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EFFECTS OF THE PYRROLIZIDINE ALKALOID SENECIONINE AND THE ALKENALS *trans*-4-OH-HEXENAL AND *trans*-2-HEXENAL ON INTRACELLULAR CALCIUM COMPARTMENTATION IN ISOLATED HEPATOCYTES

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Abstract—The pyrrolizidine alkaloid senecionine has been shown to produce an increase in cytosolic free Ca^{2+} concentration in isolated hepatocytes that correlated with an increase in cellular toxicity. The cytotoxicity was greater in the absence of extracellular Ca^{2+} than in its presence, suggesting that alterations in intracellular Ca^{2+} distribution, and not an influx of extracellular Ca^{2+}, were responsible for the senecionine-induced hepatotoxicity. The effect of senecionine, as well as the effects of *trans*-4-OH-2-hexenal (t-4HH), a microsomal metabolite of senecionine, and a related alkenal, *trans*-2-hexenal, on the sequestration of Ca^{2+} in mitochondrial and extramitochondrial compartments were examined in isolated hepatocytes. Each of the test compounds elicited a decrease in the available extramitochondrial Ca^{2+} stores that was inhibited by pretreatment with the thiol group reducing agent, dithiothreitol. Senecionine and t-4HH decreased the level of Ca^{2+} sequestered in the mitochondrial compartment of hepatocytes. The presence of a pyridine nucleotide reducing agent, β-hydroxybutyrate, inhibited this reduction. These results suggest that both senecionine and t-4HH inhibit the sequestration of Ca^{2+} in extramitochondrial and mitochondrial compartments possibly by inactivating free sulfhydryl groups and oxidizing pyridine nucleotides respectively.

Pyrrolizidine alkaloids (PAs) are native to many *Senecio* species throughout the world and are responsible for numerous livestock losses and human poisonings [1]. Senecionine, a macrocyclic PA derived from the *Senecio* species, has been shown to be hepatotoxic [2–4], genotoxic, and cytotoxic [5–8]. This toxicity is attributed to the formation of reactive pyrroles by the action of hepatic microsomal enzyme systems [9–11]; however, the biochemical mechanism for the hepatocellular toxicity remains to be elucidated. The association between the appearance of cellular necrosis and the accumulation of Ca^{2+} in necrotic cells prompted the hypothesis that the plasma membrane is the primary target in chemically-induced cell death, and a consequence of this membrane damage was an influx of extracellular Ca^{2+} into the cell [12]. Senecionine has been shown to produce peroxidation of membrane lipids in a dose-related manner in isolated rat hepatocytes [13]. However, this process could be dissociated from that of cellular toxicity, suggesting that lipid peroxidation was a secondary effect of senecionine and not the primary mechanism by which senecionine induces hepatotoxicity at the cellular level. Alterations in intracellular Ca^{2+} concentration also were examined as a possible primary mechanism of cellular toxicity. Senecionine was shown to elicit an increase in cytosolic free Ca^{2+} levels in isolated hepatocytes, when incubated in the presence of extracellular Ca^{2+}, which correlated with an increase in cellular toxicity [14]. Senecionine is more toxic to isolated hepatocytes in the absence of extracellular Ca^{2+} than in its presence [14]. This suggested that a disturbance in intracellular Ca^{2+} homeostasis, and not an influx of extracellular Ca^{2+}, may play a role in senecionine-induced hepatotoxicity.

Intracellular Ca^{2+} concentrations in hepatocytes (approximately 10^{-7} M) are controlled by the transport of Ca^{2+} across mitochondrial and extramitochondrial membranes, such as the endoplasmic reticulum and plasma membrane [15, 16]. The Ca^{2+} content of these compartments can be measured by using a non-disruptive technique based on the selective release of Ca^{2+} from mitochondrial and total cellular pools by addition of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and the ionophore A23187 respectively. In the presence of the metallochromic indicator Arsenazo III, the released Ca^{2+} can be determined spectrophotometrically [17]. Using this method we examined the effects of senecionine, *trans*-4-OH-2-hexenal (t-4HH), a microsomal metabolite of senecionine, and the related alkenal, *trans*-2-hexenal (t-2H), on mitochondrial and extramitochondrial Ca^{2+} sequestration in isolated rat hepatocytes. In additional experiments, the hepatocytes were preincubated either with dithiothreitol or the pyridine nucleotide reducing agent, β-hydroxybutyrate. The effects of these protective agents on compartmental Ca^{2+} sequestration in response to senecionine, t-4HH and t-2H were also examined.

