of control fluorescence determined for each experiment. Results were not different when data were analyzed using arbitrary fluorescence units (data not shown).

Previously, we reported that both ascorbate and reduced glutathione (GSH) prevented cytotoxicity associated with PAP (Hallman *et al.*, 2000). Therefore, we incubated 0.5 units of LDH with 1 mM concentrations of ascorbate or GSH in the presence or absence of 5 mM PAP, menadione, H_2O_2 , or cisplatin. Inclusion of ascorbate or GSH in the incubation mixture was without significant effect on LDH activity (Figure 7, open bars). LDH activity in the presence of PAP was reduced to 75% of LDH activity in the absence of PAP. Inclusion of ascorbate or GSH partially prevented PAP-associated loss of LDH activity (Figure 7, left-hatched bars). Menadione reduced LDH activity to about 40% of control activity (Figure 7, black bars). Inclusion of ascorbate was ineffective in preventing menadione-induced LDH inactivation and in fact, LDH activity was reduced to a significantly greater extent in the presence of both ascorbate and menadione than in the presence of menadione alone ($40 \pm 2.7\%$ for menadione alone vs. $19 \pm 1.1\%$ for menadione plus ascorbate). In contrast, GSH partially prevented loss of LDH activity in the presence of menadione (Figure 7, black bars). H_2O_2 reduced LDH activity to $48 \pm 9.4\%$ of LDH activity in the absence of peroxide (Figure 7, cross-hatched bars). As with menadione, ascorbate was ineffective in preventing H_2O_2 -associated LDH loss and significantly decreased LDH activity to $14 \pm 0.5\%$ of control LDH activity. In contrast, GSH completely prevented H_2O_2 -associated LDH loss (Figure 7, cross-hatched bars). Cisplatin significantly reduced LDH activity to $14.6 \pm 0.5\%$ of LDH activity in the absence of cisplatin. Again, ascorbate was ineffective in preventing cisplatin-induced LDH inactivation while GSH significantly increased LDH activity in the presence of cisplatin ($125.9 \pm 3.6\%$ of control LDH activity) (Figure 7, right-hatched bars).

DISCUSSION

LDH is a commonly used marker for lethal cell injury (Lock *et al.*, 1993; Valentovic and Ball, 1998; Lash *et al.*, 1995). We incubated LLC-PK₁ cells with a range of concentrations of either PAP or menadione and observed decreases in viability using alamar Blue fluorescence

(Figures 1 and 3). We also observed declines in the activity of LDH recovered in cell lysates but were unable to determine increased LDH activity in the medium (Figures 2 and 4). Consequently, monitoring LDH release was unsuitable in our hands as an index of cell death. This phenomenon of LDH inactivation in medium has been reported for mercury (Lash and Zalups, 1992) but to our knowledge, not for PAP or menadione. However, in investigating cytotoxicity in LLC-PK₁ cells incubated with glutathione conjugates of bromobenzene, Mertens and co-workers (1995) reported intracellular LDH activity rather than LDH release. One might speculate that these bromobenzene conjugates inactivated released LDH similar to our current observations.

Our results are at variance with other laboratories. For example, Valentovic and Ball (1998) were able to detect LDH release from rat renal slices incubated with PAP. Lash and co-workers (1995) determined LDH release from rat proximal and distal tubular cells exposed to PAP and other investigators monitored LDH release from rabbit proximal tubules (Lock *et al.*, 1993). Menadione is associated with LDH release in hepatocytes (Maellaro *et al.*, 1994; Verrax *et al.*, 2004). The range of PAP and menadione concentrations used by these other investigators is within the range of concentrations we routinely use. While we do not know why our results are at variance with others, we can offer several potential explanations. One difference between our studies and those of other investigators is related to presence or absence of protein. We routinely omit protein from our incubations whereas Lash and Zalups (1992) used 2% bovine serum albumin in their incubations and Verrax and co-workers (2004) used 10% fetal calf serum. Others have dissolved PAP in DMSO (Valentovic and Ball, 1998; Lock *et al.*, 1993), which can act as a scavenger of hydroxyl radicals (Walker and Shah, 1991; Baliga *et al.*, 1998). Investigators have used a variety of incubation media, including HBSS and Krebs-Ringer solutions (Valentovic and Ball, 1998; Lash *et al.*, 1995). We use a commercial mixture of DMEM/F-12, which may modulate interactions between toxicants and LDH. In investigating cisplatin nephrotoxicity in LLC-PK₁ cells, Townsend and co-workers (2003a) found differences in toxicity when cells were incubated in different media. However, we observed similar results when cells were incubated in a balanced salt solution (Tables 1 and 2), suggesting that differences in incubation media did not contribute to our inability to measure LDH release. We used a commercial assay for measurement of LDH activity and it is possible that menadione and PAP interfered with one or more components of the assay. However, as seen in Tables 1 and 2, we observed similar results (loss of total LDH activity without an increase in LDH activity measured in media) when using a spectrophotometric assay that measures NADH oxidation coupled to LDH-catalyzed pyruvate reduction (Bergmeyer and Bernt, 1963). Therefore, we speculated that PAP and menadione were reacting with and inactivating LDH. In order to more fully understand the interaction between PAP and menadione with LDH, we used purified enzyme for additional studies.

