Antioxidants Modify the Effect of X Rays on Blood Vessels

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Abstract. *Background: It was recently shown, with the chicken embryo chorioallantoic membrane (CAM) model, that X rays decrease the number of blood vessels within the first hours after irradiation. In the present study, the possible role of reactive oxygen species (ROS) and nitric oxide (NO) in this effect of X rays was evaluated. Materials and Methods: An area of 1 cm^2 of the CAM, restricted by a plastic ring, was irradiated at room temperature, in the presence or absence of the tested agents. The number of vessels was measured 48 h after irradiation of the tissue. Results: Superoxide dismutase and tempol, which are superoxide ion scavengers and catalase, a hydrogen peroxide scavenger, had additive effects, while dimethylsulfoxide, a hydroxyl radical scavenger, reversed the vascular targeting effect of X rays. The combination of X rays with W1400, a selective inducible NO synthase (NOS) inhibitor, had an additive effect on the decrease in number of CAM blood vessels. In contrast, L-NAME, a non-selective NOS inhibitor and D-NAME, its inactive analog, reversed the vascular-targeting effect of X rays, possibly due to their ability to act as potent hydroxyl radical scavengers. Conclusion: The above data collectively suggest that hydroxyl radicals mediate the damaging effects of X rays on CAM blood vessels, while antioxidants against other ROS do not protect against the vascular-targeting effect of X rays.*

Ionizing radiation is thought to damage the endothelium (1-3) and thus decrease the number of blood vessels (4). Although the exact mechanisms of action of ionizing radiation on tissues remain unclear, it is well known that X rays cause the production of reactive oxygen species (ROS), leading to the manifestation of tissue oxidative stress that involves cytotoxic effects (5). Major ROS are superoxide ion, hydroxyl radical and hydrogen peroxide.

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Nitric oxide (NO) is a short-lived free radical with extremely high reactivity and a variety of physiological activities. NO production from L-arginine is catalyzed by three isoforms of NO synthase (NOS), neuronal, inducible (iNOS) and endothelial (eNOS). NO seems to be pro-angiogenic both *in vivo* and *in vitro* (6-8), and to suppress apoptotic pathways at multiple levels and by several mechanisms (9). In endothelial cells, the levels of NO generated by either eNOS (10) or iNOS (11) effectively suppress the apoptosis induced by exogenous agents.

It has previously been shown, with the chicken embryo chorioallantoic membrane (CAM) model, that X rays decrease the number of CAM blood vessels, most probably due to vascular targeting within the first hours after irradiation (4, 12). In the present work, in order to evaluate the possible role of different ROS or NO in this effect of X rays, the effects of several antioxidants or NOS inhibitors were studied in combination with irradiation in the *in vivo* CAM model.

Materials and Methods

The tested agents included: superoxide dismutase (SOD) and its membrane permeable analog, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol), which remove superoxide ions (Sigma, Greece); catalase, which detoxifies hydrogen peroxide to water (Sigma); dimethylsulfoxide (DMSO), a scavenger of hydroxyl radicals (Sigma); dexamethasone, a glucocorticoid receptor agonist which inhibits iNOS mRNA transcription (Sigma); N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of NOS activity and its inactive (on NOS) analog D-NAME (Sigma); N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydro-chloride (1400W), a selective inhibitor of iNOS activity (Tocris Cookson Ltd).

CAM assay. The *in vivo* CAM model was used as previously described (8, 12). Leghorn fertilized eggs (Pindos, Greece) were incubated for 4 days at 37˚C, when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator until the ninth day of development. Substances were then applied on an area of 1 cm^2 of the CAM, restricted by a plastic ring. In a series of experiments, the area restricted by the ring was irradiated at room temperature, using an RT 50 mobile contact therapy unit (Philips, Amsterdam,

The Netherlands), with 20 KV X-rays (0.1 mm Al). The SSD was 4 cm and the dose rate 2.600 cGy per min. A single X-ray dose of 10 Gy was used, because the decrease in CAM blood vessels was maximal at this dose without affecting the viability of the embryos (4, 12). When combination experiments were performed, the substances were applied 15 min prior to irradiation.

