Optimization of Protocol for the Study of Glucose Transporters

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Abstract

The purpose of this experiment was to determine if it was possible to make changes in the current method of glucose transporter study, which is often undertaken in an attempt to find a link between glucose transporters and diabetes. In order to do this, an antibody against rat glut-4, one of the isoforms of glucose transporters was obtained from East Carolina University School of Medicine. The antibody was then purified with a Protein A column. The glut-4 glucose transporter was isolated from homogenized rat skeletal muscle. A liver sample was also taken as a negative control. The proteins taken from the homogenization of liver and muscle were immobilized on nylon paper by dot blotting. A primary antibody, antiglut-4 antibody from East Carolina University, was added to the immobilized proteins. A secondary antibody, goat anti-rabbit antibody, which was conjugated to horse radish peroxidase enzyme was added to the protein-primary antibody complex. When substrate for the enzyme was added, a color change would have indicated whether or not the glucose transporter was present. In this experiment, no color change was detected. An ELISA test was performed in an attempt to determine which step in the procedure was not working. Both the primary and secondary antibodies were found to be functional by the ELISA test. This indicates that the problem in the procedure may be in the extraction of the antigen (glucose transporter).

Purpose

The objective of this experiment is to determine whether it is possible to improve current methods used in glucose transporter study without sacrificing results. Although most researchers follow similar procedures in studying glucose transporters, there are many procedure decisions that can be made by researchers. For example, there are several options for purifying antibodies from serum. A researcher may choose to use a Protein A column, a Protein G column, or a diethylaminoethyl column.

In this experiment, the methods of separation and detection of glucose transporters will be altered. Typically in glucose transporter studies, the glucose transporter protein is isolated by polyacrilimide gel electrophoresis followed by Western Blotting. For this experiment, a blotting kit marketed a Bio-Rad Laboratories called dot blotting will be used instead of the other two procedures. The current methods of protein detection uses radioactive isotope labeled antibodies. As an alternate to this, enzyme-linked antibodies will be used. Although glucose transporters are frequently studied, no optimal procedure has yet been established. The purpose of this experiment is to determine if these alternate methods may be considered an improvement over current methods because of the safety and ease with which the alternates can be performed.

Introduction

Characteristics of Glucose Transporters -

Glucose is the major source of energy for cells and for this reason, virtually all animal cells have a facilitated type transport system. The proteins which facilitate the diffusion of glucose are called glucose transporters (Bell, 18). The natural forms of sugar which are transported by the glucose transporter are D-glucose, D-galactose, D-mannose, and D-xylose (Carruthers, 1135). So far, five forms (called isoforms) of glucose transporters have been identified and sequenced and have had their structures and functions intensely studied since their discovery in the 1980's (Glucose, 58). Each of the glucose transporters has been named numerically, from one to five, according to the order in which they were discovered (Glucose, 59). Each of the isoforms has its own set of characteristics. One characteristic property is the effect on each of the glucose transporters caused by hormones, like insulin, and metabolic conditions, like hyperglycemia or hyperglycemia (Carruthers, 1135). Each form of glucose transporter also has its own major site of expression, affinity for glucose, and supposed function (Glucose, 59). Characteristic properties also include the number of amino acids in each isoform's peptide chain and molecular weight (Bell, 18-20). These characteristic properties are important in being able to distinguish one glucose transporter isoform from another.

Glucose transporter isoform 1, abbreviated glut-1, was the first glucose transporter to be discovered. Glut-1 occurs in tissues throughout the body

(Glucose, 58). In fact, it has been found in all cell types studies to date, but is most commonly found in red blood cells and brain tissues. The affinity of glut-1 for glucose refers to the force with which glut-1 and glucose are chemically attracted to each other. The affinity measurement, called the affinity constant and abbreviated Km, is measured in units of millimoles/L -- "mM". The affinity constant, Km, for glut-1 is 1 to 2 mM. The lower the Km, the more attracted the glucose transporter and the glucose are to each other. This is true because the value given for Km is the concentration of glucose required to cause the transporter to bind and transport glucose at half its maximum value. If the Km is high, it takes a large concentration to be present before the transporter works at half its maximum value. The low Km for glut-1 shows a strong affinity for glucose (Glucose, 59). The supposed function of glut-1 is non-insulin mediated uptake of glucose in cells. There are 492 amino acids which make up the peptide chain of glut-1. The molecular weight of glut-1 is 45,000 to 55,000 daltons. The sequence conservation, the amount of identity between rat and human forms of glut-1, is 98%, which suggests that all regions of this particular transporter are important in either glucose transport or regulation of glucose transport (Bell, 18).

