NITRIC OXIDE COORDINATES DEVELOPMENT OF GENOMIC INSTABILITY IN REALIZATION OF COMBINED EFFECT WITH IONIZING RADIATION

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The aim of this study was to investigate the ability of environmental nitrogen oxides or natural nitric oxide (NO) donors to modify free radicals balance and development of genomic instability alone or in combination with ionizing radiation. Methods: Genotoxicity and cytogetic abnormalities were assessed in vitro in peripheral blood lymphocytes (PBL) isolated from healthy humans or in vivo in rats PBL. Human PBL were treated with physiologically relevant NO donor — S-Nitrosogluthathione and X-ray irradiation. The inhalation treatment of animals with NO was carried out in chamber with purified gaseous NO mixed inside with air. Levels of S-Nitrosohemoglobin and methemoglobin in the blood were assessed with electron paramagnetic resonance. The total level of reactive oxygen and nitrogen species in PBL was determined fluorometrically, and serum levels of reactive oxygen species was determined by spectrophotometric assay. DNA damages were assessed by alkaline single-cell gel electrophoresis. The frequency of chromosomal aberrations in human PBL measured with the conventional cytogenetic assay in metaphase cells on short-term (52 h) and long-term (72 h) cultures. Results: Environmental nitrogen oxides or release of NO from stable complexes with biomolecules (such as S-Nitrosothiols) intensified generation of free radicals, DNA damage and development of genomic instability alone or in combination with ionizing radiation. Treatment of PBL by S-Nitrosoglutathione caused prevalent induction of chromatid type but irradiation — chromosome aberrations. The dose dependence of chromatid-type aberrations observed in human PBL after combined influence of S-Nitrosoglutathione and ionizing radiation indicates a crucial role of NO in the formation of chromosomal instability. Conclusion: NO can deregulate free radicals balance resulted in genotoxic effect, posttranslational modification of repair enzymes and thus coordinated development of genomic instability and increase of cancer risk.

Key Words: nitric oxide, S-Nitrosothiols, nitrosative stress, ionizing radiation, DNA damage, genomic instability.

Intensification of anthropogenic activities leads to the growth of environmental pollution by chemical and physical factors that affect both the state of ecosystems and human health. Combinations of chemical and physical agents are dangerous in terms of mutagenic and carcinogenic effects. Nitrogen oxides (NOx) are one of the main air pollutants. The possible role of NOx in the formation of genomic instability is debatable due to their ability to inhibit DNA repair enzymes [1, 2]. Ionizing radiation (IR) is a powerful physical genotoxic factor that affects three related systems: redox homeostasis, cell cycle control and DNA repair [3]. Biological effects of nitric oxide (NO) are broad ranging and significantly depend on the concentration. Signal properties of NO molecules occur at physiological concentrations as low as 1–30 nM. An increase of NO concentration in 10 times activates oncogenic signaling cascades, and at NO concentrations of above 500 nM results in nitrosative stress [4]. NOx participate in all stages of neoplasia, demonstrating cytotoxic and cytostatic activities, and under certain conditions, are able to increase the effectiveness of anticancer therapy [5, 6]. Manifestation of NO cytotoxicity depends on both concentration and cumulative dose. Significant cytotoxic effects occur at concentrations exceeding 150–300 mM/min of comparable cumulative dose [7].

Relatively high NO concentrations lead to the failure of antioxidant defense and DNA repair systems accompanied by formation and accumulation of DNA strand breaks that significantly enhance the level of somatic mutations [8]. NO can cause direct or/and indirect mutagenic effects. Direct effects imply interaction of NO hydrated derivatives with DNA amino groups. Nitrosation of primary amines lead to formation of alkylation agents. Indirect mutagenic effects involve formation of N-nitrosocompounds such as N-alkylnitrosamines in reactions of NO with secondary amines and amides.

