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INTERACTIONS BETWEEN SUPEROXIDE AND NITRIC OXIDE: IMPLICATIONS IN DNA DAMAGE AND MUTAGENESIS

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1. ABSTRACT

Chronic inflammation is known to be associated with enhanced production of both nitric oxide (NO) and reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Patients with long-standing ulcerative colitis are also known to be at increased risk of developing colorectal cancer. Although NO and reactive oxygen intermediates alone have been known to damage DNA and to promote a wide array of mutagenic reactions, there is increasing evidence to suggest that the interaction between O_2^- and NO may dictate the type of mutagenic reactions produced at sites where both these free radicals are produced. In the absence of O_2^-, NO will engage in nitrosative chemistry to yield stable N-nitrosamine derivatives of secondary amines and promote nitrosative deamination of DNA bases. As the flux of O_2^- is increased, nitrosation reactions are suppressed and oxidative chemistry is enhanced. Thus, depending upon the fluxes of each radical either nitrosation or oxidation chemistry may predominate. The fundamental understanding between O_2^- and NO may provide new insight in the mechanisms responsible for inflammation-induced mutagenesis.

2. INTRODUCTION

Active episodes of ulcerative colitis are characterized by infiltration of large numbers of phagocytic leukocytes into the mucosal interstitium. This enhanced inflammatory infiltrate is accompanied by extensive injury to the mucosa. A growing body of clinical and experimental data suggests that severe long-standing inflammation of the colon is associated with an increase risk of colorectal cancer (1-3). In addition, investigators have shown that inflammation enhances the formation of colonic tumors in experimental animals given known carcinogens (4,5). Despite these studies, the mechanisms by which inflammation promotes tumor formation remain poorly understood. It has been suggested that certain leukocyte-derived products may act as endogenous carcinogens or tumor promotors *in vivo* (1). Recent studies have shown that neutrophils and macrophages are capable of generating the free radical nitric oxide (NO) via an L-arginine-dependent pathway (6-9). Nitric oxide is unstable in the presence of molecular oxygen and will rapidly and spontaneously decompose to yield a variety of nitrogen oxides that are known to be potent nitrosating agents that will N-nitrosate primary and secondary amines to yield carcinogenic nitrosamines (10). Secondary nitrosamines require metabolic activation to yield alkylating agents that have been shown to activate certain oncogenes via the covalent modification of certain DNA bases (11). Nitrosative deamination of primary aromatic amines has been another suggested pathway by which NO-derived N-nitrosating agents produce transition and transversion mutations (12). The objective of this review is to discuss the chemical interactions between superoxide (O_2^-) and NO and to exmamine how these interactions may be involved in inflammation-induced DNA damage and mutagenesis.

3. NITRIC OXIDE AND N-NITROSATION REACTIONS

Exposure of critical genes to mutagenic conditions increases the probability of tumor development. Chronic inflammation is one such environment which promotes

