THE EFFECT OF TRANS-CINNAMALDEHYDE ON NA,K-ATPASE ACTIVITY IN RABBIT NONPIGMENTED CILIARY EPITHELION

By

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The Effect of trans-Cinnamaldehyde on Na,K-ATPase Activity in Rabbit Nonpigmented Ciliary Epithelium

Brian Gerard Espiritu

The ciliary epithelium (CE), the tissue responsible for aqueous humor (AH) production, is the outer layer of the ciliary process and is composed of the pigmented epithelium (PE) and the nonpigmented epithelium (NPE). Previous studies have shown that inhibition of Na,K-ATPase in the CE by ouabain causes a 62% decrease in AH formation and that there is more Na,K-ATPase in the NPE than in the PE. In addition, cinnamaldehyde (CA) has been shown to inhibit Na,K-ATPase and other ATPases in different tissues and organisms, such as rat hepatocyte and certain bacteria.

The purpose of this study was to determine if trans-CA inhibits Na,K-ATPase in rabbit NPE (rNPE).

Na,K-ATPase activity was measured using a colorimetric method quantifying the amount of ATP hydrolyzed. Rb⁺ which is transported by Na,K-ATPase in a way similar to K⁺, was measured through atomic absorption spectrophotometry.

The present study indicates that trans-CA has a linear concentration-dependent inhibitory effect on Na,K-ATPase and non-Na,K-ATPase activity in rNPE at concentrations of 25 and 50 μM.

Trans-CA may be used as a compound to inhibit Na,K-ATPase in the CE in order to decrease aqueous humor production and intraocular pressure.
ἡ περὶ τῆς ἀληθείας θεωρία τῇ μὲν χαλέπῃ τῇ δὲ ῥαδίᾳ. Σημεῖον δὲ τὸ μὴ τ' ἀξίως μηδένα δύνασθαι θιγεῖν αὐτῆς μήτε πάντας ἀποτυγχάνειν. ἀλλ' ἐκαστὸν λέγειν τι περὶ τῆς φύσεως, καὶ [καθ' ἑνα μὲν ἢ μηθὲν ἢ μικρὸν ἐπιβάλλειν αὐτῇ,] ἐκ πάντων δὲ συναθροίζομένων γίγνεσθαι τι μέγεθος.

- Aristotle, *Metaphysics*: 993a30-993b1, 4th Century BC
INTRODUCTION

The ciliary body is a musculoepithelial structure, composed of the ciliary muscles and the ciliary processes. It is located in the posterior chamber and forms a circular boundary. The ciliary epithelium (CE), the outer layer of the ciliary process, is composed of the nonpigmented epithelium (NPE) and the pigmented epithelium (PE). Fenestrated capillaries within the ciliary stroma allow the ciliary processes to have a rich blood supply.

Fig. 1 & 2: A transverse view of the porcine ciliary process. The dark band is the cuboidal-celled PE (on both diagrams), with the inner lumen as the ciliary stroma with the fenestrated capillaries, while the outside layer of columnar cells is the NPE. Fig. 1 is from Shahidullah, 2010, unpublished data; Fig. 2 is from Bill, 1975.

The CE actively transports ions and solutes from the blood within the stroma into the posterior chamber, which leads to the formation of aqueous humor (AH), a fluid which creates intraocular pressure (IOP). First, ions and solutes are transported from the stroma into the PE via transmembrane proteins. Second, the ions and solutes diffuse across gap junctions into the NPE. Third, the ions and solutes are transported into the posterior chamber via active transport. The buildup of an osmotic gradient in the posterior chamber induces osmosis, thus forming the AH.

The nascent AH from the posterior chamber flows over the lens, past the pupil, and into the anterior chamber. It then flows over the iris, percolates through the trabecular meshwork, and
then drains into Schlemm’s canal and ultimately to the venous circulation. Through this sojourn, AH provides nutrients and oxygen to the avascular components of the eye, such as the lens, cornea, and the trabecular meshwork. Through the hydrostatic pressure (the IOP), it also maintains the correct geometry of the eye ball which is conducive to light transport and vision (Shahidullah et al., 2011 (a)).

