Lack of Tyrosine Nitration by Peroxynitrite Generated at Physiological pH*

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Nitration of tyrosine residues of proteins has been suggested as a marker of peroxynitrite-mediated tissue injury in inflammatory conditions. The nitration reaction has been extensively studied in vitro by bolus addition of authentic peroxynitrite, an experimental approach hardly reflecting in vivo situations in which the occurrence of peroxynitrite is thought to result from continuous generation of NO and O₂⁻ at physiological pH. In the present study, we measured the nitration of free tyrosine by NO and O₂⁻ generated at well defined rates from the donor compound (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]-diazene-1-ium-1,2-diolate (spermine NONOate) and the xanthine oxidase reaction, respectively. The results were compared with the established nitration reaction triggered by authentic peroxynitrite. Bolus addition of peroxynitrite (1 mM) to tyrosine (1 mM) at pH 7.4 yielded 36.77 ± 1.67 μM 3-nitrotyrosine, corresponding to a recovery of about 4%. However, peroxynitrite formed from NO and O₂⁻, which were generated at equal rates (~5 μM × min⁻¹) from 1 mM spermine NONOate, 28 millimolar/ml xanthine oxidase, and 1 mM hypoxanthine was much less efficient (0.67 ± 0.01 μM; ~0.07% of total product flow). At O₂⁻ fluxes exceeding the NO release rates, 3-nitrotyrosine formation was below the detection limit of the high performance liquid chromatography method (<0.06 μM). Nitration was most efficient (~0.3%) with the NO donor alone, i.e. without concomitant generation of O₂⁻. Nitration by NO had a pH optimum of 8.2, increased progressively with increasing tyrosine concentrations (0.1–2 mM), and was not enhanced by NaHCO₃ (up to 20 mM), indicating that it was mediated by O₂⁻ rather than peroxynitrite. Our results argue against peroxynitrite produced from NO and O₂⁻ as a mediator of tyrosine nitration in vivo.

Nitric oxide (NO) is a cellular messenger regulating numerous biological processes, including relaxation of blood vessels and neurotransmitter release in the brain, but overproduction of NO appears to contribute essentially to tissue injury in inflammatory and ischemic conditions (1). The molecular mechanisms underlying the cytotoxicity of NO are not well understood. The potent oxidant peroxynitrite, which is formed in a rapid reaction from NO and O₂⁻, is thought to be a key mediator of NO toxicity in atherosclerosis, congestive heart failure, glutamate excitotoxicity, and other disease states involving inflammatory oxidative stress (2).

Formation of peroxynitrite from NO and O₂⁻ occurs at nearly diffusion-controlled rates (4.3–6.7 × 10⁻⁷ M⁻¹ s⁻¹) (3, 4). Therefore, NO out-competes the reaction of O₂⁻ with superoxide dismutase at steady-state concentrations that are likely to occur in vivo (5). Peroxynitrite is stable at alkaline pH but has a half-life of less than 1 s at pH 7.4 (pKa = 6.8) (6). Depending on the pH, the corresponding peroxynitrosic acid either rearranges to NO₃⁻ or decomposes to NO₂ and O₂ (7).

Peroxynitrite has been shown to react with virtually all classes of biomolecules (8). The reaction with phenolic compounds, including free and protein-bound tyrosine, results in the formation of nitrated, hydroxylated, and dimeric products (9–12). The nitration of tyrosine, yielding mainly 3-nitrotyrosine, is markedly enhanced by CO₂, which reacts with peroxynitrite anion at physiological pH to form the potent nitrat-
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The flux rates of NO and O2, the oxidation of DHR, and formation of 3-nitrotyrosine were measured as described under “Experimental Procedures.” Data are mean values ± S.E. of three experiments performed in duplicate. Sper NO, spermine NONOate; DHR-ox., dihydrorhodamine oxidation; NO2-Tyr, 3-nitrotyrosine.