Exogenous NOx, after being inhaled are transferred to the blood stream. In the blood, NO binds to hemoglobin, albumin and other iron- or SH-containing proteins and compounds, that are transported through vessels to different tissues and organs [9], where NO oxidized to nitrite and/or nitrates prior removal from the body. Some part of NO reversibly binds to biological molecules thus forming S-Nitrosothiols (RSNO) and nitrated complexes of nonheme iron ((RS)2Fe(NO+)3) that provide NO stabilization and transfer from donor to target cells [10, 11]. Recent data suggest a vital role of RSNO in post-translational modification of signaling cascades in cells and modulation of biological processes and pathological states [12, 13].
Gaseous NO can induce DNA single (SSB) and double-strand breaks (DSB), DNA SOS-repair, formation of micronuclei, chromatid-type aberrations, and exchanges between sister chromatids [7, 14]. SSB formation is characteristic for NO effects, detection of which may serve as biomarker of NO genotoxicity [1].

NO plays a significant role in formation of mammalian cells sensitivity to IR both in vivo and in vitro [15], but at the genetic level the NO dose-effect dependence has not been determined yet. Many NO-dependent biological functions are directly linked with the RSNO synthesis. RSNO is a primary form of NO transport, which released under physiological conditions and is able to establish the background for appearance and accumulation of aberrant cells [6, 16].

The common mechanism of NO and IR genotoxicity is the formation of reactive oxygen (ROS) and nitrogen (RNS) species. About 80% of IR–induced damages are due to the influence of free radicals [8]. DNA-free radicals interactions lead to DNA SSB and DSB, sister chromatids exchanges, point mutations, microdeletions [17]. The reaction of NO with superoxide radicals leads to peroxynitrite formation, that causes DNA DSB [18].

Chemical agents alone or in combination with IR can cause the formation of DSB, that if unrepaird or inefficiently repaird can give rise to chromatid breaks subsequently converted into chromosomal aberrations [19].

To date, the question about duration of induced chromosomal instability and its ability to be transmitted to future generations of cells remains unanswered. This issue needed for prediction of long-term health effects as a result of combined action of mutagenic and carcinogenic factors [20]. It has been shown that radiation-induced signaling, which leads to the development of genomic instability, is inherited epigenetically [21]. Therefore, investigation of heritability of chromosomal instability under combined action of IR and NO in somatic cells is of importance.

Taking into account the multiple impact of NO and its derivatives on human health together with radiological situation after the Chernobyl accident in some regions of Ukraine, the study of the combined action of NO and IR on DNA and development of chromosomal instability is very important.

In order to evaluate the combined genotoxic potential of chemical and radiation factors and estimate the efficiency of DNA repair processes were used alkaline gel electrophoresis of isolated cells (DNA comet assay) [22, 23] and chromosomal aberrations [24]. Chromosomal aberrations are considered as integral index, which takes into account both the implementation of primary DNA damage and repair processes. An increase of chromosomal aberrations in peripheral blood lymphocytes (PBL) is a valuable biological marker for the assessment of stochastic effects, including cancer [25]. High sensitivity of chromosomal apparatus in PBL, low and quite stable level of spontaneous chromosomal aberrations in these cells, their ability to accumulate cytogenetic abnormalities, together with the natural cells synchronization led to the widespread use of PBL culture in assessing mutagenicity of physical and chemical environmental factors [26].

The aim of investigation was to study ability of environmental NO or natural NO donors to modify free radicals balance and cause development of genomic instability alone or in combination with IR.

**MATERIALS AND METHODS**

Adult random-bred male rats (120–150 g) were obtained from the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine) and kept at steady state conditions with a constant temperature and natural light. The work with animals was performed according to the rules of local Ethic Committee [27]. The animals were divided into four groups: 1) intact control (IC); 2) animals that inhaled NOx for 1 month (16 h per day); 3) animals that were fractionally irradiated with X-rays at a frequency of exposure 0.1 Gy per 3 days giving the total absorbed dose 1 Gy; 4) animals that received combined treatment of NO and IR.

Genotoxicity and cytogenetic abnormalities were assessed in PBL isolated from healthy humans for an *in vitro* study or rats treated with NO and/or IR for *in vivo* study. An informed consent of donors for taking blood samples and conducting cytogenetic studies was obtained.

