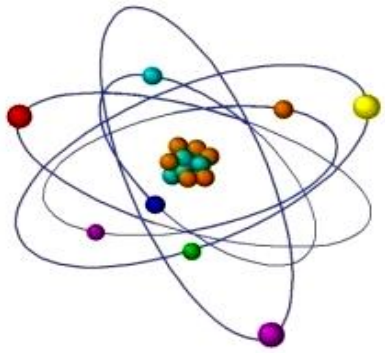
THE EFFECT OF IONIZING RADIATION ON HIPPOCAMPAL NA,K-ATPASE ACTIVITY



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ABSTRACT: *Ionizing radiation (IR) exposure initiates the rapid generation of reactive oxygen species (ROS), leading to oxidative stress and cellular damage. While ROS serve as critical signaling molecules under physiological conditions, excessive levels can disrupt membrane integrity via lipid peroxidation and impair membrane-bound enzymes such as Na^+,K^+-ATPase. In this context, our study investigates the dual effect of IR-induced ROS and endogenous ouabain on Na^+,K^+-ATPase activity. We demonstrate that different doses of IR (1 Gy and 5 Gy) initially enhance enzyme activity through reversible redox modification of thiol groups of the Na^+,K^+-ATPase complex. Simultaneously, IR stimulates the release of endogenous ouabain, which not only binds to Na^+,K^+-ATPase but also activates intracellular signaling cascades that further augment mitochondrial ROS production. This positive feedback loop—termed the "Na^+,K^+-ATPase oxidant amplification loop"—ultimately leads to irreversible oxidative modifications and enzyme inhibition under sustained oxidative stress. These findings highlight a redox-dependent biphasic regulation of Na^+,K^+-ATPase in response to IR, implicating both reversible and irreversible oxidative mechanisms in radiation-induced cellular effects.*

Keywords: Na,K-ATPase, gamma irradiation, enzyme activity, thiol groups

INTRODUCTION

Oxidative stress describes a physiological condition in which the balance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses is disrupted, leading to an excess of ROS. This state reflects an imbalance in cellular oxidation processes, where tissues experience heightened exposure to molecular oxygen or its reactive derivatives. Normally, oxygen is used in metabolism to produce energy, generating byproducts like superoxide, hydrogen peroxide, and hydroxyl radicals. When ROS levels exceed the antioxidant defenses, they can damage lipids, proteins, and DNA, potentially leading to cell dysfunction or death. This stress plays a key role in aging and chronic diseases like Parkinson's, atherosclerosis, cancer, diabetes, and eye disorders [1-3]. Diets rich in antioxidants (e.g., polyphenols, vitamin C, carotenoids) are linked to lower disease risk. Measuring oxidative damage and total antioxidant capacity helps us understand how oxidative stress contributes to pathology and how dietary antioxidants might protect against it [4 -5]. To study oxidative stress, researchers often measure (i) free radicals, (ii) damage they cause to lipids, proteins, or DNA,

and (iii) antioxidant levels or enzyme activity. Free radicals are unstable molecules naturally formed during metabolism and immune responses. While they play beneficial roles, excessive levels can damage cells by oxidizing lipids, proteins, and DNA [6]. The body counters this with antioxidant defenses, including enzymes (like SOD, catalase) and molecules (like vitamins C and E). An imbalance between radicals and antioxidants contributes to aging and diseases such as cancer and Parkinson's.

