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Diisopropylphosphorofluoridate-Induced Muscle Hyperactivity Associated with Enhanced Lipid Peroxidation *In Vivo*

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ABSTRACT. Acute exposure to acetylcholinesterase (AChE) inhibitors such as organophosphates and carbamates induces functional changes at the neuromuscular junctions, leading to fasciculations that ultimately cause muscle fiber necrosis. There is recent evidence that oxygen free radical formation may be a factor in the toxicity of these insecticides. One of the targets of free radical-induced injury is lipid peroxidation. The role of lipid peroxidation in diisopropylphosphorofluoridate (DFP)-induced muscle necrosis was investigated by quantifying two products resulting from the oxidation of lipids in muscle tissue—the thiobarbituric acid–malondialdehyde complex (TBA–MDA) and F_2-isoprostanes, the latter being a novel and extremely accurate marker of lipid peroxidation *in vivo*. When compared with control animals, significant increases in MDA of 96% and in F_2-isoprostanes of 56% were found in the diaphragms of rats treated with 2.0 mg/kg DFP after 60 min ($P < 0.01$). In rats pretreated with the neuromuscular blocking agent *d*-tubocurarine or the lazaroid U-78517F, an antioxidant, no DFP-induced increases in either MDA or F_2-isoprostanes were observed. It is suggested that the AChE inhibitor-induced cholinergic hyperactivity initiates the accumulation of free radicals leading to lipid peroxidation, which may be the initiator of the AChE inhibitor-induced cell injury. BIOCHEM PHARMACOL 52;2:357–361, 1996.

KEY WORDS. acetylcholinesterase; antioxidant; diisopropylphosphorofluoridate; F_2-isoprostanes; lipid peroxidation; oxygen free radicals

AChE§ (EC 3.1.1.7) at the neuromuscular junction is essential for the removal of ACh from the synaptic cleft. Acute inhibition of AChE by potent inhibitors such as organophosphates and carbamates, used widely as insecticides, profoundly modifies neuromuscular transmission by initiating muscle hyperactivity, and ultimately causes muscle fiber necrosis [1–6]. Attenuation of muscle hyperactivity prevents AChE inhibitor-induced muscle injury, and thus it has been suggested that muscle hyperactivity is responsible for most of the AChE inhibition-related myopathic alterations [7–9]. Little is known, however, about the basic mechanisms of muscle hyperactivity-related cell injury.

Muscle hyperactivity, which leads to intracellular ATP depletion [10, 11], enhances the generation of reactive oxygen free radicals, and these free radicals may play an important role as mediators of skeletal muscle damage and inflammation [11]. The attack of oxygen-centered free radicals on lipids in the cell membrane, referred to as lipid peroxidation, produces reactive peroxyradicals that initiate a free radical chain reaction causing further cell damage. Oxygen free radical formation and subsequent lipid peroxidation may be among the initial mechanisms causing AChE inhibitor-induced cell damage.

Recent evidence from our preliminary experiments indicates that DFP, an organophosphorus inhibitor of AChE, causes enhanced lipid peroxidation in skeletal muscle, coinciding with the appearance of fasciculations and electromicroscopic lesions [2, 12]. In these preliminary studies, lipid peroxidation was quantified in muscle tissue by measurement of F_2-isoprostanes, which are novel prostaglandin F_2-like compounds derived from the free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme. We have shown previously that measurement of these compounds provides an accurate measure of lipid peroxidation both *in vitro* and *in vivo* [13].

To assess the mechanisms by which inhibitors of AChE, such as DFP, cause cell injury and, in particular, to determine the role of lipid peroxidation in mediating this injury, we evaluated the temporal and concentration relationship

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§ *Abbreviations:* ACh, acetylcholine; AChE, acetylcholinesterase; DFP, diisopropylphosphorofluoridate; MDA, malondialdehyde; TBA, thiobarbituric acid; and TBA–MDA, thiobarbituric acid–malondialdehyde.

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between the production of F_2-isoprostanes and MDA, which is determined by the TBA–MDA method. The TBA–MDA method is, perhaps, the most widely used to quantify lipid peroxidation [14–17]. However, this method may often be inaccurate especially when applied to the quantification of lipid peroxidation *in vivo*. We have quantified morphological changes in muscle as a means to determine whether a causal relationship exists between lipid peroxidation and muscle necrosis. Furthermore, we wanted to explore whether prevention of DFP-induced muscle hyperactivity (fasciculations) similarly prevents lipid peroxidation. To this end, we used the neuromuscular blocking agent *d*-tubocurarine to prevent fasciculations [7]. In addition, we used an inhibitor of lipid peroxidation, the lazaroid U-78517F, to see whether prevention of lipid peroxidation [18, 19] could limit muscular injury.

