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Non-involvement of Glycolytic Enzymes in 2,5-Hexanedione induced Neurotoxicity - in vitro Studies

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Abstract

n-Hexane and methyl n-butyl ketone (MnBK) are industrial solvents which are metabolized in vivo to a neurotoxic γ -diketone, namely, 2,5-hexanedione. 2,5-Hexanedione is known to cause peripheral neuropathy in humans following exposure. The molecular mechanisms underlying 2,5-hexanedione induced neuropathy has been the subject of considerable investigation. Inhibition of glycolytic enzymes, in the brain and sciatic nerve following 2,5-hexanedione exposure have been proposed as a possible mechanism of action. Present study was undertaken to reinvestigate the molecular mechanisms of 2,5-hexanedione induced neurotoxicity using whole brain homogenate and sagittal slices of rat brain as an in vitro model. Glycolytic enzymes namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase were inhibited by 2,5-hexanedione at high concentration, However, the non-neurotoxic structurally related ketones, namely, acetone and methyl ethyl ketone also showed similar effects. These observations indicate that inhibition of GAPDH and enolase may not be involved in 2,5-hexanedione induced neuropathy.

Key words -

Hexanedione,
Glyceraldehyde 3-phosphate dehydrogenase,
Enolase,
Brain slices,
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Methyl ethyl ketone
2,5-Hexanedione,
Glyceraldehyde 3-phosphate dehydrogenase,
Enolase,
Brain slices,
Acetone,
Methyl ethyl ketone

Man is exposed to industrial and commercial solvents like n-hexane and methyl n-butyl ketone. Occupational exposure to these solvents causes peripheral neuropathy in humans [1], [2]. The γ -diketone, namely 2,5-hexanedione has been identified as the toxic metabolite of the ketones involved in dying back neuropathy [3]. Exposure of the γ -diketone to experimental animals resulted in the accumulation of neurofilaments both in central and peripheral nervous system [4],

[5]. Inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was proposed as a possible mechanism of action in 2,5-hexanedione induced neuropathy [6]. 2,5-Hexanedione and methyl butyl ketone have been shown to inhibit GAPDH and enolase in rat brain homogenate [7], [8], [9].

The present study was undertaken to reinvestigate effect of 2,5-hexanedione on various subcellular enzymes following incubation of the γ -diketone with rat brain homogenate and sagittal slices of rat brain. The microenvironment is better maintained in slices than in homogenate or isolated cells or in culture and hence, could be used as a model system for in vitro evaluation of neurotoxicants [10]. The effect of structurally related non-neurotoxic ketones namely, acetone and methyl ethyl ketone on brain slices and homogenate was also studied simultaneously, in order to determine the biochemical effects that were specific to the neurotoxic action of 2,5-hexanedione.

Materials and Methods

Wistar rats (3-4 months old, 200 gms) were used for all experiments. The animals had access to food (pelleted diet, Hindustan Lever Ltd., Bombay) and water ad libitum. Animals were anaesthetized with ether, decapitated, the brain quickly removed and homogenized in 0.32M sucrose (5% w/v). Homogenate was centrifuged at 1000g for 10 minutes. Supernatant was incubated with 2,5-hexanedione, acetone and methyl ethyl ketone at various concentrations for 20 minutes at 37° C. At the end of the incubation period, glycolytic enzymes namely GAPDH and enolase [9]; and lysosomal enzymes, acid phosphatase [11] and N-acetyl glycosaminidase [12] were assayed in the homogenate. Control incubations containing no neurotoxin were carried out simultaneously. All solvents were freshly distilled prior to use.

Sagittal slices of rat brain (400-500μ m thickness) were prepared using an indigenous slicer, quickly after removing brain from the animal. The slices were incubated in well oxygenated artificial CSF [13], made up to pH 7.4 by bubbling 5% carbondioxide. The slices were incubated in an atmosphere of 100% oxygen at 37° C in a shaking water bath. 2,5-Hexanedione (100mM) and acetone (100mM) were dissolved in artificial CSF and added to slices at the beginning of the experiment. At the end of 1 hour, incubations were placed in ice water bath. Slices were rinsed, blotted and homogenised in 0.32 M sucrose (5% w/v). Enzyme assays were performed after removing the cell debris by centrifugation (1000g), as mentioned earlier [10]. In addition to glycolytic enzymes, lactate dehydrogenase [14], isocitrate dehydrogenase [15], cytochrome c oxidase [16], Na^+-K^+ATPase [17], β -glucuronidase [18] and other lysosomal enzymes were also estimated. Control slices were incubated simultaneously without the ketones. Protein was estimated by dye binding method [19].

Results

Rat brain homogenate was incubated with 2,5-hexanedione at various concentrations. In order to determine which of the biochemical effects were related to neurotoxicity of 2,5-hexanedione, incubations were carried out with structurally related, non-neurotoxic ketones, namely, acetone and methyl ethyl ketone. Control incubations were carried out simultaneously in the absence of ketones.

A dose dependent decrease in GAPDH activity was seen in brain homogenate incubated with 2,5-hexanedione at various concentrations as shown in Table I. A similar dose dependent decrease of

the above enzyme was also observed with non-neurotoxic ketone, acetone, while methyl ethyl ketone inhibited GAPDH activity only at 100mM concentration. GAPDH was unaffected at lower concentrations (10mM or less) of the neurotoxicant 2,5-hexanedione and non-neurotoxic ketone.

