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RESEARCH ARTICLE

Antidiuretic hormone effect on transport of non-charged solutes (urea and glycerol) across the epithelium

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ABSTRACT

Background: The frog skin epithelium is used as a model to demonstrate the transport of various charged and non-charged solutes from the mucosal to serosal side through channels and transporters. The mucosal side, otherwise termed as the apical side or outer side, is the side which is exposed to the external environment (towards pond water). The serosal side, also known as the basolateral side, faces the internal environment of the organism. O**bjectives:** The objective of the present study is to determine the action of antidiuretic hormonal (ADH) on the movement of non-charged solutes such as urea and glycerol across the frog skin epithelium. **Materials and Methods:** For this study, the ventral abdominal skin of frog, species *Rana hexadactyla* was mounted in an Ussing's type chamber. Normal Ringer's solutions containing urea (5 mM) and glycerol (90 mM) each were placed on the mucosal side, and ADH was added either on the mucosal side or the serosal side. After the addition of ADH, the colorimetric method was used to measure the transport of urea at the absorbance of 540nM and glycerol absorbance of 505 nM using a spectrophotometer; statistical analysis was done by Wilcoxon-Signed Ranks Test, all values were expressed as a mean ± standard error of means, $n = 4$. **Results:** Addition of ADH (40 nM) on serosal side increased the urea transport from control 29.3 ± 5.1 µmol/dL to 52.3 ± 6.8 µmol/dl and increased glycerol transport from 34.8 ± 6.0 µmol/dL to 45.3 ± 6.6 µmol/dL, whereas addition of ADH on the mucosal side did not increase the glycerol transport, the control value was 14.6 ± 4.3 µmol/dL and with ADH that the value was 9.6 ± 2.9 µmol/dL. **Conclusion:** The results concluded that the addition of ADH on serosal side enhanced the transport of urea and glycerol. Addition of ADH on the mucosal side did not show any effect on transport of glycerol.

KEY WORDS: Antidiuretic hormone, Aquaporin, Frog skin epithelium, Urea and Glycerol, Spectrophotometer.

INTRODUCTION

The frog skin epithelium is used as a model to demonstrate the transport of various charged solutes (sodium and chloride) and non-charged solutes (urea and glycerol) across the mucosal and serosal membranes through channels and transporters to maintain the homeostasis of the body fluid.[1,2] Aquaporins (AQP), which exists as intrinsic membrane proteins on the plasma membrane of cells, is responsible for the transport of water molecules.[3] The mechanism of water transport across the epithelia is well known in vertebrates, invertebrates, plants, eubacteria, archaebacteria, and other microbes. Nearly, 13 AQP have been identified in mammals. The aquaglyceroporin is selectively permeated either to water as AQP, to maintain the osmolarity of the body fluid, or to water along with other solutes such as glycerol, urea, ammonia, and gases including carbon dioxide, nitric oxide, as well hydrogen peroxide. AQP-mediated urea transport involved in energy metabolism, glycerol transport involved in epidermal hydration, CO_2 and NH_3 in maintains of

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intracellular acid-base homeostasis. AQP-3, 7 and 9 permeate urea and glycerol.[4-7]

Glycerol transports are essential during periods of fasting or starving. The triglycerides degraded in adipocytes exited through AQP-7 and entered into hepatocytes through AQP-9 for gluconeogenesis to occur.[8] AQP-3 deficient mice showed dry skin with reduced skin elasticity as well as impaired epidermal biosynthesis. These Aquaglyceroporins are essential for fat metabolism and normal skin structure. The defects of these AQPs were used to study skin diseases and obesity-related to fat metabolism.[9]

Urea is an end product of nitrogen metabolism and is excreted in the urine. In general, urea passes through AQP or active transport in a hyperosmotic saline solution or dehydration. Antidiuretic hormone (ADH) has its effect on urea transport and consequently helps maintain the osmolarity of the body fluid.[10]

The objective of the present study is to prove the role of ADH in the transport of non-charged solutes urea and glycerol across the epithelium.

MATERIALS AND METHODS

The methods used were approved by the Institutional Research Committee and animal ethics committee adhered to the legal requirement of the country. Frogs of the species *Rana hexadactyla*, obtained from a local animal vendor were anesthetized with ether and pithed. The ventral abdominal skin was dissected and mounted between two Perspex half plates of an Ussing type chamber. The area of the skin exposed for the ionic transport was 1 cm^2. Initially, both sides of the skin were bathed with aerated normal ringer (NR) solution with the composition as follows (in mM): NaCl 115.0; KCl 2.5; $CaCl_2$ 1.0; $MgCl_2$ 1.0; HEPES 3.5; glucose 10.0; and pH 7.35–7.4.[2] To assess urea and glycerol transport, NR's solutions containing urea (5 mM) and glycerol (90 mM) each were placed on the mucosal side, ADH added on the serosal side. Colorimetric method was used to measure urea absorbance at 540 nm and glycerol absorbance of 505 nm. Materials used in this study include an Ussing chamber, frog skin, spectrophotometer, vertex, incubator, and test tubes (20 ml).