MATERIALS AND METHODS

Materials

DFP was purchased from the Aldrich Chemical Co. (Milwaukee, WI). U-78517F was a gift from John M. McCall, the Upjohn Co. (Kalamazoo, MI). Atropine sulfate and *d*-tubocurarine were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Animals

Male Sprague–Dawley rats weighing 200–250 g were maintained on a 12 hr light/dark cycle with food and tap water available *ad lib*.

Drug Administration and Tissue Preparation

The different DFP concentrations were prepared freshly 30 min before injection. Under a chemical hood, samples were aliquoted into ice-cold normal saline (0.9% NaCl) and kept on ice. Any remaining amount of DFP was deactivated with 2.5 N NaOH. *d*-Tubocurarine and atropine were prepared by dissolving in unbuffered water. U-78517F was dissolved in a citric acid formulation (0.02 M citric acid monohydrate, 0.0032 M sodium citrate dihydrate, 0.077 M NaCl, pH 3.0). All drugs were prepared immediately before use. The volume of injection was 0.1 mL/100 g rat. A single dose of DFP (1.0 to 2.0 mg/kg, s.c.) was given, and the rats were decapitated 30–120 min following the DFP administration. Protective agents were given i.p. (*d*-tubocurarine, 70 μg/kg; U-78517F, 7 mg/kg) 30 min prior to the DFP injection, immediately after the DFP injection, and 30 min following the DFP injection. To prevent signs of muscarinic toxicity, rats were also pretreated with atropine sulfate (2.0 mg/kg, i.p.) when *d*-tubocurarine was used.

Diaphragm muscles were used for F_2-isoprostane and TBA–MDA assays. They were removed after the decapitation and frozen immediately in liquid nitrogen.

F_2-Isoprostane Assay

F_2-Isoprostanes in muscle tissue were quantified by gas chromatography/negative-ion chemical ionization mass spectrum based on a previously described method [13]. Briefly, F_2-isoprostane compounds were extracted by solid phase techniques using a C-18 cartridge, followed by a TLC purification. Analysis by mass spectrometry was accomplished as free F_2-isoprostanes. Heptadeuterated 9α,11β-prostaglandin/F_2 was used as the internal standard. Quantification was performed by selected ion monitoring of the ratio of the M-181 (loss of $^{\cdot}CH_2C_6F_5$) ions at m/z 569 for endogenous F_2-isoprostanes and at m/z 576 for the internal standard. This method had a precision of ±11% and an accuracy of 96%.

TBA–MDA Assay

The TBA–MDA complex was determined using an HPLC with a C_{18} μBondapak column (3.9 × 300 mm, 10 μm particle size) and a fluorescence detector at Ex 515 nm and Em 550 nm [17].

Histochemical Analyses of Muscle Lesions

All rats were killed by decapitation 24 hr after the administration of DFP. Diaphragm muscles were removed, mounted in gum tragacanth, and frozen in isopentane cooled in liquid nitrogen for histological analysis. The fresh frozen muscles were then sectioned in a cryostat at a thickness of 10 μm. Whole muscle cross-sections were collected at 250-μm increments and stained with Gomori trichrome for identification of necrotic muscle fiber lesions. The total number of necrotic fibers in each muscle cross-section was counted and photographed, utilizing a light microscope.

Quantification of Muscle Activity

Electrical activity of the gastrocnemius muscle was recorded by electromyography, as described previously [4].

Statistical Evaluation

Student's *t*-test was used to establish significance at $P < 0.05$.

RESULTS

Animal Observation

Only rats injected with DFP (1.5 and 2.0 mg/kg, s.c.) exhibited signs of DFP toxicity within 5–7 min following the injection. These signs included: salivation, lacrimation, urination, and fine tremors. Within 10–30 min following the injection of DFP, the rats exhibited muscle fasciculations that lasted for about 4 hr. None of the pretreatment drugs, when given without DFP, induced any obvious toxicity signs or behavioral changes. Rats receiving atropine and

d-tubocurarine followed by DFP did not exhibit the signs described above for DFP alone.

Histochemistry

Muscles from rats having received only DFP revealed scattered necrotic fibers characterized by disruption of sarcoplasmic elements and infiltration of the fibers by macrophages or monocytes. The number of lesions found was reduced significantly when animals were pretreated with atropine and *d*-tubocurarine, or with U-78517F (Table 1).

