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Effect of Lead Exposure on the Level of Protein Kinase C Isolated from Mouse Brain

By

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ABSTRACT

Subchronic exposure of mice to different concentrations of lead has been performed for 70 days. F_1-pups were produced from different cross-mating animals of F_0 female and male mice, which have been treated by lead acetate at different concentrations of 0.0, 26, 260 and 2600 ppm. The level of protein kinase C (PKC) in brains of treated and untreated animals was determined by using immunoblot analysis on polyvinylidene difluoride (PVDF) membrane against anti-protein kinase C antibody. Immunoblot analysis showed that the level of PKC was significantly decreased in most of the treated animals either at F_0 premated females or males. At 260 and 2600 ppm, the reduction level of PKC was about 16.7 and 100% for females and 33.7 and 50% for males respectively. During mating, periods, The reduction level of this enzyme isolated from females after gestation and lactation was observed in all the exposed females and it was 100% by 2600 ppm. Furthermore, PKC level of female pups produced from treated females mated with treated or untreated males, was significantly decreased compared to those pups produced from untreated animals. There are three major reasons for the reduction of PKC whish's are could be concluded from the present

data.(a) Dramatic reduction which occurred in treated F_0 male and female mice during gestation and lactation period.
(b) The level of PKC from F_0 female after gestation and lactation and male mice after mating, was apparently reduced and (c) severe reduction in the level of this enzyme was observed in F_1-female mice produced from treated animals.

INTRODUCTION

Protein kinases are phosphoryl transferases. They transfer the terminal phosphate group of ATP to hydroxyl group of serine, threonine, and tyrosine residue of acceptor substrate protein. The study of protein kinases has resulted in their classification into two general groups based upon amino acid, which is phosphorylated from their activity, serine/ threonine kinases and tyrosine kinases. The tyrosine kinases are generally composed of kinases, which act as receptors for growth factors. Some protein kinases are known to play a role in the regulation of normal cell metabolism such glycogen synthesis, while a number of protein kinase activities were shown to be associated with growth factor receptors.

Successful regeneration in animals involves multi-complex systems that are required to ensure birth and nourishment of healthy individuals. One of the most important enzymes in the growth, development and proliferation, is the Ca^{+2}/ phospholipid-dependent protein kinase (PKC). There are circumstantial evidences to support a key role for PKC in control of many biological responses, including endocrine and exocrine secretion, neurotransmitter release, Na^{+}/H^{+} exchange, cell-cell interaction, cell surface receptors and gene expression (Nishizuka 1984). Therefore, the interruption of the enzyme was found to alter the biochemical and physiological responses as well as the behavioral development of organs function (Annika, 1997; Chen et al, 1997; Chen et al, 1998).

Until now there are lack of information regarding the effect of heavy metals such as lead, on protein kinases as a new target. Recently, it has been found that Al^{3+} inhibits human brain tau (micotubule associated

protein) protein phosphorylation at low concentrations, whereas, at concentrations higher than 100 μM caused aggregation of tau protein preventing its entry in SDS- gel electrophoresis (El-Sebae et al, 1993 and Abdel-Ghany et al, 1993). Exposure of cells to lead resulted in strong modulation of PKC activities (Tonner and Heiman 1997). In lead treated cells, cytosolic PKC activity was reduced by 48 % compared to control (Tonner et al 1997). Furthermore, chronic low-level of lead exposure to children have exhibited deficit in their memory and learning (Chen et al 1998). In addition, lead exposure was found to modulate the PKC activity in brain endothelial preparation, which may explain Pb-induced damage at the blood-brain barrier (Zhao et al 1998).

The purpose of this study is focussed on the evaluation of the level of protein kinase C activity in the male and female mice that have been exposed to lead as well as the first generation of the treated animals. Furthermore, the study is aimed to use the simplified and highly specific method based on immunoblot analysis of isolated enzyme to be implicated in toxicological assessments of heavy metals and other toxicants.