Increasing amounts of rabbit muscle LDH yielded increasing amounts of fluorescence. A fixed concentration of PAP (0.5 mM) decreased the amount of fluorescence associated with each concentration of LDH (Figure 5). Inhibition was most efficient with smaller amounts of LDH and less complete with the largest amount of LDH (1 unit/ml). These results suggested that PAP was either interfering with the assay used for LDH or that an interaction was occurring between PAP and LDH. Since we were able to detect LDH activity in lysate from cells incubated with PAP, we believed that a direct interaction was a more likely explanation.

We used a single concentration of LDH (0.5 units/ml) with varying amounts of four different toxicants: PAP and menadione to help explain our results in LLC-PK₁ cells; H₂O₂, which we assumed would oxidize LDH; and cisplatin, as an example of a metal that could interact with LDH. All four chemicals caused concentration-dependent loss of fluorescence (Figure 6). It is possible that PAP, menadione and H₂O₂ could be oxidizing NADH rather than inactivating LDH. However, cisplatin does not undergo oxidation so this potential mechanism does not

explain the decline in LDH fluorescence seen with cisplatin. To confirm that oxidation of NADH cannot account for our observations, we incubated NADH and pyruvate with 1 mM concentrations of the four toxicants. We noted no change in absorbance at 340 nm over a 60 min interval and no difference between readings obtained from incubations containing only pyruvate and NADH vs. incubations containing pyruvate, NADH and one of the toxicants (data not shown). These observations suggest that PAP and menadione are not simply interfering with the LDH assay but rather, undergo a chemical interaction with the LDH molecule. Therefore, we tested the ability of ascorbate, as an aqueous antioxidant, to prevent loss of fluorescence due these four toxicants.

Ascorbate effectively prevented PAP-induced inactivation of LDH, consistent with the ability of ascorbate to protect cells from PAP toxicity (Hallman *et al.*, 2000). In contrast, ascorbate did not prevent loss of LDH activity due to menadione, H₂O₂ or cisplatin. The results with cisplatin are consistent with lack of oxidation and suggest an interaction of cisplatin with the LDH protein. Loss of LDH activity was exacerbated when ascorbate was coincubated with menadione or H₂O₂ (Figure 7). An interaction between menadione and ascorbate wherein the combination exerted greater toxicity to isolated hepatocytes than either agent alone has been reported previously (Verrax *et al.*, 2004; Maellaro *et al.*, 1994). The mechanism of this interaction is attributed to ascorbate-dependent redox cycling of menadione generating increased amounts of H₂O₂ (Verrax *et al.*, 2004). In aqueous solution, menadione may be reduced by ascorbate, forming dehydroascorbate and semiquinone free radical. In turn, the semiquinone free radical may be oxidized back to menadione by molecular oxygen. This reaction would regenerate menadione for additional redox cycling as well as forming reactive oxygen intermediates including superoxide anion and H₂O₂ (Jarabak and Jarabak, 1995). At pharmacologic concentrations (0.3–20 mM), ascorbate exerts cytotoxicity against malignant but not normal cells (Chen *et al.*, 2005). The toxicity was attributed to generation of hydrogen peroxide in the incubation medium since peroxide scavengers prevented toxicity (Chen *et al.*, 2005). Thus, the potentiation of LDH inactivation that we saw with the combination of H₂O₂ and ascorbate may be attributed to enhanced hydrogen peroxide concentrations due to oxidation of ascorbate.

