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Toluene-Induced Oxidative Stress in Several Brain Regions and Other Organs

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ABSTRACT

The in vivo dose–response relationship between toluene and reactive oxygen species (ROS) formation in rat brain, liver, kidney, and lung, and the time-course of these effects has been characterized. The rate of oxygen radical formation was measured using the probe 2′,7′-dichlorofluorescin diacetate. In vivo exposure to various doses of toluene (0.5, 1.0, and 1.5 g/kg ip) elicited a dose-dependent elevation of ROS generation within crude mitochondrial fractions obtained from rat lung and kidney, and within crude synaptosomal fractions from cerebellum. ROS formation in crude mitochondrial fractions from liver, and crude synaptosomal fractions from striatum and hippocampus, reached a maximum value at relatively low doses of toluene. Of the brain regions, the hippocampus had the highest induced levels of ROS. In vivo exposure to a single dose of toluene (1.5 g/kg ip), revealed that toluene-induced ROS reached a peak within 2 h, which correlated directly with measured toluene blood levels. This elevated oxidative activity was maintained throughout the next 24 h, even though blood values of toluene decreased to negligible amounts. These results demonstrate that exposure to toluene results in broad systemic elevation in the normal rate of oxygen radical generation, with such effects persisting in the tissues despite a rapid decline in

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toluene blood levels. Acute exposure to toluene may lead to extended ROS-related changes, and this may account for some of the clinical observations made in chronic toluene abusers.

Index Entries: Organic solvent; neurotoxicity; toluene; reactive oxygen species; central nervous system.

INTRODUCTION

Toluene is a widely used organic solvent. Human exposure to this agent may occur when toluene is in its pure form or when it is present as a component of paints, thinners, lacquers, and adhesives. In addition to the widespread industrial uses and potential for occupational exposures to toluene, this substance is a commonly abused solvent (Press and Done, 1967). In this work environment, toluene comes into contact with the skin and the respiratory tract primarily in the form of vapors or liquid. On the average, 50% of inhaled toluene vapor is absorbed by the lung (Cohr and Stokholm, 1979). Absorbed toluene is rapidly distributed through the blood to the tissues and organs.

A large number of epidemiological and experimental investigations of toluene have reported that exposure may produce signs and symptoms of nervous system dysfunction (Kelly, 1975; Boor and Hurtig, 1977; Malm and Lying, 1980; Fornazzari et al., 1983). Several neurological deficits have been described in experimental animals following inhalation or intraperitoneal injection, including modified levels of enzymes (Korpela and Tahti, 1988), lipids (Kyrklund et al., 1987), and specific neurotransmitter receptors (Yamawaki et al., 1982; Von Euler et al., 1988). Behavioral and electroencephalic changes have also been reported (Naaslund, 1986; Rees et al., 1989).

The mechanisms by which this organic solvent produces its effects are poorly understood. Recent studies have indicated that toluene neurotoxicity may be expressed through a membrane-associated event, such as, alteration of membrane fluidity and levels of intracellular Ca^{2+} and Ca^{2+}-regulated events (LeBel and Schatz, 1989, 1990; von Euler et al., 1990). Toluene has been shown to induce morphological changes in astroglia and neurons in striatal primary cell cultures (Hansson et al., 1988). In addition, altered receptor binding and increased levels of glutamine synthetase activity have been found in the cerebellum of toluene-exposed rats (Bjornaes and Naaslund, 1988). Animal studies have suggested that extended toluene exposure may induce long-lasting morphological changes in the frontal cortex (Vasquez-Nin et al., 1980) and hippocampal and cerebellar regions (Ladefoged et al., 1991).