MATERIALS AND METHODS

Animals:

One-hundred eighty male and three-hundred twenty female mice ICR (CD-1) were obtained from High Institute of Public Health, Alexandria University, Alexandria, Egypt and used as the F_0 generation. Upon arrival, mice were examined for external signs of disease or injury. The animal room was maintained at 25°C ± 2 °C with relative humidity of 50 ± 5% for 12 hr light-dark cycle. Animals were acclimated for two weeks prior to treatments. Standard diet was offered and tap water *ad libitum*.

Chemicals :

Lead acetate trihydrate was obtained from Sigma Co. Anti-protein kinase-C (rabbit-anti-peptide monoclonal antibody) was obtained from

GIBCO-BRL. Tween-20 and goat anti-rabbit antibody conjugated to alkaline phosphatase were from Bio-Rad. Bromochloro-indolyl phosphate/nitro blue tetrazolium mixture (BCIP/NBT) as substrate for antibody conjugated alkaline phosphatase, were from Kirkegaard & Perry laboratories,USA. Polyvinylidene difluoride (PVDF) membrane was from Millipore Co.

Experimental Design:

Lead acetate trihydrate was dissolved in deionized water to give lead concentrations of 26, 260, and 2600 ppm. Acetic acid (0.00125 %) was added to help in lead, disolving. These solutions were provided as drinking water *ad libitum* beginning at the day 42 of age, which was designated as 0.0 day of treatment. Sixty male, and eighty female mice were assigned to the control group. Eighty females and fourty males were assigned to each above-mentioned concentration. Drinking water containing the different concentrations of lead was provided with glass water bottles supported by stainless steel spouts. The tested concentrations of lead were freshly prepared and rechanged each 24 hr.

Cross-mating and F_0-necropsy:

After 70 days of lead treatment, ten males and ten females from each group were randomly selected to undergo a full necropsy. The brains were removed, and kept at -20°C. For PKC preparation. The remaining F_0 females and males of control group and treated groups were mated to produce the F_1 offspring. Exposed male mice in different exposed groups were mated with unexposed female mice, unexposed male mice were mated with exposed female mice, exposed male mice were mated with exposed female mice, and unexposed male mice were mated with unexposed female as shown in the following design.

In general, two female mice were paired with one male up to 15 days without any treatment. Vaginal plugs were inspected daily, and the day that vaginal plugs observed was designated as the Day 0 of gestation. Confirmation of mating, females were removed from the male's cage and housed individually. The F_0 males were anaesthetized immediately after mating. The brains were removed for PKC preparation. Pregnant F_0

females were allowed to produce the F1 pups (without treatment during gestation and lactation periods), and when the delivery has been completed, it was designated the day one of lactation. Any female which did not deliver was considered not pregnant. After pups were weaned, the F_0 females were anaesthetized. Brains were removed and immediately kept at –20°C. for PKC preparation.

Group No.	Mice cross-mating		
1	♂c	×	♀c
2	♂c	×	♀26
3	♂26	×	♀26
4	♂26	×	♀c
5	♂260	×	♀260
6	♂c	×	♀260
7	♂260	×	♀c
8	♂c	×	♀2600

Where, the numbers shown under the signs ♂ and ♀ are referred to the lead concentration while, C is referred to the control.

F1-generation:

At 90 days of age, all F_1-females and males produced from F0-crossmating group were anaesthetized Brains were removed and kept at -20°C for PKC preparation

Protein Kinase C preparation:

The PKC was isolated from the brains of treated and untreated mice (pre- & post-mated, and F_1 males and females) as described by

Abo-El-Saad et al, 1998. Brains were homogenized in buffer containing 10 mM-Tris buffer, pH 7.4, 5 mM 2-mercaptoethanol, 25 mM sucrose, 5 mM EGTA, 5 mM EDTA and 1 mM PMSF and centrifuged at 10,000xg for 15 min at 4°C. The supernatants were used as sources of protein in immunoblot analysis. The protein concentrations were measured using a Bio-Rad protein assay based on the method of Bradford (1976).