Muscle Electrical Activity

Activity of untreated muscle was determined as zero activity. With DFP alone, there were high frequency discharges (18.0/sec). None of the prophylactic treatments produced a significant change in electrical activity before DFP. Pretreatment with atropine and *d*-tubocurarine caused a significant decrease in DFP-induced activity (2.0/sec), whereas the lazaroid U-78517F had no effect on the DFP discharge frequency.

Effect of DFP on the Formation of F_2-Isoprostanes

Rats were treated with 1.0, 1.5, or 2.0 mg/kg DFP. F_2-Isoprostane levels in the diaphragm at the above DFP concentrations and various DFP exposure times are shown in Fig. 1. Significant increases of F_2-isoprostanes over control were observed in the 1.5 and 2.0 mg/kg DFP-treated rats. These rats also exhibited fasciculations. The largest increase in F_2-isoprostanes, that being 56%, was observed in rats treated with 2.0 mg/kg DFP following a 60-min exposure ($P < 0.01$). The F_2-isoprostane level decreased when the exposure time was longer than 60 min, but it was still significantly higher than that of control. Lower doses of DFP, such as 1 mg/kg, which did not induce fasiculations, did not cause increases in the formation of F_2-isoprostanes when compared with control animals (Fig. 1).

TABLE 1. Prevention of the DFP-induced increase in TBA-MDA and muscle fiber necrosis in rat diaphragm by *d*-tubocurarine and U-78517F

Treatment*	% Difference of MDA levels from control†	Number of necrotic fibers/1000 muscle fibers
Control		4 ± 2 (0.4%)
DFP	96 ± 11‡	479 ± 45‡ (47.9%)
d-Tubocurarine/DFP	−30 ± 5	73 ± 19§ (7.3%)
U-78517F/DFP	−41 ± 6	70 ± 31§ (7.0%)

Values are means ± SEM of seven muscles.

* Treatment: DFP, 2.0 mg/kg; *d*-tubocurarine, 0.070 mg/kg; and U-78517F, 7.0 mg/kg. Atropine sulfate (2.0 mg/kg) was also given to the *d*-tubocurarine-pretreated rats. All drugs were given i.p. with the exception of DFP which was given s.c. Rats were pretreated with these compounds 30 min before DFP, at the time of DFP, and 30 min later. Rats were killed 1 hr after DFP treatment for TBA-MDA assay or 24 hr after DFP for histochemistry.

† MDA in control: 49.42 ng/mg protein.

‡ Significant difference between control and DFP-only-treated rats ($P < 0.01$).

§ Significant difference between DFP-only-treated and pretreated rats ($P < 0.01$). No significant difference existed between the pretreatment regimens.

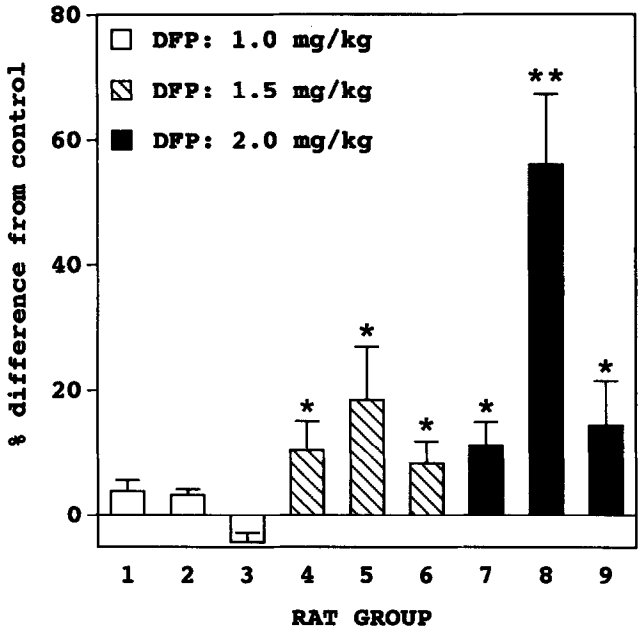

FIG. 1. Percent difference in F_2-isoprostane formation in rat diaphragm receiving DFP (s.c.) treatment versus control rats. Rat groups 1, 4, and 7: 30-min exposure to DFP; groups 2, 5, and 8: 60-min exposure to DFP; groups 3, 6, and 9: 120-min exposure to DFP. Values are means ± SEM (N = 6–10). F_2-Isoprostanes in control: 1.666 ng/g tissue. Key: (*) $P < 0.05$; and (**) $P < 0.01$ between control and treated rats.

