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THE METAL-MEDIATED FORMATION OF HYDROXYL RADICAL BY AQUEOUS EXTRACTS OF CIGARETTE TAR

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Summary: Aqueous extracts of cigarette tar produce hydroxyl radicals that are spin trapped by 5,5-dimethyl-1-pyrroline-N-oxide. The addition of catalase almost completely inhibits and superoxide dismutase partially inhibits spin adduct formation. The addition of ethylenediamine tetraacetic acid greatly increases the amount of hydroxyl radical adduct observed; in contrast, diethylenetriamine pentaacetic acid causes complete inhibition of spin adduct formation. We suggest that the hydroxyl radical arises from the metal-mediated decomposition of hydrogen peroxide, and that hydrogen peroxide is formed from the reduction of dioxygen by the semiquinones present in the cigarette tar.

Introduction: Cigarette smoking is a major cause of human lung cancer and other respiratory diseases (1,2). Cigarette smoke contains high concentrations of radicals, both in the tar and the gas-phase (3,4), and there is a large body of evidence supporting the involvement of these radicals in smoking-related diseases (5-8).

Several workers have demonstrated that gas-phase smoke is involved in biologically important reactions including the inactivation of alpha-1-proteinase inhibitor (9,10) and the production of hydrogen peroxide (11). The properties and chemical reactivity of cigarette tar, on the other hand, have received little attention. We have shown that the tar free radical is a quinone/hydroquinone redox system in a polymeric matrix (3) and the radical signal becomes associated with DNA (12). Quinone/hydroquinone polymers are known to be redox catalysts in both chemistry (13,14) and biochemistry (15).

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ABBREVIATIONS: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; SOD, superoxide dismutase; EDTA, ethylenediaminetetraacetic acid; DETAPAC, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; PBN, N-tert-butyl-a-phenyl nitrone.

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Cigarette tar has strongly reducing properties, and we have suggested that tar is capable of reducing dioxygen to the superoxide radical anion (4).

This report describes, for the first time, the spin trapping of hydroxyl radicals from buffered aqueous solutions of cigarette tar, using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as the spin trap. The trapped hydroxyl radicals are formed from the metal-mediated decomposition of hydrogen peroxide. The generation of hydrogen peroxide by cigarette tar is due, at least in part, to the reduction of dioxygen to superoxide. We believe that the formation of these active oxygen species by cigarette tar in the lung may play an important role in the onset of smoking-related diseases.

MATERIALS AND METHODS: 5,5-Dimethyl-1-pyrroline-N-oxide, catalase, superoxide dismutase, EDTA and DETAPAC were purchased from Sigma Chemical Company (St. Louis, MO). The DMPO was purified by the method of Buettner and Oberley (16). The catalase, SOD, EDTA, and DETAPAC were used as received. Research cigarettes (1R1) from the University of Kentucky, Tobacco and Health Institute, were stored in a freezer in sealed packages and conditioned at 4° over a saturated aqueous solution of ammonium sulfate for at least 48 hours before use. All other chemicals were reagent grade and were used without further purification.

The tar from two cigarettes, smoked at a continuous flow rate of 1000 mL/min, was collected on a MicronSep No. 5 cellulosic membrane filter (MSI; Honeoye Falls, NY). The filter was then extracted for 30 mins with 10 mL of Chelex-100 treated (Bio-Rad Laboratories; Richmond, CA), 0.1 M phosphate buffer, pH 7.4. Chelex-treated tar was prepared by passing the aqueous tar extract through a small column of Chelex-100 immediately before use.

All spin trapping experiments were performed with 2 mL of the buffered tar extract combined with 2 mL of 0.08 M aqueous DMPO solution. All reaction mixtures were kept for 4 hours in the dark at room temperature prior to recording the ESR spectrum.

All ESR spectra were recorded on a Bruker ER 100D spectrometer interfaced to an ASPECT 2000 computer. The spectral parameters were as follows: microwave frequency, 9.75 GHz; power, 10 dB; modulation amplitude, 0.8 G; modulation frequency, 100 kHz; time constant, 200 msec.; scan rate, 0.9 G/sec. Spectra were recorded with degassed solutions in a quartz flat cell.

RESULTS AND DISCUSSION: Previous attempts in our laboratory to spin trap radicals from cigarette tar in organic solvents using N-tert-butyl-alpha-phenyl nitrone (PBN) were not successful, due to the facile reduction of the nitroxide spin adducts to ESR inactive products by the tar (17). Recently, however, we have discovered that the tar free radical can be extracted into aqueous solutions, and that it is possible to spin trap free radicals from these aqueous extracts. We have used DMPO as the spin trap, since the hydroxyl-DMPO adduct is more stable than is the corresponding PBN adduct (18).

