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COMMENTARY

MECHANISMS OF LEAD NEUROTOXICITY

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In the central nervous system, lead toxicity is more common in children than adults and may produce either overt symptoms of acute encephalopathy such as ataxia, headache, convulsions, and coma or lesser deficits including learning disorders and hyperactive behavior [1, 2]. The symptoms of acute encephalopathy usually evolve over several days and are associated with blood levels of lead in excess of 4 μM. This degree of lead poisoning has become uncommon in recent years, probably as a result of screening and treatment of children at risk. Lower levels of lead poisoning produce more subtle neurologic dysfunction. Thus, several recent clinical studies found a significant correlation between lead exposure and learning and behavioral deficits in children [2–4]. No threshold for toxicity is apparent, suggesting that blood levels of lead currently prevalent in industrialized societies (0.2 to 1.5 μM) and previously thought to be safe may signify some degree of risk for brain injury.

The focus of this review is on the interaction between calcium and lead. We will emphasize studies that relate to the neurologic deficits observed in children poisoned by lead. Several recent reviews consider other aspects of lead toxicity [5–7].

Lead and neurotransmitter release

Many of the biological aberrations produced by lead appear related to the ability of this heavy metal to either inhibit or mimic the action of calcium. In the nervous system, calcium ions play a special role in the release of neurotransmitters from presynaptic nerve endings. Many investigators have focused on this aspect of function in describing the neurotoxic actions of lead. Experimental systems have varied from neuromuscular junction and cervical ganglia preparations to suspensions of nerve endings (synaptosomes) isolated from brain. In some experiments, the animals were exposed to lead prior to tissue preparation while in others the tissue under study was suspended in or superfused with a buffer containing lead. Results are surprisingly consistent considering the variability in source of tissue and experimental design.

At low concentrations, lead enhances the spontaneous or basal release of neurotransmitter from presynaptic nerve endings. In frog neuromuscular preparations, lead at concentrations between 5 and 50 μM increased the frequency of miniature end plate potentials (MEPPs). Exposure to lead did not alter the presynaptic potential, nor did it affect the end plate potential after direct stimulation [8, 9]. The authors concluded that lead acted directly on the release process. A similar lead-mediated increase in the frequency of MEPPs occurred in rat phrenic nerve-diaphragm preparations after a latency of 15 min [10]. This stimulation was not affected by the omission of calcium from the bath or the blockage of calcium channels by manganese. It appeared that lead either mobilized intracellular calcium stores or served as a calcium substitute. Exposure to lead enhanced basal release of neurotransmitters from cat sympathetic ganglia [11].

Lead-induced release of neurotransmitters also occurs in the central nervous system. In superfused synaptosomes prepared from rat brain, Minnema and co-workers found that micromolar concentrations of lead increased spontaneous release of dopamine [12], acetylcholine [13], and γ-aminobutyric acid [14]. In each case, the latency for activating neurotransmitter release was 15–30 sec, suggesting an intracellular site of action for lead. Lowering the concentration of calcium in the medium augmented the ability of lead to increase spontaneous release of dopamine. This suggests that external calcium is not necessary for the effect and may compete with lead for entry into the cell. In the hippocampal synaptosomes, lead increased the efflux of calcium-45 with the same kinetics that it increased acetylcholine release [13]. The enhanced calcium efflux may be explained by stimulation of a plasma membrane efflux pump or the mobilization of intracellular calcium stores.

In addition to enhancing the spontaneous release of neurotransmitters, lead blocks the release of neurotransmitters normally produced by depolarization of nerve endings. In neuromuscular preparations, this results in a diminished excitatory end plate potential [9]. Using isolated adrenal medullary cells, Pocock and Simons [15] found that lead inhibited the release of epinephrine and norepinephrine evoked by either potassium or cholinergic agonists. Lead appeared to block voltage-dependent calcium channels since the influx of calcium-45 after depolarization with potassium or activation by cholinergic agents was reduced markedly. Furthermore, omission of calcium from the medium produced an inhibition of neurotransmitter release similar to that found after exposure to lead.

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Lead inhibition of evoked release of neurotransmitters also occurs in the central nervous system. The latency for blockade is very short and consistent with a site of action on the surface of the cell. Brain cerebral cortical minces derived from lead-fed mice released less acetylcholine after potassium-evoked stimulation than nontreated animals [16]. Similarly, synaptosomes prepared from cerebral cortex of rats exposed to lead exhibited a smaller uptake of choline and a diminished potassium-evoked release of acetylcholine [16], while synaptosomes isolated from the striatum had a diminished evoked release of dopamine [17]. Evoked release of these amino acid neurotransmitters requires the presence of extracellular calcium, and lead appears to competitively block the voltage-sensitive calcium channel.