There if some evidence that toluene exerts its toxicity by way of reactive oxygen species (ROS) formation. Exposure to toluene, both in vivo and in vitro, caused a significant elevation in ROS formation within

cerebral cortical synaptosomal and microsomal fractions (Mattia et al., 1991). Reactive oxygen species have been implicated in the action of a wide range of structurally and pharmacologically diverse compounds with an equally broad range of effects (Mason, 1982; Trush et al., 1982; Halliwell and Gutteridge, 1985). Many solvents are metabolized into reactive intermediates, including epoxides and reactive oxygen species (Savolainen, 1977). Toluene is metabolized mainly through the oxidation of its methyl group by mixed function oxidases to benzyl alcohol, followed by oxidation to benzaldehyde with alcohol dehydrogenase (Agency for Toxic Substances and Disease Registry, 1987). Benzaldehyde is rapidly oxidized by aldehyde dehydrogenase to benzoic acid. However, in humans and in animals, small quantities of toluene may be converted by phenolic compounds by the enzyme arylmonooxygenase and by epoxide formation of the aromatic nucleus, leading to potentially harmful reactive intermediates (Gerarde and Ahlstrom, 1966; Bakke and Scheline, 1970; Laham, 1970; Jerina and Daly, 1974). In vitro studies of benzene and toluene in pulmonary alveolar macrophages have shown a loss of cell respiration and a dose-dependent and time-dependent increase in lipid peroxidation (Suleiman, 1987).

Experiments were undertaken to determine whether:

1. The effects of toluene, in vivo and in vitro, on the induction of ROS generation follow a dose–response relationship.
2. Lipid peroxidative events are a characteristic of toluene toxicity.
3. Toluene, in vivo, expresses regional specificity in the CNS.
4. Following a single exposure, toluene exerts its effects in the nervous system for an extended time.

METHODS

Animals and Treatment

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 175–200 g were employed in this study. Rats were housed six per cage with wood-chip bedding and maintained on a 12-h light/dark cycle in a temperature-controlled (20 ± 1°C) room. Food (Purina Laboratory Chow, St. Louis, MO) and tap water were provided ad libitum.

In the dose–response study, toluene was dissolved in corn oil and administered in single doses of 0.5, 1.0, and 1.5 g/kg body wt, ip. Animals were killed 1 h following exposure. In the time-course study, toluene in corn oil was administered in a single dose of 1.5 g/kg body wt, ip. Animals were killed 1, 2, 5, and 24 h following exposure. The rationale for the dose used was based on the results obtained from the dose–response study, where maximum ROS induction was observed at the highest dose of toluene (*see* Results).

Chemicals

Toluene was obtained from Fisher Scientific (Tustin, CA). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR) and 2',7'-Dichlorofluorescein (DCF) was purchased from Polysciences (Warrington, PA). 2-Thiobarbituric acid and cyclohexane were obtained from Sigma Chemical Co. (St. Louis, MO) and malonaldehyde *bis* (dimethylacetal), 98%, was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Preparation of Tissue Fractions

Rats were decapitated, and the trunk blood was collected into heparinized tubes and stored at 0–5°C until analysis. The brains were excised quickly on ice, and the cerebellum, hippocampus, and striatum were dissected out. In addition, the liver, kidney, and lung were excised and frozen at −70°C. All of the brain regions were placed into microcentrifuge tubes, stored at −20°C for 24 h, and subsequently stored at −70°C until preparation. This slow freezing rate was intended to maintain synaptosomal integrity (Dodd et al., 1981). Each region was weighed and homogenized in 10 vol of 0.32*M* sucrose and centrifuged at 1800*g* for 10 min. The resulting supernatant fraction was then centrifuged at 31,500*g* for 10 min to yield the crude synaptosomal pellet from brain and crude mitochondrial pellet from liver, kidney, and lung. This pellet (P2) was then resuspended in HEPES buffer to a concentration of 0.037 g-eq/mL. The composition of the HEPES buffer was (m*M*): NaCl (120), KCl (2.5), NaH_2PO_4 (1.2), $MgCl_2$ (0.1), $NaHCO_3$ (5.0), glucose (6.0), $CaCl_2$ (1.0), and and HEPES (10), pH 7.4.

Resuspending the crude mitochondrial fraction from noncerebral tissues in HEPES buffer exposes the mitochondria to higher concentrations of Ca^{2+} and Na^+ than found in the cytosol. However, incubation of this fraction in HEPES buffer together with endogenous substrates, such as glutamate, malate, and succinate, leads to an increase in the rate of oxidation of DCFH to DCF and of dihydrorhodamine to rhodamine 123 (LeBel, unpublished data). Therefore, under these conditions, the respiratory rate of mitochondrial fractions may be responsive to substrate supply, with the increase in oxidation being attributable to mitochondrial production of superoxide (Chacon and Acosta, 1991).

