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Molecular mechanisms of damage by excess nitrogen oxides: nitration of tyrosine by gas-phase cigarette smoke

Jason P. Eiserich[a,*], Veronique Vossen[a], Charles A. O'Neill[a], Barry Halliwell[a,b], Carroll E. Cross[a], Albert van der Vliet[a]

[a]*Division of Pulmonary/Critical Care Medicine, University of California, Davis, CA 95616, USA*
[b]*Neurodegenerative Disease Research Centre, King's College, University of London, Manresa Road, London SW3 6LX, UK*

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Abstract Nitric oxide (nitrogen monoxide, •NO) plays important physiological roles, but an excess can be toxic. •NO is present in cigarette smoke (CS) at up to 500 ppm, and probably represents one of the greatest exogenous sources of •NO to which humans are exposed. We show here that gas-phase CS is capable of converting tyrosine to 3-nitrotyrosine (3-NO_2-Tyr) and dityrosine, to an extent dependent on time of exposure and pH. Glutathione, ascorbic acid and uric acid decreased the CS-induced formation of 3-NO_2-Tyr and dityrosine. We suggest that nitrogen oxides in CS can modify proteins in the respiratory tract and may contribute to CS toxicity.

Key words: Cigarette smoke; Radical; Nitric oxide; Tyrosine; Nitrotyrosine; Dityrosine

1. Introduction

Cigarette smoking is associated with a variety of pulmonary and cardiovascular disorders including emphysema, atherosclerosis, and cancer [1,2]. However, the exact chemical/biochemical mechanisms underlying CS-induced effects on biological systems are incompletely understood. It has been proposed that free radicals in CS or generated by exposure to it (e.g. by CS-dependent activation of phagocytes), may be major contributory factors to CS-related diseases [3–5].

An important free radical in CS is nitric oxide (nitrogen monoxide, •NO) [6], which is present in CS at up to 500 ppm and probably represents one of the greatest exogenous sources of •NO to which humans are exposed. •NO is a species of considerable recent interest, both because of its multiple physiological roles (ranging from blood pressure modulation to neurotransmission) and because of its toxic effects when generated in excess [7,8]. The autoxidation of •NO, in air, to nitrogen dioxide ($^{\bullet}NO_2$) is well understood and proceeds with a rate that is second-order in •NO and first-order in O_2. It has been shown recently that •NO in CS is converted to $^{\bullet}NO_2$ within minutes [9]. Similarly, •NO reacts quickly with superoxide radical ($O^{\bullet}_2^-$) [10] to give peroxynitrite [11,12] and with organic peroxyl radicals (known to be found in CS, [6]) to give alkyl peroxynitrites [13], both of which are cytotoxic species.

Whereas the reactivity of •NO with most biological macromolecules is somewhat limited, $^{\bullet}NO_2$, peroxynitrite and peroxynitrates are highly reactive cytotoxic species. For example $^{\bullet}NO_2$ undergoes both addition and hydrogen abstraction reactions with alkenes and unsaturated lipids, initiating lipid peroxidation [14–16]. Peroxynitrite is a powerful oxidant towards -SH groups [17] and can decompose to a range of reactive species, possibly including $^{\bullet}NO_2$, NO_2^+ and •OH [11,12,18]. In the present investigation we show that nitrogen oxides and/or their reaction products in gas-phase CS convert the amino acid tyrosine to nitrated and oxidized products. Such reactions could cause damage to enzymes and transport proteins and would be expected to interfere with cell signalling pathways involving tyrosine phosphorylation.

2. Materials and methods

2.1. Chemicals and cigarettes

L-*p*-tyrosine, 3-nitro-L-tyrosine, *N*-acetyl-L-tyrosine (NAT), uric acid, ascorbic acid, glutathione, potassium nitrite, dithiothreitol and Dowex chelating resin were purchased from Sigma Chemical Co. (St. Louis, MO). Monobromobimane (3,7-dimethyl-4-bromomethyl-6-methyl-1,5-diazabicyclo-[3.3.0]octa-3,6-diene-2,8-dione) was purchased from Calbiochem (La Jolla, CA). Dityrosine was synthesized from tyrosine, using H_2O_2 and horseradish peroxidase and purified as previously described [19]. The cigarettes used in this study were University of Kentucky (UK) 2R1 cigarettes containing 23 mg tar and 2.2 mg of nicotine per cigarette (according to the Federal Tobacco Council). Filters were standard Cambridge filters rated to remove 99.9% of all particles >0.1 μm in diameter.

