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PROTEIN KINASE C ISOZYME PATTERN IN LIVER HYPERPLASIA

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Lead nitrate, a potent activator of protein kinase C, is able to induce reversible rat liver hyperplasia. This phenomenon shows sex-related growth differences: liver hyperplasia as well as its regression by apoptosis occurred earlier and was more pronounced in male than in female rats. Dietary choline administration to females causes a shift of growth pattern towards the male values. Analysis of protein kinase C isoenzymes with hydroxylapatite column chromatography at time points crucial for lead-induced liver proliferation in male, female and choline-treated female rats showed a significant down-regulation of ß and α PKC activities and a marked activation of epsilon PKC. The fluctuation of these activities could be related to the rates of DNA synthesis. These data suggest that the observed PKC isoenzymes could be involved in the signal transduction pathway leading to lead-induced liver proliferation.

Lead nitrate induces rat liver hyperplasia characterized by an outgrowth of an excess of tissue, whose differences from compensatory cell proliferation after partial hepatectomy in terms of growth rates and DNA synthesis kinetics are probably due to a differential selection of signal transduction and metabolic pathways (1, 2). A sexual dimorphism exists in lead-induced liver hyperplasia, being its mitogenic effect more pronounced in males (3). Such dimorphism can be consistently reduces by dietary administration of an excess of choline. This treatment was also shown to modify the female growth pattern of liver regeneration after partial hepatectomy as well as of focal growth during hepatocarcinogenesis in a male-like fashion (4,5).

It has been shown that lead is a potent activator of diacylglycerol, calcium and phospholipid-dependent protein kinase C (PKC), and that

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picomolar concentrations of this metal are equivalent to micromolar calcium in kinase activation (6, 7).

In order to clarify the molecular mechanisms leading to lead-induced - hyperplasia and the related sexual dimorphism, the pattern of PKC activity was investigated in relation to DNA synthesis. Since the family of PKC isozymes plays an important role in transmembrane signaling and in cell growth control (8), the present investigation was planned to elucidate whether a relationship could exist between lead-induced cell proliferation and the expression and activation of PKC isozymes. Since lead was shown to substitute calcium for enzyme activation, our attention has been focused on the calcium-dependent subspecies expressed in rat liver: α and ß PKC (9).

MATERIALS AND METHODS

Chemicals

6-3Hthymidine (20 Ci/mmol) was from New England Nuclear (Boston, MA); Picofluor 40 from Packard Instruments International (Zurich, Switzerland); gamma-32P ATP (3000 Ci/mmol), colloidal gold labelled protein A, IntenSE BL silver enhancement kit were from Amersham Corp. (Arlington Heights, IL); hydroxylapatite was from Bio Rad Labs. (Hercules, Ca); PKC isoform-specific policlonal antibodies were purchased from Gibco Inc. (Gaithersburg, MD); choline, calf thymus DNA from Sigma Chemical Co. (St. Louis, MO).

Animal treatment

Rats of both sexes of the Wistar strain (Charles River, Como, Italy), 6 weeks old, were maintained under standardized conditions of light (light 08:00-20:00 h) and temperature (21 ± 1°C). Food and water were ad libitum. Choline (1g/Kg/day) was given in the drinking water for 3 weeks before i.v. treatment with lead nitrate (30 mg/Kg b.w.). DNA synthesis was measured by the decay of specific DNA radioactivity (cf. 10). 600 µCi/Kg b.w. of 3H-thymidine was given to rats 22 hours after lead nitrate treatment, then animals were killed by decapitation at 24, 36, 48 and 60 hours after lead. Livers were weighed and immediately frozen at -80°C to determine PKC activity and at -20°C to measure DNA radioactivity.

DNA specific radioactivity

Livers were homogenized to 10% (w/v) in distilled water with a Polytron 10-ST apparatus (Kinemata, Luzern, Switzerland). The procedure for radioactivity measurements has been reported previously (10). DNA was assayed by the method of Burton (11), using calf thymus DNA as standard. Fractional rates of DNA synthesis (ks) were calculated by the following equation: ks = ln (specific DNA radioactivity) /t, as previously reported (10), and expressed as percentage per day.

