

Full Length Research Paper

Evaluation of TonEBP modulation on heart rate and biochemical parameters

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Abstract

The transcription factor, tonicity-responsive enhancer binding protein (TonEBP) plays a key role in the accumulation of organic osmolytes by stimulating gene expression of membrane transporters (sodium-inositol cotransporter and sodium-chloride-betaine cotransporter) and a biosynthetic enzyme (aldose reductase) that catalyzes production of sorbitol from glucose. Thus, TonEBP plays a key regulatory role in protecting the renal medulla from the deadly stress of hypertonicity. Although TonEBP plays a key role in hypertonicity induced stimulation of gene transcription in the renal medulla and T cells, TonEBP is active under isotonic conditions. These wide-ranging transcriptional targets delineate TonEBP as a critical element of osmosensory signal transduction in cells of the renal medulla. In this study, we ascertained the role of TonEBP on the heart rate in conjunction with pituitary hormone vasopressin and with vasoconstrictor endothelin antagonist, BQ-123. We also studied the effects of TonEBP modulators on glucose and ketone levels in the urine and on the blood potassium and sodium levels. We utilized, doxorubicin (Dox) and isosorbide dinitrate to modulate the levels of TonEBP endogenously.

Keywords: TonEBP, hyperosmolality, transcription, doxorubicin, heart rate.

INTRODUCTION

Hypertonicity itself alters several intracellular processes by decrease of cell volume and changes in intracellular ion concentrations. In this process, protein function as well as DNA stability are modified (Cai et al., 2005). A variety of compensatory mechanisms are essential to prevent cell death. Regulatory volume increase contributes to osmoadaptation as well as expression of heat shock proteins and solute carriers and induction of signaling pathways involved in DNA repair and cell cycle delay (Jeon et al., 2006). Many of these adaptations are mediated by the transcription factor Tonicity Enhancer Binding Protein (TonEBP) (Burg et al., 2007). TonEBP is a major transcription factor whose activity is regulated by extracellular tonicity. Results of previous studies provide evidence that p38 kinase signaling contributes to TonEBP activity (Irrarazabal et al., 2008; Küper et al., 2009). Cells in the renal medulla are bathed in hypertonic interstitium. The hypertonicity is due to hyperosmotic concentration of sodium chloride, which routinely reaches

1,000 mosmol/kgH₂O in rat inner medulla during antidiuresis (Han et al., 2004). While cultured cells die at such extreme hypertonicity, cells in the renal medulla *in situ* do not display any measurable sign of cell death (Hasler et al., 2006). The slow increase in ambient tonicity allows increased cellular accumulation of organic osmolytes (Lam et al., 2004). It has been reported that treatment with the antineoplastic drug Dox, decreases the expression of the TauT gene in cultured cardiomyocytes isolated from the neonatal rat heart. The protein level of the TonE-binding protein (TonEBP) was reduced by Dox treatment. In addition, the reduction in TonEBP protein content was suppressed by proteasome inhibitors. In conclusion, the Dox-enhanced degradation of TonEBP results in reduced TauT expression in the cardiomyocyte thus signifying a role in the cardiovascular system. There is a binding site for TonEBP in the promoter region of the AQP₂ that mediates transcriptional stimulation in response to hypertonicity (Storm et al., 2003). Likewise, the promoter of the UT-A urea transporter gene is also stimulated by TonEBP (Nakayama et al., 2000; Cha et al., 2001). Vasopressin is known to affect the activity of urea transporters. It should be pointed out that, throughout the collecting duct where AQP₂ and UT-A are

expressed, TonEBP is highly expressed, including the cortical segments (Favale et al., 2007).

On the other hand, Endothelin-1 the peptide hormone plays multiple, complex roles in cardiovascular, neural, pulmonary, reproductive, and renal physiology. It was originally identified in 1988 as an endothelin-derived factor that produces prolonged vasoconstriction and increase in arterial blood pressure. Extracellular hypertonicity also regulates ET-1 release from inner medullary collecting duct cells. Likewise, a high-salt diet increased ET-1 expression in cardiomyocytes and renal medullary thick ascending limb cells. The molecular mechanism responsible for mediating this response remains unknown. Endothelin also causes coronary vasoconstriction (Mather et al., 2012).

The present study was hitherto conducted to ascertain the effect of TonEBP modulation on the effect of vasopressin and endothelin-1 antagonist on the heart rate in experimental rats apart from the effects on biochemical parameters like the glucose and ketone levels in urine and electrolyte levels i.e plasma sodium and potassium levels. The results of our study suggest that TonEBP was able to affect the influence of vasopressin and BQ-123 on heart rate and alter the biochemical parameters *per se*.