2.2. CS exposure of N-acetyltyrosine solutions

Solutions of NAT (1 mM) were prepared in chelexed 100 mM KH_2PO_4 buffer at various pH values and exposed to gas-phase CS with slight modification of the method of Frei et al. [20]. The above solution (20 ml) was placed into a 500 ml side-arm flask and preincubated in a shaking water bath at 37°C for 5 min and a first set of samples was withdrawn. The side-arm of the filter flask was connected via a plastic Y connector to a vacuum and to a UK 2R1 standard cigarette with a Cambridge filter and the top of the filter flask was closed with a rubber stopper. The flask was evacuated and then the connector to the vacuum was clamped. The cigarette was lit, and gas-phase (filtered) CS was slowly introduced into the flask, burning about one-fifth of the cigarette. The side-arm to the cigarette was clamped and the flask was removed from the apparatus and placed into the water bath and incubated at 37°C for 1–20 min. At the end of the incubation, solutions were sampled and another 'puff' of CS was introduced into the flask. Control samples were incubated similarly, but with 'puffs' of room air instead of CS. In some experiments, solutions and the headspace of flasks were purged with N_2 prior to exposure. Glutathione (GSH), ascorbic acid and uric acid were added to the NAT solutions at final concentrations of 100 μM.

2.3. Sample preparation

Aliquots of 250 μl, sampled at various times during CS exposure,

*Corresponding author. Pulmonary Research Laboratory, c/o Med. Spon. Programs, TB 150, University of California, Davis, CA 95616, USA. Fax: (1) (916) 752 4374.

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were placed in glass vials and 15 μl of BHT (5% wt/wt in ethanol) was added to prevent further free radical reactions. Concentrated hydrochloric acid (HCl) (250 μl) was added to each sample yielding a final HCl concentration of approximately 6 M. Samples were purged with a stream of N_2 and incubated for 4 h at 110°C to hydrolyze the acetyl group and liberate tyrosine, 3-nitrotyrosine and/or dityrosine. The samples were placed in a water bath (65°C) and evaporated to dryness with a stream of N_2. Each dried sample was resolubilized in 250 μl of 50 mM KH_2PO_4/8% (v/v) methanol (pH 3.0) and analyzed by HPLC. The hydrolysis efficiency of NAT to tyrosine was approximately 90%.

2.4. Analysis of 3-nitrotyrosine, dityrosine, antioxidants and nitrite

Reconstituted samples were analyzed for 3-nitrotyrosine and dityrosine by HPLC with combined in-line UV and fluorescence detection. Briefly, the above components were resolved using a 5 μm Spherisorb ODS-2 analytical column (250 × 4.6 mm; Alltech, Deerfield, IL) equipped with a 7 μm RP-8 Aquapore guard column (15 × 3.2 mm; Dychrome Inc., Sunnyvale CA). Chromatographic separation employed an isocratic elution consisting of 50 mM KH_2PO_4–H_3PO_4 (pH = 3.0) with 8% (v/v) methanol at a flow rate of 1 ml/min. Detection of 3-nitrotyrosine was accomplished by UV absorbance at 274 nm, using a Shimadzu SPD-6AV UV detector (Shimadzu, Columbia, MD). Dityrosine was detected by fluorescence (excitation λ = 284 nm, emission λ = 410 nm) using a Waters 470 Scanning Fluorescence Detector (Waters, Milford, MA). Peaks were identified based on coelution with authentic standards and quantified by peak area using external standards. 3-Nitrotyrosine formation was confirmed by comparing absorbance spectra of the peaks with that of authentic 3-nitrotyrosine, using a Waters 996 Photodiode Array (PDA) Detector. All data were recorded and processed on a 486 IBM clone computer using Waters Millennium 2010 software.

