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^{3}H-PDBu Binding after Administration of Methylmercury to Mice

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Methylmercury has been recognized as an extremely hazardous environmental and industrial pollutant. Although it particularly accumulates in the liver, kidneys and brain, thereby causing impairment in several biochemical steps, its functional impairment in the liver and kidneys is difficult to demonstrate. Nonetheless, evidence would suggest that the primary site of action of methylmercury toxicity is limited to the neural tissues (Chang, 1977). The mechanism underlying its toxicity is apparently related to its extreme high affinity to the sulfhydryl groups of proteins (Hughes, 1957; Rabenstein, 1978). However, it is still obscure as to which biochemical or physiological pathways are primarily affected by methylmercury.

Recently, much attention has been focused on protein kinase C, which is known to be the target of tumor promoting phorbol esters including phorbol 12,13-dibutylate (PDBu) (Nishizuka, 1984), as a key enzyme in signal transduction for various biologically active substances, such as hormones, neurotransmitters, chemical mediators etc. (Nishizuka, 1986). Protein kinase C is ubiquitous in tissues and organs although the highest concentration is found in neural tissues (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981; Ashendel *et al.*, 1983). Moreover, sequence studies (Parker *et al.*, 1986; Coussens *et al.*, 1986; Ohno *et al.*, 1987; Ono *et al.*, 1987) revealed that protein kinase C is different from other protein kinases because it is rich in cysteine (14-17 cysteine residues per molecules).

Thus, it is reasonable to assume that the mode of action of methylmercury is by impairment of protein kinase C activity, thereby bringing about neuronal dysfunctions. Recent reports from this laboratory (Inoue *et al.*, 1988; Saijoh *et al.*, 1988) have demonstrated that sulfhydryl blocking reagents, such as methylmercury, inhibited PDBu binding and enzymatic activity of protein kinase C *in vitro*. In the present study, we evaluated the effects of methylmercury on protein kinase C *in vivo* by determining ^{3}H-PDBu binding in several tissues from methylmercury-administered mice.

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MATERIALS AND METHODS

The following drugs were used: ^{3}H-PDBu (15.2Ci/mmol) (New England Nuclear), leupeptin, PDBu (Sigma), diolein (Serdary), phosphatidylserine, methylmercury chloride (Tokyo Kasei), and ethylene glycol-bis(β-aminoether) N,N,N',N',-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride, polyethyleneimine and 2-mercaptoethanol (Nacalai Tesque). All other chemicals were of reagent grade.

Female mice (Jcl:ICR, 4 week-old) were housed in cages in a temperature controlled room with fixed lighting schedule and with free access to food and water. Administration of methylmercury was based according to the method of Komulainen and Tuomisto (1985) with some minor modification. Instead of gavage administration, subcutaneous injection was carried out on mice, since ^{3}H-PDBu binding in the stomach and small intestine could still be examined as well. Briefly, methylmercury chloride dissolved in physiological saline solution was administered either in doses of 1 or 5 (on every 3rd day) by subcutaneous injection at a dose of 10 mg Hg/Kg body weight. Mice were sacrificed 72 hr after injection in single dose experiments or 48 hr after the last dose in repeated dose experiments. The cerebrum, cerebellum, thymus, lung, heart, spleen, liver, kidney, stomach and small intestine were quickly removed and homogenized with 10 vols. of the homogenate buffer containing 20 mM Tris/HCl (pH 7.4), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.01 % leupeptin and 10 mM 2-mercaptoethanol.

The concentration of total mercury in the preparations were measured using a semi-automatic mercury measuring instrument (cold vapor atomic absorption method with two step gold trap; Rigaku Mercury SP™, Rigaku Ind. Corp., Japan). Detection limit was 1 ng while recovery was 98 ± 2 % (95 % confidence interval).

^{3}H-PDBu binding was determined essentially according to the method of Kikkawa *et al.* (1986). Briefly, 300 µl aliquots of homogenate (about 10 mg wet tissue) were added to a 300 µl reaction mix to make a final concentration of 5 - 50 nM ^{3}H-PDBu, 20 mM Tris/HCl (pH 7.4), 1 mM EGTA, 0.2 mM $CaCl_2$, 30 mg/ml phosphatidylserine and 4 mg/ml bovine serum albumin. After 30 min incubation at 30°, the mixture was rapidly filtered and washed on a Whatman GF/B glass filter, which had been soaked for 1 hr prior to use in a fresh 0.5 % polyethyleneimine solution. Non-specific binding was measured in the presence of 30 mM non-radioactive PDBu. Specific binding represents the difference between total and non-specific binding. Radioactivity was determined using a scintillation spectrophotometer. Binding data were analyzed by Scatchard plot and statistical analysis was performed using a two-tailed Student's t-test to determine the significance of differences between the means.

