EXPERIMENTAL EYE RESEARCH (2005) 80-4:493-499.

An adenosine agonist regulates outward active transport of fluorescein across rabbit blood retinal barrier

Kawahara, A; Hikichi, T; Kitaya, N; Takahashi, J; Mori, F; Yoshida, A

An adenosine agonist regulates outward active transport of fluorescein across rabbit blood retinal barrier

Atsushi Kawahara, MD, Taiichi Hikichi, MD, Norihiko Kitaya, MD, Junichi Takahashi, MD, Fumihiko Mori, MD, and Akitoshi Yoshida, MD

From the Department of Ophthalmology, Asahikawa Medical College, Asahikawa, Japan.

Running Title: Adenosine agonist regulates outward active transport across BRB

Text word count:

Tables: 0

Figures: 5

Section code: Physiology & Pharmacology

Commercial relationships policy: None

Keywords: Adenosine, blood retinal barrier, differential vitreous fluorophotometry, outward permeability

Corresponding author: Taiichi Hikichi, MD, Department of Ophthalmology, Asahikawa Medical College, 2-1 Midorigaoka-Higashi, Asahikawa 078-8510 Japan (telephone: +81-166-68-2543; fax: +81-166-68-2549; e-mail: hikichi@asahikawa-med.ac.jp).

Abstract

PURPOSE. To investigate the regulation of an adenosine agonist, 2-5′-N-ethylcarboxamidoadenosine (NECA) on the outward active transport of fluorescein across rabbit blood retinal barrier (BRB).

METHODS. Pigmented rabbits were given an intravitreous injection of various concentrartions of NECA or phosphate buffered saline (PBS). Sodium fluorescein was intravenously injected 180 minutes after NECA injection. Differential vitreous fluorophotometry (DVF) was performed 180 minutes after intravenous injection of sodium fluorescein to measure fluorescein (F) and fluorescein monoglucuronide (FG) concentrations in the vitreous. The F/FG ratio was calculated as an indicator of the estimated outward active transport of the BRB. Retinal detachments were experimentally produced by injection of PBS into the subretinal space. Experimental solution or PBS were injected intravitreally, and the size of blebs was monitored under masked conditions.

RESULTS. In eyes with intravitreal injection of high dose NECA, F/FG ratio was significantly lower when compared with in controls ($p<0.05$), but in eyes with low dose injection that was higher ($p<0.05$). The effect of high dose NECA on F/FG ratio was suppressed by the A1 receptor antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (CPX) and the effect of low dose NECA was suppressed by the A2 receptor

antagonist, ZM241385. The A3 receptor antagonist MRS1191 didn’t have an influence on the effect of high or low dose NECA. Intravitrteal injection of high dose NECA enhanced the removal of subretinal fluid when compared with intravitrteal injection of PBS alone.

CONCLUSIONS. These data suggest that intravitreous injection of high dose NECA accelerate the active outward transport of the BRB via A1 receptors and low dose NECA decelerate it via A2 receptors, and A3 receptors don’t contribute to the regulation of it.

Introduction

Adenosine is a purine nucleoside that is invoved in the regulation of several cellular processes. In the eye, it induces the increase of retinal blood flow, the vasodilation of retinal blood vessels, the regulation of intraocular pressure, and the promotion of corneal deturgescence.[1-4] Adenosine activates three characterized receptors on the extracellular surface (A1, A2, and A3) and regulates each action.[5] A1 and A2 receptors are coupled to adenylate cyclase; A1 receptors inhibit it, on the contrary, A2 receptors stimulate it.[6] A3 receptors are negatively coupled to adenylate cyclase and also coupled to phospholipase C.[7] A3 receptors are presently under study in order to better understand their physio-pathological functions.[8] Adenylate cyclase activity causes cyclic AMP (adenosine monophosphate, cAMP) production, and cAMP blocks water movement across retinal pigment epithelium (RPE)-choroid.[9, 10]

We already demonstrated that the F/FG ratio obtained by differential vitreous fluorophotometry (DVF) that measures F and FG concentrations in the vitreous simultaneously might be a marker to estimate the outward transport of the blood-retinal barrier (BRB).[11,12] The fluorescein (F) is metabolized to

fluorescent conjugate, the fluorescein monoglucuronide (FG).[13] F and FG in the vitreous are transported out of the eye through BRB by passive and active transport, but the active component for FG is much smaller than for F.[14-16] This suggests that F/FG ratio reflect the outward active transport function of the BRB. The low F/FG ratio indicates that the outward active transport function of the BRB is high. The high F/FG ratio indicates that the outward active transport function of the BRB is low.

