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REACTION OF INDOMETHACIN WITH SINGLET MOLECULAR OXYGEN

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Abstract—Singlet molecular oxygen is a highly reactive species and is capable of disrupting cell membranes and collagen. It may also play a role in the synthesis of prostaglandins. We show here that indomethacin can react with singlet molecular oxygen and suggest that part of the anti-inflammatory action of this drug may stem from its ability to scavenge singlet oxygen.

The molecular mechanism of action of the non-steroidal anti-inflammatory drugs, such as indomethacin, remains a subject of considerable and continued interest. Likewise, the role of the reactive forms of oxygen as mediators of inflammation is a topic of active investigation at the present time.

The reactive forms of oxygen include singlet oxygen $^1O_2$, superoxide anion radical $O_2^{\cdot-}$, hydroxyl radical OH·, hydrogen peroxide H_2O_2 and metal ion–oxygen complexes. These species are known to cause various deleterious effects. Examples of these are the development of pulmonary oxygen toxicity [1], the amplification of effects of a chemical carcinogen on the development of abnormal morphology of cultured cells [2], toxic effects of proteins, bacteria and bacteriophage [3], lipid peroxidation in biological membranes [4, 5], peroxidation of unsaturated fatty acids [6], damage to adrenal mitrochondrial membranes [7] and damage to DNA [8]. McCord [9] has presented evidence that the hydroxyl radical can mediate the depolymerization of hyaluronic acid. He suggested that this reaction may play a role in the pathogenesis of inflammatory types of arthritis. Greenwald and Moy [10] have shown that reactive oxygen species can mediate the inhibition of collagen gelation and suggested that leukocyte-derived radicals may thereby alter the integrity of cartilage.

On a molecular level, the mechanism of action of indomethacin is still unknown. Among the suggested modes of action are the uncoupling of oxidative phosphorylation [11], inhibition of histidine decarboxylase [12], stabilization of lysosomes [13], complex formation with ferrous ion [14] and sulfhydryl-disulfide stabilization [15]. Currently, the role of prostaglandins as mediators of inflammation has gained prominence and indomethacin has been shown to inhibit the enzyme prostaglandin synthetase [16]. There is evidence, pro and con, regarding the reversible or irreversible nature of this inhibition [14, 17, 18]. Rahimtula and O'Brien [19] have presented evidence that prostaglandin synthetase may act via singlet molecular oxygen.

We present evidence here that indomethacin reacts with singlet molecular oxygen. If singlet oxygen plays a role in the mediation of inflammation, indomethacin may, in part, exert its anti-inflammatory effect either directly by preventing, for example, the $^1O_2$-mediated destruction of cell membranes, or indirectly by preventing $^1O_2$ from participating in prostaglandin synthesis.

MATERIALS AND METHODS

Chemicals were purchased from the following suppliers as indicated: deuterium oxide (Bio-Rad Laboratories, Richmond, CA); rose bengal (Chemical Dynamics Corp., South Plainfield, NJ); indomethacin, nitroblue tetrazolium, D-mannitol and sodium azide (Sigma Chemical Co., St. Louis, MO); DL-methionine (Mann Research Laboratories, New York, NY); potassium superoxide (Research Organic–Inorganic Chemical Co., Belleville, NJ); dimethyl sulfoxide and H_2O_2 (Baker Chemical Co., Phillipsburg, NJ); sodium formate (Fisher Scientific Co., Springfield, NJ); and dicyclohexyl-18-crown-6-ether† (Tridom-Fluka Chemical Co., Hauppauge, NY). Superoxide dismutase was prepared from bovine erythrocytes according to the procedure of McCord and Fridovich [20].