| Conditions | Sper NO (μM) | XO (μM) | DHR-ox. (μM) | NO2-Tyr (μM) |
|------------|-------------|---------|--------------|--------------|
| SIN-1 (1 mM) | 0.02 | 5.5 | 2.16 ± 0.13 | 2.13 ± 0.17 |
| SIN-1 (1 mM) | 0.3 | 8 | 2.93 ± 0.17 | 2.97 ± 0.52 |
| SIN-1 (1 mM) | 0.5 | 15 | 3.96 ± 0.18 | 4.33 ± 0.10 |
| SIN-1 (1 mM) | 1 | 28 | 5.00 ± 0.26 | 5.13 ± 0.20 |
| SIN-1 (1 mM) | 5 | 9.5 | 9.53 ± 0.11 | 4.87 ± 0.16 |

Experimental Procedures

Materials—Spermine NONOate was from Alexis (Laufelfingen, Switzerland). Hypoxanthine and DHR were from Fluka (Vienna, Austria). NO gas (99% pure) was from Linde (Munich, Germany). SIN-1 was a generous gift from Ho¨chst Marion Roussel Inc. (Frankfurt, Germany). XO (from buttermilk, 1.4 units/mg) and all other chemicals were from Sigma.

Solutions—All solutions were prepared fresh each day. Water was ultrapure from a Barnstead Pure water apparatus. Spermine NONOate was prepared as a 10-fold stock solution in 10 mM NaOH. SIN-1 was dissolved to 10 mM at pH 5.0. DHR was dissolved to 10 mM in acetonitrile and kept in the dark until use. NO solutions were prepared as described (28). Alkaline solutions of peroxynitrite were prepared from acidified NO2 and H2O2 as described (7). Stock solutions were diluted with H2O to 10 mM (pH 12.8) immediately before the experiments and added to 50 mM K2HPO4/KH2PO4 buffer, pH 7.4, to obtain final peroxynitrite concentrations of 1 mM. Changes of buffer pH were <0.1 unit. oxyHb was prepared as described (29).

Combined NO/O2 Assay—Fluxes of NO and O2 were determined photometrically using a previously described method that is based on the simultaneous scavenging of NO and O2 by oxyHb and cytochrome c, respectively (30). The absorbance changes at 542 nm (oxidation of oxyHb), 465 nm (reduction of cytochrome c), and 525 nm (isosbestic point for both reactions) were monitored with a Hewlett-Packard 8452A diode array spectrophotometer. Flux rates of NO and O2 were calculated using extinction coefficients of 6.6 mM–1 cm–1 (542–525 nm) and 7.3 mM–1 cm–1 (465–525 nm), respectively. The signal averaging time was 0.5 s; spectra were recorded every second for 1–3 min. All measurements were performed at ambient temperature in a total volume of 0.2 ml of a 50 mM K2HPO4/KH2PO4 buffer, pH 7.4, containing 1 mM hypoxanthine, 5 μM ferrocyanochrome c, 20 μM oxyHb in the presence of XO and spermine NONOate at the concentrations indicated.

The kinetics of spermine NONOate decomposition was simulated with the software package Mathematica (Version 2.2.2., Wolfram Research Inc., Champaign, IL) based on a half-life of 230 min at 22 °C and pH 7.4 as described (31).

Tyrosine Nitration—Unless indicated otherwise, hypoxanthine (1 mM) and tyrosine (1 mM) were incubated at ambient temperature for 12 h in 50 mM K2HPO4/KH2PO4 buffer, pH 7.4, in the presence of various concentrations of XO and spermine NONOate or SIN-1 (final concentration, 1 mM). Authentic 3-nitrotyrosine was stable under these conditions (recovery, 97.2 ± 3.44% (n = 4) after 12 h of incubation). In some experiments, reaction mixtures were incubated for 1 h with a solution of authentic NO (final concentration, ~1 mM) instead of the NO donor. To study tyrosine nitration by authentic peroxynitrite, alkaline stock solutions of peroxynitrite (final concentration, 1 mM) were added dropwise under vigorous vortexing to tyrosine (1 mM) in 50 mM K2HPO4/KH2PO4 buffer, pH 7.4, followed by incubation for 1 h. The combined presence of hypoxanthine (1 mM), XO (28 millimoles/ml), and spermine NONOate (1 mM) had no effect on tyrosine nitration triggered by authentic peroxynitrite. The following buffers were used to study the pH dependence of tyrosine nitration: pH 6.0–8.0, 0.2 mM K2HPO4/KH2PO4, pH 8.2–9.0, 0.2 mM Tris/HCl; pH 10, solutions of NaOH. The effect of O2 was studied by incubation in the presence of up to 20 mM NaHCO3.

