Observational study of the effect of elevated extra-cellular potassium chloride level on the differentiation of PC12 cells towards neuron-like cells.

Sultan Zayed Alshaqsi
Third Year Medical Student
Dunedin School of Medicine
University of Otago

Sultan is currently in his third year in Dunedin. He did his research with Dr Stephen Bunn in the Centre for neuro-endocrinology after his Health Science First Year.

ABSTRACT

The nervous and endocrine systems are connected together. Chromaffin cells in the adrenal medulla display neuro-endocrine features as they secrete biological signals (hormones) in response to neuronal stimuli. Biological signals that stimulate the development of neuro-endocrine function have yet to be fully elucidated. This observational study tests the effect of depolarizing cellular membrane on the development of neuron-like cells from a population of undifferentiated PC12 cells. In this study the depolarization effect is achieved by elevated extra-cellular KCl which is a good inducer of PC12 differentiation towards neuron-like cells.

Key words

PC12, differentiation, KCl

INTRODUCTION

The human endocrine and nervous systems are interconnected and integrated together. Body organs which have both nervous and endocrine functions are the connection points between the two systems (nervous vs. endocrine) such as the adrenal gland. The adrenal medulla is thought to evolve from the nervous system and that explains why it retains some nervous functions[1] Thus, precursor cells of the adrenal medulla theoretically can differentiate into neuron-like cells or endocrine cells or a combination of both (neuro-endocrine cells). The Chromaffin cells of the adrenal medulla are neuro-endocrine cells that have they ability to receive nervous input and respond by releasing endocrine factors (hormones).

Better understanding of the complex biological mechanisms behind the development of cells from this type holds promise for future treatment of neuro-degenerative diseases such as Parkinson's disease and many other endocrine disorders such as phaeochromocytomas (tumors of adrenal gland). The factors that switch on Chromaffin cell development are not fully understood and the signaling pathways of Chromaffin cells are still to be filled. Research groups have used a cell-line from a phaeochromocytoma (PC12 cells) as a model to study neuro-endocrine cell development.

PC12 cells were derived from a rat phaeochromocytoma (adrenal gland medulla tumor) in 1976 by Greene and Tichler[2]. This line of cells is able to proliferate, divide limitlessly and avoid apoptosis untill they receive a stimulus that directs their differentiation and hence limiting their division and eventually inducing apoptosis. Furthermore, it has been used extensively in neural development studies because it has the ability to differentiate into neuron-like cells in response to external stimuli and its differentiation is detected by morphological changes. PC12 cells respond to external factors by differentiating either into endocrine-like cells or neuron-like cells. Many studies have shown that neuron-like differentiation of PC12 can be achieved by nerve growth factor (NGF) and forskolin[3]. On the other hand, endocrine-like differentiation occurs in response to glucocorticoids[3]. Morphological changes including neurite outgrowth index the differentiation of PC12 into neuron-like cells. Biological substances that control and initiate cellular differentiation of PC12 are still not fully recognized. It seems that most of these substances work co-operatively with other mechanisms in a network of pathways to switch on the cellular differentiation process. The broad goal of this research project is to examine the combinations of biological substances that will give optimal stimulation of PC12 differentiation into neuron-like cells. Many studies have suggested that elevated extracellular potassium chloride (KCL) is involved in neural differentiation via wide mechanisms and pathways[3-10]. Based on those studies, KCL is selected to be the start point biological factor that is examined in this short research. In other words, the main variable in this project is the extracellular level of KCL. Neurite outgrowth was used as an index to indicate the differentiation of PC12 towards neuron-like cells. This project provides the first step in formulating a powerful stimulant that drives PC12 neural differentiation and explores the role of KCL on neural differentiation.

