Arginine increases genotoxicity induced by methyl methanesulfonate in human lymphocytes

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Abstract Nitric oxide (NO) is a free radical that is produced in cells from L-arginine. NO is involved in the physiological control of different tissues, but it can act as a toxic mediator in the cells. In this study we investigated the effect of L-arginine on the genotoxicity induced by methyl methanesulfonate (MMS) in human lymphocytes. Blood was treated with N^G-nitro-L-arginine methyl ester (L-NAME) as an inhibitor of nitric oxide synthase for finding out the role of NO in this effect. Human whole blood was treated with L-arginine (50, 100 and 250 μM) and/or L-NAME, then it was treated in vitro with MMS after 24 h of culture. The lymphocytes were stimulated by phytohemagglutinin to find out the micronuclei in cytokinesis-blocked binucleated cells. DNA fragmentation of lymphocytes was detected by using a fluorescence microscope after propidium iodide staining. These data showed that

arginine increased the frequency of MMS-induced micronuclei in lymphocytes. However, the genotoxicity was decreased by using L-NAME. Arginine and L-NAME have not shown any DNA damage in cultured human lymphocytes. In conclusion, addition of L-arginine to MMS as an alkylating agent caused an increase of DNA damage in human lymphocytes. This enhancement of genotoxicity was reduced by NAME as NO inhibitor. It is thus cleared that an increase of DNA damage by arginine and MMS is related to NO production.

Keywords Arginine · Methyl methanesulfonate · Genotoxicity · Micronuclei · Apoptosis

Introduction

Nitric oxide (NO) is produced in the cells. NO has several physiological properties such as immunity and inflammatory response, vasodilatation, inhibition of platelet aggregation and neutrophil adhesion, scavenging superoxide (O₂⁻) formation and suppression of xanthine oxidase (XO) (Fukahori et al. 1994; Kelly et al. 1996). NO acts as a physiological intercellular mediator and sometimes it can indirectly attack macromolecules such as DNA to cause genotoxicity (deRojas-Walker et al. 1995; Pacher et al. 2007; Forstermann and Sessa 2011). NO is endogenously generated from L-arginine by NOS (nitric oxide

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synthase) isoenzymes. High concentration of NO and its metabolites have been shown to cause DNA damage and mutagenesis (Nguyen et al. 1992; Fehsel et al. 1993). However, arginine is a substrate for production of NO in the cells, there are evidenced that arginine has a protective effect against toxicity induced by oxidative stress due to increase of antioxidant enzymes in the cells (El-Missiry et al. 2004; Lin et al. 2005). Arginine could induce DNA damage with raising of NO in lymphocytes, however, this genotoxicity effect was protected by silymarin (Yurtcu et al. 2012). These studies have shown that arginine has dual effects as toxic or protective effects on the cells. It is important to find out the role of arginine as a substrate for production of NO in genotoxicity induced by hazardous chemical agents. Methyl methanesulfonate (MMS) is a toxic agent; it is widely used for genotoxicity study (Hosseinimehr et al. 2011). This studies was conducted to evaluate the triggering effect of L-arginine on the genotoxicity induced by MMS in cultured human lymphocytes. It was focused on the role of NO by using N^G-nitro-L-arginine methyl ester (L-NAME) an inhibitor of nitric oxide synthase. This study help understanding more details about NO as a mediator in the cells exposed to hazardous chemical agents.

Materials and methods

L-Arginine, cytochalasin B, L-NAME (N^G-nitro-L-arginine methyl ester) and methyl methanesulfonate (MMS) were prepared from Sigma (USA). All cell culture reagents (RPMI 1640 culture medium, fetal bovine serum, phytohemagglutinin and penicillin/streptomycin) were purchased from Gibco (UK).

Genotoxicity assay and culture set up to determine micronuclei

This study was performed after obtained permission from the ethical committee of Mazandaran University of Medical Sciences, Sari, Iran. Informed consent was obtained from three healthy male, non-smoking human volunteers, with ages between 25 and 35 years. After overnight fasting, 12 mL peripheral blood was collected in heparinized tubes. A half milliliter of each whole blood sample was added to 4.3 ml of RPMI 1640 culture medium supplemented by 10 % fetal calf