Assay for Reactive Oxygen Species

P2 fractions were kept on ice prior to incubation, diluted 1:10 with 40 m*M* Tris (pH 7.4), and loaded with 5 μM DCFH-DA (0.5 m*M* in ethanol) for 15 min at 37°C, during which time esteratic activity results in the formation of the nonfluorescent compound DCFH (Bass et al., 1983). Following loading, the formation of DCF, the fluorescent-oxidized derivative of

DCFH, was recorded and incubation continued for an additional 60 min, when the fluorescence was again determined (LeBel and Bondy, 1990). Lighting conditions were kept at a minimum to prevent autooxidation of DCFH to DCF.

Previous experiments by LeBel and Bondy (1990) demonstrated that the intermediate, DCFH, is largely trapped within the P2 fraction and that the presence of tissue was required for DCFH oxidation to occur. The rate of formation of DCF was shown to be linear during the 60-min incubation period. In the absence of tissue, DCF formation proceeded at a very slow rate. In addition, DCF formation in HEPES buffer with and without Ca^{2+} was investigated, and results showed that buffer Ca^{2+} content did not play a role in the intracellular formation of ROS in our system (LeBel and Bondy, 1990).

Fluorescence was monitored at excitation wavelength 488 nm (bandwidth 5 nm) and emission wavelength 525 nm (bandwidth 20 nm). The cuvet holder was maintained thermostatically at 37°C. Prior to calculating the rate of formation of DCF, corrections were made for any autofluorescence of fractions. This correction was always $<6\%$ of values in the presence of DCFH. The formation of DCF was quantified from a standard curve over the range of 0.05 to 1.0 μM.

Assay for Lipid Peroxidation

The formation of thiobarbiturate-reactive material was used as an index of peroxidative activity. The precise method used was based on the modification of Uchiyama and Mihara (1978). Briefly, a 3-mL portion of 1% H_3PO_4 was added to 0.5 mL of 10% homogenate in 1.15% KCl or 0.9% saline. One milliliter of 0.6% thiobarbituric acid (TBA) was then added. The mixture was heated at 90°C for 45 min. After cooling, the mixtures were centrifuged for 5 min at 22,700*g*. Color formation at 535 nm was determined in the supernatant. The molar extinction coefficient of malonaldehyde (MDA) derivative at this wavelength (1.56×10^5) was used to determine the malonaldehyde equivalent produced (Utley et al., 1967). This procedure determines the in vitro levels of peroxides rather than the rate of peroxidation in vitro, and is more sensitive to perturbation by toxic agents than the latter procedure (Mihara et al., 1980).

Blood Toluene Analysis

The method for toluene determination was a modification of the methods of Guertin and Gerarde (1959) and Ikeda and Ohtsuji (1971). Heparinized blood from 3–4 rats was pooled in a small beaker. Immediately, 1 mL of the blood was placed into a 25-mL glass-stoppered flask containing 5 mL of 0.2*M* HCl and 3.0 mL of cyclohexane and decanted for 10 min. The mixture was immediately transferred to a screw-capped,