Free radicals come from two main sources Endogenous: Formed inside the body during normal metabolic processes, enzyme activity, or metal ion reactions Exogenous: Generated from environmental exposures such as radiation, heavy metals, smoking, pollution, and poor diet. Recent studies have focused on how ionizing radiation affects the brain, suggesting that the damage involves a mix of vascular and neurodegenerative changes [6-7]. In rats, whole-brain irradiation (25 Gy) led to cognitive decline and brain tissue changes one year later, resembling accelerated brain aging seen in diseases like Alzheimer's [7]. Notably, cognitive issues have also been observed in patients receiving radiotherapy outside the brain, such as breast cancer patients, even months after treatment [8]. Radiation effects have been widely studied in whole-body or targeted treatments, but less is known about changes in distant tissues. Studies have shown that irradiating the mediastinal area in rats caused right ventricular hypertrophy and reduced cardiac Na^+,K^+-ATPase activity [9], essential for sodium balance. The enzyme's function also declined in the kidneys, likely due to increased oxidative stress in the blood [10]. Since Na^+,K^+-ATPase is sensitive to oxidative stress, its impaired activity may result from changes in its sodium and ATP binding sites. Brain tissue is especially vulnerable to ROS due to its high oxidative metabolism, abundance of polyunsaturated fatty acids in membrane lipids, presence of iron ions, and relatively low antioxidant enzyme capacity [12-13]. Studies have shown that both lethal and sublethal doses of radiation can affect sodium-potassium transport systems in neural and glial cell-enriched fractions as well as in cortical slices from rats [14]. Na^+,K^+-ATPase is essential for maintaining ion balance across the plasma membrane, supporting the resting membrane potential crucial for muscle function [15]. Skeletal muscles, which contain the most immense amount of this enzyme, rely heavily on it for normal excitability and contraction. Among its isoforms, α2 is dominant in adult muscle and adapts to activity, helping resist fatigue. This isoform is found mainly in transverse tubules and at neuromuscular junctions. Na^+,K^+-ATPase also binds cardiotonic steroids like ouabain, which at low physiological levels may act as a hormone [16-18]. Ouabain, a plant-derived cardiotonic steroid, is believed to be synthesized naturally in the adrenal cortex and hypothalamus [19]. Endogenous ouabain typically circulates at subnanomolar levels, though its concentration can rise under different physiological and pathological conditions [20-22]. While high levels of ouabain are toxic, low concentrations show potential in regulating inflammation, blood pressure, and neural activity [23-24]. This study examined whether whole-body irradiation in mice alters Na^+,K^+-ATPase activity in the brain, specifically in the hippocampus.

MATERIALS AND METHODS

Experiments were conducted on age- and weight-matched male and female mice (10 weeks old). The animals underwent a single session of total-body ionizing radiation (IR). Two experimental groups were established: one group (n = 6) received a 1 Gy dose, while another

group (n = 6) was exposed to a 5 Gy dose. A third group, which did not undergo radiation exposure, served as the control. Post-irradiation, animals were housed separately based on group and monitored.

Hippocampal tissue was extracted from each group and utilized for analysis. Tissue homogenization followed the protocol recommended by Gislaine Rezin [25]. The activity of Na^+,K^+-ATPase was assessed by quantifying the ouabain-sensitive component of total ATPase activity. The assay for total ATPase activity utilized an incubation medium comprising 140 mM NaCl, 5 mM KCl, and 50 mM Tris-HCl buffer (pH 7.7). Mg-ATPase activity was evaluated in the presence of 1 mM ouabain using a medium containing 145 mM KCl and 50 mM Tris-HCl buffer (pH 7.7). The difference between the two assays was used to calculate the activity of the Na,K-ATPase. Protein concentration was standardized to 0.014 mg/mL for all enzymatic assays.

Incubations were carried out at 37°C for 15 minutes. Enzymatic activity was determined based on the hydrolysis of ATP, with the amount of inorganic phosphate (Pi) released expressed per mg protein per hour. The liberated Pi was quantified according to the method described by Chan et al. [26]. Na,K^+-ATPase activity was calculated in nanomoles of Pi released per minute per mg of protein. Protein concentrations were measured using the Lowry method [27], employing bovine serum albumin as the reference standard.

All experimental data were statistically analyzed. Results are reported as arithmetic means ± standard error (SE). Statistical significance between two groups was determined using an unpaired, two-tailed Student's t-test, with p-values less than 0.05 considered significant.

No animals showed signs of distress prior to decapitation. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Ivane Beritashvili Center of Experimental Biomedicine.