MATERIALS AND METHODS

Animals and Housing conditions

Experiments were performed on Wistar Albino rats of either sex weighing, between 160-220g obtained from experimental animal center of Christian Medical College, Vellore, India. Animals were housed in groups of 3-4 in polypropylene plastic cages under hygienic conditions, lined with paddy-husk bedding. Animals were housed in a colony room once the experiments completed under controlled temperature (25 \pm degree), relative humidity of (60 \pm 2%) and were exposed to 12 hour light: 12 hour dark cycle, with food and water available *ad libitum*. All experiments were conducted during the light phase, between 8.00-13.00 hours. Experimental protocol was approved by Institutional Animal Ethics Committee (IAEC).

Estimation of cardiac rate

Wistar albino rats weighing between 160 to 220 g were utilized for this study. Groups of animals (n=6) were first anesthetized with ketamine (10mg/kg i.m) and left in the cage for 10 minutes. After ten minutes they were anesthetized using Diazepam (4mg/kg i.p). Electrocardiography was conducted using the limb lead II on a physiograph (INCO, India) using a speed of 10

mm/second for the control reading. Doxorubicin (2mg/kg i.p) was administered to the animals intraperitoneally 45 minutes prior to experimentation. Again Electrocardiography was conducted on a physiograph using a speed of 10 mm/second. Vasopressin (4IU/kg) was administered to the animals intraperitoneally and for each 15 minutes interval electrocardiography was conducted on a physiograph using a speed of 10 mm/second. To evaluate the effect of ET-1 antagonist, BQ123 the experiment was repeated in another groups of animals after treatment with Dox but instead of vasopressin, ET-1 antagonist, BQ123 (200 μ g/kg i.p) was administered to the animals intraperitoneally and for each 15 minutes interval electrocardiography was conducted on a physiograph using a speed of 10 mm/second. Heart rate was estimated from the ECG tracings by counting the number of 'R' waves per minute as per earlier described technique (Tyagi and Thomas, 1999).

Estimation of plasma sodium and potassium levels:

Estimation of plasma sodium and potassium levels was determined in Wistar rats. Blood was collected from the retro-orbital sinus of the animals using the Heparinised capillary tubes. 1.5-2ml of blood was collected in a small eppendorf tubes. Before collecting blood, tubes were added with 0.2ml heparin. Blood was collected in 5 tubes for control reading. Dox (2mg/kg i.p) was administered to the animals intraperitoneally and animals left for 1 hour in plastic cages. After 1 hour, blood was collected from the retro-orbital sinus of the animals using the heparinised capillary tubes. Before using capillary tubes lignocaine was added to the eyes of the animal to protect the eyes from any pain sensation. 1.5- 2.0 ml of blood was collected in a small eppendorf tubes. For another group of animals isosorbide dinitrate was administered intraperitoneally. After 1 hour blood was collected from the retro-orbital sinus of the animals using the heparinised capillary tubes. 1.5-2.0 ml of blood was collected in small eppendorf tubes. The whole blood was fractioned by centrifuging at 1500-2000 rpm for 10-15 min at room temperature. This would separate the blood into an upper plasma layer, a lower red blood cell layer, and a thin interface containing the WBCs. From the plasma obtained sodium and potassium levels were analyzed using the Electrolyte Analyzer (Clinical Biochemistry Laboratory, CMC, Vellore, India).

Estimation of urine glucose and ketone levels

Estimation of urine glucose and ketone levels was done in Wistar rats. Dox (2mg/kg i.p) was administered to the animals intraperitoneally and animals left in cage for 45 mins. After that animals were administered with saline (4ml) intraperitoneally and left for sometime in a clean

