Biochemical Pharmacology, Vol. 37, No. 22, pp. 4319–4325, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ANTIDOTAL EFFECTIVENESS OF *N*-ACETYLCYSTEINE IN REVERSING ACETAMINOPHEN-INDUCED HEPATOTOXICITY

ENHANCEMENT OF THE PROTEOLYSIS OF ARYLATED PROTEINS*†

MARY K. BRUNO,‡ STEVEN D. COHEN§ and EDWARD A. KHAIRALLAH‡‖

‡Department of Molecular and Cell Biology, and §Department of Pharmacology and Toxicology, The University of Connecticut, Storrs, CT 06268, U.S.A.

(*Received* 9 *October* 1987; *accepted* 2 *May* 1988)

Abstract—The post-arylative mechanisms by which *N*-acetylcysteine (NAC) reduces the severity of the hepatotoxicity induced by acetaminophen (APAP) were investigated in primary cultures of mouse hepatocytes. When administered at selected times immediately following removal of medium containing 10 mM APAP, 2.0 mM NAC was shown to restore glutathione levels through 16 hr of APAP pretreatment and to minimize the leakage of glutamate-oxaloacetate transaminase resulting from the first 8 hr of drug exposure. This temporal difference defined a critical period in which cells were responsive to NAC and permitted the investigation of potential post-arylative mechanisms of the antidote. In the absence of NAC during the recovery period, the cellular loss of covalently-bound APAP could be accounted for by the appearance of arylated proteins in the medium without any apparent degradation of APAP-bound proteins. By contrast, when NAC was present during the recovery period, there was a decrease in intracellular protein-bound APAP which could not be accounted for by that detected in the medium. Since during the recovery period the low residual intracellular concentration of APAP could not contribute significantly to any additional covalent binding in this system, NAC could not merely be acting as a nucleophilic trap for the reactive electrophile. Furthermore, NAC is not likely to dissociate covalently bound APAP from proteins. Hence, the overall decrease in covalent binding observed in cultures previously exposed to APAP for up to 8 hr must have arisen from an NAC-dependent enhancement of the degradation of the arylated proteins. However, after a more prolonged exposure to APAP, the ineffectiveness of NAC may have resulted from APAP-induced irreparable damage to the intracellular proteolytic system. These data suggest that the post-arylative efficacy of NAC may reside in the ability of the antidote to restore the functional capacity of the proteolytic system to rid the cells of arylated proteins.

Centrilobular hepatic necrosis induced by toxic doses of the widely used analgesic, acetaminophen (APAP), is believed to result from cytochrome P-450-dependent activation of the drug to a highly reactive metabolite, *N*-acetyl-benzoquinone-imine (NAPQI) [1] that is both a strong electrophile and a potent oxidizing agent [2]. Covalent binding of NAPQI to proteins following a significant reduction in the intracellular concentration of glutathione (GSH) has been implicated in the resulting organelle damage, enzyme leakage, and eventual cell death [3, 4]. Alternatively, NAPQI may lead to oxidative changes in critical macromolecules which may also be mechanistically involved in APAP-induced liver injury [5].

Both mechanisms for APAP-induced hepatic injury have been considered in evaluating data obtained *in vitro* [6–11] and in the development of effective antidotal therapy *in vivo* [12–17]. The effectiveness of the antidote, *N*-acetylcysteine (NAC), in reducing the severity of liver necrosis has been attributed primarily to its direct or indirect (via cysteine or GSH) nucleophilic capacity to conjugate and detoxify the reactive metabolite [10, 14, 15, 18]. This mechanism implies that NAC would be effective only as long as the reactive electrophile was still being generated. However, the antidotal efficacy of NAC and other agents, in the absence of decreased covalent binding, has also been demonstrated [19–21]. This suggests that the antidotes may also act by post-arylative mechanisms which prevent or reverse functional impairment resulting from the initial actions of NAPQI.

The present study was conducted in cultured mouse hepatocytes to determine the nature and occurrence of the post-arylative mechanisms that may be involved in the antidotal action of NAC in ameliorating alterations in cellular homeostatic processes.

MATERIALS AND METHODS

Materials. Uniformly labeled [^{3}H]APAP and L-[4,5-^{3}H(N)]leucine were purchased from New Eng-

* Presented in part at the 26th Annual Meeting of the Society of Toxicology, February 1987, Washington, DC.

† Supported in part by a grant from the National Institutes of Health (GM 31460) and the University of Connecticut Research Foundation.

‖ Correspondence should be addressed to: Dr. Edward A. Khairallah, Department of Molecular and Cell Biology, U-125, The University of Connecticut, 75 North Eagleville Road, Storrs, CT 06268.

land Nuclear-DuPont, Inc. (Boston, MA). Ring-labeled [^{14}C]APAP and NCS tissue solubilizer were obtained from Amersham-Searle (Arlington Heights, IL). Collagenase CLS-II was purchased from Organon Teknika Corp. (BCA-Cappel) (Cooper Biomedical, Inc., Westchester, PA). Eagle's Minimal Essential Medium (MEM) and fetal bovine serum (FBS) for cell culture were obtained from Flow Laboratories (McLean, VA) and Hyclone Laboratories, Inc. (Logan, UT) respectively. All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) and were of the purest grade available.

