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SALINE CONTAINING PHOSPHATIDYLCHOLINE LIPOSOMES
POSESS THE ABILITY TO RESTORE ENDOTHELIAL FUNCTION
DAMAGED RESULTING FROM G-IRRADIATION

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The protective action of passive saline filled ("empty") phosphatidylcholine liposomes (PCL) on endothelial function was examined in thoracic aortas obtained from gamma irradiated (6 Gy) Chinchilla rabbits, and then verified in experiments on non-anesthetized and anesthetized rats. Acetylcholine (ACh)-induced vascular relaxant responses in isolated vascular tissues rats were used as the test of endothelial integrity and its functional ability. It was shown that when added to the bath solution (100 µg/ml), PCL effectively restored endothelium-dependent ACh relaxations of isolated vascular rings damaged resulting from g-irradiation but had no effect on endothelium-independent vascular responses to therapeutic nitric oxide (NO) donors. The liposomes were also without protective effect when injected to the rabbits intraperitoneally (30 mg/kg) 1 hour before irradiation. In contrast, PCL, being injected at the same dose 1 hour after radiation impact, promote normalization of both endothelium-dependent vascular responses to ACh and nitric oxide (NO) donors. PCL restored also the sensitivity of vascular tissues to authentic NO (aqueous NO solution) that was surprisingly increased after irradiation, and normalized relationship between ACh-stimulated NO release and relaxant response amplitudes in irradiated aortas. Experiments on non-anesthetized and anesthetized rats demonstrated that irradiation led to significant elevation in the level of arterial blood pressure without any changes in cardiac contractility. PCL administration (25 mg/kg, i.v.) effectively normalized an increased arterial blood pressure in irradiated animals. In conclusion, it appears that PCL due to its ability to normalize NO-dependent vascular tone control mechanisms might be worthwhile therapeutic approach in case of ionizing irradiation accident. These result support the concept that the depression of endothelium-dependent vascular responses after irradiation may be result of decreased NO bioavailability due to its conversion to less potent vasodilators during irradiation-induced oxidative attack.
INTRODUCTION

Chernobyl catastrophe created a lot of problems for Ukrainian population, adjoined territory of Russia and Republic of Belarus. At the same time it presented us with a very important health information. The first surprising discovery was the fact that the most common health problem in Ukrainian population exposed to radiation was diseases of cardiovascular system, and hypertension especially (1,2). Although hypertension is common in normal population, it seen to have an ever higher incidence in survivors of the tragedy (3). It is important to note that the same phenomenon was also found in survivors of the Nagasaki and Hiroshima atomic bombs (4).

These data allow suggest that blood vessels may represent one of the main targets for ionized irradiation. A number of data demonstrate that endothelium is one of the most vulnerable and radiosensitive components of vascular wall (5,6,7,8,9).

Some years ago we have demonstrated that phosphatidylcholine liposomes (PCL) possess the ability to restore endothelium-dependent vascular responses in spontaneously hypertensive rats (10). We speculated that this effect of PCL depends on the synthesis of NO by endothelial cells. However, the precise cellular mechanisms of these restoring phenomena are not yet established till this time. Despite of the absence of such data, we nevertheless decided to test PCL as a possible restoring agent in endothelial dysfunction following ionized irradiation.

This study was designed to confirm the principal role of endothelium in vascular dysfunction and hypertension development in γ-irradiated experimental animals, and shed light on the cellular mechanisms underlying the generalized vasospasm development. The main goals are: i) to elucidate the cellular mechanisms responsible for radiation-induced endothelial dysfunction and hypertension; ii) to identify pharmacological interventions that can normalize vascular tone increased by ionizing radiation.

MATERIALS AND METHODS

Materials

1,10-phenanthroline was from Serva (Heidelberg, Germany), 3-morpholino-sydnonimine, luminol sodium salt, phenylephrine and acetylcholine chloride were obtained from Sigma (St. Louis, USA), glyceryl trinitrate was from Bio-Therabel (Brussels, Belgium).
Liposomes preparation.

