Thromboxane A_2 and TP receptors: A Trail of Research, Well Traveled

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This article was written is response to a request from the editor-in-chief as a result of my receiving the Governor's Award in Scientific Awareness. My goal is to provide a glimpse into a 40 plus year research career that was predominately focused on the role of thromboxane A_2 and its receptor (TP) in physiologic and pathophysiologic processes in the cardiovascular system. It also enumerates the lessons learned along the way that may lead to a productive research career. Those lessons were: perseverance; think out of the box; challenge the dogma; take risks; failure is not a bad thing; focus; select a good mentor(s); choose your collaborators and colleagues wisely (team science); when you start an experiment-finish it (analyze all the data); and take time for your family.

This review is written in response to a request from the editor-in-chief as a result of my receiving the Governor's Award for Excellence in Scientific Awareness. The following was adopted from the Keynote Address for the 50th Annual Perry V. Halushka MUSC Research day given on November 12, 2015.

The themes for my address were; lessons learned, team science and translational research. These 3 themes reflect the story of my career in research and contributed to receiving the Governor's award. The focus of my research has been on the cardiovascular system and the role that thromboxane A_2 plays in both physiologic and pathophysiologic processes.

Metabolism of Arachidonic Acid

In order to appreciate the role of thromboxane A_2 in physiologic and pathophysiologic processes, one needs to have a basic understanding of the formation of thromboxane A_2 from arachidonic acid. Arachidonic acid is an essential fatty acid that cannot be synthesized by the mammalian species (Figure 1). It is acquired from various food sources and is stored in membrane phospholipids and released upon activation of phospholipase A_2. Once released it is rapidly metabolized to the labile endoperoxides, PGG_2 and PGH_2 via the enzyme fatty acid cyclooxygenase. The enzyme is inhibited by aspirin and non-steroidal anti-inflammatory drugs. PGH_2 is rapidly metabolized by thromboxane synthase to thromboxane A_2, which is very labile with a $t_{1/2}$ of ~30 seconds. It is hydrolyzed to the stable but inactive thromboxane B_2. Thromboxane synthase is inhibited by imidazole and other derivatives of imidazole. Thromboxane A_2 stimulates platelets to aggregate and vascular smooth muscle cells to contract. Thus, thromboxane A_2 participates in hemostatic control of bleeding as part of a normal physiologic process. However, for example, in the setting of atherosclerotic cardiovascular disease thromboxane A_2 plays a pathophysiologic role in vascular occlusive phenomena. It is aspirin's ability to irreversibly inhibit the platelet cyclooxygenase and thus thromboxane A_2 that leads to its beneficial effects in the primary and secondary prevention of heart attacks and strokes.

Endotoxemia, Thromboxane A_2 and TP Receptors

Around 1979, James Cook, PhD and Curtis Wise, PhD brought to my attention their observation that essential fatty acid deficient rats (EFAD) were resistant to the lethal effects of endotoxin. Endotoxin is the outer wall of gram negative bacteria and is released when the bacteria are killed. Endotoxin causes a host reaction which in turn can lead to severe shock and death. This situation is mostly commonly encountered when individuals have overwhelming bacterial infections in their blood stream. As

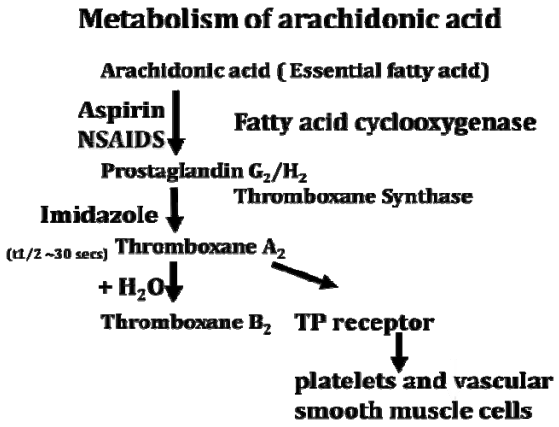

Figure 1. Metabolism of arachidonic acid. Aspirin, indomethacin and other non-steroidal anti-inflammatory drugs (NSAIDS) inhibit the enzyme, fatty acid cyclooxygenase. Imidazole and derivatives of imidazole inhibit thromboxane synthase. Thromboxane A_2 stimulates platelets to aggregate and smooth muscle cells to contract.

noted above, thromboxane A_2 is a fatty acid derived from the essential fatty acid arachidonic acid (Figure 1).