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MATERIALS AND METHODS

Animals and chemicals. Adult male Sprague–Dawley rats, 250–300 g, were obtained from Banton-Kingman Laboratories (Fremont, CA). The animals were allowed feed (Standard Ralston Purina rat chow No. 5012) and water *ad lib.* and housed under a 12-hr light/dark photoperiod. Senecionine was extracted and isolated from *Senecio vulgaris* (common groundsel) by the method of Segall [18, 19]. *trans*-4-OH-2-Hexenal was synthesized by the method of Erickson [20]. *trans*-2-Hexenal was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Collagenase Type IV, percoll, digitonin, β-hydroxybutyrate, and 1,4-dithiothreitol (DTT) were obtained from the Sigma Chemical Co. (St. Louis, MO). The cation ionophore A23187 was purchased from Calbiochem-Behring (San Diego, CA). Arsenazo III (2,2′-{1,8-dihydroxy-3,6 - disulfonaphthylene - 2,7 - bisazo)}bisbenzene-arsonic acid) and carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP) were obtained from Aldrich Chemical Co. FCCP and A23187 were dissolved in dimethyl sulfoxide. Arsenazo III was dissolved in modified Ca^{2+}-free Hanks' medium. The sources of chemicals for perfusates I and II (PF I, PF II) have been described previously [21]. All other reagents were of the highest grade commercially available.

Procedure. Rat hepatocytes were isolated according to the method of Hems *et al.* [22] with minor modifications. Briefly, the liver was perfused *in situ* with a solution containing ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) (PF I) for 10 min. The liver was then perfused with a second solution (PF II) containing $CaCl_2$ (0.74 g/100 ml) and collagenase (394 units/ml) for 15 min. The hepatocytes were centrifuged through a 36% Percoll solution in Hanks' buffer as previously described [23]. Non-viable hepatocytes were removed, and viable cells were resuspended in a Krebs–Henseleit medium supplemented with 12.6 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)(pH 7.4) at 6.0×10^6 cells/ml. Viability of the hepatocytes following the Percoll gradient was routinely 96–100%, as determined by means of the trypan blue exclusion test [15]. Following a 15-min preincubation period at 37° in a shaking water bath (200 oscillations/min), samples were taken at zero time and the test compounds were added. *trans*-4-OH-2-Hexenal and t-2H were dissolved in ethanol. Senecionine was dissolved in 0.1 M HCl and neutralized with 0.1 M NaOH prior to addition to the suspensions. The control suspensions were treated with 0.1 ml ethanol/6.0×10^6 cells.

In the study investigating the effect of a thiol group reducing agent on intracellular Ca^{2+} levels, hepatocytes were preincubated for 5 min with 2 mM 1,4-dithiothreitol (DTT). To maintain the mitochondrial pyridine nucleotides in a reduced state, hepatocytes were preincubated with 6.0 mM β-hydroxybutyrate for 5 min [24]. Hepatocyte incubation flasks were flushed with 95% oxygen:5% carbon dioxide and incubated at 37° in a shaking water bath.

Biochemical assays. Intracellular Ca^{2+} levels from mitochondrial and extramitochondrial compartments were determined by the method of Kendrick *et al.* [25] as described by Bellomo *et al.* [26]. The hepatocytes were separated from the Ca^{2+}-containing Krebs–Henseleit medium by centrifugation through a 36% Percoll solution in modified Ca^{2+}-free Hanks' buffer [17]. The hepatocytes were resuspended in modified Ca^{2+}-free Hanks' medium. Protein was determined by the method of Lowry *et al.* [27] on 0.5 ml of the hepatocyte suspension.

