The effect of fructose 2,6-bisphosphate on muscle fructose-1,6-bisphosphatase activity

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Age-related changes in the translocation of phosphatidate phosphohydrolase from the cytosol to microsomal membranes in rat liver

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The effects of oleate, spermine and chlorpromazine were assayed in the presence or absence of 0.15 M KCl on the translocation of phosphatidate phosphohydrolase activity from cytosol to endoplasmic reticulum membranes in liver homogenates obtained from rats aged 1, 30, 60, 180 and 360 days. Marked age-associated decreases in phosphatidate phosphohydrolase distribution onto the membranes were demonstrated under nearly all conditions. In liver homogenates taken from 1-day-old rats and incubated with 0.15 M KCl, most of the enzyme was active (associated with the membranes). Physiological salt concentration (0.15 M KCl) produced a 2-fold increase of oleate-induced translocation of phosphatidate phosphohydrolase activity in liver homogenates from 1-day-old rats; it had no effect on those from 60-day-old rats, and produced a notable decline in liver homogenates obtained from 180- and 360-day-old rats. The promoting effect of spermine on oleate-induced translocation of this enzyme activity was higher in younger rats when incubated in the absence of 0.15 M KCl. Chlorpromazine did not show its usual antagonizing effect on oleate-induced translocation of phosphatidate phosphohydrolase when added to homogenates taken from 1-day-old rats. The antagonizing effect was slightly apparent in liver homogenates from 30-day-old rats and was more pronounced in those from 60-day-old rats in which the values diminished to one-half and to one-third either in the presence or absence of 0.15 M KCl.

Introduction

It is well known that lipid metabolism undergoes marked age-dependent fluctuations. Savolainen et al. [1] have studied in male and female rats the postnatal development in hepatic phosphatidate phosphohydrolase (PAP) activity over 100 days, and have shown that the soluble activity of this enzyme sharply increased after birth (7-fold), returning to the adult level at the 3rd postnatal day. The changes in PAP activity were followed by similar changes in hepatic triacylglycerol concentration. Microsomal and soluble PAP activities have been studied in liver homogenates, and showed minor fluctuations with age in the microsomal activity and a peak in the cytosolic at the 58th day, followed by a progressive decline [2].

Since PAP activity seems to be regulated by its ability to interact with the membranes of the endoplasmic reticulum, and since this interaction is promoted by oleic acid [3] and a series of substances [4–6], the aim of the present investiga-

Abbreviation: PAP, phosphatidate phosphohydrolase (EC 3.1.3.4).

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tion was to test whether the modulation of this enzyme activity and its subcellular distribution could be affected by age. The oleate-induced translocation of PAP to microsomal membranes, as well as the modulating effect of substances such as spermine and chlorpromazine, were determined in liver homogenates taken from rats aged 1, 30, 60, 180 and 360 days. The effect of physiological concentrations of salt on the subcellular distribution of PAP activity was also determined by adding 0.15 M KCl to the incubation medium, since it has been shown [7] that high ionic strength can increase the binding of PAP to microsomal membranes.

Material and Methods

Reagents. Chlorpromazine (Largactil) was provided by Rhône-Poulenc Farma, Alcorcón, Madrid. Substrates and coenzymes were obtained from Sigma, St. Louis, MO, U.S.A. Enzymes were obtained from Boehringer Mannheim, F.R.G. All other chemicals, provided by Merck, were of the highest purity available commercially.

Animals and experimental design. Male albino Wistar rats were maintained on a standard laboratory diet and water ad libitum, at a constant day/night rhythm and controlled temperature (21° C). Rats were decapitated, the liver was immediately chilled in ice-cold buffer and a sample was taken for homogenization. Livers were homogenized in 4 vol. ice-cold 0.25 M sucrose containing 20 mM Hepes (pH 7.4), 0.2 mM dithioerythritol and 2 mM EDTA.

Subcellular distribution of PAP. The supernatant obtained from rat liver homogenates after centrifuging for $18\,000 \times g$ ($r = 10.8$ cm) for 10 min at 4° C in a Kontron centrifuge (Centrikon H-401) was incubated at 37° C for 10 min in the presence or absence of 0.15 M KCl with various combinations of 0.4 mM chlorpromazine, 0.5 mM oleate, 1 mM spermine. In the case of oleate, plus chlorpromazine, however, there was a previous incubation for 10 min at 37° C with 0.5 mM oleate to cause the translocation of PAP to the microsomal membranes. The concentrations of chlorpromazine, spermine and oleate [4,8,9], were chosen from previous works. The protein concentration of the postmitochondrial supernatant was about 21–25 mg/ml. The microsomal and cytosolic fractions were collected after centrifugation at $105\,000 \times g$ ($r = 5.99$ cm) in a Kontron ultracentrifuge (Centrikon T-2080) for 45 min at 4° C. Microsomal pellets were resuspended in 0.25 M sucrose/0.2 mM dithioerythritol/20 mM Hepes (pH 7.4)/2 mM EDTA. Protein concentrations were about 14, 19, 15, 17 and 13 mg/ml for the cytosolic fraction and about 4.2, 3.6, 3.7, 3.4 and 3.6 mg/ml for microsomal fraction. Both fractions were obtained from postmitochondrial supernatant of liver homogenates taken from rats aged 1, 30, 60, 180 and 360 days.

