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**c-fos and ornithine decarboxylase gene expression in brain as early markers of neurotoxicity**

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An increase of proto-oncogene c-fos expression in cerebral cortex of rats treated with subconvulsant doses of the pesticide organochlorine lindane (γ-hexachlorocyclohexane) has been detected using Northern blots. Immunohistochemical studies show that Fos protein was already increased in neuronal nuclei 3 h after treatment. The administration of the benzodiazepine diazepam prior to lindane totally blocked the activation of this proto-oncogene expression. Parallel to this increased expression of c-fos an activation of the ornithine decarboxylase (ODC) gene and enzyme was also observed. High levels of ODC mRNA and increased enzyme activity in cortex were found in rats following lindane treatment. These changes were attenuated by prior treatment of animals with diazepam. The co-induction of c-fos and ODC suggests a potential link between the ODC/polyamine system and the short-acting proto-oncogenes in stimulus-transcription coupling events.

**INTRODUCTION**

Lindane (γ-hexachlorocyclohexane) is a potent and widely used organochlorine pesticide employed in veterinary and human medicine as an ectoparasiticide. The compound is known to be able to cause hyperexcitability, tremors and convulsions in humans and other mammals. Although it is uncertain by what mechanism lindane effects excitation, enhanced synaptic release of neurotransmitters may be involved. It has been proposed that an increased level of intracellular calcium underlies this effect. Low level exposures (10⁻⁶–10⁻⁷ M) to lindane increased ⁴⁰Ca uptake into isolated rat brain synaptosomes. Direct measurement of free intrasynaptosomal Ca²⁺ revealed that lindane increases [Ca²⁺], in a dose-dependent manner. In other studies, lindane and the α and β isomers of hexachlorocyclohexane have been reported to produce both time- and dose-related increases in free intracellular calcium in a neurohybridoma cell line. Lindane may thus exert its neurotoxic effects in part by affecting calcium homeostasis in neurons.

Increases in levels of intracellular free calcium can also bring about rapid and profound changes in numerous cellular processes like protein phosphorylation and gene transcription. One of the earliest transcriptionally activated genes is the fos proto-oncogene. c-fos, together with other genes, is rapidly induced after cell activation, and is thought to take part in the early cellular response to a wide range of stimuli. Such genes may function as third messengers within an intracellular cascade linking extracellular stimuli to long-term adaptive processes. Some of these so-called early genes are components of known transcription regulation complexes and thus may be directly responsible for alterations in the expression of other target genes. A model for such a cascade has been proposed by Morgan and Curran.

Ornithine decarboxylase (ODC) catalyses the first and rate-limiting step in the synthesis of polyamines in animal cells. Polymamines are believed to be essential for DNA replication and cell proliferation. ODC is rapidly induced in many tissues by a variety of stimuli. The stimulation of neural tissue can induce this enzyme. The protein has an unusually short half-life (10–30 min) in higher organisms and its activity is subject to multiple regulatory controls. It can undergo drastic and transient elevation in activity. It is known that a common feature of lesions of the nervous system is the rapid induction of ODC mRNA and enzyme activity. Furthermore, ODC activity has also been detected at various times following the initiation of seizures. The ODC/polyamine system and the nuclear proto-oncogenes thus share some similar properties.

In a previous study electroconvulsive shock (ECS) was...
shown to result in the induction of ODC mRNA and enzyme activity as well as c-fos mRNA. In this communication we report that lindane treatment of rats results in induction of cortical c-fos mRNA followed by accumulation of Fos protein, and also in the induction of ODC mRNA and enzyme activity. The rapid induction of c-fos and ODC following neural insult precedes major morphological changes and thus they may be considered as early markers of response to neurotoxic damage.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats weighing 250–270 g were used for all experiments. Animals were kept under standardized conditions with a 12-h dark/12-h light schedule. They were given free access to food and water.

Four different treatment groups were utilized: (1) animals orally gavaged with lindane at 30 mg/kg (convulsant dose) dissolved in pure olive oil in a dose volume of 1 ml/kg; (2) animals that were orally gavaged with olive oil alone (control animals); (3) animals injected with diazepam (i.p., 20 mg/kg, 5 x SSC, 5 x Denhardt’s solution (1 x contains 0.25% SDS at room temperature for 40 min. Stringent washings were in 0.1 x SSC, 0.1% SDS at 45 °C for 30 min. The filters were exposed to AGFA CURIX RP 2 film with an intensifying screen at ~80 °C.

Northern blot analysis

All solutions contained 0.1% diethylpyrocarbonate to inhibit RNA degradation by ribonucleases during the extraction procedure. Frozen tissues were homogenized in a 3 M LiCl, 6 M urea plus 0.2% SDS solution. The homogenates were kept on ice for 4 h and RNA was then extracted by a modification of the LiCl and urea phoresis through 1.2% agarose/2% formaldehyde gels stained with UV-light source for 5 min to cross-link the RNA. They were then rinsed in 2 x SSC and air dried for 1 h and washed with PBS (0.1 M, pH 7.2). Sections were then incubated in a buffer containing 10 mM Tris-HCl, 5 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 7.4 to reduce background staining. The immunological reactions were carried out using the peroxidase–antiperoxidase (PAP) method.

RESULTS

In an earlier study, we reported increased c-fos expression after ECS-induced seizures. In this work, we have used lindane as a paradigm for c-fos induction.
Subconvulsant doses of lindane (30 mg/kg) significantly increased c-fos mRNA levels 1 h after treatment when compared to control values (Fig. 1A). Ethidium bromide staining confirmed that all lanes were equally loaded with mRNA (Fig. 1B). While administration of lindane resulted in increased expression of c-fos mRNA, there were no apparent differences between the oil-treated group (control) and those treated with diazepam (10 mg/kg, i.p.) 5 min prior to lindane administration. No induction was seen when diazepam was given before gavaging with the oil vehicle. The induction elicited by PTZ was of the same magnitude as that of lindane. Its effect was also blocked by diazepam (Fig. 1A).