Photo-oxidation was performed in an apparatus containing two 15-W Westinghouse F15T8 gold fluorescent bulbs. Light intensity was determined with a Yellow Springs Instrument Co. model 65A radiometer and YSI 6551 probe. A typical reaction mixture contained 8 μM rose bengal, 40 μM indomethacin and 10 mM potassium phosphate, pD 7.5. Unless otherwise indicated, all solutions were prepared with 99.8% D_2O. All photo-oxidations were carried out in quartz cuvettes and unless otherwise indicated were for 20 min. The potassium superoxide (KO_2) was prepared as a saturated solution in dimethyl sulfoxide (DMSO) containing 0.5% dicyclohexyl-18-crown-6-ether [21]. The DMSO was previously dried with molecular sieves 3A. The con-

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† 2,5,8,15,18,21-Hexaoxatricyclo [20, 4, 0, $0^{9,14}$]-hexanose.

centration of O_2^- was assayed by sequential 5 μl additions of KO_2 solution to a 0.1 mM solution of nitroblue tetrazolium (NBT) in 10 mM potassium phosphate, pD 7.5, prepared with 99.8% D_2O. The extinction coefficient at 560 nm for the reduction of NBT to diformazan was taken to be $3 \times 10^4\ M^{-1}\ cm^{-1}$ [22, 23].

RESULTS

In our experiments we have chosen to generate singlet oxygen photochemically, using visible light and rose bengal (RB) as a photosensitizer [24]:

$$RB \xrightarrow{h\nu} RB^1$$

$$RB^1 \longrightarrow RB^3$$

$$RB^3 + {}^3O_2 \longrightarrow RB + {}^1O_2.$$

Ground state RB is excited by the absorption of a photon and is transformed into a relatively short-lived RB singlet (RB^1). RB^1 undergoes spin inversion to a relatively long-lived RB triplet (RB^3). RB^3 is then quenched by ground state triplet molecular oxygen ($^3O_2$). This energy transfer reaction results in the formation of $^1O_2$ and ground state RB. Control experiments have shown that the light source alone (in the absence of RB) does not cause significant changes in the absorption spectrum of indomethacin; the light source alone (in the absence of indomethacin) does not cause any significant changes in the absorption spectrum of RB when irradiated for 20 min; and irradiated RB (20 min) does not cause significant changes in the absorption spectrum of unirradiated indomethacin.

Figure 1 shows that the absorption spectrum of indomethacin in a singlet oxygen-generating system (rose bengal-D_2O) changes steadily with increasing duration of irradiation. There is a progressive loss of the 318 nm peak, indicating a rose bengal-catalyzed photoreaction of indomethacin.

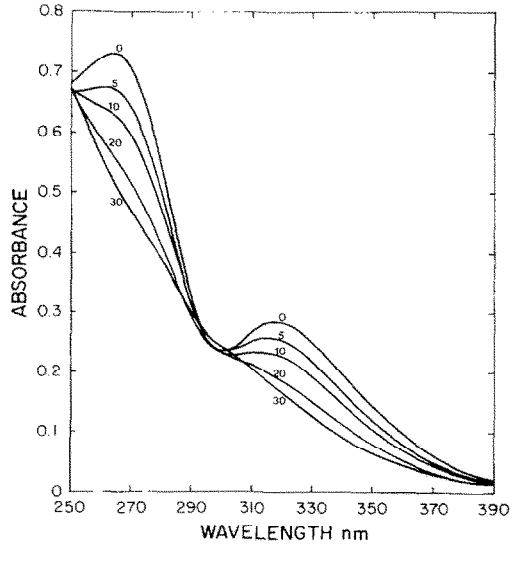

Fig. 1. Time course of photo-oxidation of indomethacin. The reaction mixture and photo-oxidation procedure are as described under Materials and Methods. The numbers on the curves indicate the duration of irradiation in minutes.

Table 1. Effects of singlet oxygen reactive compounds on the rose bengal-catalyzed photo-oxidation of indomethacin

Addition	$\% \Delta A_{320}$*
None	100
H_2O in place of D_2O	22
Methionine (2.5 mM)	37
Azide (0.2 mM)	37

* Change in absorbance is expressed as percentage of control. Each value is the average of two determinations with a control cuvette being run simultaneously. The duration of photo-oxidation was 20 min. The photo-oxidation procedure was as described under Materials and Methods.