The second isoform of glucose transporters is found mainly in liver, kidney, small intestine, and Beta-cells, cells of the pancreas responsible for making insulin. The tissues in which glut-2 is found are those tissues that are important in maintaining blood glucose homeostasis and glucose sensing. The affinity constant of glut-2 is 15 to 20 mM which indicates a low affinity.

Glut-2 is the only isoform identified so far that has a low affinity for glucose (Glucose, 60). The supposed function of glut-2 is glucose homeostatis (Glucose, 59). There are 524 amino acids in the peptide chain of glut-2. The glut-2 sequence is 55.5% identical to glut-1 and there is 82% identity between rat and human glut-2 (Bell, 19).

Glut-3, the third isoform, is found in many tissues including kidney, placenta and brain. The Km of glut-3 is less than one, indicating a great affinity for glucose. The function of glut-3 is unknown (Glucose, 59). A glut-3 peptide chain has 496 amino acids. There is 64% identity between glut-1 and glut-3 and 52% identity between glut-2 and glut-3 (Bell, 20).

Glut-4 isoform is found in muscle and fat tissues. The Km for glut-4 is approximately 5 mM. The supposed function of glut-4 is responsibility for insulin-mediated glucose uptake (Glucose, 59). There are 509 amino acids in a glut-4 peptide chain and there is 65%, 54%, and 58% identity to isoforms 1, 2 and 3 respectively. There is 95% identity between rat and human glut-4 (Bell, 20). Glut-4 is important to researchers because it appears to be a factor in insulin resistance (Glucose, 59). Insulin resistance is the body's lack of ability to respond normally to insulin and is a condition present in non-insulin dependent diabetes mellitus (or diabetes type II). Diabetes type II is a condition in which a person is insulin resistant even though insulin levels are normal or even raised (Glucose, 58). Normally, insulin causes glut-4 transporters to move from the interior of the cell to the cell membrane where they can take up glucose (Glucose, 50). Studies with two forms of low insulin conditions rats, one group of fasted rats and one group with induced diabetes

suggest that reduced glut-4 expression is responsible for lack of efficiency of insulin in diabetes type II patients (Bell, 22).

Glut-5 isoform is found in small intestine and kidney. Its affinity constant is 1 to 2. Its function is unknown (Glucose, 59). There are 501 amino acids in a glut-5 peptide chain. The sequence of glut-5 is 42%, 40%, 39%, and 42% identical to isoforms 1, 2, 3 and 4 respectively (Bell, 20).

Although glut-4 is the major suspect in insulin resistance, the study of all isoforms is important. All of the glucose transporters work towards the common goal of glucose homeostasis (Glucose, 62). Research on glucose transporters may prove to be clinically valuable for the prevention and treatment of diabetes type II, which is currently the most common form of diabetes. The disease affects 10 million people in the United States. Diabetes type II can lead to blindness, kidney failure, and cardiovascular disease (Glucose, 58).

Steps in the Procedure for Isolation and Detection of Glucose Transporters -

The steps required for the study of glucose transporters include 1) isolation of membrane proteins, 2) separation of glucose transporters from other membrane proteins and immobilization of glucose transporters, 3) detection of glucose transporters, and 4) quantitation of glucose transporters. Different tools and techniques can be used to achieve each of these steps. The purpose of this research is to change the tools and techniques to improve the method of glucose transporter study.

Isolation of Membrane Proteins -

Glut-4 is a membrane protein found in skeletal muscle and fat. This means that glut-4 is located in the cell membrane of muscle (or fat) tissue. To isolate a membrane protein such as glut-4, a sample of tissue must be taken from a sacrificed animal. The donated tissue must then be homogenized. This homogenate must then be centrifuged in an ultracentrifuge machine. The result of this centrifugation is a supernatant (liquid) waste and a pellet containing packed cell membranes and the contents of the membranes. Another centrifugation results in a superntant containing solubilized membrane proteins and a waste pellet of the lipid membrane. After this procedure has been done, glut-4 and all of the other types of membrane proteins found in the cells of the donated tissue are present together in the same pellet.