In this study, we intravitreously injected non-selective adenosine receptor agonist 2-5′-N-ethylcarboxamidoadenosine (NECA) in rabbits and explored the regulation of NECA on the outward active transport of fluorescein across rabbit BRB using DVF.

Materials and Methods

Animals

Dutch-belted rabbits weighing about 2.5 kg were used. Pentobarbital sodium (40mg / kg) and ketamine hydrochloride (10mg /kg) were administered intramuscularly for general anesthesia of the rabbits. The animals were treated according to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

DVF

The Fluorotron Master (OcuMetrics) was modified by replacing the single excitation light source with a dual blue light-emitting diode (LED) assembly. The computer-controlled software was designed to take 10-msec fluorescence readings with each LED; the values then were converted to the fluorescein (F) and fluorescein monoglucuronide (FG) concentrations using the method of McLaren and Brubaker,[17] and the F/FG ratio was calculated from the concentrations of F and FG obtained by DVF as an index of the outward active transport function of the BRB.[11,12]

Effect of adenosine agonist, NECA on the F/FG ratio

0.1ml of various concentrations [1×10^{-5}M (n=6), 1×10^{-4}M (n=12), 2.5×10^{-4}M (n=4), 5×10^{-4}M (n=6), 1×10^{-3}M (n=6) or 2×10^{-3}M (n=8)] of non-selective adenosine receptor agonist, 2-5′-N-ethylcarboxamidoadenosine (NECA) or phosphate buffered solution (PBS) (n=10) was injected into the vitreous cavity. 50mg of sodium fluorescein was intravenously injected 180 minutes after NECA injection. Differential vitreous fluorophotometry (DVF) was performed 180 minutes after intravenous injection of sodium fluorescein to measure F and FG concentrations in the vitreous. General anesthesia was undergone just before DVF.

The intravitreal injection was performed under topical anesthesia.

Effect of A1, A2, and A3 antagonist on the F/FG ratio

0.1ml of 1×10^{-3}M 8-cyclopentyl-1,3-dipropylxanthine (CPX), the A1-selective antagonist, ZM241385, the A2-selective antagonist, or MRS1191, the A3-selective antagonist, were injected intravitreously. Sixty minutes after the injection, 0.1ml of 1×10^{-5}M (low dose group: CPX n=7, ZM241385 n=7, and MRS1191 n=4) or 1×10^{-3}M (high dose group: CPX n=8, ZM241385 n=3, and MRS1191 n=4) NECA was injected intravitreously. 50mg of sodium fluorescein was intravenously injected 180 minutes after NECA injection. DVF was performed 180 minutes after intravenous injection of sodium fluorescein.

Effect of high dose NECA on experimental retinal detachment

1×10^{-3}M high dose NECA or PBS was injected intravitreously to rabbit eyes (n=4, respectively). Six hours after the injections, experimental retinal detachments were made as described previously.[18] In brief, a glass micropipette with tip diameter of 30-40 μ was advanced through a sclera slit, and across the vitreous, to penetrate the retina in the posterior pole.

Immediately upon entiring the subretinal space, 100 μl of PBS was injected by gentle air pressure to raise a dome-shaped retinal detachment (bleb). After subretinal injection, the images of experimental retinal detachments were monitored using HRA (Heidelberg Retina Angiograph). The apparent bleb size either increased or decreased monotonically as judged by the experimenter using the seven rank scale (0 ± 3) reported previously.[19] Ranks were assigned by observing the change in apparent bleb size between 0 and 120 miniutes after the injection by masked observer. A rank of −3 means that the retinal bleb was apparently flattened, -2 means that the retinal bleb shows decrease in size 50-90%, -1 means that the retinal bleb shows decrease in size 1-49%, and a 0 rank means that the apparent bleb size was unchanged.

Statistical Analysis

All comparisons between the groups were performed for statistical analysis to use the Mann-Whitney U-test. P value of 0.05 or less than 0.05 were considered statistically significant.