Effect of d-Tubocurarine and the Lazaroid U-78517F on F_2-Isoprostane Formation

Pretreatment with anticholinergic agents such as atropine and *d*-tubocurarine prevented the DFP-induced muscle hyperactivity and increases in F_2-isoprostane formation. The antioxidant U-78517F had no effect on muscle hyperactivity, but prevented the increase in F_2-isoprostane formation (Fig. 2).

Comparison of MDA Formation to F_2-Isoprostane Levels in Rat Skeletal Muscle

In addition to assessing F_2-isoprostane formation, levels of MDA in diaphragm tissue were also quantified under the same experimental conditions. As shown in Fig. 3, increases in MDA formation correlated with increases in F_2-isoprostane formation and were greatest (96% increase above baseline, $P < 0.001$) after 60 min of treatment with

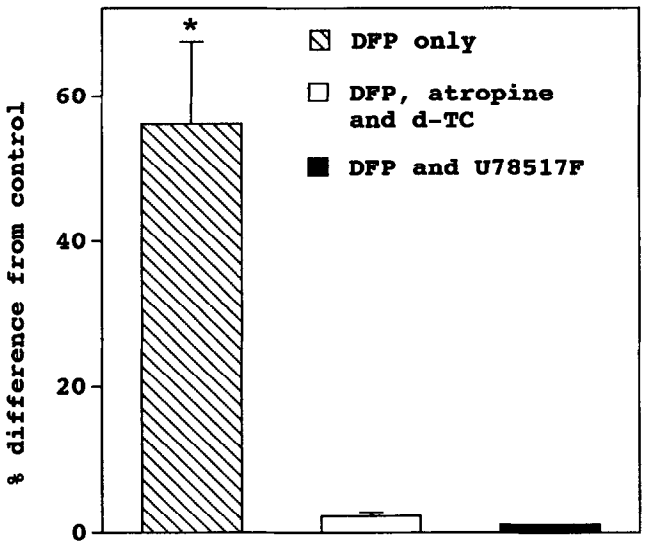

FIG. 2. Effect of pretreatment agents on F_2-isoprostanes (percent difference from control). Rats were treated with DFP (2.0 mg/kg, s.c., 60 min). Atropine (2.0 mg/kg, i.p.) and *d*-tubocurarine (0.07 mg/kg, i.p.), or U-78517F (7.0 mg/kg, i.p.), were given three times: 30 min before DFP, immediately after DFP, and 30 min after DFP. Values are means ± SEM (N = 6–10). F_2-isoprostanes in control: 1.666 ng/g tissue. Key: (*) $P < 0.01$ between control and treated rats.

DFP (2 mg/kg). Pretreatment with the neuromuscular blocking agent or the antioxidant U-78517F, which inhibited increases in F_2-isoprostane formation, also blocked increases in MDA (Table 1).

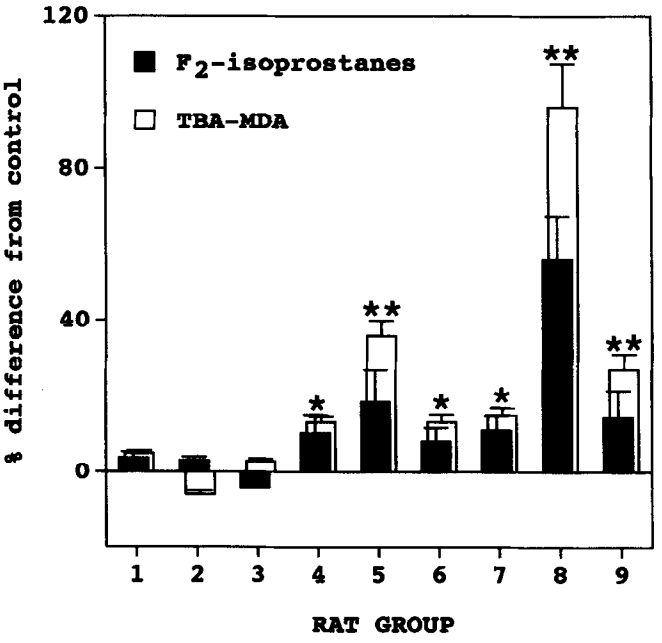

FIG. 3. Comparison of the productions of F_2-isoprostanes and the TBA–MDA complex following DFP (s.c.) treatment. Various DFP treatments for rat groups 1–9 were the same as those for rat groups 1–9 in Fig. 1, respectively. Values are means ± SEM (N = 6–10). F_2-Isoprostanes in control: 1.666 ng/g tissue; MDA in control: 49.42 ng/mg protein. Key: (*) $P < 0.01$; and (**) $P < 0.001$.