Separation of Glucose Transporters from Other Membrane Proteins and Immobilization of Separated Glucose Transporters -

The first steps to be changed in this experiment are separation and immobilization of glucose transporters. Following homogenization and ultracentrifugation, electrophoresis is then usually performed to separate the glucose transporters from the other membrane proteins collected in the pellet of the second centrifugation. Electrophoresis is a technique in which different molecules (such as proteins are placed in a gel material. The gel containing the molecules is then exposed to an electrical field. When the molecules are exposed to the electrical field, a force is exerted on the molecules. This force

causes the molecules to move within the gel. The force on each of the molecules depends on two factors: strength of the electrical field and charge of the molecules. The distance each molecule travels through the gel depends on its molecular weight, shape and size because of the effects of friction and the opposing force caused by the gel on the molecule (Dunbar, 4). Therefore, smaller or lighter molecules travel further in the gel, separating themselves from large or heavy molecules.

After electrophoresis, the next step is usually Western Blotting, the transfer of proteins from the supporting gel used in electrophoresis to nitrocellulose or nylon paper. The purpose of this transfer is to stop the proteins from continuing to move through the electrophoresis gel. Another reason for this transfer is so an antibody can be used to identify the protein. The transfer is often done electrophoretically (Dunbar, 155-156). The proteins are often blotted onto nitrocellulose paper because it binds proteins well (Bers, 278).

Because of the complexity and the time required in electrophoresis and Western Blotting, one goal of this experiment is to determine if dot blotting is easier to use. Dot blotting is an assay method introduced in the early 1980's for rapid immobilization and identification of proteins. The use of a dot blot assay eliminates the need for electrophoresis and Western Blotting. The dot blotting assay kit used in this experiment is marketed by Bio-Rad Laboratories. The kit consists of a square plastic base which is fastened to a matching plastic top. The top portion of the kit has wells made into it. Nitrocellulose, nylon, or other suitable paper is placed between the base and the top so that the

proteins will become bound to it. There is a hose connected to the kit which can be connected to a special water faucet to create a vacuum and increase the filtration rate. To use a dot blot assay kit, the proteins isolated from centrifugation must be diluted to an acceptable concentration and placed into the wells of the dot blot top. The protein solution is then filtered through the paper (nylon for this experiment), allowing the liquid to go through the base and out the hose while the proteins are trapped on the paper. This filtration can be done using gravity to pull the liquid or by creating a vacuum. After the proteins have been bound to the paper, they are incubated for a period of time. The incubation time may vary from one to several hours. After the incubation period, detection or identification of proteins can be done.

Detection of Glucose Transporters -

After the glucose transporters have been immobilized on paper by either electrophoresis and Western Blotting or dot blotting, the next step of the procedure is detection of the proteins. Detection of proteins often requires the use of an antibody. Antibodies are proteins made by a body in response to an antigen in the body. An antigen is a material foreign to the body. There are two categories of antigens. One category is called particulate and these are such things as red blood cells or viruses. The second category, soluble antigens, included proteins not usually found in the body. Antibodies usually bind by chemical attraction specifically to the antigen against which they were created (Cooper, 13). There are five classes of antibodies: IgA, IgM, IgD, IgE and IgG. IgGs are the most common class of antibodies (Cooper, 14).

The structure of antibodies consists of three regions. Two of these regions are Fab regions and one is an Fc region. Fab means "Fragment antigen-binging" and Fc means "Fragment crystllizable." The Fab region is where antigen-antibody binding occurs whereas the Fc region lacks antibody activity. The Fc region is so called because this region is crystallizable in the cold, unlike the Fab regions (Cooper, 179). A secondary antibody is an antibody which is against another (primary) antibody.