The transport of ions and solutes from the stroma into the posterior chamber is accomplished through different types of transporters on both the PE and the NPE. Currently there is still much to know about the specific and the joint functions of both cell layers (Shahidullah et al., 2011 (a)). There is evidence that both components of the CE work in a syncytium to transport ions and solutes via gap junctions (Raviola and Raviola, 1978). Ions, such as Na⁺, Cl⁻, HCO₃⁻, and solutes, such as amino acids and glucose, are actively transported by the NPE into the posterior chamber (Shahidullah et al., 2011 (a)). Research up to this point has yielded the composite picture of transporters in the CE, as shown in Figure 4.

![Diagram of the human eye](image)

**Fig. 3:** A superior, transverse view of the human eye, showing the circulation of aqueous humor (yellow arrows). From Distelhorst et al., 2003.
Acceptable levels of human IOP can range from 10.5-21 mmHg, with the average being 15 mmHg (normal IOP) (Davson, 1990; Hurvitz et al., 1991). While normal IOP is needed to maintain eye structure, high levels of IOP may lead to ocular problems. For example, continuous high IOP may damage the optic nerve and may cause the visual loss characteristic of the ocular condition glaucoma (Hernandez, 2000; Johnson et al., 1996). Glaucoma is an eye disease frequently associated with a decrease in AH filtration from the eye and is characterized by: high IOP (>21 mmHg), changes in the shape of the fundus (the area behind the lens), and visual field loss (Page, et al, 2006). Strictly, it is defined as “the loss of function in retinal ganglion cells resulting in reduced visual acuity and ultimately blindness” (Page, et al, 2006). Traditionally, high IOP is connected with open angle glaucoma, though sometimes, high IOP may not cause glaucoma, nor glaucoma be characterized by high IOP. Despite this, high IOP is still the most prevalent risk factor for glaucoma and is therefore the first target for treating the disease (Shahidullah et al., 2011 (a); Page et al., 2006). Currently, treatment of high IOP is a major preventative action and treatment for glaucoma, with reduction of degenerative visual abilities as the ultimate goal of treatment (Page et al., 2006).
Currently, lowering IOP is achieved either by: decreasing AH formation e.g. by β adrenoceptor antagonists, carbonic anhydrase inhibitors, or by increasing the drainage of AH into the canal of Schlemm (Page et al., 2006). Current clinical treatment of glaucoma that is aimed at reducing AH secretion primarily utilizes β adrenoceptor antagonists, also known as β blockers (Shahidullah et al., 2011 (a)). Clinically, timolol is the most commonly used β blocker, in the form of topical cream applied to the eye. Carbonic anhydrase catalyzes the conversion of carbon dioxide and water to carbonic acid and bicarbonate. Since bicarbonate is a major ion used in the PE and NPE to create AH, then inhibiting ocular production of bicarbonate leads to a decrease in IOP (Shahidullah et al., 2011 (a); Page et al., 2006). This successful mode of treatment demonstrates that controlling the levels of the specific ions of desired transmembrane proteins relevant to the production of AH may generally decrease IOP.

Inhibition of ciliary epithelial Na,K-ATPase with ouabain in a whole eye perfusion system leads to a 62% reduction in AH production, which indicates that this transport protein has a major role in the formation of AH (Shahidullah et al., 2003). In addition, Na,K-ATPase is expressed in greater levels in the NPE than in the PE (Ghosh et al., 1990). Because of these, targeting Na,K-ATPase exclusively in the NPE may be a method of decreasing AH production and IOP.

Cinnamaldehyde (CA) is the molecule in cinnamon (genus Cinnamomum) which gives the spice its distinctive smell and taste. The *trans* isomer of CA is the most commonly found isomer in nature and is frequently used in research. CA has been shown to be an interesting,
salubrious, and biologically-active compound. For example, it has anti-carcinogenic properties in a human melanoma SCID mouse xenograft model (Cabello et al., 2009), causes a vasodilatory effect on isolated rat aorta (Yanaga et al., 2006), and inhibits the aldose reductase in rat lens (Lee, 2002). Specifically, CA has been shown to inhibit Na,K-ATPase and other ATP dependent transmembrane proteins in different tissues, such as rat hepatocyte (Usta et al., 2003) and bacteria (Gill et al., 2006 (b)).

