HYDROGEN PEROXIDE INDUCES OXIDATIVE DAMAGE IN RAT TYPE II PULMONARY EPITHELIAL CELLS

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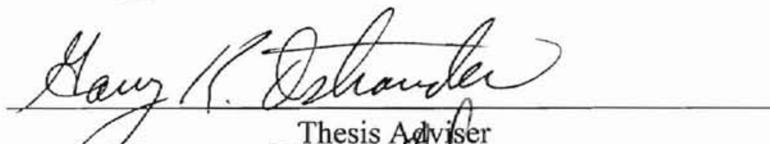

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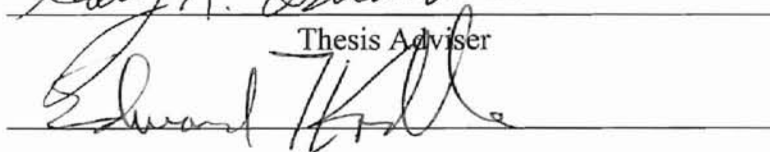

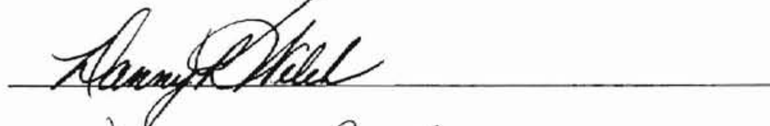

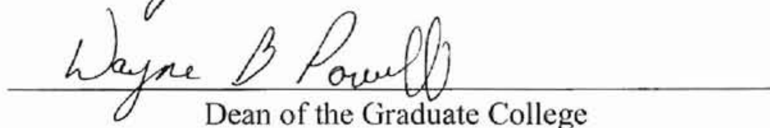

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NOMENCLATURE

A	adenine
C	cytosine
DAPI	4'-6-diamidino-2-phenylindole
FAPy-Ade	4,6-diamino-5-formamidopyrimidine
FAPy-Gua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
GC-MS/SIM	gas chromatography-mass spectrometry with select ion monitoring
G	guanine
HBSS	Hank's balanced salt solution
H_2O_2	hydrogen peroxide
$O_2^{\cdot-}$	superoxide radical
$OH^{\cdot}$	hydroxyl radical
PBS	phosphate buffered saline
2-OH-Ade	2-hydroxyadenine
5-OH-Cyt	5-hydroxycytosine
5-OH-Hyd	5-hydroxyhydantoin
5-OHMeHyd	5-(hydroxymethyl)hydantoin
5-OHMeUra	5-(hydroxymethyl)uracil
5-OH-Ura	5-hydroxyuracil

8-OH-Ade	8-hydroxyadenine
8-OH-Gua	8-hydroxyguanine

Background

Oxidative DNA damage has been correlated with mutagenesis and carcinogenesis in both animal model systems and human tissues [1-10]. For example, DNA base modifications such as 8-hydroxyguanine and 8-hydroxyadenine have been shown to occur after exposure to oxidative agents, and to be present at higher concentrations in tumor tissue than in surrounding tissue [9,10]. It has therefore been suggested that these modified products may be used as markers for predicting mutagenic and carcinogenic risk in human tissues [9]. Currently, oxidative DNA damage is detectable through a variety of labor intensive methodologies, including gas chromatography-mass spectrometry with select ion monitoring (GC-MS/SIM) [11], ^{32}P post-labeling [12], and high pressure liquid chromatography (HPLC) [13]. The original goal of my work as an M.S. candidate was to develop a novel detection methodology based on fluorescence spectroscopy, whereby oxidative DNA damage could be detected more easily and more sensitively than methods previously employed.

Work by Barcellona and Gratton [14] had shown that, upon intercalation of the fluorophore 4'-6-diamidino-2-phenylindole (DAPI) into the minor groove of calf thymus DNA, a characteristic fluorescence spectra was obtained in both continuous wave and lifetime modalities. We successfully repeated these continuous wave fluorescence experiments, and proceeded to expose calf thymus DNA to a hypoxanthine/xanthine oxidase enzyme system known to cause oxidative DNA damage [15]. Calf thymus DNA thus treated and bound to DAPI was examined using continuous wave fluorescence

spectroscopy to determine if the fluorescence spectra had been altered by the oxidatively damaged bases. No significant alteration in fluorescence spectra was detected between treatment groups and controls.

This was not an unexpected result, as continuous wave fluorescence spectroscopy has a limited sensitivity. However, we intended to utilize lifetime fluorescence spectroscopy, a much more sensitive system, to resolve the lifetimes of normal calf thymus DNA and oxidatively damaged calf thymus DNA. For several months we attempted to run lifetime measurements on DNA-DAPI conjugates using an SLM 48000 Lifetime Spectrofluorometer capable of resolving fluorescent lifetimes in the nanosecond range. Unfortunately, the SLM 48000 system available to us had technical problems that we could not resolve, and we were unable to obtain the data necessary to answer our questions.