The detection of glucose transporters is the second step to be altered in this experiment. In this experiment, a detection method called ELISA will be used. ELISA is an acronym for Enzyme Linked Immuno Sorbent Assay. In the ELISA method of detection, an antigen is detected by means of an enzyme chemically coupled to a secondary antibody against primary antibody--which is against the antigen. The presence of the antigen is signaled by a color change resulting from the addition of the substrate for the enzyme (Chambers, 100). ELISA assays are beneficial because the enzymes used require no special storage, handling or disposal (Bers, 280). The glucose transporter, bound to the nylon from dot blotting, is the antigen. The primary antibody is anti-rat glut-4 (from East Carolina University), an IgG class antibody. The anti-rat glut-4 is made by introducing rat glut-4 into a rabbit's body. Before the primary antibody can be used it must be purified using a Protein A column.

Protein A is a protein found on the surface of certain strains of Staphylococcus bacteria (Chard, 118). Because of the way Protein A reacts with antibodies, it has been used in a procedure called affinity chromatography.

The use of Protein A in affinity chromatography is done in a Protein A column. A Protein A column, a plastic column containing Protein A, is used to purify antibodies. Purification occurs by the Protein A's binding to the antibody as it is poured through the column (Dunbar, 138). The Protein A binds mostly to the Fc region of an antibody, so the antibody retains its antigen binding ability. Protein A binds to some classes of antibodies, but not to all. One class to which it does bind is class IgG, of which anti-rat glut-4 is an example. Protein A binds best to IgGs that are obtained from rabbits, guinea pigs, humans, and nonhuman primates (Dunbar, 140).

After the primary antibody has been purified, it is ready for use in detecting the antigen (rat glut-4). The primary antibody will become bound to the nylon at the site of the antigen. The secondary antibody is goat anti-rabbit IgG conjugated to horse radish peroxidase (GAR-HRP). In the ELISA method of detection, the secondary antibody-enzyme complex binds to the primary antibody where it is bound to the antigen. After the antigen-primary antibody-secondary antibody-enzyme complex is created on the nylon, substrate for the enzyme can be added to cause a color change wherever the complex is located (Walker, 100).

This method of detection is being tested as an alternative to the current practice of using radioactive isotope labeled antibodies to detect antigen presence. Commonly, a radioactive isotope of iodine, ^{125}I, is used to determine whether or not a specific protein is present in a sample. For research projects such as this one, the radioactive iodine is purchased already conjugated to a secondary antibody. The radioactive iodine is conjugated to

the secondary antibody by its incorporation into the amino acid tyrosine of the antibody protein (Osterman, 120). The secondary antibody will bind to the primary antibody at the location of the antigen. The location of the primary antibody-secondary antibody-radioactive iodine complex can be determined by putting the sample in contact with x-ray film (Osterman, 120).

Using enzyme conjugated secondary antibodies rather than radioactive iodine conjugated secondary antibodies avoids problems associated with radioactive substances. One problem with radioactive isotopes is their potential for damaging the health of the researcher. Contamination of workers with radioactive substances is one of the greatest dangers faced by biomedical researchers. Although the amounts of radioactivity used in radioactive labels are relatively small, there are still dangers in the use of radioactive labels. Radioactive contamination may cause skin death and decay. Another possible problem is ingestion of food or drink contaminated with isotopes from skin or clothes. Radioactive iodine specifically may cause severe damage to the thyroid tissue where it accumulates if ingested. Radioactivity is also carcinogenic (Chard, 244-245). Disposal of radioactive materials is also a problem because potential harm to the environment (Chard, 247).

Quantification -

Often, the final step in studying glucose transporter is quantification. For this experiment, quantification was not required because the results were

evaluated qualitatively instead of quantitatively. However, there are two ways of doing quantification whenever it is needed.

The optimal method, in terms of safety, is spectrophotometry which is used after ELISA is done for detection. In spectrophotometry, light is passed through a cuvette containing a liquid in which there is protein to be quantified. The amount of light that is absorbed by the liquid is displayed by the spectrophotometer. The amount of light absorbed is directly proportional to the amount of protein in the sample. Therefore, using a standard curve established by plotting a graph of absorption versus protein concentration for known samples, the amount of protein in the sample can be determined.