Table 1 shows the effects of $^1O_2$ reactive compounds on the RB-catalyzed photo-oxidation of indomethacin. A deuterium oxide enhancement effect is generally observed in $^1O_2$-mediated reactions provided that the $^1O_2$ concentration is rate limiting [25, 26]. The lifetime of $^1O_2$ has been determined to be 2 μsec in H_2O and 20 μsec in D_2O [25–27]. As would be expected if $^1O_2$ is the mediator, an increase of its lifetime in D_2O would increase the steady state concentration of $^1O_2$ available to react with indomethacin during irradiation and thus potentiate the rate of photo-oxidation as compared to the rate in H_2O. The shortened lifetime of $^1O_2$ in H_2O as compared with D_2O is thought to occur via the transfer of electronic energy from $^1O_2$ to the vibrational energy of H_2O [26]. As can be seen in Table 1, the reaction rate in H_2O medium is one-fifth of that in a D_2O medium.

Methionine is a well-known scavenger of $^1O_2$, with methionine sulfoxide as the major product at physiologic pH [28]. The second order rate constant for the reaction is $3 \times 10^7\ M^{-1}\ sec^{-1}$ [29]. Table 1 shows that 2.5 mM methionine causes a 63 per cent inhibition in the oxidation of indomethacin.

Azide is a well-known quencher of $^1O_2$. The rate constant for the quenching of $^1O_2$ by azide ion is

Table 2. Effects of various compounds on the photo-oxidation of indomethacin

Expt.	Addition	$\% \Delta A_{320}$*
1	None	100
2	H_2O_2, (2.5 mM)	95
3	D-Mannitol (2.5 mM)	94
4	Benzoate (2.5 mM)	95
5	Formate (2.5 mM)	97
6	EDTA (0.1 mM)	96
7	*Superoxide dismutase* (15 μg/ml)	95

* Change in absorbance is expressed as percentage of control. Each value is the average of two determinations with a control cuvette being run simultaneously. The values among the duplicates differed by less than 10 per cent. The photo-oxidation was of 20 min duration. The photo-oxidation procedure was as described under Materials and Methods.

$2.2 \times 10^8\ M^{-1}\ sec^{-1}$ [30]. Table 1 shows that 0.2 mM azide results in a 63 per cent inhibition of indomethacin photo-oxidation. It is worthwhile to note that the 10-fold difference in concentrations between azide and methionine required to give the same degree of inhibition is consistent with the relative magnitudes of the rate constants for the reactions between $^1O_2$ and either azide or methionine.

The experiments in Table 2 explore the possible roles of O_2^-, $OH\cdot$, H_2O_2 and metal ions, in mediating the photo-oxidation of indomethacin. H_2O_2 (2.5 mM) does not markedly potentiate the reaction. D-Mannitol, benzoate and formate are well-known scavengers of $OH\cdot$, a highly reactive oxidative species. The second order rate constants for the reactions of benzoate and formate with $OH\cdot$ are 3.3×10^9 and 2.5×10^9, respectively [31]. Experiments 3, 4 and 5 in Table 2 show that D-mannitol, benzoate and formate at 2.5 mM (sixty times more concentrated than indomethacin) have no effect on the photo-oxidation of indomethacin. This suggests that the reaction is not mediated by $OH\cdot$. In experiment 6, EDTA was used to test for the possible role of metal ions in the photo-oxidation. EDTA was found to be without significant effect. In addition, neither 20 μM Fe^{2+} nor 20 μM Fe^{3+} (in both the presence and absence of 0.1 mM EDTA) had a significant effect on the rate of reaction. Experiment 7 shows that superoxide dismutase, an enzyme which converts O_2^- to O_2 and H_2O_2, does not inhibit the photo-oxidation of indomethacin. Further evidence to exclude O_2^- as an initiator of the oxidation was carried out by direct addition of a KO_2 solution to a solution of indomethacin without irradiation. Two hundred microliters of the KO_2 in crown ether–DMSO solution were added slowly to 1.0 ml of a 48 μM solution of indomethacin in D_2O buffer (with magnetic stirring). The concentration of O_2^- was estimated to be 200 μM. A similar crown ether–DMSO solution without KO_2 was added to the control cuvette. No significant differences were detected between the absorption spectra of the experimental and control solutions. It has been demonstrated that under certain conditions rose bengal-mediated photo-oxidation may lead to O_2^- formation [32]. The experiments described above, however, eliminate O_2^- from contention in indomethacin oxidation.