We knew that attempting to develop a novel methodology had a relatively high likelihood of yielding negative results, and therefore had planned a second project. We intended to use GC-MS/SIM, a proven methodology, to look at the role of oxidative DNA damage in tumor formation in rainbow trout (*Oncorhynchus mykiss*). DNA was extracted from normal rainbow trout livers, as well as from liver carcinomas induced by exposure to aflatoxin B1. In addition, DNA was extracted from control rainbow trout hepatocytes and from hepatocytes exposed to varying levels of hydrogen peroxide (H_2O_2), an agent known to cause oxidative DNA damage [1]. DNA was derivitized (see Materials and Methods for details) and analyzed with GC-MS/SIM by Dr. Dilip Sensharma of the Oklahoma State University Department of Chemistry. However, the

peaks obtained using GC-MS/SIM were not consistent with previously published results [*e.g.* 11]

DNA samples were therefore sent to Dr. Miral Dizdaroglu at the Institute of Standards and Technology for analysis. In contrast to results obtained in Dr. Sensharma's laboratory, Dr. Dizdaroglu's GC-MS peaks conformed to previously published data. We concluded that there was a problem with Dr. Sensharma's GC-MS/SIM detection system at Oklahoma State. As there was no precedent for detecting this type of DNA base damage at O.S.U., we set out to modify the GC-MS/SIM system at O.S.U. in order to collect this data. We changed several parameters on Dr. Sensharma's GC-MS/SIM in an attempt to repeat Dr. Dizdaroglu's results. These attempts failed. Dr. Nicholas Basta was also kind enough to allow us to use his GC-MS/SIM system, but again we were unable to obtain the results produced by Dr. Dizdaroglu. After changing several components of Dr. Basta's GC-MS/SIM system, including the column, the project was altered as I moved on to the Penn State College of Medicine to pursue my Ph.D.

Soon after arriving at Penn State I initiated a collaboration with Dr. Eugene Rannels to determine if rat type II alveolar cells were vulnerable to oxidative DNA damage. Given their position on the surface of the lung, type II cells are very likely to be exposed to oxidative insult *in vivo*. As Penn State did not have a GC-MS/SIM system available, we arranged a collaboration with Dr. Jeremy Spencer of King's College, London. We were able to show that type II cells are indeed vulnerable to oxidative DNA damage, and what follows is a paper submitted to FEBS Letters reporting the data we have obtained.

1. Introduction

Formation of molecular oxygen as a photosynthetic by-product of ancient autotrophs is believed to have mediated the evolution of aerobes on earth. The energetic benefits of aerobic respiration are not, however, without cost. Metabolic oxygen-derived species such as hydrogen peroxide (H_2O_2) and the superoxide radical ($O_2^{\cdot}$) can form the extremely reactive hydroxyl radical ($OH^{\cdot}$) [16], which is thought to react with critical biomolecules in aerobic organisms. The resulting oxidative damage has been implicated in several human diseases [17]. DNA in particular has been shown to be vulnerable to oxygen radical insult both *in vitro* and *in vivo* [1-3]. Moreover, oxidative damage to DNA has been strongly correlated with carcinogenesis [4-10].

Type II pulmonary epithelial cells line the alveolar surface of the lung, and are exposed to a variety of potential oxidative stresses. Type II cells comprise approximately 15% of cells in the distal lung, and occupy approximately 10% of the alveolar surface [18]. Type I pulmonary epithelial cells account for the majority of alveolar surface area, but type II cells are better-characterized, primarily due to development of reliable procedures for their isolation and primary culture [19]. Type II cells have three primary functions: (a) synthesis and secretion of pulmonary surfactant, (b) maintenance of alveolar surface by proliferation and differentiation into type I cells, and (c) minimization of alveolar fluid *via* sodium transport from the apical to the basolateral epithelial surface [18].

In the present study rat type II pulmonary epithelial cells were exposed to H_2O_2 *in*

vitro. DNA was then extracted and monitored for fourteen different DNA base modifications using gas chromatography/mass spectrometry with select ion monitoring (GC/MS-SIM). Twelve of fourteen base products increased significantly above baseline levels, as detailed below. These base modifications are typical of the types of DNA damage caused by exposure to oxygen radicals [1], and several have been correlated with carcinogenesis [4-10].

2. Materials and Methods

2.1 Reagents

8-Azaadenine, 6-azathymine, 8-bromoadenine, 5-hydroxyuracil (isobarbituric acid), 4,6-diamino-5-formamidopyrimidine (FAPy-adenine), 2,5,6-triamino-4-hydroxypyrimidine, and 5-(hydroxymethyl)uracil were purchased from Sigma Chemical Co. (Poole, Dorset, UK). 8-Hydroxyguanine was from Aldrich (Poole, Dorset, UK). 6-amino-8-hydroxypurine (8-hydroxyadenine) and 2,6-Diamino-4-hydroxy-5-formamidopyrimidine (FAPy-guanine) were synthesized (courtesy of Dr. H. Kaur, King's College, London) by, respectively, treatment of 8-bromoadenine with concentrated formic acid (95%) at 150°C for 45 min with purification by crystallization from water [20], and treatment of 2,5,6-triamino-4-hydroxypyrimidine with concentrated formic acid and purification by recrystallization from water [21]. Thymine glycol was synthesized by reaction of 5-methyluracil with OsO_4 for 1 hr at 60°C, and excess OsO_4 was removed by freeze-drying [11]. 2-hydroxyadenine, 5-hydroxycysteine, and 5-