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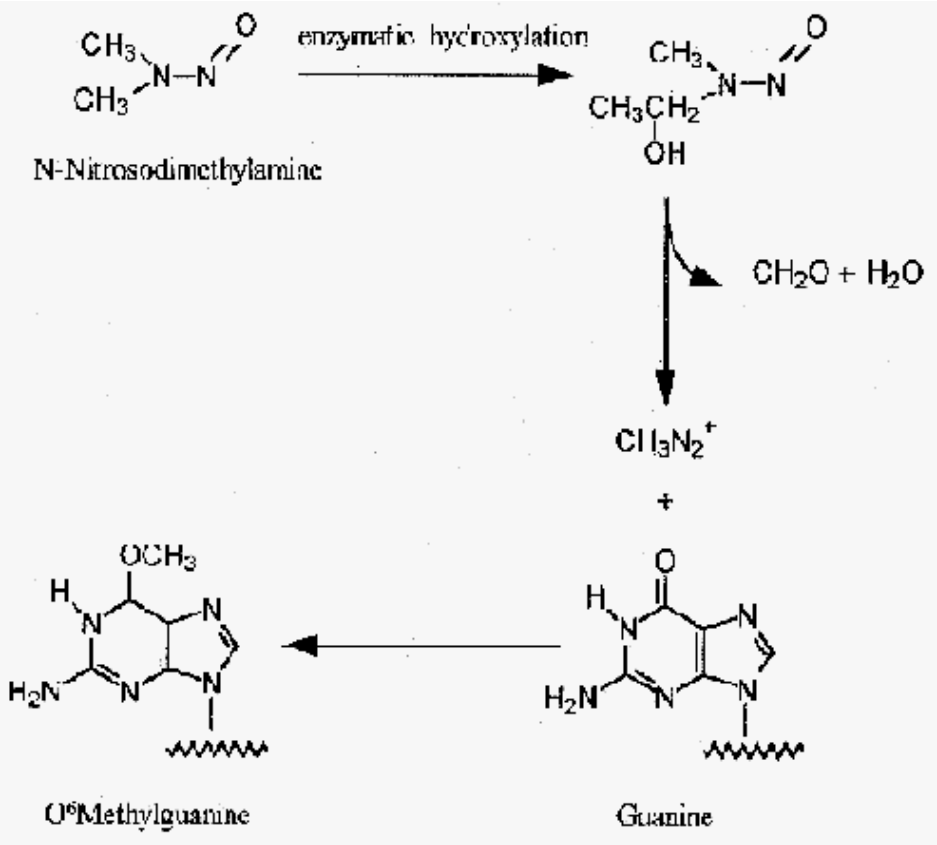

Figure 1: Nitrosamine-mediated alkylation of DNA bases. Secondary nitrosamines such as N-nitrosodimethylamine induce point mutations by alkylation of DNA bases such as guanine to form O^6methylguanine residues.

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malignant transformation. First, tissues neighboring inflammatory foci undergo increased cell division

Consequently, mutagenic effects associated with chronic inflammation can become multiplicative, as the chance of mis-repair and DNA exposure increase (13). Secondly, certain leukocyte-derived metabolites and inflammatory mediators cause genomic damage, thereby increasing the probability of nicks, deletions, and point mutations (14).

In situations of chronic inflammation, target cells may be exposed to large amounts of NO (as much as 10^4 molecules/cell/s) (15). Nitric oxide will autooxidize in the presence of molecular oxygen (O_2) to yield a variety of nitrogen oxides:

$$2NO + O_2 \rightarrow 2NO_2$$
$$2NO + 2NO_2 \rightarrow 2N_2O_3$$
$$2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

where NO_2, N_2O_3, and NO_2^- represent nitrogen dioxide, dinitrogen trioxide, and nitrite, respectively. Of these, NO_2 and N_2O_3 have drawn particular interest due to their ability to N-nitrosate certain nucleophilic substrates such as primary and secondary amines (16).

Such nitrosating species have been shown to promote the nitrosative deamination of primary aromatic amines, including purines and pyrimidines, via the formation of nitrosamine and diazonium ion intermediates (17). Deamination of cytosine, methyl cytosine, adenine, or guanine results in the formation of uracil, thymine, hypoxanthine, and xanthine respectively. Base conversion of cytosine and methyl cytosine can lead ultimately to a base pair substitution mutation, while deamination of adenine and guanine results in transversion mutations. Moreover, the instability of hypoxanthine and xanthine in the DNA structure leads to rapid depurination and consequent single strand breaks. Even crosslinking with other nucleic acids or proteins have been suggested via reaction of a nucleophilic site on an adjacent macromolecule and the diazonium ion of the modified base (18).

Nitrosation of secondary aliphatic and aromatic amines can also produce potentially carcinogenic nitrosamines. Secondary nitrosamines are more stable than their primary amine counterparts:

$$R_2NH + XNO \rightarrow R_2NNO + HX$$

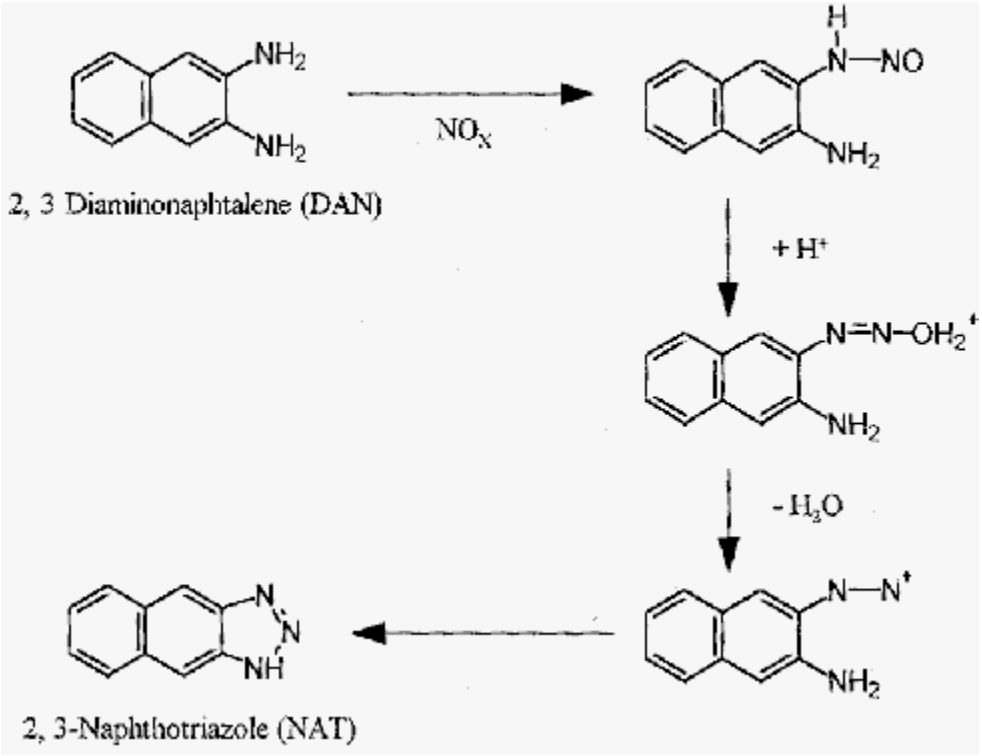

Figure 2: N-nitrosation of 2,3-diaminonaphthalene (DAN) to yield 2,3-naphthotriazole (NAT) by an NO-derived N-nitrosating agent (NO_X).

Such nitrosamines, like many chemical carcinogens, are thought to promote mutagenesis and carcinogenesis via their ability to alkylate specific sites in DNA. For example, these types of nitrosamines undergoe enzymatic alpha-hydroxylation. The alpha-hydroxy nitrosamine decomposes to form the alkyl diaznoium ion and free alkyl carbocation (Fig 1). The alkyl diazonium salt or carbocations then can react with nucleophilic sites in DNA.

To date, alkylation of DNA has been noted on the ring-nitrogen positions in the bases (adenine, guanine, cytosine, thymine), the oxygen atoms of hydroxyl or carbonyl groups (guanine, thymine, and cytosine) as well as on the phosphate groups (19).

Thus, the limitation of NO-mediated genomic damage, rests primarily on the localized diffusion of the small molecule, the degree of reactivity and nitrosation, and ultimately the cells' replicative and DNA repair machinery. However, even in the latter case, DNA repair proteins such as O6-methylguanine-DNA-methyltransferase and Fpg,

have been shown to be inhibited by NO-derived nitrosating agents such as N_2O_3 *in vitro* and *in vivo* (20, 21).

Coincident with the sustained overproduction of NO, inflammatory foci are also sites of enhanced production of reactive oxygen species, such as O_2^- and H_2O_2. Because O_2^- is known to rapidly react with NO, it was of interest to determine whether this reactive oxygen specie may modulate NO-dependent N-nitrosation of primary aromatic amines. Consequently, we have used 2,3-diaminonaphthalene (DAN) as a model to study the effect of O_2^- on the NO-dependent N-nitrosation of this primary aromatic amine. DAN is N-nitrosated by nitrosating agents derived from NO to yield its highly fluorescent triazole derivative 1-naphtho(2,3)triazole (NAT) (Fig 2).

