

Photoadducts and Peroxynitrite: Implications in Pathophysiology and Immunopathology

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Abstract

DNA adducts are a form of DNA damage caused by covalent attachment of a chemical moiety to DNA. Recent evidence suggests associations between the occurrence of adducts formed by specific compounds and various types of toxicity, such as mutation, cancer, and developmental effects. Small DNA or protein adducts can occur both from chemical exposure and from normal metabolic processes. Peroxynitrite is a relatively long-lived oxidant that may serve as an important cytotoxic agent. Its biological effects are due to its reactivity toward many molecules including lipids, amino acids and nucleic acids. *In vivo*, peroxynitrite formation represents a crucial pathological mechanism in conditions such as stroke, myocardial infarction, chronic heart failure, diabetes, inflammation, neurodegenerative disorders and cancer.

Keywords: Peroxynitrite; Nitric oxide; DNA-adducts

Abbreviations: TNF: Tumor Necrosis Factor; HPLC: High-Performance Liquid Chromatography; PON: Peroxynitrite; PKB: Protein Kinase B; NFκB: Nuclear Factor Kappa B; ETC: Electron Transport Chain; BH4: Tetrahydrobiopterin; NOS: Nitric Oxide Synthase; DHR: Dihydro Rhodamine 123; PARP: Poly-ADP Ribosyl Synthetase or Polymerase; iNOS: Inducible NO Synthase; STZ: streptozotocin; PAHs: Polycyclic Aromatic Hydrocarbon.

Introduction

DNA can be damaged by many different sorts of mutagens, which change the DNA sequence. Mutagens include oxidizing agents, alkylating agents and also high-energy electromagnetic radiation such as ultraviolet light and X-rays. DNA damage depends on the type of mutagens. For example, UV light can damage DNA by producing thymine dimers, which are cross-

links between pyrimidine bases [1]. On the other hand, oxidants such as free radicals or hydrogen peroxide produce multiple forms of damage, including base modifications, particularly of guanosine, and double-strand breaks. In each human cell about 500 bases suffer oxidative damage per day. Of these oxidative lesions, the most dangerous are double-strand breaks, as these are difficult to repair and can produce point mutations, insertions and deletions from the DNA sequence, as well as translocations. Many mutagens fit into the space between two adjacent base pairs, this is called intercalation. Most intercalators are aromatic and planar molecules, such as ethidium bromide, daunomycin, and doxorubicin. To be an intercalator to fit between base pairs, the bases must separate, distorting the DNA strands by unwinding of the double helix. This inhibits both transcription and DNA replication, causing toxicity and mutations [2]. As a result, DNA intercalators are often carcinogens, and

benzo- pyrene diol epoxide, acridines, aflatoxin and ethidium bromide are well-known examples. Nevertheless, due to their ability to inhibit DNA transcription and replication, these toxins are also used in chemotherapy to inhibit rapidly-growing cancer cells. The information carried by DNA is achieved via complementary base pairing, a cell may simply copy its genetic information in a process called DNA replication [3].

A DNA adduct is a piece of DNA covalently bonded to a (cancer causing) chemical leading to a cancerous cell or carcinogenesis [4]. DNA adducts are used as biomarkers and as such are themselves measured to reflect the amount of cancer in the subjects, i.e. rats or other animals. Under experimental conditions, example of such DNA adducts is DMBA [7, 12-dimethyl-benz [a] anthracene]. DNA and protein adduct worked as a marker of genotoxicity, determination of the interaction products (adducts) of a carcinogen with DNA or protein indicates the amounts of genotoxicity material that has reached the tissue. The cross linking of genetic material to protein is one of the fundamental lesions produced in the biological system by ultraviolet light. Lipid peroxidation products are also bound to DNA in the human liver and leukocytes. In chemical carcinogenesis, the formation of a carcinogen DNA adduct is a critical step and therefore considered an important biomarker during the initiation stage. Gene polymorphism thought to modify tobacco related cancer risk due to which of DNA adducts get complex [4]. The inducibility of DNA adducts *in vitro* appear to be a risk factor in the development of lung, head and oral cancer. Cellular toxicity occurs when adduct formation disrupts the protein structure function. DNA protein adduction induces neurotoxicity at the molecular and cellular levels. Examples of DNA adducts are: Cisplatin-DNA adduct, polycyclic aromatic hydrocarbons-DNA adduct, Mitomycin C-DNA adduct, Anthramycin-DNA adduct, and DNA-lysine adduct [5].