(hydroxymethyl)hydantoin were gifts from Dr. Miral Dizdaroglu (National Institute of Standards and Technology, Gaithersburg, MD). Soybean trypsin inhibitor, triton-X-100, phenol, and chloroform:isoamyl alcohol (24:1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $BaSO_4$, hydrogen peroxide (30%), and NH_4OH were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's minimum essential medium (DMEM), Hank's balanced salt solution (HBSS), and Joklik's modified eagle's medium (JMEM) were from GIBCO (Gaithersburg, MD, USA). Cellu.Sep dialysis membranes with relative molecular mass cut off of 3500, silylation grade acetonitrile, and bis(trimethylsilyl)trifluoro- acetamide (BSTFA) (containing 1% trimethylchlorosilane, TMS) were obtained from Pierce Chemical Co. (Rockford, IL, USA). Pentobarbitol was from Dodge Laboratories; elastase was from Elastin Products, Inc.; DNase 1 from Calbiochem (La Jolla, CA, USA); newborn calf serum from ICN Biochemicals (Costa Mesa, CA); and percoll from Pharmacia (Piscataway, NJ, USA). Dialysis membranes of molecular mass cut off of 3500 were purchased from Spectrum supplied by Pierce Chemical Co., USA.

2.2 Isolation and primary culture of type II cells.

Type II cells were isolated from lungs of male Sprague-Dawley rats (200-250 g body weight; Charles River Laboratories). Rats were anesthetized with pentobarbitol sodium (60 mg/kg body wt). Procedures for type II cell isolation are detailed elsewhere [19]. Briefly, after washout of the pulmonary circulation and repeated lavage of the airways, alveolar cells were dispersed by intratracheal instillation of Joklik's minimal

essential medium (JMEM) containing elastase and $BaSO_4$. After 30 min, proteolysis was inhibited by instillation of JMEM containing soybean trypsin inhibitor, deoxyribonuclease, and newborn calf serum. Lungs were then minced and filtered through Nitex-HC 160 nylon mesh (Tetko, Elmsford, NY); cells were collected by centrifugation and were resuspended in JMEM containing deoxyribonuclease. Type II cells were purified by density centrifugation on discontinuous Percoll gradients and by differential attachment. The final cell preparation was plated in six-well tissue culture plates (Falcon) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 2×10^6 cells/well. The day of cell isolation is designated as day 0.

2.3 Exposure of type II cells to hydrogen peroxide

H_2O_2 was diluted in Hank's balanced salt solution (HBSS) immediately prior to exposure to achieve the desired concentration. HBSS was used because the pyruvate in culture media has been shown to scavenge H_2O_2 [1]. On day 1 growth medium was removed and cells at 90% confluency were washed 2 times with sterile, filtered PBS. H_2O_2 was then added to six-well plates (2 ml per well) and cells were incubated for 60 min at 37°C.

2.4 DNA isolation

After incubation cells were immediately washed twice with sterile, filtered PBS. Cells from an entire six-well plate were pooled to obtain one sample for analysis (approximately 1.2×10^7 cells). Harvested cells were pelleted, resuspended in 1 M

NH_4OH/0.2% Triton-x-100, and left at 37°C for 30 min with periodic agitation. After extractions with phenol and chloroform:isoamyl alcohol (24:1), DNA was precipitated with 100% ethanol/0.1 M NaOAc and purity was verified using A_{260}/A_{280} ratios.

2.5 Analysis of DNA Base Modifications by Gas Chromatography-Mass Spectrometry

Derivitized samples were analyzed by GC-MS (Hewlett-Packard 5890II gas chromatograph interfaced with a Hewlett Packard 5917A mass selective detector). Preparation, derivitization, and analysis of samples were performed as described previously [15,22] with the following modifications. The injection port and the GC-MS interface were kept at 250 and 290°C, respectively. Separations were carried out on a fused silica capillary column (12 m x 0.2 mm i.d.) Coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.33 μm) (Hewlett-Packard). Helium was the carrier gas with a flow rate of 0.93 mL/min. Derivatized samples (1.0 μL) were injected into the GC injection port using a split ratio of 8:1. Column temperature was increased from 125 to 175°C at 8°C/min after 2 min at 125°C, then from 175 to 220°C at 30°C/min and held at 220°C for 1 min, and finally from 220 to 290°C at 40°C/min and held at 290°C for 2 min. Selected-ion monitoring was performed using the electron ionization mode at 70 eV with the ion source maintained at 185°C.