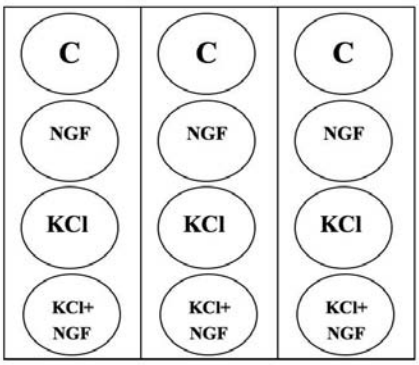
METHODS

Frozen PC12 stocks were used as a source of PC12 cells for this project. The cell culture medium was RPMI complete medium, which contains 5% fetal bovine serum (FBS), 10% horse serum(HS) and the antibiotics streptomycin and penicillin. Untreated PC12 cells were cultured in collagen-coated plastic plates for four days to allow the cells to double in number. During these four days, cells were incubated in a controlled environment with 37°C and 5% CO2 with regular removal of detached cells(dead cells). After this multiplication period, PC12 cells were sub-cultured into smaller collagen-coated wells and were treated immediately.

PC12 treatment

The first row of cells were treated with nerve growth factor(NGF) only, at a concentration of 50 ng/ml and the second row of cells with KCl only at concentration of 50 mmol/l and the third row with both agents. One row was left without treatment to act as the control. The treatments were added immediately after the subculture procedure. The treated cells were incubated for 96 hours with regular maintenance of the culture medium. This was done by the removal of 1ml of RPMI with detached cells and replaced with fresh RPMI in a daily basis.

On the 4th day of treatment, cells were processed for immunohistochemistry. First they were fixed using cold 100% methanol, then stained for total tyrosine hydroxylase enzyme using a specific monoclonal antibody (TH318 from Chemicon). In a replicated experiment, cells were stained for beta-actin protein. Immunohistochemistry enabled the visualization of the cells with microscopy and the evaluation of the morphological changes accordingly. The staining process clarified the difference between the KCl treatment and NGF treatment.



Observations

During the first 24 hours of treatment, there were not many noticeable morphological changes in the PC12 cells. However, one important observation was that the division process of PC12 cells with KCl only, NGF only and KCl/NGF treatment was reduced compared to the control cells. The culture medium became orange-yellow in colour indicating that metabolic activity was occuring. On the 2nd day of treatment, some neurite outgrowth appeared in these three treatments. PC12 cells treated with KCl appeared to be flat with undefined shape while PC12 cells treated with NGF were more spherical. During the third day, the difference in length of neuron-like processes of different treatments was very obvious. Cells treated with KCl and combination had web-like processes radiating in all directions. Furthermore, the number of processes from each cell treated with KCL and combination cells was far more than cells treated with NGF only. All the observations during the first three days were made using phase-contrast inverted-stage microscopy. Immunohistochemistry staining for TH and Beta-actin shows the clear difference between KCL treatment and NGF treatment in term of neurite outgrowth. Cells treated with combination of NGF and KCL had extensive neurite outgrowth ccompaerd to one treatment only(either NGF or KCL). In general, treated PC12 stained darker than untreated PC12.

DISCUSSION

This project supported many studies that have been done to investigate the key role of elevated KCL in neural development and PC12 differentiation. One possibility could be related to the depolarizing effect of KCl. That means the increase of KCl in the extra-cellular fluid encourages neuronal development of PC12 by triggering internal mechanisms that controls differentiation and neural development.

To begin with, scientific studies have suggested that elevated KCl exposure is time-dependant[5]. In this project, there was continuous exposure for three days which might explain the strong effect of KCL on cell differentiation. Other studies suggested that 6 hours daily exposure to elevated KCL for three days is sufficient to drive neural differentiation similar to the effect of continuous exposure[5]. In addition to exposure time, KCl effect is dependant on the stage of neural development. Experiments showed that neurons of petrosal ganglion (PG) developing during embryonic day 16 effected with elevated KCl more than neonatal PG[5]. It is hard to estimate at which stage the PC12 cells used in this project were first extracted to evaluate the observed effect. However, the response to elevated KCl indicated that they were more likely to be at early embryonic state.