Adducts induce harmful immune responses. Adduct formation results in increased secretion of messenger molecules cytokines and chemokines that mediate communication among cells and promote inflammation e.g. a cytokine called tumor necrosis factor (TNF) [5]. Several neurodegenerative diseases such as Alzheimer's and Parkinson's as well as septic shock and inflammation involve formation of reactive oxygen and nitrogen species that include peroxynitrite (PON) [6]. Peroxynitrite can also react with endogenous antioxidants. Therefore, dietary supplementation with antioxidants may help in these diseases. An exogenous antioxidant, vanillin (4-hydroxy-3-methoxybenzaldehyde), widely used as a food flavoring agent, was evaluated for its ability to scavenge peroxynitrite and inhibit peroxynitrite-mediated reactions. Nitration of tyrosine by peroxynitrite was

assessed by high-performance liquid chromatography (HPLC). This reaction was inhibited by vanillin. The oxidation of dihydrorhodamine-123 to fluorescent rhodamine-123 was also inhibited by vanillin. The products of this reaction were analyzed by HPLC, and hydroxy vanillin was identified as one of the five products with absorption at 350 nm. These data demonstrate that vanillin effectively scavenges peroxynitrite in cell-free systems. Proteolysis products of proteins damaged by glycation, oxidation, and nitration-glycated, oxidized, nitrated amino acid (glycation, oxidation, and nitration free adduct) are waste products normally excreted in urine and cleared in peritoneal dialysate [6].

Peroxynitrite as a reactive nitrogen species is a potent oxidant and nitrating species formed from the reaction between the free radical's nitric oxide and superoxide. An excessive formation of peroxynitrite mechanism contributing to cell death and in multiple cardiovascular pathologies, such as myocardial infarction, heart failure and atherosclerosis [7]. Peroxynitrite shows ability to nitrate tyrosine residues and affects cellular processes dependent on tyrosine phosphorylation. Peroxynitrite shows its effect in the redox regulation of cardiovascular homeostasis, including protein kinase B (PKB) and C (PKC), nuclear factor Kappa B (NFκB) [6]. The mode of cell death induced by peroxynitrite can be necrosis, apoptosis, or mixed types of cell death, depending on peroxynitrite concentration, on the duration of peroxynitrite exposure & on intracellular ATP levels. It has been reported that peroxynitrite can induce apoptosis in thymocytes and the toxicity of thymocytes induced by peroxynitrite can be mediated by the release of zinc from intracellular stores [7].

Free Radicals

Free radicals are atoms or molecules or ions with unpaired electrons or having open shell configurations. These unpaired electrons are usually highly reactive. Radicals play an important role in human physiology and biochemistry and other chemical processes for example, superoxide and NO regulate many biological processes, such as controlling vascular tone. Historically the term "radical" has also been used for bound parts of the molecule, especially when they remain unchanged in reactions these are called functional groups e.g. methyl alcohol consisting of methyl "radical" [8].

Formation of radical may involve of covalent bond homolytically, a process that require significant amount of energy. Homolytic bond cleavage mostly often occurs between two atoms of similar electronegativity. Radical may also be formed by single electron oxidation or reduction of an atom or molecule. An example is the production of superoxide by electron transport chain

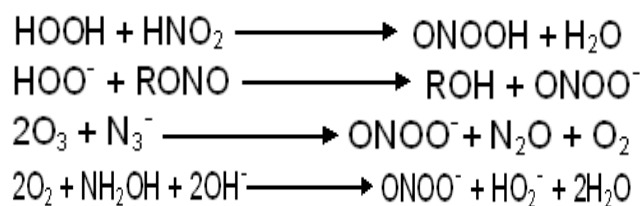
(ETC). The flammability of material is also strongly dependent on the concentration of free radical [8]. When a hydrocarbon is burned, many different oxygen radicals are involved. The first thing to form is a hydroperoxide radical which reacts into hydroperoxide that break up into hydroxide radical [8].