We have demonstrated that the addition of a O_2^- generator such as hypoxanthine/xanthine oxidase virtually eliminated the NO-dependent N-nitrosation of DAN. Inhibition was maximal when equimolar fluxes of NO and O_2^- were produced (17). We also noted that this inhibition was reversed by the addition of superoxide dismutase, but not catalase suggesting that O_2^- and not H_2O_2 was responsible for the inhibition. We proposed that at equimolar fluxes, O_2^- reacts rapidly with NO to generate products such as peroxynitrite ($ONOO^-$) or derivatives thereof - which have only a limited ability to N-nitrosate amino compounds (16,17). Although we found that O_2^- inhibits the potentially mutagenic N-nitrosation of primary and secondary amines, the formation of $ONOO^-$ could conceivably promote oxidative (and nitrative) modifications of DNA bases, switching NO-mediated DNA

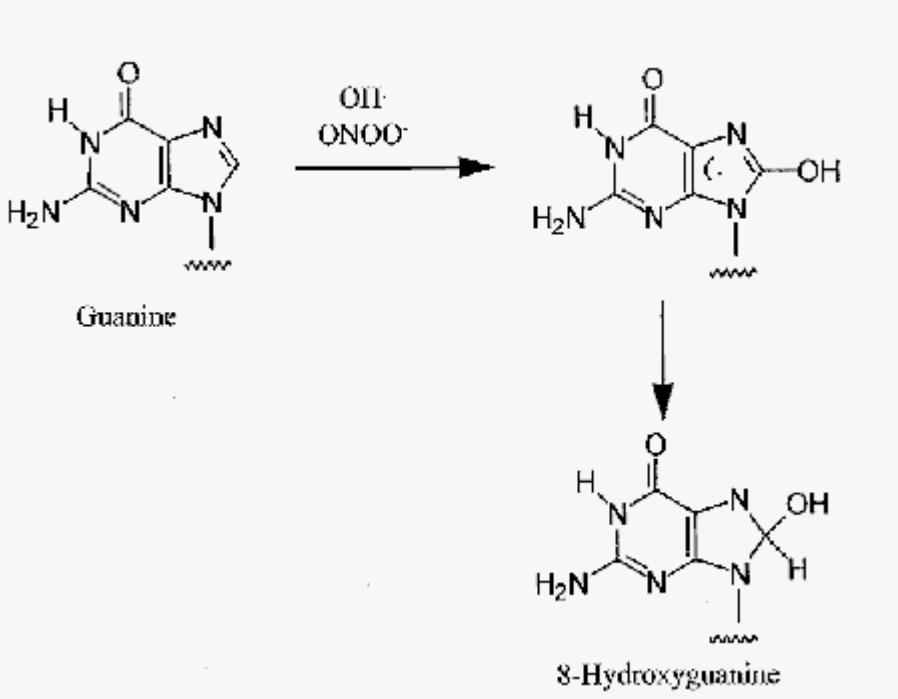

Figure 3: Hydroxyl radical (OH^-) and peroxynitrite ($ONOO^-$)-mediated oxidation of guanine.

damage from a nitrosative to a more oxidative pattern of mutagenic reactions (22).

4. SUPEROXIDE-NITRIC OXIDE INTER-ACTIONS: OXIDATIVE REACTIONS

Nitric oxide reacts with O_2^- to yield $ONOO^-$ with a second order rate constant of 6.7 x 10^9 $M^{-1}.s^{-1}$ (23). Once formed, $ONOO^-$ is protonated resulting in its rapid decomposition to nitrate (NO_3^-) in the absence of any oxidizable substrate. Therefore, O_2^- will rapidly inhibit NO-mediated N-nitrosation reactions in favor of reactions catalyzed by $OONO^-$.

Peroxynitrite exists in equilibrium with its conjugate acid peroxynitrous acid (ONOOH, pKa 6.6):

$$NO+O_2^- \rightarrow ONOO^-+H^+ \Leftrightarrow ONOOH \rightarrow ONOOH^* \rightarrow NO_3^-$$