RESULTS

Our previous study has shown [28], that IR influences ATPase activity from brain synaptic membranes. This study focused on hippocampal fraction and investigated Na,K-ATPase activity after one and two weeks of total-body IR (1Gy and 5 Gy). Fig.1 demonstrates the effect of 1Gy irradiation on Na,K-ATPase activity after 1 and 2 weeks. From Fig.1, it is clear that 1Gy irradiation increases enzyme activity after 1 week by 45%. No statistical changes occur after 2 weeks ($P>0.05$) compared with one week group, and activity remains elevated by 36% compared to the control group (Fig.1). For 5Gy irradiated groups after 1 week Na,K-ATPase activity is increased by 129% compared to the control group, and after the 2-week activity is decreased compared to 1week group by 19% but still is elevated by 87% compared to the control group (Fig.2). Various external and internal factors can lead to specific alterations in the structure and function of Na^+,K^+-ATPase, resulting in changes to its enzymatic activity [29-32]. Among these factors are ROS, a class of chemically reactive molecules formed through the incomplete reduction of molecular oxygen. These free radicals have the potential to change Na,K-ATPase activity [29]. IR possesses enough energy to remove electrons from atoms, resulting in ionization and facilitating the production of ROS. ROS primarily targets cysteine (Cys) residues within proteins, which are key sites for diverse thiol-based modifications. To test whether IR affects Cys within the enzyme, we have studied the influence of IR on p-

chloromercuribenzoic acids (PCMB) affinity. PCMB interacts with protein thiol groups, inducing conformational changes that can either activate or inhibit specific proteins, depending on their structure and function [33]. From Fig.3 it is clear, that 1Gy IR does not change the maximal velocity (V_{max}) of the enzyme. For the control group V_{max}=0.285± 0.0013; after 1 week of 1Gy irradiation V_{max}=0.286±0.0014; after 2 weeks V_{max}=0.283±0.001. PCMB inhibition constant (K_i) remained the same after 1 week but deeply increased after 2 weeks of IR. Ki = 2.69±0.12 for the control group; Ki = 2.55±0.23 after 1 week and Ki = 3.72 ±0.18 after 2 weeks; 5Gy IR also does not change the V_{max} of the enzyme after 1 (V_{max}=0.284±0.0034) and 2 weeks (V_{max}=0.293±0.0016) but increases the K_i after 2 weeks (Fig.4). Ki = 2.09±0.13 after 1 week and Ki = 3.72 ±0.18 after 2 weeks.

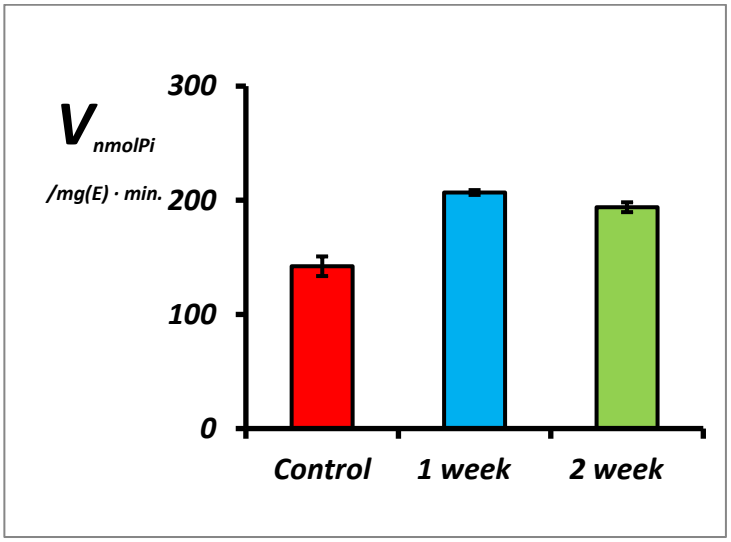

Fig.1 Dependence of hippocampal Na, K-ATPase activity on IR (1Gy). The reaction medium was [MgATP]=1.69mM; [Mg_f^{2+}]=[ATP_f]=0.31mM. (P < 0.001 vs control)