Results

Effect of NECA on the F/FG ratio

In the eyes with the intravitreous injection of NECA, the concentration from 5×10^{-4}M to 2×10^{-3}M, F/FG ratios were significantly lower compared with the eyes with the intravitreous injection of PBS ($p<0.05$). Whereas, in the concentration from 1×10^{-5}M to 1×10^{-4}M, F/FG ratios were significantly higher compared with the injection of PBS ($p<0.05$). There was not significant difference between the effects of 2.5×10^{-4}M NECA and PBS on F/FG ratios. (Figure 1)

Effect of A1, A2, and A3 antagonist on the F/FG ratio

The increased F/FG ratio induced from low dose NECA (1×10^{-5}M) injection was significantly inhibited by the injection of A2 selective antagonist ZM241385, but CPX injection or MRS1191 injection did not influence the increased F/FG ratio induced from low dose NECA injection (Figure 2). On the contrary, the decreased F/FG ratio induced from high dose NECA (1×10^{-3}M) injection was significantly inhibited by the injection of A1 selective antagonist CPX, but ZM241385 injection or MRS1191 injection did not influence the decreased F/FG ratio induced from high dose NECA injection (Figure 3).

Effect of high dose NECA on experimental retinal detachment

In eyes with intravitrteal injection of 1×10^{-3}M high dose NECA, subretinal bleb showed decreasing in size (Figure 4), consequently flattened at 120 minutes after the procedure of subretinal bleb formation, whereas in the control eyes, subretinal bleb tended to be small decrease in size at 120 minutes after the procedure of subretinal bleb formation. The results summarized in Figure 5 show a significant difference between the two groups ($p<0.02$).

Discussion

The mechanism of resolution of subretinal fluid is not fully understood. There are many transport pathways which contribute to fluid movement across RPE.[20-22] It has been reported that the administrations of NECA act on several functions via A1 receptors or A2 receptors in the eye.[1-5,23,24] It was previously reported that a high level of endogenous adenosine has been immunocytochemically localized to the human RPE using specific antisera against adenosine and RPE contains an adenylate cyclase.[25,26] Adenosine agonists activate adenosine receptors on the RPE and regulate cAMP which is produced by adenylate cyclase

accumulation and blocks apical to basal water movement across the RPE.[9]

Adenylate cyclase on the RPE cells is influenced by inhibitory (Gi) and stimulatory (Gs) GTP-binding proteins. A1 binds to Gi, but A2 binds to Gs. Therefore, A1 receptors inhibit adenylate cyclase, on the contrary, A2 receptors activate it. Cyclic AMP is increased by adenylate cyclase activation. It controls the chemical composition and the volume of the subretinal space, which prevents the absorption of retina to choroids.[10, 11] Our results indicate that the intravitreal injection of high dose NECA activates the outward transport of the BRB and the injection of low dose NECA inhibits it. Furthermore, high dose NECA accelerate the outward transport of the BRB via A1 receptors and low dose NECA decelerate it via A2 receptors, and A3 receptors don't contribute to the regulation of active transport of BRB.

Previous reports have demonstrated that NECA has more affinity for A2 receptors than A1 receptors,[27] so intravitreal injection of low dose NECA mainly activated A2 receptors and decelerate the active transport function of the BRB, whereas, high dose NECA injection activated both A1 and A2 receptors, but phenotype is related with A1 receptors and accelerate the active transport function of the BRB, then experimental retinal

detachment was reduced by high dose NECA injections in this study. The effect of NECA on the active transport function of the BRB via A1 receptors may be stronger than that via A2 receptors. The further research may apply these findings to therapeutic approach for subretinal fluid such as retinal detachment.

