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THE INTRACELLULAR DISTRIBUTION OF LIVER CELL CALCIUM IN NORMAL RATS AND ONE HOUR AFTER ADMINISTRATION OF CARBON TETRACHLORIDE

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Abstract—In order to recognize the significance of elevated liver calcium level in the early phase of carbon tetrachloride intoxication, the subcellular distribution of calcium in liver was investigated one hour after administration of CCl_4 intraperitoneally. During preparation of subcellular fractions attempts were made to prevent redistribution of calcium by adding Ruthenium Red and EGTA or $LaCl_3$ to homogenization medium, and by shortening of differential centrifugation to a minimum. The latter caused a loss of purity in sub cellular fractions which was overcome by correction of calcium values from atom absorption spectrometry by means of marker enzyme activity. The calcium levels in normal rat liver were found to be 70 nmoles/g liver wet wt in cytosol, 310 nmoles/g liver in microsomes and about 500 nmoles/g liver in mitochondria. A minor part of the latter fraction may belong to nuclei and plasma membranes. One hour after CCl_4 administration calcium levels in cytoplasma were not altered, in microsomes were decreased to 200 nmoles/g liver and in mitochondria were elevated to 2.5 μmoles/g liver. In rats pretreated with vitamin D the whole additional calcium, after carbon tetrachloride application in the range of 9 μmoles/g liver, was sequestrated in mitochondria. In the early phase of carbon tetrachloride intoxication all border membranes of liver cells have to participate in bringing about the reversible increase of liver cell calcium.

The increase of liver cell calcium in carbon tetrachloride intoxication has long been known [1, 2] and Reynolds [3] has already demonstrated the biphasic course of that increase. The enhanced level of liver calcium as early as one hour after administration of CCl_4 was indeed confirmed by Smuckler and Arcasoy [4] but little significance was attributed to the importance of this phenomenon in liver cell damage, especially that by carbon tetrachloride. The reason for this might be that the two phases of elevated liver cell calcium in carbon tetrachloride intoxication were not differentiated.

In the later phase of liver cell damage by different agents an increased calcium level was found [5], so that it was seen as the reason [5] or merely the indicator [6] for the transition to irreversible cell death. Therefore this phenomenon is not very interesting for the mechanism of cell damage. This is not true in the rise of liver calcium in the early phase of carbon tetrachloride intoxication. As the author could show [7] after pretreatment of animals with calcium gluconate or with vitamin D this rise is greatly enhanced and combined with a diminished shift of alkali ions in liver and a lesser efflux of enzymes from liver in a sense of protection. This effect is specific to carbon tetrachloride intoxication and cannot, for example, be observed in galactosamine hepatitis [8]. Calcium supply, enhanced by pretreatment with vitamin D is especially favorable in respect to the early damage of endoplasmic reticulum by CCl_4, e.g. the inhibition of protein synthesis and the inhibition of calcium sequestration by microsomes *in vitro* [9] as well as the damage of cytochrome P-450 [10].

All these observations indicate the significance of calcium in the early phase of liver cell damage by carbon tetrachloride. To integrate the effect of calcium in the "cascade phenomenon" [6], investigation of intracellular localization seems to me to be indicated as the next step. Indeed I agree with Bygrave [11] that "there seems to be no way at this stage of measuring precisely the concentration of calcium in a cell *in situ*", but also in my opinion, the use of specific inhibitors of mitochondrial calcium transport to prevent redistribution during cell disruption has been successfully attempted [12, 13]. Because the differences between treated and untreated animals are particularly more interesting than the absolute levels in the different compartments, the investigation seems all the more justified.

MATERIALS AND METHODS

Female Wistar rats (home bred), 180–220 g body wt, were used throughout. They received drinking water *ad lib.* until they were killed and the standard diet No. 1310 from Altromin (Lage, West Germany) until 20 hr before death. Carbon tetrachloride p.a. Merck (Darmstadt, West Germany), 1 ml/kg body wt diluted with a 4-fold volume of olive oil, was injected i.p. one hour before rats were killed. Some animals were pretreated with vitamin D_3. For that one ampule vigantol forte pro injectione (Bayer/Merck, Leverkusen, West Germany) was mixed with 5 ml olive oil. Three days before the experiments rats received from this mixture 5 ml/kg body wt by forced feeding, corresponding to 500,000 IU/kg.