In order to evaluate the effect of each substance in combination with irradiation on the number of blood vessels, 48 h after treatment and subsequent incubation at 37˚C, the CAMs were fixed *in situ*, excised from the eggs, placed on slides and left to air-dry. Pictures were taken through a stereoscope equipped with a digital camera and the total length of the vessels was measured, as previously described (8). The assays for each test sample were carried out three times and each experiment included at least ten eggs per data point.

Western blot analysis of iNOS. In CAM paraffin sections, iNOS is detected only on blood cells (13). Therefore, in order to increase the sensitivity of the assay for the measurement of iNOS protein levels, Western blot analysis of iNOS was performed on CAM blood cell lysates, as previously described (8). In brief, blood cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.25% SDS, 150 mM NaCl, 1 mM EDTA, 1mM PMSF, 1 μg/ml aprotinin, 1 mM Na_3VO_4) with agitation for 4 h at 4˚C. The lysates were then centrifuged at 20,000 xg for 30 min at 4˚C. The total protein concentration was determined in the supernatants, using the Bradford method (14). Equal amounts of total protein were loaded on 7.5% SDS-PAGE mini gels, analyzed and transferred to Immobilon P membranes (Millipore). Blocking was performed by incubating the PVDF membranes with 5% (w/v) non-fat dry milk in Tris buffered saline (TBS) pH 7.4, for 1 h at room temperature under continuous agitation. The membranes were then incubated with a polyclonal anti-iNOS antibody at a dilution of 1:1,000 (Upstate Biotechnologies, NY, USA) in 3% (w/v) non-fat dry milk in TBS containing 0.05% Tween-20 (TBS-T), overnight at 4˚C under continuous agitation, and then with horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma, Athens, Greece) diluted 1:2,500 in 3% (w/v) non-fat dry milk in TBS-T, for 1.5 h at room temperature under continuous agitation. The detection of immunoreactive bands was performed by Super Signal West Pico chemiluminescence substrate (Pierce, USA), according to the manufacturer's instructions. The protein levels that corresponded to the immunoreactive bands were quantified using the image PC image analysis software (Scion Corporation, Frederick, MD, USA). Normalization was performed against total protein amounts.

Measurement of nitrite levels. The CAMs of at least five eggs were dissected, cut into small pieces and washed five times with sterilized phosphate-buffered saline (PBS) pH 7.4. They were then placed in 24-well plates (each well contained approximately 1 mg protein) in Ham's F10 medium that did not contain phenol red. 1400W was added at the indicated concentrations and the CAMs were incubated for 24 h at 37˚C and 5% CO_2. After completion of the incubation, the samples were collected and centrifuged at 7,000 xg for 2 min in a microcentrifuge. Nitrites were measured in the supernatant with the use of the Griess reagent, as previously described (8). Total protein amounts were measured in the tissue pellets using the Bradford assay. The results are expressed as nmoles NO_2^-/mg total protein.