Arsenazo III (final concentration was 30 μM) was added to the remaining hepatocyte suspension (2.5 ml) in a plastic cuvette. Digitonin (2 μM) was added to make the plasma membrane permeable to Ca^{2+}. Absorbance changes at the wavelength pair 654 versus 685 nm were recorded using a Beckman DU-6 UV/VIS Scanning Spectrophotometer. FCCP (10 μM) was then added to the hepatocyte suspension and the change in absorbance (654 − 685) was recorded. After no further change in absorbance occurred, the Ca^{2+} ionophore A23187 (15 μM) was added, and Ca^{2+} release was recorded.

RESULTS

A graph of $A_{654-685}$ as a function of added Ca^{2+} was prepared to correlate changes in absorbance with changes in Ca^{2+} (1–10 nmol/ml) which had been added to hepatocytes (6.0×10^6 cells/ml) previously exposed to EGTA to remove Ca^{+} (Fig. 1). As shown by the graph in Fig. 1, the addition of EGTA did not deplete completely the hepatocellular Ca^{2+} content. This residual Ca^{2+} concentration was taken into account when calculating Ca^{2+} levels in the intracellular compartments. Absorbance changes at the wavelength pair 654−685 nm were recorded. These wavelengths, rather than 675 versus 685 nm, were used because they provided greater sensitivity.

Figure 2 shows the release of sequestered Ca^{2+} by FCCP from isolated hepatocytes treated with 240 nmol/10^6 cells of senecionine, t-4HH, and t-2H. Calcium released from hepatocytes by FCCP is associated with Ca^{2+} of mitochondrial origin [26]. The values are expressed as a percent of the initial value at time zero (7.34 ± 0.65 nmol Ca^{2+}/mg cell protein). This value represented approximately 70% of the total Ca^{2+} released from the hepatocytes.

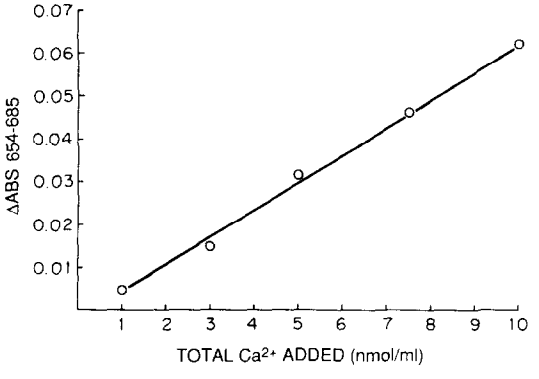

Fig. 1. Plot of $A_{654-685}$ versus the addition of known amounts of Ca^{2+} to 6.0×10^6 cells/ml of isolated hepatocytes previously depleted of Ca^{2+} by the addition of EGTA.

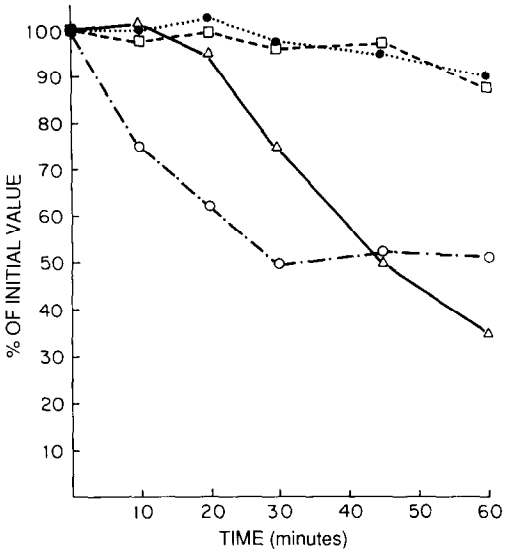

Fig. 2. Calcium released by FCCP from 6.0×10^6 cells/ml isolated hepatocytes. Hepatocytes were incubated alone (control) (●), or with one of the following: 240 nmol/10^6 cells senecionine (△), 240 nmol/10^6 cells *trans*-4-OH-2-hexenal (○), or 240 nmol/10^6 cells *trans*-2-hexenal (□). The results are expressed as a percent of the initial value at time zero (7.34 ± 0.65 nmol Ca^{2+}/mg protein). Each point represents the mean of three separate preparations. Standard deviations did not exceed 12% of the mean.