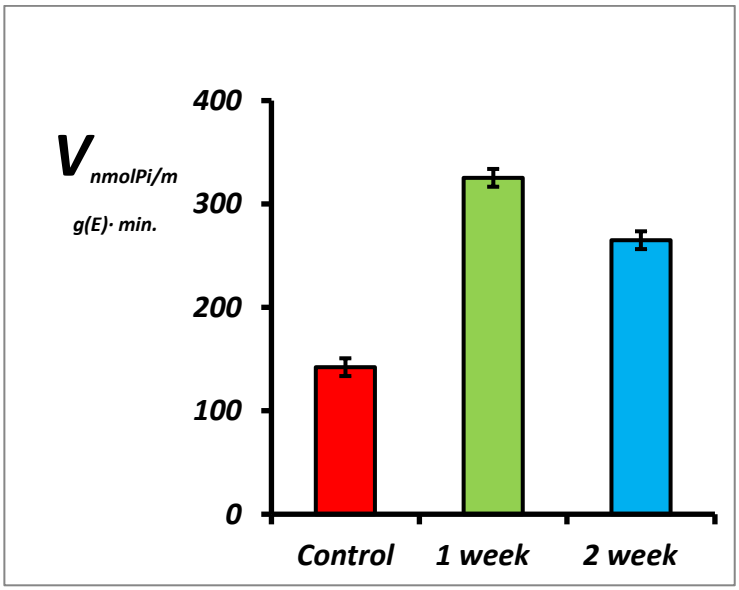

Fig.2 Dependence of hippocampal Na,K-ATPase activity on IR (5 Gy). The reaction medium was [MgATP]=1.69mM; [Mg_f^{2+}]=[ATP_f]=0.31mM.(P < 0.001 vs control)

