



PROSTAGLANDIN $F_{2\alpha}$ RISES IN RESPONSE TO HYDROXYL RADICAL GENERATED IN VIVO

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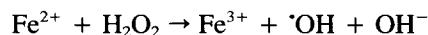
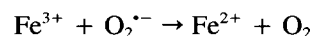
(Received 18 March 1994; Revised 22 June 1994; Accepted 5 July 1994)

Abstract—Free radicals and some free fatty acids, such as arachidonic acid metabolites, have been hypothesized to be contributors to secondary damage to the spinal cord upon injury. These two types of species may form a feedback loop in which generation of one type leads to formation of the other. In this study, to determine whether hydroxyl radical causes generation of arachidonic acid metabolites in vivo, we generated hydroxyl radical, a most reactive oxygen radical, in the rat spinal cord and measured resulting changes in levels of prostaglandin $F_{2\alpha}$, an arachidonic acid metabolite that rises following traumatic injury. The hydroxyl radical was generated in the rat spinal cord by administering H_2O_2 through one microdialysis fiber and $FeCl_2/EDTA$ through a parallel fiber. The prostaglandin $F_{2\alpha}$ in the collected microdialysates was measured by HPLC as its 3-bromomethyl-6,7-dimethoxy-1-methyl-2-(1H)-quinoxalinone derivative. Prostaglandin $F_{2\alpha}$ dramatically increased in response to hydroxyl radical generation, but declined substantially after 3 h of exposure. Prostaglandin $F_{2\alpha}$ was undetectable when either H_2O_2 or $FeCl_2/EDTA$ was administered alone in control experiments, demonstrating that its formation was caused by generated hydroxyl radical.

Keywords—Arachidonic acid metabolites, Fenton's reagents, Hydroxyl radical generation, HPLC analysis, Microdialysis, Prostaglandin $F_{2\alpha}$, Secondary spinal cord injury, Free radicals

INTRODUCTION

A variety of agents have been hypothesized to mediate secondary damage upon CNS injury, including released excitatory amino acids, elevated intracellular Ca^{2+} , free radicals, and membrane phospholipid hydrolysis and peroxidation products.^{1–3} Free radical damage is initiated by formation of reactive oxygen species (ROS). Superoxide anion radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are produced during normal metabolism, but excessive production of these species upon cell injury may damage tissue. The damage produced by $O_2^{\bullet-}$ and H_2O_2 is slight in the absence of iron ions. Injecting iron salts^{4,5} into rat brain causes transient focal epileptiform discharges and lipid peroxidation. It is hypothesized^{6–8} that excessive H_2O_2 formed upon injury is catalytically dissociated by iron ions to the highly reactive hydroxyl radical ($\bullet OH$) by the Haber–Weiss reaction:



$\bullet OH$ would be very damaging because it reacts very rapidly with almost every molecule found in living cells. Cell damage by any mechanism has the potential to accelerate free radical reactions because lysed cells release their intracellular iron pools into the surrounding environment, thereby providing a catalyst for generating $\bullet OH$.^{6–9}

Spinal cord injury is followed within a few minutes by the appearance of phospholipid degradation products.^{10,11} These are thought to be formed by lipid peroxidation and phospholipase-mediated hydrolysis of membrane lipids to liberate free fatty acids (FFAs), such as arachidonic acid.^{11–16} Arachidonic acid is rapidly converted to prostaglandins (PGs) and thromboxanes by cyclooxygenase, and to leukotrienes by lipoxygenase.

Arachidonic acid metabolites can cause generation of $O_2^{\bullet-}$, H_2O_2 , and $\bullet OH$.^{17,18} Furthermore, reactive oxygen species also cause formation of arachidonic acid

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metabolites.^{19,20} In summary, formation of ROS and arachidonic acid metabolites appears to initiate feedback loops in which generation of one leads to formation of the others.