**NO inhalation.** The inhalation treatment of animals with NO was carried out in 0.1 m³ chamber equipped with device for input of purified gaseous NO mixed inside with air. Air circulation inside the chamber allowing triple total replacement of air per 1 h. NOx concentration at the chamber’s output was 150 mg/m³ of air, 40% corresponds to NO and 60% — NO₂ of their total content. Concentration of NOx was expressed in mg of NO per m³ of air. The control of NO content in the inhalation chamber was performed as described previously [28].

**X-ray irradiation.** X-ray irradiation of rat and human PBL was performed at dose rate of 0.89 Gy/min with doses range 0.1–2 Gy. Measurements of absorbed dose were performed using an ionization chamber and ferro-sulfate dosimeter.

**Isolation of PBL.** Whole blood was diluted in an equal volume of PBS and stratified on Histopaque-1077 (“Sigma”, St Louis, MO) for lymphocyte separation according to the manufacturer’s instruction. After isolation, lymphocytes were washed in PBS, diluted in 1 ml culture medium and counted after trypan blue (“Euroclone”, Pero, IT) exclusion staining. PBL were suspended in PBS and kept at 4–6 °C before use.

**Electron paramagnetic resonance (EPR) studies.** The levels of S-Nitrosohemoglobin (HbNO) and methemoglobin (methHB) were measured by EPR in the blood of rats. EPR spectra were obtained using spectrometer RE-1307 (USSR) and E-109 (Varian, USA) in the centimeter wavelength range at T = 77K. Results
were expressed in arbitrary units (a.u.), characterizing the intensity of the EPR signal.

RSNO concentration in the blood serum of rats was determined according to [29] and expressed in nM. The total level of ROS and RNS in PBL determined fluorometrically [30]. The serum levels of ROS determined by spectrophotometric assay [31].

**Alkaline Comet Assay.** The single-cell gel electrophoresis was used for visualizing and measuring SSB and DSB DNA damage in individual cells. The method is based on detection of various mobility of damaged DNA [32]. PBL were washed in PBS and suspended in agarose gel at concentration of 0.5–0.7*10⁶ cells/ml then processed as previously described [33]. Slides were stained with SYBR Green (Sigma, 15 μg/ml). The images of comets were observed at 40x–100x magnification with a fluorescence microscope equipped with video camera (CCD, Webbers, USA). One hundred images were randomly selected from each sample and analyzed by an image-analysis program “CometScore” (TriTek Corp, Sumnerduck, VA, USA). The degree of DNA damage was estimated by the DNA percentage in the tail (%DNA%).

**Cytogenetic analysis.** The frequency of chromosomal aberrations in human PBL measured with the conventional cytogenetic assay in metaphase cells on short-term (52 h) and long-term (72 h) cultures. The frequency of chromosomal aberrations was expressed as a number of damaged chromosomes per 100 analyzed metaphases. Total chromosomal aberrations were sub classified as chromosome-type aberrations (including chromosome-type breaks, rings chromosomes, marker chromosomes, and dicentrics) and chromatid-type aberrations (including chromatid-type breaks and chromatid exchanges) [34]. As a transport form of NO, S-Nitrosoglutathione (GSNO) was used, which was added to the cell culture in the concentration range of 0.5–1.5 μM.

**Statistical analysis.** Statistical analysis was performed using Student’s t-test. Values are reported as mean ± standard error. Significance level was set at P < 0.05 [35].

**RESULTS AND DISCUSSION**

Prolonged inhalation of exogenous NOx was accompanied by significant increase of RSNO level in the blood serum of rats and by formation of a large amount of S-nitrosyl complexes of hemoglobin in erythrocytes. The reaction between NOx and hemoglobin resulted in formation of HbNO complex registered at g = 2.03 on EPR spectrum (Fig. 1). The blood of experimental animals was sampled in less than 1 hour after termination of NO treatment and showed a 3.6-fold increase in HbNO content (Fig. 2).