Quantitation of modified bases was achieved by relating the peak area of the compound with the internal standard peak area and applying the following formula:

$$\text{concn (nmol/mg of DNA)} = A/A_{IST} \times [IST] \times (1/K)$$

where K = relative molar response factor for each base, A = peak area of product, A_{IST} =the peak area of internal standard, and [IST] = concentration of the internal standard (5 nmol/mg of DNA). K constants were calculated from the slopes of the calibration curves constructed using known concentrations of internal standards and authentic compounds.

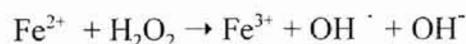
3. Results

Using *in vitro* cultures of rat type II lung epithelial cells, fourteen DNA base modifications were measured by GC/MS-SIM after exposure to H_2O_2. Twelve of fourteen base products rose significantly ($P < 0.05$, Student's t-test) upon exposure to 5.0 mM H_2O_2 for 60 min at 37°C. These twelve products were 8-hydroxyguanine (8-OH-Gua), 2,6-diamino-4-hydroxy-formamidopyrimidine (FAPy-Gua), 8-hydroxyadenine (8-OH-Ade), 4,6-diamino-5-formamidopyrimidine (FAPy-Ade), 2-hydroxyadenine (2-OH-Ade), 5-hydroxyhydantoin (5-OH-Hyd), 5-(hydroxymethyl)hydantoin (5-OHMeHyd), 5-(hydroxymethyl)uracil (5-OHMeUra), 5-hydroxycytosine (5-OH-Cyt), *cis*-thymine glycol, *trans*-thymine glycol, and xanthine. The two products that did not change significantly from baseline were 5-hydroxyuracil (5-OH-Ura) and hypoxanthine. (See Table 1). Of the products that increased in concentration, the largest net increase was seen in FAPy-Gua, which increased from 0.423 ± 0.300 to 3.865 ± 0.650 nmol/mg DNA. Increases in other base products ranged from 2.3 to 9.5-fold and final concentrations ranged from 0.100 ± 0.030 nmol/mg (5-OHMeUra) to 2.270 ± 0.150 nmol/mg DNA (*cis*-thymine glycol).

Several base modifications increased significantly from baseline levels upon exposure to comparatively low concentrations of H_2O_2, but did not incur proportional levels of damage when exposed to higher H_2O_2 concentrations. For example, xanthine concentrations rose from 1.278 ± 0.286 nmol/mg DNA to 2.289 ± 0.306 nmol/mg DNA upon exposure to 0.2 mM H_2O_2, but rose to only 2.255 ± 0.342 nmol /mg DNA after exposure to 5.0 mM H_2O_2, a value statistically equivalent to the concentration reached upon exposure to 0.2 mM H_2O_2. Similar patterns were also observed for other base modifications, including 2-OH-Ade, 8-OH-Ade, and 5-OHMeHyd. FAPy-Gua and 5-OH-Hyd diverged from this pattern and followed a more linear dose-response curve (See Figures 1-4, noting differences in scale for each figure).

4. Discussion

H_2O_2 was used for incubation because it is a by-product of normal aerobic metabolism. In addition, high local concentrations of H_2O_2 and other oxidizing agents can be formed by polymorphonuclear leukocytes (PMNs) and macrophages during inflammation [23]. H_2O_2 is known to penetrate cell membranes easily [24], and will in the presence of a transition metal react to form $OH^{\cdot}$ in a Fenton-type reaction. For example:



$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Table 1

Levels of DNA base modifications increase in rat type II lung epithelial cells following exposure to H_2O_2.

Modified Base Product	Control Levels nmol/mg DNA	+ 5.0 mM H_2O_2 nmol/mg DNA	P value
5-OH,Me-Hyd	0.095 ± 0.031	0.374 ± 0.068	0.003
5-OH-Hydantoin	0.091 ± 0.031	0.627 ± 0.061	<0.001
5-OH-Uracil	0.093 ± 0.030	0.162 ± 0.031	NS
5-OH,Me-Uracil	0.019 ± 0.006	0.100 ± 0.030	0.010
5-OH-Cytosine	0.202 ± 0.016	0.600 ± 0.100	0.002
cis-Thymine Gly	0.258 ± 0.039	2.270 ± 0.150	<0.001
trans-Thyime Gly	0.727 ± 0.171	1.707 ± 0.227	0.004
Hypoxanthine	4.367 ± 0.245	4.289 ± 0.631	NS
FAPy-Adenine	0.152 ± 0.035	1.445 ± 0.283	0.001
8-OH-Adenine	0.072 ± 0.022	0.343 ± 0.078	0.004
Xanthine	1.278 ± 0.286	2.255 ± 0.342	0.019
2-OH-Adenine	0.047 ± 0.028	0.122 ± 0.026	0.027
FAPy-Guanine	0.423 ± 0.300	3.865 ± 0.650	0.001
8-OH-Guanine	0.812 ± 0.233	2.136 ± 0.520	0.016

Cells were exposed to 5.0 mM H_2O_2. for 60 min at 37°C. Results are means of three experiments ± S.D. A Student's t-test was used to determine P values. NS = not significant at $P < 0.05$.

