Aminoguanidine ameliorates radiation-induced oxidative lung damage in rats

Celalettin Eroglu MD1
Oguz Galip Yildiz MD1
Recep Saraymen PhD2
Serdar Soyuer MD1
Eser Kilic PhD 2
Servet Ozcan PhD3

Departments of Radiation Oncology,1 Biochemistry,2 Erciyes University, Faculty of Medicine, Departments of Biology,3 Erciyes University, Faculty of Arts and Sciences, Kayseri, Turkey

Manuscript submitted 27th February, 2008
Manuscript accepted 6th May, 2008


Abstract

Purpose: To investigate the possible protective effects of aminoguanidine (AG) on lung damage in whole body irradiated rats.

Methods: To evaluate the biological damage of radiation on rat lung tissue, lipid peroxidation products were measured using biochemical parameters. Thirty Wistar albino rats were divided into three subgroups: control (C), irradiation alone (RT), and RT + AG combined. After sacrificing the rats, antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities and malondiadehyde (MDA), nitric oxide (NO) levels were evaluated in lung tissue.

Results: Administration of AG resulted in an increase in the activities of CAT, SOD and GSHPx in the lungs. All were reduced after radiatio. In addition, AG administration resulted in a decrease in both NO and MDA levels in lung compared with the irradiated group.

Conclusion: Aminoguanidine increased the endogenous antioxidant defence mechanism in rats and protected the animals from radiation-induced lung toxicity. Moreover, AG may protect against ionizing radiation-induced lung damage because of its antioxidant effect.

Radiation therapy (RT) is a crucial component of application for many thoracic malignancies. Radiation-induced lung toxicity remains a major factor that limits the ability to escalate radiation doses in the application of thoracic tumours. RT-related pulmonary symptoms occur in up to 30% of patients irradiated for lung cancer, breast cancer, lymphoma, or thymoma. Pulmonary toxicity from thoracic irradiation is also an important problem in other settings, including combined modality treatment of Hodgkin’s disease and esophageal cancer, and bone marrow transplantation with total body irradiation.

Ionizing radiation is a well-established carcinogen due to the resulting oxidative damage, and the molecule most often reported to be damaged by this physical agent is DNA. Approximately 60–70% of cellular DNA damage produced by ionizing radiation is caused by OH, formed from the radiolysis of water. Besides DNA, lipids and proteins are also attacked by free radicals induced by ionizing radiation. Lipid peroxidation is believed to be an important cause of destruction and damage to cell membranes and has been suggested to be a contributing factor to the development of oxygen radicals-mediated tissue damage.

Oxidative stress occurs when there is excessive free radical production and/or low antioxidant defence, and results in chemical alterations of biomolecules causing structural and functional
The generation of the reactive oxygen metabolites (ROMs) plays an important role in the pathogenesis of radiation-induced tissue injury. Extensive studies implicate cellular DNA as the primary target for the biological and lethal effects of ionizing radiation. Besides DNA, lipids and proteins are also attacked by free radicals induced by ionizing radiation. Thus, drugs that scavenge or inhibit the formation of ROMs may have relevance to cancer patients by ameliorating damage of normal tissues exposed to ionizing irradiation therapy.

Aminoguanidine (AG) is an inhibitor of nitric oxide synthase (iNOS), with high selectivity for the inducible isoform (iNOS). Some studies have reported that nitric oxide (NO) is a mediator of radiation-induced acute tissue damage. In addition to being an inhibitor of NOS, AG also exhibits antioxidant activity. As an antioxidant, AG could ameliorate free radical–mediated lipid peroxidation and protein modifications by binding aldehydes and other reactive oxygen species.

AG, a compound structurally similar to L-arginine, inhibits iNOS in a selective and competitive manner, leading to decreased generation of NO. In addition, AG is endowed with many other activities that together account for its beneficial effects: AG inhibits diamine oxidase; binds to sites of nonenzymatic glycosylation; and prevents further advanced glycosylation.

The present study is the first to test the effect of AG on oxidative stress in lungs due to ionizing radiation. It was initiated to investigate mechanisms based on the possible protective effects of aminoguanidine against γ-irradiation-induced oxidative damage in lung tissue.

Materials and Methods

Animals

Thirty in-bred adult male Swiss Albino rats were obtained from the Test Animals Brooding Center at Erciyes University. Animals were fed with standard feed and water under sterile hygienic conditions. They were 12 weeks-old and weighed 250 +/-15 g. The light and dark cycle was automatically regulated at 12 hr.