Our hypothesis was that endotoxin stimulates the formation of thromboxane A_2 and that it contributes to the pathophysiology of endotoxic shock. We postulated that the EFAD rats could not make thromboxane A_2 and therefore were protected from the lethal effects of endotoxin. To test the hypothesis several series of experiments were conducted; 1) measurement of plasma levels of thromboxane B_2 in control and endotoxin treated rats, 2) inhibition of the metabolism of arachidonic acid at the level of the cyclooxygenase using indomethacin, 3) at the level of thromboxane synthase using imidazole and 4) direct antagonism of the thromboxane (TP) receptor using 13- azaprostanoic acid. Endotoxin significantly increased the levels of plasma thromboxane B_2 in the rats challenged with endotoxin compared to the EFAD rats and the imidazole treated rats (Figure 2) (1). Treatment with either indomethacin or imidazole significantly improved survival of the rats treated with endotoxin (Table 1) (1). Ibuprofen also significantly improved the outcome of endotoxic shock in rats (2). Treatment of the endotoxic rats with 13-azaprostanoic acid also significantly improved survival compared to the vehicle treated group (1). Working with Dr. David Reines, a surgeon/intensive care physician, we subsequently went on to show that patients that died from overwhelming sepsis had a 10 fold greater level of plasma

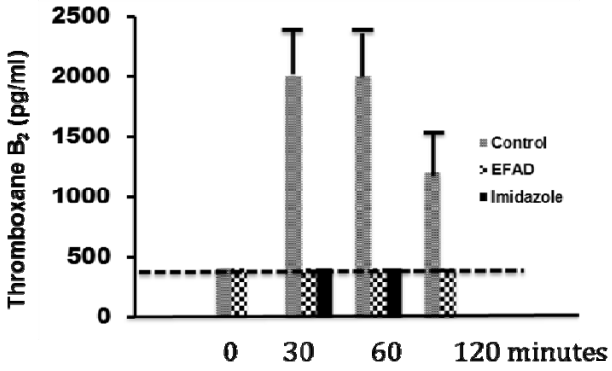

Figure 2. Plasma levels of thromboxane B_2 following a bolus injection of endotoxin in rats. The levels of thromboxane B_2 were significantly ($p<0.01$) greater in the control rats compared to those treated with indomethacin or imidazole. N=8 to 10/group. Modified from *J. Clinical Investigation* 65:227, 1981, J. Cook et al.

Table 1. Survival rates after a lethal injection of endotoxin in rats.

	Time after endotoxin (hrs)			
Group (Dead/Total)	5	12	24	48
Control	14/20	19/20	19/20	19/20
EFAD (7-8 wk old)	0/12	1/12	7/12	8/12
EFAD (12-13 wk old)	0/14	0/14	0/14	0/14
Indomethacin I.V.	0/8	0/8	1/8	1/8
Imidazole I.V.	1/10	2/10	4/10	4/10

thromboxane B_2 compare to those that survived a bout of sepsis or patients in the ICU that did not have a bout of septic shock (3). We also treated a group of septic patients with a thromboxane synthase inhibitor and showed that it was safe and effective in lowering plasma thromboxane B_2 levels (4). The company that made the thromboxane synthase inhibitor decided not to further develop it, so we were not able to conduct a large multicenter clinical trial to test its efficacy as a treatment for septic shock. The company along with several other companies may have decided to drop the development of thromboxane synthase inhibitors as antiplatelet agents based on the observations made by one of my graduate students (see below).