Ascorbic acid and uric acid concentrations were determined by an HPLC equipped with a Waters 464 Pulsed Electrochemical Detector operating at a voltage of 500 mV. Separations were performed on a Supelcosil LC-NH_2 analytical column (250 × 4.6 mm; Supelco, Bellefonte, PA) with an isocratic elution consisting of 40 mM KH_2PO_4 with 5% (v/v) methanol. Glutathione was analyzed after pre-column derivatization with monobromobimane using HPLC with fluorescence detection (excitation λ = 394 nm, emission λ = 480 nm [21]).

Nitrite (NO_2^-) concentrations were determined by a method based on the Griess reaction as previously described [22]. NO_2^-, generated from aqueous-phase autoxidation of $^{\bullet}NO$ in CS, is relatively stable in solution at neutral pH. However, under highly acidic conditions the resulting nitrous acid (HNO_2) decomposes to form $^{\bullet}NO$, $^{\bullet}NO_2$ and H_2O [23], and these products are capable of nitrating tyrosine in proteins [24]. To exclude the possibility that 3-NO_2-Tyr is formed artifactually by the above mechanism during acid hydrolysis of NAT, CS-exposed buffer solutions were added to stock solutions of *N*-acetyltyrosine, hydrolyzed, and analyzed for 3-NO_2-Tyr and dityrosine. 3-NO_2-Tyr and dityrosine were not detected in three separate experiments, thus confirming that CS-induced formation of these products was not artifactual.

3. Results

3.1. Nitration of N*-acetyltyrosine*

Initial studies with solutions of free tyrosine exposed to gas-phase CS showed that aldehydes, or other species in CS, react with the free amino group of tyrosine and caused significant losses of tyrosine and very low recoveries of its oxidized and nitrated products (data not shown). Hence, in all further experimentation we used *N*-acetyltyrosine (NAT), which has a blocked amino group and is a more representative model for tyrosine residues in a peptide sequence. When 1 mM solutions of NAT were exposed to gas-phase CS, at pH 7.4, both 3-NO_2-Tyr and dityrosine were detected following hydrolysis. Fig. 1 illustrates the formation of 3-NO_2-Tyr, dityrosine and nitrite (NO_2^-) after 18 puffs of gas-phase CS (equivalent to approximately 3 cigarettes smoked). After an initial lag phase, there was a rapid increase in 3-NO_2-Tyr formation, slowing somewhat at longer incubation times. By contrast, dityrosine concentrations increased linearly with incubation time. The overall yields of 3-NO_2-Tyr and dityrosine, after 18 puffs of CS and 20 min incubations between puffs, were 17.1 ± 0.6 and 1.5 ± 0.07 μM, respectively (n = 3). Formation of NO_2^- seemed to parallel 3-NO_2-Tyr formation (Fig. 1B). Fig. 2 shows that formation of both 3-NO_2-Tyr and dityrosine increased with increasing pH, although the yield of dityrosine was always much smaller than that of 3-NO_2-Tyr. Changing the pH of the solutions from 6.0 to 8.5 resulted in a 1.8- and 10.5-fold increase in 3-NO_2-Tyr and dityrosine, respectively, after 9 puffs of CS.