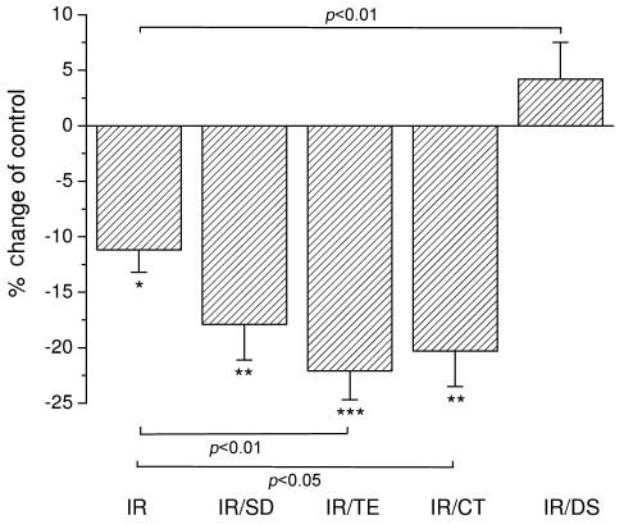

Figure 1. *Effect of ROS scavengers in combination with X rays (10 Gy) on the number of CAM blood vessels. The tested agents in the same final volume of 20 μl were applied on a CAM area of 1 cm^2 restricted by a plastic ring on day 9, 15 min before irradiation of the tissue, as described in Materials and Methods. After 48 h of incubation at 37˚C, the CAMs were fixed, excised from the eggs, photographed and the total length of the vessel network was measured using image analysis software. The results are expressed as mean ± S.E.M. of the % change of the number of vessels in treated compared with untreated, non-irradiated tissue (control). IR, irradiation; SD, SOD 300 u/cm^2; TE, tempol 580 nmol/cm^2; CT, catalase 100 u/cm^2; DS, DMSO 25 nmol/cm^2. Asterisks denote a statistically significant difference from the control. *p<0.05, **p<0.01, ***p<0.001.*

Statistical analysis. The significance of variability between the results from each group and the corresponding control was determined by unpaired *t*-test or ANOVA. Each experiment included at least triplicate measurements for each condition tested and all the results are expressed as mean±S.E.M. from at least three independent experiments.

Results

Effect of ROS scavengers in combination with X rays on the number of blood vessels in vivo. In order to evaluate the possible involvement of ROS in the effect of X rays on the number of CAM blood vessels, the influence of different antioxidants were studied in combination with irradiation. As shown in Figure 1, the combination of SOD, tempol and catalase with X rays caused a greater decrease in the number of CAM blood vessels, compared with the effect of X rays alone. In contrast, DMSO completely reversed the effect of X rays.

Effect of X rays on iNOS protein levels. The effect of X rays, on iNOS protein levels was also investigated. As shown in Figure 2, the iNOS protein levels decreased 6 and 24 h after irradiation. This effect was reversed at later time-points, similarly to the pattern observed at the mRNA level (15).

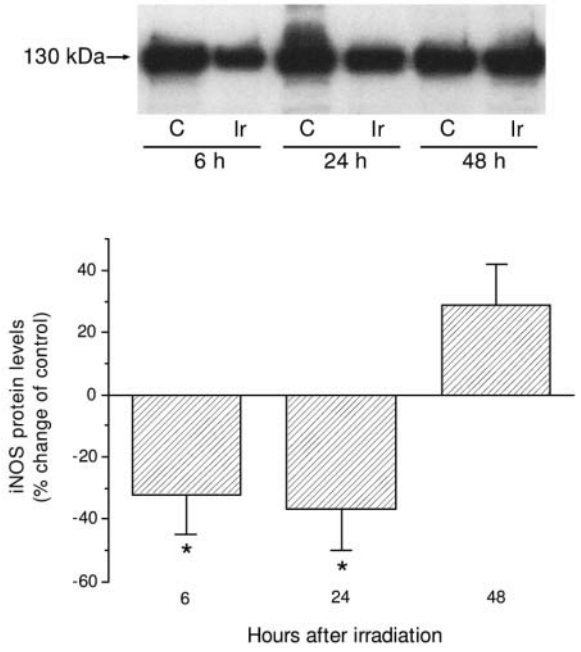

Figure 2. *Effect of X rays (10 Gy) on iNOS protein levels. Western blot analysis for iNOS 6, 24 and 48 h after irradiation of the chicken embryo CAM. Representative picture of five independent experiments. The protein amounts that corresponded to the iNOS immunoreactive band were quantified using image analysis software. The results are expressed as mean ± S.E.M. of the % change of iNOS protein amounts in irradiated compared to non-irradiated tissue at each time-point (control). C, control, Ir, irradiated CAM. Asterisks denote a statistically significant difference from the control. *p<0.05.*