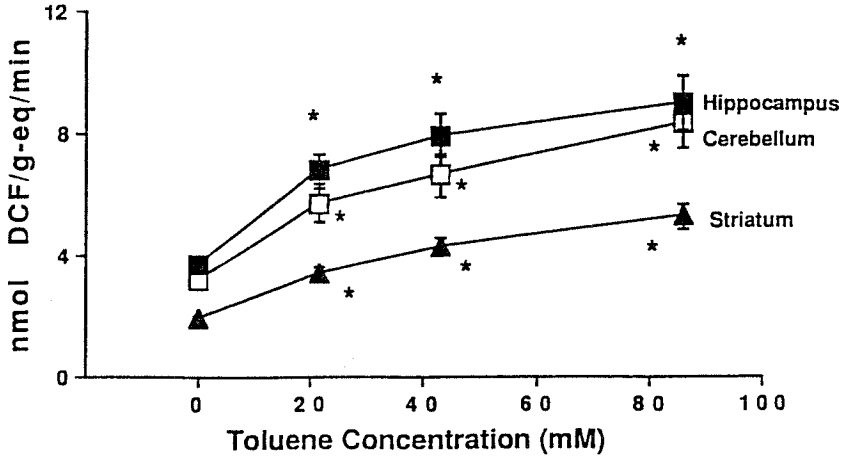

Fig. 1. The formation rate of reactive oxygen species in various brain regions following in vitro incubation with increasing concentrations of toluene. Data are presented as the mean ± SEM of 5–8 independent experiments. *Denotes statistical significance from control values; $p < 0.05$.

teflon-coated centrifuge tube and centrifuged for 15 min at 8000*g*. The cyclohexane layer was carefully transferred to a 2-mL quartz cuvet and the UV absorption spectrum of toluene was recorded on a Varian Cary 210 UV Spectrophotometer, from 240–290 nm against a cyclohexane reference. The concentration of toluene was determined by reference of the absorption peak of blood toluene at 269 nm to a calibration curve previously prepared with pure toluene.

Statistical Analyses

Differences between groups were assessed by one-way analysis of variance followed by Fisher's Least Significant Difference Test. The acceptance level of significance was $p < 0.05$ using a two-tailed distribution. Five or six rats were used in the determination of each data point.

RESULTS

The effect of toluene treatment on the rate of formation of reactive oxygen species was studied using the DCF-generating assay. In vitro exposure of the crude synaptosomal fractions from hippocampus, cerebellum, and striatum to increasing doses of toluene elicited a dose-dependent elevation in the rate of induction of ROS (Fig. 1). Equivalent concentrations of toluene have been shown not to dramatically affect membrane permeability (von Euler et al., 1990). In addition, an equivalent in vitro exposure to benzene, a structurally related organic solvent with no known neurotoxic potential, did not alter the rate of generation of

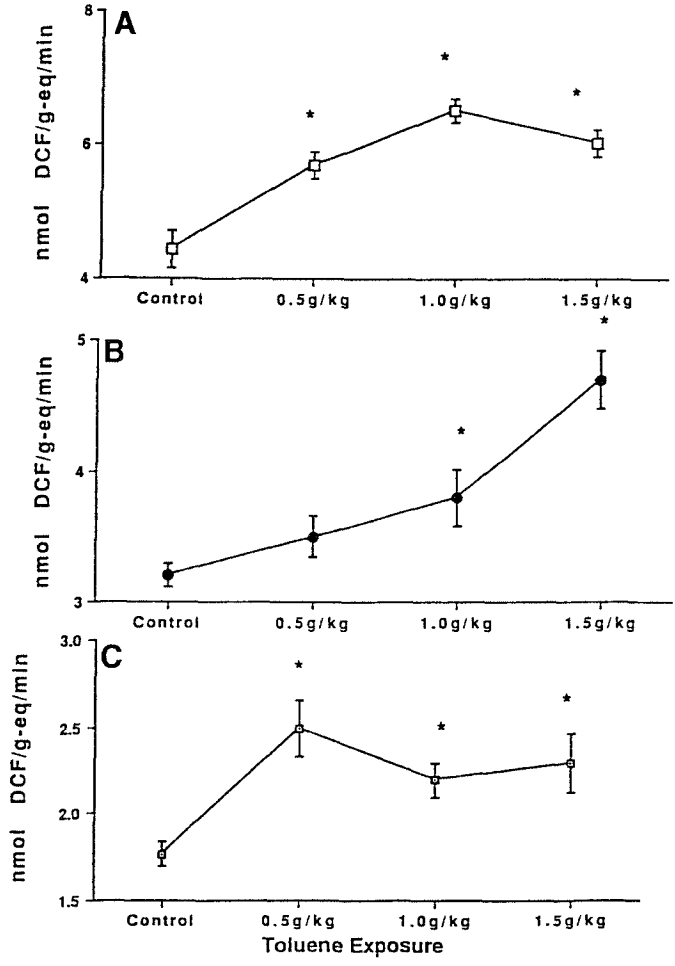

Fig. 2. The effects of in vivo exposure to various doses of toluene by ip injection on the formation rate of reactive oxygen species in crude synaptosomal fractions from hippocampus (**A**), cerebellum (**B**) and striatum (**C**). Data are expressed as the mean ± SEM derived from 5 or 6 rats. *Denotes statistical significance from control values; $p < 0.05$.

