Free Rad. Res., Vol. 21, No. 4, pp. 203-212
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NITRIC OXIDE REVERSIBLY SUPPRESSES XANTHINE OXIDASE ACTIVITY

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(Received November 29th, 1993; Accepted January 17th, 1994)

The effects of nitric oxide (NO) on xanthine oxidase (XOD) activity and the site(s) of the redox center(s) affected were investigated. XOD activity was determined by superoxide (O_2^-) generation and uric acid formation. NO reversibly and dose-dependently suppressed XOD activity in both determination methods. The suppression interval also disclosed a dose-dependent prolongation. The suppression occurred irrespective of the presence or absence of xanthine; indicating that the reaction product of NO and O_2^-, peroxynitrite, is not responsible for the suppression. Application of synthesized peroxynitrite did not affect XOD activity up to 2 μM. Methylene blue, which is an electron acceptor from Fe/S center, prevented the NO-induced inactivation. The results indicate that NO suppresses XOD activity through reversible alteration of the flavin prosthetic site.

KEY WORDS: Xanthine oxidase, Nitric oxide, Methylene blue, FAD.

INTRODUCTION

Among the diverse functions of NO in the biological system, the interference of NO with O_2^- has been a current interest. Simultaneous production of NO and O_2^- has been shown to occur in a variety of inflammatory conditions such as chronic infection, ischemia/reperfusion and sepsis.[1-3] NO may attenuate tissue injury in these conditions by directly scavenging O_2^- and reducing the accumulation of neutrophils,[4] a major source of O_2^-, thereby reducing O_2^-. An additional mechanism of the protective effect of NO against tissue injury may be the inactivation of O_2^- generation systems since a recent study revealed that NO inactivates NADPH oxidase of neutrophils[5], thus reducing O_2^-. However, the effect of NO on xanthine oxidase (XOD), which is another key source of O_2^- in inflammatory conditions,[6] has not yet been investigated.

The purpose of this study was to demonstrate the inhibitory effect of NO on XOD activity and to investigate the site of redox centers of XOD affected. The XOD activities were determined by two independent methods, one of which evaluates O_2^- generation (Figure 1, right) and the other measures uric acid formation (Figure 1, left) using xanthine as the substrate in both methods. A detailed study was performed to determine the specific site(s) of inactivation in redox centers of XOD; molybdenum, FAD and iron-sulfur (Fe/S) centers,[7] since NO is shown to

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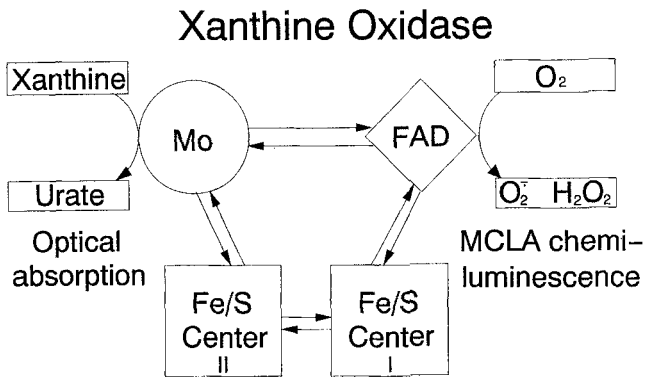

FIGURE 1 Schema of redox centers of XOD; XOD contains one molybdenum, one FAD and two Fe/S centers in each of two independent subunits.[20]

inactivate enzymes containing an Fe/S center such as NADH dehydrogenase and succinate dehydrogenase.[8] We also examined the possible contribution of peroxynitrite ($ONOO^-$) on XOD activity since NO reacts with O_2^- to yield $ONOO^-$ at a rate[9] of $6.7 \times 10^9\,M^{-1}\,s^{-1}$ which has been shown to be a strong oxidant.[10]

MATERIALS AND METHODS

Chemicals

XOD and catalase were obtained from Boehringer (Mannheim, Germany) and MCLA (2-methyl-6- [p-methoxyphenyl]-3,7-dihydroimidazo [1.2-a] pyrazin-3-one), a O_2^--specific chemiluminescence probe, from Tokyo Kasei (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan).