DISCUSSION

The results of these studies suggest that lipid peroxidation is associated with the pathogenesis of organophosphate-induced muscle damage. By measurement of either F_2-isoprostanes or TBA–MDA, significant dose-related increases in either marker of lipid peroxidation were detected after the administration of DFP to rats. The largest increase in either MDA or F_2-isoprostanes was observed after a 60-min treatment of animals with DFP at a dose of 2 mg/kg. The difference between the amounts of F_2-isoprostanes and MDA produced may be due to the fact that F_2-isoprostanes are derived only from arachidonic acid, whereas MDA is generated by other fatty acids. Thus, other fatty acids containing fewer double bonds may contribute a relatively greater fraction to the generation of MDA. In addition, other substances such as sugars and proteins can cross-react in the assay to yield TBA material. Thus, it appears that F_2-isoprostanes may provide a better index for lipid peroxidation [20]. Pretreatment with agents, such as atropine and *d*-tubocurarine, or U-78517F, prevented the DFP-induced increase in both MDA and F_2-isoprostanes. *d*-Tubocurarine blocked the muscle hyperactivity by preventing fasciculations, possibly through presynaptic regulation of ACh release [7, 9].

In the absence of direct nerve stimulation, AChE inhibitors such as DFP cause fasciculations associated with repetitive antidromic nerve action potentials. Both fasciculations as well as antidromic firing were prevented by atropine and *d*-tubocurarine in concentrations that have no effect on twitch tension or normal transmission.

Although the presynaptic effects of *d*-tubocurarine are less known than its postsynaptic actions, under appropriate conditions (low concentrations and repetitive firing), presynaptic actions of *d*-tubocurarine cause reduced transmitter release [21].

U-78517F is an antioxidant [18,19] and prevented lipid peroxidation but had no effect on the muscle hyperactivity. These findings support the role of muscle hyperactivity-induced lipid peroxidation as the initial mechanism of muscle injury. They suggest that muscle hyperactivity is the initiating event for lipid peroxidation. Once lipid peroxidation is inhibited, the injury to the muscle is reduced significantly [9].

During muscle hyperactivity, oxygen free radicals in mitochondria are produced in the ATP regeneration process through a univalent oxygen reduction pathway. The intensive muscle hyperactivity and the related increase in energy demand accelerate the conversion of CoQ to semiquinones. Semiquinones can easily reduce oxygen molecules univalently, and therefore the above univalent reduction pathway and the free radical formation are enhanced greatly [11]. Hyperactivity-induced metabolic stress also causes an increase in xanthine oxidase by conversion from its native form (xanthine dehydrogenase) to the oxidized form (xanthine oxidase). The latter utilizes oxygen molecules as the

electron acceptor, and thus the formation rate of superoxide radicals ($O_2^{\cdot -}$), which can be converted to other oxygen radicals and induce lipid peroxidation, is increased [11].

No studies to date have reported a relationship between AChE inhibitor-induced muscle hyperactivity, lipid peroxidation, and muscle lesions. From the present studies, however, it is concluded that the accumulation of oxygen free radicals and the related increases in lipid peroxidation are caused by the AChE inhibitor-induced muscle hyperactivity and that lipid peroxidation is a contributing initial factor in the mechanism of AChE inhibitor-induced cell injury.

In summary, the results of this study demonstrated that (1) the AChE inhibitor, DFP, causes lipid peroxidation and muscle necrosis, (2) prevention of DFP-induced muscle hyperactivity by *d*-tubocurarine inhibits lipid peroxidation and necrosis, and (3) prevention of lipid peroxidation by 21-aminosteroids protects the muscle during muscle hyperactivity. Thus, our studies to date provide strong evidence that lipid peroxidation is linked directly to DFP-induced cellular necrosis.