The moderate concentration of RSNO was present in the blood serum of intact rats, but after NO inhalation the level of RSNO increased in a 9.5-fold, indicating the development of nitrosative stress in organism of animals (Fig. 2). The most prevalent form of RSNO in organism is nitrosated form of glutathione — GSNO. Previous work has shown that transnitrosation between oxygenated hemoglobin and GSNO is a slow, reversible process. However, GSNO reacts with deoxygenated hemoglobin to form glutathione, NO and ferric hemoglobin (methHb). NO formed from this reaction is immediately autostabilized to form HbNO [36].

The tendency of ROS formation in serum and PBL was similar. Maximum ROS generation (1.8-fold higher than in control) was observed after prolonged inhalation of exogenous NO, while IR did not affect the ROS level. Combined action of NO and IR resulted in an insignificant increase (1.2-fold) of radicals level (Fig. 3).
in vitro gate the combined effect of NO donors and IR, we per-

3.1-fold greater than in control. The highest level of DNA damage was observed under the combined action of NO and IR (15.4 ± 0.1%) and it was 3.8 ± 0.06%.

The difference in genotoxic effect of IR in human and rat PBL can be explained by variations in species-specific radiosensitivity [39] and hypersensitivity of DNA exposed to fractionated low-dose IR [40].

The results of DNA damage study after combined treatment of PBL with IR and GSNO (1 μM) in vitro shown on Fig. 5. The treatment of PBL with GSNO alone led to 1.4-fold increase in DNA fragmentation compared with control cells. Combined treatment of PBL with GSNO and IR at dose of 0.5 Gy caused 2.2-fold increase of DNA breaks. This effect was 1.8-fold greater than the effect of IR alone, and in 1.6-fold exceeded DNA damage by GSNO alone. Increase of IR dose up to 1 Gy together with 1 μM of GSNO resulted in 3.1-fold rise of genotoxic effect (Fig. 5). But further elevation of IR dose up to 1.5 Gy combined with GSNO did not cause increase of DNA damage. On the contrary, their level dropped 1.6-fold compared with level of DNA damage induced by 1 Gy, also it remained 1.9-fold higher compared to control PBL. Thus, the genotoxic effect after combined treatment with GSNO and IR was 1.2-fold weaker than after individual action of 1.5 Gy of IR, but this effect was in a 1.4-fold stronger than individual effect of GSNO (data not shown) (Fig. 5).

The interaction of NO and DNA repair enzymes may lead to the failure of DNA repair and increase of the genotoxic effect. It is known that during the DNA excision repair SSB are formed. The replication of DNA with unrepaired SSB may cause formation of DSB, associated with development of genetic instability. Existence of DNA DSB accompanied by impaired DNA repair resulted in formation of chromosomal aberrations.

The cytogenetic effect of GSNO and IR on human PBL in vitro (Table) was evaluated under short time cultivation condition in the first cell generation. Results suggest that upon an increase of GSNO concentration from 0.5 to 1.0 μM the number of PBL with chromosome aberrations and the overall frequency of induced chromosomal aberrations are also increased (from 6.0 ± 0.2 and 7.0 ± 0.2 to 12.0 ± 1.0 and 18.3 ± 1.4, respectively).
The aberrations of chromatid type, such as deletions and exchanges, were prevalent in the spectrum of GSNO induced damage. On the contrary, irradiation of PBL mainly caused chromosomal type aberrations. The combined effect of low dose of IR (0.5 Gy) and smaller GSNO concentrations (0.5 μM and 1.0 μM) caused an increase in the number of chromosome aberrations in human PBL (Table), as well as an increase of DNA damage frequency in rat PBL (Fig. 5). The total effect does not exceed the sum of individual effects of these factors, while it was larger than the effect of a single factor. Increasing the dose of GSNO (1.0 μM) and IR (1 Gy) caused the additive effect of all studied cytogenetic parameters. The obtained data indirectly indicates inhibition of DNA repair due to combined effect of IR and GSNO, possibly because of induction of slow- or unrepairable DNA breaks, as evidenced by formation of structural chromosom complexes.