NO Solution

Saturated NO solution was made by the method of Ignarro *et al.*[11] Briefly, N_2 was bubbled through 10 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-etanesulfonic acid) buffer (pH 7.4 NaOH) for 20 min to remove dissolved O_2. Then authentic NO gas (99%, Nippon Sanso Co. Ltd., Tokyo, Japan) that had passed through 1M KOH to remove nitrogen dioxide (NO_2) was bubbled for 20 min. In each saturated NO solution pH was checked to ascertain the complete removal of O_2 and NO_2 since traces of O_2 or NO_2 produces NO_2^- and NO_3^- which lower pH.[12] Saturated NO solution was diluted to the desired concentrations using N_2-flushed 10 mM HEPES buffer. Since the concentration of NO in a dilution process can not be equal to the theoretical concentration due to the extreme reactivity of NO with O_2, the actual NO concentration of each solution was measured concomitantly in all experiments using the difference-spectrophotomeric method as previously described.[13] This method quantifies the oxidation of oxyhemoglobin to methemoglobin by recording the NO-induced spectral shift at 401 nm, taking 411 nm as the isobestic point.

Synthesis of $ONOO^-$

$ONOO^-$ was synthesized by the reaction of acidified H_2O_2 and $NaNO_2$ in a quenched flow reactor with a subsequent stabilization induced by 1.5 M NaOH as previously described.[14] Purity and concentration of $ONOO^-$ was checked by its absorption spectrometry.

Assay of XOD Activity by O_2^- Generation

O_2^- generation was measured by a sensitive and selective chemiluminescence method using a C1230 photon counter with an R550 photomultiplier (Hamamatsu Photonics K.K., Hamamatsu, Japan). The assays were conducted in a petri dish (45 mm I.D.) containing the chemiluminescence probe, MCLA (1 μM), XOD (335 pM) and xanthine (50 μM) in 10 mM HEPES buffer (pH 7.4). The rate of O_2^- generation was monitored by determination of light emission per second (c.p.s) in the presence or absence of various concentrations of NO solution. O_2^- generation rate under this condition was determined to be 285 pM/sec by the concomitantly performed cytochrome *c* method. NO solutions (18 nM, 67 nM, 1020 nM) were added via a stainless-steel needle on a gas-tight syringe.

Assay of XOD Activity by Uric Acid Formation

Unlike the assay using O_2^- generation, this method can evaluate XOD activity without the influence of O_2^- quenched by NO. The formation of uric acid was monitored at 295 nm by a Ubest-50 spectrophotometer (JASCO, Tokyo, Japan) using 10 mM HEPES buffer (pH 7.4, NaOH) containing xanthine (150 μM) and XOD (46.1 nM) in the presence of various concentrations of NO solutions (2.2–203 μM). It was necessary to increase the concentration of NO since the sensitivity of this method is lower by a factor of two orders than that of the chemiluminescence method. In order to determine whether the observed effect of NO on XOD is caused by NO itself or by the reaction product of NO and O_2^- ($ONOO^-$), uric acid formation was measured under the same condition however NO was applied prior to the addition of xanthine, in other words prior to the generation of O_2^-. The effect of synthesized $ONOO^-$ was also examined at a concentration of 2 μM. To identify the inactivation site(s), the effect of NO was examined in the presence of methylene blue (25 μM), an electron acceptor from the Fe/S center of XOD.[7]

All measurements were performed at room temperature (25°C) in triplicate; 10 mM HEPES buffer (pH 7.4 NaOH) was used in all experiments unless otherwise stated. Oxygen tension of the reaction solution was monitored by an oxygen electrode.

Absorption Spectra of Xanthine Oxidase

Absorption spectrophotometry of XOD was performed to identify flavin (peak at 450 nm) and Fe/S centers (peak at 550 nm) in the presence or absence of NO using a quartz cell. In 10 mM HEPES buffer (pH 7.4 NaOH) containing xanthine (50 μM) and XOD (2.6 μM), NO solution (600 μM) was added and absorption spectra were recorded every 5 min using Ubest-50 spectrophotometer for 20 min. XOD was then reoxidized by bubbling O_2 for 5 min and absorption spectra of reoxidized XOD was recorded again.