References

1. Braunagel SC, Xiao JG, Chiou GCY. The potential role of adenosine in regulating blood flow in the eye. *J Ocular Pharm.* 1988;4:61-73.
2. Campochiaro PA, Sen HA. Adenosine and its agonists cause retinal vasodilation and hemorrhages. Implications for ischemic retinopathies. *Arch Ophthalmol.* 1989;107:412-416.
3. Crosson CE, Gray T. Characterization of ocular hypertension induced by adenosine agonists. *Invest Ophthalmol Vis Sci.* 1996;37:1833-1839.
4. Riley MV, Winkler BS, Starnes CA, Peters M. Adenosine promotes regulation of corneal hydration through cyclic adenosine monophosphate. *Invest Ophthalmol Vis Sci.* 1996;37:1-10.
5. Daly JW, Jacobson KA. Adenosine and adenosine nucleotides. *Molecular biology to integrative physiology.* 1995; 157-166.
6. Londos C, Cooper DMF, Wolff J. Subclasses of external adenosine receptors. *Proc Natl Acad Sci U.S.A.* 1980;77:2551-2554.
7. Suh BC, Kim TD, Kim KT. Pharmacological characterization of adenosine receptors in PGT-beta mouse pineal gland tumour cells. *Br J Pharmacol.* 2001;134:132-142.
8. Baraldi PG, Cacciari B, Spalluto G. A(3) adenosine receptor

ligands: history and perspectives. *Med Res Rev.* 2000;20:103-128.

9. Miller SS, Hughes BA, Machen TE. Fluid transport across retinal pigment epithelium is inhibited by cyclic AMP. *Proc Natl Acad Sci U.S.A.* 1982;79:2111-2115.

10. Marmor ME, Negi A. Pharmacologic modification of subretinal fluid absorption in the rabbit eye. *Arch Ophthalmol.* 1986;104:1674-1677.

11. Takahashi J, Mori F, Hikichi T, Yoshida A. The effect of acetazolamide on outward permeability of blood-retina barrier using differential vitreous fluorophotometry. *Curr Eye Res.* 2001;23:166-170.

12. Takahashi J, Hikichi T, Mori F, Kawahara A, Yoshida A. Effect of Nucleotide $P2Y_2$ Receptor Agonists on Outward Active Transport of Fluorescein across Normal Blood-Retina Barrier in Rabbit. *Exp Eye Res.* (in press).

13. Chen SC, Nakamura H, Tamura Z. Studies on the metabolites of fluorescein in rabbit and human urine. *Chem Pharm Bull.* 1980;28:1403-1407.

14. Kitano S, Nagataki S. Transport of fluorescein monoglucronide out of vitreous. *Invest Ophthalmol Vis Sci.* 1986;27:998-1001.

15. Yoshida A, Ishiko S, Kojima M. Outward permeability of the blood-retinal barrier. *Grafe's Arch Clin Exp Ophthalmol.* 1992;230:78-83.

16. Larsen L. Ocular fluorometry methodological improvements and clinical studies. *Acta Ophthalmol (Copenh)* 1993;221(suppl):1-52.

17. MacLaren J, Brubaker R. Measurement of fluorescein and fluorescein glucuronide in the living human eye. *Invest Ophthalmol Vis Sci.* 1986;27:966-974.

18. Negi A Marmor MF. Quantitative estimation of metabolic transport of subretinal fluid. *Invest Ophthalmol Vis Sci.* 1986;27:1564-1568.

19. Maminishkis A, Jalickee S, Blaug SA, Rymer J, Yerxa BR, Peterson WM, Miller SS. The $P2Y_2$ receptor agonist INS37217 stimulates RPE fluid transport in vitro and retinal reattachment in rat. *Invest Ophthalmol Vis Sci.* 2002;43:3555-3566.

20. Frambach DA, Marmor MF. The rate and route of fluid resorption from the subretinal space of the rabbit. *Invest Ophthalmol Vis Sci.* 1984;22:292-302.

21. Negi A, Marmor MF. Effects of subretinal and systemic osmolality on the rate of subretinal fluid resorption. *Invest*

Ophthalmol Vis Sci. 1984;25:616-620.

22. Negi A, Marmor MF. Quantitative estimation of metabolic transport of subretinal fluid. *Invest Ophthalmol Vis Sci.* 1986;27:1564-1568.

23. Friedman Z, Hackett SF, Linden J, Campochiaro PA. Human retinal pigment epithelial cells in culture possess A2-adenosine receptors. *Brain Res.* 1989;492:29-35.

24. Mino RP, Spoerri PE, Grant MB. Adenosine receptor antagonists and retinal neovascularization in vivo. *Invest Ophthalmol Vis Sci.* 2001;42:3320-3324.