The biphasic response of neurotransmitter release to lead with stimulation of the basal rate and inhibition of the depolarization-induced fraction may have special relevance to the immature nervous system. The continuous release of subthreshold amounts of neurotransmitter into the synaptic cleft is thought to have a trophic influence on maintaining the efficiency of a synaptic connection and the survival of the postsynaptic cell. Depolarization-induced release of presynaptic neurotransmitter, on the other hand, is responsible for producing the signal summation that excites (or inhibits) the postsynaptic cell. The trophic and functional events that these two types of activity produce in neural networks are particularly important during development [18].

The number of synaptic connections established during the first several years of life greatly exceeds the number that are present in the adult brain [19]. Pruning of excess synapses begins early in life and appears to be influenced by the experiences of the infant as represented by the amount and pattern of activity in specific neural networks [20, 21]. The precision of this relationship between experience and neuronal activity would be disrupted if lead increases basal neurotransmitter release and decreases the response of activated circuits in children. Since the pattern of neuronal activity appears to drive the pruning process, exposure to lead early in life may have a lasting adverse effect upon synaptic anatomy and brain function [22]. Such changes may underlie the effects of low level lead exposure upon the learning skills and behavior of young children in the absence of overt pathologic damage.

Lead and calcium metabolism

We have discussed studies which suggest that lead modulates neurotransmitter release by altering calcium metabolism—either by competing with calcium for entry into the cell or by increasing intracellular calcium levels. Lead also modulates calcium metabolism in nonexcitable tissue. A marked accumulation of calcium occurs when isolated or cultured cells are exposed to lead. This response is found in a number of different cell and tissue types including isolated rat brain microvessels [23], arterial smooth muscle [24], rat hepatocytes [25] and mouse bone cell cultures [26]. In rat brain capillaries, the increase in calcium-45 accumulation was attributed to an inhibition of the active efflux pump as opposed to a stimulation of uptake [23]. This inhibition was not due to ATP depletion or a nonspecific membrane perturbation since potassium uptake in lead-treated microvessels was unimpaired. In rat hepatocytes, the highest levels of calcium-45 were found in the mitochondria although increases were also observed in other cellular compartments [25]. Isotopic analysis is sometimes difficult to interpret because of multiple calcium pools. This problem was circumvented in a recent report by measuring calcium directly with ^{19}F nuclear magnetic resonance. In a cultured osteoblastic cell line, exposure to 25 μM lead increased the free concentration of calcium by 120% [27].

Lead entry into cells

The mechanism of lead uptake has been studied in both excitable and non-excitable cells. Simons and Pocock [28] suggest that lead enters adrenal medullary cells through calcium channels. They found that potassium, carbachol, and veratridine stimulated both calcium and lead uptake. Calcium acted as a competitive inhibitor of lead uptake. The K_m for calcium uptake was similar to the K_i for inhibition of lead uptake by calcium. Further, lead uptake was blocked by the calcium channel blockers D-600 and nifedipine. In keeping with a role for the calcium channel, lead uptake was enhanced by the channel agonist Bay K 8644. Inhibitors of sodium, potassium or anion transport were ineffective in these epithelial cells. By contrast, inhibitors of anion transport block lead uptake in erythrocytes [29]. Thus, the entry of lead into epithelial and mesenchymal cells appears to occur by different mechanisms.

Effects of lead on calcium channels

There is an approximately 10,000-fold difference between the concentration of calcium in the extracellular fluid and the cytosol of most cells. This gradient is maintained by the limited passive permeability of the plasma membrane for calcium entry combined with active transport mechanisms that move calcium either out of the cell or into specific organelles. Neural tissues and transporting epithelium have voltage-dependent calcium channels regulating calcium entry through the plasma membrane. In the brains of rats treated with lead, an increase in the number of nitrendipine (a voltage-dependent calcium antagonist) binding sites was observed [30]. In addition, lead was more effective than calcium in increasing nitrendipine binding sites in synaptosomes exposed to the metals *in vitro*.

In adrenal medullary cells, calcium and lead regulate the activity of the calcium channel, although the nature of the regulatory activity exerted by these metals appears to differ [28]. In the presence of 1 mM $CaCl_2$, evoked uptake of calcium reached a maximum within a relatively short time and declined more than 50% in 10 min. In contrast, evoked uptake of lead continued for more than 45 min without a measurable decline. The results suggest that lead is a less effective regulator of channel closure which

may explain, in part, the higher permeability of these cells to lead.

Proteins that bind lead

Given the preferential incorporation of lead into growing bone and its ability to use the absorption and transport systems that normally distribute calcium throughout the body [31], it is not surprising that lead can substitute for calcium as an intracellular second messenger. The best studied example concerns the ability of lead to bind and activate calmodulin.