One possible mechanism is the role of KCl in the control of trans-membrane calcium channels which are known for their differentiation and apoptotic effects by switching on the expression of genes that are involved in[5-9]. That means KCl controls the opening and closing of Ca2+ channels and thus the influx of Ca2+ to the cytoplasm of neural cells. The use of L-channels inhibitory molecules such as Nifedipine caused the loss of differentiation even with higher doses of stimulating KCL in the extracellular environment[9]. In another study, the use of selective K1+ blocker(Tetra-ethyl ammonium) which blocks Kv1.3 and Kv3.1 trans-membrane channels showed increased proliferation of neural progenitor cells(NPGs) and loss of differentiation process[8]. This gave first signs that KCl is a key controller of neural differentiation in many cell lines including PC12.

The genetic expression that is turned on by KCl induction involves very complex pathways and neuroscientists just start to appreciate its complexity.

One well established pathway is the Mitogen-activated protein kinase and extracellular signal-regulated kinase pathway (MAPK/ERK pathway). Both MAPK and ERK are activated by elevated KCL in the extracellular fluid. Activated MAPK and ERK drive the activation of G-proteins Ras and Rap1 via protein kinase (PKA)-dependant mechanisms[11]. The use of MAPK inhibitor (PD98059) and ERK inhibitor (H89) showed loss of KCL effect on the neural differentiation and disappearance of neurite outgrowth[6]. The end result of this pathway is the expression of genes that control neural differentiation.

The genes which are activated by MAPK/ERK pathway are not all known. One possible gene that is switched on by the KCL-sensitive MAPK/ERK pathway is NID67 which is discovered using representational difference analysis. This gene seems to be a differentiation primary response gene which is induced by elevated KCL[9]. The function of NID67 gene is not fully understood but it controls the expression of K-sensitive trans-membrane channels which controls Ca2+ ionic influx[9].

Another gene that is switch on by KCL-induced differentiation is Hst8Sia III. This gene controls the expression of Beta-tubulinIII which is a main component of neurite processes[12]. This might explain the longer processes induced by elevated KCL in PC12 cells.

One interesting point about KCL-induced neural-like PC12 is that, the cells stayed sensitized to subsequent exposure up to one week after the removal of KCL from the extracellular environment. Furthermore, a study used tyrosine hydroxylase (TH) expression as marker for neural differentiation of PC12 showed that pre-exposed PC12 cells showed four folds increased TH expression than 1st time exposed cells[5,6].

CONCLUSION

While this project was limited in scope, it gives a good starting point for further biomedical research. For future research, it would be useful to use biochemical analysis such as Western-blotting to test the effect of KCl treatment on neuron specific markers such as beta-tubullinIII, synapsin I and neuron-specific enolase (NSE). Also, it would be a future project focus to be able to culture PC12 on cover slips and use immune-florescent staining to visualize different proteins at one time.

Some limitations of this short project are the difficulty of growing PC12 cells and also the competence of PC12 to represent actual neuron-like cells is still in question.

There are many questions still to be answered about the role of KCl on cellular and tissue development and many neural differentiation pathways to be filled in.

ACKNOWLEDGMENT

I would like to thank Dr Stephen Bunn for this great opportunity to work in his laboratory and his continuous support. Also, I would like to thank Mrs Maureen Buchan and Mrs Shirley Douglas for their technical support.

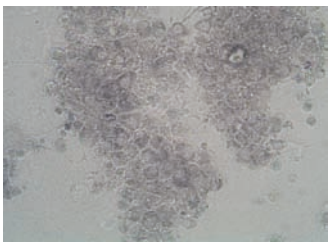


Figure 1: PC12 cells before treatment (phase contrast)

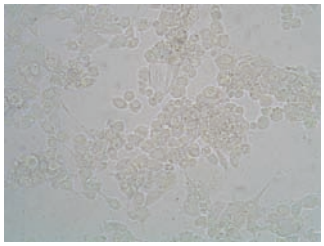


Figure 2: PC12 cells with NGF day two (phase contrast)