In the present investigation, it is reported that trans-CA inhibits Na,K-ATPase in transformed rabbit NPE (rNPE) in a concentration-dependent manner.

**MATERIALS AND METHODS**

**Cell culture**

Simian virus 40-transformed rabbit NPE was used for all experiments. The cell line was a gift from Dr. Miguel Coca-Prados (Head of Glaucoma Research Group, FIO-Fernandez-Vega Ophthalmological Institute, Spain) and was developed in accordance with ARVO guidelines for use of animals in research. The cells were grown in cell media, composed of 1x DMEM (+4.5 D-glucose, +L-Glutamine, +25 mM HEPES, -sodium pyrate; Gibco Life Technologies Corp., Grand Island, NY), 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 0.1% epidermal growth factor (Sigma-Aldrich, St. Louis, MO), and 0.1% Gentamicin (Sigma-Aldrich, St. Louis, MO). The cells were incubated in a humidified CO₂ incubator, grown until confluence, and were trypsinized using 0.25% Trypsin-EDTA (Gibco Life Technologies Corp., Grand Island, NY). Trypsin was neutralized with 1:1 FBS/NCS (Atlanta Biologicals, Lawrenceville, GA). Cells were then plated onto either 60 mm plastic petri dishes for the Na,K-ATPase activity assay (Corning,
Tewksbury, MA) or onto 24-well plates for the rubidium uptake measurement (Becton Dickinson Labware, Franklin Lakes, NJ). In both experiments, confluent monolayers of rabbit NPE in the 26th passage were used.

**Krebs Solution Composition**

The Krebs solution used in these experiments was constituted of the following (in mM): 119 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 25 NaHCO₃, 5.5 D-(+)-glucose, 1.0 MgCl₂, 2.5 mM CaCl₂, resulting in 300 mOsm. The solution was then bubbled with 5% CO₂ for 35 minutes, adjusted to pH=7.4, and then placed in an incubator to reach 37°C. All materials were purchased from Sigma-Aldrich, St. Louis, MO.

For the rubidium uptake study, rubidium-containing Krebs solution was used after pre-incubation with the Krebs solution described above. The rubidium-containing Krebs solution had the following differences: KCl was replaced with 4.7 mM RbCl and KH₂PO₄ was replaced with 1.2 mM NaH₂PO₄.

**2x ATPase Buffer Solution Composition**

The 2x ATPase buffer solution used in these experiments constituted of the following (in mM): 200 NaCl, 20 KCl, 6 MgCl₂, 2 EGTA, and 1% Halt Protease Inhibitor Cocktail EDTA-free (Thermo Scientific, Rockford, IL). The buffer was made at twice the necessary concentration so that after addition of ATP and other reagents to the assay mixture, the final concentration of the buffer for the ATPase assay would be at the correct level. The 2x ATPase buffer was kept at 0°C throughout the experiment. All salts were purchased from Sigma-Aldrich, St. Louis, MO.
Pre-Na,K-ATPase Sample Preparation

The confluent and monolayered rNPE was washed with Krebs solution and incubated for one hour. Stock solutions of trans-CA in DMSO (Sigma-Aldrich, St. Louis, MO) were added to the cells to obtain 10, 25, and 50 μM trans-CA. Control cells were exposed to 0.2% DMSO. The cells were incubated with DMSO or trans-CA for 30 minutes at 37°C. The drug solution was then removed and the cells washed with cold Krebs solution. The Krebs solution was aspirated and the cells were scraped in 500 μL of ice cold 2x ATPase buffer and homogenized using a Misonix Sonicator 3000 (QSonica, Newtown, CT) at 3.5 W at four pulses of sonication with 15 second intervals. The samples were then centrifuged at 14,000 RPM for 25 minutes in 4°C using an Eppendorf Centrifuge 5415C. The supernatant was then separated.

