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Effects of radiation on SGLT1 gene expression in LLC-PK₁ porcine kidney cells

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**EFFECTS OF RADIATION ON SGLT1 GENE EXPRESSION
IN LLC-PK₁ PORCINE KIDNEY CELLS**

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Randal Akio Mihara

August 1997

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ABSTRACT

EFFECTS OF RADIATION ON SGLT1 GENE EXPRESSION IN LLC-PK₁ PORCINE KIDNEY CELLS

by Randal Akio Mihara

LLC-PK₁ porcine kidney cells were exposed to doses of ionizing radiation from a ¹³⁷Cs source ranging from 5 Gy to 60 Gy. The glucose concentrating capacity of the cells was then up-regulated by decreasing the concentration of glucose in the extracellular medium. Northern blot analysis was used to quantify the expressed amounts of the mRNA encoded by the high-affinity, low-capacity glucose cotransporter gene (SGLT1). The dose response characterization revealed that the quantities of the transcribed SGLT1 mRNA were not significantly inhibited by the doses of ionizing radiation. This suggests that the dose-dependent inhibition of the glucose concentrating capacity for the cells is due to post-transcriptional effects rather than a decrease in the level of transcription.

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LITERATURE REVIEW

Radiation treatment of abdominal tumors has often involved inadvertent irradiation of neighboring non-cancerous normal tissues. The kidney is a radiosensitive organ that would limit radiotherapy of adjacent neoplasms because of manifestation of late damage. Functional failure or reduced functional capacity of the kidney often involves the reduced ability to reabsorb solutes back into the circulatory system. Elucidation of the mechanism by which ionizing radiation causes functional damage would be an invaluable step towards understanding mechanisms of late radiation effects in organs and tissues at risk.

Ionizing Radiations

The spectrum of electromagnetic radiations can be divided into two major subcategories, the non-ionizing spectrum which includes electric waves, radio waves, and the optical spectrum, and the ionizing spectrum (Alpen, 1990). Electric waves and radio waves are low energy emissions such as FM broadcast radiowaves and microwaves. The optical spectrum is higher energy than the electric waves and radiowaves. This spectrum spans energies from as low as infrared light to as high as ultraviolet light, and includes the visible spectrum. The ionizing spectrum contains the most energetic radiations and includes x-rays, gamma rays and energetic charged particles. Ionizing radiations can be defined as high energy electromagnetic rays or particles that have sufficient energy to ionize atoms and molecules with which they interact. These radiations are emitted by disintegrating radioactive nuclides and are produced in man-made devices such as x-ray machines and particle accelerators. The particles and rays comprising ionizing radiation from radioactive nuclides can be separated into four classes: α , β , γ and neutron radiation.

Alpha radiation is the emission of helium nuclei with two positive charges from a nuclear disintegration of a radioactive nuclide. These relatively large, charged particles do not penetrate very far and are thus stopped by the epidermis. Since the epidermis is the outermost layer of the skin and is composed of dead cells, energy deposition by alpha radiation in this tissue layer usually does not have noticeable biological effects. Their biological effects are only realized when their parent nuclides are inhaled or ingested and incorporated into tissue cells. When these nuclides decay in the body, the alpha particles may cause damage to tissues directly adjacent to them. It is important to note that the relatively large mass and energy of alpha particles means that they have a great potential for depositing a large amount of energy in a small volume, and are therefore termed high linear energy transfer (high LET) radiation.

Beta decay involves the emission of electrons. The energies of these electrons is dependent on the characteristic emissions of the nuclide of interest. In general, most beta particles are attenuated rapidly in environments other than a vacuum. Their charged nature provides a high probability of interaction with matter they encounter. Therefore, many beta emissions are absorbed at the epidermis just like alpha particles, and do not enter the body. However, higher energy beta particles stand a better chance than alpha particles of penetrating beyond the epidermis because of their small mass and charge.

Gamma emissions, on the other hand, are deep penetrators because they are electromagnetic rays, as are x-rays. Their lack of charge and no mass give them a reduced probability of interacting with matter that is not dense. Hence, gamma emissions easily traverse the epidermis and are often used to treat tumors deep within the body. But since they must travel through neighboring normal tissues to reach the tumor, these neighboring tissues are affected as well as the tumor.

Neutron radiation can possess both penetrating ability and high energy. It has a relatively large mass that can potentially deposit a large amount of energy, and since neutrons are not charged, they can penetrate quite deeply into tissues. Neutrons are a form of high LET radiation because of elastic collisions which transfer energy to atomic nuclei, producing relatively heavy charged particles with sufficient energy to produce dense ionizing tracks.

In addition to the direct interactions of the particles and rays with target atoms, energy may be deposited indirectly. High energy particles and rays commonly interact with matter, causing the ejection of an electron. This electron contains some of the energy from the original impacting emission and may have enough energy to cause subsequent ionizations and excitations. Additionally, the original impacting emission may retain enough energy after the collision to cause many more interactions. This chain reaction means that a single, high-energy electron generated from a primary energy transfer process can cause thousands of indirect ionizations.

Mechanisms of Energy Deposition in Biological Systems

Ionizing radiation transfers energy by initially interacting with an electron in the medium which gains high kinetic energy. The electrons undergo coulombic interactions with molecules, causing ionizations and excitations. Ionization can be caused directly when one of these electrons interacts with cellular components; however, since cells are mostly composed of water, most interactions are with water rather than directly with target biological molecules. Depending on the amount of energy transferred by the radiation, water can be either excited or ionized. In both cases, the most important products that arise to cause cellular damage are $\text{H}\cdot$ and $\text{OH}\cdot$ radicals, and e^-_{aq} (aqueous

electrons)(Figure 1). Of these, the OH• radical is thought to be the most damaging (Alpen, 1990). The majority of the cellular damage due to ionizing electromagnetic

Figure 1. Water radiolysis products.

radiation is caused indirectly by these radicals and aqueous electrons. Since H•, OH• and e⁻_{aq} have very short lifespans before interacting with other molecules, they tend to localize their damage in a very small target volume. The mechanisms by which these cause damage in molecules include extraction of hydrogen atoms, dissociative reactions, and addition reactions (Figure 2). Hydrogen radicals can extract a hydrogen atom from a target molecule, making the target molecule a radical and producing hydrogen gas.

Extraction of Hydrogen Atoms

Dissociative Reactions

Addition Reactions

Figure 2. Mechanisms of damage caused by water radiolysis products.