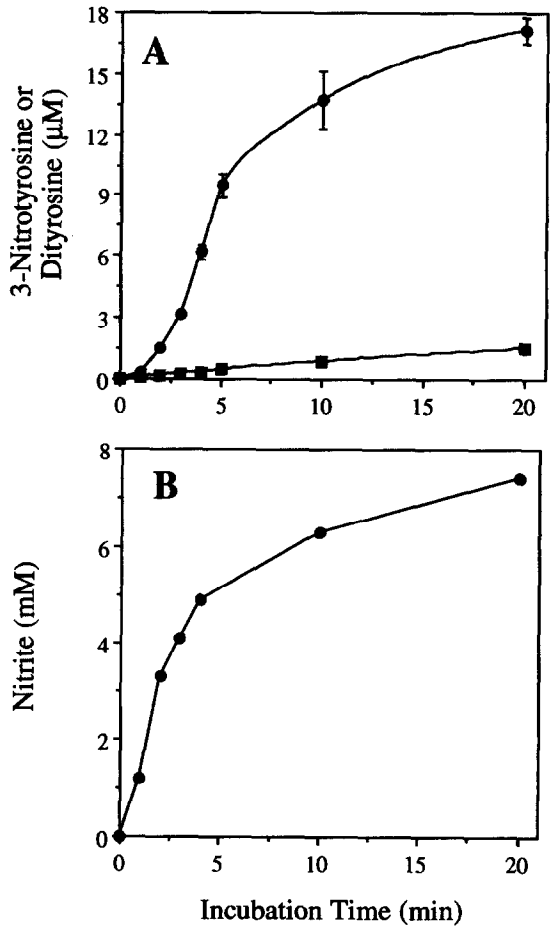

Fig. 1. (A) Formation of 3-nitrotyrosine (●) and dityrosine (■) by gas-phase cigarette smoke (CS) as a function of incubation time. (B) Formation of nitrite (NO_2^-) by gas-phase CS. Solutions of *N*-acetyltyrosine (1 mM) were exposed to a total of 18 puffs of CS and incubated for various periods of time after each puff of CS, as described in section 2. Each data point represents the mean ± S.D. of 3 separate experiments, or the average of 2 separate experiments for NO_2^-.

3.2. Effect of antioxidants

The effect of uric acid, ascorbic acid and reduced glutathione (GSH) is shown in Fig. 3. GSH, ascorbic acid and uric acid (100 μM final concentrations) inhibited the formation of 3-NO_2-Tyr in solutions of NAT exposed to 18 puffs of gas-phase CS; the mean % inhibitions were 24, 38 and 77%, respectively. GSH, ascorbic acid and uric acid inhibited the formation of dityrosine in the same experiments by 8, 45 and 78%, respectively. Complete depletion of GSH and ascorbate occurred within 3 and

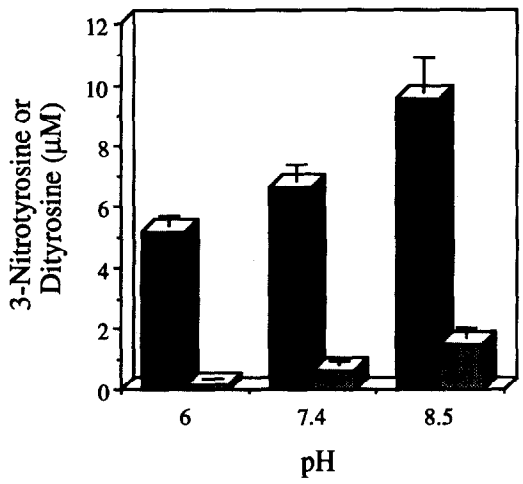

Fig. 2. pH dependent formation of 3-nitrotyrosine (dark bars) and dityrosine (shaded bars) by gas-phase cigarette smoke. Solutions of *N*-acetyltyrosine (1 mM) were exposed to a total of 9 puffs of CS and incubated for 5 min between puffs. Each data point represents the mean ± S.D. of 3 separate experiments.

6 puffs, respectively, whereas uric acid was only depleted by approximately 60% after 18 puffs of CS. The abilities of ascorbic acid and uric acid to inhibit nitration and oxidation of tyrosine were comparable until 6 puffs of CS were administered. However, after complete depletion of ascorbic acid, the rate of formation of 3-NO_2-Tyr and dityrosine paralleled that of the control without added antioxidant.

3.3. Effect of anaerobic conditions

Purging the NAT solutions and the headspace of the exposure flasks with N_2 before CS exposure and successive purging of the headspace of the flask between puffs of CS, inhibited the formation of 3-NO_2-Tyr by a mean value of 52% after 18 puffs of CS. By contrast, dityrosine formation was not affected by purging with with N_2 (Table 1).