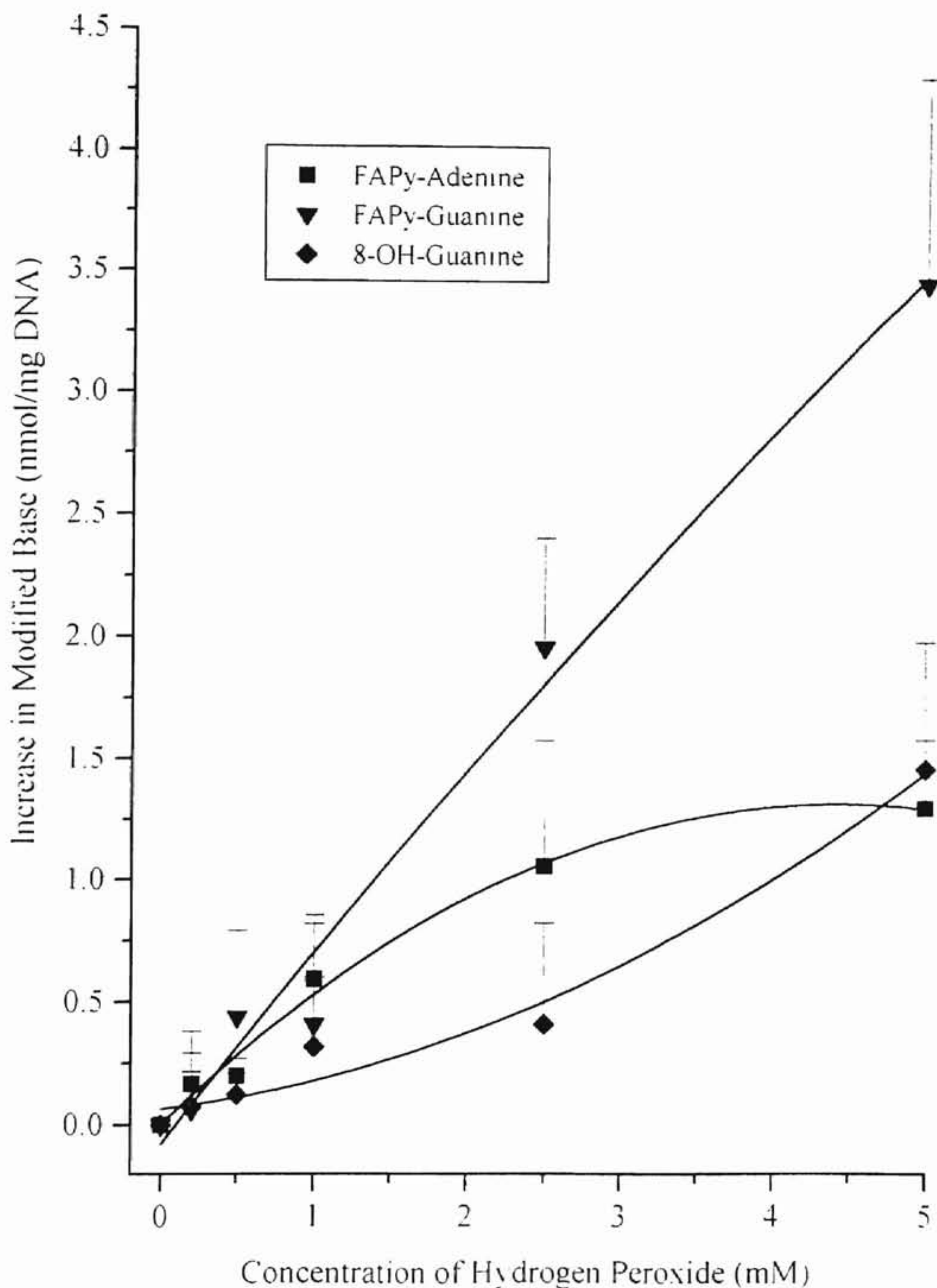


Figure 1. Effect of increasing H_2O_2 concentration (mM) on the concentration of DNA base modifications (nmol/mg DNA, background concentrations subtracted) in cells exposed to H_2O_2 for 60 min. Experiments were conducted as described in section 2. Data points are mean ± S.D. (n = 3).