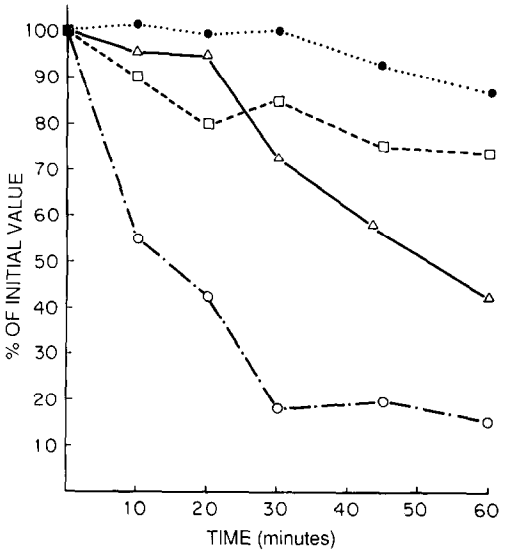

Fig. 3. Calcium released by FCCP–A23187 from 6.0×10^6 cells/ml isolated hepatocytes. Hepatocytes were incubated alone (control) (●), or with one of the following: 240 nmol/10^6 cells senecionine (△), 240 nmol/10^6 cells *trans*-4-OH-2-hexenal (○), or 240 nmol/10^6 cells *trans*-2-hexenal (□). The results are expressed as a percent of the initial value at time zero (2.08 ± 0.11 nmol Ca^{2+}/mg protein). Each point represents the mean of three separate preparations. Standard deviations did not exceed 26% of the mean.

trans-4-OH-2-Hexenal decreased FCCP-releasable Ca^{2+} levels to approximately 50% of the initial value by 30 min and remained at this level until 60 min. In contrast, senecionine-treated hepatocytes initially exhibited an FCCP-releasable pool similar to that of the control hepatocytes. At 60 min, the Ca^{2+} pools decreased to approximately 35% of initial values. *trans*-2-Hexenal did not produce a significant decrease in FCCP-releasable Ca^{2+} stores in the hepatocytes.

Release of Ca^{2+} from hepatocytes treated with 240 nmol/10^6 cells of senecionine, t-4HH, and t-2H by the ionophore A23187 after addition of FCCP is shown in Fig. 3. The results were obtained by subtracting the FCCP releasable Ca^{2+} from the Ca^{2+} released by addition of A23187. Calcium released from hepatocytes by A23187 is representative of total releasable cellular Ca^{2+} [28]. The values obtained in Fig. 3 are associated with Ca^{2+} sequestered in extramitochondrial compartments of hepatocytes. Initial values taken at time zero were 2.08 ± 0.11 nmol Ca^{2+}/mg cell protein. This level remained within 88% of the initial values over 60 min in control hepatocytes incubated with ethanol only. Hepatocytes treated with t-4HH elicited a rapid decrease in A23187—FCCP releasable Ca^{2+} reaching 18% of initial values by 30 min. Senecionine-treated hepatocytes produced a more gradual time-dependent decrease in Ca^{2+} reaching 42% of initial values by 60 min. *trans*-2-Hexenal decreased hepatocellular A23187–FCCP Ca^{2+} levels to 73% of initial values by 60 min.