ODC mRNA expression in the different treatment groups is presented in Fig. 2. In a previous study we had determined time courses for ODC and c-fos mRNA following ECS. ODC mRNA increased until 5 h post-stimulus where it reached a plateau. In the present study we have chosen this time to study ODC mRNA levels, which were also elevated after treatment with lindane (Fig. 2). Basal levels of ODC mRNA were found in those animals pretreated with diazepam. Variations in ODC mRNA levels correlated with changes in ODC enzyme activity (Fig. 3). Levels of ODC activity increased 2.8-fold 5 h after lindane treatment. However this increase was only 1.6-fold in rats treated with diazepam before lindane (Fig. 3).

Immunohistochemical studies were carried out to evaluate any changes in Fos protein levels and distribution in the cortex. Although Fos-positive neurons were observed throughout the brain 1 h after treatment with lindane (data not shown), we preferred to sacrifice the animals 3 h postadministration because by that time essentially all neurons in the cortex were labeled. Fig. 4 shows a sequence of images corresponding to the cingulate cortex of brains from the different treatment groups. The immunostaining was highly specific, being abolished when the antibody was either omitted or pre-absorbed with the Fos peptide. There was intense staining of cell nuclei in those animals treated with lindane, with far weaker staining in the cytoplasm (Fig. 4a–c). The nucleolus did not appear to be stained. This pattern was also true for other brain areas such as different cortical
areas, the hippocampus and the amygdala (data not shown). Animals treated with the oil vehicle showed no nuclear staining (Fig. 4e). This result was consistent with the lack of induction of c-fos mRNA seen in the Northern blots. The administration of diazepam before treatment with lindane decreased protein levels to basal values (Fig. 4d).

**DISCUSSION**

Lindane treatment has been shown to elevate the expression of proto-oncogene c-fos. This effect has been described for other seizure-eliciting agents like PTZ or ECS. Under the conditions described here this induction occurred in the absence of convulsions. Nevertheless, we do not exclude that undetectable seizures might be taking place. Diazepam completely blocked the induction elicited by lindane although the administration of diazepam alone did not modify the level of expression of c-fos. Administration of lindane solvent (olive oil) alone also did not induce c-fos. Thus, we infer that the increased levels of c-fos mRNA that we find are attributable to the action of lindane.

Fos protein displayed a similar induction pattern to that already observed for the mRNA. There was an intense nuclear staining involving only neural cells. In all Fos-positive cells, the nucleoli appeared as immunonegative translucent vacuoles. There was also no staining in the perinucleolar heterochromatin. Fos-like immunoreactivity has been reported to be exclusively associated with dispersed chromatin (euchromatin), where all actively transcribing DNA sequences are thought to
The fact that diazepam counteracts the convulsant effects of zodiazepines are neuropharmacological agents that have the GABA receptor-chloride ionophore complex. The CNS 18 ·19 ·26 . The anti-convulsant effects via their agonistic interaction with the GABA receptor-chloride ionophore complex 32 . Lindane seems to act on the picrotoxin component of this receptor which may subsequently interfere with the inhibitory neuro-physiological action of GABA in opening the chloride channel14 . Since GABA is the principal inhibitory neurotransmitter in the mammalian brain, lindane might exert its convulsant effects through ‘inhibition of inhibition’, leading to increased activation of neurons. A similar pattern of action has been proposed for another convulsant, PTZ, widely used as experimental paradigm to study the activation of proto-oncogene c-fos in the CNS18 ·19 ·26 . The effects of both lindane and PTZ are abolished by the administration of diazepam. The benzodiazepines are neuropharmacological agents that have anti-convulsant effects via their agonistic interaction with the GABA receptor-chloride ionophore complex. The fact that diazepam counteracts the convulsant effects of lindane and PTZ may indicate that the effects of these agents all center upon the GABA receptor complex.

It is still unclear how these convulsants bring about changes in early gene expression. Several studies with PTZ have reported the coincidence of the distributions of Fos-like immunostaining and the N-methyl-D-aspartate (NMDA) type of glutamate receptor in the CNS15 ·17 ·26 . ODC expression after ECS is also partially inhibited by MK-801, an NMDA receptor antagonist34 . A partial inhibition by MK-801 has also been described for c-fos induction after PTZ26 . Although the NMDA receptor may only constitute a portion of the pathway of early gene activation, the remarkable resemblance that its distribution in the brain bears with that of Fos suggests its participation in triggering the nuclear event18 . This type of glutamate receptor can gate calcium entry into the cell. Thus, calcium could play the role of the common intracellular mediator of transcriptional activation6 ·7 . Studies conducted with calcium channel agonists and antagonists demonstrate the importance of calcium in the onset of early gene transcription16 ·17 . As already mentioned, lindane increases calcium uptake into neurons, their synaptosomes and possibly other cells3 ·11 . Furthermore, influx of calcium through cell membrane channels has been linked to glutamate neurotoxicity5.

A neurotoxic agent has here been shown to activate both the proto-oncogene c-fos and the ODC/polyamine system. Although such activation is not temporally coincident, this suggests that ODC may be regulated by a gene comprising part of the early response of the cell to an external stimulus. The polyamines may modulate the expression of proto-oncogene target genes as well as having their own target sites. Both systems may be part of the initially activated cellular machinery implicated in the coupled stimulus-transcription cascade that is ultimately responsible for long-term adaptive changes.

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