Peroxynitrous acid is very unstable and at physiological pH decomposes to form derivatives with potent oxidizing properties. This may involve the homolysis of ONOOH to nitrogen dioxide radical ($NO_2^{\cdot}$) and hydroxyl radical (OH^-) within a solvent cage, the two free radicals diffusing out of the solvent cage to mediate oxidation reactions (24). Hydroxyl radical is an extremely reactive species, interacting with virtually all biomolecules at diffusion limited rates (~10^7 -10^9 $M.sec^{-1}$) (25). Nitrogen dioxide can initiate lipid peroxidaton and N-nitrosate certain amines to yield nitrosamines (26). However, a second more probable mechanism that may account for the oxidative properties of $ONOO^-$ is suggested to involve the formation of an activated isomer of peroxynitrous acid, ONOOH* which would possess $NO_2^{\cdot}$ and OH-like properties (27). From the standpoint of biologically relevant reactions, $ONOO^-$ is a potent oxidizing, hydroxylating, and nitrating agent. In regard to DNA modifications, $ONOO^-$ has been found to oxidize and nitrate isolated DNA resulting in DNA strand breaks (28).

Little is known regarding the detailed reactions of $ONOO^-$ with DNA. Because of the multiplicity of DNA modifications produced during oxidative reactions, it has been difficult to establish the specificity of mutations engendered by individual oxidants such as $ONOO^-$. Among oxidative-mediated DNA base modifications, a major product is 8-hydroxydeoxyguanosine (8-OHdG) (Fig 3) (29). The occurrence of this alteration has been associated with a number of conditions leading to increased oxidative stress including higher basal metabolic rate, gamma-irradiation, and hydrogen peroxide-mediated oxidative stress (30). Nitric oxide and iron have also been implicated in the formation of 8-OHdG in asbestos-treated human lung epithelial cells (31). Peroxynitrite also mediates the oxidation of deoxyguanosine (32). In all of those conditions, the formation of 8-OHdG might lead to mutations by inducing misreading of the base itself and of the adjacent bases (29) which may represent an important source of mutations (33). Peroxynitrite induces G:C toT:A mutations for the supF gene in *E. coli* and in human AD293 cells (34).

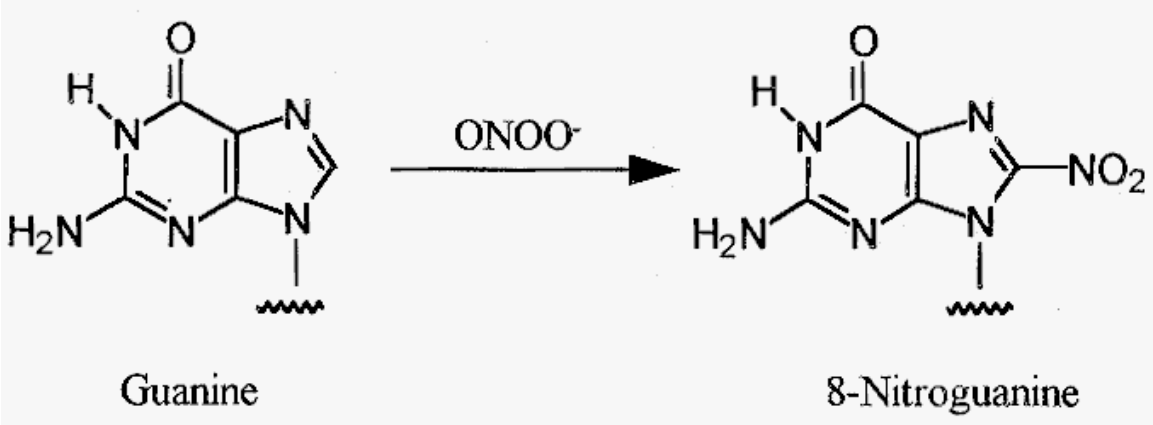

Figure 4: Peroxynitrite ($ONOO^-$)-mediated nitration of guanine to form 8-nitroguanine.

In addition to oxidative reactions, recent data suggest that the interaction of $ONOO^-$ with DNA results in the nitration of guanine to form 8-nitroguanine (Fig 4) (35). This modification is potentially mutagenic, the epurination of 8-nitroguanine yielding apurinic sites with the resultant possibility of G:C to T:A transversions (36). However, whether $ONOO^-$ mediates such DNA damage in cells and tissues is yet to be determined. The identification of 8-nitroguanine as a marker of $ONOO^-$ -induced nitration may represent an important tool for the detection of such alteration *in vivo*.