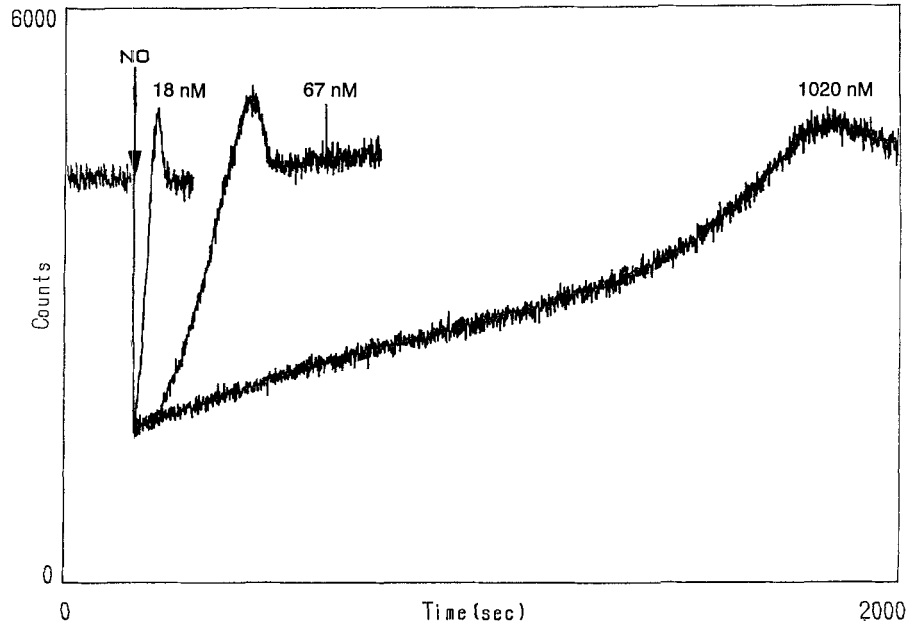

FIGURE 2 Time-course of MCLA chemiluminescence: NO solution (18, 67, 1020 nM) was added to 10 mM HEPES buffer containing 50 μM xanthine, 335 pM XOD and 1 μM MCLA.

ESR Measurements

ESR spectra of XOD were recorded on a JEOL JES-RE3X ESR spectrometer (JEOL Co. Ltd., Tokyo, Japan) equipped with a liquid He variable temperature controller, in the presence or absence of NO in attempt to demonstrate the formation of iron-nitrosyl complex or to show the disappearance of the spectrum of Fe/S centers in the presence of NO. Briefly, XOD solution (15 μM) was reduced by excess sodium dithionite and then NO solution (900 μM) or control solution was added. The solution was then transferred to an ESR cell and immediately frozen by liquid nitrogen and ESR spectra was recorded. 10 mM HEPES buffer (pH 7.4 NaOH) was used in all experiments unless otherwise stated.

RESULTS

Time-course of MCLA Chemiluminescence

In the presence of MCLA and xanthine, the addition of XOD demonstrated steady-state O_2^--dependent chemiluminescence. With the addition of NO, the emission decreased abruptly but gradually recovered and then returned to the previous level (Figure 2). Both the initial decrease and the time to recovery of emission were concentration dependent. The initial and abrupt decrease can be attributed to the direct quenching of O_2^- by NO but the prolonged attenuation which lasted beyond the half-life of NO (6 sec) can not be explained by the quenching effect. A plausible explanation is a transient reduction in the generation of O_2^- caused

by the reversible suppression of XOD activity. Catalase (70 ng/ml), NO_2^- (100 μM) and NO_3^- (100 μM) had no effect on the time course of the NO-induced chemiluminescence change (excluding the effects of the dismutation product of O_2^- and the decomposition products of NO in the reaction). NO-induced decomposition of MCLA could not be considered as a cause for the reduced emission because the emission subsequently returned to the initial level. In addition, it was revealed that NO did not destroy MCLA at concentrations lower than 2 μM (data not shown). Inactivation periods (time for the emission to return to the control level) were 288 sec and 1690 sec with NO concentrations of 67 nM and 1020 nM, respectively.