ROS in crude synaptosomal fractions (LeBel and Bondy, unpublished data). In vivo exposure to various doses of toluene (0.5, 1.0, and 1.5 g/kg body wt, ip) resulted in a dose-dependent elevation of ROS generation within crude mitochondrial fractions obtained from rat kidney and lung, and from crude synaptosomal fractions prepared from cerebellum (Figs. 2 and 3). The rate of ROS generation in liver, hippocampus, and striatum was not linear with the doses of toluene tested, but reached maximal values at lower toluene concentrations (Figs. 2 and 3). In in vivo and in vitro studies, both the basal rate of DCF generation and the toluene-induced increase were highest in the hippocampus and lowest in the striatum.

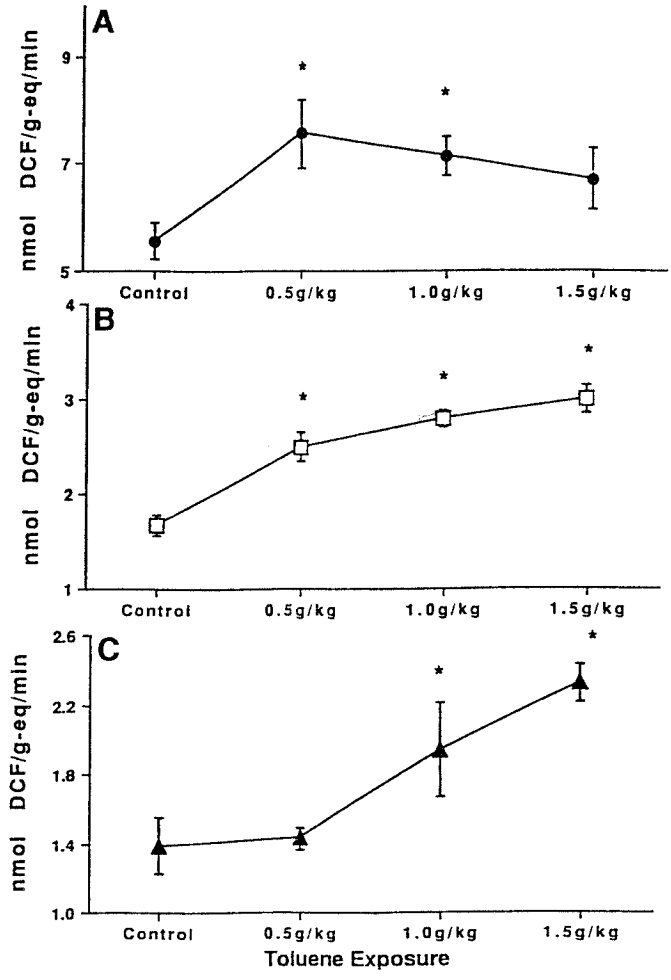

Fig. 3. The effects of in vivo exposure to various doses of toluene by ip injectin on the formation rate of reactive oxygen species in mitochondrial fractions from liver (**A**), kidney (**B**), and lung (**C**). Data are expressed as the mean ± SEM derived from 5 or 6 rats. *Denotes statistical significance from control values; $p < 0.05$.