It is important to note that the evaluation of $ONOO^-$-mediated mutagenic properties has been assessed *in vitro* using bolus amounts of chemically generated oxidant. However, it is becoming increasingly evident that the formation of $ONOO^-$ at sites where both O_2^- and NO are produced may depend upon the relative fluxes of NO and O_2^-. Using the hypoxanthine/ xanthine oxidase system to generate both O_2^- and H_2O_2 and the spermine/NO adduct to generate various fluxes of NO, we found that the simultaneous production of equimolarfluxes of O_2^- and NO dramatically increased the oxidation of the oxidant-sensitive probe dihydrorhodamine (DHR) (22). The oxidation of DHR was inhibited by superoxide dismutase but not catalase suggesting that O_2^- and not NO interacted with NO to form $ONOO^-$ (25). In these same experiments, $ONOO^-$-mediated oxidation of DHR was quenched by excess formation of either NO or O_2^- suggesting that excess production of either radical may act as an endogenous modulator of $ONOO^-$ chemistry. Subsequent experiments demonstrated that NO (or O_2^-) interacted with and decomposed $ONOO^-$. Although this hypothesis suggests that NO and O_2^- may modulate steady state concentrations of $ONOO^-$, there has yet to be any direct evidence demonstrating such modulation of $ONOO^-$-mediated oxidative and/or nitrating reactions under physiological conditions.

5. SUPEROXIDE, FENTON CHEMISTRY AND NITRIC OXIDE

In general, O_2^- per se is not thought to be highly toxic to cells and tissues since it is a better reducing agent than an oxidant (37). However, O_2^- will rapidly dismutate to form H_2O_2. Although H_2O_2 is an oxidizing agent, most of the H_2O_2-dependent oxidizing activity is mediated by secondary radicals such as OH^- generated from metal (Mn)-catalyzed reactions. It is known that O_2^- and H_2O_2 interact with chelates of iron or copper to yield the potent oxidant OH^- or OH^--like species via the superoxide-driven Fenton reaction:

$$O_2^- + Mn^{+2} \rightarrow O_2 + Mn^{+1}$$
$$H_2O_2 + Mn^{+1} \rightarrow OH^- + OH^- + Mn^{+2}$$

The interaction of free radicals derived from superoxide-driven Fenton reactions with DNA has received considerable interest (38). Recent reports have focused on the ability of copper to participate in mutagenic reactions *in vivo* via Fenton-catalyzed reactions. Copper is an important structural metal in chromatin (39) that in fact induces more DNA bases damage in the presence of H_2O_2 than does iron (40).

There is now evidence to suggest that NO may modulate Fenton-driven oxidative reactions. We have recently investigated the ability of different fluxes of nitric oxide to modulate iron complex (10) and hemoprotein-catalyzed oxidative reactions (41). We found that generation of O_2^- and H_2O_2 in the presence of 5 µM Fe^{+3}-EDTA stimulated oxidation of DHR producing approximately 15 µM rhodamine (10). Catalase and superoxide dismutase were both effective at inhibiting this classic Fenton-driven reaction. Addition of NO increased DHR oxidation such that a 2:1 NO/ O_2^- ratio increased DHR oxidation by 30%. As the ratio NO/ O_2^- further increased to 4.5, DHR oxidation was reduced by 40%. Kanner *et al.* (42) proposed that NO may inhibit iron-mediated