GSH protects cells from toxicity due to PAP (Hallman *et al.*, 2000), menadione (Nath *et al.*, 1995), H₂O₂ (Zager and Burkhart, 1998), and cisplatin (Ishikawa and Ali-Osman, 1993). In addition, GSH prevented inactivation of LDH by these chemicals (Figure 7). An interaction between glutathione and cisplatin has been described and may account for some of the selective renal toxicity of cisplatin (Townsend *et al.*, 2003a). However, cisplatin also forms glutathione conjugates spontaneously in aqueous solution (Townsend *et al.*, 2003b). Protection of LDH activity by glutathione is consistent with the presence of cysteine residues in the LDH molecule. LDH from rabbit muscle is a tetrameric protein with a molecular weight of about 140,000 (Lovell and Winzor, 1974). A monomer of LDH contains six cysteine residues out of a total of 571 amino acids (www.expasy.org, primary accession number Q8X666). Thus, there are multiple sites within LDH capable of accepting a reactive oxygen intermediate in much the same way as glutathione.

In summary, we found that several common toxicants interact with LDH in incubation media. In contrast, LDH in cells seems to be preserved from inactivation, possibly by the numerous other reactants available within cell cytoplasm. The phenomenon of LDH inactivation may be more common than previously thought, and investigators should be aware that LDH released into the incubation medium may be subject to chemical inactivation.

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Figure 1.

Cell viability following PAP incubation. LLC-PK₁ cells were incubated with PAP (0–10 mM) for 4 h at 37 °C. At the end of the incubation, half of the wells were washed with HBSS and 5% alamar blue was added. Fluorescence was determined at the beginning and end of a 2 h interval. After the final fluorescence reading, the remaining media was recovered and cells lysed. LDH activity was determined in media and lysate using the CytoTox-ONE assay kit. Each point represents mean $\pm$ SE of 3 independent experiments. Asterisks indicate mean values that are significantly different from control cells incubated in the absence of PAP ($p < 0.05$).

Figure 2.

Fluorescence data of media and lysate from cells incubated with PAP (0–10 mM) for 6 h. Fluorescence in control media was low and significantly decreased rather than increased in the presence of PAP (inset). In contrast, fluorescence in the lysate (representing cells remaining intact) dropped significantly when cells were incubated with PAP (open circles). Each point represents mean $\pm$ SE of 3 independent experiments. Asterisks indicate mean values that are significantly different from control cells incubated in the absence of PAP ($p < 0.05$).

Figure 3.

Cell viability following menadione incubation. LLC-PK₁ cells were incubated with menadione (0–1000 μM) for 4 h at 37 °C. At the end of the incubation, half of the wells were washed with HBSS and 5% alamar blue was added. Fluorescence was determined at the beginning and end of a 2 h interval. After the final fluorescence reading, the remaining media was recovered and cells lysed. LDH activity was determined in media and lysate using the CytoTox-ONE assay kit. Each point represents mean ± SE of 3 independent experiments. Asterisks indicate mean values that are significantly different from control cells incubated in the absence of PAP ($p < 0.05$).

Figure 4.

Fluorescence data of media and lysate from cells incubated with menadione (0–1000 μM) for 6 h. Fluorescence in control media was low and unchanged by the presence of menadione (inset). In contrast, fluorescence in the lysate (representing cells remaining intact) dropped significantly when cells were incubated with menadione (open circles). Each point represents mean ± SE of 3 independent experiments. Asterisks indicate mean values that are significantly different from control cells incubated in the absence of menadione ($p < 0.05$).

Figure 5.

Effect of PAP on LDH activity. LDH (0.1 to 1 unit) from rabbit muscle was incubated for 1 hour in the presence or absence of 0.5 mM PAP. Increasing amounts of LDH yielded increasing amounts of fluorescence and PAP significantly decreased fluorescence. Data represent means $\pm$ SE of 3 independent experiments and are expressed as arbitrary fluorescence units (AFU). Asterisks indicate mean values that are significantly different from mean LDH fluorescence in the absence of PAP ($p < 0.05$).

Figure 6.

Effect of toxicants on LDH activity. LDH (0.5 units) from rabbit muscle was incubated for 1 hour in the presence or absence of PAP, menadione, H₂O₂, or cisplatin (each at 1, 5 and 10 mM). Data represent means $\pm$ SE of 4 independent experiments and are expressed as a percentage of fluorescence observed in the absence of toxicants. Fluorescence with 0.5 units of LDH was about $40,000 \pm 1100$ AFU. The asterisk indicates that all treatment means are significantly different from mean LDH activity in the absence of toxicants ($p < 0.05$). Within a single concentration of chemical, means with the same letter are not significantly different.