Partial purification and assay of PKC subspecies from rat livers

PKC subspecies were partially purified with DEAE/Hydroxylapatite cromatography and assayed from 3 grams of liver tissue has previously described (12, slightly modified as in 9). For enzymatic characterization of 80P kinase, peak fractions of females, males and choline treated females 36, 24 and 20 h after lead treatment, respectively, were tested for kinase activity in the presence of diacylglycerol and calcium, or of the two separate PKC activators.

Western blot analysis of PKC

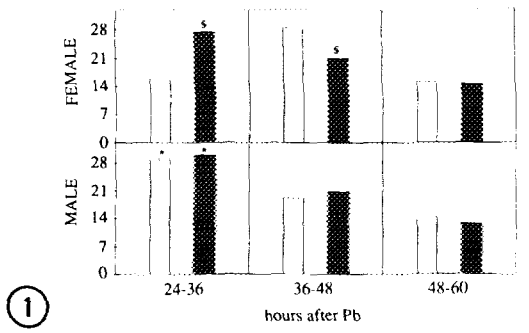
Immunological identification of eluted peak fractions was performed as previously described (9), on 80P peak fractions of females, males and choline-treated females, 36, 24 and 24 h after lead administration, respectively. A polyclonal antibody raised against the epsilon isoform of PKC was used for Western blot analysis. To confirm that 80 kDa immunoreactive bands corresponded to the epsilon subspecies of PKC, the above described peak fractions were pooled and loaded in the indicated lane. Separately, the same Western analysis was performed in the presence of the specific epsilon peptide against which the rabbit polyclonal antibody was raised, according to manufacturer's instructions.

RESULTS AND DISCUSSION

As previously reported, an anticipation of the proliferative response to lead was seen in males when compared to females, the DNA synthesis rates being higher and earlier (peak at about 24h after treatment) in males when compared to females (peak at about 36h after treatment; Fig. 1; 3). An excess of choline partially corrected this sexual dimorphism (Fig. 1; 3), according to previous data on liver regeneration after partial hepatectomy (4).

In normal conditions, hydroxylapatite column chromatography of liver homogenates, allows the separation of calcium-dependent isoforms of PKC, which are resolved into two distinct peaks of calcium-activated, phospholipid-diacylglycerol dependent histone kinase activity. We and others have demonstrated that these peaks, eluted at about 55 and 115 mM potassium phosphate, in our condidions correspond to ß (minor peak) and α (major peak) PKC respectively (9, 13). Fig. 2 shows a representative elution pattern of these isoenzymes from whole, untreated rat liver. A peculiar sexual dimorphism was observed in the activity of these two isoforms (PKC α and ß), under the experimental conditions of liver hyperplasia induced by lead nitrate. At the time points corresponding approx. to the S phase, the peaks corresponding to α and ß PKC were markedly reduced at 24 hours after lead in males, at 36 hours in females (Fig. 3). Interestingly, the peak of DNA synthesis shows in female rats a delay of about 12 hours relative to males (Fig. 1). The elution profiles were qualitatively similar to controls: in female livers at 24 hours after lead and at 36 hours in male livers (Fig. 3).

In male livers, 24 hours after lead administration, a third distinct peak was eluted between α and ß PKC at about 80 mM potassium phosphate



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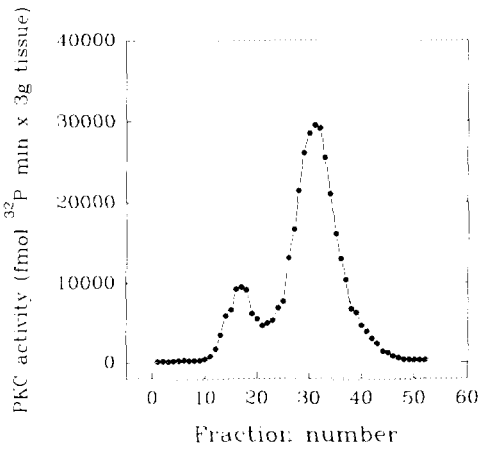

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Fig. 1. DNA synthesis rates during lead-induced liver hyperplasia. 3H-thymidine (600 µCi/Kg. b.w.) was given at 22 h and animals were killed at 24, 36, 48 and 60 h after lead nitrate. Fractional rates of DNA synthesis are expressed as %/day. Significance of the differences: * = $P < 0.05$ versus females; § = $P < 0.05$ choline females versus control females. Each time-point corresponds to 4-5 animals; controls = (□), choline = (■).