To characterize an agent's ability to cause secondary damage, we have established an experimental model. The model combines sampling and agent administration by microdialysis with electrical recording (to examine whether electrical conduction is blocked by the administered substance), neurochemical analysis (to evaluate the biochemical changes caused by this substance), and postmortem histological examination of the tissue around the site of administration (to determine the extent of damage to the tissue). This enables us to correlate electrophysiological, histological, and neurochemical parameters in the same experiment to determine whether a substance causes secondary damage, and, if so, to study mechanisms whereby it produces damage.²¹

Most evidence for a role of oxygen radicals in tissue damage is indirect. To study damage caused by $\cdot\text{OH}$, we developed a double fiber technique to generate $\cdot\text{OH}$ in rat spinal cord.²² The generation of $\cdot\text{OH}$ was demonstrated by administering phenylalanine and detecting o-, m-, and p-hydroxyphenylalanine in dialysates.²² Using our experimental model, we characterized the damage caused to neurons by $\cdot\text{OH}$ generated in vivo in rat spinal cord.²³ In those experiments, $\cdot\text{OH}$ blocked electrical conduction, killed neurons, and released amino acids. We also demonstrated that methylprednisolone, a drug clinically efficacious in ameliorating spinal cord injury, significantly reduces excitatory amino acid²⁴ and prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$)⁴⁴ release.

In this study, we attempt to determine whether $\cdot\text{OH}$ causes release of arachidonic acid products in vivo. We did this by (1) generating $\cdot\text{OH}$ radical in vivo in rat spinal cord^{22,23} by administering H_2O_2 and $\text{FeCl}_2/\text{EDTA}$ through two parallel microdialysis fibers so they mixed in the cord, and (2) analyzing $\text{PGF}_{2\alpha}$ in microdialysates in response to $\cdot\text{OH}$ generation by HPLC.

MATERIALS AND METHODS

Animal preparation and microdialysis fiber insertion

Male Sprague-Dawley rats (350–400 g) were used. Animals were anesthetized with pentobarbital (35 mg/kg IP) followed by urethane (780 mg/kg IP). Anesthesia was considered to be adequate when there was no flexor withdrawal upon noxious foot pinch. When the rat was fully anesthetized, its back was shaved and a laminectomy performed from vertebra T12 to vertebra L3. The animal was then clamped in a frame by attachments to its dorsal vertebral processes. To minimize possible protective effects of pentobarbital against

damage, that agent was used only for initial anesthesia of the animal. Anesthesia was maintained with urethane. Body temperature was maintained at 37–38°C throughout the experiment utilizing feedback from a rectal probe to a heating blanket.

Microdialysis fiber preparation and placement are described in more detail elsewhere.^{25,26} Briefly, we utilized Cuprophan dialysis fibers (Spectrum Industries) of 220 μm external diameter including their coating. Except for a 2-mm dialysis zone, fibers were coated with a thin layer of silicone rubber. A 1-mm wide ink mark extending away from the trailing edge of the dialysis zone was made on the fiber before applying the silicone rubber. The fiber was pulled through the cord until the mark just disappeared into the cord. This placed the dialysis zone in the gray matter of the cord. The pin was then cut off and the fiber attached to a syringe pump with a length of PE 50 tubing. The technique for preparing double fibers is described elsewhere.²¹ When double fibers were used, they were glued together with their dialysis zones aligned. Ringier's solution was pumped through the fibers at a rate of 5 $\mu\text{l}/\text{min}$. It contained (in mM) 147 NaCl, 4 KCl, 2 CaCl_2 , and its pH was 7.2. All of the solutions were bubbled with 95% $\text{O}_2/5\%$ CO_2 just before each experiment.

One or two microdialysis fibers glued together were inserted laterally through the cord at L3-L4 when the animal was clamped in the frame. After the fiber was inserted, the exposed cord was covered with warm mineral oil.