Determination of 3-Nitrotyrosine—HPLC analysis of 3-nitrotyrosine was performed on a C18 reversed phase column with 50 mM KH2PO4/H3PO4 buffer (pH 3) containing 10% (v/v) methanol at 1 ml/min and detection at 274 nm as described (32). Calibration curves were recorded daily with authentic 3-nitrotyrosine (0.06–5 μM for experiments with ‘NO/O2’ and NO, and 10–100 μM for experiments with authentic peroxynitrite).

Oxidation of DHR—DHR oxidation was measured at 501 nm with a Shimadzu 160A spectrophotometer at ambient temperature in a total volume of 0.2 ml of a 50 mM K2HPO4/KH2PO4 buffer, pH 7.4, as described (33, 34). The amount of oxidized DHR was calculated using an extinction coefficient of 78.78 mM–1 cm–1. With authentic peroxynitrite, the recovery of DHR oxidation was 37.4 ± 1.43% (n = 4). Spermine NONOate (1 mM) or hypoxanthine/XO (28 millimoles/ml) alone led to DHR oxidation rates of <0.06 and <0.3 μM min–1, respectively.

RESULTS

Peroxynitrite Formation—We examined the effects of peroxynitrite produced by reaction of NO with O2 at physiological pH. Co-incubation of the NO donor spermine NONOate with hypoxanthine/XO allowed the simultaneous generation of NO and O2 at identical rates. As shown in Table I, the fluxes of ‘NO/O2’ were increased from ~2 to ~5 μM min–1 when the concentrations of spermine NONOate and XO were increased from 0.2 to 1 mM and from 5.5 to 28 millimoles/ml, respectively. The simultaneous generation of NO and O2 led to oxidation of DHR, strongly suggesting that peroxynitrite had been formed. The rates of DHR oxidation were approximately 30% of the corresponding ‘NO/O2’ fluxes. Authentic peroxynitrite oxidized DHR with a yield of 37.4 ± 1.43%, indicating that 72.5 ± 2.57% of the ‘NO/O2’ formed by the spermine NONOate/XO system had been converted to peroxynitrite. The transition metal ion chelator diethylenetriaminepentaaetic acid (0.1 mM) had only minor effects (up to 20% inhibition) on the rates of DHR oxidation (data not shown). Decomposition of the sydnoneamine SIN-1 (1 mM) also resulted in the generation of NO and O2 and consequent oxidation of DHR with an approximate yield of 80%.

Tyrosine Nitration—Formation of 3-nitrotyrosine was measured by incubation of spermine NONOate and hypoxanthine/XO for 12 h in the presence of 1 mM tyrosine. Increasing the fluxes of ‘NO/O2’ from ~2 to ~5 μM min–1 led to a progressive increase of 3-nitrotyrosine formation from 0.08 ± 0.03 to 0.67 ± 0.01 μM, corresponding to 0.04–0.07% of the maximally releasable ‘NO/O2’ (1 mM). Incubation of SIN-1 (1 mM), releasing ~6 μM NO min–1 and ~5 μM O2 min–1, resulted in the formation of 0.80 ± 0.20 μM 3-nitrotyrosine (~0.08% of the maximally releasable ‘NO/O2’). The same concentration of authentic peroxynitrite led to formation of 36.77 ± 1.67 μM 3-nitrotyrosine, corresponding to ~4% of the total amount of peroxynitrite added (Table I).
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Fig. 1. Time course of 3-nitrotyrosine formation by simultaneous generation of 'NO and O₂⁻. Tyrosine (1 mM) was incubated at ambient temperature in 50 mM K₂HPO₄/KH₂PO₄ buffer, pH 7.4, in the presence of spermine NONOate (1 mM), XO (28 milliunits/ml) (giving release rates of ~5 μM × min⁻¹ for both 'NO and O₂⁻), and hypoxanthine (1 mM). Samples were analyzed for 3-nitrotyrosine at the indicated time points by HPLC as described under "Experimental Procedures." Data are mean values ± S.E. of three experiments performed in duplicate.

which 3-nitrotyrosine formation appeared to level off, the rate of 'NO release was still 1 μM × min⁻¹, with an 'NO steady state concentration of ~3 μM (data not shown). Together, these results suggest that the low yields of tyrosine nitration cannot be explained by a falling-off of the 'NO/O₂⁻ release rates after the initial period.