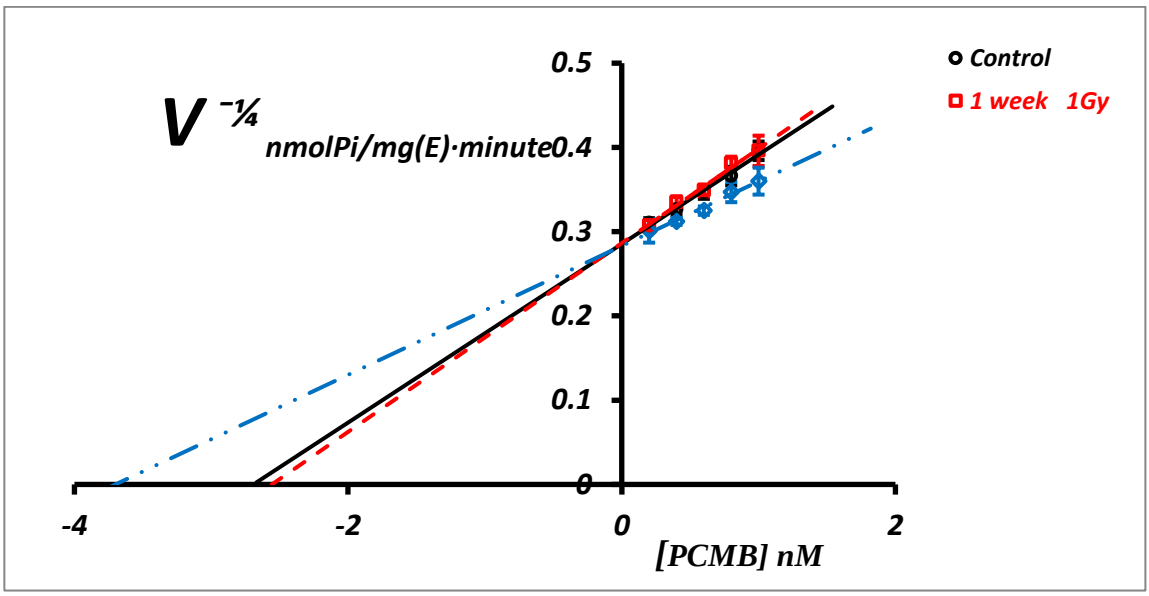

Fig.3. Dependence of hippocampal Na, K-ATPase activity on PCMB concentrations in the $V^{-1/4}$=f(PCMB) coordinate system. The reaction medium was [MgATP]=1.69mM; $[Mg_f^{2+}]=[ATP_f]\leq 0.31$mM. (P < 0.01vs control; n = 3).IR=1Gy
I -Control $V^{-1/4}$=0.106x + 0.285 Ki = 2.69±0.12 V_{max}=0.285± 0.0013 x=[PCMB]
II- 1week $V^{-1/4}$=0.112x +0.286 Ki = 2.55±0.23 V_{max}=0.286±0.0014
III –2 week $V^{-1/4}$=0.076x + 0.283 Ki = 3.72 ±0.18 V_{max}=0.283±0.001

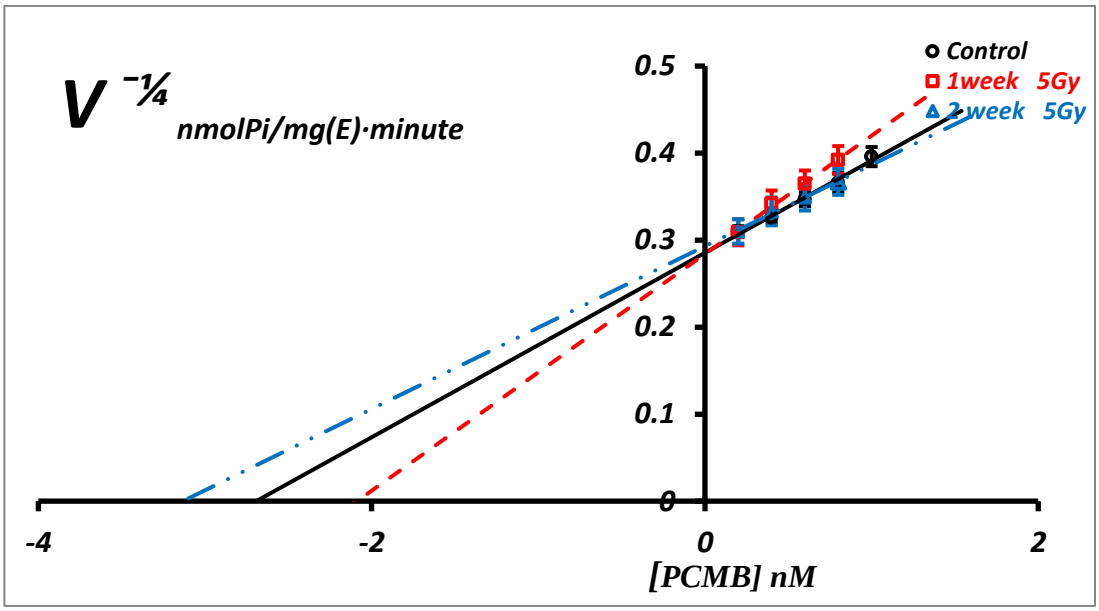

Fig.4 Dependence of hippocampal Na, K-ATPase activity on PCMB concentrations in the $V^{-1/4}$=f(PCMB) coordinate system. The reaction medium was [MgATP]=1.69mM; $[Mg_f^{2+}]=[ATP_f]\leq 0.31$mM. (P < 0.01vs control; n = 3).IR=5Gy
I -Control $V^{-1/4}$=0.106x + 0.285 Ki = 2.69± 0.12; V_{max}=0.285± 0.0013 x=[PCMB]
II- 1week $V^{-1/4}$=0.136x +0.284 Ki = 2.09±0.13 V_{max}=0.284±0.0034
III –2 week $V^{-1/4}$=0.093x + 0.293 Ki = 3.15 ±0.11 V_{max}=0.293±0.0016