serum, 100 µL phytohemagglutinin, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine. These samples were treated with 100 µl of arginine (5, 100 and 250 µM) and/or NAME. All cultures were set up in duplicate and incubated at 37 ± 1 °C in a humidified atmosphere of 5 % CO₂/95 % air. MMS samples were treated with methyl methanesulfonate at 24 h after cell culture, at a final concentration of 30 µg/mL (Hosseinimehr et al. 2011). Cytochalasin B, at a final concentration of 6 µl/mL was added after 44 h to the culture. After 72 h of incubation, the cells were collected by centrifugation for 8 min at 1,000 rpm and re-suspended in 0.075 M potassium chloride for 8 min. Cells were fixed immediately in fixative solution 3 times (methanol: acetic acid, 6:1), and were then dropped on to clean microscopic slides, air-dried and stained with a Giemsa solution. All slides were coded and evaluated by light microscopy for micronuclei frequency in cytokinesis-blocked binucleated cells with well-preserved cytoplasm. Criteria for scoring of micronuclei (MN) were diameter between 1/16th and 1/3rd of main nuclei, nonrefractile, not linked to main nuclei and not overlap the main nuclei (Fenech 2000). For each sample, 1,000 binucleated cells (BN) were examined from cultures done in duplicate, and then the frequency of micronuclei was recorded.

DNA fragmentation

Whole blood treated with arginine and/or NAME, and then samples were cultured and exposed to MMS after 24 h of cell culture. In this assay, cytochalasin B was not used. Cells were collected according to the above protocol for micronuclei assay. For fluorescence microscopy, the fixed cells were washed with PBS and then re-suspended in PBS. An aliquot was stained with propidium iodide (PI) at 40 µg/ml for 15 min in the dark, and then cells were observed using a fluorescence microscope (Nikon Japan). DNA fragmentation in lymphocytes was counted with changing of nuclear morphology as chromatin condensation and fragmentation with shrinkage.

Statistical analysis

For each volunteer, at each blood collection time, the incidence of MN/1000BN was recorded. One-way ANOVA analysis followed by Tukay's HSD post hoc test was used for multiple comparisons of data.

Results

Micronuclei assay

The percentage of MN/1000BN in control samples without any treatment was 0.9 ± 0.36 (Table 1). A significant increase in the percentage of MN/1000BN in lymphocytes treated with MMS was observed, compared to control. The frequency of micronuclei was $9.77 \% \pm 0.96$ in samples treated with MMS alone. The frequencies of micronuclei in the lymphocytes treated with MMS and arginine were $8.93 \% \pm 0.72$, $10.07 \% \pm 0.55$ and $12.20 \% \pm 0.57$ at concentrations of 50, 100 and 250 μM , respectively. The frequency of micronuclei in the human lymphocytes treated with MMS and arginine (250 μM) was significantly higher than when treated with MMS alone ($p < 0.05$). The frequency of micronuclei was $6.03 \% \pm 1$ in the lymphocytes treated with MMS plus arginine (250 μM) and NAME, interestingly, it was significantly lower than the MMS and arginine treated cells ($p < 0.01$).

DNA fragmentation

Chromatin changing is one of the characteristics of a serious DNA damage. DNA fragmentation can be observed in fluorescence microscopy after propidium iodide staining. These chromatin changes led to cell

damage and death (Fig. 1). The percentage of DNA fragmentation is shown in Fig. 2 for lymphocytes. A significant increasing in the percentage of DNA fragmentation and shrinkage was seen in MMS treated cells compared to the control ($p < 0.0001$). Arginine (250 μM) did not lead to an increase in DNA fragmentation in lymphocytes; even the were co-treated with MMS and arginine in the lymphocytes ($p < 0.01$). Arginine and NAME alone did not show any increase in frequency of DNA fragmentations.

Discussion

In this study, we studied the effects of L-arginine as a substrate for NO production on genotoxicity induced by methyl methanesulfonate in human lymphocytes, as well as the effect of L-NAME as NOS inhibitor on this response. We found that arginine increased DNA damage in human lymphocytes induced by MMS. Several studies showed that arginine has protective effects against oxidative stress through increase of antioxidant enzymes (Lin et al. 2005; Lanteri et al. 2006; Acquaviva et al. 2009). For example, arginine has a preventive effect on pulmonary and diabetes related to oxidative stresses; it is associated to the increase of superoxide dismutase, glutathione peroxidase and glutathione in the cells. These enzymes have defense roles against oxidative stress (El-Missiry et al. 2004;

Table 1 The percentages of micronuclei induced in vitro by methyl methanesulfonate on cultured blood lymphocytes from human volunteers treated with arginine and/or NAME