Method to Measure Urea Transport

There were three samples which were collected at different intervals during the period of the experiment.

Blank (B)

NR solution was placed on both sides of Ussing chamber and was kept for an hour. After an hour, 200 µl of the sample was collected from the serosal compartment.

Test sample. 1 (T.1)

The mucosal solution was replaced with 5 mM urea dissolved in NR and after half an hour, 200 µl of the sample was collected from the serosal compartment.

Test sample. 2 (T.2)

Subsequent to this ADH (40 nm) was added in the serosal compartment, and 200 µl of the sample was collected after an hour.

Urea Estimation was Done by Diacetyl Monoxime-Thiosemicarbazide Method[11]

Principle

Urea reacts directly with diacetyl monoxime under strongly acidic conditions to give a yellow condensation product. This reaction was intensified by the presence of ferric ions and thiosemicarbazide; less concentrated sulfuric acid (H_2SO_4) was then needed, and the resulting red colored complex was a measure of the urea concentration.

Procedure

The samples and the reagents (diacetyl monoxide, ferric chloride, thio semi-carbazide, and H_2SO_4) were mixed well using a vertex, 100 µl of the sample was added to a test tube (volume of 10 mL), and 1 mL of reagent was added to the same. Glass marbles were kept on the mouth of the test tubes to avoid evaporation, and all the tubes were placed in a vigorously boiling water bath for 20 min. The tubes were brought to the room temperature by keeping them in cold water. Urea transport was measured at an absorbance of 505 nm using the spectrophotometer. The instrument was adjusted in such a manner that the optic density (O.D) of the blank solution (B) was zero. Then, the O.D. readings for the standard and test were taken.

Calculation:

$$\text{The actual transport of urea } (\mu\text{mol / dL}) = \frac{\text{Optic density of test}}{\text{Optic density of standard}} \times \text{Dilution factor } (100)$$

Method to measure glycerol transport

There were three samples which were collected at different intervals during the period of experiment.[12]

Blank (B)

NR solution was placed on both sides of Ussing chamber and was kept for an hour. After an hour, 200 µl ml of the sample was collected from the serosal compartment.

Test sample.1 (T.1)

The mucosal solution was replaced with 100 mM glycerol dissolved in ¼ of NR and after half an hour, and 200 µl of sample was collected from serosal compartment.

Test sample.2 (T.2)

ADH was added on the serosal compartment, and 200 µl of sample was collected after an hour.

Procedure

The samples and the reagents (Pipes buffer, 4 - chlorophenol, Magnesium ion, ATP, lipase, peroxidase, glycerol kinase, 4-amino antipyrine, glycerol 3-phosphate oxidase, detergents, preservative, and stabilizer) were mixed well using a vertex. They were incubated at 37°C for 10 min, and glycerol transport was measured at an absorbance of 505 nm using the spectrophotometer.

Pipette into tube marked	Blank	Standard	Test
Sample	-	-	10 µl
Reagent 2	-	10 µl	-
Reagent 1	1000 µl	1000 µl	1000 µl

Calculation:

$$\text{Glyceride } (\mu\text{mol/dL}) = \frac{\text{Optic density of test} \times \text{Dilution factor } (100)}{\text{Optic density of standard}}$$

Statistical Analysis

Estimated by Wilcoxon-signed ranks test. All values were given as mean ± standard error of mean (SEM), $n = 4$. Probability values of $P < 0.05$ were considered significant.

Chemicals

NaCl and KCl were procured from SD Fine Chemicals, $CaCl_2$ from Merck (Mumbai, India); $MgCl_2$, glucose, urea, and glycerol and HEPES were obtained from Sigma Chemical, USA. ADH (Pitressin - lysine vasopressin) from Hanlim Pharm Co. Ltd, Seoul, Korea.

RESULTS

Effect of ADH on Urea Transport Across the Frog Skin

The 5 mM urea dissolved in NR solution placed on the mucosal side considered as control, and NR was placed on the serosal side. Addition of ADH (40 nM) on serosal side increased the urea transport from control 29.3 ± 5.1 µmol/dL to 52.3 ± 6.8 µmol/dL (values were shown as mean ± SEM, $n = 4$, $P < 0.05$ by Wilcoxon-signed ranks test).