'NO/O₂⁻ Ratios—To further investigate the origins of the tyrosine nitration that we observed in the 'NO/O₂⁻-generating system, we studied the effects of varying the relative rates of 'NO and O₂⁻ release (Fig. 2). At a fixed rate of 1.9 ± 0.09 μM O₂⁻ × min⁻¹ (5.3 milliunits XO/ml), formation of 3-nitrotyrosine increased progressively with increasing concentrations of spermine NONOate. Maximal recovery (2.38 ± 0.15 μM) was obtained in the presence of 1 mM spermine NONOate (4.88 ± 0.17 μM 'NO × min⁻¹), and half-maximal formation of 3-nitrotyrosine was observed at an 'NO to O₂⁻ ratio of approximately 2:1 (Fig. 2A). Similar results were obtained with 'NO released at a fixed rate of 4.88 ± 0.17 μM × min⁻¹ from 1 mM spermine NONOate in the presence of increasing concentrations of XO (Fig. 2B). Maximal recovery of 3-nitrotyrosine (3.07 ± 0.27 μM) was obtained with spermine NONOate alone, whereas co-incubation with XO (3–42 milliunits/ml) led to a progressive decrease in tyrosine nitration. Again, half-maximal effects were observed at an 'NO to O₂⁻ ratio of approximately 2:1.

Tyrosine Dependence—The finding that tyrosine nitration due to spermine NONOate alone was more efficient than in the presence of hypoxanthine/XO implied that the nitration was triggered by 'NO or a product of 'NO autoxidation rather than by peroxynitrite. We next characterized the tyrosine dependence of the nitration reaction due to spermine NONOate compared with that caused by authentic peroxynitrite. In the presence of 1 mM spermine NONOate, formation of 3-nitrotyrosine increased progressively with increasing tyrosine concentration up to 2 mM (Fig. 3A), whereas the reaction triggered by authentic peroxynitrite reached a maximum at 0.5 mM tyrosine (39.17 ± 3.48 μM 3-nitrotyrosine) (Fig. 3B) and then declined at higher concentrations (20.53 ± 1.61 μM 3-nitrotyrosine at 2 mM).

pH Dependence—Another characteristic of tyrosine nitration by authentic peroxynitrite is its pH dependence (10, 12, 35). We therefore studied the pH dependence of 'NO-mediated tyrosine nitration for comparison. To avoid complications arising from the known pH sensitivity of 'NO release from spermine NONOate (36), we used an aqueous solution of 'NO gas as the 'NO source. The pH dependence of tyrosine nitration by this solution showed a sharp maximum at pH 8.2; in contrast, tyrosine nitration triggered by authentic peroxynitrite was most efficient at pH 7.0, in agreement with the previous studies (Fig. 4) (10, 12, 35). At pH 7.4, addition of the 'NO solution (final concentration, ~1 mM 'NO) to 1 mM tyrosine yielded 7.37 ± 0.47 μM 3-nitrotyrosine. This represents about a 2-fold higher yield than was observed with spermine NONOate.

CO₂ Dependence—Finally, because nitration by authentic peroxynitrite is characteristically dependent on CO₂, we compared the effects of CO₂, delivered from NaHCO₃, on tyrosine nitration due to spermine NONOate, 'NO/O₂⁻ and authentic peroxynitrite. The concentrations of authentic peroxynitrite (50 μM) and spermine NONOate (1 mM) were chosen to yield similar amounts of 3-nitrotyrosine in the absence of CO₂; the 'NO/O₂⁻-generating system was used at a release rate of ~4 μM × min⁻¹. NaHCO₃ (10 and 20 mM) increased the nitration by authentic peroxynitrite about 1.7-fold but did not stimulate nitration by spermine NONOate or by peroxynitrite generated in situ from 'NO/O₂⁻ (Fig. 5). The presence of NaHCO₃ (up to 20 mM) affected neither the flux rates of 'NO/O₂⁻ nor the rates of DHR oxidation (data not shown), demonstrating that CO₂ did not interfere with the generation of peroxynitrite from 'NO/O₂⁻ under the experimental conditions.

