INDUCTION OF A SISTER-CHROMATID EXCHANGE BY NITROGEN OXIDES AND ITS PREVENTION BY SOD

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ABSTRACT — Observations were made on the increase in the frequency of sister-chromatid exchanges (SCEs) induced by a nitric oxide (NO) releaser (NOR4) and NaN02 in Chinese hamster lung cells (CHL/IIU). During these observations, NaN02 did not have any effect on SCE induction. NOR4- and NaN02- induced SCE frequencies decreased due to treatment with bovine serum (10%), bovine serum albumin (BSA, 0.1%, 1.0%), oxyhemoglobin (Hb, 10 μM), and superoxide dismutase (SOD, 250U/ml), but not with glutathione (oxidized and reduced forms), cysteine, cystine and catalase. NO concentrations decreased with Hb, but not with any other agent, indicating that NO and/or NO had a strong binding reaction with Hb. The mechanism for a decrease in genotoxicity due to SOD is still unclear. However, it would appear that S-nitrosothiols in the cells can be stabilized by SOD in consideration of the S-nitrosothiol stabilizing effect of SOD reported by Kowaluk et al. (1990). In the presence of NO and superoxide anions, genotoxicity seemed to be decreased by catalase and SOD, since the former decreases the superoxide anion-induced SCE frequency, and the latter, the NO-induced frequency.

KEY WORDS: Nitric oxides, Genotoxicity, Sister-chromatid exchange, SOD

INTRODUCTION

Nitrogen oxides (NOx) are known as potent environmental pollutants because of their genotoxic properties. Isomura et al. reported the induction of mutation and chromosome aberration in rat lung cells with nitric oxide gases (NO and NO2) in vivo (Isomura et al., 1984). Recently, NO was identified as having an endothelium-derived relaxing effect. Many papers have also reported its pathophysiological role in normal and abnormal conditions in humans. During infection with bacteria, viruses or other microorganisms, NO may be synthesized in large amounts for prolonged periods of time. During such situations, NO and its metabolites (NOx) could have potential genotoxic effects on the hosts, and increase the risk of cancer, especially in the chronic process (Ohshima and Bartsch, 1994). Regarding DNA damage with NO, recent papers have reported deamination of the DNA bases (Wink et al., 1991). Furthermore, the oxidative damage to DNA was identified in combination with the oxygen radicals (deRojas-Walker et al., 1995). With high dose of NO (100 μM, 1mM), a DNA cleavage was reported by Sugiura and Matsumoto (1995) with NO attacks in the order of G>C>A>T. This information showed a high risk of cancer with endogeneously produced NOx for many morbidity conditions. Therefore, we used the SCE analysis, a more sensitive method than the chromosomal aberration test, as a factor of genotoxicity in vitro to investigate the genotoxicity of low doses of NOx. We also reported on its protection with sulfhydrls containing amino acids and proteins because NO binds with sulfhydrls.
MATERIALS AND METHODS

Sister-chromatid exchange (SCE) analysis in vitro

CHL/IU cells were obtained from Dainippon Pharmaceutical Co. (Osaka). These cells were seeded at a density of $10^5$ cells/ml in rectangular bottles, and then incubated for about 20 hr at 37°C in an Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo) supplemented with 10% calf serum (new calf serum; Nacalai tesque, Kyoto).

After washing with a phosphate-buffered saline (PBS, 0.01M, pH7.4), the cells were exposed to agents and/or NOx in MEM not containing phenol red and serum for 5 hr at 37°C. Then the medium was changed to phenol red- and serum-contained MEM. Cells were incubated with 5-bromo-2'-deoxyuridine (10 μM BrdU; Sigma, St. Louis, MO) in complete darkness for 24 hr at 37°C, and colcemid (0.2 μg/ml, GIBCO Laboratories, New York) was added to each bottle 4 hr before the end of the incubation period. At the end of the incubation period, the cells were harvested by an addition of trypsin (Merck, Darmstadt), and subsequently centrifuged (112 × g, 5 min). After treating the cells with 0.04 M KCl for 15 min at 37°C, chromosomes were obtained by centrifugation (174 × g, 5 min) and fixed with methanol-acetic acid (3:1 v/v). These procedures were performed during a dark condition.