Recombined experiments. Postmitochondrial supernatant obtained from homogenates of 1 day-old livers were centrifuged at $105\,000 \times g$ for 45 min and soluble and microsomal fractions were isolated. Soluble fraction was passed through a Sephadex G-25 column and the microsomal fraction was washed twice with the homogenising medium. Both fractions were recombined and incubated either in the presence or absence of 0.5 mM oleate. Afterwards, the recombined sample was centrifuged ($105\,000 \times g$ for 45 min) and PAP activity was assayed either in the resuspended pellet (microsomes) or in the supernatant (soluble).

Preparation of phosphatidate as a substrate for PAP activity. [^{3}H]Phosphatidate substrate (0.4 Ci/mol) was prepared in a Ca^{2+}-free form treating with chelating resin and then sonicated with phosphatidylcholine in EGTA and EDTA [10].

Analytical methods. PAP activity was assayed as previously described by Martin et al. [10]. Each assay contained in a final volume of 0.1 ml: 100 mM Tris buffer adjusted to (pH 7.4) with HCl, 1 mM dithioerythritol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.2 mg of fatty acid-poor serum albumin/ml, 60 nmol of [^{3}H]phosphatidate (0.4 Ci/mol) and 40 nmol phosphatidylcholine. Cytosolic and microsomal fractions were diluted to about 1 mg of protein/ml and mixed with the buffer. The reaction was started by adding the [^{3}H]phosphatidate containing phosphatidylcholine, EDTA, EGTA and fatty acid-poor bovine serum albumin. The reaction was stopped with 2 ml of chloroform/methanol (95 : 5, v/v) containing 0.08% olive oil, followed immediately by adding 1 g of dry aluminium oxide and the amount of [^{3}H]diacylglycerol was determined according to

Martin et al. [10]. Lactate dehydrogenase activity, as a cytosolic marker, was determined following Saggerson and Greenbaum [11]. Rotenone-insensitive NADPH–cytochrome-*C* reductase, as a microsomal fraction marker, was determined by the method of Sottocasa et al. [12]. Protein concentrations were determined essentially according to Bradford [13] using bovine serum albumin as a standard.

Results and Discussion

In liver homogenates obtained from 1-day-old rats and incubated in the presence of 0.15 M KCl, almost all PAP activity seemed to be associated with the microsomal membranes. In Figs. 1, 2 and 3, it is possible to observe that the percentage of distribution of PAP activity onto the membranes, in the presence of 0.15 M KCl, was over 80% under all experimental conditions assayed, which include oleate, spermine, chlorpromazine and no addition. However, in the same liver homogenates, but in the absence of salt, oleate did not induce the translocation of this enzyme, but diminished it. Figs. 1 and 3 showed that the decrease in PAP activity bound to membranes, induced by oleate, in homogenates from 1-day-old rats, was significant if compared to none versus oleate (65 → 43%, $P < 0.001$) and chlorpromazine versus chlorpromazine plus oleate (74 → 58%, $P < 0.001$).

In order to investigate the negative effect of oleate on PAP translocation in liver homogenates from 1-day-old rats, the postmitochondrial fraction was centrifuged and the soluble and microsomal fractions were isolated before incubation. The soluble fraction was passed through a Sephadex G-25 column and the microsomal pellet was washed twice. Both fractions were then recombined and incubated in the presence or absence of 0.5 mM oleate. PAP activity was assayed in both fractions after centrifuging the recombined sample. The results obtained (Fig. 4) demonstrated that some substance, present in the soluble frac-

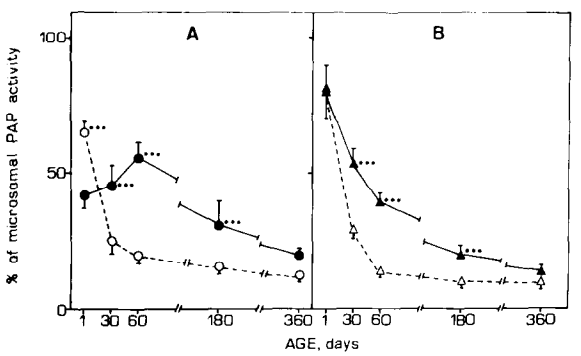

Fig. 1. Age-dependent changes on the oleate-induced translocation of PAP onto the endoplasmic reticulum membranes. Results, expressed as relative distribution of PAP activity into microsomes (%), are the mean of four experimental observations. S.E.M.s are indicated by vertical bars. $*P < 0.05$; $***P < 0.001$. (A) without KCl: ○, none; ●, oleate. (B) with 0.15 M KCl: △, none; ▲, oleate.

