Impairment of lipoglycoprotein metabolism in rat liver cells induced by 1,2-dichloroethane

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Abstract

Background—1,2-Dichloroethane (DCE) is a volatile liquid readily absorbed through dermal, digestive, or inhalatory routes. After inhalation or oral administration to rats, death occurs within a narrow range of concentrations (six hour $LC_{50} = 5100 \text{ mg/m}^3$). Exposure to single high doses of DCE resulted in adverse effects on the central nervous system, liver, kidneys, adrenals, and lungs. The liver showed fatty changes and hepatocellular necrosis with haemorrhage. These injuries are probably related to changes in several cell functions and constituents. Therefore, it was decided to investigate whether DCE was capable of impairing the secretion of hepatocellular lipoglycoproteins acting both at the level of the Golgi apparatus and endoplasmic reticulum.

Methods—Isolated hepatocytes of Wistar rats were prelabelled with two precursors of lipoglycoproteins ^3H -Na-palmitate and ^{14}C -glucosamine, and then exposed to concentrations of DCE from mean (SD) 4.4 (0.03) to 6.5 (0.02) mM for different durations ranging from five to 60 minutes. To measure lipid and sugar bound radioactivity, a preliminary separation of cell homogenate, cytosol, total microsomes, Golgi apparatus, and lipoglycoproteins secreted into cell suspension medium was carried out.

Results—After five minutes of exposure, DCE did not induce obvious changes in cell viability or lactic dehydrogenase leakage, but a significant ($p < 0.01$) depletion of reduced glutathione content was seen ($40.10 (4.3) \text{ nM}/10^6 \text{ cells}$). Furthermore, the cells poisoned by DCE started to show noticeable accumulation of ^3H -Na-palmitate in the Golgi apparatus after five minutes ($5103 (223) \text{ dpm}/10^6 \text{ cells}$) and in the microsomes after 15 minutes ($85\,470 (7190) \text{ dpm}/10^6 \text{ cells}$). There was a simultaneous significant increase in ^{14}C -glucosamine content in the Golgi apparatus ($690 (55) \text{ dpm}/10^6 \text{ cells}$) and the microsomes ($15\,975 (2035) \text{ dpm}/10^6 \text{ cells}$). The specific radioactivity of lipid and sugar moieties incorporated in secreted lipoglycoproteins was already significantly reduced after only five minutes of exposure ($480 (57) \text{ dpm}/10^6 \text{ cells}$ for lipids, and $315 (45) \text{ dpm}/10^6 \text{ cells}$ for sugars).

Conclusions—Overall, DCE, like other

haloalkanes, produces a block of secretion of hepatocellular lipoglycoproteins as early as five minutes after poisoning. The simultaneous percentage increases into Golgi apparatus and microsomes of lipid and sugar bound radioactivity suggest that lipid retention at the sites of processing of lipoglycoproteins would probably play an important part in the early stages of cellular accumulation of fat after exposure to DCE.

(*Occup Environ Med* 1994;51:281-285)

1,2-Dichloroethane (DCE) is a colourless, flammable, and volatile liquid extensively used as an intermediate in the synthesis of halogenated hydrocarbons, particularly vinyl chloride.¹ Other minor uses are as an industrial solvent and a fumigant.² Occasional and prolonged human exposure to this xenobiotic compound is increasing worldwide and occurs within and near production facilities. Data concerning levels of exposure are, however, scarce.^{3,4}

Several experimental and clinical investigations have shown that, after inhalation and oral ingestion, the liver seems to be the major target organ.⁵ Exposure of rats to single high doses of DCE resulted in adverse effects on the central nervous system, liver, kidneys, adrenals, and lungs (six hour $LC_{50} = 5100 \text{ mg/m}^3$).⁶ When given to rodents, DCE is quickly absorbed and biotransformed, mainly by the liver, into urinary metabolites through two known pathways. One of these includes a P-450 mediated microsomal hydroxylation to 2-chloroacetaldehyde and 2-chloroethanol,⁷ and the other a conjugation with the reduced form of glutathione.⁸ This conjugation is widely influenced by the centrolobular O_2 mean partial pressure (PO_2).⁹ Thus it is reasonable to suspect that the liver zonal changes induced by DCE, consisting of either fat storage or necrosis,^{5,6} may depend on critical depletion of intracellular reduced glutathione content.¹⁰