Calmodulin has several binding sites for calcium. After their occupancy, the tertiary configuration of calmodulin is changed so that it can interact with a number of enzymes and transporters [32, 33]. For example, calcium–calmodulin stimulates specific protein kinases, cyclic AMP phosphodiesterase, and a calcium pump that decreases the concentration of calcium in the cytosol and terminates the activation of calmodulin. In red blood cells, activation of calmodulin by calcium causes the opening of potassium channels and a marked efflux of this ion [34, 35].

Calmodulin exhibits a higher affinity for lead than it does for calcium [36, 37]. Lead can substitute for calcium in at least two calmodulin-dependent processes—activating a calmodulin-dependent phosphodiesterase, and the opening of potassium channels in red blood cells [38]. The ability of lead to interact with calmodulin and other calcium binding sites may relate to the similarity of the hydrated radius of these two divalent cations [39]. In this regard, lead and calcium are closer in radius than other divalent cations. This may explain why barium, cobalt, mercury, arsenic and other heavy metals do not act as calcium agonists and have a different pattern of tissue toxicity.

Lead and protein kinases

Protein kinases regulate a number of cellular events. One family of protein kinases is activated by calcium–calmodulin [40]. A member of this family, calmodulin protein kinase II, is highly enriched in neural tissue [41] and is proposed to have a role in the release of neurotransmitters. The state of phosphorylation of a synaptic vesicle protein, synapsin I, is increased by a variety of agents which regulate neurotransmitter release [42]. Many of these agents activate the calmodulin-dependent kinase. In a model proposed by Greengard and colleagues, synapsin I separates from the synaptic vesicle after it is phosphorylated and the vesicle is then more likely to fuse with the plasma membrane and release neurotransmitters [42]. If activation of the calmodulin-dependent protein kinase by lead results in phosphorylation of synapsin I, this sequence may explain how lead increases the basal rate of neurotransmitter release.

Protein kinase C is activated by second messengers formed by the hydrolysis of inositol phospholipids [43]. In the phosphatidylinositol transduction system, ligand receptor binding activates phospholipase C and liberates inositol triphosphate and diacylglycerol. The inositol triphosphate causes a transient elevation in the cytosolic concentration of calcium by releasing calcium from intracellular stores. Diacylglycerol in the presence of calcium activates protein kinase C. The protein substrates of protein kinase C play important regulatory roles in cell function including control of cell proliferation and differentiation.

In the nervous system, protein kinase C appears to regulate long-term potentiation since activators of the enzyme enhance [44] and inhibitors block [45] this process. Since long-term potentiation may be the functional equivalent of memory storage, interference by lead could relate to the learning and behavioral deficits found in poisoned children. Lead can substitute for calcium in the activation of protein kinase C [46]. In fact, lead exhibited a much higher affinity for the enzyme than calcium. Lead activation did not change the requirements for diacylglycerol. The kinetics of protein kinase C activation by lead were similar to that of calcium, suggesting that these two metals interact with protein kinase C in a similar fashion.

Although lead can interact with several arms of the signal transduction system (phosphodiesterases, kinases) in cell-free preparations, it is important to determine whether lead is active in the intact cell. Earlier we discussed the effects of lead upon neurotransmitter release. It appears that the opening of voltage-sensitive calcium channels is blocked by lead causing inhibition of evoked neurotransmitter release. Inside the nerve terminal, lead may act as a calcium agonist and induce spontaneous release of neurotransmitters. There have also been reports on the effects of lead on signal transduction mechanisms in nonexcitable tissue. For example, in rat hepatocytes, pyruvate kinase activity is inhibited after glucagon or epinephrine stimulation although the enzyme activity eventually returns to basal levels. Lead was found to retard the return to basal levels if the inhibition was mediated by epinephrine but not glucagon [47]. At the time this work was completed, it was believed that epinephrine-mediated events were calcium dependent and glucagon events were cAMP dependent [48]. The argument was raised that lead interfered with calcium-mediated events. Presently, these differences are less distinct. Epinephrine modulates cAMP levels in certain systems [49, 50], and glucagon can augment calcium levels in hepatocytes [51]. Therefore, the ability of lead to interfere with epinephrine-mediated events in hepatocytes may not be explained simply by the type of second messenger which is generated. On the other hand, the ability of lead to interfere with second messenger dependent events appears to be dependent, at least in part, on the nature of the primary signal.