25. Zarbin MA, Brass K, Newby A, Green WR, Snyder SH. Localization of adenosine and adenosine receptors in the retina. *Invest Ophthalmol Vis Sci.* 1976;27(Suppl):185.

26. Friedman Z, Hackett SF, Campochiaro PA. Characterization of adenylate cyclase in human retinal pigment epithelial cells in vitro. *Exp Eye Res.* 1987;44:471-479.

27. Camaioni E, Francesco ED, Cristalli G. Adenosine receptor agonists: synthesis and biological eveluation of the diastereoisomers of 2-(3-hydroxy-3-phenyl-1-propyn-1-yl) NECA. *Bioorganic & Medicinal Chemistry.* 1997;5:2267-2275.

Legends

Figure 1. Effect of NECA on the F/FG ratio. The F/FG ratios of NECA were significantly lower from 5×10^{-4}M to 2×10^{-3}M, whereas those were significantly higher from 1×10^{-5}M to 1×10^{-4}M when compared with PBS. Data are the mean ± SD (standard deviation). ＊Significant low ($p< 0.05$) when compared with PBS. #Significant high ($p< 0.05$) when compared with PBS.

Figure 2. Effect of A1, A2, and A3 antagonist on the F/FG ratio after low dose NECA administration. The F/FG ratio of A2 selective antagonist ZM241385 injected group was significantly lower than that of eyes with low dose NECA injection. Data are the mean ± SD (standard deviation). ＊Significant low ($p< 0.05$) when compared with low dose NECA alone.

Figure 3. Effect of A1, A2, and A3 antagonist on the F/FG ratio after high dose NECA administration. The F/FG ratio of A1 selective antagonist CPX injected group was significantly higher than that of eyes with high dose NECA injection. ＊Significant high ($p< 0.05$) when compared with high dose NECA alone.

Figure 4. Image of experimental retinal detachment monitored

using HRA. (A) Immediately after formation of experimental retinal detachment in eye with intravitrteal injection of high dose NECA. (B) At 60 minutes after the procedure in the samne eye, subretinal bleb showed apparently decreasing in size.

Figure 5. Effect of high dose NECA on experimental retinal detachment. Rank of grading scale for decrease in subretinal bleb size was significantly smaller in eyes with intravitrteal injection of high dose NECA than that in controls ($p<0.02$).