Several investigators have shown that a block of synthesis, processing, and secretion into the bloodstream of hepatic lipoglycoproteins, and particularly of the very low density fraction, may represent the crucial mechanism underlying toxic fatty liver. Different steps of the secretory pathway are probably involved in the expression of such damage, which occurs very soon after poisoning.¹¹⁻¹³

Both in vitro studies with isolated rat liver

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Accepted 9 August 1993

cells and experiments carried out with whole animals, provided direct evidence that ethanol¹⁴ and carbon tetrachloride (CCl₄)¹⁵ might impair the intracellular traffic of lipoglycoprotein micelles, and also indicated a probable derangement of the functions of the Golgi apparatus.¹⁶ Furthermore, the amounts in the Golgi apparatus of the dolichols, a family of long chain polyisoprenoid molecules that play a key part in the glycosylation processes, decrease early compared with amounts in total microsomes, in particular on the secretory side (F₁) of the Golgi apparatus.^{17,18}

The purpose of this study was to investigate the pathogenesis of impairment of the Golgi apparatus that actually takes place within five minutes of exposure to DCE in the intact rat hepatocyte model. To assess the part played by the block of the secretory pathway of hepatic lipoglycoprotein in fat accumulation, the changes induced by DCE in the cell uptake and release of two prelabelled precursors of lipoglycoproteins, ³H-Na-palmitate and ¹⁴C-glucosamine, were evaluated.

Materials and methods

ANIMALS, DIET, AND REAGENTS

Adult male rats of the Wistar strain (obtained from S Morini sas, S Polo d'Enza, Reggio Emilia, Italy), weighing 250–300 g, were used. They were maintained under a 12 hour light/12 hour dark cycle with controlled feeding (a standard semisynthetic antioxidant free diet from Italiana Mangimi, Settimo Milanese, Milano, Italy), and free access to water for one week before use. The DCE and reduced glutathione were obtained from E Merck, Darmstadt, Germany. Trypan blue and collagenase type I were obtained from Sigma Chemical Co, St Louis, Missouri, USA; lactic dehydrogenase was evaluated with kits from Poli Diagnostici, Industria Chimica Spa, Milano, Italy. The 9-10-³H-palmitic acid (specific activity 60 mCi/mM) and D-¹⁴C-glucosamine (specific activity 57 mCi/mM) were from the Radiochemical Centre, Amersham, Bucks, UK; and liquid scintillation fluid (Pico-Fluor TM40) from Packard Chemicals, Groningen, The Netherlands. All other chemicals were at least of reagent grade and purchased from BDH Italia, Milano, Italy or from E Merck, Darmstadt, Germany.

PREPARATION OF ISOLATED HEPATOCYTES

Rat liver cells were isolated by the Berry and Friend method,¹⁹ successively modified.²⁰ The buffers and medium used during hepatocyte isolation and incubation procedures were prepared according to Poli *et al.*¹⁵ The liver of rats, previously anaesthetised with sodium pentobarbitone (50 mg/kg of body weight), were first perfused through the portal vein with 100 ml of warm (37°C) saline buffer A. This contained 0.143 M NaCl, 7 mM KCl, 10 mM HEPES NaOH buffer pH 7.4. Then, the livers were perfused with 100 ml of warm (37°C) saline buffer B (0.1 M NaCl, 7 mM