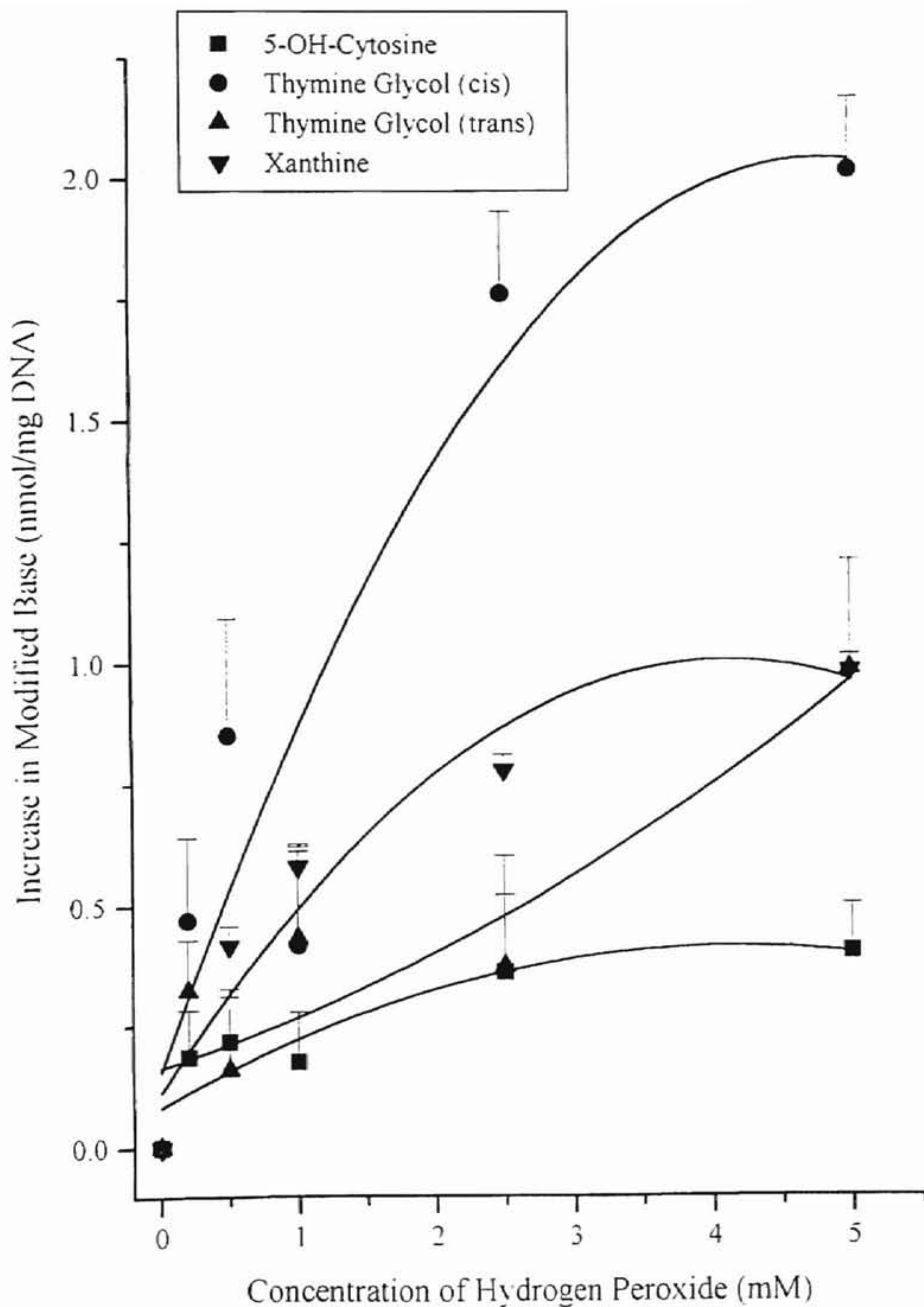


Figure 2. Effect of increasing H_2O_2 concentration (mM) on the concentration of DNA base modifications (nmol/mg DNA, background concentrations subtracted) in cells exposed to H_2O_2 for 60 min. Experiments were conducted as described in section 2. Data points are mean ± S.D. (n = 3).