Hepatocyte isolation and culture. Hepatocytes were isolated by collagenase perfusion [22, 23] from 3-month-old male C57/Bl6 mice fed *ad lib.* Cells were cultured at a density of 750,000 cells per 35 mm Falcon tissue culture dish in 2 ml of MEM supplemented with the non-essential amino acids (1 mM each), 10% FBS, 2 μM insulin, 10 μM dexamethasone, 15 mM nicotinamide, 200 units penicillin/ml, 200 μg streptomycin/ml and 50 μg gentamicin/ml. Hepatocytes were maintained in culture for at least 18 hr prior to the initiation of experiments in fresh medium lacking nicotinamide. The binding of ligands, such as nicotinamide, to cytochrome P-450 has been reported to maintain the concentration of the enzyme in cultured hepatocytes [24, 25]. However, it was necessary to remove nicotinamide from the culture medium during the experimental conditions so as to prevent any possible interference with cytochrome P-450-dependent activation of APAP.

Covalent binding of acetaminophen. Covalent binding was determined in cultures at 4, 8, and 16 hr following the addition of 10 mM [^{3}H]APAP (0.1 μCi/μmol) solubilized in medium. This concentration was selected based upon earlier dose–response studies which demonstrated cytotoxicity in cultured mouse hepatocytes within a 24-hr exposure period [22]. At selected times, APAP-exposed cultures were either harvested immediately (treatment phase) or washed twice with 2 ml of fresh medium without APAP and divided into two groups for further incubation with or without 2 mM NAC for an additional 12 hr (recovery phase). Cells from all three groups were washed twice with 2 ml of ice-cold phosphate-buffered saline prior to harvest into a total volume of 1.2 ml of hypotonic buffer (15 mM KCl, 10 mM Tris–HCl, 1.5 mM magnesium acetate, pH 7.5). Cells were homogenized with ten strokes of a Teflon–glass homogenizer, and isotonicity was restored by the addition of one-tenth the volume (120 μl) of a hypertonic buffer containing 200 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 1.2 mM KCl, and 50 mM magnesium acetate, pH 7.5. To permit a quantitative comparison between the covalently bound metabolite present in cells following the initial exposure to APAP and that measured 12 hr after APAP removal, medium samples from the recovery phase cultures were also analyzed for arylated proteins released from the cells. Aliquots from the homogenate or medium samples were precipitated with perchloric acid (PCA) at a final concentration of 1.0 N. The precipitated protein pellets were collected by centrifugation for 5 min at 12,000 *g* in an Eppendorf model 5412 microfuge and were subsequently extracted three times with 80% methanol [3] containing 10 mM unlabeled APAP. To minimize partial solubilization of some proteins in acidic methanol [26], the methanol suspension was adjusted to pH 8 by the addition of 20–40 μl of 1.0 N NaOH to each pellet. Samples were then digested in NCS tissue solubilizer, and the radioactivity was quantitated by liquid scintillation spectrometry (Beckman model LS 3801) using an external standard for quench correction. Total APAP binding to proteins is represented as that amount measured in cells alone at the end of the treatment phase or as the sum of that determined in cells and medium following the recovery phase. The data are expressed as nanomoles APAP bound per milligram of total cellular protein per plate.

Intracellular concentration of acetaminophen after drug removal. To determine the intracellular concentration of unbound APAP after drug removal, hepatocytes were exposed to 10 mM [^{14}C]APAP (0.1 μCi/μmol) for 8 hr. Following removal of the radiolabeled drug, cells were washed twice with 2 ml medium and either harvested immediately (t = 0) or incubated in APAP-free medium for selected times up to 2 hr. Cultures were washed twice again with 2 ml of phosphate-buffered saline and homogenized directly in 1.0 N PCA. Acid-soluble extracts from both cells and medium were neutralized with 3 M $KHCO_3$ prior to the quantitation of radioactivity. The intracellular concentration of APAP and APAP conjugates was estimated on the basis of an 85% cellular water content (5.4 μl/mg protein) after correction for any residual extracellular medium contamination remaining on the monolayers [27].

