molecular weights of FVIII/vWf and hemophiliac material may be much less than the upper limit given. Moreover, there may not be any simple relation between the molecular weights of FVIII/vWf and hemophiliac material of similar dimensions. Hence, these results do not necessarily conflict with the observation that both hemophiliac FVIII and normal FVIII have molecular weights of at least 1.2 million and behave similarly on gel filtration (*9*). Thus, the primary distinction appears to be one of a different quarternary structure for normal compared to hemophiliac material. Some corroboration of this might be indicated by the difference between internal images of normal and hemophiliac material on low-exposure microscopy. Whether this difference, derived from concentrated purified FVIII/vWf in buffer, is present in vivo cannot be ascertained. It is not possible, at present, to derive further information from the available data without overinterpretation of essentially morphological differences. We caution that far greater numbers of normal and abnormal plasmas, purified by different techniques, must be examined before significant differences may be claimed. We suggest that the combination of techniques used in our study should be useful in the analysis of normal and disease states associated with the FVIII/vWf molecule.

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References and Notes

1. O. D. Ratnoff, L. Kass, P. D. Lang, *J. Clin. Invest.* **48**, 957 (1969); M. E. Legaz, R. B. Schmer, R. B. Counts, E. W. Davie, *J. Biol. Chem.* **248**, 3946 (1973).
2. G. A. Shapiro, J. C. Andersen, S. V. Pizzo, P. A. McKee, *J. Clin. Invest.* **52**, 2198 (1973).
3. S. L. Marchesi, N. R. Shulman, H. R. Gralnick, *ibid.* **51**, 2151 (1972).
4. M. E. Switzer and P. A. McKee, *ibid.* **57**, 925 (1976).
5. E. L. Benedetti and P. Favard, *Freeze-Etching, Techniques and Applications* (Societe Francaise de Microscopie Electronique, Paris, 1973).
6. Quartz crystal thin-film monitor QSG 201 (Balzars, Liechtenstein) was used in conjunction with electron beam evaporation equipment EVM 052 (Balzers) and electron beam gun EK 552 (Balzers) for precise control and monitoring of carbon platinum film layer. All freeze-etch procedures were carried out in a Balzers BAF 300 high-vacuum freeze-etch unit equipped with a turbomolecular pump and examined with a Zeiss EM 10 electron microscope (Carl Zeiss, Oberkochen, West Germany).
7. R. C. Williams and H. W. Fisher, *J. Mol. Biol.* **52**, 121 (1970).
8. J. C. Andersen and P. A. McKee, *Circulation* **46** (Suppl. 2), 52 (1972).
9. L. Kass, O. D. Ratnoff, M. A. Leon, *J. Clin. Invest.* **48**, 351 (1969).

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Cigarette Smoke Activates Guanylate Cyclase and Increases Guanosine 3′,5′-Monophosphate in Tissues

Abstract. *The gaseous phase of cigarette smoke induced a 2- to 36-fold increase in the activity of guanylate cyclase in supernatant and particulate fractions from various rat and bovine tissues over basal activity. The characteristics of this phenomenon paralleled those of the activation of guanylate cyclase by nitric oxide, which is a component of tobacco smoke.*

Guanylate cyclase (E.C. 4.6.1.2), the enzyme that catalyses the formation of guanosine 3′,5′-monophosphate (cyclic GMP) from guanosine triphosphate and its product are incompletely understood. Although the concentrations of guanylate cyclase and cyclic GMP are increased in tumors and proliferating tissues (*1*) and in tissues exposed to certain smooth muscle relaxing agents, including sodium nitroprusside and nitroglycerin (*2*), the physiological implications of these observations are unclear. Compounds capable of forming nitroso compounds, compounds containing nitroso groups (some of which are carcinogenic), and nitric oxide (NO) gas are all able to increase guanylate cyclase activity and cyclic GMP levels (*2–4*). The common mechanism for this activation is thought to be the formation of NO (*4*).

The gas phase of fresh cigarette smoke contains NO but minimal amounts, if any, of the higher oxides of nitrogen (*5*). We report here that the gas phase of cigarette smoke causes a 2- to 36-fold increase in the activity of guanylate cyclase in preparations of broken cells from various rat and bovine tissues, and increases cyclic GMP but not adenosine 3′,5′-monophosphate (cyclic AMP) in minced rat lung. The characteristics of this activation are similar, both qualitatively and quantitatively to those reported for NO (*4*) with respect to the extent of activation, tissue specificity, and activity in the presence of various compounds that alter the activation of guanylate cyclase by NO. Other workers have demonstrated the induction of aryl hydrocarbon hydroxylase in lung preparations by particulate components of cigarette smoke (*6*). However, our experiments suggest that NO is the component of cigarette smoke vapor that activates the guanylate cyclase.