Fig. 2. Elution profiles of PKC activity from hydroxylapatite column chromatography of normal rat liver.

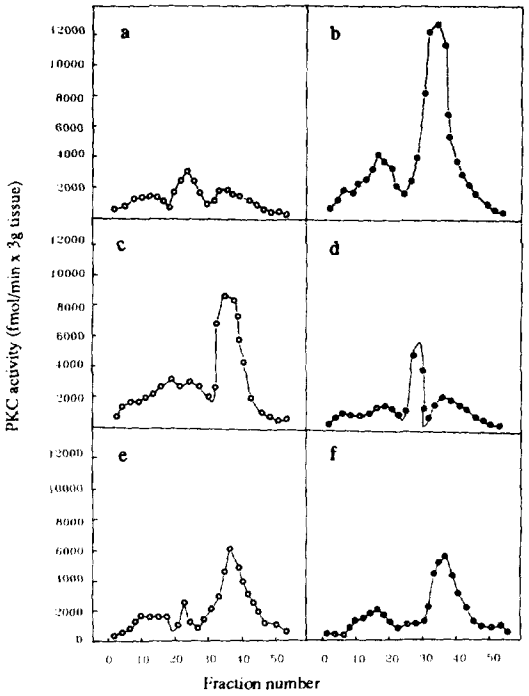

Fig. 3. Elution profiles of PKC activity from hydroxylapatite column chromatography of lead-nitrate-treated males (a, b), females (c, d) and choline-treated females (e, f), 24 (○) and 36 (●) hours after lead.

(Fig. 3). This peak will be referred throughout the text as 80 P. The same unidentified kinase 80 P was eluted, at the same position of that of males, in females livers, but at 36 hours after lead. At 24 hours, in females, a small shoulder, at the same elution position of 80 P, could be observed, probably suggesting an early sign of the induced activation/synthesis of this enzyme (Fig. 3). These data suggest that a correlation could exist between the observed modification of PKC isoenzyme profile and the sex-related changes of growth parameters.

An excess of choline was able to attenuate the sex-related delay of liver proliferation in females, leaving unaffected the male pattern, in agreement with our previous data (4). Down regulation of PKC α and ß in choline-receiving females was not seen at the same extent neither at 24 nor at 36 hours, probably suggesting a sustained activation of the enzyme due to a relative increase in the content of phosphatidylcholine (14), being this molecule the major source of the PKC activator diacylglycerol in long-term cellular responses (15). Choline treatment caused the activation of the unknown kinase activity (80 P) whose expression followed the choline shift of female growth parameters toward that of males. In fact, this peak appared in choline-receiving females at 24 hours in association with the highest proliferation rates, as it occurred in untreated male at the same time period of 24 hours (Fig. 3).

In order to elucidate the biochemical properties of the unknown kinase (80 P), enzymatic assay was performed on peak fractions collected from

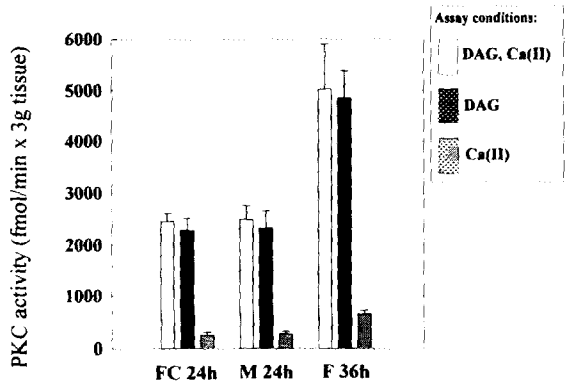

Fig. 4. Enzymatic characterization of 80 P kinase; peak fractions from chromatographed choline-treated females, males and females 24, 24 and 36 hours after lead administration, respectively, were assayed for kinase activity in the presence of diacylglycerol and calcium (DAG, Ca(II)); diacylglycerol alone (DAG) or calcium alone (CaII), as indicated in the methods section.