DISCUSSION

The nitration of free tyrosine by 'NO and O₂⁻ generated at physiological pH was compared with the nitration reaction caused by bolus addition of alkaline solutions of authentic peroxynitrite. Surprisingly, tyrosine nitration by 'NO/O₂⁻ was found to be about 100-fold less efficient than the reaction triggered by authentic peroxynitrite, even though oxidation of...
DHR indicated that peroxyxinitrite had been formed. The effective (corrected) recovery of DHR oxidation by NO/O₂⁻ was somewhat lower (72.5 ± 2.57%) than the measured NO/O₂⁻ release rates, pointing to scavenging of some of the formed peroxyxinitrite in the reaction mixture, e.g. by the urate produced by XO (33). Varying the ratio of NO/O₂⁻ release showed that tyrosine nitration was most efficient with spermine NONOate alone, i.e. in the absence of simultaneously generated O₂⁻, indicating that the nitration was caused by NO or an intermediate of NO autoxidation rather than by peroxyxinitrite.

NO does not directly nitrate tyrosine (2, 37) but can cause nitration in the presence of additional oxidants generating tyrosyl radicals. The second order reaction of NO with tyrosyl radicals (k > 10⁹ M⁻¹ s⁻¹) (38) leads to formation of C-nitroso and/or O-nitrosotyrosine products that can be converted to 3-nitrotyrosine in a two-electron oxidation reaction (39, 40). It is unlikely that this mechanism accounts for tyrosine nitration under our experimental conditions, i.e. in the absence of additional oxidants required for tyrosyl radical formation and oxidative rearrangement of the intermediate(s). The recovery of 3-nitrotyrosine was considerably higher when a high initial concentration of NO was applied as a bolus as compared with the continuous release of NO from spermine NONOate giving low steady state concentrations of free NO. This observation hints at an essential involvement of NO autoxidation, a reaction that follows second order kinetics with respect to NO and, therefore, occurs at significant rates only at relatively high NO concentrations (41). The initial product of NO autoxidation, NO₂, was shown to nitrate free tyrosine and tyrosine residues of proteins in aqueous solution (12, 42, 43). The pH dependence of tyrosine nitration caused by NO (Fig. 4) resembles that obtained by others with authentic NO (42), suggesting that the nitration of tyrosine observed under our experimental conditions was mediated by NO₂ formed in the course of NO autoxidation.

The most interesting finding of this study was the apparent lack of significant tyrosine nitration by peroxyxinitrite generated from NO and O₂⁻ at physiological pH, even though alkaline solutions of peroxyxinitrite efficiently nitrated tyrosine under identical conditions. These results suggest that the peroxyxinitrite formed from NO and O₂⁻ at physiological pH differs from the species present in alkaline solutions. The efficiency of tyrosine nitration triggered by alkaline solutions of peroxyxinitrite is markedly enhanced by CO₂, which reacts with peroxyxinitrite anion to give the potent nitrating species ONO₂CO₂⁻ (13, 15, 35, 44–46). We observed that NaHCO₃ led to an about 2-fold increase in tyrosine nitration by alkaline peroxyxinitrite, a result that agrees well with previous data from other laboratories (14, 35, 45, 46). However, Berlett et al. (47) have recently suggested that formation of ONO₂CO₂⁻ is obligatory for peroxyxinitrite-mediated nitration (47). The apparently CO₂-independent reaction observed in the absence of added bicarbonate would accordingly be due to contaminating bicarbonate present in appreciable concentrations (0.1–0.2 mM) in buffer solutions (35).