Addition of ADH (40 nM) on the serosal side, increased the urea (5 mM) transport from mucosal to serosal side (mean ± SEM, $n = 4$).

Glycerol Transport Across the Frog Skin with Addition of ADH on Serosal Side

Glycerol (90 mM) was dissolved in ¼ diluted NR solution and was placed on the mucosal side as control, and NR was placed on the serosal side. Addition of ADH (40 nM) on the serosal side increased the glycerol transport from 34.8 ± 6.0 µmol/dL to 45.3 ± 6.6 µmol/dL (values were shown as mean ± SEM, $n = 4$, $P < 0.05$ by Wilcoxon-signed ranks test).

Addition of ADH (40 nM) on serosal side had increased the glycerol transport from mucosal to serosal side (mean ± SEM, n = 4).

Glycerol Transport Across the Frog Skin with Addition of ADH on Mucosal Side

Glycerol (90 mM) was dissolved in ¼ diluted NR solution and was placed on mucosal side as control and NR was placed on the serosal side. Addition of ADH (40 nM) to the mucosal side did not show an increase in the glycerol transport from mucosal to serosal side. Initially, the glycerol transport was 14.6 ± 4.3 µmol/dL and with the addition of ADH that the glycerol transport was 9.6 ± 2.9 µmol/dL (mean ± SEM, $n = 4$).

Addition of ADH (40 nM) on the mucosal side had no effect on glycerol transport from mucosal to serosal side (mean ± SEM, $n = 4$).

DISCUSSION

The novel focus of this study is to demonstrate the transport of urea and glycerol across the frog skin in association with ADH stimulation. The present study proved that the transport of urea was minimal without ADH and it was significantly increased after the addition of ADH. The ADH influenced the transport of urea was well demonstrated in the species *R. hexadactyla* [Figure 1]. The rate of glycerol transport was minimal without ADH, significantly increased with ADH addition on the serosal side [Figure 2]. In general, the location of a receptor is determined using hormones with demonstrating a dose-response curve. This study used another method to find out the availability of the receptors by the addition of hormone on both sides of the membrane separate. The addition of ADH on the mucosal side did not show any effect on the glycerol transport [Figure 3]. It confirmed the presence of a receptor for ADH on the serosal side.

In mammals, nearly 13 AQPs have been identified. AQP-0 is present in the lens to maintain fluid balance. AQP-1 is for the formation of aqueous humor in the eye, the formation of cerebrospinal fluid in the brain, hydration of airways in the lungs, and most importantly leads to water reabsorption in the proximal tubules and thin descending loop of Henle.

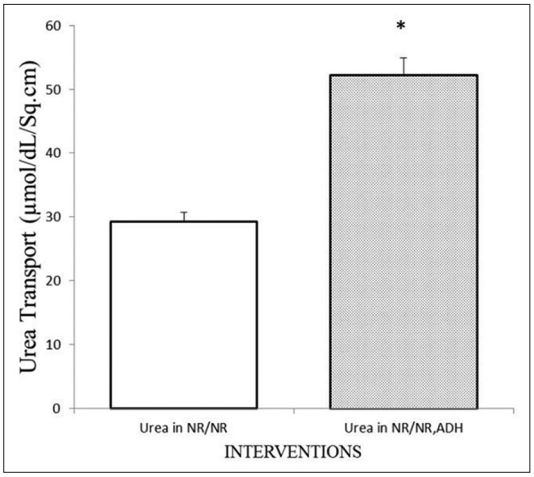

Figure 3: Addition of ADH on mucosal side for glycerol transport across the frog skin

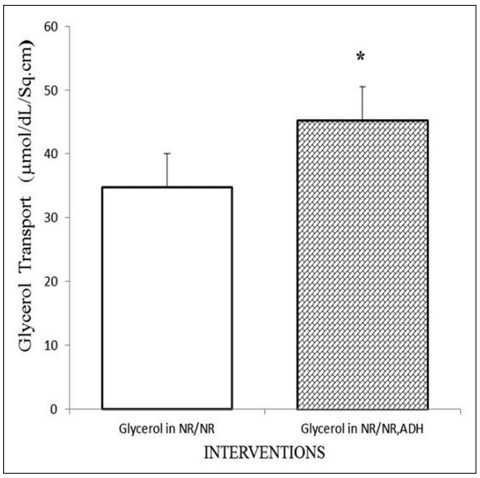

Figure 2: Glycerol transport across the frog skin with addition of ADH on serosal side