Table 1
Effect of air or nitrogen atmospheres on the CS-induced formation of 3-nitrotyrosine and dityrosine

Puffs of CS	Air	Nitrogen
3-Nitrotyrosine (μM)		
0	n.d.*	n.d.*
6	3.6 ± 0.1	2.2 ± 0.9
12	5.7 ± 0.9	3.2 ± 1.3
18	8.9 ± 0.5	4.3 ± 1.5
Dityrosine (μM)		
0	0.04 ± 0.01	0.02 ± 0.01
6	0.30 ± 0.07	0.32 ± 0.06
12	0.35 ± 0.07	0.41 ± 0.05
18	0.42 ± 0.09	0.54 ± 0.12

Solutions of *N*-acetyltyrosine (1 mM) in 100 mM KH_2PO_4 buffer (pH 7.4) were exposed to 18 puffs of gas-phase CS with 5 min incubations following each puff. Initially, solutions were either purged with air or N_2, exposed to CS and the headspace was purged with N_2 or air for 15 s before each subsequent puff of CS. Data are expressed as mean ± S.D. from three separate incubations.
*n.d. = not detected.

4. Discussion

There is considerable current interest in the toxicity of excess oxides of nitrogen [7,25–27] and CS is one of the major sources of $^{\bullet}NO$ to which humans are exposed. We show here that gas-phase CS is capable of nitrating and oxidizing *N*-acetyltyrosine in aqueous solutions. The relatively low yields of dityrosine compared to 3-NO_2-Tyr could be due to competitive reaction of the tyrosyl radical with the plethora of other radicals produced in CS [6].

The inhibitory effects observed with the antioxidants GSH, ascorbic acid and uric acid are consistent with free radical mechanisms of both 3-NO_2-Tyr and dityrosine formation by CS. These water soluble antoxidants are present in the respiratory tract lining fluids, and thus may help to protect against CS-induced damage [28,29].

The exact species responsible for nitration of tyrosine by CS has not been identified in our experiments, but it is likely to be one or more of the reaction products of $^{\bullet}NO$. $^{\bullet}NO_2$ may be involved, since oxidation of $^{\bullet}NO$ to $^{\bullet}NO_2$ in CS has been shown to occur within minutes [9]. For instance, exposure of various peptides and proteins to $^{\bullet}NO_2$ causes damage, including the formation of 3-NO_2-Tyr and dityrosine in amounts increasing

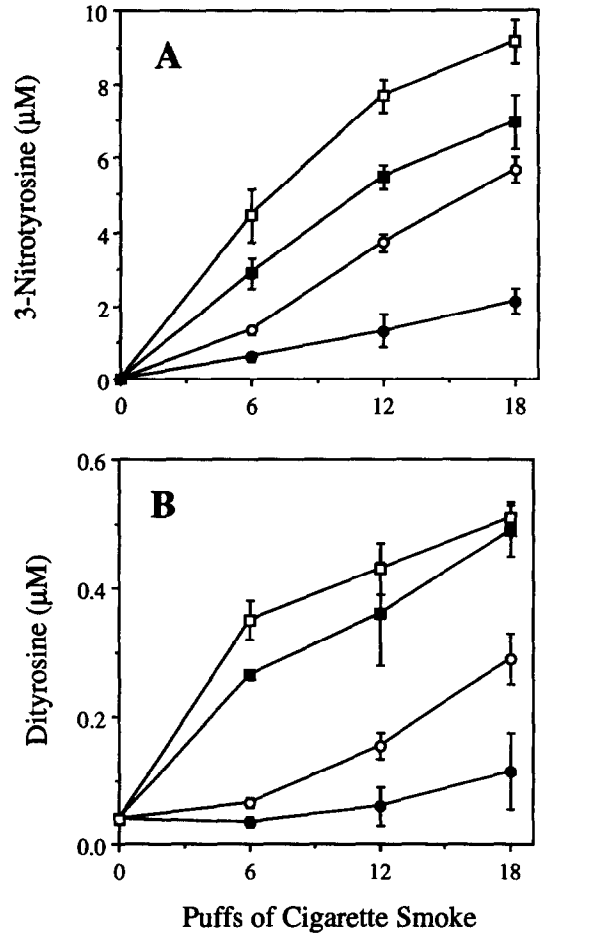

Fig. 3. Inhibition of cigarette smoke-induced formation 3-nitrotyrosine (graph A) and dityrosine (graph B) by glutathione (■), ascorbic acid (○) and uric acid (●). Control samples without added antioxidant are represented by (□). Each data point represents the mean ± S.D. of 3 separate experiments.

with pH [30–32]. Purging the NAT solutions and headspace with N_2 prior to CS exposure inhibits formation of 3-NO_2-Tyr and suggests that formation of a nitrating species in CS is O_2-dependent.