Immunoblotting and detection of protein kinase-C with enzyme-labelled reagents:

Isolated Proteins, 5µg from each treatment were dot-blotted onto PVDF membrane following standard conditions (Abo-El-Saad and Wu 1995). The non-binding sites of membrane were blocked with 2.5% BSA (fraction V) for an hour in TTBS (0.1% Tween 20, 50 mM Tris-HCl, pH 7.5 and 0.15 M NaCl). Monoclonal anti-Protein protein Kinase-C antibody was applied to the membrane at 0.5 µg/ml in TTBS with 1% BSA. Two hours later, the membrane was washed at room temperature four times, for 5 min each time, in TTBS with gentle shaking. The bound antibody was detected after a 1h. incubation in affinity-purified goat anti-rabbit immunoglobulin coupled to alkaline phosphatase (Diluted 1:3000 in TTBS with 1% BSA) and washed as above. The dots were developed by adding 10 ml color development reagent (BCIP/NBT) for 5-10 min at room temperature with agitation until the bands were suitably dark.

Calculation of relative dot blot darkness:

The relative and darkness degree of dot blot were calculated using the method of Georghiou and Saitso (1983) in which the sign of (+) was used to indicate the darkness level of each dot blot on the membrane. For example, the control have three dark dot blots, each of which was given two signs of (+), thus the number of signs in control is equal to 6 (three replicates). However, the light dot blots in treated samples was given single sign of (+). While, the disappeared dot blot was given no sign. The following equation was used for calculation of the relative dot blot darkness.

$$RDBD\ (\%) = \frac{NST}{NSC} \times 100$$

Where:
RDBD = relative dot blot darkness.
NSC = number of sings in control.
NST = number of sings in treatment.

RESULTS AND DISCUSSIONS

Effect of lead exposure on the level of PKC isolated from premated male and female mice:

Mortality rates in the 260, and 2600 ppm treated groups for males and 2600 ppm treated group for females were significantly increased compared to the control in the premating period beginning in the 5th and 8th treated weeks for males and females, respectively (Table 1). While the mortality rates increased up to 41.18% in the high treated group of F_0 males after mating, there was no significant differences in the mortality rates in the remaining groups (26, and 260 ppm). No mortality in any treated females in F_0 generation during gestation and lactation periods was noted. All males that have been exposed to 2600 ppm lead, were mated with females and then some died after the mating period. Thus, the data showed that the male mice after mating were highly affected by lead exposure. Furthermore, it seems that the mating of animal mice could play a role in sensitivity enhancement of male mice toward lead exposure because many of biological responses including endocrine secretion and neurotransmitter release during animal mating are controlled by PKC regulation (Nishizuka 1984, Downey and Han 1998).

Table (2) showed the immunoblot analysis of PKC isolated from the treated F_0 female and male mice after mating compared to the control. The data showed that the treated animals by 26 ppm had no effect in the density of the dot blots

Table (1): Mortality Rates (%) of F_0-Male and Female Mice During Premating and Postmating Period.

Mortality				
Lead conc.	Males		Females	
(ppm)	No	%	No	%
F_0 generation (premating)[a]				
0.00	0/60	0.00	0/80	0.00
26	0/40	0.00	0/80	0.00
260	6/40	15.00*	0/80	0.00
2600	13/40	32.50*	16/80	20.00*
F_0 generation (postmating)[b]				
0.00	0/50	0.00	0/70	0.00
26	0/30	0.00	0/70	0.00
260	0/24	0.00	0/70	0.00
2600	7/17	41.18*	0/54	0.00

[a]No.of females or males dead during treatment / total No. of females or males at day 0 of treatment.

[b]No.of females or males dead after mating / total No. of females or males used for mating.

- Significantly different from the control value

However, by increasing the concentration of lead, the dot-blot density apparently decreased by 33.7% and 16.7% at 260 ppm in both F_0 males and females. While, at 2600 ppm, the level of PKC was strongly decreased by 50% and 100% for males and females respectively .