Measurement of protein degradation. Four hours after plating, hepatocytes were prelabeled with [^{3}H]leucine (0.5 μCi/ml) for 15 hr. Following removal of the radioactive medium, the cultures were first washed once and incubated with 2.5 ml of nicotinamide-free medium supplemented with 10 mM unlabeled leucine (chase medium) for an additional 15 min to deplete the radioactive tracer from the intracellular amino acid pool [28]. Following removal of this medium, the cultures were then exposed to 10 mM APAP in chase medium for selected times up to 12 hr. Parallel cultures incubated in chase medium alone served as controls. Each time point was terminated by removing the chase medium, rapidly washing the cells with 2 ml of ice-cold phosphate-buffered saline, followed by direct homogenization of the cells in a total volume of 1.2 ml of 1.0 N PCA. The chase medium was also acidified to a final concentration of 1.0 N PCA. The precipitated protein pellets were collected by centrifugation for 5 min at 12,000 *g* and were washed three times with 1.0 N PCA containing 10 mM unlabeled leucine prior to digestion in NCS tissue solubilizer. The corresponding acid supernatant fractions from the cells and medium were neutralized with 3 M $KHCO_3$ prior to liquid scintillation spectrometry. Protein degradation was expressed as the percentage of acid-soluble radioactivity released into the medium relative to the total radioactivity recovered per plate.

Analytical procedures. Protein content was deter-

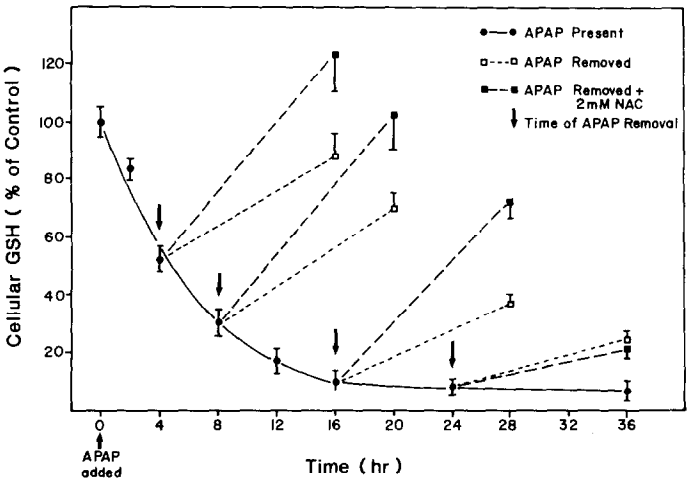

Fig. 1. Recovery of GSH in the presence and absence of *N*-acetylcysteine (NAC) following APAP exposure. Primary cultures of mouse hepatocytes either were continuously exposed to 10 mM APAP for up to 36 hr (●——●) or, for the recovery experiments, cultures were washed free of drug at the times indicated by the arrows, and incubated for an additional 12 hr in the presence (■-----■) or absence (□-----□) of 2 mM NAC. The data are expressed as a percentage of the concentration of GSH determined in control cells (21.8 ± 1.5 nmol/mg cell protein) and are the average ± SE of three to seven experiments. The addition of NAC did not alter significantly the GSH content in control cultures.

mined by the method of Lowry *et al.* [29] using bovine serum albumin as a standard. Cellular GSH content was assayed by the method of Griffith [30] following neutralization with 3 M $KHCO_3$ of the acid supernatant fractions isolated as described above. The functional integrity of the plasma membrane was assessed by determining the percentage of glutamate-oxaloacetate transaminase (GOT) released into the medium. Hepatocytes were ruptured by hypotonic lysis, and the activity of GOT in both cells and medium was determined by the colorimetric assay of Reitman and Frankel (see legend of Table 1 for details) [31]. Medium samples were appropriately corrected for endogenous GOT activity present in FBS.

RESULTS AND DISCUSSION

Effects of NAC addition after acetaminophen removal on GSH content and GOT release. Cultured mouse hepatocytes continuously exposed to 10 mM APAP for up to 36 hr exhibited a progressive decline in the intracellular concentration of GSH (Fig. 1). By 16 hr, the cellular content of GSH in APAP-treated cultures had been reduced to 10% of control. Removal of APAP at either 4, 8, 16, or 24 hr resulted

Table 1. Effect of 2.0 mM NAC on GOT release during the recovery phase following APAP removal

Length of initial exposure (hr)	Initial GOT release (%) Control	Initial GOT release (%) APAP	Additional GOT release after 12-hr recovery (%) −NAC	Additional GOT release after 12-hr recovery (%) +NAC	Extent of improvement with NAC (%)
4	2.2 ± 0.4	6.1 ± 1.8	6.6 ± 1.3	1.4 ± 0.4	78.8 ± 10.3*
8	6.2 ± 0.8	12.0 ± 2.8	8.0 ± 1.5	4.3 ± 0.7	46.3 ± 6.1†
16	9.7 ± 3.8	21.5 ± 4.3	10.6 ± 1.2	10.7 ± 1.3	
20	13.7 ± 5.7	32.5 ± 7.6			
28	19.8 ± 3.5	62.0 ± 3.5			