The protective effects of the thiol group reducing agent, DTT, on compartmental Ca^{2+} sequestration in hepatocytes treated with 240 nmol/10^6 cells senecionine, t-4HH, and t-2H are shown in Tables 1 and 2. Hepatocytes pretreated with DTT and incubated with the test compounds exhibited both FCCP and A23187 Ca^{2+} levels similar to those observed with the control hepatocytes. Results of Ca^{2+} release by hepatocytes pretreated with β-hydroxybutyrate to maintain the mitochondrial pyridine nucleotides in a reduced state, and then treated with the test compounds, are shown in Tables 3 and 4. The presence of β-hydroxybutyrate markedly inhibited loss of FCCP-releasable Ca^{2+} from both senecionine- and t-4HH-treated hepatocytes. Loss of Ca^{2+} from FCCP-releasable stores also was inhibited in t-2H-treated hepatocytes in the presence of β-hydroxybutyrate. However, t-2H did not appear to affect FCCP-releasable Ca^{2+} stores in hepatocytes without the pyridine nucleotide reductant (Fig. 2). Loss of Ca^{2+} from the A23187–FCCP stores of hepatocytes was decreased slightly for each test compound in the presence of β-hydroxybutyrate; however, the difference between the presence of β-hydroxybutyrate and its absence was not significant.

DISCUSSION

The results of the experiments suggested that senecionine and t-4HH decreased both the mitochondrial and extramitochondrial Ca^{2+} pools in hepatocytes, whereas t-2H decreased only the extramitochondrial pools. Calcium released by the protonophore FCCP from isolated hepatocytes, by collapsing the proton

Table 1. FCCP releasable Ca^{2+} from isolated hepatocytes following treatment with senecionine, *trans*-4-OH-2-hexenal and *trans*-2-hexenal in the presence and absence of 2.0 mM dithiothreitol (DTT)

Chemical	Time (min)	FCCP-releasable Ca^{2+}* (nmol Ca^{2+}/mg protein)	
		Without DTT	With DTT
Control†	30	7.13 ± 0.84	7.45 ± 0.37
	60	6.61 ± 0.99	7.39 ± 0.59
Senecionine	30	5.51 ± 0.44	7.33 ± 0.44
(240 nmol/10^6 cells)	60	2.57 ± 0.21	7.19 ± 0.86
trans-4-OH-2-Hexenal	30	3.67 ± 0.44	7.37 ± 0.81
(240 nmol/10^6 cells)	60	3.75 ± 0.52	7.37 ± 0.51
trans-2-Hexenal	30	7.06 ± 1.27	7.40 ± 0.37
(240 nmol/10^6 cells)	60	6.43 ± 0.58	7.36 ± 0.29

* Values represent the mean ± SD of three separate preparations.
† Solvent control—ethanol only.

electrochemical gradient across the mitochondrial membrane, is associated with the mitochondrial compartment [17]. Values for mitochondrial Ca^{2+} content obtained with FCCP and Arsenazo III have been shown to be comparable to values obtained with atomic absorption spectrometry following rapid cell disruption [17]. The remaining Ca^{2+} released from the hepatocytes by A23187, after the initial release by FCCP (A23187–FCCP), is suspected of originating from the extramitochondrial compartments. The amount of Ca^{2+} released from hepatocytes by this sequential treatment correlates well with those values obtained with rapid cell disruption techniques and atomic absorption for extramitochondrial compartments [17].

The values obtained for the amount of Ca^{2+} released by the different intracellular compartments were higher than those previously reported. Bellomo *et al.* [26] found approximately 2 nmol/10^6 cells for the mitochondrial compartment and approximately 1 nmol/10^6 cells for the extramitochondrial compartments. Isolated hepatocytes, incubated in the presence of extracellular Ca^{2+}, have been shown to sequester more than 90 nmol Ca^{2+}/10^6 cells without adversely affecting cell viability [17]. This additional Ca^{2+} is thought to be sequestered in the mitochondrial and extramitochondrial compartments. Prior to measuring Ca^{2+} release, our hepatocytes were incubated in a Krebs–Henseleit buffer containing 2.8 mM $CaCl_2$. It is possible that hepatocytes sequestered some of this extracellular Ca^{2+}, which would explain the greater release of Ca^{2+} from each intracellular compartment. In contrast to t-4HH, senecionine exhibited a lag period prior to decreasing the available pools of sequestered Ca^{2+}. Senecionine has been shown previously to require metabolism by the cytochrome P-450 monooxygenases of isolated hepatocytes to produce cellular toxicity, whereas t-4HH and t-2H do not appear to require metabolic activation to produce cellular toxicity [8]. This requirement for activation may explain the initial lag observed in Figs. 2 and 3 with senecionine.