Free radical plays an important role in many biological processes, some of which are necessary for life such as intra cellular killing of bacteria by neutrophil granulocytes. Many forms of cancer are thought to be the result of reaction between free radical and DNA, resulting in mutations that adversely affect the cell cycle and potentially lead to malignancies [8]. Free radical contributes to alcohol induced liver damage. Radicals in cigarette smoke leads to adverse effects in the lung. This process promotes the development of emphysema. The body has many mechanisms to minimize free radical induced damage and to repair damage which is takes place by the enzyme super oxide dismutase, catalase, glutathione peroxidase and glutathione reductase. In addition, antioxidants play a key role in these defense mechanisms. These are three vitamins A, C, and E and polyphenol in addition bilirubin and uric acid can act as antioxidant to neutralize certain free radicals. Bilirubin comes from breakdown of RBC's contents while uric acid is a breakdown product of purines, increase in bilirubin leads to jaundice, which can damage uric acid cause gout [9].

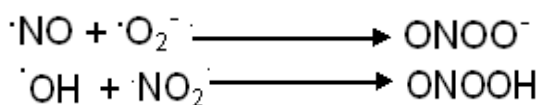
Formation of Peroxynitrite

Peroxynitrite is formed when carbon monoxide gets bind with heme proteins such as mitochondrial cytochrome C oxidase leading to nitrosative stress and cardiac manifestation include myocardial ischemia, and cardiogenic pulmonary edema [9]. Moreover, up regulation of NADH oxidase leads to increased presence of superoxide & peroxynitrite. Excessive amounts of NO are known to be able to produce peroxynitrite, an important reactive nitrogen compound, by reacting with superoxide [10]. Peroxynitrite formation increased after myocardial infarction indicating strong interaction between NO and superoxide [12].

Peroxynitrite is formed in nitrate salts or nitrate-containing solutions when exposed to ionizing radiation or ultraviolet light [13]. Solutions can also be prepared by a variety of chemical reactions, including the reaction of hydrogen peroxide with nitrous acid reaction of the hydro peroxide anion with organic and inorganic nitro sating agents, reaction of ozone with the azide ion or apparently [14], reaction of O₂ with compounds capable of generating the nitroxyl anion [10].



These preparations invariably contain unreacted materials or decomposition products, particularly nitrite ion, which can significantly modulate the peroxynitrite chemical reactivity [15]. Peroxynitrite is also formed in radical-radical coupling reactions, notably superoxide with nitric oxide and hydroxyl radical with nitrogen dioxide [16].



Peroxynitrite has been isolated as the tetramethylammonium salt in liquid ammonia. Formation of peroxynitrite in both solids and solutions is indicated by the appearance of yellow coloration, which is due to ultraviolet absorption bands into the visible region [17].

Peroxynitrite is a powerful oxidant that has been shown to react with a wide variety of inorganic and organic reductants. Nitric oxide and superoxide are generated in the bloodstream, neuronal tissues, and phagocytic cells of animals in sufficient quantities to form peroxynitrite [17]. Peroxynitrite oxidant shows its major role in diseases and tissue damage associated with oxidative stress. In natural cellular defense mechanisms, it acts against microbial infection. Within the central nervous system, tetrahydrobiopterin (BH₄) is an essential cofactor for dopamine & serotonin synthesis. BH₄ is an essential cofactor for all iso-forms of nitric oxide synthase (NOS) [18]. NOS may generate superoxide whilst other BH₄ saturated NOS enzymes may be generating NO, thus leading to favor peroxynitrite generation. If the peroxynitrite is not scavenged (for e.g. by antioxidants such as reduced glutathione) irreversible damage to critical cellular enzymes like alpha ketoglutarate dehydrogenase & possibly pyruvate dehydrogenase could occur leading to neurodegenerative conditions such as Parkinson's disease & Alzheimer's disease [18].

Peroxynitrite Biochemistry

Peroxynitrite, the reaction product of nitric oxide and superoxide anion, and a powerful oxidant, was found to nitrate as well as oxidize adenine, guanine, and xanthine nucleosides [19].