Hydroxyl radicals react similarly, except the product is water instead of hydrogen gas. Target molecules can also be attacked by the radicals and aqueous electrons in a dissociative process where a functional group, such as an amine group, is removed from the target molecule. Addition reactions involve the radicals binding to the target molecule, and the target molecule becoming a radical because of the unpaired electron. All these reactions cause the target molecules to become radicals. The three processes that determine the fate of these radicals are restoration, damage fixation, and restitution (Figure 3). Restoration can occur if the products of the reaction are still located close to

Restoration

Damage Fixation

Restitution

Figure 3. Mechanisms determining fate of biological radicals.

each other and they recombine to their original forms. Damage fixation usually occurs if the newly-formed radicals combine with oxygen to form peroxides, since subsequent reactions usually lead to irreversible damage. Restitution of the target molecule to its original form takes place if radical scavengers, such as glutathione found in cells, interact with the newly-formed radicals.

Three of the important targets of ionizing radiation are DNA, membranes, and proteins. Since the DNA of cells is the template for the expression of cellular components, any damage caused to DNA has a great probability of manifesting observable effects. Cell survival models such as the multi-target single hit (MTSH) model and the linear quadratic model have been developed to explain the interactions of radiation with these targets and

DNA in particular. DNA damage includes base damage, single strand breaks, and double strand breaks. Base damage occurs when radiation alters a single base on one strand of the DNA double helix which may either prevent transcription or cause the template to be misread. This damage may also block DNA replication at the lesion or cause a mutation to be formed by mispairing if replication occurs past the lesion. This kind of damage is repaired efficiently by DNA repair enzymes that quickly identify these lesions. However, lack of repair may result in blocked or incorrect transcription and replication. Also of great importance is the interaction of ionizing radiation with the backbone of the DNA which links the individual nucleotide bases together. The backbone of the DNA consists of the deoxyriboses of each nucleotide linked together with phosphates. A phosphodiester bridge joins the 5'-hydroxyl of one deoxyribose with the 3'-hydroxyl of the adjacent deoxyribose. A single-strand break can occur if ionizing radiation breaks a phosphodiester linkage. Since the second strand is still intact and hydrogen bonding between the corresponding base pairs is present, the two nucleotides directly adjacent to the scission are still in close proximity. DNA ligase can easily repair the DNA back to its original state. However, if a double-strand chain scission occurs and a lesion is created in each strand, the DNA can separate into two fragments. Although it is possible that DNA ligase might recombine the fragments, the separation distance between fragments and the relatively low concentration of DNA ligase makes repair very unlikely. Double-strand scissions of this kind are thought to be a major cause of clonogenic death, chromosomal aberrations, and functional damage to cells (Ward, 1988)(Figure 4).

Figure 4. Mechanisms of DNA Scissions.

Lipid membranes can also be altered by ionizing radiation. Peroxidation of polyunsaturated lipids in bilayers has been shown to reduce membrane fluidity (Edwards et al., 1984; Spector and Yorek, 1985). The reduced fluidity is the result of the loss of cis-double bonds in the phospholipids that compose the membrane (Edwards et al., 1984). Of the products produced from the peroxidation of polyunsaturated fatty acids, malonaldehyde is the most significant, and has been demonstrated to cause damage to DNA and proteins (Reiss et al., 1972).

Proteins are also potential targets of ionizing radiation. The two amino acid residues which contain sulfur side chains are cysteine and methionine. Approximately 80% of all sulfur in cells is in the form of these amino acids. Methionine contains a methyl group attached to the sulfur, so it is not very reactive. However, cysteine contains a sulfhydryl group which is highly reactive. Hydroxyl radicals can interact with this sulfhydryl group to form thiol radicals. If two thiol radicals interact with each other, they form a disulfide linkage in a protein chain (Figure 5). Disulfide linkages, in conjunction

Figure 5. Sulfur-containing amino acids and disulfide linkage formation.

with covalent interactions, determine the tertiary structure of proteins. Therefore, radiation-induced modification of disulfide linkages can alter protein shapes and lead to functional instability. However, this mechanism of ionizing radiation damage is not considered very important, since enormous doses of radiation would be required to overcome the rather small probability of damaging a significant number of target proteins.

Biological Manifestation of Damage

Depending upon the dose of ionizing radiation, the effects on cellular systems can range from functional impairment to cell killing. Cell death can take the form of clonogenic death or functional death. Clonogenic death is the inability of cells to reproduce; whereas, functional death (interphase death) is the inability of cells to

satisfactorily carry out their cellular processes. Usually functional death requires a significantly greater dose than that required for clonogenic death to occur. Presumably, lower doses of ionizing radiation can damage the mechanism required for cellular reproduction, while not significantly impairing functional processes.

Dose fractionation experiments have demonstrated that DNA undergoes repair over time. By fractionating a dose into smaller doses with time delays between exposures rather than a single massive dose, a greater number of cells survive. It is believed that single-strand breaks and base pair damage can be repaired during these time delays. Hence, cells are able to withstand a greater subsequent dose if DNA repair occurs than if DNA repair does not occur (Elkind and Sutton, 1959). Since neoplastic tissue is more sensitive to ionizing radiation than normal tissue, fractionation has become a common method for preferentially destroying tumor cells while allowing normal cells to survive.

Radiation effects on biological systems can be categorized as non-stochastic effects and stochastic effects. Non-stochastic effects are effects resulting from a dose greater than some threshold value. Doses below the threshold value do not produce noticeable effects, while increasing doses larger than the threshold dose produces an increasing severity of the effects. Stochastic effects are effects that are based on probability and lack a threshold dose. For even small doses, there is a detectable non-zero incidence of the effects. Also, the effects are usually "all or none". That is, the severity of the effects is not dependent on the magnitude of the dose.

Non-stochastic effects include acute radiation-induced effects and many late effects of normal tissues. Acute effects are observed within a few days to a few weeks after irradiation; whereas, late effects are manifested many weeks to many months or years after irradiation. Acute responses are attributed to the depletion of clonogenic stem cells

so that the renewal of the normal cell population is at risk. It has been suggested that late effects are due to either cell depletion or damage leading to vascular insufficiency.

Stochastic effects include carcinogenesis and genetic mutations. The latency period to observe the effects of carcinogenesis in humans may be as short as 2 years to as long as 30 years. It has been suggested that this latency period is required so that a number of mutations and cellular changes can occur that are necessary for the development of a neoplasm. Since there is no threshold dose, there is always a probability of carcinogenesis even with the smallest doses. Additionally, the ultimate severity of a tumor forming is not dependent on the magnitude of the dose; however, the probability of forming a tumor at all is proportional to the dose. Genetic mutations are similar to carcinogenesis in that there is always a probability of radiation causing a mutation even with the smallest doses. The effects of genetic mutations are usually observed as late effects, since a viable mutation in a single stem cell requires time to reproduce and to form a population of gametes possessing the mutant trait. Also, sometimes the mutation is not expressed until after the first few generations since many are usually recessive.