Lessons learned: Choose your collaborators and colleagues wisely (Team Science) (Dr. Cook and I have shared an RO1 for 33 years.) and think out of the box.

Platelet Thromboxane Formation and Platelet Aggregation

Dr. John Colwell, a diabetologist at MUSC, found that diabetic patients' platelets were more sensitive to aggregating agents compared to the appropriate controls (5). Given the role of thromboxane A_2 in promoting platelet aggregation, we collaborated to determine the formation of thromboxane A_2 by platelets from diabetic and control subjects. We found that diabetic patients' platelets made significantly more thromboxane A_2 and at a significantly greater rate compared to the control subjects' platelets (6). In collaboration with Ron Mayfield, also a diabetologist, we found that improving glucose control in the diabetic patients decreased the formation of thromboxane A_2 by their platelets (7).

In the late 70s there was a reigning hypothesis that platelet thromboxane A_2 synthesis was a major contributor to platelet aggregation and that inhibition of its synthesis would confer significant inhibition of platelet aggregation. Along with a couple of my colleagues, we decided to synthesize a series of thromboxane synthase inhibitors as potential drugs to act as inhibitors of platelet aggregation. 7-IHA was one of those compounds. We asked Linda Grimm a graduate student at the time to test its antiplatelet effects and its ability to inhibit thromboxane A_2 synthesis. Soon after she finished the first experiment assessing arachidonic acid induced platelet aggregation, she came to my office and concluded that 7-IHA did not inhibit thromboxane A_2 synthesis because it did not inhibit arachidonic acid induced platelet aggregation. I asked her if she had measured thromboxane B_2. She said that she had not. So I told her to come back to me once she had measured thromboxane B_2. Much to her amazement, 7-IHA significantly inhibited thromboxane synthesis, yet did not inhibit platelet aggregation. Her results completely disproved the reigning hypothesis. The saga continues because, when we submitted the paper for publication, the review came back with a rejection and my take on the review was that it was pejorative and written by the person who was promulgating the hypothesis. I appealed to the editor and he agreed to have the manuscript reviewed by different reviewers. Within about 2 weeks, the manuscript came back accepted as is (8).

Lessons learned: When you start an experiment, finish it with a complete analysis of the data, challenge the dogma and perseverance.

"When you come to a fork in the road, take it", Yogi Berra

We had conducted many studies demonstrating a role for thromboxane A_2 in physiologic and pathophysiologic processes and decided that it was time to start to characterize the binding characteristics of thromboxane A_2 (TP) receptors and their signaling mechanisms. However, there were no radioactive ligands available that could be used for characterization of the binding characteristics and regulation of their expression. So along with our chemistry colleagues, we synthesized a compound 13-cisAPO, a derivative of 13-azaprostanoic acid, that could be radiolabelled with ^{125}I using a simple one-step process. With the compound in hand, we took a 1 year sabbatical in London, England to work with Dr. John MacDermot, to begin to characterize the binding characteristics of the TP receptor using ^{125}Iodo-cisAPO. Little did we know that we would run into many challenges, the most critical one was that ^{125}Iodo-cisAPO had a very low affinity for the receptor, making it very difficult to get specific binding to the platelet TP receptor. Nonetheless, with John MacDermot's guidance, we persevered and were able to ultimately demonstrate specific binding (9) and establish the concept of making ^{125}I labeled ligands for the characterization of TP receptors (10-12).

Lessons learned: Think out of the box, Choose your collaborators and colleagues wisely, Failure is not a bad thing, Take risks, Focus and Perseverance.

Characterization of the TP Receptors

With a generous gift of a chemical precursor from the ONO pharmaceutical company, Dale Mais, a postdoctoral fellow, and ultimately assistant professor, synthesized a series of thromboxane receptor antagonists. Using these compounds, we characterized the TP receptor in platelets and blood vessels and demonstrated that they represented two distinct classes of

receptors (13-15). The receptor was cloned by Narumyia et al, and since they could not find a second transcript, they concluded that there was only one class of receptors (16). However, a splice variant was subsequently discovered (17) and we also generated additional data to support the notion that there were two classes of receptors (18, 19).