Figure 1a shows the ESR spectrum of the spin adducts that are formed when DMPO is allowed to stand with an aqueous tar extract for 4 hours in the dark. This complex spectrum results from three different spin adducts. Two of the adducts show the typical

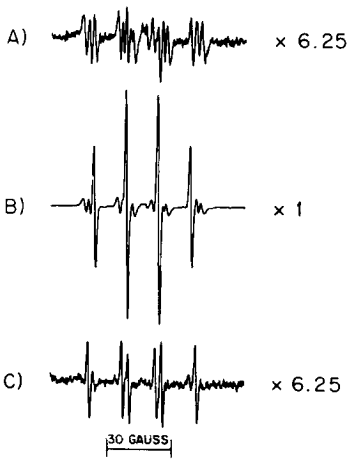

Figure 1: Examples of the DMPO spin adduct spectra obtained from aqueous extracts of cigarette tar. The numbers to the right of each spectrum indicate the relative instrument gain setting. A) Spectrum of the 3 spin adducts obtained from cigarette tar with 0.04M DMPO. The alkyl radical adduct has A_N=15.9G and A_H=22.7G. The hydroxyl adduct has A_N=A_H=14.9G. The third adduct has A_N=15.6G and A_H=18.6G which is consistent with the trapping of the radical anion of carbon dioxide. B) Same solution as in A except with 0.03M EDTA added. Note change in gain. Also note that all three spin adducts are observable, however the hydroxyl adduct is now much stronger. C) Spectrum obtained from an aqueous solution of cigarette tar which was passed through a Chelex-100 column before mixing with the DMPO. Note here that only two spin adducts are seen, the alkyl radical spin adduct is not observed.

DMPO spin adduct spectrum of six lines; the third adduct has only four lines, with A_N = A_H = 14.9 G, characteristic of the DMPO hydroxyl radical spin adduct (19). On the basis of the hyperfine splitting constants, the other two adducts appear to arise from the trapping of an alkyl radical and either an acyl radical or the radical anion of carbon dioxide (20).

The ability of the hydroxyl radical to react rapidly with ethanol to form alpha-hydroxyethyl radicals, which can then be trapped, can be used to confirm the presence of free hydroxyl radicals (20,21). The results in Table 1 show that the addition of a small amount of ethanol causes a decrease in the hydroxyl spin adduct signal and a concomitant increase in the signal due to an alkyl radical that is the alpha-hydroxyethyl spin adduct.

As can be seen from Table 1, catalase greatly reduces the amount of free radicals that are spin trapped. We find that both aqueous and organic solutions of cigarette tar consume oxygen and in the process produce hydrogen peroxide (22). Thus, it is probable that the hydroxyl radicals we detect from tar are produced from the decomposition of

TABLE 1: Relative DMPO spin adduct concentration for aqueous solutions of cigarette tar with various additives. (a,b)

TAR	COMPOUND ADDED	TRAPPED RADICAL HO•	$CO_2^{\bar{}}$	R•(c)
NONE	NONE	6	0	0
NONE	EDTA (d)	7	0	0
NONE	EDTA + ETHANOL (e)	5	0	0
NON-CHELEX TREATED TAR				
(j)	NONE	11	11	10
(j)	ETHANOL	3	9	17
(j)	EDTA	181	19	19
(j)	EDTA + ETHANOL	56	6	216
(j)	EDTA + CATALASE (f)	2	6	1
(j)	EDTA + SOD (g)	113	19	19
(j)	EDTA + SOD (h)	88	13	13
(j)	EDTA + SOD (i)	88	13	13
(j)	DETAPAC (d)	0	0	0
CHELEX TREATED TAR				
(k)	NONE	7	19	0
(k)	ETHANOL	2	14	8
(k)	EDTA	34	12	4
(k)	EDTA + ETHANOL	17	11	15

a) Relative intensity measured as the peak height of the lowest field peak for each spin adduct. Values are accurate to 10%. b) All solutions are 0.04 M in DMPO. c) This represents the alkyl radical trapped from cigarette tar or the sum of the alkyl radical from tar plus the hydroxyethyl radical in the cases where ethanol was added. d) EDTA and DETAPAC are each 0.03 M. e) Ethanol concentration is 0.21 M. f) Catalase is present at 1 mg/mL. g) 10 ug/mL of SOD was added. h) 100 ug/mL of SOD was added. i) 1 mg/mL of SOD was added. j) The tar from two 1R1 research cigarettes was extracted into 10 mL of 0.1 M, pH 7.4 phosphate buffer and then 2 mL of that solution was mixed with 2 mL of a 0.08 M solution of DMPO. k) Same as (j) except the tar solution was passed through a Chelex-100 column before mixing with the DMPO.

hydrogen peroxide. A number of workers have shown that iron-EDTA complexes can catalyze the formation of hydroxyl radical from hydrogen peroxide (23,24), while DETAPAC complexes inhibit this reaction (23). Cigarette tar contains a number of metals, including iron (1); therefore, if the hydroxyl radicals result from an iron-mediated decomposition of hydrogen peroxide, then EDTA should increase the intensity of the DMPO hydroxyl radical spin adduct and DETAPAC should cause it to decrease in intensity. Comparison of Figure 1b with 1a shows the dramatic increase in hydroxyl radical spin adduct formation caused by the addition of EDTA to the reaction solution. The addition

of DETAPAC, however, completely inhibits adduct formation (Table 1). The addition of SOD to the reaction mixtures also decreases the concentration of spin adducts formed, although SOD does not completely inhibit spin adduct formation.