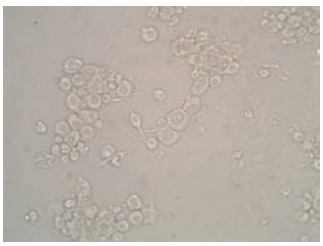


Figure 3: PC12 cells with KCL day two (phase contrast)

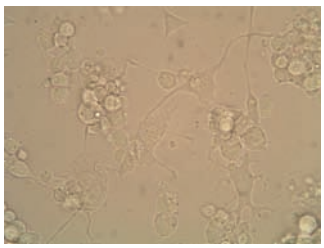


Figure 4: PC12 cells with KCL+NGF day two (phase contrast)

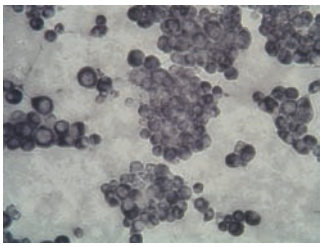


Figure 5: untreated PC12 stained for TH day four

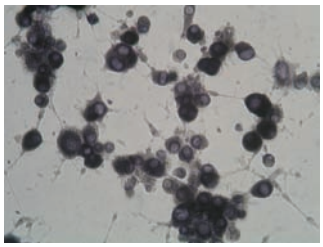


Figure 6: PC12 cells with NGF stained for TH day four

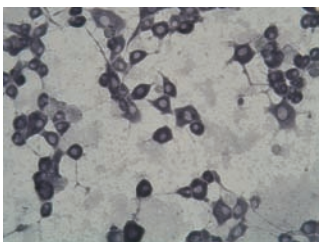


Figure 7: PC12 cells with KCL stained for TH day four

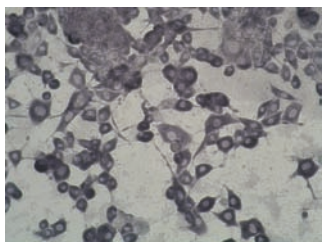


Figure 8: PC12 cells with KCL+NGF stained for TH day four

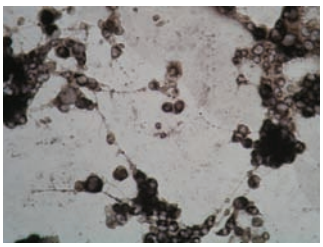


Figure 9: PC12 cells with KCL stained for beta-actin day four

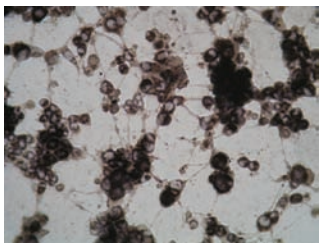


Figure 10: PC12 cells with KCL+NGF stained for beta-actin day four

REFERENCES

1. Ball.J.
Understanding disease.
Blackdown publications,UK,1990.

2. Green,L.A.,Tischler,A.S.,
Establishment of noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor.
Proc. Natl. Acad. Sci. USA ,1976, vol:73, 2424-2428.

3. Beaujean.D,Rosenbaum.C.
Combinatorial code of growth factors and neuropeptides define neuroendocrine differentiation in PC12 cells.
Experimental Neurology Journal,2003, vol:184, 348-358.

4. Mandela.P,Ordway.G
KCL stimulation increases norepinephrine transporter function in PC12 cells.
Journal of neurochemistry,2006, vol:98 issue:5 page:1521

5. Brosentisch.T, Salgado.D,Kunze.D.
A role of L-type calcium channels in developmental regulation of transmitter phenotype in primary sensory neurons.
The journal of neuroscience, Feb1998, vol:18(3): 1007-1055.

6. Thomas.V, Hansen.O, Jens.F, Rehfeld.C, Finn.N
KCL induces PC12 cell neurite outgrowth via protein kinase A and extracellular signal-regulated kinase signaling pathway.
Neuroscience letters, August 2003,vol:347(1): 57-61.