Cytotoxicity was determined by the release of GOT from cultured mouse hepatocytes exposed to APAP for selected times up to 28 hr. Following each period of exposure, drug-containing medium was removed, and the cells were washed and incubated for an additional 12-hr recovery period in the presence or absence of NAC. Enzyme leakage was expressed as a percentage of GOT activity released into the medium during either the initial APAP exposure or recovery periods relative to the total enzyme activity per plate. Each plate contained approximately 1 mg of protein and exhibited GOT activity capable of converting 0.3 to 0.4 μmol of alpha-keto-glutarate to glutamate/min at 25°, which was measured by the rate of formation of the 2,4-dinitrophenylhydrazone at 546 nm [31]. The enzymatic activity in the medium was corrected for endogenous GOT activity initially present in FBS. The data are expressed as the mean ± SE for three to five experiments. The extent of improvement with NAC was compared to that observed in the absence of NAC, and the statistical significance of the differences was evaluated by Student's paired *t*-test.

*† Comparisons of GOT release during the recovery phase in the presence and absence of NAC are denoted as significant at $^*P < 0.01$ or $^\dagger P < 0.05$.

in the return of GSH over the ensuing 12 hr to 90, 70, 40, and 17% of control levels respectively. Thus, the extent of GSH repletion upon removal of APAP appeared to be dependent upon the duration of exposure and the resultant extent of prior GSH depletion. However, the rates of GSH repletion (estimated from Fig. 1) for exposures of 4, 8, or 16 hr appeared similar (i.e. 0.5 to 0.7 nmol/hr/mg cell protein) with a lower rate estimated (0.2 nmol/hr/mg cell protein) after a 24-hr exposure. Addition of NAC during the recovery period nearly doubled the rate of GSH accumulation in cells exposed for 4, 8, or 16 hr. By contrast, NAC had no significant effect on the rate of GSH repletion after 24 hr of exposure to APAP. This suggests that, between 16 and 24 hr of exposure, APAP-dependent damage to the GSH biosynthetic process has progressed to an irreversible stage.

Cytotoxicity, as evidenced by GOT release into the medium, also increased with the duration of APAP exposure (Table 1). Within 4 hr after the addition of APAP, 6% of the total GOT activity was in the medium and after 28 hr of continuous drug exposure the proportion of enzyme released was 62% of total. In contrast, GOT release from control cultures was 2.2% at 4 hr and increased only to 19.8% by 28 hr. On removal of APAP following selected times of exposure, the extent of additional GOT release during the recovery period was not significantly different from the incremental increase in enzyme released from control cells. Comparison of total GOT release (initial + recovery) for cells washed free of APAP with that for cells continuously exposed for the same total period revealed that removal of APAP alone significantly diminished GOT release during the subsequent 12 hr. For example, the data in Table 1 indicate that, by 16 hr, the total release of GOT was 21.5% for cells continuously maintained in APAP, yet the comparable value was only 12.7% for cultures pretreated with the drug for 4 hr and then placed in fresh medium without APAP for an additional 12 hr (6.1% released during the treatment phase and 6.6% during the 12-hr recovery period).

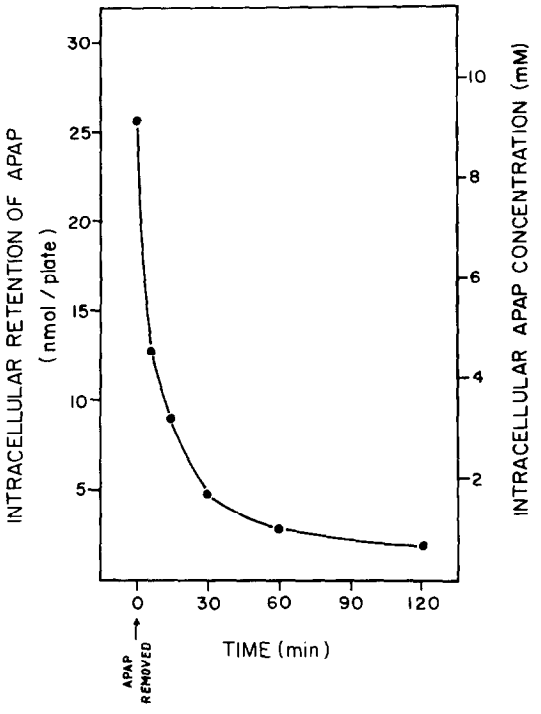

Fig. 2. Intracellular concentration of APAP in cultured mouse hepatocytes after drug removal. Hepatocytes cultured in 2 ml medium were treated with 10 mM [^{14}C]APAP (0.1 μCi/μmol) for 8 hr. Following removal of the drug, cells were washed twice with 2 ml medium and either were harvested immediately (t = 0) or incubated for the times shown to determine the rate of exchange between the cellular pool of drug and the fresh medium lacking APAP. Cultures were washed twice with phosphate-buffered saline and harvested directly into 1.0 N PCA. Radioactivity was quantitated in neutralized acid-soluble extracts from both cells and medium. The intracellular concentration of APAP was calculated on the basis of an 85% cellular water content (5.4 μl cellular water/mg protein) after correction for residual extracellular contamination of the monolayers [23]. Less than 5% of the intracellular acid-soluble radioactivity was conjugated to low molecular weight adducts (data not shown).