All experimental procedures were approved by UTMB's Animal Care and Use Committee and were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Sampling and hydroxyl radical generation by microdialysis

$\text{Fe}^{2+}/\text{EDTA}$ is a good agent for generating $\cdot\text{OH}$ from H_2O_2 .⁷ Our double fiber technique allows us to administer H_2O_2 through one microdialysis fiber and $\text{FeCl}_2/\text{EDTA}$ through a parallel fiber. These two agents were mixed in tissue in the overlapping dialysis zones around the two fibers, and $\cdot\text{OH}$ radical was generated.^{22,23} We also administered H_2O_2 and $\text{FeCl}_2/\text{EDTA}$ each alone as controls.

The double fibers were also used to sample the release of substances caused by the administration of damaging agents. When we used double fibers for sampling, sample collection was begun 2 h after fiber insertion; when a single fiber was used, collection was started 75 min after insertion of the fiber into the cord. These delays permitted release of $\text{FGF}_{2\alpha}$ induced by

the insertion of the fiber to decline to a stable baseline. Fluid was collected continuously from the outlet end of the fibers in plastic tubes in ice. After collecting two 15-min control samples, the perfusion solutions were switched to ones containing the agents. Agents were administered and samples collected for the next 4 h.

To administer H_2O_2 , 50 mM H_2O_2 was prepared in Ringer's solution and the pH adjusted to 7.2–7.4 with NaOH. To prepare the FeCl_2 /EDTA solution, NaCl, KCl, CaCl_2 , and EDTA were dissolved in a solution of NaH_2PO_4 (10 mM) and NaOH (20 mM). This gave a pH of 9.4–9.7. The solution was degassed by sonication for 15 min, followed by bubbling with argon for 15 min. Aliquots were then sealed under argon. FeCl_2 was added to this solution at the start of each experiment. The final pH of the FeCl_2 solution was 7.2–7.4, and the final Na^+ , K^+ , and Ca^{2+} concentrations were those in regular Ringer's solution. The EDTA concentration was 8.2 mM, and the Fe^{2+} concentration 5 mM. Unless the Ringer solution was degassed as described and addition of FeCl_2 delayed until the start of an experiment, a brown precipitate formed. A precipitate also quickly formed when EDTA was omitted.

We have found using double fibers that the K^+ concentration outside an administering microdialysis fiber is 10 times lower than it is inside the fiber.²¹ Administered kainate also produce a tenfold concentration gradient.^{45,46} In the experiments presented here, 5 mM FeCl_2 /8.2 mM EDTA was given through one fiber and 50 mM H_2O_2 through the second fiber. According to the estimated gradient across the fiber wall, the concentrations near the fibers in the tissue are assumed to be 0.5 mM FeCl_2 /0.82 mM EDTA and 5 mM H_2O_2 . We use the latter concentrations in the following in describing experimental conditions. However, there was actually a gradient of decreasing concentration of the applied agents with increasing distance from the fiber.

Analysis of prostaglandin $\text{F}_{2\alpha}$ by HPLC

Prostaglandin $\text{F}_{2\alpha}$ was analyzed as its 3-bromo-methy-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (Br-DMEQ, Sigma) derivative.²⁷ The $\text{PGF}_{2\alpha}$ was extracted from the microdialysates with ethyl acetate at pH 3.0–3.5, following which the extracts were evaporated to dryness. The residue was derivatized by adding acetonitrile, potassium hydrogen carbonate (KHCO_3), Br-DMEQ, and 18-crown-6 ether (Sigma) sequentially at 50°C in the dark.²⁷

The derivatives were analyzed on a Beckman dual pump high pressure liquid chromatograph (HPLC) equipped with a Waters autosampler and a Shimadzu RF-10 fluorescence detector (excitation wavelength

370 nm, emission wavelength 455 nm). A C8 cartridge column (Phase Separations; 150 × 4.6 mm, 5 μm particle diameter) was used. The components of the elution system were CH_3CN : CH_3OH : H_2O (30:10:60 for mobile phase component A and 35:30:35 for B). Isocratic elution was used to elute $\text{PGF}_{2\alpha}$ (A:B = 97:3); the ratio A:B was then changed to 50:50 to wash the column. Each run lasted 60 min. The flow rate was 2 ml/min.