Application categories

They were divided into three groups, each consisting of 10 animals. The control group (C), received only saline solution equal in volume to AG administration intraperitonealy (n= 10). The RT group received 800 cGy radiation to total body (n= 10) in one fraction, and the last group received both RT and AG. Aminoguanidine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in saline (0.9% NaCl w/v) and 200 mg/kg was administered by intraperitoneal daily injection. The third application category, received RT + AG using the same dose of radiation and AG as in the single application categories (n= 10). External total-body irradiation was given with a special 30 x 30 x 5 cm animal-fixing box using gamma rays from the Co60 teletherapy machine (Theratron 780 C) with parallel-opposed field at the mean dose rate 3.13 cGy/MU. Radiation for total body was 800 cGy. Animal fixing box contained five rats for each irradiation. The dose was calculated as Dmax dose at 2.5 cm depth for SSD 80 cm.

Analysis of lipid peroxidase level

After decapitation, the rat lungs were removed promptly, 72 hr after the last application under general anesthesia with intraperitoneally ketamine (50 mg/kg). The lungs were weighed and chilled in ice-cold 0.9 % NaCl. After washing with 0.9 % NaCl, tissue homogenates were prepared in 6 ml 1.15 % KCl buffer solution by using Edmund Buhler 7400 Tubingen HO4 homogenizer.

Since Kohn and Liversedge described the colorimetric reaction of thiobarbutiric acid (TBA) with an unknown substance formed during the aerobic incubation of tissue homogenates. This was later identified by Patton and Kurtz as MDA, a secondary product of...
lipid peroxidation, the reaction of lipid peroxides with TBA has been widely adopted as a sensitive assay method for lipid peroxidation in animal tissue. The reaction mixture contained 0.2 ml of sample, 0.2 ml of 8.1 % sodium dodecyl sulfate (SDS), 1.5 ml of 20 % acetic acid solution (pH:5.5) and 1.5 ml of 0.8 % aqueous solution of TBA. The mixture was made up to 4.0 ml with distilled water and heated at 95 °C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) were added and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbency of the layer (upper layer) was measured at 532 nm by spectrophotometer. The level of lipid peroxides was expressed in terms of nmol MDA/ g wet-tissue, which was calculated from the absorbance at 532 nm.

**NO Determination**

Since NO measurement is very difficult in biological specimens, tissue nitrite (NO₂⁻) and nitrate (NO₃⁻) levels were estimated as an index of NO production. The method for lung nitrite and nitrate levels was based on the Griess reaction. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite and nitrate) was measured after conversion of nitrate to nitrite by copperized cadmium granules by a spectrophotometer at 545 nm (Ultraspec Plus, Pharmacia LKB Biochrom Ltd, England). A standard curve was established with a set of serial dilutions (10⁻⁸–10⁻³ mol/L) of sodium nitrite. Linear regression was done by using the peak area from nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as nmol per gram tissue protein.

**Determination of CAT activity**

CAT activity was determined according to Aebi’s method. The principle of the method is based on the determination of H₂O₂ decomposition rate at 240 nm. The results were expressed as U/mg protein.

**Determination of SOD activity**

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. The method is based on inhibition of Nitro Blue Tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the lysate after 1.0 ml of ethanol–chloroform mixture (5: 3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as U mg/ protein.

**Determination of GSH-Px activity**

GSH-Px, (EC 1.6.4.2) activity was measured by the method of Paglia and Valentine. The enzymatic reaction in the tube containing NADPH, reduced glutathione (GSH), sodium azide and glutathione reductase was initiated by addition of H₂O₂, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per gram of protein (U/g protein).

**Determination of protein content**

Protein measurements were made at all stages according to Lowry’s method.

**Statistical analysis**

Data were analysed using a commercially available statistics software package (SPSS® for Windows v. 13.0, Chicago, USA). All groups showed normal distribution, so parametric statistical methods were used to analyse the data. A one-way ANOVA test was performed and post hoc Tukey HSD multiple comparisons, were made using least-squares differences. Comparisons with the control group were performed using the Dunnett test. Results are presented as mean ± standard deviation (SD) ; P< 0.05 was regarded as statistically significant.
Results
Catalase activity was found to be decreased in RT group in comparison with group C and it increased again in the RT + AG groups. CAT activity was different among groups, \( (P < 0.05) \). Activity of GSH-Px in C and RT + AG groups increased compared with RT group \( (P < 0.001) \). In addition, SOD activity in C and RT + AG groups increased compared with RT group \( (P < 0.002) \).

The level of MDA was higher in lung tissue homogenate of RT group than in both C and RT + AG groups. There was also a difference in MDA levels between C and RT + AG groups \( (P < 0.001) \). The level of NO was higher in the RT group than in the C group and RT + AG group. There was no difference between C and RT + AG group \( (P > 0.05) \).

Discussion
The present study demonstrates that whole-body irradiation in rats causes tissue damage in the lung as assessed by increased antioxidant enzyme activities, lipid peroxidation and NO levels.