Figure 1

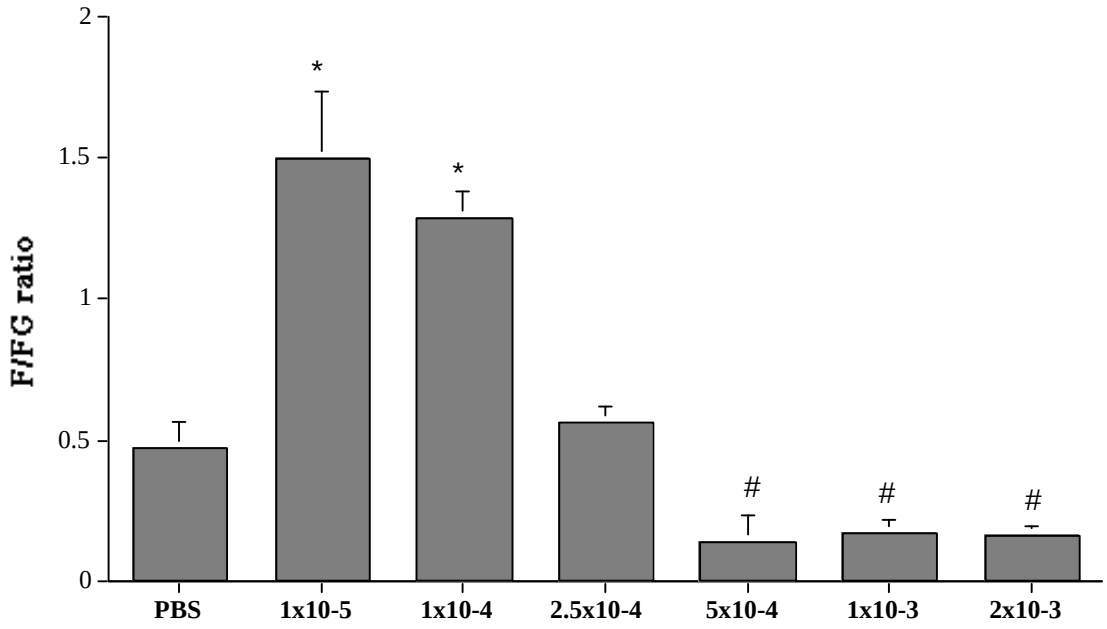


Figure 2

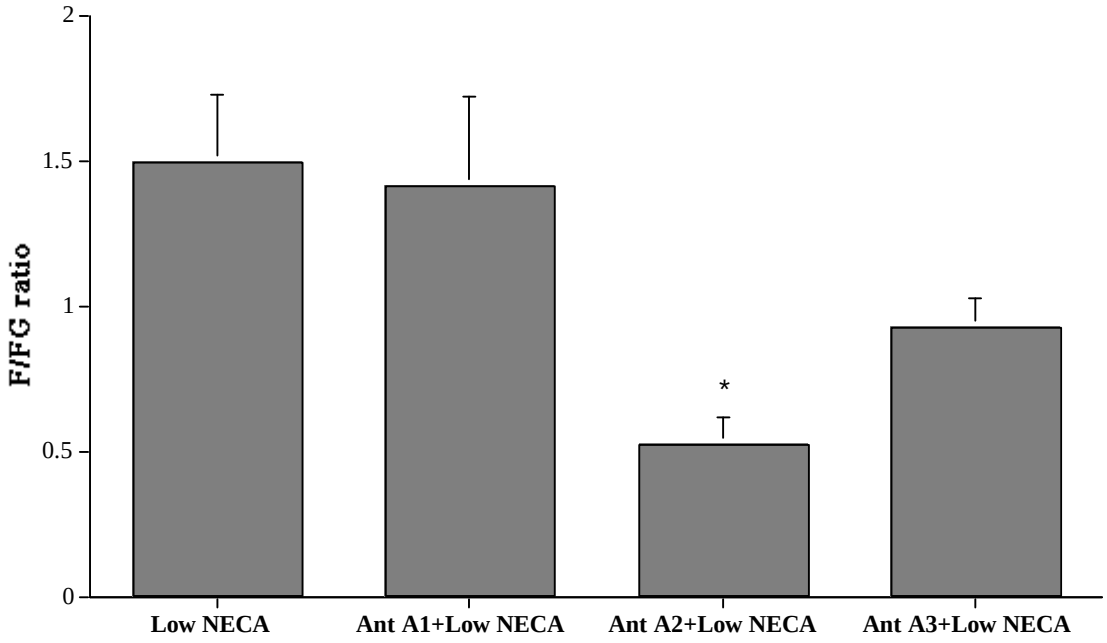

F/FG ratio
2
1.5
1
0.5
0
*
Low NECA
Ant A1+Low NECA
Ant A2+Low NECA
Ant A3+Low NECA