7. Seo.M, Kim.Y, Lee.Y, Kim.S, Ahn.Y, Kang.U, Roh.M, Kim.Y, Yuhnn.Y.
Membrane depolarization stimulates the proliferation of SH-SY5Y human neuroblastoma cells by increasing retinoblastoma protein(RB) phosphorylation through activation of cyclin-dependant kinase 2 (CdK2).
Neuroscience letters, august 2006, vol: 404(1-2) 87-92.

8. Liebus.M, Propper.C, Brockers.T, Lehamann.H.
Selective blockage of Kv1.3 and Kv3.1 channels increases neural progenitor cell proliferation.
Journal of neurochemistry, October 2006, vol: 99(2): 426-37.

9. Consuelo.M, Valle.M, Leticia.V, Diaz.O, David.E
The role of voltage-gated Ca2+ channels in neurite growth of cultured chromaffin cells induced by extremely low frequency(ELF) magnetic field stimulation.
Cell and tissue research, Jan 1998, vol:291(2): 217-230.

10. Vician.L, Silver.A, Farias.E, Eisner.R, Herschman.H.
NID67, a small putative membrane protein is preferentially induced by NGF in PC12 cells.
Journal of neuroscience Res, April 2001, vol:1562(2):108-20.

11. Yutaro.O, Angela.M, Horgan.F, Philip.J, Stork.S.
The requirement of Ras and Rap1 for the activation of ERKs by c AMP, PACAP and KCL in cerebellar granule cells.
Journal of neurochemistry, 2007, vol: 101(2): 470.

12. Kim.S, Chung.T, Jin.U, Suh.S, Lee.Y, Kim.C.
Molecular mechanisms involoved in transcriptional activation of human hST8Sia III gene induced by KCL in human glioblastoma cells.
Biochem biophys Res comman, June 2006, vol:16(344): 1057-64.

HealtheX

The inaugural exposition celebrating student research

Divya Dhar
Fifth Year Medical Student
Auckland School of Medicine
University of Auckland

Divya is studying medicine in Auckland was the chair of HealtheX 2007. She was also the driving force in organising this unique student research forum.

Event Synopsis

On Saturday 8th September 2007, over 90 students from the Faculty of Medical and Health Sciences at the University of Auckland got the chance to present their research in either oral or poster format to supervisors, family, friends and a panel of judges.

Vision

The vision for this conference went far beyond celebrating student research. It served to excite and inspire students in paving a pathway to revolutionise health care both in New Zealand and around the globe.

This event sought to pull together the multitude of threads involved in developing a truly world class health system, from developing the spatial configuration of a radical new drug, to formulating bold health policies that will shift the paradigm togiveyoung scientific minds the possibility to explore new passions and horizons.

HealtheX showcased discoveries, creations and innovations made by students from a diverse array of disciplines be it medical, pharmacy, nursing, biomedical sciences or population health. It presented a platform where both undergraduate and postgraduate students could gather under the same slogan of young budding researchers, providing opportunities for not only mentoring but, more importantly, camaraderie.

HealtheX is a celebration of the future face of health care and delivery.

In the next two to three years HealtheX hopes to expand by inviting student presenters from all around New Zealand.

Proceedings of the day

HealtheX was officially opened by the Dean of Medical and Health Sciences, Professor Iain Martin.

He was followed by the key note speaker Professor Robert Beaglehole who recently retired in his role as Director of Non-communicable Diseases and Health Promotion in the World Health Organization 2004-2007. Since his return to New Zealand he has been assisting the Parliamentary Select Committee on Obesity and Type 2 Diabetes acting as an international public health consultant. He is passionate about public health and especially the prevention of chronic diseases. Professor Beaglehole gave an inspiring address, encouraging students to examine the application and dissemination of research as a crucial, if not the most important part of research.

Over 50 students had qualified in the initial judging round to present their research in the oral format. These students then split into three morning and afternoon sessions run concurrently in the categories of Biomedical and Pharmaceutical, Population Health and Education, and Clinical research. At least two judges, both of whom were experts in that category of research were also present in the room. A moderator judge was asked to judge a couple of presentations in each session for scaling purposes.