Table (2): Immunoblot analysis of PKC isolated from the brains of F_0-female and male mice,after premating period

Lead conc. (ppm)	Darkness degree of dot blot		Relative dot blot darkness (%)		%Reduction of PKC level	
	♂	♀	♂	♀	♂	♀
None	++++++	++++++	100.0	100.0	0.0	0.0
26	++++++	++++++	100.0	100.0	0.0	0.0
260	++++	+++++	66.3	83.3	33.7	16.7
2600	+++	none	50.0	0.0	50.0	100.0

The data obviously indicated that the density of dot blots was completely abolished by using higher concentrations of lead which would reflect the level of PKC because the used monoclonal antibody of PKC in the experiments is highly specific against the enzyme. The results of reduction in the level of PKC followed by cell dysfunction would explain why do number of animals have died during the treatment period at high concentrations of lead. The results also are consistent with the well known key role of PKC as second messenger in cell function. In addition, the results are consistent with the previous data reported by several investigators (Tonner and Heiman, 1997, Chen et al 1997, Tonner et al

1997 and Ueda et al 1998) in which the activity of PKC from treated mice by lead was obviously decreased. However, Olivi et al 1996 reported that the exposure to lead resulted in an increase in the activity of PKC. This discrepancy might be due to the source of the enzyme and the tested concentration of lead. Besides, their study was focussed on the activity of the enzyme, while, the present investigation was mainly on the level of PKC. Moreover, the data indicated that the female mice were more sensitive to the exposure of lead than the male mice at the high concentration of 2600 ppm, where the level of PKC from females was almost completely abolished. Thus, the difference of lead effect on the level of PKC might be sex dependent, besides being dose dependent.

Level of PKC from treated female mice after postmating, gestation, and lactation periods:

Table (3) summarized the immunoblot analysis of the proteins isolated from the brain of F_0-generation female mice after gestation and lactation . The data showed that the density of dot blots of the enzyme from $_{26}$ (treated female by 26 ppm lead) mated with $_{26}$ was strongly decreased and it was similar in darkness with those obtained from $_{26}$ mated with $_{C}$ (untreated female). In the meantime, the reduction of PKC level of all above mated animals was 50% at 26 ppm lead concentration. When the female mice treated by 260 ppm lead, then mated with $_{260}$ (treated male by 260 ppm lead) or mated with $_{C}$, the density of dot blots was dramatically decreased. The reduction of PKC level which correspond to the concentration of 260 ppm in case of ($_{C}$X $_{260}$) was about 33.3 %. However, the dot blots of the enzyme isolated from untreated female mated with treated male by 260 ppm, showed no apparent effect compared to control female. In addition, the dot blots of PKC isolated from treated female by 2600 ppm was completely disappeared, thus the reduction of PKC level was 100%. These results revealed a significant point, that by increasing the lead concentration to 2600 ppm, the reduction of PKC was 100%.

The present data are consistent with the results previously reported by Laterra at al 1992, who suggested that lead cause microvascular dysfunction in the brain by interfering with PKC modulation.

Table (3): Immunoblot analysis of PKC isolated from the brains of F_0- female mice after gestation and lactation periods.

Mice cross-mating			Darkness degree of dot blot	Relative dot blot darkness (%)	% Reduction of PKC level
♂c	×	♀c	++++++	100.0	0.0
♂c	×	♀26	++++	66.7	33.30
♂26	×	♀26	+++	50.0	50.0
♂26	×	♀c	++++	66.7	33.3
♂c	×	♀260	++++	66.7	33.3
♂60	×	♀c	++++++	100.0	0.0
♂c	×	♀2600	none	000.0	100.0

C = control

* The numbers shown under the signs ♂ and ♀ are referred to the lead concentration.

Effect of lead exposure on F_0-postmated male mice and its level of PKC:

Table (4) showed the reduction and the dot blot density of PKC isolated from F_0 male mice after mating. The dot blot density of this enzyme at 26 ppm apparently decreased and the reduction level of PKC was 33.3 %. While, the dot blot density was completely abolished in the 260 ppm lead treated group, and the reduction of the enzyme level was 100%. However, the results revealed that the high concentrations of lead, was strongly effective on male mice and its level of PKC.

Table (4): Immunoblot analysis of PKC isolated from the brains of F_0- male mice,after mating.