The second method of quantification is called autoradiography. Autoradiography is done by putting a sample containing a radioactive iodine conjugated secondary antibody and x-ray film together in a container so that they can touch each other and be kept in the dark (Osterman, 120). The radioactive iodine in the sample decays and emits radiation which causes a chemical conversion of silver halides in the film to metallic silver. The metallic silver shows up as a dark spot on the x-ray film (Dunbar, 117). The size and density of the resulting spots is directly affected by the amount of radioactivity present at any location in the sample (Chard, 66). Because autoradiograph is done after radioactive labels have been used, the same safety hazards discussed with radioactive labels apply. Another problem is that background control is more difficult in autoradiography than in spectrophotometry (Bers, 280).

Procedure

The first step in this experiment was to purify the rabbit anti-rat glut-4 antibody received from East Carolina University School of Medicine. This was done with a Protein A column. Before the column could be used, buffer solutions has to be made. The first solution made was Phosphate Buffered Saline (PBS) which was used to store the antibodies after they had been purified. To make the PBS, the following ingredients were mixed together: .10g of Sodium Monobasic Phosphate, .600g of Sodium Dibasic Phospate, 2.550g of NaCl, and 300 ml of water. The pH required for PBS was 7.2. When checked with the pH meter, the pH was 7.14. To raise the pH 6 Normal NaOH was used. 6 Normal NaOH was made by adding .6 moles of NaOH to .1L water. After one drop was added, the pH was 7.30. To lower the pH to 7.2 prepared 1 Normal HC1 was used.

Next, the binding buffer was prepared. The binding buffer is used to create a binding atmosphere in the Protein A column. To make this buffer, 31.4g of buffer mix, packaged with the column, was added to enough water to make 100ml total volume. This mixture was stirred for 10 minutes. The solution was then filtered with an aspirator flash and the pH was adjusted to 9.0 ± .02 in the same manner as above.

Next, the elution buffer, which was necessary to wash the purified antibody out of the Protein A column was made. This was made by mixing 2.2g of elution mix with enough water to make 100ml total volume. It was then stirred for 10 minutes, filtered, and the pH was adjusted to 3.0± .2.

Before the crude antibody sample could be put into the Protein A column, the salt had to be removed from the sample. This was done with the 10 DG Desalinating column provided with the Protein A column. 20 ml of PBS was added to the column to push out the waste with which the column is packed. When the PBS had pushed out the waste and was below the frit, the result was a binding atmosphere in the desalting column. The column instruction said to use 3 ml of sample. Since only 9µl of antibody sample was available for this experiment, it was diluted with 2.1ml of binding buffer to make 3ml. The first 3ml to be pushed out of the column were discarded, so the PBS and sample were then in the column below the frit. The sample was then eluded with 3ml of binding buffer. Binding buffer was used instead of elution buffer so the sale bound to the column would not be eluted with the sample. The sample with the salt removed was the "prepared Sample." The prepared sample was then ready to e purified. (The desalting column was washed with 20ml of .02% Sodium Azide so it could be reused).

The protein A column contained a packing buffer which had to be removed before the column could be used. This was poured off the top. The column was then equilibrated with 10ml of binding buffer. 2.0ml of the prepared sample (which contained PBS and antibody) was added to the column. 30ml of binding buffer were added. The antibodies were then washed out of the column with 10ml of elution buffer, resulting in the "purified sample." The 3ml of sample were then added to 8ml of PBS and this was put into a column (not a Protein A column) and divided between 10 cuvettes, each receiving 1ml of the PBS-sample. This was done to avoid

having all the antibodies in one sample. An absorption reading was done with a spectrophotometer to see if any proteins were present in the liquid in the cuvettes. The absorption reading for a cuvette with distilled water was .30. The readings for cuvettes 1-10 (containing the sample) were: .145, .112, .113, .146, .151, .108, .216, .123, .135, and .138 respectively. This indicated that there probably was some protein in the fractions.

After the antibodies were purified, the Pilch's Homogenization Buffer required for homogenization of the rat muscle and liver was made. This buffer was made by adding together 1.49g of Hepes, .37g of EDTA, .98g of benzamidine, 17µl of leupeptin, 17µl of pepstatin, 25µl of aprotinin and .087g of phenylemethylosulfonyl flouride dissolved in .5ml dimethyl sulfoxide. The phenylmethylsulfonyl flouride was added last, as the instructions indicated. To make the correct concentration of aprotinin, 1g was added to 153ml of water. Three one thousandths of a gram of leupeptin was added to 100µl of water to make the correct concentration of it. Seven one-thousandths of a gram of pepstatin was added to 10ml of water for the appropriate concentration.