Multilamellar vesicles were prepared from egg phosphatidylcholine according to the thin film method. The lipid film was hydrated under mechanical stirring with sterile saline for 1 hour to final concentration of 50 mg/ml. Samples of liposome were subjected to ultrasound treatment for 5 min using a probe bath sonicator (Fisher sonic dismembrator, Model 300, Artek systems Co., USA). The resultant suspensions were sterilised by sequential extrusion under pressure through Durapore filters under pressure (0.45 µm, then 0.2 µm pore diameter) and kept at -20°C until used.

Animals irradiation and tissues preparation

The main part of the study was performed on Chinchilla rabbits weighing 3.0-3.5 kg and divided into four groups in 5 animals in each. Group I was a control group. Group II received a whole body irradiation in dose of 6 Gy. Group III underwent to 6 Gy irradiation combined with PCL injected intraperitoneally (30 mg/kg) 1 hour before irradiation impact. Group IV - experienced by 6 Gy dose irradiation that also accompanied with PCL treatment at the same dose but PCL were administered 1 hour after irradiation. In a special group of experiments the PCL were added directly to the bath solution (100 µg/ml). All experiments presented in this part of the study were performed on the 9th day of the post-irradiated period.

Whole body irradiation was performed with gamma rays delivered at a rate of 0.80-0.84 Gy/min from a cobalt" source (TGT ROCUS M, Russia). During irradiation rabbits were restrained in a plastic box specifically designed for this study and radiation beam was focused to animals chest. There was no change in housing, standard food or drink following irradiation. All animals survived throughout the experimental period (up to 180 days).

Systolic arterial blood pressure was measured in non-anaesthetized rats using cuff tail sphygmomanometer S-2 (Hugo Sachs Electronik, Germany).

Contractile recording experiments were performed on the ring preparations of thoracic aortas that were obtained from healthy and irradiated rabbits. The rings of aorta were prepared with special care in order to keep the endothelium intact.

Cardiovascular responses to irradiation and the effects of intravenous injection of PCL on cardio-hemodynamic were studied using anaesthetized rats. All in vitro experiments and experiments on anaesthetized rats were performed on the 9th day of postirradiated period.

Contractile force recordings

Aortic rings were mounted isometrically in a tissue bath between a stationary stainless steel hook, and an isometric force transducer (AE 801, SensoNor A/S, Norten, Norway) was coupled to a chart recorder (model 202, Cole-Parmer Instrument Company, USA). The rings were equilibrated for 1 hour with a resting tension of 30 mN. Experiments were made at 37°C in modified Krebs bicarbonate buffer solution of the following composition (in mM): NaCl, 133; KCl, 4.7; NaHCO₃, 16.3; NaH₂PO₄, 1.38; CaCl₂, 2.5; MgCl₂, 1.2; glucose, 7.8; pH, 7.4.

Chemiluminescence detection of nitric oxide

The detection was based upon chemiluminescence reaction between NO and the luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) - H₂O₂ system as described (8) with some modifications to prevent buffer solution components and agonists interfering with the test compounds. The reaction solution contained 0.2 mM luminol, 250 µM 1,10-phenanthroline, 50 mM H₂O₂ and 4 mM potassium carbonate. For the chemiluminescence assay the tissue bath effluent (2 ml/min, flow rate)
was mixed with a luminol-containing solution (0.8 ml/min) in a special reactor cell connected to a flow cell-type chemiluminescence detector with a photomultiplier tube (model R 2693, Hamamatsu). The limit of NO determination was approximately $5 \times 10^{-11}$ M (linear range of $10^{-10}$ M - $3 \times 10^{-9}$ M). NO concentrations were detected within 5 seconds after the superfusate exit from the tissue bath.

Standard aqueous NO solution (authentic NO) was prepared according to the following procedure. Deoxygenated water was prepared by flushing O$_2$-free argon for 3 h through a sealed bottle containing HPLS-grade H$_2$O. Then NO gas was injected into the deoxygenated water. The saturated solution of NO (1.91 mM at 20°C) was obtained by adding 5 ml NO gas to 20 ml deoxygenated water.