Supplementing APAP-pretreated cultures with 2 mM NAC during the recovery phase further decreased the release of GOT after APAP removal. The effectiveness of the antidote also depended upon the length of prior drug treatment. For example, the addition of NAC to cells exposed to APAP for 4 hr reduced the proportion of enzyme released during the 12-hr recovery period from 6.6 to 1.4%, corresponding to a 78% improvement. By contrast, after a 16-hr APAP pretreatment period, NAC was no longer effective in further decreasing enzyme leakage beyond the reduction observed by removal of APAP alone. Although the incremental increase in GOT release was quantitatively similar in control and treated cultures, NAC was only effective in preventing the increase in APAP-exposed cells. This indicated that the mechanisms responsible for GOT release were different. The gradual increase in release from control cells was probably reflective of the age of the cultures, whereas the additional release subsequent to APAP exposure was more likely the result of APAP-initiated events. Thus, these data suggest that the presence of NAC during the recovery period specifically interfered with the progression of cell injury initiated by prior exposure to APAP.

Collectively, these data suggest that NAC intervention in cultured mouse hepatocytes was beneficial with respect to GSH resynthesis through 16 hr of APAP exposure. However, with respect to interference with the progression of toxicity, as evidenced by GOT release, NAC was only effective for APAP exposures of less than 16 hr. This suggests that some irreversible damage to the plasma membrane integrity [32] had occurred during the exposure interval between 8 and 16 hr.

Clearance of intracellular acetaminophen after removal of acetaminophen from the culture medium. A possible complicating factor in the interpretation of the above results would arise if residual APAP remained within the hepatocytes subsequent to removal of APAP-containing medium and washing the cells. Preliminary investigations revealed that,

in cultured cells washed free of APAP-containing medium, approximately 85–90% of the total radiolabeled APAP within the intracellular pool was acid soluble. Since the interpretation of cellular recovery studies could be confounded by the residual intracellular pool of unbound APAP, additional studies were undertaken to determine the clearance of unbound APAP from the cultured cells. Hepatocytes, pretreated with 10 mM [^{14}C]APAP for 8 hr, were washed free of radioactive medium and further incubated for selected times up to 120 min. From the specific radioactivity of the drug (0.1 μCi/μmol) and the estimated volume of the water space within the hepatocytes (5.4 μl/mg cellular protein) [27], one can calculate the concentration of intracellular APAP that might be available for further metabolism. Immediately upon removal of the APAP-containing medium, the intracellular concentration of free APAP (8.8 mM) approximated the 10 mM concentration that was originally present extracellularly. There was a rapid decline to 1.5 mM by 30 min (Fig. 2) with a slower decline to 0.5 mM by 2 hr after APAP removal. These data indicate that residual APAP was cleared rapidly from the cells upon changing the medium, and that any contribution of residual intracellular APAP to the toxicity after APAP removal would be minimal. For example, if one extrapolates the intracellular APAP concentration depicted in Fig. 2 for the remaining 10 hr of the recovery period and integrates the area under the curve, then the maximal intracellular concentration throughout the 12-hr recovery period would be equivalent to less than 20 additional min of exposure to 10 mM APAP. Whereas cultured mouse hepatocytes treated with 0.5 mM APAP or less did not demonstrate significant covalent binding or depletion of GSH [22], the possibility remains that a persistent low intracellular concentration of APAP may be of toxicological significance to cells previously exposed to higher APAP concentrations and already containing significant amounts of NAPQI-arylated proteins. It is possible that a low level of electrophile production following APAP removal may result in the arylation or oxidation of critical target proteins which could be involved in the progression of cell damage. Therefore, one purpose of the following experiments was to determine if there were any detectable continued arylation of proteins after APAP removal.

Effect of NAC on covalent binding after removal of acetaminophen. Cultured mouse hepatocytes continuously exposed to 10 mM [^{3}H]APAP for up to 16 hr demonstrated a progressive increase in the amount of reactive metabolite covalently bound to proteins (Table 2, column A). During the first 16 hr of drug exposure, the rate of covalent binding averaged approximately 0.4 nmol of APAP bound per mg cellular protein per hr. Comparison between the amount of protein covalent binding during the treatment period and after recovery in the absence of NAC indicated that total (cells + medium) binding did not increase during the 12-hr period but remained equivalent to that initially present at the time the drug was removed (compare columns A and D). Based on the estimated amount of the residual intracellular APAP concentration during the recovery period (i.e. the equivalent of 20 min of 10 mM), the maximum amount of additional covalent binding that could have occurred would be no greater than 0.14 nmol bound/mg cellular protein. Thus, if there were any continued formation of NAPQI during the recovery period from any residual intracellular APAP, it could not have contributed significantly to the total extent of covalent binding.