KCl, 5 mM CaCl₂, 50 mM HEPES NaOH buffer pH 7.6) containing 0.050% collagenase type I. After this, they were removed and dispersed into 200 ml of medium C (60 ml NaCl, 40 mM KCl, 50 mM HEPES NaOH buffer pH 7.4, 1 mM CaCl₂, 2 mM MgSO₄, 1 mM Na₂ HPO₄, 5 mM glucose, 0.58 mM amino acid mixture). The hepatocyte suspension, filtered through a 200 µm mesh, was centrifuged at 400 g for four minutes, and the pellet was resuspended in medium C. After counting with a haemocytometer, the cells were diluted with the same medium to 5–7 × 10⁶ cells/ml. Cell yields ranged from 200–300 × 10⁶ cells per liver. Hepatocyte viability was routinely assessed by the trypan blue exclusion test²⁰ and by colorimetric measurement of release of lactate dehydrogenase in a suspension medium.¹⁵ The extracellular enzyme activity was expressed as a percentage of total (intracellular plus extracellular) enzyme activity determined after cell destruction with 0.5% Triton X100. Hepatocyte preparations with viability ranging from 83% to 86% were used.^{21,22}

PRELABELLING OF HEPATOCYTES

The 9-10-³H-palmitic acid was converted to 9-10-³H-Na-palmitate with NaOH. Successively, the liver cells in medium C were prelabelled with 1.51 µCi/ml of 5% bovine albumin-bound 9-10-³H-Na-palmitate for 45 minutes and with 0.16 µCi/ml of ¹⁴C-glucosamine for 15 minutes. After this time, the cell suspension was diluted 1:10 with medium C and centrifuged at 400 g for four minutes. Both the ³H-Na-palmitate and ¹⁴C-glucosamine labelled hepatocytes were suspended in medium C to give 5–7 × 10⁶ cells/ml.

POISONING OF ISOLATED HEPATOCYTES WITH DCE

Three ml aliquots of cell suspension were placed into the main compartment of 50 ml flasks fitted with a centre well, and closed with a screw cap. Then DCE was added to the centre well and allowed to diffuse in the closed system. The flasks were incubated for five to 60 minutes at 37°C in a Dubnoff bath. Control cells were incubated in the same way, but without DCE. By adding 5.5 µl (1 µl = 1.2569 mg) of DCE, the actual concentration of this haloalkane was determined on a Varian 3500 capillary gas chromatograph, equipped with electron detector. Columns were fused silica 30 m × 0.25 mm internal diameter DBWAX from J and W Scientific (Folsom, CA, USA).

REDUCED GLUTATHIONE ASSAY

Intracellular reduced glutathione content was measured by a fluorimetric method,²³ and is expressed as nM/10⁶ cells.

PREPARATION OF SECRETED

LIPOGLYCOPROTEIN FRACTION

The secreted lipoglycoprotein fraction was obtained by adding one part of 2% sodium phosphotungstate (pH 7.6) in 1 M MgCl₂ to nine parts of cell incubation medium C. The

Table 1 Time course of DCE concentration in cell suspension medium (determined after adding 5.5 µl (1 µl = 1.2569 mg) DCE in the centre well of flasks)

	Time (min)			
	5	15	30	60
DCE (mM)*	4.4 (0.03)	5.8 (0.01)	6.5 (0.02)	6.5 (0.02)

*Values are mean (SD) for four to six experiments.

samples were left overnight at 4°C, and then the precipitates were reharvested after centrifugation at 10 000 g for 45 minutes.²⁴

DETERMINATION OF LIPID AND SUGAR BOUND SPECIFIC RADIOACTIVITY

To evaluate the incorporation of ³H-Na-palmitate and ¹⁴C-glucosamine into the subcellular fractions and the concentrations in the lipoglycoproteins secreted into suspension medium C, the aliquots were processed as follows: Samples of cell homogenate, cytosol, total microsomes, and Golgi apparatus to be counted were processed by addition of cold (4°C) 1% phosphotungstic acid in 20% trichloroacetic acid. The precipitates were washed twice with 5% trichloroacetic acid and once with distilled water. Aliquots of the trichloroacetic acid washed precipitates were washed three times in 2:1 by volume chloroform-methanol mixture, and the protein residue, after lipid extraction, was dissolved in Insta-Gel (Packard). Specific radioactivity was determined with a Packard TriCarb liquid scintillation counter (model 4430), and expressed as dpm/10⁶ cells.