Effect of NO on the Formation of Uric Acid

As shown with the chemiluminescence, NO dose dependently suppressed uric acid formation (Figure 3a). To demonstrate the time course of the recovery of uric acid formation more precisely, the rate of uric acid formation is shown in Figure 3b. It is evident that the formation rate decreased quickly upon addition of NO with peak suppression at 4 min, and then the rate gradually approached the control value. The percent of peak suppression as a function of NO concentration could be fitted to an exponential curve (Figure 4). The reversible inactivation of XOD by NO was confirmed using this uric acid formation method in which the direct reaction of NO and O_2^- is not involved. Similar suppression of XOD activity occurred when NO was applied to XOD 5 min before the addition of xanthine. Addition of synthesized $ONOO^-$ did not affect the XOD activity at the concentration of 2 μM, which is the highest concentration to be theoretically formed in this condition, indicating that NO itself, and not the reaction product of NO and O_2^-, is responsible for the inactivation.

In the uric acid formation study, it was necessary to increase the concentrations of both XOD and NO to approximately 100-folds the concentrations used in the chemiluminescence study to deal with the low sensitivity of the spectrophotomeric method. The high concentrations of XOD and NO consumed O_2 rapidly and resulted in low O_2 (125 μM). Thus it might be argued that the observed suppression of uric acid formation is due to the reduction in O_2 concentration. However this possibility was denied by a preliminary experiments,in which XOD activity remained unaltered with an O_2 concentration as low as 100 μM as produced by bubbling N_2 which is lower than the level of the present study (data not shown). To determine the affected site(s) in the redox centers of XOD, uric acid formation was measured in the presence of methylene blue. Uric acid formation was not suppressed by NO in the presence of methylene blue (Figure 5). Direct interaction between NO and methylene blue was excluded because no change was detected in the optical spectra of methylene blue with the addition of NO. Thus it was shown that electrons can be transferred to methylene blue through Fe/S centers, indicating that at least one Fe/S center is intact.

Effects of NO on the Optical Spectra of XOD

Oxidized XOD (2.6 μM) showed optical absorption attributed to FAD moiety and Fe/S centers at 450 nm and 550 nm, respectively. Incubation of XOD with xanthine (50 μM) caused a decrease in absorption by partial reduction of these redox centers. This decrease in absorption could be recovered by reoxidation with bubbling O_2 for 5 minutes (Figure 6a). However, the decrease in absorption which