Figure 7.

Effect of antioxidants on chemical-induced decreases in LDH activity. LDH (0.5 units) from rabbit muscle was incubated for 1 hour in the presence or absence of four toxicants (PAP, menadione, H₂O₂ and cisplatin, each at 5 mM). In addition, some incubations contained ascorbate or reduced GSH (each at 1 mM). Data represent means $\pm$ SE of 3 (cisplatin) or 7 (PAP, menadione, H₂O₂) independent experiments and are expressed as a percentage of fluorescence observed in the absence of toxicants or antioxidants. Fluorescence due to 0.5 units of LDH was about 37,000 $\pm$ 1600 arbitrary fluorescence units. Fluorescence in the presence of ascorbate or GSH was not different (36000 $\pm$ 1100 AFU and 40,000 $\pm$ 1000 AFU, respectively). Asterisks indicate mean values that are significantly different from mean LDH activity in the absence of toxicants or antioxidants. Daggers indicate values that are significantly different from LDH activity in the presence of toxicant alone.

Table 1LDH release and Total LDH Activity in LLC-PK1 cells incubated with *para*-aminophenol.

[PAP] (mM)	NADH oxidation		Resorufin formation	
	LDH release (% of total)	Total LDH (% control)	LDH release (% of total)	Total LDH (% control)
0	4.6 ± 4.6	100 ± 0	7.9 ± 2.3	100 ± 0
0.1	7.9 ± 4.8	101.9 ± 3.0	4.5 ± 0.9	97.6 ± 4.3
0.5	6.9 ± 3.5	83.0 ± 2.6 [*]	2.9 ± 1.6	71.2 ± 8.5 [*]
1	7.0 ± 2.8	85.0 ± 6.7 [*]	2.0 ± 1.0	65.1 ± 8.9 [*]
5	2.7 ± 2.7	76.0 ± 2.3 [*]	1.9 ± 1.0	67.7 ± 6.8 [*]
10	0 ± 0	65.8 ± 5.4 [*]	2.6 ± 1.5	67.7 ± 5.4 [*]

Cells were incubated for 4 h with PAP (0–10 mM) dissolved in HBSS. For NADH oxidation, cells were plated in 60 mm dishes and LDH was measured as the change in absorbance at 340 nm. For resorufin formation, cells were incubated in 24-well plates and LDH activity was determined using the CytoTox-One assay protocol. Data are expressed as means ± SE of 3 independent experiments.

* Significantly different from control (0 mM PAP) by one-way ANOVA with SNK post-hoc test ($p < 0.05$).

Table 2

LDH release and Total LDH Activity in LLC-PK1 cells incubated with menadione.

[menadione] (μM)	NADH oxidation		Resorufin formation	
	LDH release (% of total)	Total LDH (% control)	LDH release (% of total)	Total LDH (% control)
0	1.0 ± 0.3	100.0 ± 0.0	$7.0 \pm 1.4^{\dagger}$	100.0 ± 0.0
10	1.3 ± 0.5	98.4 ± 2.5	$6.1 \pm 0.9^{\dagger}$	98.1 ± 1.0
50	1.0 ± 0.6	101.7 ± 4.7	5.8 ± 0.8	87.5 ± 0.8
100	1.5 ± 0.3	97.7 ± 6.0	$6.4 \pm 0.6^{\dagger}$	$75.0 \pm 4.2^{*\dagger}$
500	1.6 ± 0.6	$72.0 \pm 4.1^*$	$6.9 \pm 0.4^{\dagger}$	$59.3 \pm 4.8^*$
1000	$3.2 \pm 0.6^*$	$75.3 \pm 5.2^*$	$8.2 \pm 1.1^{\dagger}$	$56.8 \pm 7.8^{*\dagger}$

Cells were incubated for 4 h with menadione (0–1000 μM) dissolved in HBSS. For NADH oxidation, cells were plated in 60 mm dishes and LDH was measured as the change in absorbance at 340 nm. For resorufin formation, cells were incubated in 24-well plates and LDH activity was determined using the CytoTox-One assay protocol. Data are expressed as means $\pm$ SE of 3 independent experiments.

* Significantly different from control (0 mM menadione) by one-way ANOVA with SNK post-hoc test ($p < 0.05$).

† Significantly different from value determined by NADH oxidation at the same concentration of menadione.