Table 2 Effects of exposure to DCE on cell viability, lactate dehydrogenase leakage, and reduced glutathione content of isolated rat hepatocytes

	Time (min)			
	5	15	30	60
Cell viability [†] (%):				
Control	86 (6)	85 (4)	84 (8)	83 (4)
DCE	84 (7)	83 (2)	62 (7)*	53 (6)*
Lactate dehydrogenase (%):				
Control	19 (3)	19 (4)	20 (3)	21 (4)
DCE	22 (3)	36 (5)*	54 (6)*	68 (7)*
Reduced glutathione (nM/10 ⁶ cells):				
Control	60.07 (7.4)	59.10 (6.3)	57.3 (5.20)	56.10 (6.35)
DCE	40.10 (4.3)*	39.12 (4.5)*	23.1 (4.10)*	11.30 (2.10)*

*p < 0.01 v control.

†Values are mean (SD) for four to six experiments.

Table 3 Distribution of ³H-Na-palmitate (dpm/10⁶ cells)[†] in homogenate and fractions of control and DCE treated hepatocytes

	Time (min)			
	5	15	30	60
Homogenate:				
Control	245 450 (26 350)	245 320 (27 200)	245 350 (30 140)	245 210 (31 044)
DCE	249 328 (29 340)	257 698 (31 210)	270 431 (33 240)	285 382 (29 250)**
Cytosol				
Control	85 500 (10 700)	85 450 (10 200)	87 420 (14 200)	86 420 (12 210)
DCE	86 340 (6310)	90 870 (9574)	92 146 (9560)	98 203 (10 320)**
Total microsomes:				
Control	71 500 (9620)	71 300 (9400)	73 210 (10 050)	70 350 (10 500)
DCE	76 135 (695)	85 470 (7190)**	93 224 (9830)**	106 395 (11 250)**
Total Golgi apparatus:				
Control	3920 (420)	3840 (340)	3721 (410)	3721 (310)
DCE	5103 (223)**	5404 (443)**	5649 (507)**	6403 (851)**
Secreted lipoglycoproteins:				
Control	1060 (128)	2110 (275)	3280 (358)	4220 (518)
DCE	480 (57)**	910 (115)**	1150 (135)**	1610 (186)**

**p < 0.01 v control.

†Values are mean (SD) for four to six experiments; cells were incubated for 5–60 minutes with the label.

STATISTICAL EVALUATION

All experiments were measured at least in triplicate. The results are expressed as the means (SD) of four to six different preparations of isolated rat hepatocytes. To compare the relation between the ³H-Na-palmitate and ¹⁴C-glucosamine bound specific radioactivity secreted into suspension medium C and the time of incubation, we performed linear regression analysis with the method of least squares, and then calculated the correlation coefficient (*r*). The differences were determined by Student-Fisher's *t* test, and were considered significant when *t* values corresponded to *p* < 0.01.

Results

Table 1 shows that the actual concentration of DCE (mM) in the cell suspension medium increased from 4.4 (0.03) to 6.5 (0.02) mM, reaching a plateau after 30 minutes.

Table 2 shows hepatocyte viability, evaluated by the trypan blue exclusion test, lactate dehydrogenase leakage, and intracellular reduced glutathione content after different times of incubation (from five to 60 minutes). Cell preparations with viability ranging from 83% to 86% were used. In agreement with several authors,^{25,26} a 15%–18% range of damaged cells did not significantly interfere with reproducibility of experimental data. In untreated hepatocytes, both cell viability and reduced glutathione content remained unchanged for 60 minutes, confirming that cell integrity did not change after the isolation procedures. After five minutes of exposure, DCE did not induce obvious changes in cell viability and lactate dehydrogenase leakage. Thereafter, these data showed significant changes (*p* < 0.01), pronounced after 60 minutes of incubation (53% (6%) for cell viability and 68% (7%) for leakage of lactate dehydrogenase compared with control values).