These results can be rationalized as shown below, where Q, QH_2, and QH$^{\bullet}$ are quinone, hydroquinone and semiquinone groups, respectively, in the tar.

$$Q + QH_2 \longrightarrow 2QH^{\bullet} \quad [1]$$

$$QH^{\bullet} + Fe^{III} \longrightarrow Fe^{II} + Q + H^{+} \quad [2]$$

$$QH^{\bullet} + O_2 \longrightarrow Q + O_2^{\bullet -} + H^{+} \quad [3]$$

$$Fe^{II} + O_2^{\bullet -} + H^{+} \longrightarrow Fe^{III}\text{-}OOH \quad [4]$$

$$2O_2^{\bullet -} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad [5]$$

$$Fe^{II} + Fe^{III}\text{-}OOH + H^{+} \longrightarrow 2Fe^{III} + H_2O_2 \quad [6]$$

$$Fe^{II} + H_2O_2 \longrightarrow Fe^{III} + HO^{\bullet} + HO^{-} \quad [7]$$

$$DMPO + HO^{\bullet} \longrightarrow HO\text{-}DMPO^{\bullet} \quad [8]$$

The results shown in Table 1 using Chelex-100 treated tar solutions suggest the involvement of metal ions in the various reactions, since the total concentration of spin adducts is decreased when the tar solutions are treated with Chelex. Clearly, metals are implicated in these reactions, but we have no evidence that iron is the specific metal involved. Also, it is not likely that free metal ions are involved; it is more probable that the metals are complexed, either with added chelator such as EDTA, or with components of the cigarette tar.

We have already shown that the tar radical is a quinone/hydroquinone complex held in a tarry matrix (3,4), and it is known that semiquinones can reduce ferric to ferrous ion (25) and dioxygen to superoxide (26). The inhibitory effect of SOD (Table 1) shows that superoxide is on the reaction path to hydrogen peroxide in this system, eqs 3-5. However, the addition of relatively large amounts of SOD does not completely inhibit hydroxyl adduct formation. This could be because a portion of the hydrogen peroxide is

formed via a peroxo complex, eqs 4 and 6, which SOD cannot inhibit. Alternatively, superoxide may be formed within the matrix of the tar and be inaccessible to SOD.

The ability of iron complexes to catalyze hydroxyl radical production, eq 7, is well established (23,24). In the absence of added chelator, it is likely that iron is complexed with some component of the tar, e.g. orthoquinones, and this tar-iron complex can decompose hydrogen peroxide, as shown in eq 7. Apparently the iron-EDTA complex is more effective at catalyzing eq 7 than is the tar-iron complex, because the addition of EDTA leads to a much higher concentration of hydroxyl spin adduct. Of course, an EDTA-metal complex could also accelerate other reactions in Scheme 1 that involve metal ions, leading to more hydrogen peroxide.

DETAPAC is known to inhibit reaction 7. Consistent with our mechanism, no hydroxyl spin adducts are observed in the presence of DETAPAC. The other spin adducts also are not observed, implying that the alkyl radical and the carbon dioxide anion radical result from hydroxyl radical reactions. Hydroxy-substituted alkyl radicals could be formed as a result of the reaction of hydroxyl radical with aliphatic alcohols in the tar. The anion radical of carbon dioxide could be formed by the reaction of hydroxyl radical with formate; formate is present in tar (27) and it is known that formate ions react with hydroxyl radicals to produce the carbon dioxide anion radical (20).

The results obtained using Chelex-100 treated tar support the involvement of metal ions in the various reactions shown in the scheme. Chelex treated tar yields only the hydroxyl radical and the carbon dioxide anion radical spin adducts (Fig 1c); the alkyl radical adduct concentration is too low to be measured. The addition of EDTA increases the overall concentration of the spin adducts, but the total concentration of the three adducts still is much lower than that of the spin adducts observed in the spectrum of the non-Chelex treated tar with EDTA.

In conclusion, we have shown that aqueous solutions of cigarette tar reduce dioxygen and produce hydrogen peroxide. The mechanism by which hydrogen peroxide is produced involves, at least in part, the formation of superoxide, probably by redox cycling of the semiquinone radicals present in tar. Tar also is capable of decomposing the hydrogen peroxide to form hydroxyl radicals by a metal-mediated process. We believe that the formation of these active oxygen species by tar-oxygen reactions may be

significant in smoking-related diseases. When tar is deposited in the lung matrix, these active-oxygen species could be produced and could react with biomolecules, leading to biological damage.

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