**Experiments on anesthetized rats**

The experiments were performed in adult Wistar-Kyoto rats weighing from 200 to 400 g and anesthetized with intraperitoneal chloralose (4 mg per 100 g body weigh) plus urethane (1:10). Intravenous injection of heparin (50 ED/100 g body weigh) was made. The rats were undergone tracheotomy for intermittent mandatory ventilation. Abdominal aorta was cannulated through a femoral artery and used for blood pressure measurement (pressure transducer ISOTEC coupled to DBA amplifier (Hugo Sachs Elektronik, Germany). Micro-tip catheter pressure transducer SPR-249 (Millar Instruments, Inc., USA) was inserted into left ventricle using retrograde method via a. carotis communis dextra to measure systolic and end-diastolic pressures in the left ventricle and to calculate LV dp/dt. Computer program HAEMODYN (Hugo Sachs Elektronik, Germany) has been utilized for data collection, storing and calculations.

**Restricted clinical observations**

Clinical observations of arterial blood pressure were made on two groups of human volunteers aged 42-50 years: 1) survivors of Chernobyl accident (so-called liquidators), and 2) control group of healthy men from non-contaminated areas of Ukraine.

**Statistics**

The data are shown as means±SEM; n indicates the number of vascular tissue preparations (or anesthetized animals as well human volunteers) tested. Concentration-response curves were assessed by one way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. EC$_{50}$ values and maximal relaxation (curves were fitted to a logistic equation) were compared by the Student's $t$-test for unpaired data. In experiments measuring NO release curves were fitted to a linear equation. Differences were considered to be statistically significant when $p$ was less than .05.

All procedures were carried out according to EU directives and reviewed by local ethical committee.

**RESULTS**

A non-invasive measurements of systolic blood pressure using a cuff tail sphygmomanometer S-2 in irradiated (6 Gy) rats showed that on the 9$^{th}$ day of post-irradiated period blood pressure significantly increased from 125±9 to 185±10 mm Hg (P<0.05, n=14). Although repeated measurements 6 months later
demonstrated a decrease in blood pressure to 165±12 mm Hg, it was significantly higher (P<0.05, n=14) than in control group (132±8 mm Hg, Fig. 1).

Clinical observations on human volunteers clearly demonstrated that the mean of arterial blood pressure in the group of survivors of Chernobyl accident was significantly higher as compared to control group of patients from non-contaminated areas, 132±6 and 156±8 mm Hg, respectively (P<0.05, n=24).

Taking together these data confirm the previous studies that postulated appearance of radiation-induced hypertension (3), and arise the question of the search of pharmacological interventions for the treatment of these vascular abnormalities.

Fig. 2 shows the effect of PCL intraperitoneally injected (30 mg/kg) to the rabbits on relaxant responses of thoracic aortas to ACh and therapeutic NO donors. It is clear that PCL almost completely restored ACh-induced relaxation and significantly improved relaxant responses to NO donors when added 1 h after irradiation. But they had no a similar effects being administered 1 hour before irradiation.

When added directly to the bath solution, PCL (100 µg/ml) restored damaged endothelium-dependent responses to ACh but was without effect on endothelium-independent vascular responses to NO-donors (Fig. 2). To make the choice of PCL dose we used our previous investigations on vascular smooth muscle from

![Figure 1](image-url)
spontaneously hypertensive rats in which its restoring effect was plateaued in a range of 40-100 µg/ml (10).

When administered to the rabbit 1 hour after irradiation impact, PCL normalized also the sensitivity of thoracic aorta to authentic NO (aqueous NO solution) that was significantly increased after irradiation (Fig. 3). The $EC_{50}$s for healthy and irradiated vascular tissue for NO in vascular tissue before PCL administration were $1.5\pm0.8\times10^{-6}$ M and $2.8\pm0.8\times10^{-7}$ M, respectively ($p<0.05$, $n=14$). It is important to note that the amplitude of maximal ACh relaxation ($R_{\text{max}}$) to NO was without significant difference in both kinds of tissue ($R_{\text{max}}$ were $77.8\pm10.0\%$ and $87.1\pm3.9\%$, respectively, $p>0.05$, $n=14$). Under PCL action $EC_{50}$ for irradiated tissue had increased and became very close to normal value - $1.3\pm0.6\times10^{-6}$ M ($p>0.05$); $R_{\text{max}}$ $77.6\pm6.7\%$ ($p>0.05$, $n=12$).