The results of the dose–response study led us to examine the effects of a single dose of toluene (1.5 g/kg body wt, ip) over varying time intervals. In vivo exposure to toluene showed that toluene-induced ROS generation peaked within 2 h, in all organs studied (Figs. 4 and 5), and this statistically significant elevation in oxidative activity was maintained throughout the next 24 h in the striatum, hippocampus, and cerebellum (Fig. 4). Spectral analysis of the blood revealed a rapid rise in toluene concentration 1 h following exposure (155 ppm), which reached a peak within 2 h (167 ppm). Blood toluene at 5 h following exposure decreased to 116 ppm, and subsequently declined to negligible levels by 24 h. No

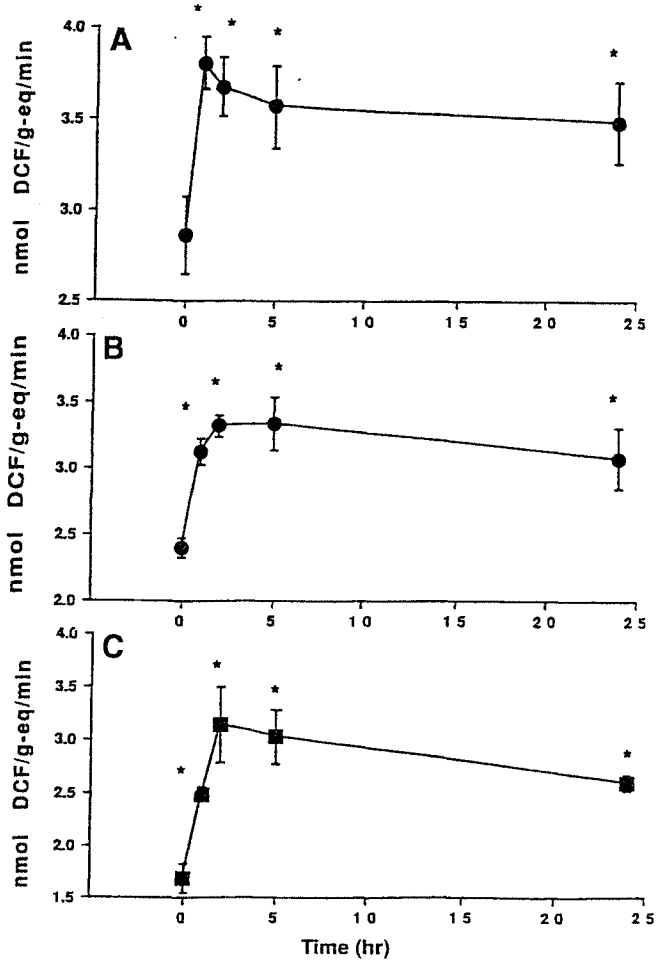

Fig. 4. The effects of in vivo exposure to a single dose of toluene (1.5 g/kg, ip), on the formation rate of reactive oxygen species in crude synaptosomal fractions from hippocampus (**A**), cerebellum (**B**), and striatum (**C**) after various time-points. Data are expressed as the mean ± SEM from 5 or 6 rats. *Denotes statistical significance from control values; $p < 0.05$.

toluene was detectable in the blood of control rats. These results suggest that the effects of toluene on ROS formation persist in the brain despite prior elimination of toluene from the blood. The kidney exhibited a relative rapid decline in the rate of generation of ROS, returning to control levels following the first 5 h after administration of toluene.

The effects of toluene administration on rat striatal, hippocampal, kidney, and liver lipid peroxidation were also studied by quantitation of thiobarbiturate-reactive substance (TBARS) (Table 1). The striatum showed no appreciable increase in lipid peroxidative activity following exposure to toluene (1.0 g/kg body wt, 1 h), whereas the hippocampus exhibited a