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In ether anesthesia livers were perfused with ice cold sucrose (0.25 mole/l) from the portal vein, removed, weighed, minced and homogenized with an eightfold volume of medium A or B in a Potter–Elvehjem homogenizer. This as well as all further steps were done at 0°. Medium A contained sucrose, 0.25 mole/l; EGTA (bis-(amino-ethyl)-glycol ether-*N,N,N'N'*-tetraacetic acid) 2.10^{-3} mole/l which was made to pH 7.4 by Tris (tris hydroxymethyl-aminomethan) and Ruthenium Red (Merck) 20 mg/l. Medium B contained sucrose 0.25 mole/l and $LaCl_3$, $1 \cdot 10^{-3}$ mole/l. Ruthenium Red was not recrystallized. According to the formula given by Fletcher *et al.* [14] and to the molar extinction coefficient of 68,000 at 533 nm given by Luft [15] the content of the drug was 45%.

In the first series of experiments nuclei were spun down in a Sorvall refrigerated centrifuge, rotor SS 34 for 5 min at 500 *g* and then mitochondria for 10 min at 6000 *g*. In a second series of experiments one part of the homogenates was centrifuged for 5 min at 15,000 *g* and the rest for 20 min at 100,000 *g* in a Beckman ultracentrifuge Spinco L 2. The sediments were resuspended in suitable volumes of water and homogenized twice for 30 sec in an Ultra Turrax (Jahnke u.Kunkel, Staufen, West Germany).

Two portions of each tissue fraction with addition of sulphuric and nitric acid were condensed in a heated aluminium block and finally ashed with addition of perhydride. The determination of calcium was done with an atom absorption spectrometer, Perkin–Elmer, model 300, for details see [7]. In the tissue fractions were also determined: the DNA level by the method of Burton [16], the activity of succinate dehydrogenase (succinate: acceptor oxydoreductase, EC 1.3.99.1) with INT (2-*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium) as hydrogen acceptor and the activity of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) with addition of tartrate to inhibit acid phosphatases according to [17].

RESULTS

The distribution of liver cell calcium among nuclear, mitochondrial and postmitochondrial supernatant fraction in rats treated with CCl_4 (one hour) and pretreated with vitamin D (three days) as well as in untreated animals is given in Table 1. The nuclear fraction contains 97.5% of total DNA but also an appreciable amount of mitochondria. Therefore the calcium level of this fraction as well as that of the supernatant fraction is corrected according to the succinate dehydrogenase activity in those fractions. The resulting calcium level of nuclei in the experiments with medium including EGTA is not significantly different from zero. If bound calcium is not detached in a complexed form as in the investigation with La^{3+} ions, 12.5% of liver cell calcium in control animals remains in the corrected nuclear fraction. In rats treated with vitamin D and CCl_4 the corresponding value is low in relation to the much elevated total calcium level. Therefore it is no longer demonstrable in that way.

In the postmitochondrial supernatant of untreated rats calcium levels of 0.33 μmole/g liver and 0.27 μmole/g liver are found in the experiments with EGTA + Ruthenium Red and with La^{3+} ions respectively. The difference between those two values theoretically should be equivalent to the calcium of microsomes in the mitochondrial fraction (see below). In treated animals the relation between extramitochondrial and mitochondrial calcium is unfavourable. So the additional calcium in the postmitochondrial supernatant need not result from the microsomal or cytoplasmic calcium level increased by CCl_4 but may be a consequence of calcium efflux from mitochondria during fractionation especially from those damaged by homogenization. Therefore, in a first approximation all the additional liver cell calcium of rats treated with vitamin D and CCl_4 is stored in mitochondria whereas in control animals the mitochondria contain only little more than half the liver calcium.