Effect of NOS inhibitors in combination with X rays on the number of blood vessels in vivo. The combination of dexamethasone, which inhibits iNOS mRNA transcription and activity in the CAM (8), with X rays, caused a greater decrease in the number of CAM blood vessels compared with the effect of X rays alone (Figure 3). In contrast to the effect of dexamethasone, the combination of the non-selective NOS inhibitor L-NAME with X rays completely reversed the effect of the latter (Figure 3). D-NAME produced results similar to those observed for L-NAME (Figure 3), although it had no effect on CAM iNOS activity (8). In order to explain this, 1400W, a selective iNOS inhibitor, was employed. 1400W decreased both iNOS activity and the number of CAM blood vessels in a dose-dependent manner (Figure 4). This effect was not due to toxicity, as verified on CAM paraffin sections stained with eosin-hematoxylin (data not shown). The combination of 1400W with X rays caused a greater decrease in the number of CAM blood vessels, compared with the effect of X rays alone (Figure 3).

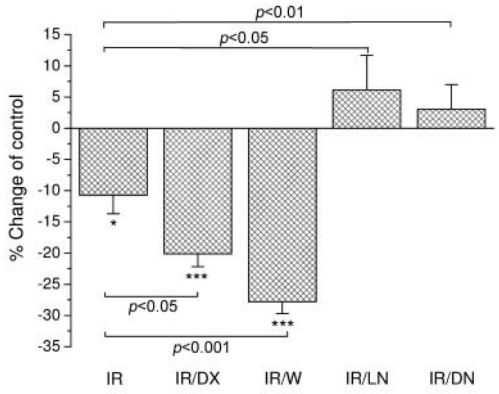

Figure 3. *Effect of NOS inhibitors in combination with X rays (10 Gy) on the number of CAM blood vessels. The tested agents in the same final volume of 20 µl were applied on a CAM area of 1 cm^2, restricted by a plastic ring on day 9, 15 min before irradiation of the tissue, as described in Materials and Methods. After 48 h of incubation at 37°C, the CAMs were fixed, excised from the eggs, photographed and the total length of the vessel network was measured using image analysis software. The results are expressed as mean ± S.E.M. of the % change of the number of vessels in treated compared with untreated, non-irradiated tissue (control). IR, irradiation; LN, L-NAME 2 µmol/cm^2; DN, D-NAME 2 µmol/cm^2; DX, dexamethasone 80 nmol/cm^2; W, 1400W 200 nmol/cm^2. Asterisks denote a statistically significant difference from the control. *p<0.05, ***p<0.01.*

Discussion

We have previously shown that X rays initially decrease the number of CAM blood vessels due to increased apoptosis within the first hours after irradiation (4, 12). In the present study, it was shown that this effect of X rays seems to be due to the production of hydroxyl radicals, since it was reversed by the hydroxyl radical scavenger DMSO. This notion is in line with our previous results, showing that the effect of X rays on CAM blood vessels was reversed by amifostine (16), a well established hydroxyl radical scavenger (17) and is in agreement with data suggesting that the production of hydroxyl radicals by X rays correlated with radiation-induced apoptosis (18).

Hydroxyl radicals have also been reported to inhibit NOS activity (19) and may further be responsible for the X ray-induced decreases in iNOS expression and activity. The subsequent increase in iNOS expression (this study and 15) may be one of the mechanisms that the tissue utilizes in order to achieve cellular free radical homeostasis (19) and restore normal function.