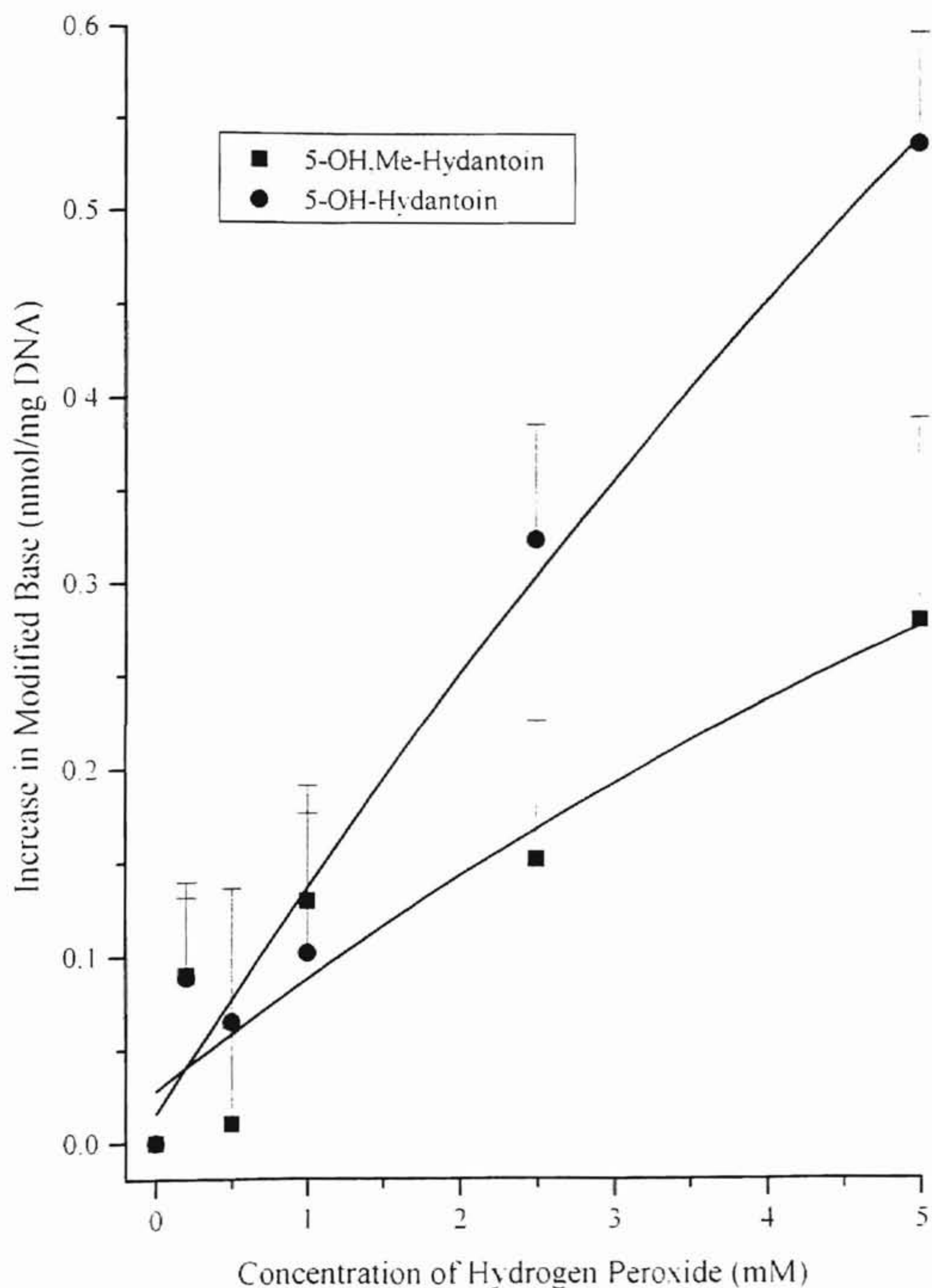


Figure 3. Effect of increasing H_2O_2 concentration (mM) on the concentration of DNA base modifications (nmol/mg DNA, background concentrations subtracted) in cells exposed to H_2O_2 for 60 min. Experiments were conducted as described in section 2. Data points are mean ± S.D. (n = 3).

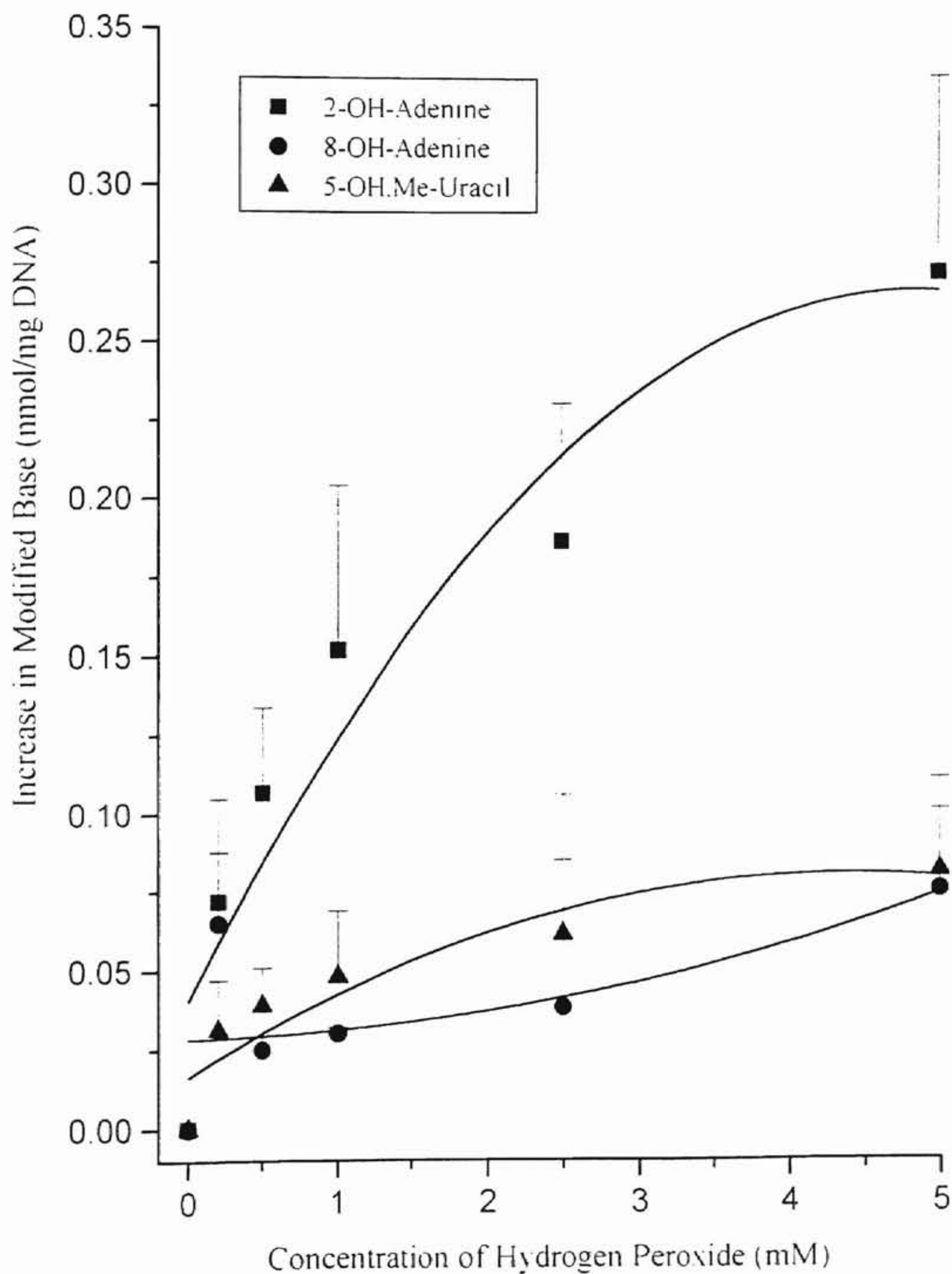
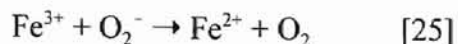