The recovery upon extraction was determined by the formula:

$$\text{recovery} = (\text{A}_1 - \text{A}_2) / \text{A}_3 \times 100\%$$

A_1 = The area of the HPLC peak obtained from an extract of a microdialysate to which a known amount of $\text{PGF}_{2\alpha}$ standard (Sigma) was added. The extract was derivatized as described earlier.

A_2 = The area of an HPLC peak in which the same amount of microdialysate as in A_1 , but without added standard, was extracted and derivatized.

A_3 = The area of a peak obtained by directly derivatizing the amount of $\text{PGF}_{2\alpha}$ standard added in A_1 without extraction.

RESULTS

The time course of changes in levels of extracellular $\text{PGF}_{2\alpha}$ during 'OH generation by $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ administration is given in Figure 1. Figure 2 illustrates typical chromatograms of the dialysates collected from one animal. $\text{PGF}_{2\alpha}$ was undetectable when Ringer solution alone was pumped through the fibers; therefore, the basal level was unavailable. At time = 0, H_2O_2 (50 mM) and FeCl_2 (5 mM)/EDTA (8.2 mM) started passing through the microdialysis fibers and mixed in overlapping dialysis zones in the cord to generate 'OH. $\text{PGF}_{2\alpha}$ increased dramatically in response to 'OH generation from undetectable (basal level) to about 333 ± 166 nM (SD, $N = 5$) in 90 min. 'OH was generated continuously for 4 h, but the concentration of $\text{PGF}_{2\alpha}$ decreased substantially after 3 h. During administration of either FeCl_2 /EDTA or H_2O_2 alone, $\text{PGF}_{2\alpha}$ was undetectable in microdialysates at the detection limit of the method.

The maximum concentration of $\text{PGF}_{2\alpha}$ reached in one experiment was about 584 nM, and the maximum average concentration measured from microdialysates was 333 nM. The time to reach the maximum concentration of $\text{PGF}_{2\alpha}$ in response to 'OH generation varied among animals. Therefore, the average $\text{PGF}_{2\alpha}$ levels display large error bars at the higher concentrations. The average recovery of $\text{PGF}_{2\alpha}$ from microdialysates