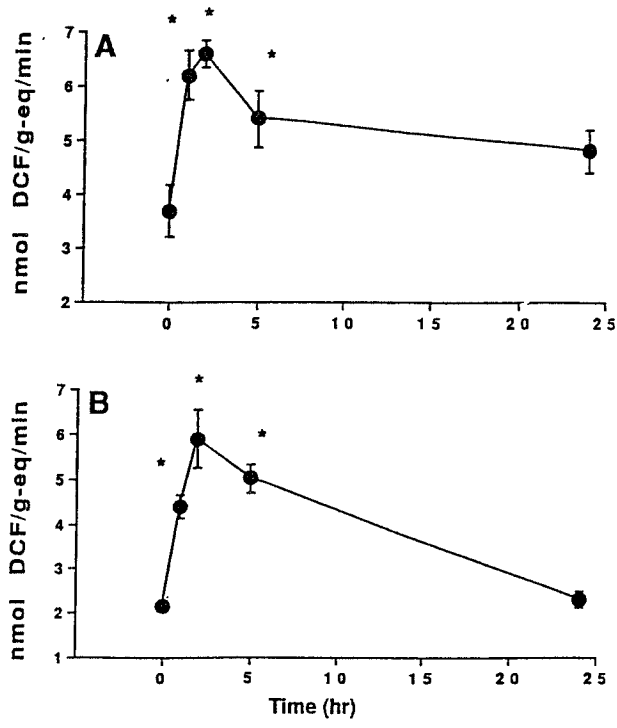

Fig. 5. The effects of in vivo exposure to a single dose of toluene (1.5 g/kg, ip), on the formation rate of reactive oxygen species in mitochondrial fractions from liver (**A**) and kidney (**B**) after various time-points. Data are expressed as the mean ± SEM from 5 or 6 rats. *Denotes statistical significance from control values; $p < 0.05$.

Table 1
Measurement of Thiobarbituric Acid Reactive Substances in Various Tissues Following Exposure to Toluene

Tissue type	Dose, g/kg	nmol MDA/g tissue[a]
Striatum	0.0	31.6 ± 3.4
	1.0	35.3 ± 3.1
Hippocampus	0.0	66.9 ± 3.1
	1.0	78.1 ± 3.1*
Kidney	0.0	127.2 ± 2.8
	0.5	124.0 ± 4.8
	1.0	120.6 ± 8.4
	1.5	135.0 ± 4.9
Liver	0.0	201.2 ± 8.3
	0.5	212.0 ± 8.1
	1.0	258.0 ± 4.0*
	1.5	234.0 ± 4.9*

[a] Data are expressed as mean ± SEM from 5 or 6 rats.
*Denotes statistical significance from control value; $p < 0.05$.

minor but significant increase in the formation of TBARS. There was no difference in lipid peroxidation between crude mitochondrial fractions isolated from the kidneys of control animals or those from animals treated with varying doses of toluene (0.5, 1.0, and 1.5 g/kg, body wt, 1 h). The corresponding liver fraction, however, showed a significant increase compared to control values in the levels of TBARS following higher doses of toluene.

DISCUSSION

The main purpose of the present study was to characterize further the effect of toluene on the induction of reactive oxygen species within mammalian tissues. Earlier we found that toluene has the ability to induce ROS, in vivo and in vitro, and that benzaldehyde may be the metabolite responsible for this induction of oxidative activity (Mattia et al., 1991). In the present studies, we have demonstrated that the lung, kidney, and cerebellum show a relationship between dose of toluene and rate of formation of ROS. The stimulatory effects of toluene on oxidative activity in the liver, hippocampus, and striatum reach their highest values at submaximal doses of toluene after which higher doses have no additional effects. The hippocampus demonstrated the highest level of inducation of ROS formation in the CNS.

Studies have shown that following intraperitoneal injection of toluene, it is taken up rapidly, within 1 h, by the brain, lung, liver, kidney, and blood (Savolainen, 1978). The liver, brain, and blood consistently exhibit the highest levels of toluene (Bruckner and Peterson, 1981). Gospe and Calaban (1988) demonstrated toluene to be differentially distributed to various brain regions and that the initial regional concentrations following exposure to toluene were significantly correlated with regional lipid content. These pharmacokinetic uptake and distribution data correlate with our time-course findings on blood toluene levels and the induction of oxidative activity. All tissues examined showed an increase in ROS generation at the time of peak toluene blood levels (2 h after dosing). The blood toluene values reached in the current study, parallel those previously reported following a similar dosing regimen (LeBel and Schatz, 1988). This prior report also showed that brain levels of toluene are very similar to concurrent blood levels suggesting very rapid diffusion into the CNS. In addition, the rat blood levels reported here are in the same range as those reported by Benignus (1981) after inhalation exposures of 2000 ppm toluene for 2 h. Thus, levels of toluene within tissues in our study are likely to be attainable following toluene inhalation.