However, further increase of IR dose up to 1.5 Gy combined with GSNO treatment (1.0 μM) led to considerable decrease of cytogenetic effects, in particular, the number of cells with chromosome aberrations and the total frequency of chromosomal aberrations (Table). The overall aberrations rate decreased 1.8-fold compared to cells treated with IR (1 Gy) and GSNO (1 μM). Chromosome-type aberrations were the prevalent damage type, while the number of chromatid-type aberrations dropped to the level of individual impact of 1.5 Gy of IR, and decreased 2-fold compared to 1.0 μM of GSNO.

As shown on Fig. 5, the number of DNA breaks increased with increasing of IR dose up to 1.5 Gy. However, the number of DNA breaks in PBL treated with IR at the dose of 1.5 Gy and GSNO at the concentration of 1.0 μM decreased compared with effect of IR and GSNO lower dose as well as with effects of individual factors. The common trend in results of molecular and cytogenetic studies suggests the direct link between the frequency of chromosomal aberrations and the level of DNA damage. Reduction of induced cytogenetic effect can be attributed to elimination of cells with heavily damaged DNA, in addition experiencing deficiency in DNA repair and, as a result, more loaded with chromosomal rearrangements.

In order to confirm this assumption we have studied the qualitative and quantitative characteristics of chromosomal instability of PBL at long-term cultivation (72 h, Fig. 6–8). Decrease of overall frequency of chromosome aberrations (1.5-fold) in irradiated PBL treated with GSNO was observed mainly in combination with low doses of IR, but the changes of the spectrum of chromosome aberrations were found throughout whole dose range of IR (Fig. 6).

The radiation effects in human PBL were characterized by linear dose dependence of the chromosome-type aberrations frequency, which is a good marker of the IR impact, but additional influence of GSNO caused marked decrease of cytogenetic effect without dependence on the IR dose (Fig. 7).

The frequency of chromatid-type aberrations, which are cytogenetic markers of chemical mutagens influence, in human PBL treated with GSNO and IR increased linearly with IR dose (Fig. 8). The treatment with IR alone was characterized by dose-independent decrease of number of chromatid-type aberrations.

These results suggest elimination of PBL with chromosome-type aberrations occurred due to DNA DSB. The contribution of chromatid-type aberrations, which are arising mainly due to DNA SSB, in the development of genetic instability at long-term cultivation of PBL was more significant. Accumulation of SSB in a number of cell generations plays a crucial role in the development of chromosomal instability under the combined influence of IR and GSNO.

<table>
<thead>
<tr>
<th>№</th>
<th>Groups</th>
<th>Cytogenetic parameters (per 100 analyzed metaphase)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Frequency of aberrant cells, %</td>
</tr>
<tr>
<td>1</td>
<td>Intact control</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>IR, 0.5 Gy</td>
<td>11.0 ± 1.3</td>
</tr>
<tr>
<td>3</td>
<td>IR, 1.0 Gy</td>
<td>18.0 ± 1.4</td>
</tr>
<tr>
<td>4</td>
<td>IR, 1.5 Gy</td>
<td>26.0 ± 1.8</td>
</tr>
<tr>
<td>5</td>
<td>GSNO, 0.5 μM</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>GSNO, 1.0 μM</td>
<td>12.0 ± 1.0</td>
</tr>
<tr>
<td>7</td>
<td>IR, 0.5 Gy + GSNO, 0.5 μM</td>
<td>20.0 ± 1.4</td>
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<tr>
<td>8</td>
<td>IR, 0.5 Gy + GSNO, 1.0 μM</td>
<td>24.0 ± 1.6</td>
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<tr>
<td>9</td>
<td>IR, 1.0 Gy + GSNO, 0.5 μM</td>
<td>27.0 ± 1.5</td>
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<tr>
<td>10</td>
<td>IR, 1.0 Gy + GSNO, 1.0 μM</td>
<td>37.0 ± 1.8</td>
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<tr>
<td>11</td>
<td>IR, 1.5 Gy + GSNO, 0.5 μM</td>
<td>35.0 ± 2.0</td>
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<tr>
<td>12</td>
<td>IR, 1.5 Gy + GSNO, 1.0 μM</td>
<td>23.0 ± 1.4</td>
</tr>
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</table>