The chromosomal preparations described above were stained with a 2% Giemsa solution (Merck, Darmstadt) prepared in 0.3M Na2HPO4 at pH 10.4 for 30 min (Ikushima and Wolff, 1974). With this technique, chromatids that had incorporated with BrdU were darkly stained. The results were recorded as the frequency of SCE/metaphase cell for chromatids that had undergone two replication cycles (Tanaka and Amano, 1989).

Determination of nitric oxide

We measured the level of NO released from NO releaser NOR4, (±)-N-[(E)-ethyl-2-[(Z)-

![Graph](image)

Fig. 1. Time course of the NO released from NOR4 (NO releaser) in MEM and PBS at 37°C. NOR4 was dissolved in DMSO, and about 10 μm of this solution was added in 10 ml of the medium. Final concentration of NOR4 was 100 μm.

The concentration of NO was measured as NO$_2^-$ using the Griess reagent as described in the text.
hydroxyimino]-5-nitro-3-hexene-1-yl]-3-pyridine carboxamide (Wako, Osaka) as NO\textsuperscript{2-} using a Griess reagent, because NO in an aqueous solution containing oxygen is oxidized primarily to NO\textsuperscript{2-} with little or no formation of NO\textsuperscript{3-} (Geng et al., 1994). NOR4 is an analog of NO releaser FK409 (Fukuyama et al., 1995). The Griess reagent consists of 0.5% sulfanilamide, 0.05% N-[(1-naphthyl)ethylene-diamine-dihydrochloride and 2.5% H\textsubscript{3}PO\textsubscript{4}. Briefly, 1 ml of the sample was mixed with 1 ml of the Griess reagent. After an incubation of 15 min at room temperature, the absorbance was read at 550 nm. The NO\textsuperscript{2-} concentration was calculated from an NaNO\textsubscript{2} standard curve (10\textsuperscript{-6} M ~ 10\textsuperscript{-4} M).

Statistics: Data were expressed as mean ± S.E. A student's paired t-test or unpaired t-test was used for statistical analysis of the results.

RESULTS

Induction of SCE with NO\textsubscript{x}

To determine the induction of SCE with NO\textsubscript{x}, NOR4 was used as the NO releaser. NaNO\textsubscript{2} or NaNO\textsubscript{3} was used as NO\textsuperscript{2-} or NO\textsuperscript{3-}. NOR4 was dissolved in DMSO, and 10 μl of this solution was added in 10 ml of the medium. Spontaneous release of NO from NOR4 can be seen in Fig. 1. NO release (measured as NO\textsuperscript{2-}) from 100 μM NOR4 was about 20% or 5% of the MEM or PBS, respectively, during the incubation period of 5 hr at 37°C.

The frequencies of NOR4-, NaNO\textsubscript{2}- and NaNO\textsubscript{3}-induced SCE in the metaphase cells are shown in Fig. 2. A similar effect of NaNO\textsubscript{2} to NOR4 was detected. SCE frequencies increased with NOR4 and NaNO\textsubscript{2}. A significant increase was observed at a concentration of more than 3 μM of NaNO\textsubscript{2} and NO\textsuperscript{2-} converted from the NO released from NOR4 compared with 0 μM.

On the other hand, the SCE frequency was not affected by NaNO\textsubscript{3}. Even at 100 μM of NaNO\textsubscript{3}, no significant increase in the SCE frequency was observed compared with 0 μM.

![Graph showing SCE frequencies induced by different concentrations of NO\textsuperscript{2-} and NO\textsuperscript{3-}](chart)

**Fig. 2.** Frequencies of SCE induced by NOR4 (NO releaser), NaNO\textsubscript{2} and NaNO\textsubscript{3}. The CHL/IU cells exposed to NOR4, NaNO\textsubscript{2} and NaNO\textsubscript{3} in MEM did not contain phenol red and serum for 5 hr. The number of recorded metaphase cells were 80-100 with NOR4, and 50-60 with NaNO\textsubscript{2} or NaNO\textsubscript{3}.

* *p<0.01 vs 0 μm concentrations of NO\textsuperscript{2-} or NO\textsuperscript{3-}.