Male Sprague-Dawley rats were decapitated, and the tissues, except as indicated in Table 1, were processed as described (*4*) to obtain supernatant and particulate fractions (separated at 105,000*g*). Bovine pulmonary structures were obtained at a local abattoir and were processed in the same way. Guanylate cyclase and adenylate cyclase (E.C. 4.6.1.1) activities were determined as described (*4*) in 100-μl reaction mixtures containing enzyme, 1 m*M* guanosine triphosphate or adenosine triphosphate, 4 m*M* $MnCl_2$, 50 m*M* tris-HCl buffer (*p*H 7.6), 10 m*M* theophylline, 15 m*M* creatine phosphate, 20 μg of creatine phosphokinase (E.C. 2.7.3.2; 120 to 130 unit/mg) and other compounds at the concentrations indicated. Nitric oxide gas (Matheson Gas Products) was introduced 1 cm above some reaction mixtures as reported previously (*4*). Various brands of filter cigarettes were held in a plastic pipette tip containing a loosely packed, 40-mg glass wool plug. Smoke was drawn from the cigarette through the glass wool plug and Millipore AP 200 2500 glass fiber discs in order to eliminate the particulate fraction of smoke. The fresh gas phase of the smoke was introduced 1 cm above the reaction mixture contained in closed tubes (10 by 75 mm). The atmosphere in the tubes was 98 to 99 percent smoke vapor and 1 to 2 percent room air. The design of the system for the delivery of smoke is similar

Table 1. Effect of cigarette smoke vapor and nitric oxide gas on cyclic GMP and cyclic AMP concentrations in rat lung. Fresh rat lung was placed in cold Krebs-Ringer solution containing glucose (2 mg/ml) and 50 m*M* tris-HCl, *p*H 7.6. Minced tissue (0.3 by 0.3 mm) was prepared with a McIlwain chopper and placed in fresh Krebs-Ringer solution for 15 minutes. The minced tissue containing 2 to 5 mg of protein was then transferred to tubes (15 by 85 mm) containing 1 ml of fresh medium and incubated for 3 minutes. Some tubes were exposed to 100 percent N_2 for 10 seconds, some to 100 percent N_2, 417 μl of NO, and 100 percent N_2 for 10 seconds each, and some to 98 percent cigarette smoke vapor from brand D for 30 seconds. All tubes were capped for the first 30 seconds to confine the desired atmosphere. Reactions were terminated with 0.5 ml of 18 percent trichloroacetic acid. The supernatant fractions were extracted and assayed for cyclic nucleotides (*4*, *9*). All experiments were done in triplicate, and the values are the means ± standard error of three determinations.

Addition	Cyclic nucleotides (pmole/mg protein)	
	GMP	AMP
None	0.08 ± 0.01	2.30 ± 0.35
NO	6.38 ± 0.73*	2.01 ± 0.01
Smoke	1.06 ± 0.17*	2.21 ± 0.02

*$P < .02$.

in principle to the systems described by others (*7*) except that smoke reservoirs, that would have resulted in accelerated NO oxidation, were not used. Oxides of nitrogen were assayed in samples of smoke (*8*). Cyclic nucleotides were determined by the radioimmunoassay method of Steiner *et al.* (*9*) as reported previously (*4*).

All cigarettes tested increased guanylate cyclase activity, with maximum activation occurring after 10 seconds of smoke exposure (Fig. 1). Activity with each of eight brands of cigarette correlated with the NO concentration of the smoke gas phase ($r = 0.85$, $P < .01$). Nicotine (0.1 μM to 10 mM) had little or no effect on guanylate cyclase activity. Nitric oxide was neither removed by filters present on the cigarettes nor by our smoke-delivering device. These filters remove the particulate phase of smoke and may remove acrolein, HCN, and NO_2 vapors but not NO or CO_2 (*10*). Carbon monoxide (98 to 100 percent) increased the guanylate cyclase activity in crude preparations of lung and liver homogenates by two to three times, but had no effect on partially purified supernatant fractions. Thus, these effects of CO were relatively small. Since the concentration of CO in cigarette smoke is only several percent (*11*) it probably had little effect in our system.