In the second experimental series performed with medium containing Ruthenium Red and EGTA the correspondence between calcium levels of 15,000 *g* sediment and 100,000 *g* sediment as well as between calcium levels of 15,000 *g* and 100,000 *g* supernatant in the different experimental groups is striking (see Table 2). There are about 50% of the microsomes in the 15,000 *g* supernatant according to glucose-6-phosphatase activity and in the 100,000 *g* supernatant there are hardly any microsomes. Therefore microsomal calcium complexed with EGTA has to be quantitatively included in the supernatants. The resulting sum of microsomal and cytoplasmic calcium in untreated control animals (0.37 μmole/g liver wet

Table 1. Calcium levels in liver cell fractions from normal rats and from rats treated with CCl_4 (1 hr) and pretreated with vitamin D (3 days)*

Homogenized in medium	A	A	B	B
Treatment with vitamin D + CCl_4	−	+	−	+
μmoles Ca/g liver in nuclear fraction	0.222 ± 0.041	2.617 ± 0.941	0.305 ± 0.090	2.268 ± 1.019
μmoles Ca/g liver in mitochondrial fraction	0.235 ± 0.031	5.402 ± 1.939	0.341 ± 0.024	5.985 ± 2.486
μmoles Ca/g liver in postmitochondrial supernatant	0.360 ± 0.070	1.328 ± 0.443	0.303 ± 0.082	1.177 ± 0.480
μmoles Ca/g liver in nuclei, corrected	0.032 ± 0.038	0.307 ± 0.329	0.116 ± 0.065	0.047 ± 0.569
μmoles mitochondrial Ca/g liver total	0.450 ± 0.080	8.187 ± 2.578	0.540 ± 0.054	8.797 ± 3.513
μmoles Ca/g liver in postmitochondrial supernatant, corrected	0.331 ± 0.063	0.898 ± 0.377	0.266 ± 0.077	0.585 ± 0.350
Total Ca in μmoles/g liver	0.817 ± 0.105	9.347 ± 2.959	0.923 ± 0.181	9.430 ± 3.866

* Values shown are means ± S.E. of at least 6 observations.

Table 2. Calcium levels and activity of glucose-6-phosphate in liver cell fractions and rats treated with CCl_4 for 1 hr*

Pretreatment with vitamin D	−	−	+	+
Treatment with CCl_4	−	+	−	+
μmoles Ca/g liver in 15,000 *g* sediment	0.533 ± 0.196	2.141 ± 1.390	0.763 ± 0.222	7.573 ± 3.077
μmoles Ca/g liver in 15,000 *g* supernatant	0.422 ± 0.102	0.414 ± 0.080	0.587 ± 0.114	0.886 ± 0.216
μmoles Ca/g liver in 100,000 *g* sediment	0.590 ± 0.151	2.258 ± 1.379	0.809 ± 0.092	8.667 ± 3.777
μmoles Ca/g liver in 100,000 *g* supernatant	0.421 ± 0.075	0.395 ± 0.102	0.487 ± 0.060	0.917 ± 0.285
μmoles Ca/g liver in 15,000 *g* supernatant, corrected	0.371 ± 0.088	0.256 ± 0.040	0.521 ± 0.117	0.239 ± 0.090
μmoles Ca/g liver in 100,000 *g* supernatant, corrected	0.365 ± 0.063	0.238 ± 0.057	0.418 ± 0.069	0.272 ± 0.106
Mitochondrial Ca in μmoles/g liver	0.584 ± 0.216	2.298 ± 1.484	0.827 ± 0.234	8.219 ± 3.301
U G-6-Pase/g liver, total activity	20.083 ± 1.721	10.815 ± 1.720	12.222 ± 1.760	8.578 ± 0.860

* One half of the animals were pretreated with vitamin D for 3 days.
Preparation of subcellular fractions was done with medium A, containing EGTA and Ruthenium Red.
Values shown are means ± S.E. of at least 6 observations.

wt) is in good accordance with the value from the first series (0.33 μmole/g liver). In rats pretreated with vitamin D the corresponding levels are distinctly higher in the 15,000 *g* supernatant (0.52 μmole/g liver), even significantly. The difference between this value and that from the 100,000 *g* supernatant (0.42 μmole/g liver) is not significant.

One hour after CCl_4 administration the sum of cytoplasmic and microsomal calcium is decreased to 0.25 μmole/g liver wet wt, independent of whether rats are pretreated with vitamin D or not. The discrepancy between this value and the corresponding one of the first experimental series should depend on experimental reasons described above. Therefore the value of 0.25 μmole calcium/g liver appears realistically. All other comparable values of both experimental series are in very good accordance.