Measurement of Na,K-ATPase Activity

Na,K-ATPase activity was measured using a modification of a method used by the Delamere Lab (Shahidullah et al., 2011 (b)). Proteins were measured using the bicinchoninic acid assay, with bovine serum albumin as a standard (Smith et al., 1985) (Thermo Scientific Rockford, IL). The supernatants were diluted with 2x ATPase buffer so that there was about 40 μg of protein per 200 μL of sample. 400 μL of each sample was placed into two tubes so that there were two sets of tubes per sample, each tube containing 200 μL. 5 μL alamethicin (Santa Cruz Biotechnology, Dallas, TX) was added to each tube to ensure that the ions and ATP could reach the membrane vesicles. Then 80 μL of 0.2 M L-histidine (Sigma-Aldrich, St. Louis, MO) was added (final concentration 40 mM). Cold double distilled water (ddH₂O) was added to each set so that at incubation, the total volume of liquid would be 400 μL. Half of the tubes received
ouabain (final concentration 500 μM) and both sets were allowed to pre-incubate with shaking at 37°C for 5 minutes in a shaking hot tub (Boekel Scientific, Feasterville, PA). 40 μL of ATP was then added (final concentration 2.0 mM) to both sets and they were allowed to incubate for 30 minutes at 37°C. At this step, the total volume per tube was 400 μL; therefore the 2x ATPase buffer was diluted to the correct concentration.

After 30 minutes, 150 μL of cold 15% trichloroacetic acid was added to stop the ATPase reactions. The samples were shaken well and placed in a Beckman GPR Centrifuge (Beckman Coulter) at 4°C at 4000 RPM for 15 minutes. Standards of inorganic phosphate (using NaH2PO4) were prepared: 0, 10, 62.5, 125, 250, 500 nmol of PO₄³⁻ served as the standards. 400 μL of the supernatant was placed in new glass tubes. 400 μL of a mixture of 4.0% FeSO₄ solution in ammonium molybdate (7.88 g ammonium heptamolybdate tetrahydrate per 100 mL 2.5 N sulfuric acid) was added to each sample and standard and shaken well. 250 μL from each sample and standard were placed in 96-well plates within 15 minutes and the absorbance was read at 750 nm using a Perkin Elmer 1420 Multilabel Plate Reader, Victor³V (Perkin-Elmer Corp., Shelton, CT). The values measured were the amount of ATP hydrolyzed and the final data was expressed in nanomoles of ATP hydrolyzed per milligram of protein per 30 minutes.

Samples without ouabain were considered the total ATPase activity. Samples with ouabain were considered the non-Na,K-ATPase activity. The difference between the two values (ouabain-sensitive) was considered the Na,K-ATPase activity. Results were analyzed using Student’s $t$-test and significance was set at $p<0.05$. 


Measurement of Rubidium Uptake

The confluent and monolayered rNPE within the 24-well plates was washed twice with 0.3 mL Krebs solution and were incubated for one hour for equilibration. The wells were designated for four conditions: control, 100 µM ouabain, trans-CA (25 or 50 µM), and 100 µM ouabain and trans-CA (25 or 50 µM). After one hour, each plate was treated with the above conditions using Krebs solution for ten minutes. After ten minutes, the solutions were removed and replaced with new solutions of the same conditions using rubidium-containing Krebs solution and were then allowed to incubate for 30 minutes. The solutions were then removed and the entire plate submerged and washed four times in cold, isotonic MgCl₂ (100 mM, pH=7.4) to remove any extracellular rubidium. 300 µL of ddH₂O was added to each well and the plates were allowed to stand at room temperature for at least 30 min. To minimize evaporation, the plates were wrapped with aluminum foil.