With the help of sensitive reverse-phase HPLC method having the nitro product at the first electrode and detects the reduced product by oxidation at the second electrode, it also helps to detect femtomole levels of 8-nitroguanine and 8-nitroxanthine. This method was used to separate and identify nitration and oxidation product formed from the reactions of nucleosides with peroxynitrite [16]. Peroxynitrite nitrates deoxyguanosine to give the very unstable 8-nitrodeoxyguanosine, in addition to 8-nitroguanine & 8-Nitrodeoxyguanosine. A decrease in the reaction pH resulted in a decrease in the level of C8-nitration [20]. Peroxynitrite also oxidizes deoxyguanosine in a pH-dependent manner, to give 8-oxodeoxyguanosine [13].

Guanosine and xanthosine exhibit reactivity like that of deoxyguanosine toward peroxynitrite at neutral pH, producing the 8-nitronucleosides as well as 8-nitroguanine and 8-nitroxanthine, respectively. 8-nitroguanosine found to be more stable than 8-nitrodeoxyguanosine. In contrast to guanine nucleosides, adenine nucleosides undergo peroxynitrite-mediated C8 oxidation to give the corresponding 8-oxoadenine nucleosides [13].

Peroxynitrite decomposes rapidly to nitrite and nitrate as a stable product [6]. Reactive nitrogen species are now considered to play an important role in various pathologies [21]. Peroxynitrite has ability to react with any component of the cell including lipids, proteins and DNA [6]. It also causes DNA damage and contributes to carcinogenic processes. Peroxynitrite triggers a signaling pathway that finally leads to cytotoxicity [6]. It is also well established that peroxynitrite is extremely short lived at physiological pH values and the formation of 3-nitro tyrosine by peroxynitrite reaction with tyrosyl residues which is used as a stable marker [9]. Indirectly peroxynitrite formation can be measured by use of fluorescent probe dihydro rhodamine 123(DHR) which accumulate in the mitochondria when oxidized by various reactive species including peroxynitrite, tert-butyl hydrogen peroxides an agent resulting in formation of endogenous peroxynitrite. Under these conditions DHR was not directly oxidized by peroxynitrite [7]. Nitric oxide (NO) is a free radical whose inappropriate formation might cause deleterious effect in human pathologies such as acute endotoxemia, neurological disorders, atherosclerosis and ischemia/reperfusion [22]. Nitrite also combines with amines in stomach to form nitrosamines which are potent cancer-causing agents [22].

Peroxynitrite is an extremely reactive entity and has *in vivo* existence. Its interaction with biomolecule may cause oxidation and nitration. H2A histone exposed to peroxynitrite mediated structural changes in histone that were studied by ultraviolet and fluorescence spectroscopy, high performance liquid chromatography,

and polyacrylamide gel electrophoresis. Analysis of results revealed that carbonyl, nitro tyrosine and dityrosine contents were significantly increased in peroxynitrite-modified H2A compared with native H2A. Rabbits with peroxynitrite-modified H2A induced high titer antibodies. The immunogenicity of peroxynitrite-modified H2A was directly proportional to protein nitro tyrosine content and induced antibodies for the immunogen and good cross-reaction with nitrated epitopes of other modified proteins [6].

Free radicals like peroxynitrite create oxidative stress which may damage the biological macromolecules & causes malfunctioning of cellular growth and development [23]. Peroxynitrite is produced by the body in response to a variety of toxicologically relevant molecules including environmental toxins. It is also produced by the body in reperfusion injury and inflammation [24]. Peroxynitrite is a potent trigger of DNA strand breakage and activates nuclear enzyme poly-ADP ribosyl synthetase or polymerase (PARP) resulting in severe energy depletion and necrosis of the cells. Peroxynitrite decomposition catalysts suggest that neutralization of peroxynitrite is of significant therapeutic benefit to a various environmental toxin as well as in a variety of inflammatory and reperfusion disease conditions [25].