In a third experimental series performed with medium containing La^{3+} ions a calcium level of 0.21 μmole/g liver was found in the 15,000 *g* supernatant of untreated rats after correction for mitochondrial calcium via activity of succinate dehydrogenase. The difference between this value and the corresponding one of the second series (0.37 μmole/g liver) should be equivalent to the calcium content of that portion of microsomes which according to glucose-6-phosphatase activity remains in the mitochondrial fraction. That calcium is obviously recorded in the supernatant fraction when medium with EGTA is used. Some difficulties arise in estimating that portion of calcium by an arithmetical correction when medium containing La^{3+} ions is applied. The difference of calcium levels in 15,000 *g* supernatant and 100,000 *g* supernatant is theoretically equivalent to the microsomal calcium in the 15,000 *g* supernatant and could be calculated to 100% according to glucose-6-phosphatase activity. In practice, however, the admixture of mitochondrial calcium interferes, especially in treated animals.

To overcome these difficulties we have calculated a corrective factor, dividing the calcium contents in 15,000 *g* supernatant corrected by the calcium contents in 15,000 *g* supernatant not corrected. Now we have corrected separately the admixture of mitochondrial calcium to the cytoplasmic part of calcium (100,000 *g* supernatant) and to the microsomal part of calcium (15,000 *g* supernatant minus 100,000 *g* supernatant) by that factor. The microsomal calcium contents found out in such a way was calculated to 100% according to distribution of glucose-6-phosphatase activity.

The result is a microsomal calcium level of 0.31 μmole/g liver wet wt in untreated controls and a clearly lower level of 0.20 μmole/g liver in rats treated with CCl_4 for one hour. If one adds to these values the level of cytoplasmic calcium corrected with the same factor: 0.07 or 0.06 μmole/g liver respectively, the resulting sums of cytoplasmic and microsomal calcium of 0.38 μmole/g liver in control rats and of 0.26 μmole/g liver in CCl_4 poisoned

Table 3. Calcium levels in liver cell fractions from normal rats and from rats treated with CCl_4 for 1 hr*

Treatment with CCl_4	−	+
μmoles Ca/g liver in 15,000 *g* sediment	0.545 ± 0.067	2.065 ± 1.318
μmoles Ca/g liver in 15,000 *g* supernatant	0.259 ± 0.045	0.371 ± 0.126
μmoles Ca/g liver in 100,000 *g* sediment	0.746 ± 0.039	2.451 ± 1.371
μmoles Ca/g liver in 100,000 *g* supernatant	0.087 ± 0.025	0.138 ± 0.054
Microsomal Ca total (μmoles/g liver)	0.307 ± 0.070	0.221 ± 0.226
Cytoplasmic Ca (μmoles/g liver)	0.070 ± 0.023	0.058 ± 0.051
Sum of microsomal plus cytoplasmic Ca (μmoles/g liver)	0.377 ± 0.084	0.280 ± 0.272

* Preparation of subcellular fractions was done with medium B containing $LaCl_3$.
Values shown are means ± S.E. of at least 6 observations.

animals conform optimally with the corresponding values of the second experimental series, where investigation was done with Ruthenium Red and EGTA in the medium. Thus the data received in the three experimental series can easily be integrated in a scheme of intracellular distribution of liver calcium: In untreated rats there is in cytoplasma 0.07 μmole calcium/g liver, in microsomes 0.31 μmole/g liver and in mitochondria the rest, about 0.5 μmole calcium/g liver wet wt.

One hour after administration of CCl_4 the level in cytoplasma is practically unaltered, in microsomes the calcium level is decreased to 0.20 μmole/g liver and the rest in a range of 2.5 μmole/g liver is accumulated in mitochondria. That distribution is not altered by additional pretreatment of animals with vitamin D. Because the sum of cytoplasmic and microsomal calcium is the same as that in rats treated with CCl_4 only, the distribution to microsomes and cytoplasma may also be equal to that in animals treated with CCl_4 only. The remaining calcium in rats treated with CCl_4 and vitamin D in a range of 9 μmoles/g liver is also sequestrated in mitochondria.

In parentheses the effect of carbon tetrachloride intoxication on glucose-6-phosphatase activity and the influence of pretreatment with vitamin D on this effect should be mentioned (Table 2). One hour after administration of CCl_4 the activity decreases by 46%, in rats pretreated with vitamin D the decrease of activity amounts to only 30%.