Autoxidation of $^{\bullet}NO$ to NO_2^- in aqueous solutions has been observed [25] and is thought to proceed via a steady-state concentration of the reactive intermediates $^{\bullet}NO_2$ and nitrous anhydride (N_2O_3) [33]. It is possible, then, that aqueous autoxidation of CS-derived $^{\bullet}NO$ produces a species capable of nitrating tyrosine. Formation of 3-NO_2-Tyr paralleled very closely the formation of NO_2^- in solutions exposed to CS (Fig. 1B) and may indicate a temporal correlation between aqueous autoxidation of $^{\bullet}NO$ to NO_2^-, and subsequent formation of 3-NO_2-Tyr.

Other contributing reactions may include reaction of $^{\bullet}NO$ with superoxide ($O_2^{\bullet-}$) and organic peroxyl radicals ($ROO^{\bullet}$), both of which are formed in CS, to form peroxynitrite (^-OONO) and organic peroxynitrites (ROONO) [6,11,13]. Superoxide can be additionally produced by CS-induced activation of neutrophils and macrophages in the lungs of smokers, potentially increasing the formation of ^-OONO. In aqueous solutions both ^-OONO and ROONO decompose into $^{\bullet}NO_2$, and possibly NO_2^+; both would be capable of reacting with tyrosine to form 3-NO_2-Tyr [11]. Indeed, addition of ^-OONO to human plasma produces nitration of aromatic amino acids [34].

Nitration of tyrosine can damage protein function, e.g. it can inactivate human α-1-proteinase inhibitor [35], an element in CS-induced emphysema [36]. Exposure of pulmonary surfactant protein A to ^-OONO caused significant nitration of tyrosine and decreased the ability of this protein to act synergistically with surfactant proteins B and C in lowering surface tension of surfactant lipids [37]. CS-induced nitration of tyrosine residues in respiratory tract proteins may inactivate critical enzymes and membrane transport/receptor proteins, impair functions of surfactant proteins, and interfere with cell signalling pathways involving tyrosine phosphorylation. Such reactions may be a significant mechanism by which CS causes its deleterious effects. They are also relevant to other situations in which excess exposure to $^{\bullet}NO$ causes toxicity in humans, e.g. in chronic inflammatory disease [38], atherosclerosis [39] and septic shock [7]. Indeed, the use of 3-NO_2-Tyr as a 'marker' to assess ^-OONO formation in humans is becoming increasingly popular [38,39] and the potential confounding effects of cigarette smoking on this 'marker' warrant further investigation.