Signal Processing Damage

Since ionizing radiation can affect the fluidity of membranes, it is possible that the functioning of intramembrane components may be impaired. Of special importance are the signaling components such as adenylate cyclase, protein kinase C, and arachidonic acid. Impairment of these signaling and regulatory elements can cause a cascade effect along the regulatory pathway ultimately leading to major gene expression alterations. Also, adverse effects to membrane-bound receptors can prevent proper regulation of gene expression as well. Protein kinases have been viewed as targets that may have widespread effects in cells, since they catalyze numerous different regulatory reactions. If damage is

occurring to protein kinases, it will be very difficult to pinpoint the specific reactions responsible for the gene expression effects.

Originally, it was hypothesized that alterations of gene expression were due to radiation directly affecting specific genes in the genome of cells. While there is little doubt that DNA is affected by ionizing radiation, it seems unlikely that each cell in a population would have a specific gene affected. Since the target volume occupied by a gene is relatively small compared to the other cellular components, it is improbable that an alteration in the gene is responsible for the overall abnormal behavior of the population of cells. It is more probable that many sites of damage of cellular components including numerous DNA alterations are responsible for observed gene expression effects.

It has been suggested that cell cycle regulation may be implicated in gene expression alterations observed in irradiated cells (Weichselbaum et al., 1994). Cellular replication can be separated into five main stages: G_1 , G_0 , S, G_2 , and M. The G_1 phase involves the growth of the cell and the manufacture of new proteins. If there is no signal to initiate cell division, cells in the G_1 phase may enter a resting G_0 phase. If a signal to stimulate cell division occurs, the cell enters the S phase where the DNA is replicated. Then, during the G_2 phase, the cell prepares to divide both the cytoplasm and the duplicate sets of DNA. In the M phase, the cell divides into two daughter cells which enter G_1 phases. Cells that are in the late G_2 phase and M phase have been observed to be the most sensitive to ionizing radiation damage. This is likely due to much of the cell's resources being used for cell division rather than cellular maintenance and repair. It has been demonstrated, for example, that irradiation can induce expression of the p53 gene and result in a G_2 -phase delay in eukaryotic cells. Cells which remain in the G_2 phase for extended periods have been shown to possess increased radioresistance, and hence, increased survival rates (Bernhard et al., 1994).

Glucose Transport in the Kidney

The kidneys act to filter the blood, excrete waste products, and maintain appropriate concentrations of metabolites within the body. The functional unit of the kidney is the nephron. There are approximately one and a quarter million nephrons per kidney in humans. The nephron is composed of six regions: glomerulus, Bowman's capsule, proximal convoluted tubule, loop of Henle, distal convoluted tubule, and collecting ducts. Blood enters the glomerulus via an afferent arteriole, and the plasma component is filtered across to the Bowman's capsule with a net filtration pressure of approximately 10 mmHg. The larger blood components, such as erythrocytes and large proteins, do not cross into the Bowman's capsule and instead return to the circulatory system via an efferent arteriole. The filtrate from the Bowman's capsule that enters the proximal convoluted tubule contains solutes such as glucose, sodium, amino acids and urea. For the current research, the proximal convoluted tubule is of greatest interest. In normally functioning kidneys, this region reabsorbs virtually all of the glucose back into the circulatory system. Detection of glucose in the urine is often used as diagnostic evidence of diabetes. The proximal convoluted tubule is composed of cuboidal epithelial cells which have a brush-border on their luminal side. The brush-border is composed of numerous microvilli which increase the surface area that comes in contact with the filtrate. Glucose is both actively and passively transported through these epithelial cells back to the circulatory system.

On the luminal membrane of the proximal convoluted tubule cells, D-glucose is actively transported into the cells by two distinct species of Na⁺/glucose cotransporters, SGLT1 and SGLT2. The SGLT2 gene encodes the low affinity high capacity Na⁺/glucose cotransporter; whereas, the SGLT1 gene encodes the high affinity low capacity Na⁺/glucose cotransporter (Pajor et al., 1992; Lee et al., 1994). While the SGLT2

cotransporter is only expressed in the proximal convoluted tubules of the kidneys, the SGLT1 cotransporter has been isolated from both the kidney and epithelium of the small intestine (Coady et al., 1990). In LLC-PK₁ pig kidney cells, the SGLT1 gene exhibits a 76% identity with the SGLT2 gene (Mackenzie et al., 1994). Studies have shown that the SGLT2 cotransporters are responsible for the reabsorption of approximately 90% of the glucose filtered into nephrons. The SGLT1 cotransporters are responsible for the reabsorption of the remaining 10% of the glucose (Kanai et al., 1994). The SGLT2 gene has been determined to encode a 660 amino acid protein with a Na⁺ and glucose stoichiometry of 1:1 (Kanai et al., 1994; Mackenzie et al., 1994). In contrast, the stoichiometry of Na⁺ and glucose uptake by the SGLT1 cotransporter was determined to be a 2:1 ratio (Koepsell et al., 1990; Lee et al., 1994). For the LLC-PK₁ porcine kidney cell line that is used in this study, the stoichiometric Na⁺:D-glucose ratio is 2:1, and therefore, the SGLT1 cotransporter is the predominant glucose cotransporter present (Sanders and Misfeldt, 1981; Moran et al., 1982; Ohta et al., 1990). Another significant difference between the two cotransporters is that while the SGLT1 cotransporter can translocate D-galactose, the SGLT2 cotransporter cannot (Kanai et al., 1994). Sequencing has shown that the SGLT1 gene encodes a protein subunit which contains 662 amino acids and has a molecular mass of about 73 kD. The SGLT1 cotransporter has consistently been shown to have a molecular mass around 300 kD. Studies have determined that a regulatory subunit (RS1), which is composed of 623 amino acids and has a molecular mass of 66,832 D, is present in the expressed SGLT1 cotransporter, and acts to increase the rate of transport. Glycosylation occurs at the Asn²⁴⁸ residue, and accounts for approximately 15 kD of mass. The most probable hypothesis currently suggests that the Na⁺/glucose cotransporter is composed of two SGLT1 subunits and two RS1 subunits (Wright et al., 1992; Koepsell and Spangenberg, 1994).

On the serosal membrane of proximal convoluted tubule cells, D-glucose is passively transported by two facilitated glucose carriers encoded by the GLUT1 and GLUT2 genes. For LLC-PK₁ cells, the GLUT1 transporter is the major facilitated carrier of D-glucose. Since the patterns of expression for SGLT1 and GLUT1 differ significantly, it has been suggested that the two transporters are regulated independently (Ohta et al., 1990). Furthermore, no homology has been found between the mammalian Na⁺/glucose cotransporter and the facilitated glucose carrier (Hediger et al., 1987).