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Effect on NOx-induced SCE

NO is known to bind with sulfhydryls in amino acids. So several agents containing sulfhydryl were used to detect the protective effects regarding the NO⁻ and NO₂⁻ induced SCE frequency. These agents were added immediately before the addition of NOx into the medium, and the cells were incubated for 5 hr. In this procedure, phenol red and calf serum were not contained in the medium so as to exclude the influence on the NO₂⁻ determination and on the effect of the agents.

As shown in Table 1, significant decreases in NOR4- and NaNO₂-induced SCE were observed with the addition of SOD (250 U/ml, Sigma, St. Louis, MO), Hb (20 μM, Funakoshi, Tokyo), BSA (0.1% and 1%, Sigma, St. Louis, MO) and calf serum (10%) but not with catalase (1000 U/ml, Merck, Darmstadt), calf serum (1%), glutathione (oxidized and reduced form, 20 μM), cysteine (20 μM) and cystine (20 μM). In those experiments, the same concentrations of SOD and catalase as in the previous study were used regarding the effect on superoxide anions-induced SCE (Tanaka and Amano, 1989), and other agents were maximum non-toxic concentrations. The concentration of NO₂⁻ decreased by only incubation with Hb.

Effect of superoxide anion and NO/NO₂ on SCE frequency

As the superoxide anion generator, paraquat (PQ) was used. In the previous study (Tanaka and Amano, 1989), we reported an increase in the SCE frequency by 0.4-20.0 μM PQ. So we used 4.0 μM PQ in this study. NO and/or NO₂⁻ released from NOR4 in the presence of superoxide anions, released from PQ (4 μM), induced a higher frequency of SCE than that with the absence of PQ (Fig. 3). The frequency of PQ-induced SCE decreased with the use of catalase (1000 U/ml) but not with SOD even at 1000 U/ml (Table 2).

DISCUSSION

In this study, NO and NO₂⁻ caused an increase in the SCE frequency (Fig. 2). This increase was inhibited by treatment with calf serum, BSA, Hb and SOD, but not with glutathione (oxidized and reduced form), cysteine and cystine (Table 1). From these results it seems that proteins having many sulfhydryls bind with NO or NO₂⁻ resulting in the produc-

<table>
<thead>
<tr>
<th>NO₂⁻ (μM)</th>
<th>SOD (250U/ml)</th>
<th>Hb (20 μM)</th>
<th>Catalase (1000U/ml)</th>
<th>Calf serum (1%)</th>
<th>Calf serum (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.76±0.17</td>
<td>4.09±0.24*</td>
<td>3.67±0.18*</td>
<td>5.45±0.18</td>
<td>5.28±0.16</td>
</tr>
<tr>
<td>NOR4 16</td>
<td>5.30±0.20</td>
<td>4.27±0.21*</td>
<td>3.53±0.16*</td>
<td>4.95±0.15</td>
<td>4.92±0.24</td>
</tr>
<tr>
<td>NaNO₂ 10</td>
<td>5.28±0.20</td>
<td>4.27±0.21*</td>
<td>3.53±0.16*</td>
<td>4.95±0.15</td>
<td>4.92±0.24</td>
</tr>
</tbody>
</table>

**Table 1.** Effects of agents regarding SCE frequency induced by NOR4 and NaNO₂.

<table>
<thead>
<tr>
<th>NO₂⁻ (μM)</th>
<th>SOD (0.1%)</th>
<th>BSA (1%)</th>
<th>BSA (1%)</th>
<th>GSH (20 μM)</th>
<th>GSSG (20 μM)</th>
<th>Cysteine (20 μM)</th>
<th>Cystine (20 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.83±0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOR4 16</td>
<td>5.20±0.17</td>
<td>3.98±0.18*</td>
<td>3.93±0.18*</td>
<td>5.31±0.29</td>
<td>5.11±0.16</td>
<td>5.21±0.19</td>
<td>5.67±0.16</td>
</tr>
<tr>
<td>NaNO₂ 10</td>
<td>4.96±0.17</td>
<td>3.88±0.19*</td>
<td>2.89±0.14*</td>
<td>4.90±0.17</td>
<td>5.02±0.18</td>
<td>4.71±0.16</td>
<td>5.10±0.18</td>
</tr>
</tbody>
</table>

* p < 0.01 vs controls n=50-70

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tion of nitrosothiols. However, other compounds such as glutathione and cysteine did not seem to produce any stable nitrosothiol compounds. In this paper, it is very interesting to note that SOD was effective in decreasing NO and NO\textsuperscript{2-} -induced SCE at low concentrations. Binding of peroxynitrate to SOD has been reported (Beckman et al., 1992). However, the mechanism for a decrease in genotoxicity due to SOD is still unclear.