After the buffer was made, the muscle and liver was cut out of the sacrificed rat. The muscles taken out of the rat were the gastrocnemius and the rectus femoris. The liver was taken to be used as a negative control. The liver does not contain glut-4 so the substrate should not have produced a color change on the nylon paper where the liver was. The muscle samples that were taken from the rat were labeled by number and then weighed. Ml weighed 192mg, M2, M3, and M4 weighed 192mg, 211mg, 200mg, and 194mg

respectively. The liver was also labeled and weighed. The weights of M1-M4 were 210mg, 203mg, 208mg, and 212mg respectively. The muscle and liver tissues were then frozen with liquid nitrogen so they could be ground into a powder. Each muscle and liver sample was then homogenized in 2ml Pilch's buffer with the Polytron machine at speed 70 for 15 seconds. While being homogenized and in subsequent steps, the tissues had to be kept on ice or refrigerated. The homogenate was then centrifuged in the ultracentrifuge at 40,000RPM (100,000g) for 1 hour at 4°C. This centrifuge packed all of the cell membraned into a pellet in each of the tubes with a tissue sample. Since the glut-4 is a membrane protein, it would be in these pellets. Since the material of interest was in the pellets, they were saved and the supernatant was discarded. The pellets were then loosened from the centrifuge tubes, 360µl of Pilch's buffer was added to each tube, and the contents of each was homogenized again with the Polyton at speed 60. 40µl of 10% Triton X was added to each tube. The 10% Triton X was made by adding 1ml 100% Triton X to ml water. The contents of the tubes were then mixed with a vortex machine and kept on ice for 1.5 hours. The samples were then centrifuged at 40,000RPM for 1 hour at 4°C. This centrifugation packed the membranes and solubilized the cell proteins. The desired proteins were in the supernatant which was saved. The pellets were discarded. The supernant tubes were then labeled and kept frozen until needed again.

The next step was to do the dot blot assay. Solutions for this were made first. Tris buffered saline (TBS) was the first solution made. This was done by dissolving 4.84g tris and 58.48g NaCl in 1.5L of water, the pH was brought to

7.5 and the final volume was raised to 2L. The next solution made was Tween TBS (TTBS). This was made by adding .5ml Tween 20 detergent to 1L of the TBS. A blocking solution was made by adding 1g Bovine Serum Albumin to 100ml TBS. An antibody solution was made by adding 2g Bovine Serum Albumin to 200ml TTBS. The instructions with the dot blot did not provide information on what concentration of protein sample to use, so several concentrations were made and used to determine which works best. The proteins were diluted by adding 250µl of TBS to the sample labeled M1. This mixture was vortexed and 250µl was taken out of it and added to a tube labeled M1D2 (first muscle sample, second dilution). 250µl of TBS was added to this, it was vortexed, and 250µl was taken out and added to M1D2. This process was carried out with each of the 4 muscle samples and the 4 liver samples until there were six different concentrations for each sample. When the dilutions were complete, 250µl of the samples were put into each of the wells on the dot blot assay kit. Underneath the tray with the wells was a plastic base where filtered liquid would be collected. Between the top tray with wells and the bottom tray was a piece of nylon paper to which the proteins would stick. The liquid in the wells was allowed to filter by gravity at first, but the higher concentrations would not filter so the vacuum system was used. The vacuum system was necessary to get the liquid out of the wells so when the top tray was removed, the liquid would not flow onto the proteins on the nylon. When the vacuum was turned on, the empty wells and the wells in which filtration was complete had to be covered to avoid drying of the proteins. The concentration that worked best was a 1:10 ratio of

sample to TBS. Higher concentrations filtered too slowly. After filtration was completed, 200µl of blocking solution was added to each well and allowed to remain for 1 hour. This was followed by two washes with 200 µl TTBS in each well. After this step was completed, the proteins were immobilized on the nylon paper. The proteins then were incubated in a Blotto solution overnight. The Blotto solution is 5% Nonfat Dry milk in water.