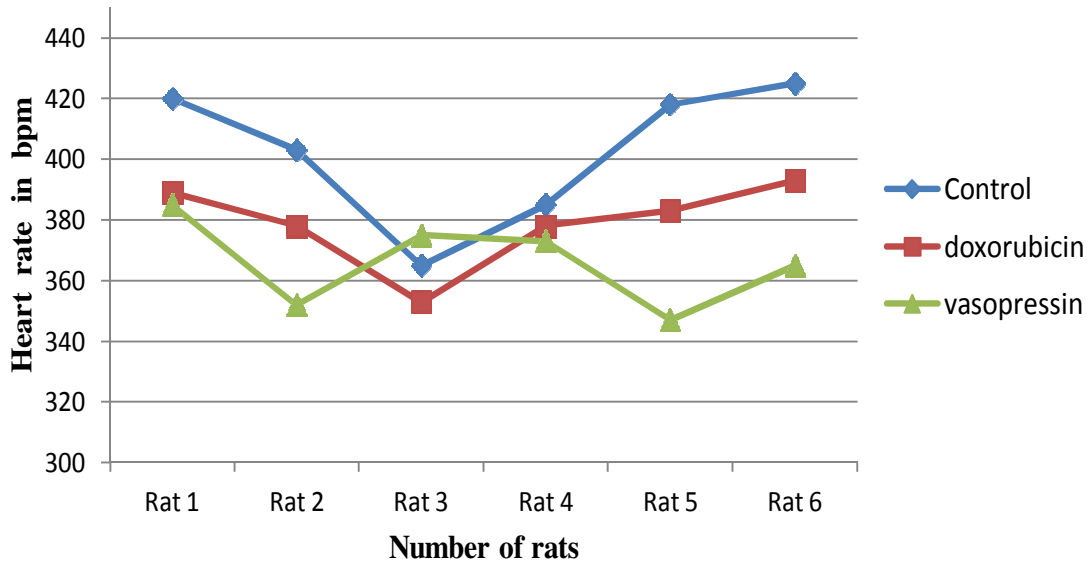


Figure 1. Effect of Doxorubicin (2mg/kg i.p) and vasopressin (4IU/kg i.p) on heart rate in Wistar rats (n=6)

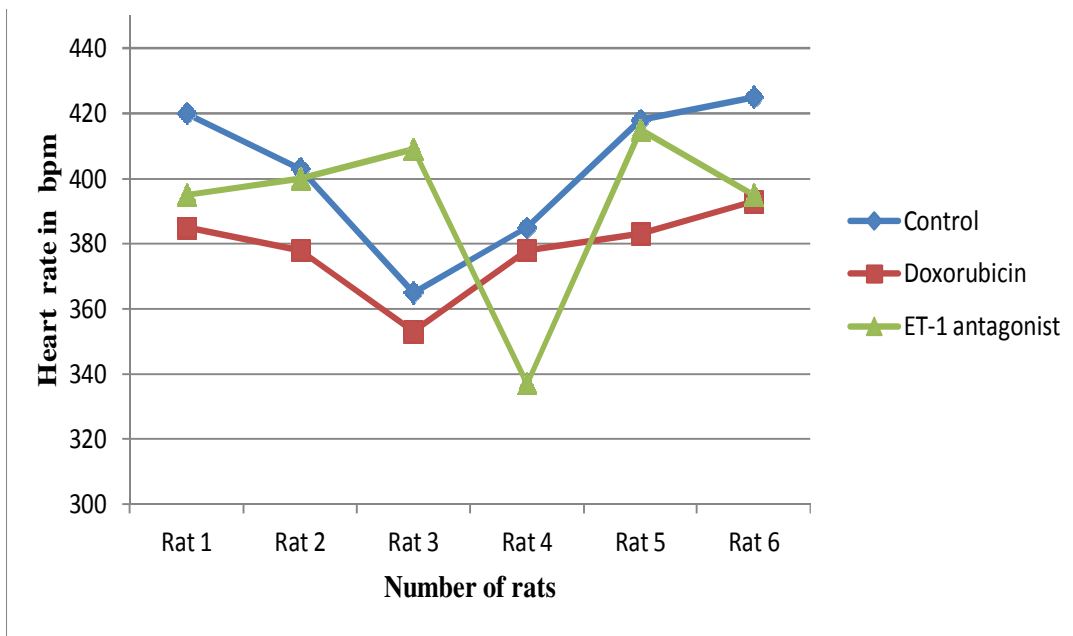


Figure 2. Effect of Doxorubicin (2mg/kg i.p) and BQ-123 (200µg/kg i.p) on heart rate in Wistar rats (n=6)

tray. The animals micturated after some time. Glucose and Ketone levels were analysed by dipping glucose sticks in urine (0.2 ml). Glucose level changes were seen after 30 sec and ketone level changes were seen after 40 sec using the Diastix (Bayer Ltd., India).

RESULTS

The results of this study are depicted in figures 1 to 4 and

Table 1. In this study the two TonEBP modulators, Dox (2mg/kg) and Isosorbide dinitrate (20 mg/kg) were used. The control heart rate was 403 beats per minute (b.p.m), while after doxorubicin it decreased to 378 b.p.m (6.20 %) and after treatment with vasopressin (4 IU/kg i.p) and BQ-123 (200µg/kg) altered it to 364 b.p.m and 395 b.p.m respectively a change of 9.67 % and 1.99 %. These data are shown in figures 1 and 2. On the other hand, Dox treatment (2mg/kg i.p) treatment did not alter appreciably the glucose levels in urine, however there was detection