DISCUSSION

In recent years the significance of calcium to non-excitable tissues (e.g., liver) has been recognized [18]. Therefore, interest in the role of this element in the regulation of liver metabolism is growing enormously. Nevertheless, the conceptions referring to this are at least incomplete [19]. The regulation of the relatively low concentration of calcium in cytoplasma by storage in mitochondria is probably an important phenomenon [11, 20, 21] but also for liver microsomes an energy dependent calcium sequestration activity could be demonstrated [22]. A further important factor in calcium transport and therefore in regulation of calcium concentration in liver cells has to be the cell membrane, especially important to our problem, the reversible increase of total liver calcium at carbon tetrachloride intoxication.

The total system is still more complicated, because calcium efflux from one compartment and calcium influx in that compartment can take place by different ways as was distinctly shown in mitochondria [20, 21]. It may be that in different controlling processes primarily the flux in but one direction between two compartments is altered; nevertheless, *in vivo* a feedback will be effective by the physiological connection between the particular components of cellular calcium regulation system. If this feedback is abolished by investigating isolated systems *in vitro* one may easily get contradictory results, as the following observation shows [23]: The activity of isolated mitochondria to take up calcium *in vitro* as well as the ability of retention of calcium is enhanced by prior perfusion of the liver with phenylephrine or glucagon. Nevertheless, simultaneously the calcium level of mitochondria of liver perfused with phenylephrine or glucagon is distinctly diminished compared to mitochondria from livers perfused without any hormone.

Whether in this connection the more complex model of isolated cells is generally preferable to the system of isolated mitochondria [24] which is easier to survey, seems questionable. Van Rossum *et al.* [13] have shown that the compartmentation of calcium is shifted in favour of mitochondria by a temporary cooling, because calcium uptake by mitochondria at 0° is less inhibited than the "extruding system(s)". The shift of calcium between the cell compartments at 0° can but partly be reversed by incubation at 38°.

The significance of this phenomenon becomes evident, if one compares the mitochondrial calcium levels to values in literature. The values taken from Table 1 related to protein content of mitochondria (not shown) amount to 5.4 nmoles calcium/mg protein at isolation with Ruthenium Red—EGTA and to 6.2 nmoles calcium/mg protein at isolation with La^{3+} ions. Van Rossum *et al.* [13] give a value of 7.3 ± 0.6 nmoles calcium/mg protein in mitochondria isolated from tissue not incubated. Taylor *et al.* [20] find a value of 8.7 ± 0.5 nmoles calcium/mg protein after liver perfusion for 15 min and isolation with EGTA. Claret-Berthon *et al.* [25] in their investigation to identify calcium pools of rat liver by kinetic analysis give a value of 13.1 ± 1.1 nmoles calcium/mg mitochondrial protein. This value from longer lasting perfusion experiments corresponds to the given calcium level of total liver of 2.2 μmoles/g wet wt, which also is 2.5 times higher than levels found by us in this and a previous investigation [7]. From this very point of view, results of such investigations [24, 25] seem problematic.

It cannot be said whether redistribution could really be avoided in the present investigation. The fact, however, that the results obtained in several experimental series with various methods unconstrainedly fit in a scheme of intracellular distribution of liver calcium, seems to indicate that this scheme may resemble the conditions *in vivo*. Surely the perfusion of liver with sucrose is problematical because it should be done quickly but also quantitatively. If perfusion is not successful, the value of cytoplasmic calcium of 70 nmoles/g liver is elevated by addition of extracellular calcium. Based on previous investigations of extracellular space and of calcium concentration in serum [7, 26] the given value corresponds to 13% of extracellular calcium. On the other hand, extracellular calcium bound to cell membranes could be found in nuclear fraction (Table 1, difference between column 1 and 3).

Concerning the influence of carbon tetrachloride on intracellular distribution of calcium there is a good correspondence between the decrease of calcium level in microsomes and the drop in calcium uptake activity of that fraction previously described [9, 27]. In this respect, however, alterations of cell membrane and of mitochondria by CCl_4 may be more important. Besides an isolated and reversible damage of cell membrane, a combination of a primary and limited damage of cell membrane with a

secondary disturbance of mitochondrial function could explain the reversible increase of calcium in the early phase of carbon tetrachloride intoxication.

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