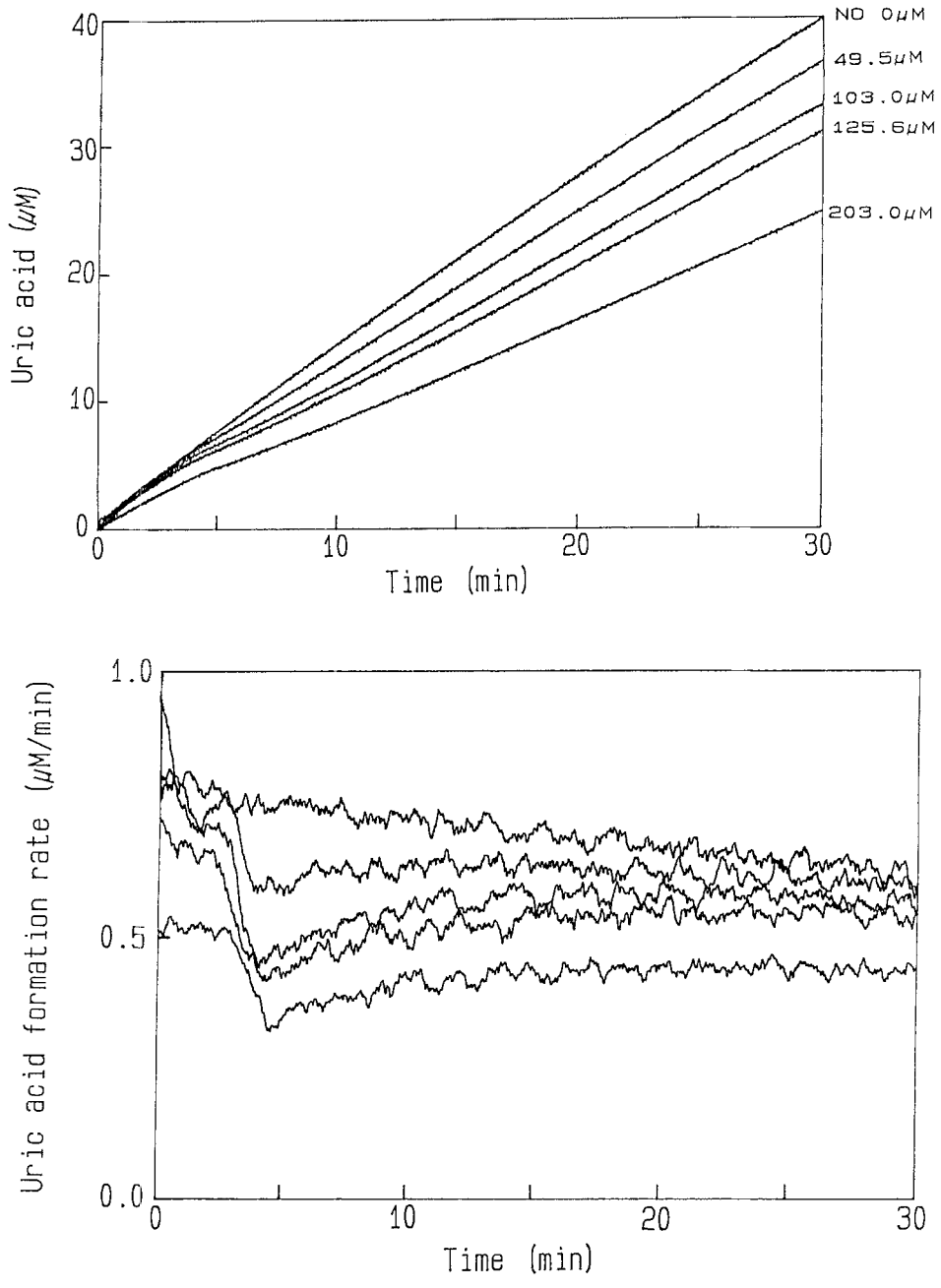

FIGURE 3 (a) Time-course of uric acid formation: NO solutions (0–203 μM) were added to 10 mM HEPES buffer containing xanthine (150 μM), XOD (46.1 nM) and absorption at 295 nm was recorded. (b) The rate of uric acid formation. NO concentrations were 0, 49.5, 103, 203 μM from the top to the bottom.

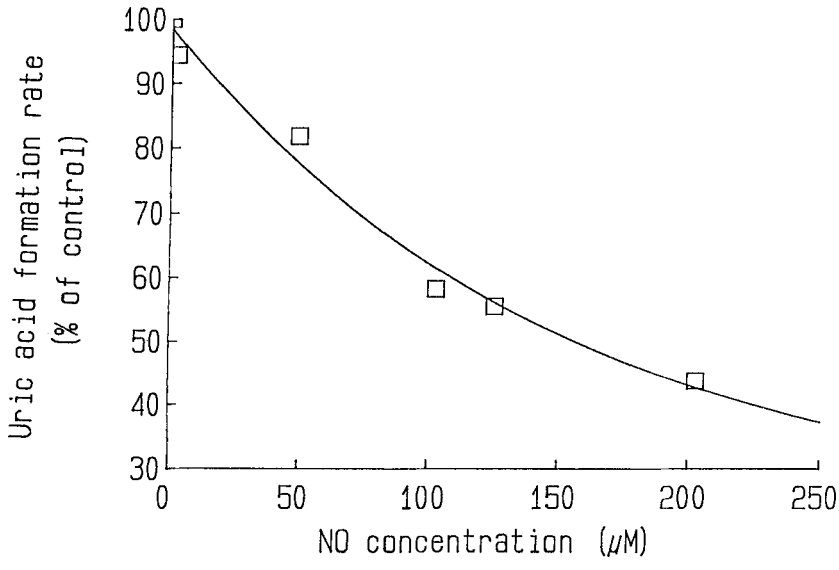

FIGURE 4 Suppression of uric acid formation by NO: Values were obtained at 4 min after the addition of NO. NO concentrations depicted in each experiment were determined by methemoglobin formation from HbO_2.