Poster presentations were judged during the lunch hour where presenters were asked to stand by their posters in case judges had any questions.

The Roche Prizegiving ceremony was hosted by the Mayor of Auckland City, Dick Hubbard, who beganworking out of a small Onehunga factory with just two staff but is now the third biggest cereal company in New Zealand with a turnover of around $38 million per annum. Mayor Dick Hubbard gave an address on the 'Role of young

Key note speaker, Professor Robert Beaglehole.

Format	Place	Prize	Category and Winners		
Oral	1st	$750	*Biomedical and Pharmaceutical* Ursula Byrne	*Population Health and Education* Jennifer Utter	*Clinical* Amy Chan
	2nd	$250	*Biomedical and Pharmaceutical* Carthur Wan	*Population Health and Education* Serena Park	*Clinical* Catherine Bacon
Poster	1st	$750	*From all:* Group project: Megan Murphy, Hsin Yao Chang, Jae Young Han and Abir Ibrahim		
	2nd	$250	*From all:* Stefan Oehlers		
Grand Prize	Overall HealtheX Winner	$1000 additional	*From 1st prize winners in either Oral or Poster presentations* Amy Chan		

researchers in tomorrow's health' and presented HealtheX prizes including the Overall Grand Prize Trophy to Amy Chan.

Categories and Prize Winners

Presentations could be given in poster format, A0 size, or oral format, 10 minute Powerpoint with 5 minutes for questions. There were three categories for oral presentations: Biomedical and Pharmaceutical, Population Health and Education, and Clinical. Prizes were distributed accordingly.

Another 20 poster and 10 oral postgraduate presentations were selected to represent the Faculty of Medicine and Health Sciences at the university-wide postgraduate research competition, EXPOSURE.

Entertainment

All refreshments throughout the day were complimentary for presenters. Entertainment was provided by the Faculty of Medical and Health Sciences Strings music group which consists of staff and students from the FMHS 'self-contained' orchestra who played while the judges deliberated and the audience enjoyed a glass of wine and nibbles.

HealtheX Committee

The HealtheX committee convened a year ago consisting initially of a group of friends with a shared vision of hosting a prestigious conference which would enabled undergraduate students from all areas of health in Australasia to gather together. Early in the year of 2007 we went about seeking support for our proposal from the five Heads of Schools including Medicine, Pharmacy, Nursing, Biomedical Sciences and Population Health, as well as the Associate Dean of Research and Dean of Faculty of Medical and Health Sciences.

The vision for the conference evolved over this period to involve both undergraduate and postgraduate students in order to encourage mentorship, and to start small in the inaugural year by only involving University of Auckland students.

The committee consisted of both undergraduate and postgraduate students from all five schools and we werefortunate to also have the support of very passionate staff members of the FMHS - and a huge thank-you to the Committee!

Sponsors

HealtheX would not exist without the support of our sponsors. We would like to thank our Gold sponsors Maurice and Phyllis Paykel Trust, Roche Pharmaceuticals and Abacus ALS. Our silver sponsors Auckland Medical Research foundation and bio strategy. Our supporting sponsors Global Science online and Soar printing.

Conclusion

It is our hope that all medical students will have an interest in either conducting or being informed about research. It is undoubtedly an exciting pathway and as future leaders in health in our communities, we should constantly be seeking new hopes for our patients through research.

We received outstanding feedback and would like to thank everyone who helped make this event a spectacular success.

For further information or feedback please check out www.health.auckland.ac.nz/healthex or contact us at healthex-info@auckland.ac.nz

It has been encouraging to see the success of an idea that sprung from a conversation I had with a friend at the Australian Medical Students' Association's (AMSA) Leadership Development Seminar. Our shared passion led to the development of a research conference at our respective universities, University of Western Australia and University of Auckland and to witness its fruition was extremely rewarding. We would like to challenge you to follow your passions, wherever they may take you!

Mayor Dick Hubbard with Amy Chan, winner of the Grand Prize

The HealheX committee