In previous studies, senecionine and t-4HH markedly decreased cellular glutathione, ATP, and NADPH levels and increased $NADP^+$ levels in a time- and dose-related manner in isolated hepatocytes [14]. In this experiment, preincubation of the hepatocytes with DTT, a thiol reductant, prevented the loss of Ca^{2+} from both the mitochondrial and

Table 2. A23187–FCCP releasable Ca^{2+} from isolated hepatocytes following treatment with senecionine, *trans*-4-OH-2-hexenal and *trans*-2-hexenal in the presence and absence of 2.0 mM dithiothreitol (DTT)

Chemical	Time (min)	A23187–FCCP releasable Ca^{2+}* (nmol Ca^{2+}/mg protein)	
		Without DTT	With DTT
Control†	30	2.07 ± 0.12	2.28 ± 0.18
	60	1.83 ± 0.15	2.25 ± 0.24
Senecionine	30	1.51 ± 0.31	2.19 ± 0.09
(240 nmol/10^6 cells)	60	0.90 ± 0.20	2.24 ± 0.13
trans-4-OH-2-Hexenal	30	0.38 ± 0.10	2.08 ± 0.16
(240 nmol/10^6 cells)	60	0.31 ± 0.06	2.60 ± 0.20
trans-2-Hexenal	30	1.77 ± 0.21	2.23 ± 0.27
(240 nmol/10^6 cells)	60	1.52 ± 0.20	2.23 ± 0.24

* Values represent the mean ± SD of three separate preparations.
† Solvent control—ethanol only.

Table 3. FCCP releasable Ca^{2+} from isolated hepatocytes following treatment with senecionine, *trans*-4-OH-2-hexenal and *trans*-2-hexenal in the presence and absence of 6.0 mM β-hydroxybutyrate (BOH)

Chemical	Time (min)	FCCP releasable Ca^{2+}* (nmol Ca^{2+}/mg protein)	
		Without BOH	With BOH
Control†	30	7.13 ± 0.86	7.18 ± 0.43
	60	6.61 ± 0.99	7.09 ± 0.57
Senecionine	30	5.51 ± 0.44	6.99 ± 0.70
(240 nmol/10^6 cells)	60	2.57 ± 0.21	6.54 ± 0.26
trans-4-OH-2-Hexenal	30	3.67 ± 0.44	6.92 ± 0.96
(240 nmol/10^6 cells)	60	3.75 ± 0.52	6.83 ± 0.89
trans-2-Hexenal	30	7.06 ± 1.27	7.00 ± 0.63
(240 nmol/10^6 cells)	60	6.43 ± 0.58	6.96 ± 0.76

* Values represent the mean ± SD of three separate preparations.
† Solvent control—ethanol only.

extramitochondrial pools for each of the test compounds. The presence of β-hydroxybutyrate, a reductant of pyridine nucleotides via β-hydroxybutyrate dehydrogenase, prevented the loss of Ca^{2+} from the mitochondrial compartment in hepatocytes treated with the test compounds; however, it had only a minimal protective effect on the extramitochondrial Ca^{2+} stores.