In the present studies, the CNS maintained a significant elevation in ROS generation for 24 h after toluene treatment, a time when blood levels of toluene diminished to undetectable concentrations. Therefore, this

induced and oxidative stress persisted beyond the period where the initiating xenobiotic agent was present, implying that tissue injury may be prolonged even after a transient event. In agreement with our findings, Savolainen (1978) also demonstrated that toluene is rapidly removed from most organs: Only very low concentrations of toluene were detected in the brain, lung, liver, and kidneys 24 h after exposure. Bruckner and Peterson (1981) found that concentrations of toluene initially fell more rapidly in liver than in brain. This was attributed to the higher lipid content of the brain and to more extensive metabolism of toluene by liver.

Following exposure of rats to toluene, the liver initially exhibited a rapid increase followed by a slower decline in the rate of ROS induction. In contrast, the kidney demonstrated a rapid increase, followed by a steep decline in ROS generation. This may be owing to the rapid clearance of the metabolites of toluene from the kidney. This clearance may also account for our failure to detect a toluene-induced increase in renal lipid peroxidation. However, more extended exposure to toluene is known to be toxic to the kidney. For example, acute toluene "sniffing" has been shown to cause renal tubular acidosis (Taher et al., 1974). It is unknown whether such renal toxicity is in part related to induction of oxidative stress. However, ROS have been shown to play an essential role in the mechanisms of experimental models of ischemic acute renal failure, acute glomerulonephritis, and toxic renal diseases (for review, *see* Baud and Ardaillou, 1986).

Previous studies have indicated that exposure of rat and rabbit pulmonary alveolar macrophages to toluene, in vitro, induced lipid peroxidation (Suleiman, 1987). These results parallel our findings of toluene-induced increases in TBARS in the liver. Our findings indicate that toluene induces lipid peroxidation in the hippocampus; however, peroxidative damage was not detected in the straitum or kidney. The hippocampus has low intrinsic superoxide dismutase activity and high basal TBARS levels compared with frontal lobe, cerebellum, and parietal/occipital lobe (Sutherland et al., 1991). This may result in a selective vulnerability toward oxidative stress-related injury within the hippocampus. This is suggested further since the hippocampus exhibited a region-specific elevation in TBARS following a 10-min ischemic insult (Sutherland et al., 1991). The hippocampus is distinctively susceptible to a wide range of environmental agents, drugs of abuse, nutritional deficiencies, and neurodegenerative disorders (for review, *see* Walsh and Emerich, 1988). The regional susceptibility to induction of lipid peroxidation that has been found in the current study may bear a relation to the persistence of excess ROS generation that characterizes some organs and brain regions.

The fluorometric techniques employed here appear to be more sensitive in detecting significant changes in oxidative stress, and in a wider variety of tissues, than traditional methods for measuring lipid peroxidation events. The connection between ROS formation and lipid peroxida-

tion is tenuous. It is possible that the tissues that demonstrate prolonged ROS-related changes and no lipid peroxidative damage have been more successful in adapting to those changes than have tissues that exhibit simultaneous elevations of ROS generation and lipid peroxidation. The results in this study suggest that macromolecular damage is not inevitable when the rate of ROS formation is elevated.

In conclusion, exposure to toluene results in broad systemic elevation in the normal rate of oxygen radical generation. The ROS-promoting effects of toluene can be detected long after the solvent has been eliminated from the tissue, suggesting that acute exposure to this organic solvent can lead to extended ROS-related changes. Future studies are essential to establish the extent to which free radicals contribute to the toxicity of toluene. Since toluene is metabolized by liver and excreted by kidney and the primary route of exposure is by inhalation, it is possible that damage could occur in these organs as a result of high level or prolonged exposure to toluene. The evidence supporting this possibility is limited, and more studies are needed to elucidate the toxic effects of toluene in liver, kidney, and lung. Presently, the products of toluene catabolism and the specific enzymes involved are being examined for their ROS-enhancing potential.

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