Figure 4. Effect of increasing H_2O_2 concentration (mM) on the concentration of DNA base modifications (nmol/mg DNA, background concentrations subtracted) in cells exposed to H_2O_2 for 60 min. Experiments were conducted as described in section 2. Data points are mean ± S.D. (n = 3).

Fe^{3+} can be recycled back to Fe^{2+} by O_2^- or other reducing agents:



$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \qquad [25]$$

The types of DNA modifications that occur during $OH^\cdot$ exposure depend upon reaction conditions [1]. For example, iron-ion dependent $OH^\cdot$-generating systems result in different patterns of DNA damage than systems dependent on copper ions [3]. Similarly, OH-adducts such as 8-OH-Gua are favored under oxidative conditions while ring-opening products such as FAPy-Gua are favored under reductive conditions [10]. Investigators have thus noted the importance of screening DNA for several modified base products rather than testing for one particular biomarker of oxidative stress [1]. We have therefore looked at fourteen different modified bases in this study.

The observed increases in modified base products indicate that type II pulmonary epithelial cell DNA is vulnerable to oxidative damage. While it is clear that oxidative damage is correlated with risk of mutagenesis and carcinogenesis, it is more difficult to interpret the meanings of specific patterns of DNA base damage. 8-OH-Gua has been studied intensively and demonstrated to be mutagenic. Several groups have shown that DNA templates with 8-OH-Gua adducts incorporated are misread by polymerases, both at the site of adduct incorporation and at adjacent bases [26-29]. G→T transversions are the most commonly observed mutations, but other point mutations have been documented. Increases in 8-OH-Gua levels have also been correlated with carcinogenesis in numerous studies [30-34]. Therefore the 2.6-fold dose-dependent increase of 8-OH-Gua levels observed in type II cells exposed to H_2O_2 suggests that they

have been predisposed to mutagenesis.

Insertions of 8-OH-Ade into DNA templates have induced mammalian polymerases to misincorporate bases, resulting in A→G transitions and A→C transversions [35]. The mutagenic potential of 8-OH-Ade suggests that the 4.7-fold increase in 8-OH-Ade observed in type II cells increases mutagenic risk. In contrast to OH-adducts, ring-opened FAPy residues appear to block DNA synthesis [36] and mRNA transcription [37], and are putatively non-mutagenic. Correlations exist between carcinogenesis and levels of 5-OH-Cyt [38,39], 5-OH-Hyd [39,40], and 5-OHMeUra [41], but little is known about molecular mechanisms involved. The question of whether these base modifications are mutagenic or simply occur concomitantly with other mutagenic lesions remains unanswered. Similarly, while increases in levels of thymine glycol have been linked to oxidative insult and carcinogenesis [1,41], molecular mechanisms have yet to be elucidated. One mechanism of mutation that all oxidative DNA damage is likely to induce is DNA repair. Modified bases are subject to repair in living cells, and DNA repair is more error-prone than DNA replication by orders of magnitude [34].

Recent studies of human tissues suggest a complex pattern of relationships between oxidative base damage and oncogenic potential. In a study of human breast carcinoma tissues, levels of 8-OH-guanine were found to be higher than in normal tissues; however, FAPy-Ade residues in the same breast carcinomas were significantly lower than in normal tissues [9]. In contrast, metastatic breast tissue was found to have elevated levels of FAPy-adenine compared to non-metastatic breast tumor tissue [10],

suggesting that conditions favoring tumor formation differ from conditions favoring tumor progression [42]. As it is difficult to interpret patterns of oxidative damage at this time, we conclude simply that type II cells are vulnerable to oxidative damage. Further *in vivo* studies will be necessary to correlate patterns of base damage to mutagenicity and carcinogenicity.

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VITA

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