DISCUSSION

The elucidation of the mechanism of action of indomethacin on a molecular level is a fundamental problem in rheumatology. While it is known, for example, that the acetyl group of aspirin is transferred to prostaglandin synthetase [18], experiments with indomethacin labeled in various positions of the molecule have not demostrated a similar transfer of part or all of the indomethacin molecule to protein [33].

As discussed in the beginning of the paper, there is a growing body of evidence suggesting that the reactive forms of oxygen may be implicated in the pathogenesis of inflammation, and act as mediators of damage to the proteins and lipids of cell membranes and to nucleic acids. In our studies we have focused on singlet molecular oxygen, a species which exists when one of the two unpaired electrons in ground state triplet oxygen undergoes spin inversion. Venkatasubramanian and Joseph [34] have shown that singlet oxygen can damage collagen, and Rahimtula and O'Brien [19] have presented evidence that singlet oxygen may play an important role in the mechanism of action of prostaglandin synthetase. Thus, singlet oxygen may be a mediator of degenerative inflammatory arthritic disease on either a primary or secondary basis.

In vivo, $^1O_2$ may be generated in a number of ways. Polymorphonuclear leukocytes are known to generate O_2^-. This species will dismutate either spontaneously or enzymatically via superoxide dismutase to form H_2O_2 and O_2, as seen in Reaction 1. It has been hypothesized that, in the spontaneous dismutation, the molecular oxygen product is in the singlet state [35, 36] whereas the enzyme catalyzed dismutation leads to ground state oxygen [35]:

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2. \quad (1)$$

It has been also suggested that under certain conditions O_2^- may react with H_2O_2 to form $^1O_2$ and $OH\cdot$ as seen in Reaction 2 [6]:

$$O_2^- + H_2O_2 \rightarrow OH^- + OH\cdot + {}^1O_2. \quad (2)$$

The second order rate constant for Reaction 2 is $0.13\ m^{-1}\ sec^{-1}$ and it would, therefore, be expected to occur at an extremely slow rate [37]. An adaptation of this reaction which involves metal ion catalysis has been suggested [38, 39]. Reaction 2 may then represent the net of the metal catalyzed reaction sequence.

Arneson [40] has proposed that $^1O_2$ may be generated from a reaction between O_2^- and OH as seen in Reaction 3. This reaction has not as yet been demonstrated under biological conditions:

$$O_2^- + OH\cdot \rightarrow {}^1O_2 + OH^-. \quad (3)$$

Myeloperoxidase is an enzyme known to exist in polymorphonuclear leukocytes, and utilizes H_2O_2 and a halide ion such as Cl^- as substrates [41]. The enzyme generates the hypochlorite ion, OCl^-, as an intermediate, and it has been proposed that $^1O_2$ is a product of the enzymatic reaction [42]. Others, however, have indicated that the evidence for $^1O_2$ production from the myeloperoxidative reaction is not unequivocal [43]. It has also been reported that $^1O_2$ is formed from the hemoprotein-catalyzed decomposition of lipid peroxides [44].

The experiments in Table 1 (potentiation of oxidation by D_2O as compared with H_2O and inhibition of oxidation by methionine and azide), combined with the changes seen in Fig. 1, indicate that indomethacin can react with singlet molecular oxygen to form a spectrally distinguishable product(s). The experiments outlined in Table 2 exclude the other reactive oxygen species ($OH\cdot$, O_2^-, H_2O_2, metal ion–oxygen complex) as the mediators of the photo-oxidation.

Taken collectively, these results indicate that indomethacin can react with singlet oxygen, suggesting the possibility that if singlet oxygen is generated *in vivo*, at least part of the anti-inflammatory effect of indomethacin may result from this scavenging reaction. This is not to say that all singlet

oxygen scavengers or quenchers will be anti-inflammatory. To be anti-inflammatory they must get to the correct site or locus; specificity in terms of location may be an important and crucial factor [45]. It is important to remember that singlet oxygen has a lifetime of 2 μsec in aqueous solution [25–27]. Thus, even if some metabolites of indomethacin were shown to be singlet oxygen reactive, they might not be anti-inflammatory because they could not reach the correct locus of action.

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