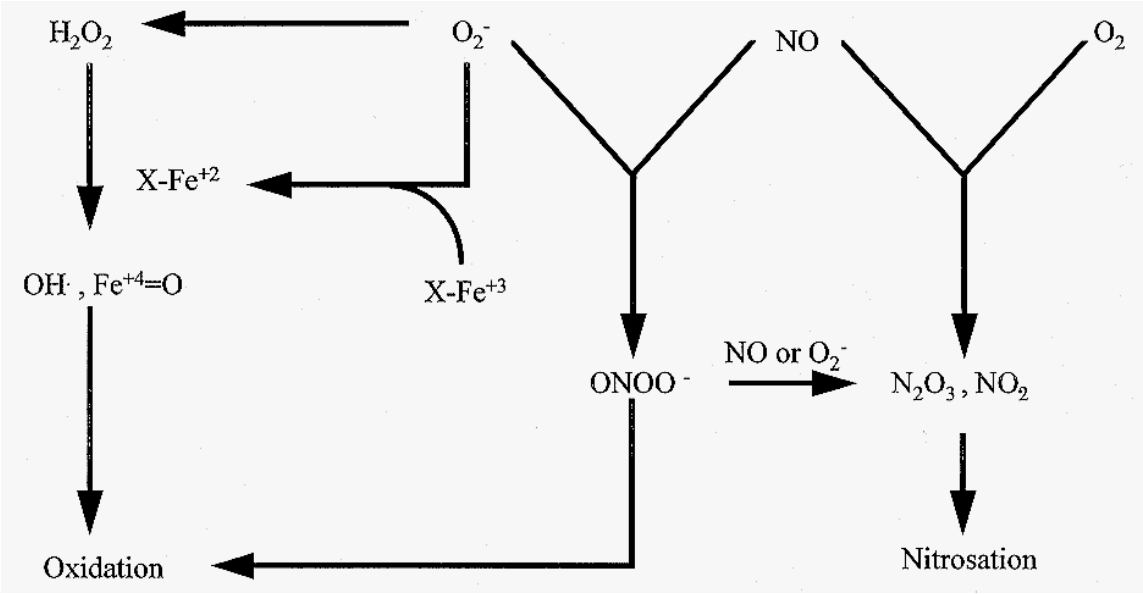

Figure 5: Modulation of oxidative and nitrosative reactions by NO and O_2^-.

oxidative reactions by forming nitrosyl complexes with ferrous iron:

$$Fe^{+3}\text{-EDTA} + O_2^- \rightarrow Fe^{+2}\text{-EDTA} + O_2$$
$$Fe^{+2}\text{-EDTA} + NO \rightarrow NO\text{-}Fe^{+2}\text{-EDTA}$$
$$NO\text{-}Fe^{+2}\text{-EDTA} + H_2O_2 \rightarrow Fe^{+3}\text{-EDTA} + HNO_2 + OH^-$$

The efficiency of such interaction may explain the decrease in DHR oxidation obtained in the presence of high fluxes of NO. We have also assessed the ability of myoglobin to oxidize DHR in the presence or the absence of O_2^-, H_2O_2 and /or NO (33). In the presence of equimolar fluxes of H_2O_2 and O_2^-, the addition of myoglobin dramatically enhanced DHR oxidation via the formation of ferryl (Fe^{+4}) myoglobin. This oxidative reaction was as expected inhibited by catalase but not Superoxide dismutase. Addition of NO to this system further enhanced DHR oxidation which was inhibited by superoxide dismutase suggesting that O_2^- reacted with NO to form a potent oxidant such as peroxynitrite in addition to the H_2O_2-dependent formation of ferryl myoglobin. Further increases in NO flux dramatically inhibited DHR oxidation which was found to be due to the NO-mediated reduction of ferryl heme to the met form. Taken together, these data suggest that NO may enhance or inhibit iron complex and hemoprotein-catalyzed oxidative reactions depending upon the relative fluxes of O_2^-, H_2O_2 and NO. In accordance with our results, Pacelli *et al*. have shown that NO can inhibit DNA strand breaks induced by H_2O_2 and certain transition metals (43).

6. CONCLUSION

It is well known that chronic intestinal inflammation is associated with an increased risk of malignancy (1-3). This pathological condition might represent one prime example in which the chemistry we have described may play an important role. Indeed, the phagocytic leukocytes that accumulate within the chronically inflamed colon produce large amounts of O_2^- and NO which may mediate mutagenesis and possibly malignant transformation (1). Thus, the fundamental understanding of the interplay between O_2^- and NO may give us new insights into the chemical role that these two free radicals play during mutagenesis. In the absence of O_2^-, NO will N-nitrosate certain primary and secondary amines to yield potentially mutagenic nitrosamine intermediates (see Fig 5). In the presence of both NO and O_2^-, N-nitrosation chemistry may be suppressed but oxidation reactions may predominate.

7. ACKNOWLEDGMENTS

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