Lead conc. (ppm)	Darkness degree of dot blot	Relative dot blot darkness (%)	%Reduction of PKC level
None	++++++	100.0	0.0
26	++++	66.7	33.3
260	none	000.0	100.0
2600	none	000.0	100.0

Effect of lead exposure on the level of PKC from the brain of F_1-female mice:

Table (5) showed the level of PKC isolated from the brain of F_1-female mice after the exposure of their parents to the different concentrations of lead. The dot blot density strongly decreased and the reduction level of PKC was 33.3 , 83.3 and 16.7 in $_{C}X_{26}$, $_{26}X_{26}$ and $_{26}X_{C}$ respectively. This data clearly indicated that the levels of the enzyme were dramatically decreased when both female and male mice were treated by lead prior to their mating. By increasing the concentration of lead to 260 ppm, no significant difference in the reduction level of PKC was observed compared to the animals that were treated by 26 ppm. Moreover, the dot blot density of PKC isolated from F_1-female mice treated by 2600 ppm was apparently decreased and the reduction level of PKC was 50%. Generally, the level of PKC in F1-female mice was strongly affected by previous exposure of their parents to different concentrations of lead. Therefore, the gender effect on the level of PKC had an important role during the gestation period. Moreover, the substantial mobilization of lead bone in previously exposed females (before pregnancy), during gestation and lactation periods may be explained by the previous finding reported by (Watson et

al.,1993, 1994).The present data might be reflected that the control of genetic program of cell had affected when the animals were exposed to lead pollution. Therefore, this finding might be correlated to apoptosis (genetic program of cell death).

Table (5): Immunoblot analysis of PKC isolated from the brain of F_1- female mice.

Mice cross-mating			Darkness degree of dot blot	Relative dot blot darkness (%)	% Reduction of PKC level
♂c	×	♀c	++++++	100.0	00.0
♂c	×	♀26	++++	66.7	33.3
♂26	×	♀26	+	16.7	83.3
♂26	×	♀c	+++++	83.3	16.7
♂c	×	♀260	++++	66.7	33.3
♂260	×	♀c	++++	66.7	33.3
♂c	×	♀2600	+++	50.0	50.0

Comparative effect of lead on the level of PKC from brains of pre-mated male and female mice after mating period:

Fig.(1) showed the level of PKC from untreated and treated male mice by lead in both premated and postmated animals. As shown from the Figure, that the level of the enzyme was 100% in the untreated animals, while at 26 ppm no decreasing in its level was observed in the premated males. However, at the same concentration the

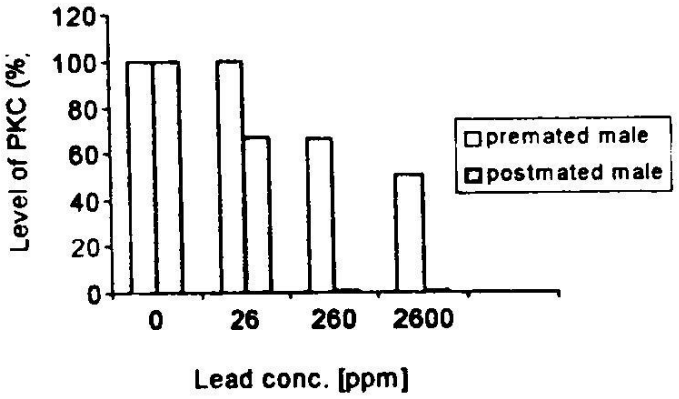

Fig.(1):Level of PKC isolated from brains of pre- and postmated male mice have been treated by different concentrations of lead.

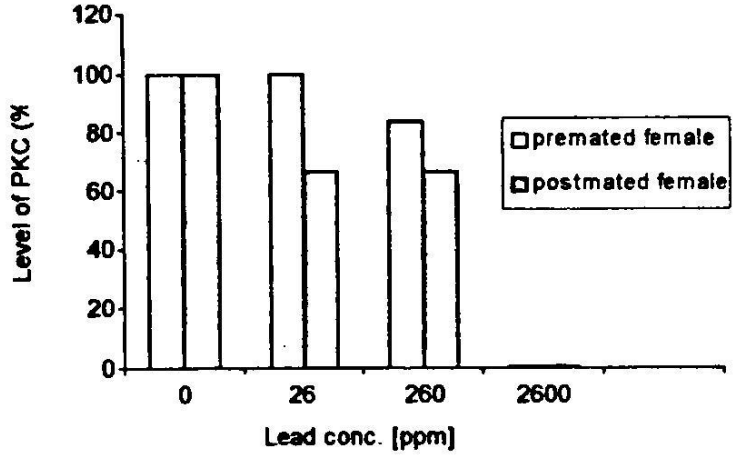

Fig.(2):Level of PKC isolated from brains of premated mice and female mice have been treated by lead and mated with untreated male.