Detection of ACh-stimulated NO release by chemiluminescence assay showed no significant difference between NO released by 1 mg aortic tissues per minute under ACh treatment before and after radiation impact: $2.7\pm0.9\times10^{-11}$ M and $3.4\pm0.8\times10^{-11}$ M, respectively ($P>0.05$, $n=11$).

![Figure 2. Effects of phosphatidylcholine liposomes on relaxant responses of isolated aortic rings obtained from healthy and irradiated (6 Gy; 9 days after irradiation) rabbits to acetylcholine (1 µM) and NO-donors (GTN and SIN-1, both 10 µM). Liposomes were administered intraperitoneally (30 mg/kg) 1 hour before or after irradiation. Additionally, in a special series of experiments liposomes were added directly to the buffer solution (100 µg/ml) 15-30 minutes before acetylcholine action. The tissues were pre-constricted with phenylephrine (10 µM). Relaxations are expressed as percent decrease in tension evoked by phenylephrine. Data shown as mean±S.E.M. from 12 experiments. The asterisks indicate a statistically significant difference between parameters ($P<0.05$).]
Radiation reversed also the relationship between the ACh-stimulated increment of NO concentration in buffer solution and the corresponding relaxant responses in thoracic aortas. Contrary to a positive correlation between these parameters in healthy animals, a negative unusual correlation in irradiated tissues ensues. Intraperitoneally injected PCL almost completely normalized above-mentioned relationship making it very close to the control (Fig. 4).

Experiments on anesthetized rats have confirmed that ionized irradiation in dose 6 Gy led to significant increase of blood pressure in left ventricle and was without effect on cardiac contractility (cardiac index). Cardiac output that reflects cardiac pump function appears to be increased due to an increased arterial blood pressure. When injected intravenously in dose 30 mg/kg 1 h after irradiation, PCL

Figure 3. Effects of phosphatidylcholine liposomes treatment on sensitivity of isolated thoracic aortas obtained from healthy and irradiated (6 Gy) rabbits to authentic NO. Open circles - control, closed circles - 6 Gy irradiation, triangles - 6 Gy irradiation + PCL (liposomes were administered intraperitoneally (30 mg/kg) 1 hour after irradiation.

Figure 4. Relationship between apparent ACh-stimulated NO release and corresponding relaxations in different samples of thoracic aorta from healthy (open circles) and irradiated rabbits before (closed circles) and after PCL administration (triangles). PCL were administered in dose of 30 mg/kg i.p., 1 h after irradiation.
significantly decreased blood pressure in the left ventricle and had no effect on cardiac contractility (Fig. 5).

**DISCUSSION**

A number of vascular disorders are associated with an abnormal endothelial function. From a pathological viewpoint, it is notable that arteries with damaged endothelium may contract in response to vasoactive substances, which normally in intact arteries cause vasodilatation (10,11). Unwanted local vasoconstriction may be a cause of myocardial ischaemia or even myocardial infarction (12), and if endothelial damage is widespread the resulting vasoconstriction may promote hypertension (13).

Just recently we postulated that the loss of endothelial integrity and related function in post-irradiated period is one of the most common effects of ionized irradiation. We demonstrated a significant impairment of endothelium-dependent relaxation occurred as early as 9 days post-irradiation and persisted or increased further over the experimental period (30 days). Contrary to endothelium-dependent responses, endothelium-independent responses to NO donors were only transiently reduced, with recovery observed later in the experimental period (14).

The novel finding of above experiments is that external beam radiation causes depression of endothelium-dependent relaxation mainly due to the loss of

![Figure 5](image-url)  
*Figure 5. Effects of radiation and the treatment with phosphatidylcholine liposomes (25 mg/kg, injected intravenously 1 h after irradiation) on blood pressure in left ventricle (A), cardiac index (B) and cardiac output (C) in anaesthetized rats.*
EDRF/NO, but not EDHF-dependent component of ACh-induced relaxation. The next surprising discovery is that there was no significant difference in ACh-stimulated NO release in both irradiated and healthy tissue. It is important to note that sensitivity of smooth muscle to authentic NO was not diminished. Taking together these data suggest that NO released by endothelial cells in irradiated vascular tissues under ACh stimulation might be someway eliminated from sub-endothelial space. Experiments with antioxidant α-tocopherol acetate allow suggest that an inhibition of EDRF/NO-dependent component may be due to accelerated inactivation of endothelium-derived NO by oxygen free radicals, thereby decreasing the bioavailability of NO (15).