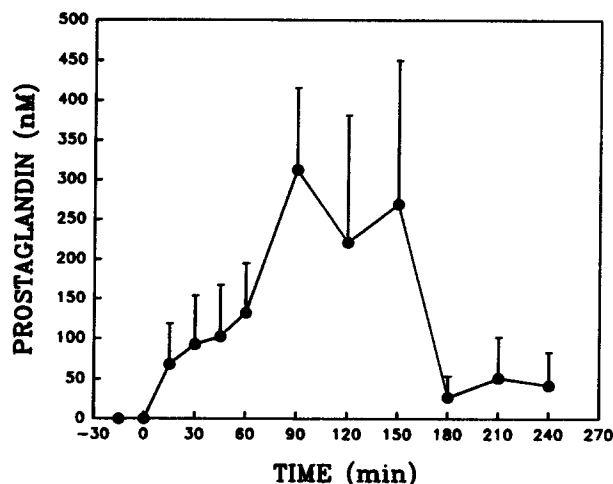


Fig. 1. Changes in $\text{PGF}_{2\alpha}$ levels in microdialysates in response to administration of H_2O_2 and Fe^{2+} /EDTA to generate $\cdot\text{OH}$. Fiber preparation and placement are described in the text. When double fibers were used to generate $\cdot\text{OH}$ in vivo, 50 mM H_2O_2 was administered through one fiber and 5 mM FeCl_2 /8.2 mM EDTA through the second fiber so they mixed in the tissue to generate $\cdot\text{OH}$. These concentrations gave estimated concentrations of 5 mM H_2O_2 , 0.5 mM FeCl_2 and 0.82 mM EDTA in the tissue near the fibers (see text). Ringer solution was pumped through the fiber until time = 0. At $t = 0$, solutions containing the agents administered began passing the dialysis zone. The time course of $\text{PGF}_{2\alpha}$ release in response to $\cdot\text{OH}$ generation was determined from microdialysate collected from the fiber in which Fe^{2+} /EDTA were administered (mean \pm SEM, $N = 5$). Administration of 5 mM H_2O_2 or 0.5 mM FeCl_2 /0.82 mM EDTA alone did not cause detectable release.

in three parallel extracts was $50\% \pm 1.5\%$ (SD); therefore, the maximum average concentration in the microdialysates was 666 nM.

The identity of $\text{PGF}_{2\alpha}$ was verified by varying the composition of the mobile phase for HPLC analysis and the flow rate of elution. The peak for the $\text{PGF}_{2\alpha}$ standard always coincided with the peak that we measured in the microdialysates.

DISCUSSION

Microdialysis has been used to demonstrate that FFAs are released in response to forebrain ischemia²⁸ and acute penetration injury of brain²⁹ in vivo. In this study, we used microdialysis both to administer Fenton's reagents to generate $\cdot\text{OH}$ in vivo in the gray matter of the rat spinal cord and to sample the resulting release of $\text{PGF}_{2\alpha}$. $\text{PGF}_{2\alpha}$ levels began to increase immediately following the start of the administration of Fenton's reagents and reached maximum concentrations of about 333 nM in 90 min. Administration of H_2O_2 or FeCl_2 /EDTA alone did not cause a detectable rise in control experiments, demonstrating that $\cdot\text{OH}$ caused the increase. Although the results do not define the

mechanism whereby $\cdot\text{OH}$ causes the $\text{PGF}_{2\alpha}$ increase, this is the first evidence in vivo that $\cdot\text{OH}$ causes increased production of arachidonic acid metabolites. This is consistent with the in vitro evidence provided by Chan et al.^{19,20}

Initial trauma is followed by several secondary events: (1) Hydrolysis of membrane phospholipids—PGs dramatically increase following spinal cord^{1,11,12,14,30} and brain^{31,32} injury. This is attributed to activation of phospholipases that hydrolyze membrane phospholipids to liberate arachidonic acid. Its metabolites enhance injury-associated ischemia by reducing circulation and induce brain edema.^{33–35} (2) Peroxidation of membrane lipids^{10,14,36–38}—Detection of 4-hydroxy-2,3-trans-nonenal and malonaldehyde, end products of phospholipid peroxidation, demonstrates that lipid peroxidation occurs during ischemia in the brain.³⁷ (3) Formation of oxygen free radicals^{39–41}—Free radicals attack membrane lipids, thereby damaging cell membrane and impairing cell function.^{10,14,36–38}

There are interactions among those secondary events: $\text{O}_2^{\cdot-}$ causes significant release of endogenous arachidonic acid from membrane lipids in cultured tissue¹⁹ and in cultured cells,²⁰ so reactive oxygen species generate arachidonate metabolites. These substances cause formation of free radicals,^{17,18,42} which in turn cause added lipid peroxidation.⁴² Phospholipid peroxidation products can be formed by free radical attack on cell membrane lipids. $\cdot\text{OH}$ abstracts a hydrogen from polyunsaturated fatty acid side chains, and reaction of the resulting radical with O_2 generates peroxy-lipid radicals. These in turn abstract hydrogens from adjacent fatty acid side chains to propagate free radical chain reactions. Thus, one $\cdot\text{OH}$ triggers the conversion of many membrane lipids into lipid hydroperoxides and can severely disrupt membrane function.⁸ Because the membrane lipids of neurons are rich in polyunsaturated fatty acid side chains, neural tissue is especially prone to radical damage. Protection against ischemic damage by the carbon-centered radical spin trapping agents t-phenyl- α -butyl-nitrone and 5,5-dimethyl-1-pyridine-N-oxide⁴³ supports the occurrence of radical chain reactions in injured tissue. Thus, CNS injury is hypothesized to produce a cascade containing feedback loops among secondary damage factors, such that generation of one leads to formation of the others.