hydroxylapatite colums and obtained from liver of males and choline-receiving females at 24 hours, and of untreated females at 36 hours after lead. This assay revealed that withdrawal of calcium in the reaction mixture did not affect the kinase activity shown by 80 P. On the contrary, lack of activation was observed in the absence of diacylglycerol in the reaction mixture, thus stating the diacylglycerol-dependent, calcium-independent nature of the purified kinase (Fig. 4).

In order to immunologically identify 80P, peak fractions from the three experimental groups expressing the novel kinase were subjected to western blot analysis with isoform-specific policlonal antibodies, raised against calcium-independent, diacylglycerol-activated δ (not shown in this paper) and epsilon PKC. All three peak fractions reacted with antibodies raised against epsilon PKC, whereas no immunoreactivity was found with δ antibodies. Lack of immunoreactivity was also found when the pooled peak fractions from the three tested samples were blotted in the presence of the epsilon antigen peptide against which the rabbit policlonal antibody used in this experiment was raised (Fig. 5). The elution position of epsilon PKC in our samples is in agreement with the observations of Koide (16), stating that this isoform elutes just after ß and before α PKC. Our cumulative data, at time points crucial for lead nitrate liver hyperplasia, indicate the following points: I) a peculiar sexual dimorphism associated with a down regulation of α and ß PKC appears during the S phase (24 hours in males and, with a delay of 12 hours, at 36 hours in females); II) an excess of choline partially modified the female to a PKC male pattern, since no down regulation of α and ß PKC isoform was observed at 36 hours, as it was seen in untreated females; III) an unknown kinase activity (80 P) was stimulated in all observed samples, at time points corresponding presumably to the S phase of cell cycle, and could be eluted between the two PKC isoforms; it was identified as epsilon PKC.

In normal conditions, as well as in proliferating livers after surgical hepatectomy, epsilon PKC is not present in rat livers in a

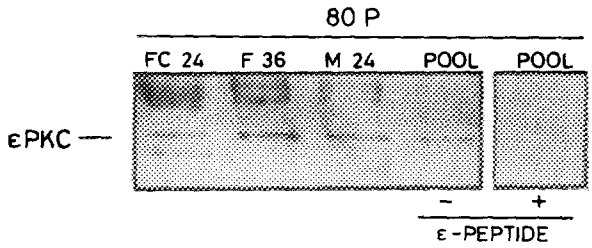

Fig. 5. Western blot analysis of chromatographed 80 P peak fractions, from males, females and choline-treated females 24, 36 and 24 h after lead nitrate, respectively. Tested peak fractions were pooled and blotted in the absence (epsilon-peptide " - ") or, separately, in the presence (epsilon-peptide " + ") of the antigen peptide against which the polyclonal antibody used in this investigation was raised.

detectable amount (9, 17, 18). However, Ono et al. (19) demonstrated in rat liver the presence of genomic sequences encoding for this isoenzyme, although it could not be found at the protein level. Recent data showed that epsilon PKC expression can be induced in CCl_4-treated rat livers (20). Since CCl_4 induces a compensative growth response due to the necrogenic effect of the compound, it is possible to speculate that the expression of epsilon PKC in rat livers could be associated with hepatocyte growth in the presence of liver-toxic compounds (21, 22). Furthermore, epsilon PKC expression has been recently associated with increased cell growth rates in transfected rat fibroblasts (22).

In conclusion, our data show that the activation of PKC during hepatic cell proliferation induced by lead nitrate is differently modulated by the hormonal asset of the animal (e.g. the pattern of sexual dimorphism) and by biochemical manipulation (e.g. the modifying effect of choline); of interest, an unknown DAG-activated kinase (80 P) becomes apparent; it was identified as epsilon PKC. The activation/expression of this PKC isoform could be related to lead-induced liver cell growth stimulation.

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