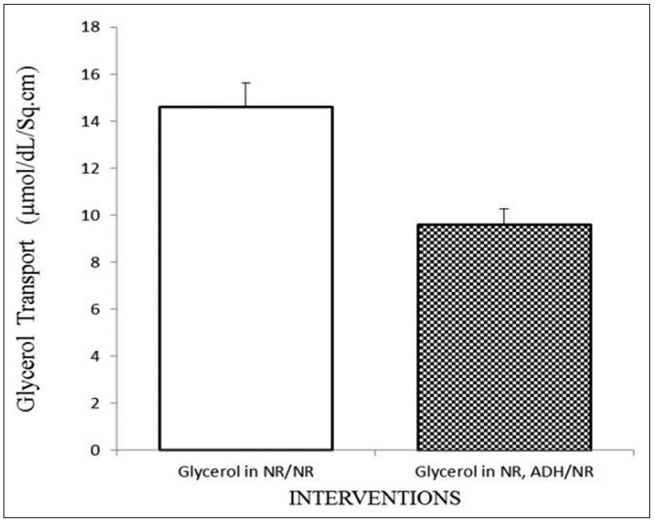

Figure 3: Addition of ADH on mucosal side for glycerol transport across the frog skin

ADH activates AQP-2 to be inserted on the luminal side as well AQP-3 and AQP-4 on the serosal side of the collecting duct of kidney. AQP-3 is located in many organs such as kidney, airways, skin, and eye. AQP-4 is also present in brain, kidney, lungs, and eye.[13-16] AQP-5 is associated with secretion of fluid in exocrine tissues. Location of AQP-6 is not apparently localized. AQP-7 is found on sperms, and AQP-8 is responsible for the secretion of pancreatic juice.[3] Expression of AQP-9 in leukocytes suggests that the protein may be pharmacologically important during the treatment of promyelocytic leukemia with arsenite. The cellular localization and functions of AQPs 10–13 are not clear.[17] Ward *et al*. had discussed the ADH regulation of water, sodium, and urea transport.[18] In the absence of ADH, the epithelia of collecting duct exhibited very low permeabilities to sodium, urea, and water and allowed the excretion of the large volume of urine. Whereas in the presence of ADH the apical membrane of the collecting duct cell showed increased permeability of water through AQP, sodium through apical sodium channels, and urea through vasopressin-regulated urea transporters on the apical membrane of epithelium which, in turn, involved in excretion of less volume of urine. Urea is transported through a membrane transport protein. Two types of urea transport proteins are present in humans and other mammals, UT-A and UT-B. The renal urea handling is by UT-A proteins and regulated by ADH.[19,20] Inhibition of UT-A results in diuresis due to urea-induced osmosis in the collecting ducts of the kidney. UT-B is expressed at the basolateral and apical regions of the descending vasa recta.[21] AQP-3 allows water and glycerol flux at the pH range of 5.8–6.2, which is required to open AQP-3 channels for the transport of glycerol but not water.[22] AQP-3 is commonly seen in mammalian structures such as epithelium of cornea and conjunctiva of eye, kidney, stomach, spleen, intestine, and erythrocytes.[23,24] Hormonal influence on the expression of various AQPs is well known. For example, aldosterone influences the occurrence of AQP-3 in rats; meanwhile, aldosterone deficiency significantly reduced the presence of AQP-3. Similarly, AQP-2, AQP-3, and AQP-4 are expressed to regulate water permeability in the epithelium of principal cells in the collecting duct and the connecting tubule segment in the presence of ADH.[25] In Xenopus oocytes, AQP-7 was involved in the transport of glycerol, urea, and water.[26] AQP-7, originally cloned from rat testis, is a member of the aquaglyceroporin family and expressed in several organs including adipose tissue and brush-border membrane of the proximal tubule of the kidney.[27,28] The development of AQP-7 knockout mice has reinforced the understanding of the physiological role of AQP-7. The research in AQP-7 knockout mice proved that this AQP-7 was primarily involved in the transport of glycerol rather than urea and water.[29] In Xenopus oocytes that the expression of AQP-7 enhanced the permeability to arsenite, indicating that it also serves as a route for the uptake of arsenite in mammalian cells.[30]

The strength of this experimental model is used to study the role of ion channels and transporters of gastric mucosa, small intestine, colon, gall bladder, various sections of vertebrate nephron, airway epithelia, corneal epithelium of the eye,

choroids plexus, exocrine glands, etc., and also useful for screening the drugs on above preparations.

The remarkable limitation of this study is that the restricted arose on the studies of the animal model by the wildlife protection act due to ecology concern. Hence hands-on experiences with an animal model for the scholars are restricted.

CONCLUSION

This study demonstrated enhanced transport of urea and glycerol by the addition of ADH on serosal side. Addition of ADH on the mucosal side did not show any effect on the transport of glycerol, whereas addition on the serosal side showed its effect. Hence, this study further proved the presence of ADH receptors on the serosal side.

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