Both the fresh gas phase of cigarette smoke and NO significantly increased cyclic GMP in lung minces without affecting levels of cyclic AMP (Table 1).

Cigarette smoke increased guanylate cyclase activity in supernatant and particulate fractions of a variety of tissues as shown in Table 2. The degree of activation depended on the type of tissue, and ranged from 2 to 36 times among the different tissue preparations. This apparent lack of tissue specificity parallels that of NO and sodium nitroprusside (*4*). The characteristics of guanylate cyclase activation by NO and cigarette smoke gas have several other similarities. First, oxidizing agents [$K_3Fe(CN)_6$ and methylene blue] inhibited stimulation both by cigarette smoke and NO, whereas some reducing agents (thiols and ascorbate) either had no effect or enhanced the stimulation by both agents (data not shown). Second, the effect of cigarette smoke gas was not additive to that of NO or sodium nitroprusside (Table 3), suggesting that cigarette smoke and these compounds activate the enzyme by a similar mechanism. These and other nitroso activating agents alone or in combination produce less stimulation when their maximally effective concentrations are exceeded (*4*). Third, native guanylate cyclase prefers Mn^{2+} as the sole cation cofactor with Mg^{2+} being about 10 percent as effective. In contrast, this preference is abolished in the presence of nitrocompounds or those capable of releasing nitric oxide; the NO activated enzyme can use either cofactor equally well for the formation of cyclic GMP and can also form cyclic

Table 2. Effect of cigarette smoke on guanylate cyclase activity in various tissues. Supernatant (S) and particulate (P) preparations of guanylate cyclase from rat tissues except where indicated were assayed as described in the text. Some reaction mixtures were exposed to smoke from brand D for 30 seconds prior to the addition of GTP and Mn^{2+}. Results are means of two or three determinations. The enzyme activity is expressed as picomoles of cyclic GMP formed per milligram of protein per minute.

Tissue	Fraction	Guanylate cyclase activity		
		No smoke (A)	Smoke (B)	Ratio of B to A
Heart	S	21.2	330.9	15.6
Heart	P	2.2	4.3	2.0
Lung	S	217.4	2489.7	11.5
Lung	P	59.2	260.3	4.4
Liver	S	42.8	790.2	18.5
Liver	P	6.5	31.1	4.8
Kidney	S	125.5	2443.2	19.5
Kidney	P	9.1	56.9	6.2
Cerebral cortex	S	91.7	2057.3	22.4
Cerebral cortex	P	19.3	294.2	15.2
Cerebellum	S	56.6	2019.2	35.7
Cerebellum	P	19.7	246.7	12.5
Bovine tracheal mucosa	S	2.8	53.3	19.0

Table 3. Effect of cigarette smoke, NO, and sodium nitroprusside on guanylate cyclase activity in the supernatant fraction (105,000g) of rat liver, determined after exposure of the fraction to the fresh gas phase from brand D cigarettes for 30 seconds. Values are means of two or three determinations.

Addition	Concentration (mM)	Cyclic GMP (pmole/mg protein/min)		
		No smoke (A)	Smoke (B)	Ratio of B to A
None		32.4	802.4	24.8
NO		521.6	675.0	1.3
Sodium nitroprusside	0.1	61.0	488.9	8.0
Sodium nitroprusside	1.0	319.3	191.3	0.6