Recent studies indicate that cells are endowed with cytosolic amplification mechanisms involving reactive oxygen and nitrogen species (ROS/RNS) and being responsive to low doses of ionizing radiation. A primary component of the ROS/RNS signal generated by radiation exposure is the \( \text{Ca}^{2+} \)-dependent activation of nitric oxide synthase and the formation of NO. Some studies have reported that NO is an important mediator of radiation-induced acute tissue damage.\(^{21}\) NO has been shown to have a cytotoxic function and the toxicity of NO is due to both NO itself and NO-derived reactive oxidants.\(^{22}\) Activated macrophages produce both NO and ONOO⁻ Under conditions where the superoxide anion \( (\text{O}_2^-) \) is generated, NO is rapidly consumed to produce the highly reactive ONOO⁻, a potent oxidizing agent known to initiate lipid peroxidation of biological membranes, hydroxylation, and nitration of aromatic amino acid residues and sulfhydryl oxidation of proteins.\(^{23}\)

Paredi et al.\(^ {24}\) reported that considerable focus has been on reactive nitrogen species (RNS), including NO, peroxynitrite \( (\text{ONOO}^-) \), and nitrogen dioxide in the respiratory tract. It has been suggested that neutrophils-and macrophage-derived RNS play an important role in cytotoxicity and in the pathogenesis of lung disease such as asthma, fibrosing alveolitis, and bronchiectasis.\(^ {25}\)

To control the flux of ROS, aerobic cells have developed their own defense system, the antioxidant system, which includes enzymatic and non-enzymatic components. The antioxidant system consists of low-molecular-weight antioxidant molecules, such as glutathione (GSH) and various antioxidant enzymes. Superoxide dismutase (SOD), the first line of defense against oxygen-derived free radicals, catalyzes the dismutation of superoxide anion into \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) can be transformed into \( \text{H}_2\text{O} \) and \( \text{O}_2 \) by catalase (CAT). Glutathione peroxidase (GSHPx) reduces lipidic or

### TABLE 1. CAT, SOD activities and MDA and nitric oxide levels in C and RT and RT + AG groups (mean ± SEM, n=10)

<table>
<thead>
<tr>
<th></th>
<th>CAT (U/mg protein)</th>
<th>GSH-Px (U/g protein)</th>
<th>SOD (U/mg protein)</th>
<th>MDA (nmol/mg protein)</th>
<th>NO (nmol/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Control</td>
<td>8.49 ± 0.51</td>
<td>80.00 ± 2.35</td>
<td>0.78 ± 0.02</td>
<td>2.43 ± 0.51</td>
<td>1.40 ± 0.63</td>
</tr>
<tr>
<td>II-RT*</td>
<td>7.26 ± 0.26</td>
<td>31.92 ± 2.16</td>
<td>0.41 ± 0.06</td>
<td>4.11 ± 0.59</td>
<td>2.56 ± 0.37</td>
</tr>
<tr>
<td>III-RT + AG**</td>
<td>9.81 ± 1.77</td>
<td>48.09 ± 2.09</td>
<td>0.87 ± 0.05</td>
<td>3.02 ± 0.32</td>
<td>1.57 ± 0.50</td>
</tr>
</tbody>
</table>

**P values**

<table>
<thead>
<tr>
<th></th>
<th>I-II</th>
<th>I-III</th>
<th>II-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>0.042</td>
<td>0.027</td>
<td>0.001</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>SOD</td>
<td>0.001</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>MDA</td>
<td>0.001</td>
<td>0.032</td>
<td>0.763 (NS)</td>
</tr>
<tr>
<td>NO</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Radiotherapy
** Aminoguanidine
nonlipidic hydroperoxides as well as H₂O₂ while oxidizing GSH.²⁶

On the other hand, the decrease in GSH content, SOD, and GSHPx activities, and the increase in MDA and total nitrate/nitrite (NO) levels in liver and lung tissues postirradiation as recorded in the present study are in agreement with those recorded by some authors.²⁷ They recorded a significant depletion in the antioxidant system accompanied by enhancement of lipid peroxides after whole body gamma-irradiation. Under normal conditions the inherent defense system including glutathione and antioxidant enzymes protect against oxidative damage.