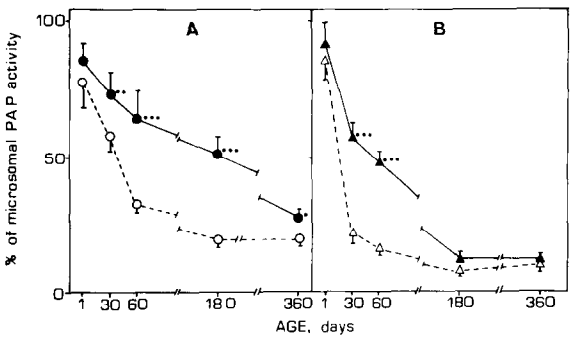

Fig. 2. Age-dependent changes on the effect of spermine on the oleate-induced translocation of PAP onto the endoplasmic reticulum membranes. Results, expressed as relative distribution of PAP activity into microsomes (%), are the mean of four experimental observations. S.E.M.s are indicated by vertical bars. $**P < 0.01$; $***P < 0.001$. (A) without KCl: ○, spermine; ●, spermine + oleate. (B) with 0.15 M KCl: △, spermine; ▲, spermine + oleate.

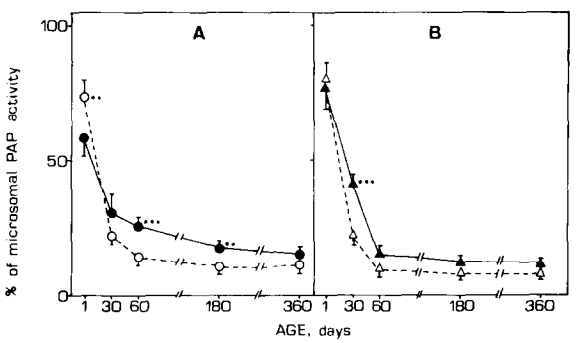

Fig. 3. Age-dependent changes on the effect of chlorpromazine on the oleate-induced translocation of PAP onto the endoplasmic reticulum membranes. Results, expressed as relative distribution of PAP activity into microsomes (%), are the mean of four experimental observations. S.E.M.s are indicated by vertical bars. $**P < 0.01$; $***P < 0.001$. (A) without KCl: ○, chlorpromazine; ●, chlorpromazine + oleate. (B) with 0.15 M KCl: △, chlorpromazine; ▲, chlorpromazine + oleate.

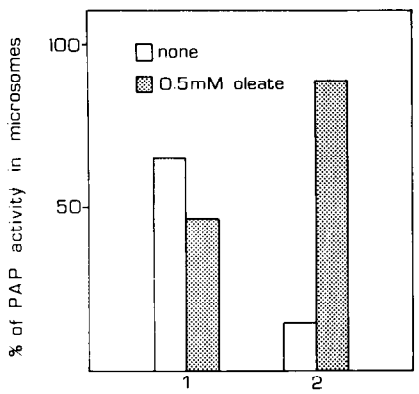

Fig. 4. PAP activity (%) into microsomal membranes in liver homogenates from 1-day-old rats. (1) Postmitochondrial fraction incubated in the absence □ or presence □ of 0.5 mM oleate. (2) Soluble fraction (passed through a Sephadex G-25 column) and microsomal fraction recombined and incubated in the absence □ or presence □ of 0.5 mM oleate.

tion, which was removed when passed through the column, is responsible for the observed negative effect of oleate. This substance could be non-esterified fatty acids usually present in large concentrations in plasma of weaning mammals [14]. Further investigations are necessary to verify the exact identity of this substance. Therefore, in the presence of high concentrations of free fatty acids, when ionic strength was low, the addition of 0.5 mM oleate does not induce a further translocation of PAP. Under these conditions, unspecific proteins are attached to microsomal membranes preventing the binding of PAP. A physiological concentration of salt (0.15 M KCl) added to the incubation medium increased the response to oleate in liver homogenates obtained from 1-day-old rats from 43 to 80% ($P < 0.001$). The same salt concentration had little effect in liver homogenates taken from 60-day-old rats (46 to 53%) and produced a notable decline in those from 180- and 360-day-old rats (31 to 20%, $P < 0.01$ and 20 to 14%, $P < 0.05$, respectively). These results demonstrate that in younger rats, ionic strength increased to a maximum the oleate-induced translocation of PAP activity onto the membranes (Fig. 1). When the interactions between soluble proteins and membranes are studied, it is particularly interesting to maintain the salt concentration at the physiological level [15]. This is because unspecific binding of soluble proteins to membranes is then reduced [7], which favours the specific attachment of PAP to the membranes.