Inducible NOS is the only NOS isoform detected to date in the CAM (8, 20), and seems to play a significant role in the formation and/or stability of blood vessels under physiological angiogenesis of the tissue (8). The data from this study support this notion, since the specific iNOS inhibitor, 1400W, also inhibited angiogenesis to a similar

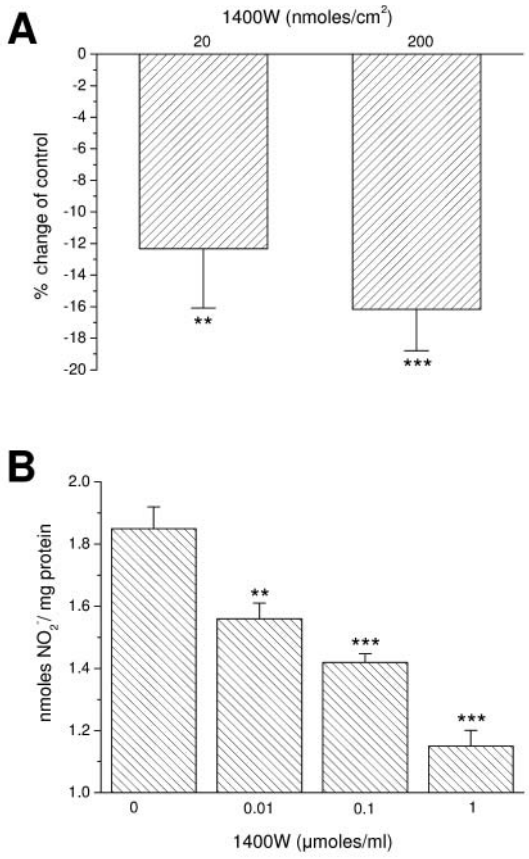

Figure 4. *(A) Effect of 1400W on CAM angiogenesis. Various amounts of 1400W in the same final volume of 20 µl were applied on a CAM area of 1 cm² restricted by a plastic ring on day 9, as described in Materials and Methods. The total length of the vessel network was measured after 48 h of incubation at 37˚C. The results are expressed as mean ± S.E.M. of the % change of the number of vessels in treated compared to untreated tissue. (B) Effect of 1400W on nitrite production. Nitrites were measured 24 h after application of 1400W to the chicken embryo CAM, as described in Materials and Methods. The results are expressed as nmoles NO_2^-/mg of total protein ± S.E.M. of three independent experiments performed in quadruplicate. Asterisks denote a statistically significant difference from the untreated tissue. **p<0.01, ***p<0.001.*

degree with L-NAME or dexamethasone (8). The combination of iNOS inhibitors with X rays further decreased the number of CAM blood vessels, although their mechanism of action seems to be different. Inducible NOS inhibitors inhibited CAM angiogenesis without being toxic or inducing apoptosis (this study and 8), while the effect of X rays is probably due to vascular targeting rather than to inhibition of angiogenesis (4, 12, 15).

Scavengers for superoxide or hydrogen peroxide decrease CAM angiogenesis through, at least partly, the down-regulation of iNOS expression and activity (8). In accordance with the discussion above on the role of iNOS is blood vessel formation and stability, as well as the effect of iNOS inhibitors in combination with X rays on the number of CAM blood vessels, the down-regulation of iNOS by superoxide or hydrogen peroxide scavengers could also explain their synergistic effect on the decrease in CAM blood vessels when administered in combination with irradiation.

In contrast to the effect of dexamethasone or 1400W on the X ray-induced decrease in the number of CAM blood vessels, L-NAME completely reversed the effect of irradiation. This effect does not seem to be due to NOS inhibition, since the inactive analog D-NAME had a similar effect at the same concentration, although it did not affect CAM angiogenesis or NOS activity (8). The effect of L-NAME and D-NAME on the X ray-induced decrease in the number of CAM blood vessels is probably due to the ability of both agents to act as potent hydroxyl radical scavengers (21).

In conclusion, the results of the present study suggest that hydroxyl radicals mediate the early effects of X rays on CAM blood vessels *in vivo*.

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