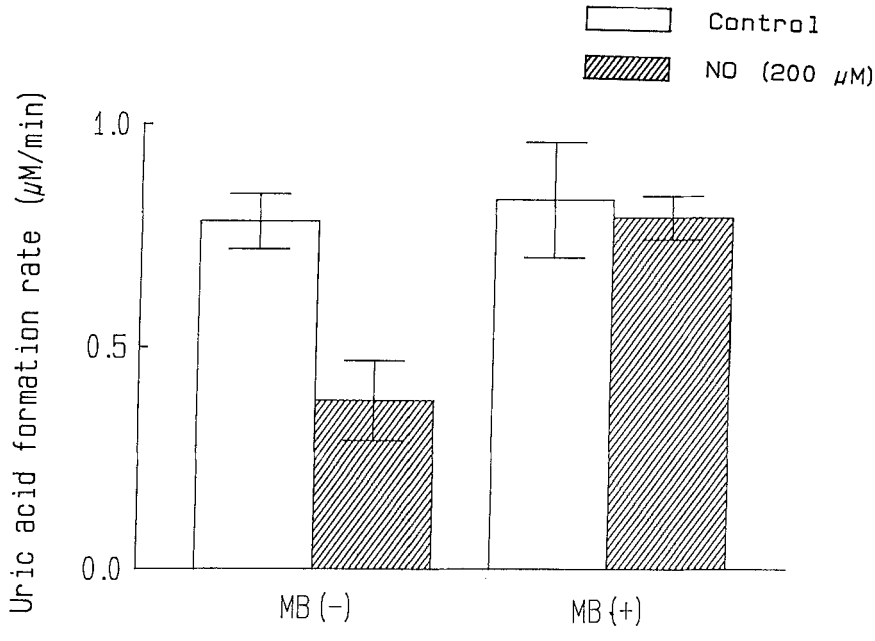

FIGURE 5 The effect of methylene blue (MB) on NO-induced inactivation: XOD (46.1 nM) and methylene blue (25 μM) were incubated for 5 min and then NO (200 μM) or N_2-flushed solution and xanthine (150 μM) were added sequentially. Data represent the mean ± SD of three separate experiments.

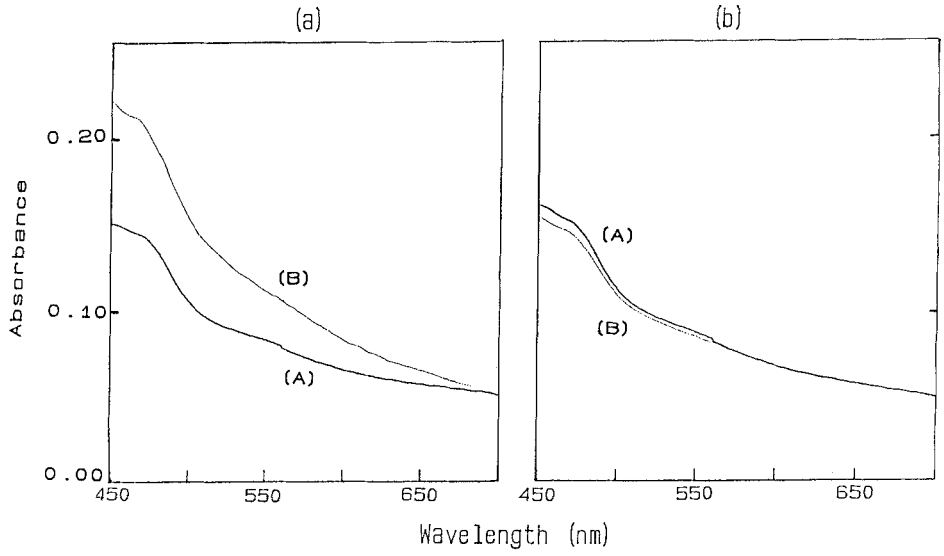

FIGURE 6 The optical spectra of XOD in the absence (a) and presence (b) of NO (600 μM). Thick line shows the spectra of XOD which was incubated with xanthine (50 μM) for 20 min. Thin line shows the spectra after the subsequent bubbling of oxygen for 5 min. Experiments were performed while the inactivation still persisted. (a) control XOD spectra. A: reduced XOD by xanthine. B: reoxidized XOD by O_2 (b) XOD spectra with NO (600 μM). A: reduced XOD by xanthine. B: no change by reoxidation with O_2.

was induced by the simultaneous addition of NO with xanthine could not be recovered by bubbling O_2 (Figure 6b).