It is well known that spermine increases the proportion of PAP that is metabolically active through its binding to microsomal membranes [16]. In the present investigation, it was shown (Fig. 2) that this increase was more pronounced in liver homogenates taken from 1-day-old rats. The promoting effect of spermine on oleate-induced translocation maintained values as high as 51% in homogenates from liver of 180-day-old rats, which decreases to less than one-quarter (51 → 12%, $P < 0.001$). Also in Fig. 2, it can be observed that the translocation of PAP activity, promoted by spermine, declined markedly in liver homogenates from 30- and 60-day-old aged rats (58 to 22%, $P < 0.001$ and 32 to 16%, $P < 0.001$, respectively). In the absence of KCl, spermine by itself produced a noticeable enhancement in the translocation of this enzyme in liver homogenates taken from liver of rats aged 1, 30 and 60 days (65 to 77%; 25 to 58%, $P < 0.001$ and 20 to 32%, $P < 0.001$, respectively).

Chlorpromazine, an amphiphilic amine, antagonizes the effect of oleate in that it displaces PAP activity from the endoplasmic reticulum membranes [4]. The negative effect of chlorpromazine on oleate-induced translocation of PAP was very clear in liver homogenates from 60-day-old rats either with or without KCl. In both cases, the translocation decreases to less than one-half, either in the presence or in the absence of KCl (56 to 26%, $P < 0.001$ and 39 to 15%, $P < 0.001$, respectively). The effect of chlorpromazine added to liver homogenates from 1-day-old rats, when KCl was present, slightly increased the oleate-induced translocation of PAP activity (43 to 58%, $P < 0.001$).

Total PAP activity (soluble plus microsomal) was assayed in liver homogenates obtained from 1–360-day-old rats and expressed as nmol of diacylglycerol per min and per mg of protein, either in the presence or absence of oleate and/or KCl. In Fig. 5, a sharp decline can be observed from 1–30 days followed by a slight recuperation of activity from 30–60 days, which was maintained only when physiological concentrations of KCl were present in the incubation medium. The positive effect of ionic strength on total PAP activity

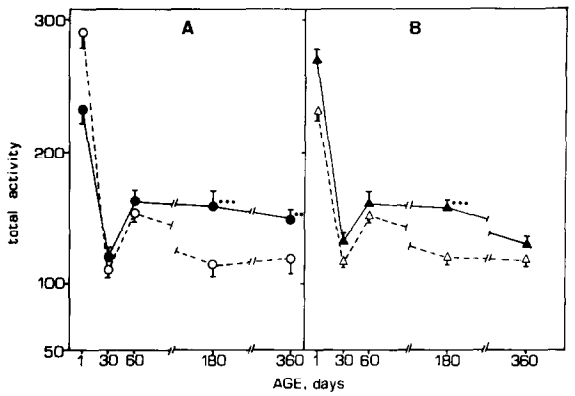

Fig. 5. Age-dependent changes on total PAP activity. Results, expressed as nmol of diacylglycerol formed per min, are the mean of four experimental observations. S.E.M.s are indicated by vertical bars. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (A) none, ○; none + 0.15 M KCl, ●. (B) oleate, △; oleate + 0.15 M KCl, ▲.

was manifested in homogenates from 180-day-old rats.

It is noteworthy to point out that in 1-day-old rats, the total PAP activity was the highest and the enzyme was localized mostly in the microsomes. These results can be explained by the higher concentration of circulating non-esterified fatty acids in suckling rats mainly because of the very high milk fat content. Thus, the concentration of plasma non-esterified fatty acids in 24-h-old rats is 1 mM [17], while in adult rats (3 months), this value has been reported to be 0.16 mM; [18]. The higher non-esterified fatty acid uptake by the liver will increase its esterification, which agrees with the increased total PAP activity and the localization of the activity mainly in the microsomes, where the enzyme is really active. On the other hand, in the younger rats, ionic strength activated the binding of PAP activity with the membranes of endoplasmic reticulum, and in mature and older rats, ionic strength produced an opposite effect. It summary, the binding of PAP to the membranes decreases progressively with aging. This decrease could be a consequence of a diminished affinity of this enzyme due to an age-related change in the fluidity of the membranes [19], and is correlated with a decrease in its activity together with a decline in the synthesis of triacylglycerol.

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