level of the enzyme was 66.7 % in the males after mating. By increasing of the concentration to 260 ppm, the level of the enzyme completely depressed, where it reached to the level of zero in males after mating, and it was 66.3% in the males before mating. Furthermore, the high concentration of lead, 2600 ppm was highly effective on the level of PKC, since it was completely decreased to the level of 50 and 0.0 % in premated and postmated mice. The results clearly showed that the postmated male mice had exhibited the lowest level of the enzyme compared to the level of PKC from premated male mice. On the other hand, Fig. (2) summarized the difference between the levels of PKC for female mice before mating and after gestation and lactation periods. The level of the enzyme in the case of female mice after these periods was dramatically decreased compared to premated female mice, while the level of PKC was completely abolished at 2600 ppm in females, however, it was 50 % for premated female mice. This difference may be explained by the possibility that the enzyme from females would be affected by gestation and lactation periods, as a condition of calcium stress, which may induce changes in the PKC, because the enzyme is strictly dependent on calcium.

It could be fairly concluded that the level of PKC in male, female mice and their offspring was strongly affected by lead exposure. This finding may reflect an important information not only to prevent the adult toxicity but also to avoid the lead exposure, especially for the children.

Since the most well known knowledge in this angle have supported the idea of the ability of child cells for lead up-taking is highest than those cells for adults.

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الملخص العربى

تأثير التعرض للرصاص على مستوى أنزيم بروتين كينيز المعزول من مخ الفئران

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تم تعريض فئران التجارب لجرعات تحت مميته من الرصاص فى صورة خلات رصاص لمدة ٧٠يوم و فى سلسلة من التركيزات صفر ؛ ٢٦ ؛ ٢٦٠ ؛ ٢٦٠٠ جزء فى المليون. وقد تم التزاوج بين الذكور و الإناث المعاملة و الغير معاملة للحصول على الجيل الأول٠ و بإستخدام طرق التحليل المعتمد على الأجسام المضادة المناعية والذى يطلق عليه Immunoblot analysis و بإستخدام أجسام مضادة متخصصة من النوع الـ monoclonal لإنزيم Protein Kinase-C أوضحت النتائج أن مستوى هذا الأنزيم إنخفض بدرجة واضحة فى كل من الإناث و الذكور قبل عملية التزاوج حيث وصل مستوى الإنخفاض فى مستوى الإنزيم عند تركيز ٢٦٠ , ٢٦٠٠ جزء فى المليون إلى حوالى ١٧% ؛ ١٠٠% فى حالة الإناث و ٣٤% ؛ ٥٠% فى حالة الذكور على التوالى و قد أوضحت النتائج أيضا أن مستوى الإنزيم إنخفض أيضا فى الإناث بعد عملية التزاوج و أثناء الحمل و الرضاعة حيث وصل الإنخفاض فى مستوى الإنزيم الى ١٠٠% عند التركيز ٢٦٠٠ جزء فى المليون ٠ وفى الجيل الأول أظهرت النتائج أن مستوى الإنزيم فى إناث الجيل الأول الناتجة من تزاوج إناث وذكور معاملة إنخفضت بدرجة واضحة وبناء عليه يتضح أن هناك ثلاث تأثيرات أساسية لمثل هذه الدراسة (١)أنخفاض شديد فى مستوى أنزيم الـ Protein Kinase-C فى كل من الذكور و الإناث و ذلك قبل التزاوج (٢) أنخفاض شديد فى مستوى أنزيم الـ Protein Kinase-C فى كل من الذكور و الإناث و ذلك بعد التزاوج٠ (٣) تأثير واضح فى إنخفاض مستوى الإنزيم لإناث الجيل الأول٠