Description of the LLC-PK₁ Cell Model System and Radiation Response

The LLC-PK₁ juvenile pig kidney cell line has been used extensively as a model for human kidney cells. This cell line is derived from proximal convoluted tubule cells of the nephron. In culture, these cells form a dense monolayer and form tight junctions between cells. Cell division is inhibited once a monolayer is formed. Only about 2-5% of the cells in confluent LLC-PK₁ monolayers are in the S-phase (Moran et al., 1986). Therefore, at confluency, most of the cells are in the G₀ phase of the cell cycle and exhibit uniform sensitivity to ionizing radiation. The serosal (basolateral) membranes of the cells bind to the surface of tissue culture flasks, while the luminal (apical) membranes contact the growth medium. The LLC-PK₁ cell line was selected because it retains the Na⁺/D-glucose cotransport and concentrating activity of its ancestral counterpart (Misfeldt and Sanders, 1982). This active transport of glucose is driven by the Na⁺ gradient which is maintained primarily by Na⁺/K⁺ ATPase activity in the serosal membrane (Rabito and Ausiello, 1980). After a monolayer is formed, the cells transport glucose, ions, and water across the serosal membrane where cell adherence is weak, and dome-shaped hemicysts are formed (Rabito et al., 1980). The level of glucose-concentrating activity in established confluent LLC-PK₁ cells is inversely dependent on

the concentration of glucose in the growth medium. Furthermore, by adjusting the glucose concentrations, established confluent cells may be induced to up-regulate or down-regulate SGLT1 cotransport (Moran et al., 1986). A low-glucose medium stimulates up-regulation, whereas a high-glucose medium induces down-regulation. Cells which are up-regulated exhibit a greater glucose concentrating capacity than those which are down-regulated, because of an increased number of transporters. Up-regulation has been measured to be a rapid process, reaching maximum capacity within 5 days; however, down-regulation has been found to be a slow process requiring much more time to reach minimal transport rates.

Moran et al. (1986) have demonstrated that ionizing radiation can inhibit the up-regulation ability of the LLC-PK₁ cells. If this inhibition were due to DNA damage, fractionated doses of radiation would be expected to produce a sparing effect due to DNA repair between doses. However, no sparing effect was observed with respect to the up-regulation ability. This suggests that the DNA is not likely to be the most important target of ionizing radiation for functional impairment (Parkins et al., 1989). But if damage to the DNA had triggered an irreversible progression of processes analogous to carcinogenesis or apoptosis, the lack of a sparing effect may be explained. That is, repair of the DNA would be inconsequential once the process had been initiated.

The regulation of the Na⁺/D-glucose cotransporter expression has been found to involve regulatory enzymes including adenylate cyclase, protein kinase A, protein kinase C, and cyclic AMP (Morgan, 1989) (Figure 6). Stimulation of adenylate cyclase and protein kinase A in differentiating cells has been shown to increase the expression of the cotransporter (Valentich et al., 1979; Amsler and Cook, 1982); whereas, stimulation of protein kinase C has been shown to decrease the expression of the cotransporter (Shioda et al., 1994). Alterations anywhere along either of these regulatory pathways

would likely affect expression. Transcription factors and post-transcriptional-processing proteins are also part of the regulatory pathways for the cotransporter, and therefore are also possible targets which could cause functional impairment of the cotransporter.

Figure 6. Protein kinase A and protein kinase C regulatory pathways.

The next logical step towards understanding how ionizing radiation interacts with LLC-PK₁ cells was to select a point along the regulatory pathways, and to determine whether damage was occurring upstream or downstream from that point. The research presented in this paper focuses on the messenger RNA which encodes the Na⁺/D-glucose cotransporter protein (SGLT1). A kinetics study of the baseline expression of the SGLT1 mRNA was completed to determine the optimal time during up-regulation to measure the SGLT1 mRNA. A radiation dose response of the cells was established to compare the relative quantities of the SGLT1 mRNA within a range of doses. By measuring the relative

quantities of the SGLT1 mRNA through Northern blot analysis, it was possible to determine whether the functional damage caused by ionizing radiation involved the level of transcription or involved post-transcriptional effects.

MATERIALS AND METHODS

Cell Culture

The LLC-PK₁ porcine kidney cells (passages 196 to 210)(American Type Tissue Culture Collection , Bethesda, MD) were cultured in Dulbecco's modified Eagles medium (DMEM) (Gibco, Grand Island, NY). The medium included 5% fetal calf serum, 2 mM glutamine, 100 μ units/ml penicillin, and 100 mg/ml streptomycin. D-glucose was added to the medium to produce either a 5 mM or a 25 mM D-glucose concentration for low-glucose or high-glucose media, respectively. Incubation of the cells was conducted in a HEPA Filtered, IR Incubator (Forma Scientific, Inc., Marietta, OH) at 37°C in air supplemented with 5% CO₂. After reaching confluency, the cells were dispersed using 0.025% trypsin and 0.02% EDTA and counted. Approximately 2×10^5 cells were seeded into each 9.4 cm² flask (Corning, Inc., Corning, NY). The cells were grown to confluency in a 25 mM D-glucose medium. Confluency was reached within 3 days of subculturing. The medium was changed every two days, making sure not to dislodge cells from the bottom of the flasks. Up-regulation was accomplished by growing and maintaining monolayers in a 25 mM D-glucose medium (high glucose) for at least five days after reaching confluency, and then switching to a 5 mM D-glucose medium (low glucose).

Radiation Dosimetry

A Mark I Model 25 ¹³⁷Cs irradiator (J.L. Shepherd and Associates, Glendale, CA) was used to provide doses of ionizing radiation to the cells. Ferrous sulfate dosimetry (Fricke dosimetry) was used to estimate the doses used for the experiments. The dose rate was measured to be approximately 8.0 Gy/min. The LLC-PK₁ cell monolayers were irradiated with doses ranging from 5 Gy to 60 Gy.