Assuming that formation of ONO₂CO₂⁻ is indeed essential for peroxyxinitrite-triggered tyrosine nitration as suggested by Ber-
let al. (47), our findings suggest that the peroxynitrite species that is generated from 'NO and O$_2$ at physiological pH does not react with CO$_2$. This would explain both the inability of NO/O$_2$ to nitrate tyrosine and the lack of effect of bicarbonate on the observed residual nitration reaction. We can only speculate about the nature of the two postulated peroxynitrite species. The most obvious assumption is the involvement of the cis- and trans-rotamers. Interestingly, the cis- and trans-isomers exhibit clearly different pK$_a$ values of 6.8 and 8.0 (48, 49), respectively, and data obtained by Raman spectroscopy indicate that peroxynitrite is present exclusively in the cis-conformation in alkaline solution (48). Based on this information, we propose the scheme shown in Fig. 6 as a hypothetical explanation of our data. It is postulated that trans-peroxynitrite is the initial product of the reaction between 'NO and O$_2$ at pH 7.4. Because CO$_2$ reacts with peroxynitrite anion but not with peroxynitrous acid (13), protonation of trans-peroxynitrite at pH 7.4 (pK$_a$ = 8.0) is expected to prevent formation of the nitrating ONO$_2$CO$_2$ adduct. In contrast, ~80% of the cis-isomer (pK$_a$ = 6.8) exists as anion at physiological pH allowing for the reaction with CO$_2$ and consequent tyrosine nitration.

What are the alternative possibilities to explain our observations? Peroxynitrite in situ nascenti might represent a novel as yet unrecognized form of this molecule, but there are no indications that such a species exists. Alternatively, peroxynitrite generated from 'NO and O$_2$ at physiological pH could react with CO$_2$ to yield an unreactive form of ONO$_2$CO$_2$. Formation of unreactive ONO$_2$CO$_2$, kinetically distinguishable from the reactive form, has been reported (50), but we cannot prove or disprove formation of such a species under our experimental conditions. Finally, the obligatory role of ONO$_2$CO$_2$ in peroxynitrite-triggered tyrosine nitration is not generally accepted. Thus, it cannot be excluded that peroxynitric acid acts as nitrating species even in the complete absence of CO$_2$. In this case, the explanation of our data would require the additional assumption that the cis- but not the trans-form of peroxynitric acid nitrates tyrosine.

Our data demonstrate that the simultaneous generation of 'NO and O$_2$ does not cause tyrosine nitration under physiological conditions. Together with the unequivocal demonstration of highly elevated nitrotyrosine levels in several human disease states (21, 24, 51, 52), these surprising results raise the question, again, of what nitrates tyrosine in vivo (55). 'NO$_2$ formed by autoxidation of 'NO may have acted as nitrating species in our experiments with authentic 'NO and spermine NONOate, but it is unclear whether 'NO autoxidation could account for the extensive nitration of tyrosine residues observed in tissues. At micromolar concentrations of 'NO, the third-order autoxidation reaction is certainly not fast enough to generate sufficient 'NO$_2$ (42). However, recent data indicate that the reaction of 'NO with O$_2$ is accelerated about 300-fold in biological membranes, rendering hydrophobic compartments of cells important sites for the formation of NO-derived reactive species (54). Thus, it is conceivable that high concentrations of 'NO$_2$ are formed in such compartments and cause tyrosine nitration of adjacent membrane proteins under certain conditions.

Several studies hint at peroxynitrite-independent pathways of tyrosine nitration in tissues. Myeloperoxidase, which is secreted by phagocytic neutrophils in inflammatory conditions, could be a key player in these events. It generates tyrosyl radicals (55) that can be trapped by NO, leading to formation of 3-nitrotyrosine in the presence of additional oxidants, e.g. H$_2$O$_2$ (40). In the presence of H$_2$O$_2$, myeloperoxidase may also cause nitration by oxidation of NO$_2$ to 'NO$_2$ (56). Finally, myeloperoxidase catalyzes formation of hypochlorous acid (HOCl), which reacts nonenzymatically with NO$_2$ to form the potent nitrating agent nitryl chloride (NO$_2$Cl) (57, 58). Thus, tyrosine nitration may become significant at sites where 'NO synthase is activated together with oxidative pathways generating tyrosyl radicals and H$_2$O$_2$ or other oxidants. The presence of myeloperoxidase appears to be important but would not be obligatory if other enzymatic pathways acting in a similar manner were activated together with 'NO synthase in tissue inflammation.

In conclusion, our results show that peroxynitrite generated from 'NO and O$_2$ at physiological pH does not nitrate tyrosine. This forces us to question the relevance of previous reports on protein nitration by bolus addition of alkaline peroxynitrite. It should also strongly stimulate new efforts to discover the true mechanism responsible for tyrosine nitration in inflammatory and infectious disease.