We observed that AG pre-application lowered radiation-induced lipid peroxidation in terms of MDA. Inhibition of lipid peroxidation in biomembranes can be caused by antioxidants. In addition, whole body gamma-irradiation of rats at 8 Gy enhanced the formation of NO. Similar results have been reported by Gorbunov et al.²⁸ Gamma-irradiation may enhance endogenous NO biosynthesis in liver, intestine, lung, kidney, brain, spleen or heart of the animals, presumably by facilitating the entry of Ca²⁺ ions into the membrane as well as the cytosol of NO-producing cells though irradiation-induced membrane lesions.²²

Nitric oxide is a volatile diatomic free radical that plays physiological roles in normal³⁰ as well as tumour tissues.³¹ Soloviev et al.³² postulated that the loss of endothelial integrity and related function in post-irradiated period is one of the most common effects of ionized irradiation. Hopkins et al.³³ reported that inflammation in the lung can lead to increased expression of inducible nitric oxide synthase (iNOS) and enhanced NO production. The resultant highly reactive NO metabolites may have an important role in host defense, although they might also contribute to tissue damage. Ibuki and Goto³⁴ suggested that whole body irradiation with low dose gamma rays (4 Gy) activates macrophages indirectly and consequently enhances NO production from macrophages, by which the self-defense systems such as the tumoricidal activity will be augmented. The enhancement of NO production following exposure to a high dose (6 Gy) of gamma rays was attributed to high levels of expression of the iNOS.³⁴ They suggested that DNA strand breaks caused by hydroxyl radicals formed inside the cells by gamma-irradiation, or strand breaks caused by radiation, plays an important role in the enhancement of NO production, but peroxidation of cell membranes has little effect.

AG has several biological activities that together account for its beneficial effects. AG inhibits diamine oxidase¹³, which catalyzes degradation of biologically active diamines such as histamine and putrescine. AG inhibits iNOS in a selective manner, leading decreased generation of NO.²⁷ Previous studies pointed out the beneficial antioxidant effects of AG and scavenger effects of ONOO⁻ which is a reactive oxidant produced from NO and O²⁻ in various forms of tissue injury.³⁵ Yildiz et al.³⁶ also found that AG had direct scavenging activities against HO. Recently, Giardino et al.³⁷ reported that AG acts as an antioxidant in vivo, preventing ROS formation and lipid peroxidation in cells and tissues, thus preventing oxidant-induced apoptosis. In addition, AG may inhibit lipid peroxidation by inducing GSH-Px and SOD, or scavenging and inactivating H₂O₂ and HO.³⁸ More recently, it was shown that AG improved GSH content and inhibited lipid peroxidation after single-dose AK-induced renal injury,³⁹ and this is consistent with the above-mentioned reports.

The protective effects of AG have been previously addressed in other models of cell damage induced by drugs.⁴⁰ The beneficial effects of AG in various experimental models of inflammation have also been reported.⁴¹ Recently, Mansour et al.³⁶ reported that AG protects against nephrotoxicity induced by cisplatin (CDDP) in rats. The beneficial antioxidant effects of AG and the scavenger effects of ONOO⁻, which is a reactive oxidant produced from NO and superoxide (O²⁻), have been observed in various forms of tissue injury.

AG was prepared more than 100 years ago. During the last 10 years, two important effects have been
discovered which have attracted a lot of interest. First, AG inhibits, in vitro and in vivo, the formation of highly reactive advanced glycosylation end products (AGEs) associated with the pathogenesis of secondary complications in diabetes and with cardiovascular changes in aging. AG ameliorates various complications in diabetes and prevents age-related arterial stiffening and cardiac hypertrophy, effects probably dependent on the inhibition of AGEs formation. Second, AG inhibits NO synthase, particularly the inducible NO synthase isoform, making it an important pharmacological tool. Inducible NO synthase isoform is associated with the production of large quantities of NO synthase in response to, for example, cytokines. Yildiz et al. also found that AG has direct scavenging activities against hydroxyl radicals. Recently, Giardino et al. reported that AG acted as an antioxidant in vivo, preventing ROS formation and lipid peroxidation in cells and tissues, preventing oxidant-induced apoptosis. It has been proposed that antioxidants maintain the concentration of reduced GSH and may restore the cellular defense mechanisms and block lipid peroxidation, thus protecting against the toxicity for wide variety of nephrotoxic chemicals.

In conclusion, GSH-Px, CAT and SOD protect cells against ROS. We found decreased activity of the key antioxidants, GSH-Px, CAT and SOD, in the lung of rats treated with RT. This indicates that the increase in MDA in the lung of rats treated with RT may be related to the decrease in the activity of SOD, CAT and GSH-Px, which scavenge hydroperoxides and lipid peroxides. Our data indicate that AG could increase the endogenous antioxidant defense mechanism in rats and protect the animals from radiation-induced lung toxicity. Moreover, AG may protect against ionizing radiation-induced lung damage, because of its antioxidant effect.

References

© 2008 CIM

Correspondence to:
Oguz Galip Yildiz MD
Departments of Radiation Oncology
Erciyes University, Faculty of Medicine, 38039 Kayseri/TURKEY
e-mail: yildizog2003@yahoo.com

© 2008 CIM
Clin Invest Med • Vol 31, no 4, April 2008 E188