Isolation of Total RNA from LLC-PK₁ Kidney Cells

Each 9.4 cm² monolayer was rinsed three times using 2 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄•7H₂O, 1.4 mM KH₂PO₄). Total RNA was isolated from the cells using a modified Peppel-Baglioni method (Peppel and Baglioni, 1991). Each monolayer was lysed using 750 µl of SDS/EDTA solution (2% SDS; 1 mM EDTA; 200 mM Tris/HCl; pH 7.5), and transferred to a 1.7 ml siliconized microcentrifuge tube. Genomic DNA and cell membranes were precipitated by the addition of 225 µl of a potassium acetate solution (4.37M K-acetate; 11.2% acetic acid). Total RNA was further purified by extraction using 500 µl of a chloroform:isoamyl alcohol (24:1) solution. The aqueous phase containing the total RNA was then precipitated by addition of 800 µl of isopropanol, and incubated at -20°C for one hour. The mixture was then centrifuged at room temperature for 15 minutes. The resulting RNA pellet was rinsed using 1 ml of 70% ethanol at -20°C. After drying the pellet in a vacuum chamber, the RNA was resuspended in 30 µl of TE buffer (10 mM Tris•Cl; 1 mM EDTA; pH 7.4), and stored at -135°C in a Queue Cryostar freezer (Queue Systems, Parkersburg, WV). This protocol has proven to be a simple and effective method for producing total RNA yields as high as 100 µg per 9.4 cm² monolayer. Precise quantification of the total RNA was measured by using a UV160U Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). An absorbance ratio for 260 nm and 280 nm wavelengths (A₂₆₀/A₂₈₀) was calculated to determine the purity of the RNA.

DNA Probe for the Detection of the mRNA Encoding the SGLT1 Gene

A pBSK(-) plasmid containing a 1.5 kbp insert encoding portions of the SGLT1 gene of LLC-PK₁ pig kidney cells was obtained from Dr. David Rhoads of the Massachusetts General Hospital Cancer Center, Harvard Medical School. A JM109 strain

of *Escherichia coli* bacteria was transfected with the plasmid by using an EZ-Comp kit (5 Prime → 3 Prime, Inc., Boulder, CO). The bacteria were grown in a G24 Environmental Incubator Shaker (New Brunswick Scientific Company, Inc., Edison, NJ) at 37°C and with constant shaking in Luria-Bertani media supplemented with 100 µg/ml ampicillin to select for bacteria containing the plasmid, since the plasmid contained an ampicillin resistance gene. While the bacteria were still in the exponential growth phase, the plasmids were isolated using a QIAprep-spin Plasmid Kit (QIAGEN, Inc., Chatsworth, CA). The resulting plasmid DNA pellet was resuspended in TE buffer, pH 7.5, and stored at -135°C. The plasmids were then cut using the PstI restriction enzyme to produce two fragments: pBSK(-) vector (~2.9 kbp) and the SGLT1 insert (~1.5 kbp). The fragments were random-prime labeled with [α -³²P]-dCTP using a Rediprime DNA Labeling System (Amersham, Arlington Heights, IL), and used immediately for Northern blot analysis.

Quantification of mRNA Encoded by the SGLT1 Gene Using Northern Blot Analysis

Total RNA samples were denatured in 1X MOPS (3-[N-morpholino]-propanesulfonic acid), 6.5% formaldehyde, 50% formamide at 55°C for 10 minutes. Approximately 25 µg of each total RNA sample (44.7 µl volume) was mixed with 8.9 µl of RNA loading buffer (80% vol/vol formamide, 1 mM EDTA, pH 8.0, 0.1% Bromphenol Blue, 0.1% Xylene Cyanol) and loaded in corresponding wells of a 1.2% formaldehyde-agarose gel (1.1% formaldehyde). A sample of total RNA isolated from L929 mouse fibroblast cells was included as a control. The samples were electrophoresed for one hour and 15 minutes at 80V in an E-C Minicell Electrophoresis Gel System and an E-C135 Power Supply (E-C Apparatus Corporation, St. Petersburg, FL) using 1X MOPS running buffer. The RNA bands were transferred to a nitrocellulose membrane using a

TurboBlotter Rapid Downward Transfer System (Schleicher and Schuell, Inc., Keene, NH) and 20X SSC buffer (3M NaCl, 0.3M Na₃citrate•2H₂O, pH 7.0) for approximately 16 hours. The membrane was then gently rinsed in 2X SSC buffer and baked in a Thelco Model 19 vacuum oven (Precision Scientific Company, Chicago, IL) at 80°C for one hour. A Spectrolinker XL-1500 (Spectronics Corporation, Westbury, NY) was used to crosslink the RNA to the nitrocellulose with an optimal energy dose of 120 µJ/cm² from 254 nm UV emissions (Church and Gilbert, 1984). The membrane was placed in a Hybridization Cassette (Scotlab, Inc., Shelton, CT), and immersed in 5 ml of Rapid-Hyb buffer (Amersham Life Science, Arlington Heights, IL) for one hour at 55°C. The prehybridization solution was discarded, and the membrane was immersed in the hybridization solution (5 ml Rapid-Hyb buffer, 25 µl SGLT1 probe [~25 µCi]) for 2 hours at 55°C. The hybridization solution was discarded, and the membrane was rinsed three times with 50 ml of 2X SSC, 0.1% SDS at 25°C for one minute with shaking. The membrane was then rinsed with 50 ml of 1X SSC, 0.1% SDS at 65°C for ten minutes with shaking. After the membrane was rinsed with 50 ml of 0.1X SSC, 0.1% SDS at 65°C for 15 minutes with shaking, it was wrapped in Saran wrap and placed in a Hypercassette with a Kodak BioMax MS Intensifying Screen (Amersham Life Science, Arlington Heights, IL). A 5"x7" sheet of Kodak BioMax MS film was exposed at -80°C for 16 hours, and developed using Kodak GBX Developer and Fixer. The visualized RNA bands on the autoradiograph were video captured to an Apple PowerMac 7200/75 (Apple Computer, Inc., Cupertino, CA) using a Gel Doc 1000 CCD camera and fluorescent lightbox (Bio-Rad Laboratories, Hercules, CA). The Molecular Analyst software package was used to estimate the relative quantities of RNA present.

RESULTS

Preparation of the Probe for the Detection of the SGLT1 mRNAs

The PstI restriction enzyme was used to excise the 1.5 kbp SGLT1 insert from the pBSK(-) vector in the amplified plasmid. To verify that adequate restriction digestion had occurred, the DNA fragments were electrophoresed in a 1.0% agarose gel, and stained with ethidium bromide (Figure 7). Fragments from lambda phage DNA which was cut with HindIII and EcoRI were used as a reference for size estimation of the digested plasmid fragments. Undigested plasmid DNA was used as a control. Both the supercoiled and nicked circular conformations were present. Various concentrations of PstI-digested plasmid DNA were loaded in other lanes, as well as one sample which used twice as much PstI restriction enzyme. The PstI enzyme preferentially cut the plasmid DNA which was in the nicked circular conformation. The pBSK(-) vector and the 1.5 kbp SGLT1 insert can be seen along with some undigested supercoiled DNA.