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REFERENCES

1. Mayer, B., and Hemmens, B. (1997) Trends Biochem. Sci. 22, 453–498
2. Beckman, J. S., and Koppenol, W. H. (1996) Am. J. Physiol. 270, C1424–C1437
3. Hsie, R. E., and Padmaja, S. (1993) Free Radic. Res. Commun. 16, 193–199
4. Goldstein, S., and Czapski, G. (1993) Free Radical Biol. Med. 17, 12078–12084
5. Malinski, T., Bailey, F., Zhang, Z. G., and Chopp, M. (1993) J. Cereb. Blood Flow Metab. 13, 355–358
6. Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992) Chem. Res. Toxicol. 5, 834–842
7. Pfeiffer, S., Gerren, A. C. F., Schmidt, K., Werner, E. R., Hansert, B., Bohle, D. S., and Mayer, B. (1997) J. Biol. Chem. 272, 3465–3470
8. Pryor, W. A., and Squadrito, G. (1998) Am. J. Physiol. 12, L699–L722
9. Halfpenny, E., and Robinson, P. L. (1952) J. Chem. Soc. 939–946
10. Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992) Arch. Biochem. Biophys. 296, 438–445
11. van der Vliet, A., O'Neill, C. A., Halliwell, B., Cross, C. E., and Kaur, H. (1994) FEBS Lett. 339, 89–92
12. van der Vliet, A., Eisircher, J. P., O'Neill, C. A., Halliwell, B., and Cross, C. E. (1995) Arch. Biochem. Biophys. 319, 341–349
13. Lymar, S. V., and Hurst, J. F. (1995) J. Am. Chem. Soc. 117, 8867–8868
14. Lymar, S. V., Jiang, Q., and Hurst, J. F. (1996) Biochemistry 35, 7855–7861
15. Gao, A., Duran, D., Thom, S. R., and Ischiropoulos, H. (1996) Arch. Biochem. Biophys. 332, 42–48
16. Xie, Q. W., and Nathan, C. (1994) J. Leukocyte Biol. 56, 576–582
17. Coyle, J. T., and Puttfarken, P. (1993) Science 262, 689–695
18. Bagasra, O., Michaels, F. H., Zheng, Y. M., Bobroski, L. E., Spitzn, S. V., Fu, Z. F., Tawadros, R., and Koprowski, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 12041–12045
19. Haddad, I. Y., Ischiropoulos, H., Holm, B. A., Beckman, J. S., Baker, J. R., and Matalon, S. (1993) Am. J. Physiol. 265, L555–L564
20. Beckman, J. S., Carson, M., Smith, C. D., and Koppenol, W. H. (1993) Nature 364, 584
21. Beckman, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994) Biochem. Hoppe-Seyer 375, 81–88
22. Mannick, E. E., Bravo, L. E., Zarama, G., Realpe, J. L., Zhang, X. J., Ruiz, B., Fonham, E. T., Mera, R., Miller, J. J., and Correa, P. (1995) Cancer Res. 56, 3238–3243
23. Akaike, T., Noguchi, Y., Ijiri, S., Setoguchi, K., Suga, M., Zheng, Y., Dietzschold, B., and Maeda, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2448–2453
24. Kaur, H., and Halliwell, B. (1994) FEBS Lett. 350, 9–12

$^3$ Dr. Willem H. Koppenol, personal communication.
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25. Feelisch, M., Ostrowski, J., and Noack, E. (1989) J. Cardiovasc. Pharmacol. 14, 13–22.

26. Miles, A. M., Bohle, D. S., Glassbrenner, P. A., Hansert, B., Wink, D. A., and Grisham, M. B. (1996) J. Biol. Chem. 271, 40–47.

27. Wink, D. A., Cook, J. A., Kim, S. Y., Vodovotz, Y., Pacelli, R., Krishna, M. C., Russo, A., Mitchell, J. B., Jourdain, D., Miles, A. M., and Grisham, M. B. (1997) J. Biol. Chem. 272, 11147–11151.