RESULTS AND DISCUSSION

No signs of toxicity were observed three days after the administration of a single dose (10 mg/kg) of methylmercury. On the other hand, after a cumulative dose of 50 mg/Kg was administered, hind

Table 1.The mercury concentration in tissues after methylmercury administration to mice

	Control	Methylmercury	
	(8)	Single dose (6) (ppm)	Repeated doses (8)
cerebrum	0.09 ± 0.01	2.36 ± 0.22	6.31 ± 0.48
cerebellum	0.08 ± 0.03	2.54 ± 0.13	5.81 ± 0.53
thymus	0.06 ± 0.02	2.31 ± 0.28	7.88 ± 0.58
spleen	0.21 ± 0.07	4.22 ± 0.21	15.24 ± 1.29
small intestine	0.09 ± 0.04	2.03 ± 0.18	20.27 ± 3.09
stomach	0.08 ± 0.05	2.31 ± 0.28	10.87 ± 0.73
lung	0.15 ± 0.04	3.45 ± 0.14	13.34 ± 0.97
kidney	0.09 ± 0.01	35.98 ± 2.15	77.64 ± 3.34
heart	0.03 ± 0.01	3.29 ± 0.38	9.63 ± 0.78
liver	0.08 ± 0.04	9.46 ± 0.22	27.07 ± 1.74

Each value is the mean ± S.E. (n).

limb paralysis was not yet detectable, although a decrease in voluntary movement, piloerection and broad-based and mild staggering gait were observed. Body weight of the mice decreased by 8.2 % ($P<0.05$) after the fifth dose, whereas tissue weight did not vary significantly. The symptoms observed in the present study seemed to be less severe than those reported in rats (Komulainen and Tuomisto, 1985), which were administered with the same doses of methylmercury by gavage. It is tempting to assume that variabilities in administration and animal species used brought about the differences in toxicity manifestations. In our study, subcutaneous injection was used since we also intended to measure ^{3}H-PDBu binding in the stomach and small intestine. Subsequently, concentrations of mercury in the tissues were also determined.

Methylmercury significantly accumulated in all the tissues examined even after a single dose. As shown in Table 1, the highest mercury concentration in tissues was observed in the kidneys, both after single and repeated doses. The concentration after repeated doses was about twice as much as that after a single dose. The concentration of mercury in other tissues were lower than that in the kidneys, both after single and repeated doses. However, all other mercury concentrations except in the small intestine increased approximately three fold after repeated doses than after a single dose. In the small intestine, mercury concentration increased ten times higher after repeated doses than after a single dose. This may be due to the reuptake of methylmercury which is excreted into bile (Norseth and Clarkson, 1971). Although mercury concentrations in the cerebrum and cerebellum were low as compared with those in the peripheral organs, methylmercury significantly accumulated in the cerebrum and cerebellum. Its accumulation in the brain was about 6 ppm = 3 x 10^{-5} M, which already appeared to evoke signs of neurotoxicity.

The IC_{50} value *in vitro* for the enzymatic activity of methylmercury

Table 2. Effects of single administration of methylmercury on ^{3}H-PDBu binding

	Control	Methylmercury
	(pmol/mg protein)	
cerebrum	42.0 ± 0.2	37.8 ± 1.0
cerebellum	12.0 ± 2.0	14.1 ± 1.0
thymus	14.4 ± 2.0	10.8 ± 1.1
spleen	5.4 ± 1.5	7.4 ± 0.8
small intestine	4.3 ± 0.3	3.5 ± 0.4
stomach	3.1 ± 0.7	3.0 ± 0.9
lung	2.4 ± 0.1	2.6 ± 0.2
kidney	1.5 ± 0.1	1.1 ± 0.1
heart	1.3 ± 0.3	1.2 ± 0.1
liver	4.8 ± 0.5	4.2 ± 0.2

Each value is the mean ± S.E. of 8 animals.

was reported to be 1.3 x 10^{-6} M (Inoue *et al.*, 1988), which corresponds to 0.26 ppm. Although the tissue concentration does not represent the intracellular free concentration, it seems possible that methylmercury accumulated in the nervous tissues, thereby affecting protein kinase C.