Separation of Total RNA

RNA samples were electrophoresed on a 1.2% formaldehyde-agarose gel, and stained with ethidium bromide to verify that the isolation procedure was producing intact RNA from the cell culture samples (Figure 8). The presence of well-defined 28S and 18S ribosomal RNA bands was evidence of only minimal degradation. Two SGLT1 mRNA bands (3.9 kb and 2.2 kb) were expected to be located on the Northern blots between the 28S and 18S ribosomal RNA bands, the larger fragment being located very close to the 28S rRNA band and the smaller fragment being located very close to the 18S rRNA band. The 3.9 kb mRNA differs from 2.2 kb mRNA only in the length of the 3' untranslated sequences. Additionally, the presence of a third band (Band 3) was anticipated midway between the 28S and 18S rRNA bands. This band has been observed in previous

Figure 7. Restriction digest of pBSK(-)/SGLT1 plasmid with PstI

Figure 8. Electrophoresed total RNA stained with ethidium bromide

experiments (Shioda et al., 1994), but has not been characterized. The appearance of this band is likely a cell culture artifact caused by the particular isolate of the LLC-PK₁ cells used, or a result of a component present in the growth medium (D. Rhodes, personal communication). However, the band is probably just a modified version of the other two SGLT1 mRNA transcripts. The intensity of the band appears to be proportional to the intensity of the 3.9 kb and the 2.2 kb mRNA bands. Since the 3.9 kb and 2.2 kb mRNA bands are located close to the 28S and 18S rRNA bands, overestimation of the expression of these two mRNAs can occur if residual probe remains hybridized to the rRNAs. Hence, Band 3 is probably a more reliable measure of the overall SGLT1 mRNA expression, and was used for the data analyses of these experiments. Data were collected from all bands when possible; however, in some blots, the 2.2 kb mRNA was too faint to quantify accurately, and was not included. Total RNA collected from L929 mouse fibroblast cells was used as a control. The densities of the corresponding regions of the control lane were subtracted from the band densities of the LLC-PK₁ sample lanes to correct for the error caused by residual probe remaining bound to RNAs other than the SGLT1 mRNAs. Relative increases in mRNA expression compared to the down-regulated state were calculated.

Kinetics Study

Two experimental data sets were collected for the kinetics study. The cell culture growth conditions were the same for both experiments. Cells either remained down-regulated or were up-regulated for periods of time ranging from 1 day to 6 days. Autoradiographs for the experiments are shown in Figures 9 and 10. Graphs of the time-dependent expression of the SGLT1 mRNA after switching from high glucose to low

Figure 9. Northern blot autoradiograph for Experiment 1 of the kinetics study.

Figure 10. Northern blot autoradiograph for Experiment 2 of the kinetics study.

Figure 11. Kinetics study SGLT1 mRNA expression data. The data for Experiments 1 and 2 show a rapid increase in expression initially and maximum expression by 5 days after up-regulation was initiated.

glucose (up-regulation) are provided in Figure 11. The data for the experiments indicate a rapid increase in SGLT1 mRNA expression soon after application of the up-regulation stimulus. The SGLT1 mRNA concentrations reached a maximum at about day 4 or 5. The noticeable decrease in concentration at day 4 of Experiment 2 is inconsistent with the trend of the rest of the data and is most likely a result of experimental error. The major contributing factors to the experimental error between samples were pipet volume inconsistencies and non-uniform transfer of the RNA from the electrophoretic gel to the Northern blot membrane. The experimental error is estimated to be as great as 20%. The maximum levels of SGLT1 mRNA expression were over three times the expression of the down-regulated state. A rapid decline in mRNA concentration was observed for day 6 of Experiment 1; however, this trend is not certain since the data for Experiment 2 does not corroborate this observation. While Experiment 1 demonstrated a rapid increase in expression within a day of up-regulation, such an increase by day 1 in Experiment 2 was not observed. Additional test runs are required to determine whether or not there is a time lag of a day or two before a significant increase in expression occurs. Since both experiments indicated a maximum SGLT1 mRNA expression at about day 4 or 5 after up-regulation had been initiated, total RNA samples were collected at these times for the dose response experiments in order to observe the greatest measurable effects on expression.

Dose Response Study

Four experimental data sets were collected for the dose response study. Experiments 3, 4, and 5 used cells that were exposed to radiation doses ranging from 5 Gy to 20 Gy. The cells used in Experiment 6 were exposed to radiation doses ranging from 5 Gy to 60 Gy. In Experiments 3 and 4, total RNA was isolated from the cells four days

after up-regulation was initiated. Most of the cell samples were irradiated immediately before up-regulation was initiated; however, one of the samples was irradiated with 20 Gy two days after up-regulation was initiated for Experiments 4, 5, and 6. Experiments 5 and 6 utilized similar experimental conditions as described above, except the total RNA was isolated five days after up-regulation was initiated, and one of the samples receiving the largest dose was irradiated three days after up-regulation was initiated.

The Northern blot autoradiographs for experiments 3, 4, 5, and 6 are shown in Figures 12, 14, 16, and 18, respectively. Corresponding bar graphs illustrating the relative increase or decrease of SGLT1 mRNA expression compared to the expression measured for the down-regulated state are included in Figures 13, 15, 17, and 19. All the SGLT1 mRNA concentrations measured for the up-regulated samples were greater than for the down-regulated state. However, no correlation between dose and SGLT1 mRNA expression is apparent. For Experiment 5, the variation of expression among up-regulated samples was quite limited, only varying from approximately 52% to 72% increases.

† Dose was administered two days after up-regulation was initiated. Total RNA was isolated four days after up-regulation was initiated.

Figure 12. Northern blot autoradiograph for Experiment 3 of the dose response study. The cells were irradiated with doses ranging from 5 Gy to 20 Gy.

† Dose was administered 2 days after the up-regulation stimulus was applied.
Total RNA was isolated 4 days after the stimulus was applied.

Figure 13. Dose response study SGLT1 mRNA expression data for Experiment 3. Although all up-regulated samples exhibited greater expression levels than the down-regulated samples, no apparent dose-dependent correlation was observed.

† Dose was administered two days after up-regulation was initiated. Total RNA was isolated four days after up-regulation was initiated.

Figure 14. Northern blot autoradiograph for Experiment 4 of the dose response study. The cells were irradiated with doses ranging from 5 Gy to 20 Gy.

† Dose was administered 2 days after the up-regulation stimulus was applied.
Total RNA was isolated 4 days after the stimulus was applied.

Figure 15. Dose response study SGLT1 mRNA expression data for Experiment 4. Although all up-regulated samples exhibited greater expression levels than the down-regulated samples, no apparent dose-dependent correlation was observed.