These data arise the question of antioxidant administration to improve vascular function after radiation attack. We decided to test PCL, which possesses both antioxidant effects (16) and demonstrated ability to restore endothelial function (10).

It is well known that endothelium controls vascular smooth muscle tone by secreting some relaxing and contracting factors. A number of vascular disorders are associated with abnormalities of endothelium-dependent relaxation. These abnormalities have been suggested to be related to changes in synthesis and/or release of EDRF/NO, changes in sensitivity of smooth muscle cells to NO, alteration of the enzyme NO synthase or oxidative attack of the NO in vascular wall with following its fast conversion to peroxynitrite (ONOO').

It is generally accepted that exposure of vascular tissue to ionizing irradiation leads to formation of reactive oxygen species that are associated with radiation-induced cytotoxicity. It was shown that ionized radiation produced both dose-and oxygen-dependent non-saturated fatty acids peroxidation with following formation of toxic products (17,18). If radiation impact represents itself a variety of oxidative stress, suppression of the system of oxidant defense appears to be involved in vascular injury under irradiation. So, we hypothesise that vascular tissues under irradiation suffer first of all from oxidative attack.

All above-mentioned data allow suggest that side by side with well known genetic target for ionised irradiation, DNA, another possible and highly sensitive target in living cells is exist. It is phospholipids of plasma membrane peroxidation of which triggers the chain reaction of free radical oxidation. Multiple processes may lead to endothelial damage under irradiation but the generation of oxygen free radicals and following lipid peroxidation may be one of the key components in this cascade of events.

When added to the bath solution in vitro experiments or injected to the rabbits intraperitoneally just after irradiation, PCL effectively restored endothelial function. It is interesting that being injected before irradiation PCL had no any protective effect suggesting that they can not prevent the development of oxidative attack initiated by irradiation but possess the ability effectively operate in already damaged tissue keeping or restoring their physiological functions.
Experiments on anesthetized rats have confirmed that PCL possess the ability selectively normalized vascular control mechanisms damaged resulting from ionized irradiation. This conclusion based on data that irradiation increased arterial blood pressure mainly due to the vascular component, and that of PCL, in turn, effectively diminished an increased arterial blood pressure without any effect on cardiac contractility.

It is known that all normally functioning living systems possess effective protective mechanisms against free radicals action - antioxidant system of defense. The properties of antioxidants possess substances of different chemical origin: superoxide dismutase, catalase, tocopherols, carotene, glutathion, ascorbic acid, cystein, SH-group-containing proteins, and others. Protective properties against free radicals demonstrate also phospholipids of membrane complexes that act as the component of the system of oxidant defense, mainly due to their carbohydrate bonds (19). It was postulated before in experiments on anesthetized animals that PCL possess its own antioxidant activity and could effectively decrease lipid peroxidation products content in tissues during hypoxia (16).

We have no absolutely persuasive explanations of the repairing effect of PCL in radiation-induced vascular lesions but nevertheless we consider necessary to give some probable speculations. It might be explained by the ability of PCL to liquidate the aftereffects of oxidative attacks. It is likely that PCL could act as the agents repairing damaged plasma membranes and restoring a normal function of endothelial cells under oxidative stress. Another possible mechanism for repairing effect of PCL may be related to its possible action as scavengers and/or targets for oxygen-derived free radicals and products of lipid peroxidation. Repairing effect of PCL in irradiated tissue is similar to the effect of well-known antioxidant, α-tocopherol acetate as we postulated before (14), suggesting that PCL exhibit an antioxidant activity.

Thus, the data obtained suggest that phosphatidylcholine liposomes cannot prevent irradiation-induced vascular lesions but they possess the ability to restore damaged endothelial function in irradiated blood vessels, i.e. liposomes act as therapeutic but not preventive cure and can be useful in case of ionizing irradiation accident or in clinic during radiation therapy.

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