Table 2. Comparison of covalent binding of APAP in the presence and absence of NAC following removal of APAP from the culture medium

Length of initial APAP exposure (hr)	Covalent binding after initial APAP exposure A		Covalent binding 12 hr after APAP removal		
			In cells B	In medium C	Total D
4	2.68 ± 0.32	−NAC	2.11 ± 0.11	0.77 ± 0.12	2.88 ± 0.14
		+NAC	1.50 ± 0.12	0.33 ± 0.08	1.84 ± 0.15
		% Change with NAC	28.9 ± 1.7	57.1 ± 4.8	36.1 ± 2.1*
8	4.42 ± 0.42	−NAC	3.09 ± 0.29	1.20 ± 0.23	4.30 ± 0.11
		+NAC	2.62 ± 0.37	0.80 ± 0.14	3.43 ± 0.31
		% Change with NAC	15.2 ± 2.1	33.3 ± 4.1	20.2 ± 3.1†
16	7.47 ± 0.97	−NAC	5.94 ± 0.94	1.39 ± 0.13	7.34 ± 0.89
		+NAC	5.37 ± 1.06	1.00 ± 0.11	6.38 ± 1.02
		% Change with NAC	9.6 ± 1.6	28.0 ± 3.8	13.1 ± 2.2

The covalent binding of APAP was determined in cultures treated as described in Table 1. The values are expressed as nanomoles of [^{3}H]APAP bound per milligram of total cellular protein per plate during either the initial APAP treatment or in both cells and medium during the 12-hr recovery periods after APAP removal. The extent of improvement upon addition of 2.0 mM NAC was compared to that observed in the absence of the antidote. Data are expressed as the mean ± SE for three to five experiments. Statistical significance of the differences was evaluated by Student's paired *t*-test.

** Comparisons between covalent binding in the presence and absence of NAC were denoted as significant at *$P < 0.01$ or †$P < 0.05$.

Closer inspection of the data in Table 2 revealed that, in the absence of NAC, the amount of arylated intracellular proteins was reduced by approximately 25% during the 12-hr recovery period (compare columns B and A). This decrease could readily be accounted for by the amount of arylated proteins detected in the medium (column C). Since the percentage of total GOT activity released during the recovery period remained below 11% (Table 1), the proportion of arylated proteins in the medium during this period may not simply be attributed to altered plasma membrane permeability but may represent a protective mechanism by which the cells attempt to dispose of the arylated proteins. Although we cannot exclude the possibility that the electrophile may escape from the cell and subsequently bind directly to serum proteins in the medium, the highly reactive properties of NAPQI [33, 34] make this an unlikely possibility.

NAC appeared to accelerate the clearance of protein-bound APAP from the hepatocytes. The effectiveness of NAC diminished as a function of the duration of the initial drug exposure. In contrast to the unsupplemented cultures, the decrease in intracellular covalent binding during the recovery period could not be accounted for by the amount of covalently-bound proteins detected in the medium. In fact, the amount of arylated proteins in the medium of NAC-supplemented cultures actually decreased relative to that in unsupplemented cultures (column C). Thus, the addition of NAC was effective in reducing the total amount of arylated proteins recovered from cultures previously exposed to APAP for 4 and 8 hr, but NAC had little effect in cultures pretreated for 16 hr (compare columns D and A). Since the continued generation of NAPQI during the recovery phase is likely to be minimal, and since it is unlikely that covalently bound APAP can be readily released from its protein conjugates, these data suggest that the loss in total protein-bound APAP resulted from the selective degradation of the arylated proteins to soluble products. In addition, the ineffectiveness of NAC supplementation in decreasing total covalent binding when the APAP exposure was increased to 16 hr is consistent with the inability of NAC to prevent further GOT release after this duration of APAP exposure. These observations suggest that APAP may selectively affect different cellular processes and imply that, although the cellular concentration of GSH was partially restored and enzyme leakage reduced, a prolonged initial drug exposure may have resulted in irreparable damage to the proteolytic system.

The suggestion that the proteolytic system may be impaired following APAP exposure is supported by our finding that, within 4–8 hr of continued APAP exposure, the degradation of prelabeled proteins in cultured hepatocytes was reduced significantly (Fig. 3) and was accompanied by an accumulation of enlarged autophagic vacuoles containing undegraded cellular organelles [35]. Maximal differences in the relative rates of degradation were calculated from the percentage of acid-soluble radioactivity released into the medium between 4 and 12 hr as 1.7%/hr compared to 1.0%/hr for control and APAP-treated cells respectively. Since the majority of liver protein