† Dose was administered three days after up-regulation was initiated. Total RNA was isolated five days after up-regulation was initiated.

Figure 16. Northern blot autoradiograph for Experiment 5 of the dose response study. The cells were irradiated with doses ranging from 5 Gy to 20 Gy.

† Dose was administered 3 days after the up-regulation stimulus was applied. Total RNA was isolated 5 days after the stimulus was applied.

Figure 17. Dose response study SGLT1 mRNA expression data for Experiment 5. Percent increases in expression for the up-regulated samples compared to the down-regulated samples only ranged from 52% to 72%.

† Dose was administered three days after up-regulation was initiated. Total RNA was isolated five days after up-regulation was initiated.

Figure 18. Northern blot autoradiograph for Experiment 6 of the dose response study. The cells were irradiated with doses ranging from 5 Gy to 60 Gy.

† Dose was administered 3 days after the up-regulation stimulus was applied.
Total RNA was isolated 5 days after the stimulus was applied.

Figure 19. Dose response study SGLT1 mRNA expression data for Experiment 6. No apparent dose-dependent expression effects were observed.

DISCUSSION

Preliminary studies have characterized the development of hexose transport in differentiating cells as they reach a confluent state. Newly seeded cells do not initially express hexose transport activity, but gain transport capacity once confluent monolayers are formed (Amsler and Cook, 1982; Cook et al., 1982). Studies of the SGLT1 mRNA expression have also characterized the development as cells make the transition from a cycling state to a non-cycling state (confluent). The undifferentiated cells do not express detectable quantities of the SGLT1 mRNA. However, an abrupt increase in the SGLT1 mRNA expression has been demonstrated once confluency has been reached (Shioda et al., 1994; Yet et al., 1994). Processes responsible for the development of the Na⁺/D-glucose cotransporter expression (differentiation) and the regulation of its expression (up or down regulation) in established confluent monolayers may be exclusive of each other. Hence, it is important to note that the current research focuses on the fully differentiated cells rather than subconfluent monolayers. Monolayers used for the current studies were maintained for five days in high glucose medium after reaching confluency and would have reached a steady state of SGLT1 mRNA expression.

The published results of both Na⁺/D-glucose cotransporter activity and SGLT1 mRNA expression demonstrate that a time lag exists between increases in SGLT1 mRNA expression and increases in Na⁺/D-glucose cotransporter activity. This likely reflects the time necessary for post-transcriptional events to produce new cotransporters or activate existing cotransporters.

Up-regulation has proved to be a valuable technique for studying SGLT1 mRNA expression and hexose cotransport. Cells grown and maintained in a high glucose (25 mM) medium exhibit a low glucose concentrating capacity. Exchanging the high glucose medium with a low glucose (5 mM) medium induces up-regulation.

Up-regulated cells exhibit an increased glucose concentrating capacity, presumably to compensate for a decreased number of glucose molecules available for transport across the apical membrane. Whereas most preliminary studies have looked at SGLT1 mRNA expression as a result of cells reaching confluency, the present research examines the up-regulation process in established confluent monolayers to determine whether or not ionizing radiation has an inhibitory effect on SGLT1 mRNA expression.

Our kinetics study with unirradiated cells clearly indicates that the optimum expression of the SGLT1 mRNA occurs approximately 4 to 5 days after up-regulation is initiated. The initial increase in transcript probably does not start immediately after the high glucose medium is replaced with low glucose medium. The increase is likely delayed, since time is required for chemical signals to be sent along the regulatory pathways to activate gene transcription. To resolve this time lag, future experiments should measure the SGLT1 mRNA concentrations at times on the order of hours after up-regulation is initiated. Moran et al. (1986) have shown that maximal up-regulated expression of the cotransporters requires 6 to 9 days. Therefore, it appears that increases in SGLT1 mRNA expression precede corresponding increases in cotransporter expression by about one or two days.

Published radiation studies demonstrated that doses of ionizing radiation ranging from 5 Gy to 50 Gy have an inhibitory effect on up-regulation of hexose concentrating capacity (Moran et al., 1986). Furthermore, irradiation must occur within 4 days of initiation of up-regulation in order to observe the full radiation sensitivity of the cells. No inhibitory effect by radiation was observed for samples irradiated 6 days or more after up-regulation was initiated.

When cells are irradiated with fractionated radiation doses rather than a single massive dose, a "sparing" effect is observed which increases the surviving fraction of cells.

This effect is thought to be a direct result of DNA repair during the time periods between doses. If the inhibitory effect of ionizing radiation on up-regulation is a result of damage incurred by DNA, a sparing effect would be expected when fractionated radiation doses are administered. However, fractionated radiation dose experiments have shown a lack of a sparing effect (Parkins et al., 1989). This suggested that the inhibition of Na⁺/D-glucose cotransport up-regulation is a consequence of damage to cellular mechanisms independent of those involved in cell replication. A goal of the current research was to establish whether the ionizing radiation is disrupting the regulatory processes upstream or downstream of mRNA transcription.

The results of our radiation dose response study suggest that the expression of the SGLT1 mRNA is independent of dose with doses ranging from 5 Gy to 60 Gy. While an increase in expression was consistently measured for the up-regulated states, no apparent dose-dependence was observed. Experiments in which samples received a dose 3 days after up-regulation (Figure 17) showed no difference in mRNA expression compared to cells irradiated immediately. If radiation were inhibiting transcript expression, then one would expect that cells irradiated 3 days after up-regulation to have a greater level of expression than cells irradiated immediately. However, this was not the case and we can conclude that radiation does not appear to be inhibiting the up-regulation of SGLT1 mRNA expression as it does cotransport expression.

In some of the cells grown in high glucose, SGLT1 mRNA appeared to be abnormally high. An explanation for this may be that those samples have depleted their glucose after 2 days and have become partially up-regulated. However, in all cases, up-regulation induced increases in mRNA transcript expression. In future studies, changing the growth medium more frequently to prevent glucose depletion and inadvertent up-regulation, will likely demonstrate more dramatic expression differences.

Parkins et al. (1989) used ionizing radiation doses in the range of those used for the current study to confirm that glucose transport capacity was inhibited by those ionizing radiation doses. In the current investigation, the lack of an inhibitory effect of ionizing radiation on the SGLT1 mRNA expression implies that the radiation-induced reduction of glucose transport capacity of LLC-PK₁ cells results from post-transcriptional effects. Studies involving the Na⁺/glucose cotransporter (SGLT1) in ovine small intestine tissue suggest that the level of expression of the SGLT1 mRNA is not significantly affected by the extracellular concentration of glucose per se, but that SGLT1 activity is primarily controlled by post-transcriptional events (Smith et al., 1992; Freeman et al., 1993; Lescale-Matys et al., 1993).