Evidence for the preceding mechanisms has been largely indirect. Our finding that $\cdot\text{OH}$ can cause prostaglandin release in in vivo experiment extends our work demonstrating that $\cdot\text{OH}$ damages spinal cord neurons²³ to supporting that formation of arachidonic acid metabolites in response to reactive oxygen species could be part of the cascade producing secondary damage upon CNS trauma.

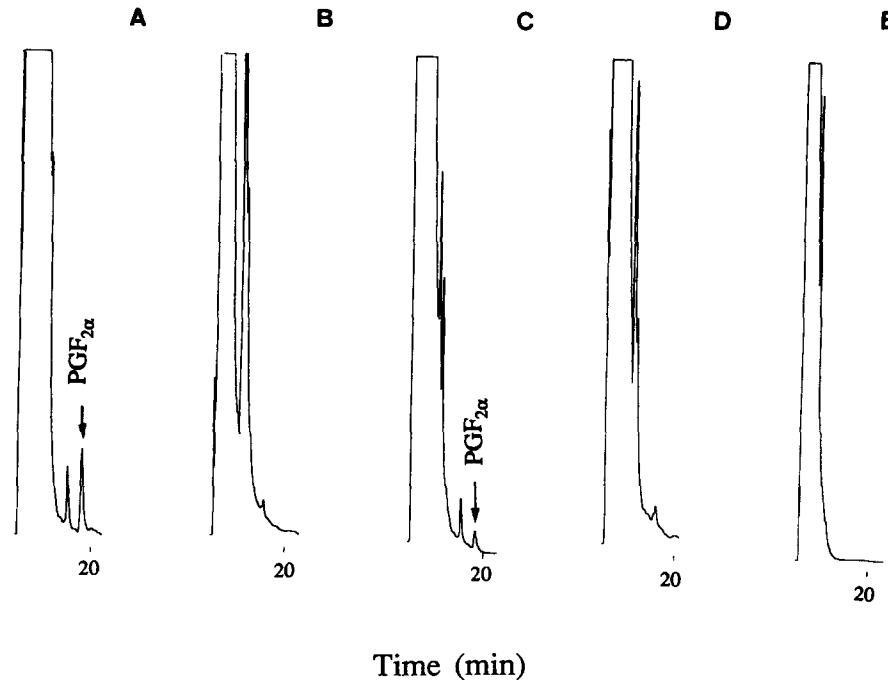


Fig. 2. Typical chromatograms of prostaglandin $F_{2\alpha}$ in microdialysates. (A) $PGF_{2\alpha}$ standard (2.36 pmol). (B) Sample collected before administration of \cdot OH-generating agents to determine the $PGF_{2\alpha}$ basal level. (C) Microdialysate collected after administering Fenton's reagents to generate \cdot OH. (D) Sample collected following administration of only H_2O_2 . (E) Sample collected following the administration of $FeCl_2/EDTA$.

Acknowledgements — The authors thank David J. McAdoo for suggested revisions of this article and Debbie Pavlu for typing. This work was supported by the Paralyzed Veterans of America Spinal Cord Research Foundation (grant #1025) and the American Paralysis Association (LB1-9104-1).

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ABBREVIATIONS

FFA—free fatty acid
 •OH—hydroxyl radical
 PG—prostaglandin
 PGF_{2α}—prostaglandin F_{2α}
 ROS—reactive oxygen species