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References

[1] Evans, M.D. and Pryor, W.A. (1994) Am. J. Physiol. 266, L593–L611.
[2] Haapanen, A., Koskenvuo, M., Kaprio, J., Kesaniemi, Y.A. and Keikkila, K. (1989) Circulation 80, 10–16.
[3] Leanderson, P. and Tagesson, C. (1992) Chem. Biol. Interact. 810, 197–208.
[4] Cross, C.E., O'Neill, C.A., Reznick, A.Z., Hu. M., Packer, L. and Frei, B. (1993) Ann. NY Acad. Sci. 686, 72–90.
[5] Church, D.F. and Pryor, W.A. (1985) Environ. Health Perspect. 64, 111–126.
[6] Pryor, W.A. and Stone, K. (1993) Ann. NY Acad. Sci. 686, 12–28.
[7] Moncada, S. and Higgs, A. (1993) N. Engl. J. Med. 329, 2002–2012.
[8] Änggård, E. (1994) Lancet 343, 1199–1206.
[9] Cueto, R. and Pryor, W.A. (1994) Vibrational Spectroscopy 7, 97–111.
[10] Huie, R.E. and Padmaja, S. (1993) Free Rad. Res. Commun. 18, 195–199.
[11] Beckman, J.S., Chen, J., Ischiropoulos, H. and Crow, J.P. (1994) Methods Enzymol. 233, 229–240.
[12] Koppenol, W.H., Moreno, J.J., Pryor, W.A., Ischiropoulos, H. and Beckman, J. (1992) Chem. Res. Toxicol. 5, 834–842.
[13] Padmaja, S. and Huie, R.E. (1993) Biochem. Biophys. Res. Comm. 195, 539–544.
[14] Gallon, A.A. and Pryor, W.A. (1994) Lipids 29, 171–176.
[15] Halliwell, B., Hu, M.-L., Louie, S., Duvall, T.R., Tarkington, B.K., Motchnik, P., Cross, C.E. (1992) FEBS Lett. 313, 62–66.
[16] Thomas, H.V., Mueller, P.K. and Lymann, R.L. (1967) Science 159, 532–534.
[17] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) J. Biol. Chem. 266, 4244–4250.
[18] van der Vliet, A., O'Neill, C.A., Halliwell, B., Cross, C.E. and Kaur, H. (1994) FEBS Lett. 339, 89–92.
[19] Amadò, R., Aeschbach, R. and Neukom, H. (1984) Methods Enzymol. 107, 377–388.
[20] Frei, B., Forte, T.M., Ames, B.N. and Cross, C.E. (1991) Biochem. J. 277, 133–138.
[21] Cotgreave, I.A. and Moldeus, P. (1986) J. Biochem. Biophys. Methods 13, 231–249.
[22] Schmidt, H.H., Nau, H., Wittfort, J., Gerlach, J., Prescher, K.E., Klein, M.M., Niroomana, F. and Oohme, E. (1988) Eur. J. Pharmacol. 154, 213–216.
[23] Park, J.-Y. and Lee, Y.-N. (1988) J. Phys. Chem. 92, 6294–6302.
[24] Knowles, M.E., McWeeny, D.J., Couchman, L. and Thorogood, M. (1974) Nature 247, 288–289.
[25] Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E. and Byrns, R.E. (1993) Proc. Natl. Acad. Sci. USA 90, 8103–8107.
[26] Gaston, B., Drazen, J.M., Loscalzo, J. and Stamler, J.S. (1994) Am. J. Respir. Crit. Care Med. 149, 538–551.
[27] Wink, D.A., Darbyshire, J.F., Nims, R.W., Saavedra, J.E. and Ford, P.C. (1993) Chem. Res. Toxicol. 6, 23–27.
[28] Slade, R., Crissman, K., Norwood, J. and Hatch, G. (1993) Exp. Lung Res. 19, 469–484.
[29] Cross, C.E., van der Vliet, A., O'Neill, C.A., Louie, S. and Halliwell, B. (1994) Environ. Health Perspect, in Press.
[30] Prütz, W.A., Mönig, H., Butler, J. and Land, E.J. (1985) Arch. Biochem. Biophys. 243, 125–134.
[31] Kikugawa, K., Kato, T., Okamoto, Y. (1994) Free Rad. Biol. Med. 16, 373–382.
[32] Hood, D.B., Gettins, P. and Johnson, D.A. (1993) Arch. Biochem. Biophys. 304, 17–26.
[33] Lewis, R.S. and Deen, W.M. (1994) Chem. Res. Toxicol. 7, 568–574.
[34] van der Vliet, A., Smith, D., O'Neill, C.A., Kaur, H., Darley-Usmar, V., Cross, C.E. and Halliwell, B. (1994) Biochem. J, 303, 295–301.
[35] Mierzwa, S. and Chan, S.K. (1987) Biochem. J. 246, 37–42.
[36] Carp, H. and Janoff, A. (1978) Am. Rev. Respir. Dis. 118, 617–621.
[37] Haddad, I.Y., Ischiropoulos, H., Holm, B.A., Beckman, J.S., Baker, J.R. and Matalon, S. (1993) Am. J. Physiol. 265, L555-L564.
[38] Kaur, H. and Halliwell, B. (1994) FEBS Lett. 350, 9–12.
[39] Beckman, J.S., Ye, Y.Z., Anderson, P.G., Chen, J., Accavitti, M.A., Tarpey, M.M. and White C.R. (1994) Biochem. Hoppe-Seyler 375, 81–88.