The importance of the glycosylation of the Na⁺/D-glucose cotransporter has been investigated through the use of tunicamycin, but discounted as a cause of glucose cotransport impairment (Wu and Lever, 1994). Tunicamycin is an antibiotic that blocks the first step in the dolichol pathway which is required for the synthesis of the core sequences of N-glycosidically linked oligosaccharides. Inhibition of glycosylation does not affect the functioning or insertion of the Na⁺/D-glucose cotransporters into the membrane of living LLC-PK₁ cells (Wu and Lever, 1994).

Numerous components of the regulatory mechanisms governing the activity and expression of the Na⁺/D-glucose cotransporter are possible targets of ionizing radiation damage. Both cyclic AMP (cAMP) and protein kinase A (PKA) are involved in the stimulation of the expression of the Na⁺/D-glucose cotransporter and SGLT1 mRNA. PKA activity or cAMP levels may be reduced as a result of ionizing radiation, and may account for the apparent up-regulation inhibition of cotransport activity that was observed by Moran et al. (1986) and Parkins et al. (1989).

Chemical inhibitors and stimulators have been used to characterize these regulatory mechanisms in unirradiated cells. Inhibitors, such as H-7 and staurosporine, have been used to study the role of protein kinase C (PKC). Activation of PKC is necessary for the decrease of the SGLT1 mRNA (Shioda et al., 1994) in dedifferentiated cells (cycling cells). Ionizing radiation has been found to activate PKC. For example, it has been found that radiation-induced apoptosis of thymocytes requires activation of PKC to cause DNA fragmentation. However, modulation of PKA does not significantly affect radiation-induced DNA fragmentation (Ojeda et al., 1992).

The half-life of the SGLT1 mRNA may be an important factor involved in post-transcriptional regulation of the Na⁺/D-glucose cotransporter expression. It has been established that when differentiated cells are dedifferentiated by reseeding at subconfluent densities, the half-life for the reduction of the SGLT1 mRNA transcript is approximately 1.5 hours (Shioda et al., 1994). PKC activity has been shown to be responsible for reduction in SGLT1 mRNA half-life. PKA activity and cAMP appear to be necessary to activate a protein factor that binds to a uridine-rich region in the mRNA, and to lengthen the mRNA half-life by increasing its stability. It has been suggested that the transcription rate of the SGLT1 mRNA may be responsible for the crude control of the Na⁺/D-glucose cotransporter expression; whereas, modulation of the half-life of the mRNA may cause fine adjustments of the expression (Freeman et al., 1993; Lescale-Matys et al., 1993). These mechanisms may be targets of radiation action that would inhibit the expression of SGLT1 cotransporter expression on the apical membranes.

A wide range of chemical compounds have been used that may provide clues about radiation effects on Na⁺/D-glucose cotransport regulation at various points along its regulatory pathway. Phosphodiesterase, which normally inhibits the activity of cAMP, is one such point. Hexamethylene bisacetamide (HMBA) and 3-isobutyl-1-

methylxanthine (IBMX), phosphodiesterase inhibitors, have been shown to increase the expression of the SGLT1 mRNA in LLC-PK₁ cells (Yet et al., 1994). Theophylline and MIX (1-methyl-3-isobutyl xanthine), also phosphodiesterase inhibitors, have been used as well to stimulate SGLT1 mRNA expression and Na⁺/D-glucose cotransport (Hilden and Sacktor, 1979). Chemical compounds have also been used to modulate the regulatory pathways at the cotransporter level. N⁶, O^{2'}-dibutyryl adenosine 3':5'-cyclic monophosphate (dbcAMP) has been used to stimulate Na⁺/D-glucose cotransport activity. The dbcAMP functions like cyclic AMP, but has butyryl groups that prevent it from being broken down by phosphodiesterase. Hence, it has a stimulatory effect on the PKA regulatory pathway, and increases cotransport activity (Valentich et al., 1979).

Another post-transcription effect which may be involved in the radiation-induced inhibition of Na⁺/D-glucose cotransport activity is alteration of the sodium gradient which drives the Na⁺/D-glucose cotransporters. Ouabain, which inhibits Na⁺/K⁺ ATPase activity, has been used to reduce the sodium gradient. Therefore, it has an inhibitory effect on the cotransport activity (Valentich et al., 1979).

Another potential cause of the observed radiation-induced Na⁺/D-glucose cotransport inhibition may be the release of arachidonic acid from the phospholipid bilayer of cell membranes when exposed to ionizing radiation. Free arachidonic acid may be metabolized through the cyclooxygenase pathway or the lipoxygenase pathway to form eicosanoids. Eicosanoids have been demonstrated to have a role in maintaining homeostasis and pathological roles in inflammation and disease (Walden, 1987). Some of the hundreds of different potential eicosanoid products may have direct or indirect effects on the Na⁺/D-glucose cotransporter and SGLT1 mRNA expression.

A DNA repair mechanism which may have indirect effects on Na⁺/D-glucose cotransport is the 5'-diphospho-5-β-ribosyl transferase (ADPRT) mechanism (Berger et

al., 1983). The enzyme is activated by DNA damage, and it forms poly(ADP)ribose chains attached to the proteins associated with the DNA strands. This ribosylation causes the chromatin to uncoil so that repair may be more efficient. However, the ribosylation utilizes NAD^+ , depleting the supply needed for glycolysis and oxidative phosphorylation. As a result, a reduced amount of ATP is produced. A reduction of such a key energy source as ATP has implications throughout the cell. The extent of the ribosylation and NAD^+ depletion is dependent on the amount of DNA damage that has been inflicted.

Although evidence suggests that ionizing radiation is acting at a post-transcriptional level, wide-spread damage throughout the genome may be a possible mechanism as well. An explanation for the abnormal gene expression effects seen in irradiated cells may be related to cell cycle arrest, carcinogenesis and apoptosis. Since the probability of ionizing radiation interacting with a single specific gene in every cell in the population is not very great, it is likely that multiple targets within the genome are potential influencers of these processes. That is, damage to any of these targets may initiate these processes. Damage may also be additive, so that the greater the DNA damage, the greater the severity of the effects. Disruption of the various regulatory pathways by this damage may be an explanation for the altered gene expression effects. In *E. coli*, an SOS response exists that causes specific alterations in gene expression in response to massive DNA damage (Walker, 1984). If an analogous response exists in eukaryotic cells, then some of the gene expression effects observed in ionizing radiation studies may be due to the triggering of this response.

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