The cells were then homogenized using a Misonix Sonicator 3000 (QSonica, Newtown, CT) at 3.5 W for 30 seconds. The homogenate was transferred into a 1.7 mL Eppendorf tube and further homogenized for 15 seconds at 4.5 W using the same sonicator. ddH₂O was added to the wells to catch any remaining rubidium and transferred into the Eppendorf tube, thus reaching a final volume of around 1 mL. Cells treat rubidium similarly to potassium as a substrate of transmembrane proteins. Therefore, the activity of the Na,K-ATPase and any other transmembrane protein utilizing potassium can be quantified by measuring the intracellular content of rubidium.
The amount of rubidium was determined using a Perkin Elmer AAnalyst 100 Atomic Absorption Spectrometer (Perkin-Elmer Corp., Norwalk, CT). Rubidium chloride solutions were prepared: 3.125, 6.25, 12.5, 25, 50 µM of Rb⁺ served as the standards. The data was standardized using the amount of proteins detected (using the bicinchoninic acid assay, as described above). The data was expressed as µmoles of rubidium per milligram of protein and was analyzed using Student’s t test and significance was set at p<0.05.
RESULTS

Using the ATPase assay described above, it was determined that trans-CA had an inhibitory effect on both ATPase ouabain-sensitive and ouabain-insensitive activity. Since both activities decreased, total ATPase activity decreased as well. In the concentrations investigated, the highest percent inhibition was less than 50%, indicating that although trans-CA has inhibitory properties, it is not as high or lethal as ouabain, which is a known potent and full inhibitor of Na,K-ATPase.

**Fig. 5.** Total ATPase activity, ouabain-insensitive (non-Na,K-ATPase), and ouabain-sensitive ATPase activity (Na,K-ATPase activity) in rNPE significantly decreased when incubated with 25 or 50 μM trans-cinnamaldehyde for thirty minutes. Values are mean ± SEM of 5-6 individual samples and * (P<0.05), ** (P<0.01), or *** (P<0.001) indicate significant inhibition as compared to the corresponding control.
Percent Inhibition

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<td>25 µM CA</td>
<td>-9.53 ± 1.86 **</td>
<td>-8.10 ± 2.24*</td>
<td>-15.41 ± 2.37 *</td>
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<td>50 µM CA</td>
<td>-25.84 ± 1.76 ***</td>
<td>-21.69 ± 1.54 ***</td>
<td>-42.96 ± 7.41**</td>
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Table 1. Percent inhibition by trans-CA on total ATPase activity, ouabain-insensitive, and ouabain-sensitive ATPase activity (Na,K-ATPase activity). *, **, *** indicate significant differences in activity from control, p<0.05, p<0.01, and p<0.001 respectively.

It was also determined that the effect of trans-CA in the concentrations investigated is concentration-dependent. Both the total ATPase activity and the non-Na,K-ATPase activity were the most linear (R²= 0.99) while the Na,K-ATPase activity was less linear (R²= 0.94).

![ATPase Activity of rNPE with trans-CA](image)

Fig. 6. The linear concentration-dependency of the inhibition caused by trans-CA.

In the rubidium uptake study, the rubidium content of the cells exposed to just ouabain significantly decreased by 15%, which is a lower level of inhibition in comparison to other types.
of cells exposed to the same concentration of ouabain. There was also a significant inhibition in cells exposed to 50 µM trans-CA alone, ouabain plus 25 µM trans-CA, and ouabain plus 50 µM trans-CA and the levels of inhibition were 15%, 17%, 26%, respectively. Though the inhibition by ouabain did not seem to fully occur, a significant decrease by 50 µM trans-CA when compared with the control and a further decrease in activity in the ouabain and 50 µM trans-CA group when compared with the ouabain group, indicates that trans-CA, under this condition, inhibits Na,K-ATPase to some extent. Though it is difficult to ascertain the quantitative inhibition by trans-CA, some level of inhibition to Na,K-ATPase must occur, since most of the intracellular content of potassium (rubidium) is due to transport by Na,K-ATPase. Therefore this effect can be ascribed to some extent to inhibition of Na,K-ATPase.