Groups ^a	Binucleate cells with micronuclei (%) ^b
Control	0.90 ± 0.36
NAME	0.97 ± 0.15
Arg100	0.57 ± 0.06
Arg250	0.57 ± 0.21
Arg100 + NAME	0.83 ± 0.15
Arg250 + NAME	0.60 ± 0.17
MMS	$9.77 \pm 0.96^*$
Arg50 + MMS	8.93 ± 0.72
Arg100 + MMS	10.07 ± 0.55
Arg250 + MMS	$12.20 \pm 0.82^{**}$
Arg250 + NAME + MMS	$6.03 \pm 1.00^{***}$

* $p < 0.0001$; MMS group compared to control group, ** $p < 0.05$; Arg250 + MMS group compared to MMS, *** $p < 0.01$; Arg250 + NAME + MMS compared to Arg250 + MMS

^a NAME (N^G -nitro-L-arginine methyl ester), Arg (L-arginine), MMS (methyl methanesulfonate)

^b 1,000 BN cells were examined in each culture. The data represent average $\pm$ standard deviation of three human volunteers

Fig. 1 Typical apoptotic cells induced by treatment of human lymphocytes with methyl methanesulfonate at 72 h of incubation. Apoptotic cells were determined with fluorescence

microscope with propidium iodide staining for highlighting chromatin condensation and nuclear fragmentation

Fig. 2 DNA fragmentation in human lymphocytes induced by methyl methanesulfonate (MMS) with/without treatment by arginine (Arg) and/or N^G-nitro-L-arginine methyl ester (NAME). $p < 0.05$, Arg + NAME + MMS group as compared to Arg + MMS group

Lin et al. 2005). In this study, we have not observed any protective effects by arginine. Instead, the frequency of micronuclei increased in the cells treated with arginine plus MMS. This effect is not related to improvements of antioxidants levels. Arginine alone did not cause any genetic damage to human lymphocytes; for its genotoxicity, MMS is needed to be added. NO is a free radical that is produced from L-arginine by NO synthases (NOSs). NO can react with oxygen, superoxide anion ($O_2^{\bullet-}$) and reducing agents to give products. These products show selective chemical reactivity towards various cellular targets (Hughes 2008). S-nitrosoglutathione as an NO donor increased

toxicity on brain tumor cells induced by alkylating agents on brain tumor cells (Yang et al. 2007). Didier et al. (1999) showed that UVA irradiation produced NO in the presence of arginine which induced DNA damage in human keratinocyte cells. This genotoxicity was mitigated with treatment of L-thiocitrulline (L-Thio) an irreversible inhibitor of NOS. UVA-arginine stress decreased antioxidant enzymes such as glutathione peroxidase and catalase (Didier et al. 1999). Besides cytotoxic effects of NO, nitric oxide plays an important role in the protection against the onset and progression of cardiovascular diseases. The cardio-protective effect of NO is control of blood pressure

and vascular tone, inhibition of platelet aggregation and of leukocyte adhesion and prevention of smooth muscle cell proliferation (Tripathi et al. 2010; Habib and Ali 2011). This dual role of NO is dependent on its concentration. NO can protect cells at a low-level; however, at higher levels, it is a known cytotoxin, having been involved in tumor angiogenesis and progression (Paradise et al. 2010). With the importance of NO in the cellular toxicity, human lymphocytes were treated with NAME as an NOS inhibitor that inhibited NO production. NAME significantly reduced DNA damage induced by arginine in the presence of MMS treatment. On inhibition of NO by NAME, NO is participating in the increase of genotoxicity induced by arginine with MMS. In our results we have observed a dose-dependent effect of arginine on increased genotoxicity in human lymphocytes induced by MMS. It may be related to the enhancement of NO levels produced by arginine. However, for assessment of the apoptosis process it is necessary to perform cell cycle analysis and phase distribution. Acquaviva et al. (2009) showed that NO acted as a mediator of cytotoxicity in neuronal cells by exhausting GSH as a cellular defense (Aquilano et al. 2011). It has been reported the level of GSH in blood leukocytes is a useful biomarker for the detection of redox imbalance in inherited disorders affecting mitochondrial function (Atkuri et al. 2009).

As a conclusion, addition of L-arginine to methyl methanesulfonate as an alkylating agent caused an increased DNA damage in human whole blood cultures. This genotoxicity was reduced by NAME as an NO inhibitor. Increased DNA damage induced by arginine in the presence of MMS is related to NO production.

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Conflict of interest The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this study.

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