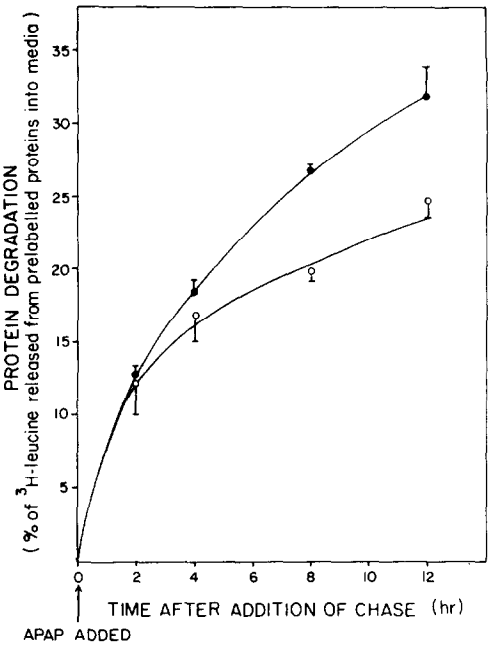

Fig. 3. Effect of APAP exposure on the degradation of prelabeled proteins. Hepatocytes were prelabeled with [^{3}H]leucine (0.5 μCi/ml medium) for 15 hr in MEM supplemented with the non-essential amino acids, 2 μM insulin, 10 μM dexamethasone, 10% FBS, 15 mM nicotinamide, 200 units penicillin/ml, 200 μg streptomycin/ml, and 50 μg gentamicin/ml. Following removal of the radioactive medium, cultures were washed and incubated for 15 min in nicotinamide-free medium supplemented with 10 mM unlabeled leucine (chase medium). Cultures were then incubated in the presence (○——○) or absence (●——●) of 10 mM APAP solubilized in chase medium for the times shown. Protein degradation was determined from the accumulated release of acid-soluble [^{3}H]leucine into the medium and is expressed as a percentage of the total radioactivity recovered per plate (40–50,000 dpm/plate = ~1 mg protein). The data are the average ± SE for three experiments.

catabolism occurs within the lysosomal compartment [36–38], these observations suggest that APAP administration may have disrupted lysosomal function. It is well documented that the lysosomal thiol proteases (Cathepsins B, H, and L) require GSH or cysteine for catalytic activity and are considered to be rate-limiting enzymes for proteolysis within the compartment [39]. It has also been reported previously that as much as 50% of the intracellular cysteine for GSH biosynthesis in hepatocytes can be derived from protein catabolism [40]. Thus, it is likely that, when proteolysis is reduced, cysteine may become rate-limiting for cellular biosynthetic or catalytic functions. This could explain the inability of cultured hepatocytes in the present study to decrease the amount of covalently modified proteins in the absence of NAC. However, in the presence of NAC, sufficient cysteine would become available to maintain biosynthetic and catabolic reactions and facilitate the catabolism of arylated proteins until such a time as the proteolytic enzymes are themselves damaged and beyond repair.

Alternatively, APAP alterations in the cellular redox state may also play an important role in the impaired clearance of arylated proteins. APAP-induced decreases in the protein sulfhydryl content in cultured hepatocytes have been attributed to the oxidative properties of NAPOI and have been correlated with the extent of cell damage [41]. Furthermore, both lysosomal and nonlysosomal proteolytic functions may be regulated by the intracellular thiol redox state [42, 43]. Thus, the effectiveness of NAC in facilitating the catabolism of arylated proteins may also be mediated through GSH-dependent restoration of the intracellular thiol redox state.

In summary, the ability of antidotal treatments to reduce the severity of hepatic necrosis following APAP exposure has been attributed to mechanisms which both precede [14, 15] and follow [19–21] arylation. Data obtained in this study provide additional evidence for a post-arylative mechanism and suggest that the protective action afforded by NAC may be mediated, in part, by restoring the activities of the intracellular proteolytic systems and imply that the normal functioning of this system in eliminating the arylated proteins may be essential for cellular homeostasis. Efforts are currently directed toward elucidating the mechanisms responsible for the elimination of the arylated proteins.