**Fig. 7.** Rubidium uptake in rNPE treated with 100 µM ouabain, and/or 25 or 50 µM trans-CA. Each bar is the mean ± SEM of 6-18 individual samples. *** (P<0.001) indicates significant inhibition compared to the corresponding control. Note that cells treated with both ouabain and 50 µM trans-CA were significant (P<0.001) as compared to cells treated with just ouabain. Note that even though ouabain did not show a strong inhibition, the decrease was statistically significant.
DISCUSSION

To recapitulate, *trans*-cinnamaldehyde was shown to have a linear concentration-dependent inhibitory effect on non-Na,K-ATPase and Na,K-ATPase activity in transformed rabbit nonpigmented ciliary epithelium.

The rubidium uptake study indicates that there is some level of inhibition of Na,K-ATPase (and possibly other potassium-using transmembrane proteins) in cells treated with 50 µM *trans*-CA.

Current research of the effects of CA specifically on the eye has not been fully explored. CA has an inhibitory effect on the aldose reductase in rat lens, which suggests a possible use of the compound to treat diabetic-induced cataracts (Lee, 2002). Their data suggests that CA has a saturation point around 0.4 mM and that the relationship is a concentration-dependent one.

Some research has been done on CA in other tissues. In rat hepatocytes, CA dissolved in ethanol has an inhibitory concentration-dependent effect on Na,K-ATPase, with the strongest effect occurring around 2.5-7.5 mM CA and 50% inhibition occurring at 4.7 ± 0.04 mM CA (Usta et al., 2003). CA significantly decreased ATPase activity in *E. coli* (at 0.1-10 mM) and *L. monocytogenes* (at 10 mM), in a concentration-dependent manner (Gill et al., 2006(b)). Also, at these inhibitory concentrations, CA is lethal to the cell, but it is also the concentration at which membrane disruption occurs; therefore, inhibition of ATPases is not the main mechanism of death (Gill et al., 2006 (a)).
Since the experiments in this investigation indicate that trans-CA inhibits Na,K-ATPase in a linear concentration-dependent manner in much lower concentrations than previous literature and since this transmembrane protein is a crucial protein in AH formation, trans-CA or cinnamon in general, may prove to be a natural method of treating glaucoma, via the control of the levels of sodium and potassium.

It has been considered that since Na,K-ATPase is an integral transmembrane protein responsible for viable physiological levels of sodium and potassium and correct intracellular voltage for cell survival, inhibition with trans-CA may be lethal. The data of this investigation suggests that in NPE the effective inhibitory concentration is much lower than the level in which it was suggested that the compound is lethal to the cell (Gill et al., 2006 (a,b)). In addition, the fact that the NPE was still able to collect rubidium at the highest concentration in this investigation (50 μM) indicates that the cell is still viable to some extent. In order to ensure non-lethal and correct use of the compound, it is therefore important that if trans-CA were to be used as a method of treatment, it must be directed carefully and specifically to the NPE. In addition, the experimentally-determined percent inhibitions suggest that trans-CA is not as potent as a full Na,K-ATPase inhibitor, such as ouabain. Though the data shows that the percent inhibition of trans-CA is low, it is nevertheless significant.

In regards to the nature of ophthalmic drugs, Page and co-workers list major characteristics that are necessary. If drugs are topically applied to the cornea, they must be lipophilic and uncharged in order to cross the cornea (Page et al., 2006). Trans-CA is a largely lipophilic molecule (as shown by the difficulties in creating a homogenous solution during the
experiments), which indicates that \textit{trans}-CA may be best applied topically or locally close to the eye. Its low solubility in water indicates that it may be difficult to administer orally or intravenously and expect to achieve a specific effect in the NPE, since it must overcome both the lipophobicity of blood and other bodily fluids and it must specifically reach the NPE. In regards to charge, \textit{trans}-CA has no ionizable functional groups and no favorable charged resonance structures, indicating that the compound has no major acid-base properties and remains largely uncharged.

Since \textit{trans}-CA has been shown to inhibit Na,K-ATPase in the NPE, future experiments should be aimed at investigating whether or not this inhibition leads to actual IOP decrease. Whole eye perfusion might be used for this purpose (Wilson et al., 1993).
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Si ego ultra vidi, stando super umeros gigantum est. - Sir Issac Newton, 1676
LITERATURE CITED