Lessons learned: Believe in your data based on first principles.

The final vignette deals with the earlier observations that some young male athletes abusing anabolic steroids were suffering strokes and heart attacks in their 20s (20) and that male rat aortas were more sensitive to rabbit aorta contracting substance (21), subsequently shown to be thromboxane A_2, and that in laboratory animals, testosterone pretreatment enhanced thrombogenic stimuli (22). We hypothesized that testosterone may increase the expression of TP receptors and in turn is responsible in part for these observations. This hypothesis was tested in cultured vascular smooth cells, HEL cells (model for megakaryocytes), rats and ultimately in normal male volunteers and patients with prostate cancer. Collectively, these studies demonstrated that in both pharmacologic doses as well as endogenous levels, testosterone may regulate the expression of TP receptors (23-26).

Throughout my research career, I have learned many lessons that help to make one more successful. They are enumerated below.

Dr. H's "TOP 10" lessons for success

- Perseverance
- Think out of the box
- Challenge the dogma
- Take risks
- Failure is not a bad thing
- Focus
- Select a good mentor(s)
- Choose your collaborators and colleagues wisely (team science)
- When you start an experiment-finish it (analyze all the data)
- Take time for your family

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Notes and References

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1. Cook, J.A., Wise, W.C., Halushka, P.V. Elevated Thromboxane levels in the rat during endotoxic shock: protective effect of imidazole, 13-azaprostanoic acid or essential fatty acid deficiency. J Clin Invest 65: 227-230, 1980.
2. Wise, W.C., Halushka, P.V., Cook, J.A. Ibuprofen, Methylprednisolone - Sodium Succinate and Gentamicin as Conjoint Therapy in Septic Shock. Circ Shock 17: 59-71, 1985.
3. Reines, H.D., Halushka, P.V. Cook, J.A., Wise, W.C., Rambo, W.. Plasma thromboxane concentrations are raised in patients dying with septic shock. Lancet, ii 174-175, 1982.
4. Reines, H.D., Halushka, P.V., Olanoff, L.S., Hunt, P.S. Dazoxiben in human sepsis and Adult Respiratory Distress Syndrome. Clin Pharmacol Ther 37: 391-395, 1985.
5. Colwell, J.A., Halushka, P.V., Sarji. K.E., Sagel, J. Platelet function and diabetes mellitus. Med Clin North America 62: 753-766, 1978.
6. Halushka, P.V., Rogers, C., Loadholt, C.B., Colwell, J. Increased platelet thromboxane synthesis in diabetes mellitus. J Lab Clin Med 97: 87-96, 1981.
7. Mayfield, R.K., Halushka, P.V., Wohltmann, H.J., M. Lopes-Virella, J.K. Chambers, Loadholt, C.B., Colwell, J.A. Platelet function during continuous insulin-infusion treatment in insulin-dependent diabetic patients. Diabetes 34: 1127-1133, 1985.
8. Grimm, L., Knapp, D.R., Halushka, P.V. Inhibition of platelet thromboxane synthesis by 7-(1-imidazolyl) heptanoic acid: Dissociation from inhibition of aggregation. Thromb Res 24: 307-317, 1981.
9. Halushka, P.V., MacDermot, J., Knapp, D.R., Eller, T., Saussy Jr., D.L., Mais, D., Blair, I.A., Dollery, C.T. A novel approach for the study of thromboxane A_2 and prostaglandin H_2 receptors using an 125-I labelled Ligand. Biochem Pharmacol34: 1165-1170, 1985.