Reactivity of Peroxynitrite

Peroxynitrite is particularly effective oxidant of aromatic molecules that include ethers & organosulphur compounds [25]. The oxidation of NADH by peroxynitrite takes place indirectly by the radical intermediate which forms during the self-decomposition of peroxynitrite. Reaction of peroxynitrite anion with CO₂ results in the formation of an unstable nitrosoperoxy carbonate anion adduct. The major product of this reaction is ethyl 2-nitroacetoacetate [25]. Peroxynitrite shows reaction with cocoa procyanidin oligomer and tyrosine residues to give 3-nitrotyrosine. It induced oxidation of plasma lipids enhanced in stable hemodialysis patients. Peroxynitrite thought to contribute β -cell destruction during type-1 diabetes. Respiratory burst is (sometimes called oxidative burst) rapid release of reactive oxygen species (superoxide radical & hydrogen peroxide) from different types of cells. Peroxynitrite mediates the oxidation of thiol group of cysteine and glutathione [26].

Cytotoxic Effect of Peroxynitrite

Peroxynitrite is produced by increased expression of inducible NO synthase (iNOS) & oxidative stress [24]. Nitrosative stress causes cardiovascular depression in streptozotocin (STZ) diabetic rats [26]. Cultured vascular endothelial cell (EC) exposed to a steady

laminar shear stress results in peroxynitrite formation intramitochondrial and inactivation of the electron transport chain leading to suppression of respiration intracellularly [25].

Oxidative stress is caused by the imbalance between the production of reactive oxygen and a biological system that can readily detoxify the reactive intermediates or easily repair the resulting damage [27]. Disturbances in this normal redox state can cause toxic effects through the production of peroxides & free radical that damage all components of the cell [28]. In human's oxidative stress involved in many diseases such as atherosclerosis, Parkinson's disease, heart fatigue, myocardial infarction, Alzheimer's disease & chronic fatigue syndrome, but it may be important in prevention of ageing by induction of a process named mitohormesis [29]. Reactive oxygen species are also used in cell signaling, this is dubbed redox signaling. More severe oxidative stress can cause cell death & even trigger apoptosis while more intense stress causes necrosis [28]. More destructive aspect of oxidative stress is production of reactive oxygen species including free radical & peroxides. More aggressive radical species can cause extensive cellular damage [29]. ROS maintain a reducing environment within their cell [46].

In Fenton's and the Haber-Weiss reaction, hydroxyl radical is produced from reduce iron and H_2O_2 [13]. The hydroxyl radical leads to the modification of amino acid (e.g. meta-tyrosine, ortho-tyrosine formation from phenylalanine) and carbohydrate. Quinone catalyzes the production of superoxide from dioxygen and H_2O_2 from superoxide [29]. The immune system uses lethal effects of oxidants by the production of oxidizing species which accelerate phagocytes producing both ROS and RNS, inducing superoxide superoxide, nitric oxide and their reactive product peroxynitrite [6]. These highly reactive compounds in the cytotoxic response of phagocytes causes damage to host tissues. Spices rich in phenolics, act as potential protectors against the peroxynitrite [7]. The reperfusion injury is due to inflammatory response of damaged tissues. Damage to the cell membrane causes the release of more free radical. Leukocytes may also build up in small capillaries, obstructing them and leading to more ischemia. Reperfusion plays a part in Brain's ischemic cascade which is involved in stroke and brain trauma. Repeated bouts of reperfusion and ischemia leads to the formation and failure to heal of chronic wounds, such as pressure sores and diabetic foot ulcers. Hyperthermia has been shown to minimize a patient's production of deadly free radicals during reperfusion [29].

Different DNA-Protein Adducts Formed in the Body

Chemical modification of bases in DNA or amino acids in protein by toxic chemical lead to the formation of adducts. These chemicals may be carcinogenic in humans and cause oxidative damage to genomic DNA [30].

The environmental contaminants 3-nitrobenzathrone was recently shown to be a very strong bacterial mutagen, a class of mutagenic compounds presents in airborne particulate matter & diesel exhaust. By using ^{32}P -post labeling assay *in vitro* adduct formed by 3-nitrobenzathrone can be investigated [31]. 3-nitrobenzathrone binds covalently to DNA after metabolic activation, forming multiple adducts *in vitro*, all of which are reduction products [32]. These adducts may contribute to the known genotoxicity & carcinogenicity of extracts from airborne particulate [30]. DNA adducts are also formed by polycyclic aromatic hydrocarbons such as aromatic-DNA adducts in target tissues & WBC's. In target organs, highest DNA adducts levels were observed in skin & lowest in stomach after oral administration of benzo [a] Pyrene (B [a]P) [33].