Table I - Effect of 2,5-hexanedione, acetone and methyl ethyl ketone on glycolytic and lysosomal enzymes in rat brain homogenate

Table I - Effect of 2,5-hexanedione, acetone and methyl ethyl ketone on glycolytic and lysosomal enzymes in rat brain homogenate

Rat brain homogenate was incubated with various concentrations of 2,5-hexanedione, acetone and methyl ethyl ketone as indicated above at 37° C for 20 minutes. Glycolytic and lysosomal enzyme assays were performed after incubation. Controls were incubated simultaneously. Enzyme activities are expressed as per cent of control values.

ND=Not determined

Control values

Control values

Rat brain enolase was unaffected both with 2,5-hexanedione and non-neurotoxic ketones even at 100 mM concentration, when ketone to protein molar ratio was low (ranging from 0.2μ moles/mg brain tissue). However, a dose-dependent inhibition of enolase activity was observed (data not shown) at a high ketone to protein ratio (ranging from 60μ moles-120μ moles/mg brain tissue).

Lysosomal enzymes, namely, N-acetyl glucosaminidase and acid phosphatase were also unaffected by toxic as well as nontoxic ketones indicating that these enzymes may not be involved in 2,5-hexanedione-induced neuropathy.

Brain slices were incubated in artificial CSF containing neurotoxin 2,5-hexanedione. Control slices contained ACSF alone. Slices were also incubated, with acetone, a non-neurotoxic ketone.

The marginal inhibition of GAPDH activity following incubation of rat brain slices in 100 mM 2,5-hexanedione was observed (Table 2). The GAPDH activity was 85% of the control value in slices incubated with 2,5-hexanedione. However, acetone, a non-neurotoxic ketone also produced similar inhibition (78%) in GAPDH activity. Similarly, enolase was also inhibited by 2,5-hexanedione and acetone. Incubation of brain slices, in either 2,5-hexanedione or acetone had no effect on the lysosomal enzymes namely acid phosphatase and β -glucuronidase; lactate dehydrogenase (cytosolic enzyme) and Na^+-K^+ ATPase (membrane enzyme). Mitochondrial enzymes, namely, isocitrate dehydrogenase and cytochrome c oxidase were also unaffected in rat brain slices following incubation with 2,5-hexanedione or acetone.

Table II - Comparison of the effect of 2,5-hexanedione and acetone on enzyme activities in rat brain slices

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Brain slices were incubated in artificial CSF containing 100 mM 2,5-hexanedione or acetone for 1 hr at 37° C. Control incubations were carried out in artificial CSF alone. Enzyme activities are

expressed asμ moles of product formed hr^{-1} mg^{-1} protein. Values expressed as Mean ± S.D. (n=5 for lactate dehydrogenase and Na^{+} -K^{+} ATPase, for other enzymes n=10 -20)

Discussion

Present study demonstrates the inhibition of glyceraldehyde-3-phosphate dehydrogenase both with 2,5-hexanedione and non-neurotoxic ketones in rat brain homogenate and in brain slices. Since non-neurotoxic ketones also decrease GAPDH activity at similar doses, inhibition of GAPDH may not be involved in 2,5-hexanedione induced neuropathy. Earlier studies demonstrating inhibition of GAPDH in vitro [7], [9] had used a very high ratio of ketone to protein (0.8-4.0μ moles/mg brain tissue) and such high concentrations are not encountered in vivo in the nervous system, where conversion of hexane to 2,5-hexanedione is very slow [21]. Further, long preincubation of 2 hours with 2,5-hexanedione was required to inhibit GAPDH in rat brain homogenate [8]. Another study had used crystalline GAPDH to study the toxic effect of acetone [7], while the effect of acetone on tissue GAPDH was not examined. Hence, even though various studies have demonstrated the inhibition of GAPDH by 2,5-hexanedione, considering the slow rate of metabolism of n-hexane to 2,5-hexanedione, and the high concentrations of neurotoxin used in these studies, our results at low ketone to homogenate molar ratio may be of immense significance.

As GAPDH is known to exist in a single form only [7], the possibility of 2,5-hexanedione and methyl ethyl ketone inhibiting different forms of the enzyme is ruled out. Thus, both 2,5-hexanedione and methyl ethyl ketone are probably inhibiting of the one and only form of GAPDH.

In the present study, enolase was unaffected even at 100 mM concentration of 2,5-hexanedione in whole brain homogenate. However, 2,5-hexanedione inhibited activity of enolase when a very high molar ratio of ketone to protein was used (data not shown). Hence, only high concentrations of neurotoxin inhibits enolase and such concentrations are not found in vivo. Even though studies with brain slices showed decrement of enolase, acetone also produced similar effects, indicating that the inhibition of enolase may not be related to neurotoxic effect of 2,5-hexanedione.

Mitochondrial, membrane, lysosomal and cytosolic enzymes remain unaffected by 2,5-hexanedione even at 100 mM concentration, indicating that these enzymes are not involved in neurotoxicity of 2,5-hexanedione. Hence, the molecular mechanisms underlying the neural tissue damage by γ -diketones may be different from peripheral neuropathies caused by acrylamide, where inhibition of glycolytic and lysosomal enzymes is selective and takes place at lower concentration of acrylamide [9], [10]. Hence, 2,5-hexanedione induced neuropathy may be attributed to alterations in cytoskeletal proteins as indicated in certain recent studies [22], [23].

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