10. Mais, D.E., Knapp. D., Halushka, P., Ballard, K., Hamanaka, N. Synthesis of thromboxane receptor antagonists with the potential to radiolabel with 125I. Tetrahedron Letters 25: 4207-4210, 1984.
11. Mais, D.E., Burch R.M., Saussy, Jr., D.L., Kochel, P.J., Halushka, P.V. Binding of a thromboxane A_2/prostaglandin H2 receptor antagonist to washed human platelets. J Pharmacol Exp Ther 235: 729-734, 1985.
12. Morinelli, T.A., Oatis, Jr., J.E., Okwu, A.K., Mais, D.E., Mayeux, P.R., Masuda, A., Knapp, D.R., Halushka, P.V., Characterization of an [125I]-labelled thromboxane A_2/prostaglandin H2 receptor agonist. J Pharmacol Exp Ther 251:557-562, 1989.
13. Mais, D.E., Saussy, Jr., D.L., Chaikhouni, A., Kochel, P.J., Knapp, D.R., Hamanaka, N., Halushka, P.V. Pharmacologic characterization of human and canine thromboxane A_2/prostaglandin H2 receptors in platelets and blood vessels: evidence for different receptors. J Pharmacol Exp Ther 233: 418-424, 1985.
14. Mais, D.E., DeHoll, D., Sightler, H., Halushka, P.V. Different pharmacologic activities for 13-Azapinane thromboxane A_2 analogs in platelets and blood vessels. Eur J Pharmacol 147: 309-316, 1988.
15. Mais, D.E., Dunlap, C., Hamanaka, N., Halushka, P.V. Further studies on the effects of epimers of thromboxane A_2 antagonists on platelets and veins. Eur J Pharmacol 111: 125-128, 1985.
16. Hirata, M., Hayashi, F., Ushikubi, Y., Yiokota, R., Kageyama, S., and Narumiya, S. Cloning and expression of cDNA for a human thromboxane A_2 receptor. Nature 349: 617-620,1993.
17. Raychowdhury, M.K. Yukawa, M., Collins, L.J., McGrail, S.H., Kent, K.C. and Ware, J.A. Alternative spicing produces a divergent cytoplamsmic tail in the human endothelial thromboxane A_2 receptor. J. Biol Chem 269: 19256-61. 1994
18. Allan, C.J., Higashiura, K., Martin, M., Morinelli, T.A., Kurtz, D.T., Geoffreoy, O., Meier, G.P., Halushka, P.V. Characterization of the cloned HEL cell thromboxane A_2 receptor: Evidence that the affinity state can be altered by G□13 and G□q. J Pharmacol Exp Ther 277: 1132-1139, 1996.
19. Becker, K.P., Garnovskaya, M., Gettys, T., Halushka, P.V. Coupling of thromboxane A_2 receptor isoforms to G□13: Effects on ligand binding and signaling. Biochem Biophys Acta 1450:288-296, 1999
20. Mochizuki, R.M., Richter, K.J. Cardiomyopathy and cerebrovascular accident associated with anabolic-androgen steroid use. Physician and Sports Medicine 116: 109-112, 1988:
21. Piper, P.J. and Vane, J.R. Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. Nature 223: 29-35. 1969
22. Penhos, J., Rabbni, F., Meyers, A., and Ramey, E. The role of gonadal steroids in arachidonate-induced mortality in mice. Proc Soc Exp Biol 167: 98-100, 1981

23. Masuda, A., Mathur, R., Halushka, P.V. Testosterone increases thromboxane A_2 receptors in cultured rat aortic smooth muscle cells. Circ Res 69: 638-643, 1991
24. Matsuda, K., Mathur, R.J., Duzic, E., Halushka, P.V. Androgen regulation of thromboxane A_2/prostaglandin H2 receptor expression in human erythroleukemia cells. Am J Physiol 265: E928-E934, 1993.
25. Matsuda, K., Ruff, A., Morinelli, T.A., Mathur, R.S., Halushka, P.V. Testosterone increases thromboxane A_2 receptor density in rat aortas and platelets. Am J Physiol 267: H887-H893, 1994.
26. Ajayi, A.A.L., Mathur, R., Halushka, P.V. Testosterone increases human platelet thromboxane A_2 receptor density and aggregation responses. Circulation 91: 2742-2747, 1995.