Polycyclic aromatic hydrocarbons (PAHs) is found in complex mixture in environment such as automobile exhaust, cigarette smokes, foods, water & urban air. PAHs elicit cancer via covalent interaction with DNA, called PAH-DNA cancers [33]. In adducts PAH exerts their carcinogenic activities at the site of application. Respiratory tract seems to be most predominant site of (B (a) P) induced cancers, in addition to inhalatory smokers or in occupationally exposed workers [34]. Other exposure route especially via food also appear important for adduct formation in WBC [34]. Consumption of PAHs containing foods enhanced DNA adduct levels in lymphocytes and excretion of 1-hydroxypyrene in urine. Benzo[a] Pyrene is a procarcinogen, a mutagen, this molecule intercalates with DNA by covalently bonding to the nucleophilic guanine nucleotide at the N2 position. X-ray crystallography and NMR studies shows that this binding distorts the DNA, inducing mutation by perturbing double helical DNA structure. This disrupts normal process of copying DNA and induces mutation, which explains occurrence of cancer. This mechanism is like that of aflatoxin which binds to the N-7 position of guanine base. Benzo[a] Pyrene diol epoxide inactivates the tumor suppression activity in certain cells leading to cancer [35].

Effects of Peroxynitrite on DNA-Protein Adduct

Peroxynitrite is extremely short lived. It reacts with tyrosyl residues and to form the 3-nitrotyrosine. Reactive nitrogen species are appreciated as signaling molecule. Nitric oxide causes deleterious effects in various human pathologies such as acute endotoxemia, neurological disorder, atherosclerosis, & ischemia or reperfusion [36]. Peroxynitrite affect hepatic DNase, *in vitro* and *in vivo*. Exposure of amino acids, peptides & proteins to gamma radiation & peroxynitrite in presence of oxygen, gives high yields of hydrogen peroxide. Hydrogen peroxide formation on nuclear protein results in oxidative damage to associated DNA. These hydrogen peroxide derived radicals react with pyrimidine DNA bases & nucleosides leads to form adduct species. These adduct causes base transversions [37].

DNA-Arginine Adducts

Adducts arise from the chemical modification of bases in DNA or amino acids in protein by toxic chemicals & high energy UV radiation [38]. Arginine is an amino acid that has potential to participate in DNA-protein photo cross linking [39]. Determination of the interaction product (adduct) of a carcinogen with DNA or protein indicates the amount of genotoxicity [40]. DNA adducts give further information to the DNA damage levels as low as one adducts per 10⁸ nucleotides can be measured [40]. Protein adducts formed as a molecular mechanism in neurotoxicity. Cellular toxicity occurs when adduct formation disrupts protein structure and function, which secondarily cause damage to sub membrane organelles, metabolic pathways, or cytological processes [41]. Neurotoxicants can form adducts with nucleophilic residues on proteins. Proteins adducts play a causal role in generation of neurotoxicity. Adduct formation also leads to neurotoxicity [40].

Harmful Effects of Peroxynitrite on Human Body

Peroxynitrite is highly reactive compound with various harmful effects on cells & could therefore be an important microbicidal compound [27]. In addition to beneficial effects in host defense mechanism, the anion may have deleterious effects on host tissues. It causes number of disorders, examples are human asthma, acute lung injury, idiopathic pulmonary fibrosis, inflammatory bowel disease and animal models of septic shock [38]. The relative balance between these radicals have important implications for vascular pathophysiology in hypertension & other vascular states. Peroxynitrite can also be produced from human

internal mammary artery (radial arteries & cephalous vein) [42].

A change in the structure of DNA could either be due to radiation or interaction with free radicals. Radical that make native DNA immunogenic is peroxynitrite that modified native DNA leads to production of anti-DNA auto antibodies in SLE [7]. Peroxynitrite directly inhibits mitochondrial respiratory chain enzymes acute exposure of peroxynitrite selectively damages neurons [42].

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