Changes in the concentration of reduced glutathione in hepatocytes were already found at five minutes (40.10 (4.3) nM/10⁶ cells). Successively, the depletion of reduced glutathione increased abruptly, in particular after 60 minutes of incubation (11.30 (2.10) nM/10⁶ cells).

Tables 3 and 4 show the time course of incorporation of ³H-Na-palmitate and ¹⁴C-glucosamine into subcellular fractions and secreted lipoglycoproteins. In cells poisoned by DCE there was a large decrease in secretion of lipoglycoproteins with a concomitant progressive accumulation of prelabelled precursors inside the cells. In total Golgi apparatus and microsomes ³H-Na-palmitate specific radioactivity showed a significant increase (*p* < 0.01), starting after five minutes (5103 (223) dpm/10⁶ cells), and 15 minutes (85 470 (7190) dpm/10⁶ cells) reaching a peak at 60 minutes (6403 (851) dpm/10⁶ cells in the Golgi apparatus, and 106 395 (11 250) dpm/10⁶ cells in the microsomes). At the same time the lipid bound radioactivity was significantly raised (*p* < 0.01) in homogenates

Table 4 Distribution of ^{14}C -glucosamine (dpm/ 10^6 cells)* into homogenate and fractions of control and DCE treated hepatocytes

	Time (min)			
	5	15	30	60
Homogenate:				
Control	75 430 (5800)	75 210 (6250)	74 250 (7210)	76 320 (7410)
DCE	77 840 (7120)	80 842 (8570)	84 938 (9740)	87 812 (10 720)*
Cytosol:				
Control	37 420 (3121)	40 500 (5600)	42 324 (6050)	41 340 (5700)
DCE	39 510 (3570)	42 771 (5210)	44 615 (5730)	46 245 (6270)
Total microsomes:				
Control	13 131 (1350)	14 320 (2200)	15 030 (1970)	14 900 (2030)
DCE	14 435 (1430)	15 975 (2035)*	18 846 (1970)*	20 060 (2275)*
Total Golgi apparatus:				
Control	551 (42)	685 (75)	720 (85)	750 (80)
DCE	690 (55)*	810 (60)*	965 (115)*	1082 (125)*
Secreted lipoglycoproteins:				
Control	650 (73)	1380 (157)	2100 (256)	2980 (365)
DCE	315 (45)*	560 (69)*	980 (118)*	1530 (179)*

* $p < 0.01$ v control.

†Values are mean (SD) for four to six experiments; cells were incubated for 5–60 minutes with the label.

(285 382 (29 250) dpm/ 10^6 cells) and cytosol (98 203 (10 320) dpm/ 10^6 cells). Moreover, we found a significant increase ($p < 0.01$) of ^{14}C -glucosamine content in total Golgi apparatus (690 (55) dpm/ 10^6 cells) and microsomes (15 975 (2035) dpm/ 10^6 cells) at five and 15 minutes (table 4). Thereafter, the sugar bound radioactivity in these subcellular fractions steadily increased, reaching a peak 60 minutes after exposure to DCE. Tables 3 and 4 report experiments in which the specific radioactivity of lipid and sugar moieties incorporated into secreted lipoglycoproteins was determined. Interestingly, as early as five minutes after poisoning, when DCE does not induce obvious changes in cell viability, we already found a significant block of secretion of both ^3H -Na-palmitate and ^{14}C -glucosamine in the medium (480 (57) dpm/ 10^6 cells for palmitate, and 315 (45) dpm/ 10^6 cells for glucosamine). To confirm these results, the slope of the straight line relating lipoglycoprotein bound radioactivity to the time of incubation was calculated (figs 1 and 2). The decreased slope of the straight lines in DCE exposed v untreated cells shows a significant block of lipoglycoprotein secretion ($p < 0.01$) as early as five minutes after exposure.