The Effect of NO on the ESR Spectra of Reduced XOD

The ESR spectra of XOD (15 μM), which was reduced by excess sodium dithionite, were recorded in the presence or absence of NO over the temperature range of 4K to 293K. The spectra characteristic of two reduced Fe/S centers[15] were observed near 22K. The spectral features were not affected by the addition of NO. The spectrum of iron-nitrosyl complex was not observed under these conditions.

DISCUSSION

This study shows that NO reversibly suppresses XOD activity and that NO_2^-, NO_3^- and $ONOO^-$ are not responsible for the inactivation. The inactivation of XOD by NO may be of physiological significance at the locus of inflammation where a marked increase in XOD activity occurs[6] and generated O_2^- is shown to be responsible for tissue injury.[16] The relevance of this finding is that significant inhibition occurs at nanomolar concentrations of NO which are well within the reported rate of intraluminal NO production, 8 μM/min.[10] We also measured the NO concentration from rat aorta using an NO-electrode to validate the NO concentration of this study and confirmed the 100 nM range.[17] The micromolar concentration used

in the uric acid formation study does not indicate the necessity of high levels of NO for inactivation since the concentration of XOD was also increased by 100-fold in order to deal with the low sensitivity of the spectrophotomeric method.

Two recent studies[5,18] have shown XOD activity in the presence of NO, although the original aim of these studies was not the examination of XOD activity in the presence of NO. Both studies demonstrated that NO attenuates O_2^- through O_2^- quenching effect and that inactivation of XOD to decrease O_2^- generation was not shown. The discrepancy between their results and those of the present study depends largely on the difference in the methods of O_2^- measurement; both studies used cytochrome *c* method whereas we used the MCLA chemiluminescence method. The cytochrome *c* method is not sufficient from the view points of sensitivity and time-resolution for the evaluation of the effect of NO on the time course of O_2^- generation in comparison with the chemiluminescence method. Therefore, in the cytochrome *c* method transient inactivation might be overlooked. Additional reasons appear to be that the observation periods were not adequate to follow the entire time courses in these studies.

XOD is a complex metalloflavoprotein in which redox centers behave as an electron pool. H_2O_2 which is self-generated or exogenously-added was shown to inactivate XOD through alteration at the site of FAD.[19] However H_2O_2 was excluded as an inactivator candidate with the conditions of the present study. The role of $ONOO^-$ could be also neglected, and NO itself was shown to be the responsible molecule for the inactivation. The underlying chemical mechanisms for the NO-induced inactivation are still unknown since studies using optical spectra and ESR spectra of XOD did not directly identify the altered site(s) in redox centers. However, the protection against NO-induced inactivation by methylene blue indicated that at least one Fe/S center remains intact. Persistent spectra of reduced FAD and Fe/S even after bubbling O_2 does not necessarily mean that all of these redox centers were altered by NO. Namely, the alteration in FAD alone can produce the persistent reduction in both FAD and Fe/S centers. If FAD were altered by NO and there was no electron acceptor from an Fe/S center, reoxidation of the Fe/S center could not occur. Taken together, FAD is the most plausible site of NO-induced alteration. The present observation demonstrated a new function of NO in O_2^--related injury.

Acknowledgements

We gratefully thank Dr. M. Kohno (JEOL Co. Ltd., Tokyo, Japan) for the use of his ESR spectrometer. This work was supported in part by a Grant-in-Aid from The Tokyo Biochemical Research Foundation and Tokai University School of Medicine Research Aid.

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Accepted by Professor T. Yoshitawa