In order to confirm this assumption we have studied the qualitative and quantitative characteristics of chromosomal instability of PBL at long-term cultivation (72 h, Fig. 6–8). Decrease of overall frequency of chromosome aberrations (1.5-fold) in irradiated PBL treated with GSNO was observed mainly in combination with low doses of IR, but the changes of the spectrum of chromosome aberrations were found throughout whole dose range of IR (Fig. 6).

Fig. 6. The total frequency of chromosome aberrations (per 100 analyzed metaphase) in human PBL treated with GSNO and IR. Data shown are the mean ± standard error of the mean.

The radiation effects in human PBL were characterized by linear dose dependence of the chromosome-type aberrations frequency, which is a good marker of the IR impact, but additional influence of GSNO caused marked decrease of cytogenetic effect without dependence on the IR dose (Fig. 7).

Fig. 7. The frequency of chromosome type aberrations (per 100 analyzed metaphase) in the human PBL treated with GSNO and IR. Data shown are the mean ± standard error of the mean.
Nitric oxide and cancer: the emerging role of genomic instability in mammals. One of these effects of radiation-induced effects, including development of chromosomal instability. Analysis of literature showed that NO may mediate crucial in the formation of chromatid-type aberrations. 

Exogenous NO not only directly provide increase of NOx negatively affected T-cells and caused decrease and reached the control level at 12-th day. 

We showed that prolonged inhalation intake of exogenous NO resulted in the development of nitrosative stress in rats accompanied by formation of large amount of methHb (68-fold of control value, data not shown), HbNO and increased level of RSNO in the blood. The formation of HbNO complexes and RSNO was of a specific interest due to possibility of reversible release of NO. As expected, the level of these compounds in the blood was highest immediately after exposure to NO, then their concentration gradually decreased and reached the control level at 12-th day. 

Exogenous NO not only directly provide increase of RSNO level, but also affect the processes associated with production of endogenous NO. In our previous study was shown that long-term exposure to exogenous NOx negatively affected T-cells and caused hyper activation of peritoneal macrophages [28].

Obtained data confirm that formation of chromosomal aberrations is typical for radiation-induced genotoxic effects. Effects associated with NO are crucial in the formation of chromatid-type aberrations. Analysis of literature showed that NO may mediate radiation-induced effects, including development of genomic instability in mammals. One of these effects is the formation of DNA DSB and decreased efficiency of repair system, which cumulatively lead to formation of chromosome aberrations.

Thus, we showed that the combined influence of NO and IR caused destabilization of genetic apparatus in PBL, the degree of manifestation of which depended on the doses of these damaging factors.

Occurrence of chromosomal aberrations is considered as a characteristic feature of neoplastic cells. To date, more than 600 tumor-associated specific chromosomal rearrangements are observed in most cases of cancer. Carcinogenesis associated with chromosomal aberrations can be induced by deregulation (usually overexpression) of the normal gene, or by formation of hybrid gene from the fragments of aberrated chromosomes [41]. There is a distinct link between the total incidence of cancer and the presence of chromosomal aberrations [42]. Since the appearance of chromosomal changes is considered to be potentially carcinogenic [43, 44], our data demonstrate the possibility of increasing carcinogenic risk under the combined influence of NO and IR.

Thus, the combined effect of NO and IR caused the formation of DNA SSB and DSB with the further development of chromosomal instability in PBL, the degree of manifestation and nature of which depends on the doses of these factors. The dose dependence of chromatid-type aberrations observed in human PBL after combined influence of IR and GSNO indicates a crucial role of NO in the formation of chromosomal instability. Release of NO from stable complexes with biomolecules (such as GSNO and GbNO) can deregulate free radicals balance resulted in genotoxic effect, posttranslational modification of repair enzymes and thus coordinate development of genomic instability and increase of cancer risk.

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