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References

1. Laskowski MB and Dettbarn W-D, The pharmacology of experimental myopathies. *Annu Rev Pharmacol Toxicol* **17:** 387–409, 1977.
2. Laskowski MB, Olson WH and Dettbarn W-D, Ultrastructural changes at the motor endplate produced by an irreversible cholinesterase inhibitor. *Exp Neurol* **47:** 290–306, 1975.
3. Wecker L, Kiauta T and Dettbarn W-D, Relationship between acetylcholinesterase inhibtion and the development of a myopathy. *J Pharmacol Exp Ther* **206:** 97–104, 1978.
4. Misulis KE, Clinton ME, Dettbarn W-D and Gupta RC, Differences in central and peripheral neural actions between soman and diisopropyl fluorophosphate, organophosphorus inhibitors of acetylcholinesterase. *Toxicol Appl Pharmacol* **89:** 391–398, 1987.
5. Clinton ME, Misulis KE and Dettbarn W-D, Effects of phenytoin, ketamine, and atropine methylnitrate in preventing neuromuscular toxicity of acetylcholinesterase inhibitors soman and diisopropylphosphorofluoridate. *J Toxicol Environ Health* **24:** 439–449, 1988.
6. Wecker L and Dettbarn W-D, Effects of denervation on the production of an experimental myopathy. *Exp Neurol* **57:** 94–100, 1977.
7. Patterson GT, Gupta RC, Misulis KE and Dettbarn W-D, Prevention of diisopropylphosphorofluoridate (DFP)-induced skeletal muscle fiber lesions in rat. *Toxicology* **48:** 237–244, 1988.
8. Sket D, Dettbarn W-D, Clinton ME, Misulis KE, Sketelj J, Cucek D and Brzin M, Prevention of diisopropylphosphorofluoridate-induced myopathy by botulinum toxin type A blockage of quantal release of acetylcholine. *Acta Neuropathol (Berl)* **82:** 134–142, 1991.
9. Yang ZP and Dettbarn W-D, Diisopropylphosphorofluoridate induced cholinergic hyperactivity and lipid peroxidation. *Toxicol Appl Pharmacol*, in press.
10. Gupta RC and Dettbarn W-D, Alterations of high-energy phosphate compounds in the skeletal muscles of rats intoxicated with diisopropylphosphorofluoridate (DFP) and soman. *Fund Appl Toxicol* **8:** 400–407, 1987.
11. Sjodin B, Westing YH and Apple FS, Biochemical mechanisms for oxygen free radical formation during exercise. *Sports Med* **10:** 236–254, 1990.
12. Yang ZP, Wu A, Morrow J, Roberts LJ II and Dettbarn W-D, Increases in malondialdehyde-thiobarbituric acid complex (MDA–TBA) and F_2-isoprostanes (PGF_2) in diisopropyl fluorophosphate (DFP) induced muscle hyperactivity. *FASEB J* **9:** A694, 1995.
13. Morrow JD, Awad JA, Boss HJ, Blair IA and Roberts LJ II, Non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) are formed *in situ* on phospholipids. *Proc Natl Acad Sci USA* **89:** 10721–10725, 1992.
14. Sinnhuber RO and Yu TC, 2-Thiobarbituric acid method for the measurement of rancidity in fishery products: II. The quantitative determination of malondialdehyde. *Food Technol* **12:** 9–12, 1957.
15. Largilliere C and Melancon SB, Free malondialdehyde determination in human plasma by high-performance liquid chromatography. *Anal. Biochem.* **170:** 123–126, 1988.
16. Draper HH and Hadley M, Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* **186:** 421–431, 1990.
17. Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S and Hadley M, A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. *Free Radic Biol Med* **15:** 353–363, 1993.
18. Braughler JM, Hall ED, Jacobsen EJ, McCall JM and Means ED, The 21-aminosteroids: Potent inhibitors of lipid peroxidation for the treatment of central nervous system trauma and ischemia. *Drugs Future* **14:** 143–152, 1989.
19. Hall ED, Braughler JM, Yonkers PA, Smith SL, Linseman KL, Means ED, Scherch HM, Von Voigtlander PF, Lahti RA and Jon Jacobsen E, U-78517F: A potent inhibitor of lipid peroxidation with activity in experimental brain injury and ischemia. *J Pharmacol Exp Ther* **258:** 688–694, 1991.
20. Longmire AW, Swift LL, Roberts LJ II, Awad JA, Burk RF and Morrow JD, Effect of oxygen tension on the generation of F_2-isoprostanes and malondialdehyde in peroxidizing rat liver microsomes. *Biochem Pharmacol* **47:** 1173–1177, 1994.
21. Hartman GS, Fiamengo SA and Riker WF, Succinylcholine: Mechanisms of fasciculations and their prevention by *d*-tubocurarine or diphenylhydantoin. *Anesthesiology* **65:** 405–413, 1986.