Figure 3

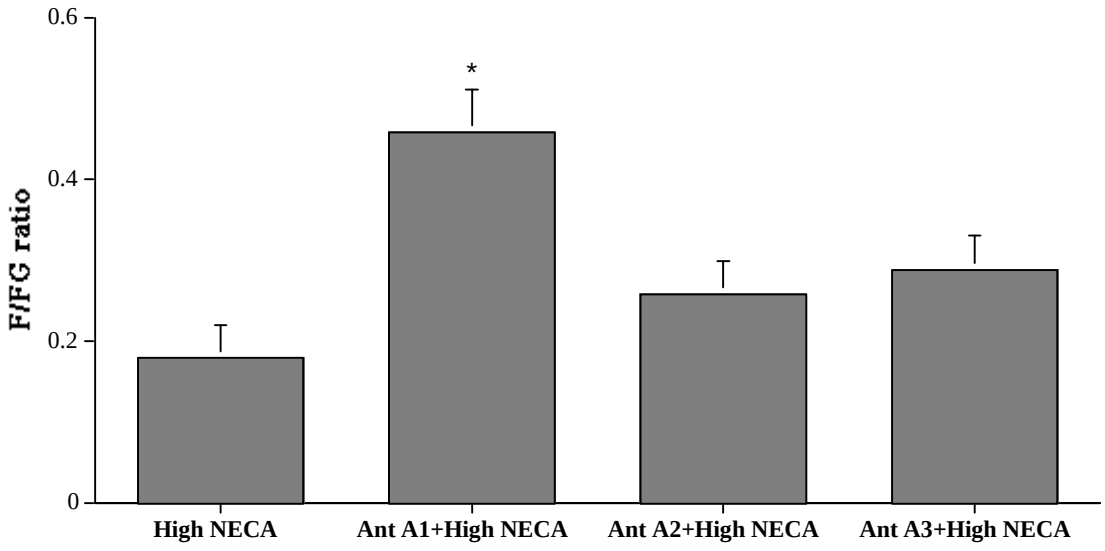
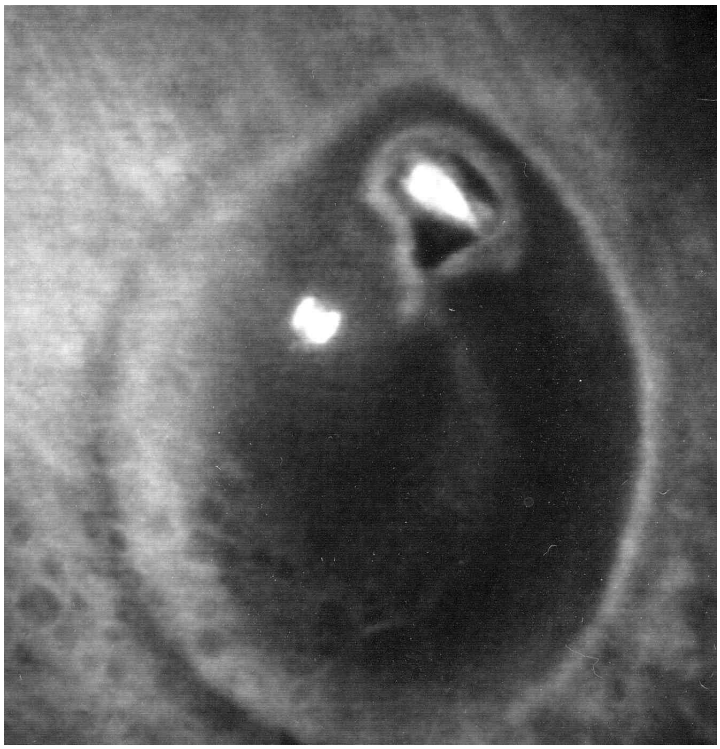

0.6
0.4
0.2
0
F/FG ratio
*
High NECA
Ant A1+High NECA
Ant A2+High NECA
Ant A3+High NECA

Figure 4.

(A)



(B)

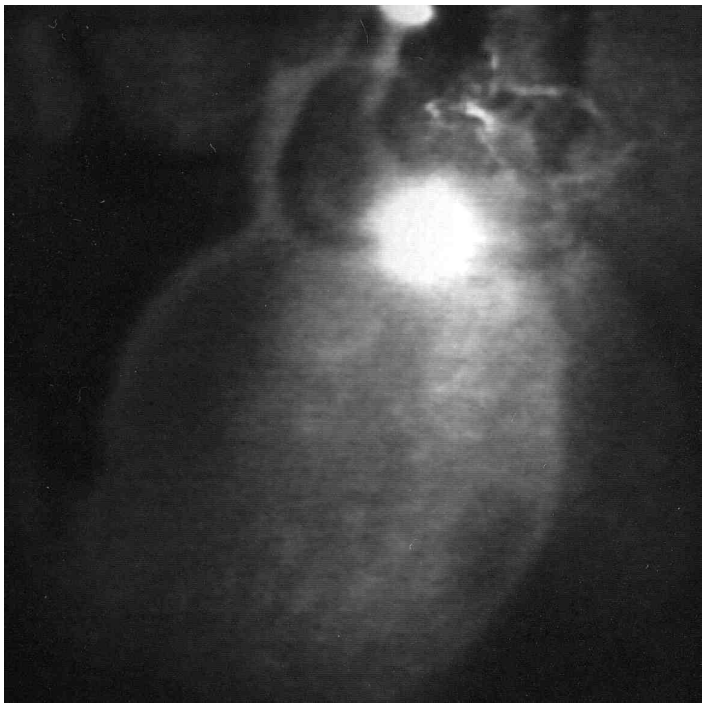


Figure 5