Discussion

Studies from different laboratories have stressed that the use of intact hepatocytes

Figure 1 Relation between the lipoglycoprotein bound radioactivity of ^3H -Na-palmitate secreted into the medium and the time of incubation in control and DCE treated cells.

Figure 2 Relation between the lipoglycoprotein bound radioactivity of the ^{14}C -glucosamine secreted into the medium and the time of incubation in control and DCE treated cells.

represents a useful integration of experimental systems for in vitro studies about the derangement of secretion of lipoglycoproteins and the pro-oxidant effects of several drugs and xenobiotics.^{27–29}

Our results have shown that hepatocytes, in single cell suspensions, can mimic in part the whole liver with regard to some responses to DCE poisoning. In isolated hepatocytes¹⁰ and in vivo,⁵ DCE induces an early fat accumulation within the cells, probably through an impairment of the secretion of lipoglycoproteins. When DCE is added to the suspension of hepatocytes there is not only a dramatic decrease in cell trypan blue exclusion, but also a leakage of lactate dehydrogenase. Moreover, in hepatocytes poisoned with this xenobiotic compound there was a striking depletion of intracellular reduced glutathione content, already significant after only five minutes of exposure. Hence, as the reduced glutathione may function as a substrate for the quenching of reactive oxygen species,^{30–31} these findings support the hypothesis that depletion of reduced glutathione can play a crucial part in the pathogenesis of liver injuries induced by DCE. In this study, isolated hepatocytes prelabelled with ^3H -Na-palmitate and ^{14}C glucosamine at the level of the lipoglycoprotein fraction, showed a significant block of secretion of lipoglycoprotein as early as five minutes after DCE poisoning. Identical experiments of cell prelabelling, looking this time also at the total lipid and sugar content of the Golgi apparatus not only confirmed the secretory block but also showed evidence of an increase of labelled material into the total Golgi apparatus of the poisoned cells with respect to the untreated ones, early after exposure to DCE. In fact, at 15 minutes the secretion of ^3H -Na-palmitate was reduced by 57% and ^{14}C -glucosamine was reduced by 60% compared with controls. At the same time, the percentage increase of intracellular lipid and sugar bound radioactivity was already significant with respect to controls in total Golgi apparatus and microsomes but not in cytosol. These findings suggest that lipid retention at the sites of processing of lipoglycoprotein would play a key part in

the early stages of cell fat storage after exposure to DCE.

Tables 3 and 4 show the time course of incorporation of prelabelled lipid and sugar precursors in intracellular and secreted lipoglycoproteins. In cells treated with DCE there is a striking impairment of secretion of lipoglycoproteins with a concomitant, progressive accumulation of ^3H -Na-palmitate and ^{14}C -glucosamine inside the cells. These results indicate that DCE produces liver cell steatosis in vitro. Furthermore, there is a good quantitative relation between the increased intracellular radioactivity, and the decrease in secretion of prelabelled precursors. These experimental findings are in agreement with the results of previously reported studies concerning poisoning with CCl_4 . In fact, Poli *et al.*, have pointed out that this haloalkane, as early as five minutes after exposure, is able to impair the secretory subfraction (F1) of the Golgi apparatus purified from total microsomes, and at 15 minutes both to impair the intermediate (F2) and the formative (F3) sides of the apparatus, which play a pivotal part in the secretion of lipoglycoproteins outside the cell.³²

In summary, our results indicate an impairment of Golgi apparatus in the liver during early phases of acute poisoning with DCE. Hence, even if our results do not exclude a favouring, indirect part played by a possible increase of uptake of fatty acid by hepatocytes treated with DCE in the pathogenesis of toxic fatty liver, the direct damage of such cells leading to an early block of secretion of lipoglycoproteins seems to be of paramount importance.

This work was supported in part by a grant from National Research Council (CNR) targeted project "Prevention and control of disease factors", subproject "SP2 9200 143 PF 41, Rome, Italy, and by a grant from ACNA CO, Cengio (Sv), Italy.

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