Blood–brain barrier

The major pathologic finding in acute lead encephalopathy is brain edema [52–54]. The swelling is predominately in the interstitial fluid space and indicates a breakdown in the function of brain microvessels. Under normal circumstances, the endothelial cells in brain microvessels are circumferentially sealed together by continuous tight junctions. The tight junctions are of the complex type and consist of multiple interlocking bands and grooves [55]. This arrangement produces a cellular

layer of endothelial cells that restricts the passage of most polar molecules between the blood stream and the interstitial fluid of the brain. The blood–brain barrier excludes plasma proteins, most organic molecules, and limits even the passage of ions such as sodium and potassium [56]. Further, certain ions, organic acids, and neuroactive amino acids are pumped from brain to blood across the endothelium [57]. This permeability barrier differs from that present in other microvascular beds where ions and small organic molecules readily diffuse into tissue spaces and albumin traffics across the endothlium to be removed by the lymphatic circulation. In the brain, essential molecules such as D-glucose and large neutral amino acids cross the endothelium by means of stereospecific transporters present in the plasma membrane of the endothelial cell. In other organs, these substrates appear to diffuse in a non-selective manner between the endothelial cells into the tissue spaces.

High dose exposure to lead (i.e. blood levels in excess of 4 μM) disrupts the blood–brain barrier. Molecules such as albumin that normally are excluded, freely enter the brain of immature animals exposed to these concentrations of lead [52, 53]. Ions and water follow and edema is produced. Since the brain does not have a well-developed lymphatic system, the clearance of plasma constituents is slow and dependent upon the flow of interstitial fluid into the cerebrospinal fluid. Intracranial pressure rises as edema accumulates in the brain because of the physical restraint of the skull. When the intracranial pressure approaches the systemic blood pressure, cerebral perfusion decreases and brain ischemia occurs.

Two factors are important in considering the mechanisms underlying acute lead toxicity in the brain microvasculature. First is the marked difference in vulnerability associated with maturity. Infants and toddlers are much more susceptible to acute encephalopathy than adults [3]. This age dependence is also found in experimental toxicity and is best demonstrated in the suckling rat [52, 53]. Second, when lead-poisoned rats are examined pathologically, little in the way of overt endothelial injury is seen despite the major disruption of barrier function and the presence of large amounts of brain edema [52, 53]. This finding suggests a functional change in the state of the endothelium rather than cell necrosis. One interpretation is that the endothelial cells lose their brain specific differentiation and revert to a more systemic type of permeability which no longer restricts the movement of plasma into the brain.

The signals that initiate and maintain the expression of the blood–brain barrier phenotype by brain endothelial cells appear to originate in the astrocytes [56, 58]. Foot processes extend from astrocytes and almost completely ensheathe the microvascular wall. Several lines of evidence implicate the astrocyte in the induction of the blood–brain barrier. Interestingly, astrocytes appear particularly vulnerable to the toxic effects of lead [59, 60]. Injury to astrocytes, therefore, may play a role in the loss of the special barrier properties normally expressed by brain endothelial cells.

Another possibility is a direct effect of lead upon intracellular second messenger activity in the endothelial cells. Protein kinase C may be involved in the differentiation of brain endothelial cells. When assayed in immature microvessels isolated from rat brain, almost all of the activity of protein kinase C is present in the cytosol [61]. In contrast, the enzyme is membrane bound when assayed in microvessels isolated from the brain of adult rats. In other systems, this change in distribution from cytosol to membrane is associated with activation of the enzyme [62, 63]. Exposure of microvessels isolated from brain of immature rats to lead results in an apparent translocation of protein kinase C from the cytosol to the membrane [64]. This activation of protein kinase C may be mediated by the ability of lead to mimic or mobilize calcium. If a similar premature activation of this enzyme occurs *in vivo*, the sequence of proliferation and differentiation of the brain endothelial cells may be disrupted and could explain in part the defects in barrier function that occur in acute lead encephalopathy.

Summary

During the past several years, there has been a renewed interest in the mechanisms by which lead poisoning disrupts brain function. In part, this is related to clinical observations that imply an absence of threshold for toxicity in the immature brain.

Many of the neurotoxic effects of lead appear related to the ability of lead to mimic or in some cases inhibit the action of calcium as a regulator of cell function. At a neuronal level, exposure to lead alters the release of neurotransmitter from presynaptic nerve endings. Spontaneous release is enhanced and evoked release is inhibited. The former may be due to activation of protein kinases in the nerve endings and the latter to blockade of voltage-dependent calcium channels. This disruption of neuronal activity may, in turn, alter the developmental processes of synapse formation and result in a less efficient brain with cognitive deficits.

Brain homeostatic mechanisms are disrupted by exposure to higher levels of lead. The final pathway appears to be a breakdown in the blood–brain barrier. Again, the ability of lead to mimic or mobilize calcium and activate protein kinases may alter the behavior of endothelial cells in immature brain and disrupt the barrier. In addition to a direct toxic effect upon the endothelial cells, lead may alter indirectly the microvasculature by damaging the astrocytes that provide signals for the maintenance of blood–brain barrier integrity.

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