REFERENCES

1. D. C. Dahlin, G. T. Miwa, A. Y. Lu and S. D. Nelson, *Proc. natn. Acad. Sci. U.S.A.* **81**, 1327 (1984).
2. I. A. Blair, A. R. Boobis, D. S. Davies and T. M. Cresp, *Tetrahedron Lett.* **21**, 4947 (1980).
3. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 195 (1973).
4. A. J. Streeter, D. C. Dahlin, S. D. Nelson and T. A. Baillie, *Chem. Biol. Interact.* **48**, 349 (1984).
5. M. Moore, H. Thor, G. Moore, S. Nelson, P. Moldeus and S. Orrenius, *J. biol. Chem.* **260**, 13035 (1985).
6. H. Thor, P. Moldeus and S. Orrenius, *Archs Biochem. Biophys.* **192**, 405 (1979).
7. T. E. Massey and W. J. Racz, *Toxic. appl. Pharmac.* **60**, 220 (1981).
8. A. R. Boobis, L. B. G. Tee, C. E. Hampden and D. S. Davies, *Fd chem. Toxic.* **24**, 731 (1986).
9. A. W. Harman and G. Self, *Toxicology* **41**, 83 (1986).
10. M. G. Miller and D. J. Jollow, *Toxic. appl. Pharmac.* **83**, 115 (1986).
11. L. B. G. Tee, A. R. Boobis, A. C. Huggett and D. S. Davies, *Toxic. appl. Pharmac.* **83**, 294 (1986).
12. A. R. Buckpitt, D. E. Rollins and J. R. Mitchell, *Biochem. Pharmac.* **28**, 2941 (1979).
13. J. O. Miners, R. Drew and D. J. Birkett, *Biochem. Pharmac.* **33**, 2995 (1984).
14. B. H. Lauterberg, G. B. Corcoran and J. R. Mitchell, *J. clin. Invest.* **71**, 980 (1983).
15. G. B. Corcoran, W. J. Racz, C. V. Smith and J. R. Mitchell, *J. Pharmac. exp. Ther.* **232**, 864 (1985).
16. J. G. Gerber, J. S. MacDonald, R. D. Harbison, P. J. Villenueve, A. J. J. Wood and A. S. Nies, *Lancet* **1**, 657 (1977).
17. L. W. Whitehouse, L. T. Wong, C. J. Paul, A. Pakuts and G. Solomonraj, *Can. J. Physiol. Pharmac.* **63**, 431 (1985).
18. L. F. Chausseaud, *Biochem. Pharmac.* **23**, 1133 (1974).
19. D. Labadarios, M. Davis, B. Portmann and R. Williams, *Biochem. Pharmac.* **26**, 31 (1977).
20. E. Piperno, A. H. Mosher, D. A. Berssenbruegge, J. D. Winkler and R. B. Smith, *Pediatrics, N.Y.* **62**, 880 (1978).
21. J. L. Devalia, R. C. Ogilvie and A. E. McClean, *Biochem. Pharmac.* **31**, 3745 (1982).
22. M. K. Bruno, J. B. Bartolone, S. D. Cohen and E. A. Khairallah, *Pharmacologist* **27**, 482 (1985).
23. P. O. Seglen, *Meth. Cell Biol.* **13**, 29 (1976).
24. A. J. Paine, L. J. Williams and R. F. Legg, *Life Sci.* **24**, 2185 (1979).
25. A. J. Paine, P. Villa and L. J. Hockin, *Biochem. J.* **188**, 937 (1980).
26. E. A. Khairallah and G. E. Mortimore, *J. biol. Chem.* **251**, 1375 (1976).
27. M. K. Bruno, *Masters Thesis*. University of Connecticut (1986).
28. P. O. Seglen, B. Grinde and A. E. Solheim, *Eur. J. Biochem.* **95**, 215 (1979).
29. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
30. O. W. Griffith, *Analyt. Biochem.* **106**, 207 (1980).
31. S. Reitman and S. Frankel, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), Vol. 2, pp. 735–739. Academic Press, New York (1974).
32. C. D. Klaassen and N. H. Stacey, in *Toxicology of the Liver* (Eds. G. Plaa and W. R. Hewitt), pp. 147–149. Raven Press, New York (1982).
33. D. C. Dahlin and S. D. Nelson, *J. med. Chem.* **25**, 885 (1982).
34. E. Albano, M. Rundgren, P. J. Harvison, S. D. Nelson and P. Moldeus, *Molec. Pharmac.* **28**, 306 (1985).
35. M. K. Bruno, S. D. Cohen and E. A. Khairallah, *Fedn Proc.* **45**, 1932 (1986).
36. C. M. Schworer, K. A. Shiffer and G. E. Mortimore, *J. biol. Chem.* **256**, 7652 (1981).
37. H. Glaumann, J. Ahlberg, A. Berkenstam and F. Henell, in *Intracellular Protein Catabolism* (Eds. E. A. Khairallah, J. S. Bond and J. W. C. Bird), pp. 417–426. Alan R. Liss, New York (1985).
38. E. A. Khairallah, *Biochem. Soc. Trans.* **13**, 1012 (1985).
39. N. Katunuma and E. Kominami, in *Current Topics in Cellular Regulation* (Eds. B. L. Horecker and E. R. Stadtman), Vol. 22, pp. 71–101. Academic Press, New York (1983).
40. D. J. Reed and S. Orrenius, *Biochem. biophys. Res. Commun.* **77**, 1257 (1977).
41. R. Birge, J. Bartolone, M. Bruno, S. Cohen, J. Mangold, E. Nishanian and E. Khairallah, *Toxicologist* **7**, 115 (1987).
42. D. L. Schneider, J. Burnside, F. R. Gorga and C. J. Nettleton, *Biochem. J.* **176**, 75 (1978).
43. D. Di Cola and P. Sacchetta, *Fedn Eur. Biochem. Soc.* **210**, 81 (1987).