Therefore, further studies will be needed. However, it would appear that S-nitrothiols in the cells may be stabilized by SOD in consideration of the S-nitrosothiols stabilizing effect of SOD. Nitrosothiols are potent vasodilators in vivo and in vitro, but the mechanism of action is still not clear. Dowaluk and Fung (1990) reported that the spontaneous NO release from S-nitroso-N-acetylpenicillamine (SNAP) and S-nitro-gluthione (GSNO) was inhibited in the presence of 100 U/ml SOD, and the relaxant effects of SNAP and GSNO were enhanced. Therefore, if the production of nitrosothiol in these experiments occurred, SOD can be seen as a very effective agent for the protection of genotoxicity with active vasodilatinge properties. Kharitonov et al. (1995) reported on the nitrosothiol formation of glutathione, cysteine and BSA with NO. However, the protective activity of SCE formation could not be observed with glutathione and cysteine in our study. With the Hb treatment, the NO\textsuperscript{2-} concentration decreased but not for the BSA, calf serum and SOD treatment in spite of the decreasing effect regarding the NO or NO\textsuperscript{2-} -induced SCE frequency. In this experiment, protein precipitation was not carried out. Therefore, it seems that Hb can strongly bind with NO and/or NO\textsuperscript{2-}.

Jenkins et al. (1995) suggested dual pro- and antitumor action of NO depending on the its local concentration. NO has an antitumor action due to the inhibition of cell proliferation (Garg and Hassid, 1990) by the inhibition of DNA synthesis and cell damage. However, NO is carcinogenic due to the induction of SCE and DNA strand breaks, (Radons et al., 1994) and deamination of DNA (Nguyen et al., 1992). Its carcinogenic action seems to be possible in situations such as chronic infection and inflammation. From our results, NO and NO\textsuperscript{2-} seem to be carcinogenic but not NO\textsuperscript{2-} due to its SCE inducible action.

In the present paper, the effects of NO\textsuperscript{2-} and NO regarding SCE induction are mixed.

![Fig. 3.](image)

Fig. 3. Frequencies of SCE induced by NOR4 (NO releaser) and paraquat (PQ, superoxide anion generator).
The CHL/IU cells were exposed to NaNO\textsubscript{2} and/or PQ in MEM not containing phenol red and serum for 5 hr. Numbers of recorded cells are 50-60. \(^{a}p<0.01\) vs control.
Victorin (1994) has described, in the genotoxicity of NO\textsuperscript{x}, that NO\textsuperscript{y} is most toxic, and has the highest genotoxicity of NO\textsuperscript{x} in vitro. It also reports that NO and NO\textsuperscript{y} attack DNA, and induce SCE in their low concentrations. In their higher concentrations, DNA cleavage can occur. These actions of NO\textsuperscript{x} are similar to these of the superoxide anions.

In the presence of NO/NO\textsuperscript{y} and superoxide anions, an increase in the SCE frequency is additional (Fig. 3). Therefore, it seems that there is genotoxicity in both the presence of NO/NO\textsuperscript{y} and superoxide anions, and genotoxicity decreases due to catalase and SOD, since the former decreases superoxide anion-induced SCE frequency, and the latter NO/NO\textsuperscript{y}-induced frequency (Table 2). In other words, the combination treatment of catalase and SOD would be effective in preventing genotoxicity in a situation in which both NO\textsuperscript{x} and superoxide anions were generated.

### REFERENCES


| Table 2. Effects of SOD and catalase regarding SCE frequency induced by paraquat. |
|-----------------|----------------|----------------|
| PQ(μM)          | Control        | SOD(1000U/ml)  | Catalase(1000U/ml) |
| 0               | 3.20±0.14      |                |                  |
| 4               | 4.57±0.17      | 4.46±0.19      | 3.25±0.20*       |

CHL/IU cells were incubated with SOD or catalase and paraquat in MEM not containing phenol red and serum for 5 hr.
PQ: Paraquat
*p < 0.01 vs control n=50～60