The mitochondrial and extramitochondrial Ca^{2+} pools are thought to be regulated by different mechanisms. Mitochondrial Ca^{2+} sequestration is thought to be regulated by an electroneutral $Ca^{2+}/2H^+$ antiport carrier located in the inner membrane of the mitochondria and modulated by the redox state of intramitochondrial pyridine nucleotides [29–31]. An increase in the ratio of oxidized/reduced pyridine nucleotides could promote release of Ca^{2+} from the mitochondrial matrix, whereas a reduced state could inhibit Ca^{2+} efflux [29]. Reduction of oxidized mitochondrial pyridine nucleotides by reversal of electron transport is dependent on the presence of ATP [32]. Decreased levels of ATP would favor oxidation of the pyridine nucleotides. Intracellular Ca^{2+} pools in extramitochondrial compartments such as the endoplasmic reticulum and plasma membrane are regulated by ATP-dependent Ca^{2+} translocases which have been found to depend on free sulfhydryl groups for activity [17, 26, 28, 33]. Oxidation of these sulfhydryl groups appears to inhibit sequestration of Ca^{2+} in the plasma membrane and endoplasmic reticulum. The previously observed decrease in NADPH/$NADP^+$ levels in isolated hepatocytes treated with senecionine and t-4HH [14] and the observed protective effects of β-hydroxybutyrate in this study suggest that senecionine and t-4HH may be inhibiting sequestration of mitochondrial Ca^{2+} via oxidation of pyridine nucleotides. The previously observed decrease in hepatocellular ATP levels in response to the test compounds may contribute to the oxidation of the pyridine nucleotides and the observed inhibition of Ca^{2+} sequestration in the mitochondria. Senecionine and t-4HH may be inhibiting sequestration of Ca^{2+} in the extramitochondrial compartments of hepatocytes via inactivation of free sulfhydryl groups critical for ATP-dependent Ca^{2+} translocase activity. In addition to the previously reported decrease in cellular glutathione in hepatocytes treated with the

Table 4. A23187–FCCP releasable Ca^{2+} from isolated hepatocytes following treatment with senecionine, *trans*-4-OH-2-hexenal and *trans*-2-hexenal in the presence and absence of 6.0 mM β-hydroxybutyrate (BOH)

Chemical	Time (min)	A23187–FCCP releasable Ca^{2+}* (nmol Ca^{2+}/mg protein)	
		Without BOH	With BOH
Control†	30	2.07 ± 0.12	2.11 ± 0.07
	60	1.83 ± 0.15	2.07 ± 0.12
Senecionine	30	1.51 ± 0.31	1.63 ± 0.22
(240 nmol/10^6 cells)	60	0.90 ± 0.20	1.14 ± 0.23
trans-4-OH-2-Hexenal	30	0.38 ± 0.10	0.52 ± 0.12
(240 nmol/10^6 cells)	60	0.31 ± 0.06	0.41 ± 0.10
trans-2-Hexenal	30	1.77 ± 0.21	1.75 ± 0.18
(240 nmol/10^6 cells)	60	1.52 ± 0.20	1.68 ± 0.50

* Values represent the mean ± SD of three separate preparations.
† Solvent control—ethanol only.

test compounds [14] and the observed protective effect of DTT on intracellular Ca^{2+} levels, the *trans*-4-OH-2-alkenals and the reactive metabolites of the PAs have been shown to act as reactive electrophilic agents forming covalent bonds with cellular macromolecules [3, 34–36]. Dithiothreitol may be protecting the mitochondrial Ca^{2+} pool by preventing the oxidation of thiol groups associated with the mitochondrial translocases. Another possibility may be that DTT prevented the oxidation of NADPH, although the detailed mechanism by which DTT exerts this effect is presently unclear.

Inhibition of Ca^{2+} sequestration from both the mitochondrial and extramitochondrial compartments may be a predominant mechanism for senecionine- and t-4HH-induced hepatotoxicity. *trans*-2-Hexenal, which elicited a decrease in the extramitochondrial compartments but not in the mitochondrial compartment, has been shown previously to be significantly less cytotoxic than either t-4HH or senecionine [8]. A number of studies have indicated that the mitochondrial compartment contains 60–80% of the total cellular Ca^{2+}, and it may have a greater capability to buffer the cytosolic free Ca^{2+} concentration than the other Ca^{2+} storage compartments [17, 37]. Calcium released into the cytosol, from extramitochondrial compartments whose translocases have been damaged by compounds that inhibit critical thiol groups, may be taken up by the mitochondria. However, inhibition of sequestration in the mitochondria could produce an irreversible rise in the cytosolic free Ca^{2+} concentration leading to the activation of hydrolases. Increases in cytosolic Ca^{2+} above 10 μM correlate with the appearance of lipid peroxidation and cell membrane damage [37].

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