In order to determine the effect of methylmercury accumulation in tissues, ^{3}H-PDBu binding of protein kinase C was examined. Specific ^{3}H-PDBu binding activity has been found in all the mouse tissues listed in Table 2 and 3. The neural tissues contained the highest concentrations of ^{3}H-PDBu binding, followed by the spleen and thymus. ^{3}H-PDBu binding in the lung, heart, kidneys and liver was low. The Kd values were almost the same among the tissues (Table 3). Distribution of ^{3}H-PDBu binding was in accordance with that obtained by other authors (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981; Ashendel *et al.*, 1983).

Although, even after a single dose, mercury concentrations in all tissues determined seemed to be sufficient to inhibit the enzymatic activity of protein kinase C, methylmercury still did not affect ^{3}H-PDBu binding in any tissues (Table 2). Even after repeated administrations, neither the binding affinity (Kd) nor the number (Bmax) of protein kinase C labelled with ^{3}H-PDBu changed significantly in any of the tissues evaluated (Table 3). What remains controversial is whether sensitivity to methylmercury is different among any neuronal pathways. For example, the presynaptic mechanism of cholinergic neuron was more labile to methylmercury than that of dopaminergic neuron *in vitro* (Saijoh *et al.*, 1987 a;b). On the other hand, the level and turnover rate of acetylcholine was decreased in mice which were administered with 5 mg/Kg/day x 14 (Kobayashi *et al.*, 1981). Mature dopaminergic pathways seem to be not particularly sensitive to methylmercury (Komulainen and Tuomisto, 1985), although the turnover rate of dopamine is said to be impaired in rats after by gavage administration of 10 mg/Kg of methylmercury x 5 (Sharma *et al.*, 1982). Thus, the findings that methylmercury induced no alteration in the level of protein kinase C

Table 3. Effects of repeated administrations of methylmercury on ^{3}H-PDBu binding

	Control		Methylmercury	
	Kd (nM)	Bmax (pmol/mg protein)	Kd (nM)	Bmax (pmol/mg protein)
cerebrum	18.9 ± 3.2	93.0 ± 6.9	17.9 ± 1.4	91.6 ± 3.8
cerebellum	17.6 ± 1.2	28.7 ± 2.3	17.6 ± 0.9	34.4 ± 2.4
thymus	23.0 ± 2.7	19.7 ± 1.7	23.8 ± 2.1	20.3 ± 1.7
spleen	19.5 ± 1.4	14.3 ± 1.7	20.0 ± 1.4	18.8 ± 1.8
small intestine	20.4 ± 3.9	12.9 ± 4.1	21.0 ± 2.1	9.6 ± 0.8
stomach	18.5 ± 3.0	6.6 ± 1.1	19.9 ± 2.6	7.5 ± 0.8
lung	21.0 ± 1.6	6.4 ± 1.0	24.4 ± 3.0	6.7 ± 0.7
kidney	23.0 ± 2.0	2.9 ± 0.4	23.5 ± 1.8	3.4 ± 0.4
heart	20.4 ± 1.7	2.0 ± 1.2	21.7 ± 2.5	3.4 ± 1.2
liver	22.5 ± 3.6	1.5 ± 0.2	23.5 ± 1.3	2.9 ± 1.0

Each value is the mean ± S.E. of 8 animals.

even after the neuronal symptoms were apparent by methylmercury administration, might explain the complexity in sensitivity of neuronal pathways to methylmercury, and *vice versa.*

Moreover, ^{3}H-PDBu binding remained unaffected in any of the peripheral tissues evaluated, including the kidney where methylmercury accumulated up to 77 ppm corresponding to approx. 4×10^{-4} M. Functional impairment in non-neuronal tissues is difficult to demonstrate with regard to methylmercury intoxications (Chang, 1977). The ineffectiveness of methylmercury on the number of protein kinase C might in part cause the symptoms to become silent.

Consequently, methylmercury did not markedly affect ^{3}H-PDBu binding in any of the tissues determined after repeated doses of administration provoked signs of neurotoxicity. This finding is taken to suggest that methylmercury had no apparent effects on the turnover of protein kinase C.

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