28. Kukovetz, W. R., and Holzmann, S. (1989) J. Cardiovasc. Pharmacol. 14, S40–S46.

29. Mayer, B., Klatt, P., Bo¨hme, E., and Schmidt, K. (1992) J. Neurochem. 59, 2024–2029.

30. Kelm, M., Dahmann, R., Wink, D., and Feelisch, M. (1997) J. Biol. Chem. 272, 9922–9932.

31. Schmidt, K., Desch, W., Klatt, P., Kukovetz, W. R., and Mayer, B. (1997) Naunyn-Schmiedeberg’s Arch. Pharmacol. 355, 457–462.

32. Pfeiffer, S., Leopold, E., Hemmens, B., Schmidt, K., Werner, E. R., and Mayer, B. (1997) Free Radical Biol. Med. 22, 787–784.

33. Kooy, N. W., Royall, J. A., Ischiropoulos, H., and Beckman, J. S. (1994) Free Radical Biol. Med. 16, 149–156.

34. Crow, J. P. (1997) Nitric Oxide 1, 145–157.

35. Lemercier, J. N., Padmaja, S., Cueto, R., Squadrito, G. L., Uppu, R. M., and Pryor, W. A. (1997) Arch. Biochem. Biophys. 345, 160–170.

36. Maragos, C. M., Morley, D., Wink, D. A., Dunama, T. M., Saavedra, J. E., Hoffman, A., Rove, A. A., Isaac, L., Hrabe, J. A., and Keeler, L. K. (1991) J. Med. Chem. 34, 3242–3247.

37. Beckman, J. S. (1996) Chem. Res. Toxicol. 9, 836–844.

38. Eiserich, J. P., Butler, J., Van der Vliet, A., Cross, C. E., and Halliwell, B. (1995) Biochem. J. 310, 745–749.

39. Gunther, M. R., Hsi, L. C., Curtis, J. F., Gierse, J. K., Marnett, L. J., Eling, T. E., and Mason, R. P., and Marnett, L. J. (1998) J. Biol. Chem. 273, 8903–8909.

40. Khartitonov, V. G., Sundquist, A. R., and Sharma, V. S. (1994) J. Biol. Chem. 269, 5881–5883.

41. Pruitt, W. A., Minig, H., Butler, J., and Land, E. J. (1985) Arch. Biochem. Biophys. 243, 125–134.

42. Kigugwa, K., Kata, T., and Okamoto, Y. (1994) Free Radical Biol. Med. 16, 373–382.

43. Lymar, S. V., and Hurst, J. K. (1996) Chem. Res. Toxicol. 9, 845–850.

44. Denicola, A., Freeman, B. A., Trujillo, M., and Rady, R. (1996) Arch. Biochem. Biophys. 333, 49–58.

45. Uppu, R. M., Squadrito, G. L., and Pryor, W. A. (1996) Arch. Biochem. Biophys. 327, 335–343.

46. Berlett, B. S., Levine, R. L., and Stadtman, E. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3784–2789.

47. Tsai, J. H. M., Harrison, J. G., Martin, J. C., Hamilton, T. P., Woerd van der, M., Jablonsky, M. J., and Beckman, J. S. (1994) J. Am. Chem. Soc. 116, 4115–4116.

48. Crow, J. P., Spruell, C., Chen, J., Gunn, C., Ischiropoulos, H., Tsai, M., Smith, C. D., Radi, R., Koppenol, W., and Beckman, J. S. (1994) Free Radical Biol. Med. 16, 331–338.

49. Lymar, S. V., and Hurst, J. K. (1998) Inorg. Chem. 37, 294–301.

50. Kooy, N. W., Royall, J. A., Ye, Y. Z., Kelly, D. R., and Beckman, J. S. (1995) Am. J. Respir. Crit. Care Med. 151, 1250–1254.

51. Saleh, D., Barnes, P. J., and Giad, A. (1997) Am. J. Respir. Crit. Care Med. 155, 1763–1769.

52. Halliwell, B. (1997) FEBS Lett. 411, 157–160.

53. Liu, X. P., Miller, M. J. S., Joshi, M. S., Thomas, D. D., and Lancaster, J. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2175–2179.

54. Heinecke, J. W., Li, W., Daehnke, H. L., III, and Goldstein, J. A. (1993) J. Biol. Chem. 268, 4069–4077.

55. van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) J. Biol. Chem. 272, 7617–7625.

56. Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B., and van der Vliet, A. (1996) J. Biol. Chem. 271, 19199–19208.

57. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) Nature 391, 393–397.