After the proteins were incubated, the nylon paper was washed with TBS. This was done by adding enough TBS to cover the paper in a plastic tray with sides. The plastic tray was put on a Red Rocker agitation machine for 10 minutes. This process was repeated with TTBS. To bind the primary antibody to the glut-4 proteins on the nylon TTBS, 10ml 1% Blotto and 1ml of purified antibody was put into a tray with the nylon. The correct amount of antibody, according to the dot blot instructions can only be determined experimentally for each individual project. The nylon remained in the antibody solution for 2 hours. After 2 hours, the nylon was washed twice in TTBS in the manner described earlier. The next step was to bind the secondary antibody, goat anti-rabbit IgG (GAR), to the primary antibody. To do this 32µl GAR and 100µl 1% Blotto was put into a tray with the nylon for 1 hour. The small amount of GAR was used so that background could be minimized. During this hour, the color developer was made by adding 900ml water the bottle of HPR color buffer supplied with the GAR. This color buffer was added to 600µl of the supplied color reagent B. 20 ml Color reagent A was added just before the color developer was needed. The reagents A and B together made the substrate for the enzyme.

Results

When color developer was added, no color change occured, therefore the test results for the presence of glut-4 were negative. Since the results for the dot-blot test revealed a problem in the procedure, steps were taken to determine which part of the procedure went wrong. The substrate and enzyme were tested first, then the secondary and primary antibodies.

To determine if the problem was in the substrate-enzyme complex and color developer, all three of these were added to the microplate wells used in the dot-blot assay. When the three were added, there was a color change which indicated that the substrate and enzyme were working properly.

Next the secondary and primary antibodies were tested to see if they worked properly. This was done with the ELISA test. The procedure for this required that solutions be made. The solutions made were PBS, PBS-TWEEN, blocking solution, diluted secondary antibody solution, and substrate solution. The PBS was made by adding .105g of Sodium Monobasic Phoshate, .600g of Sodium Dibasic Phosphate, 2.55g of Sodium Chloride, and 300ml of water. The pH was adjusted to 7.2 with Sodium Hydroxide. PBS-TWEEN was made by adding .1ml of tween to 200ml of PBS. The blocking solution was .3g of Serum Bovine Albumin in 30ml of PBS. The secondary antibody was diluted 1:3000 (33µl; 99ml) in PBS-TWEEN. The substrate for the enzyme was made by adding 5ml of Solution A and 5ml of Solution B, both of which were supplied with the dot-blot kit. To ensure freshness, the substrate was not made until just before it was needed. The primary antibody

was added to the wells of a microtitration plate. Some wells received 100µl, some 200µl and some 300µl of the primary antibody. 100µl of secondary antibody was also placed in two separate wells. The plate was incubated for one hour at room temperature and was then flick washed once using PBS. Blocking solution was added, 300µl to each well, to stop non-specific binding. It was incubated for 30 minutes at room temperature, and flick washed with PBS-TWEEN three times. The secondary antibody was added to each well in which there was not already some bound, it was incubated at room temperature for one hour, and flick washed with PBS-TWEEN four times, and once with PBS. The substrate was made at this point, and 100µl was added to each well. The plate was then incubated at room temperature for 30 minutes or until color developed, whichever was first.

In each of the wells there was a color development. Since the secondary antibody conjugated with enzyme reacted with the substrate and caused color, it can be concluded that the secondary antibody worked properly.

The wells with primary antibodies also developed a color change, indicating that the primary antibody had bound to the plate, the secondary antibody had bound to the first antibody, and the substrate reacted with the enzyme bound to the secondary antibodies. This proved that the primary antibody was working properly.

Conclusion

Since the substrate, enzyme, secondary and primary antibodies were all proven to be working properly, the negative results were caused by failure to effectively isolate the antigen--glut-4. Mistakes were probably made in the isolation of the antigen from the rat tissue. For example, the temperature in the ultra centrifuge during one centrifugation was too high for several minutes. Such an increase in temperature could result in failure to isolate the glucose transporter from the rat tissue. Although the isolation did not work, the other variable procedures did provide useful information. The dot-blot proved to be easy enough to be used in subsequent research. Also, the ELISA procedures were found to be a safe and effective alternative to other more dangerous procedures. Most importantly, in addition to being easier and/or safer than current methods, the procedures tested in this experiment (dot blot and ELISA) are just as effective, and can therefore be considered optimal for use in glucose transporter study.

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