DISCUSSION

It is well established that exposure to IR immediately triggers the generation of free radicals, initiating the production of ROS and subsequently leading to cellular damage. Under normal physiological conditions, ROS act as important signaling mediators [34], and their levels are tightly regulated by endogenous antioxidant systems, such as thiol-containing molecules. These antioxidants neutralize excess radicals, preventing oxidative damage. However, IR-induced elevation in free radical production disrupts this balance, promoting lipid peroxidation—a process that compromises membrane integrity [6]. Alterations in the oxidative state and lipid composition of membranes can profoundly impact the functional activity of membrane-bound enzymes such as Na^+,K^+-ATPase [29]. Notably, a rise in lipid peroxidation byproducts has been directly associated with the suppression of Na^+,K^+-ATPase activity [29]. Additionally, IR exposure has been shown to elevate levels of endogenous ouabain [35], a cardiotonic steroid known for its high-affinity interaction with Na^+,K^+-ATPase. While ouabain is classically recognized as an inhibitor of the enzyme, at physiologically relevant (low) concentrations—comparable to endogenously released levels—it may also exert activating effects on the pump [36–38]. Endogenous ouabain is synthesized from cholesterol in the zona glomerulosa of the adrenal cortex [17]. The stress response induced by IR activates both the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis, thereby modulating ouabain synthesis and secretion [17]. Interestingly, while ROS typically suppress Na^+,K^+-ATPase function, they are also implicated in a self-perpetuating oxidative stress signaling cascade [17]. The binding of ouabain to Na^+,K^+-ATPase can initiate multiple intracellular signaling pathways, including those that stimulate mitochondrial ROS production—thereby creating a feedback loop that further modulates Na^+,K^+-ATPase function [17].

This redox-based signaling involves the reversible covalent modification of cysteine residues located in the catalytic or regulatory domains of proteins. ROS-induced oxidation of these cysteines leads to the formation of disulfide bonds (R–S–S–R) through a sulfenic acid (R–SOH) intermediate in a biologically reversible manner [39]. These disulfides may be intramolecular, intermolecular, or occur between a protein thiol and glutathione (S-glutathionylation). Redox-regulating proteins such as thioredoxins (Trx) and glutaredoxins (Grx) catalyze the reduction of these disulfide bonds, allowing for controlled signal transduction. However, further oxidation by hydrogen peroxide (H_2O_2) can irreversibly convert sulfenic acids into sulfinic ($R–SO_2H$) and sulfonic ($R–SO_3H$) acids [39].

At early stages following 1 Gy and 5 Gy IR exposure, increased ROS production and elevated endogenous ouabain levels may initially stimulate Na^+,K^+-ATPase activity via reversible redox modification of cysteine residues in its subunits (Fig. 1; Fig. 2). These early effects appear to enhance enzyme function. However, as oxidative pressure intensifies, ouabain-induced activation of signaling pathways further amplifies mitochondrial ROS production, perpetuating the so-called "Na^+,K^+-ATPase oxidant amplification loop." This continued oxidative modification leads to irreversible thiol oxidation and subsequent enzymatic inhibition (Fig. 1).

Na^+,K^+-ATPase is a heterotrimeric protein complex composed of three subunits: the large catalytic α subunit (100–113 kDa), responsible for ion transport and ATP hydrolysis; the β

subunit (~55 kDa), which plays a regulatory and structural role; and the FXYD family of small regulatory proteins (7–11 kDa), expressed in a tissue-specific manner [40]. Each of these subunits contains cysteine residues susceptible to redox modification. In particular, the β subunit includes a single accessible thiol group located at the membrane interface, which transitions in and out of the membrane during enzyme cycling [40]. The FXYD subunit possesses two reactive thiols [40], while the α subunit contains 23–24 thiol groups, depending on the isoform [40]. These residues represent prime candidates for ROS-mediated redox regulation. The changes in pCMB affinity induced by IR confirm the involvement of regulatory thiols in this mechanism (Fig.3; Fig.4).

Therefore, our experimental findings suggest that IR-induced ROS formation and ouabain elevation initially lead to enhanced Na^+,K^+-ATPase activity via reversible thiol oxidation. However, sustained oxidative stress results in irreversible enzyme inhibition.

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