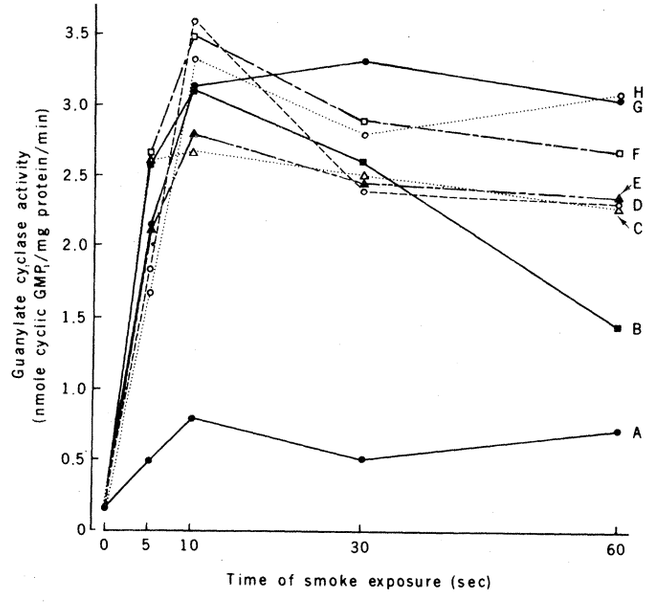

Fig. 1. The activation of guanylate cyclase by the fresh gas phase of cigarette smoke. Guanylate cyclase activity in the 105,000g supernatant fraction of bovine lung was determined as described in the text after exposing the reaction mixtures to cigarette smoke for the times indicated. Eight different brands of cigarettes are designated A through H. Stimulation of guanylate cyclase after a 10-second exposure correlated with NO concentration in the filtered gas phase with each brand of cigarette ($r = 0.850$, $P < .01$). Results are means of three determinations from a representative experiment.

AMP from adenosine triphosphate (*12*). Exposure of enzyme to the fresh gas phase of cigarette smoke resulted in responses similar to these. That is, when smoke was present, either Mn^{2+} or Mg^{2+} was effective as the sole cation cofactor. Also guanylate cyclase from the supernatant fraction of lung homogenates catalyzed the formation of cyclic AMP as well as cyclic GMP in the presence of the appropriate nucleotide substrate and 4 m*M* Mn^{2+} (56 and 818 pmole per milligram of protein per minute, respectively).

Our results thus suggest that the fresh gas phase of cigarette smoke activates guanylate cyclase and increases tissue levels of cyclic GMP by a mechanism involving the NO in cigarette smoke. However, we cannot rule out the contribution of other nonfilterable components in the gas phase of cigarette smoke to the stimulation of guanylate cyclase.

In view of the emerging role of cyclic GMP in tissue proliferation, and the recent evidence suggesting that the tumorigenic agents 1-methyl-1-nitrosourea and nitrosamines activate guanylate cyclase (*3*), it is possible that NO in cigarette smoke may be in some way related to the suspected carcinogenicity of cigarette smoking. Furthermore, some of the effects of cigarette smoke on ciliary function and mucosal secretion in lung may in part be attributable to cyclic nucleotide metabolism (*13*).

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References and Notes

1. E. W. Thomas, F. Murad, W. B. Looney, H. P. Morris, *Biochim. Biophys. Acta* **297**, 564 (1973); F. Murad, H. Kimura, H. A. Hopkins, W. B. Looney, C. J. Kovacs, *Science* **190**, 58 (1975); H. Kimura and F. Murad, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1965 (1975).
2. K. D. Schultz, K. Schultz, G. Schultz, *Nature (London)* **265**, 750 (1977); S. Katsuki and F. Murad, *Mol. Pharmacol.* **13**, 330 (1977).
3. F. R. DeRubertis and P. A. Craven, *Science* **193**, 897 (1976); D. L. Vesely, L. E. Rovere, G. S. Levey, *Cancer Res.* **37**, 28 (1977).
4. S. Katsuki, W. Arnold, C. Mittal, F. Murad, *J. Cyclic Nucleotide Res.* **3**, 23 (1977); W. P. Arnold *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3203 (1977); C. K. Mittal, H. Kimura, F. Murad, *J. Biol. Chem.* **252**, 4384 (1977).
5. A. J. Haagen-Smit, M. F. Brunelle, J. Hara, *AMA Arch. Ind. Health* **20**, 399 (1959); V. Norman and C. H. Keith, *Nature (London)* **205**, 915 (1965).
6. T. Okamoto, P. Chan, B. T. So, *Life Sci.* **11**, 733 (1972); P. G. Holt and D. Keast, *Experientia* **29**, 1004 (1973).
7. G. M. Powell and G. M. Green, *Biochem. Pharmacol.* **21**, 1785 (1972).
8. *Selected Methods of Measuring Air Pollutants* (World Health Organization, Geneva, 1976), pp. 73–76.
9. A. L. Steiner, C. W. Parker, D. M. Kipnis, *J. Biol. Chem.* **247**, 1106 (1972).
10. D. Tiggelbeck, *J. Natl. Cancer Inst.* **48**, 1825 (1972).
11. J. C. Robinson and W. F. Forbes, *Arch. Environ. Health* **30**, 425 (1975).
12. H. Kimura, C. K. Mittal, F. Murad, *J. Biol. Chem.* **251**, 7769 (1976); C. K. Mittal and F. Murad, *J. Biol. Chem.* **252**, 3136 (1977).
13. J. Iravani, *Respiration* **29**, 480 (1972).

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Free Radical Increases in Cancer: Evidence That There Is Not a Real Increase

Abstract. *The controversial finding of an increase in free radicals with the development of cancer was restudied with Walker 256 carcinosarcoma cells. It was confirmed that such an increase appears to occur, but it was also demonstrated that it is not a real increase. With growth of the tumor, the electron spin resonance lines for lyophilized samples became narrower, resulting in an increase in peak-to-peak height measurements, but there was no change in the total number of spins. The signals for lyophilized tumor samples were different from those for the same samples before lyophilization. Changes in line shape indicated that lyophilized tumor samples contain a different mixture of radicals than lyophilized samples of normal tissue.*

There have been conflicting reports on the free radical content of tissues during the development or growth of malignant tumors (*1*). In a number of studies, usually with lyophilized samples (*2*, *3*), an increase in free radical content was found during the early phases of tumor growth, with a subsequent fall in concentration as the tumors become large. Studies in which tumor tissues were frozen but not lyophilized indicated that free radical concentrations decrease during tumor growth (*4*). It is important to resolve this conflict because several hypotheses, including some suggested new treatment schemes, associate free radicals with cancer, partly on the basis of increased free radical contents during tumor growth.

To our knowledge, no studies have been reported in which both methods of sample preparation were used for the

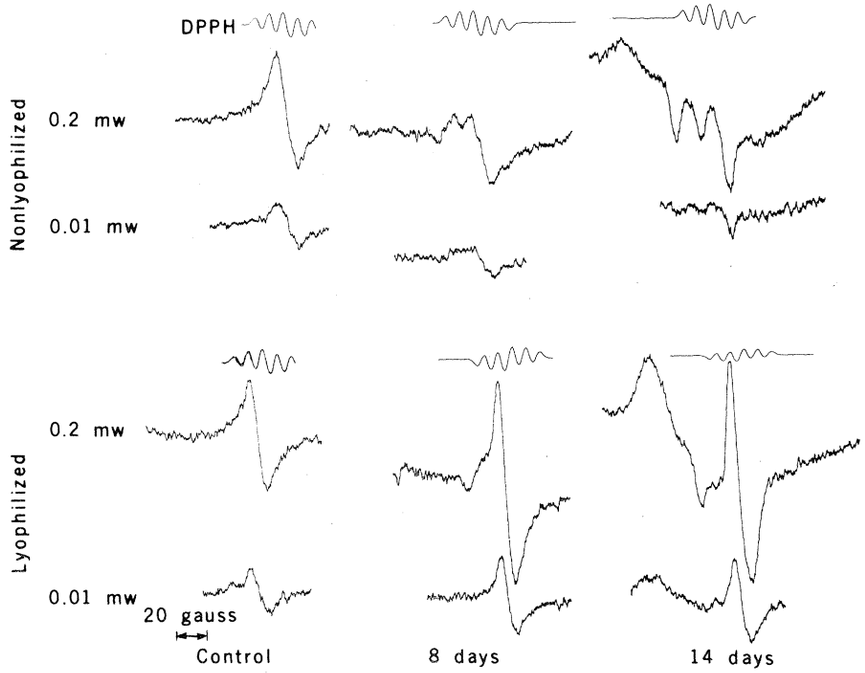

Fig. 1. Representative ESR spectra of nonlyophilized and lyophilized samples. Conditions: temperature, 77°K; incident microwave power levels to a TE_{104} cavity in a Varian E-9 spectrometer, as indicated; frequency, 9.1 Ghz; modulation, 8 gauss at 100 khz; relative signal amplitudes, all the same; the spectra of the DPPH (α,α-diphenyl-β-picrylhydrazyl) standard (g = 2.0036) indicate the g-factor. Spectra are shown for samples taken at 0, 8, and 14 days after inoculation with 10^4 Walker tumor cells, before and after lyophilization. After lyophilization the samples retained the same configuration as in the nonlyophilized condition and were transferred to liquid nitrogen with minimal exposure to air. Care was taken in the lyophilization procedure to avoid artifacts that could appear in this type of preparation (*11*).