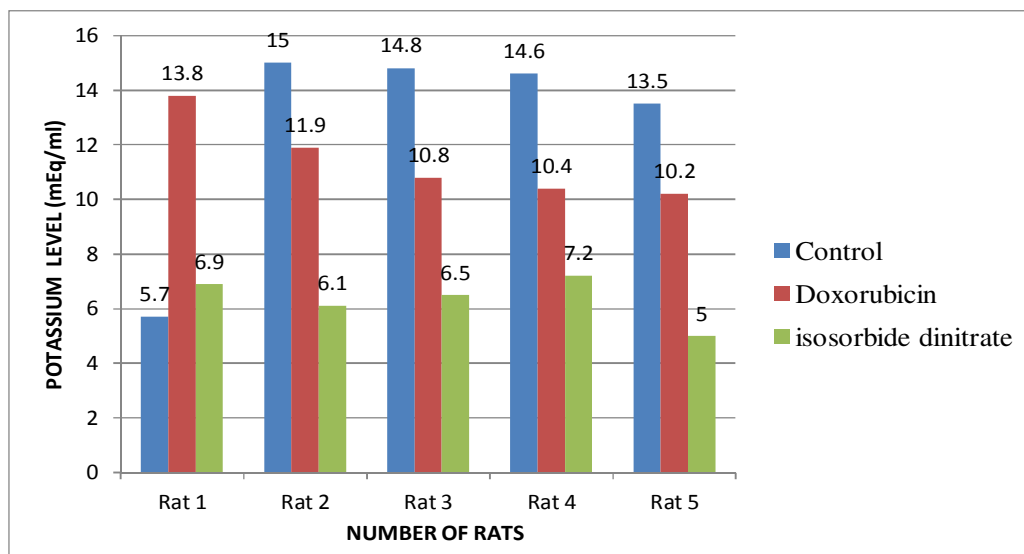


Figure 3. Effect of Doxorubicin (2mg/kg i.p) and isosorbide dinitrate (20mg/kg i.p) on plasma potassium levels

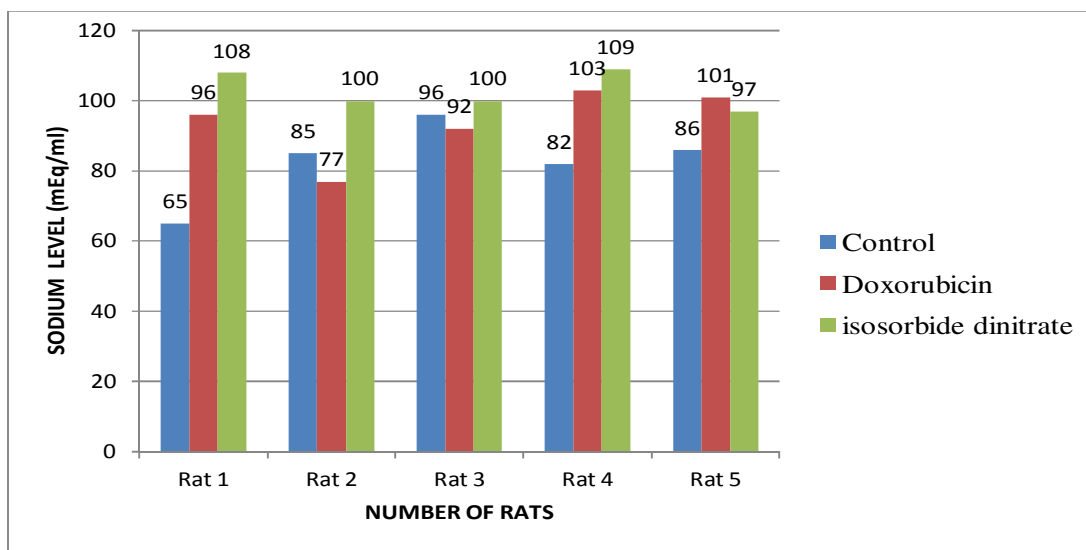


Figure 4. Effect of Doxorubicin (2mg/kg i.p) and isosorbide dinitrate (20mg/kg i.p) on plasma sodium levels

of ketone bodies in a range of 5-15 mg in urine. The two TonEBP modulators, Dox and isosorbide dinitrate also significantly altered potassium and sodium levels in blood as shown in figure 3 and 4.

DISCUSSION

TonEBP was first found in heart by immunohistochemistry. Although there was a high level of expression, no physiological function was described. Navarro and Chiong (Navarro et al., 2008) with other

colleagues found out that hypertonicity induced both TonEBP mRNA and protein in cardiomyocytes; and increases in mRNA and protein for TonEBP target enzymes were also found.

In this study, we utilized Dox and Isosorbide dinitrate the two known modulators of TonEBP to elucidate the effects on the heart rate and biochemical parameters. It has been shown that the protein level of the TonEBP was reduced by Dox treatment. In addition, the reduction in TonEBP protein content was suppressed by proteasome inhibitors. As shown in the results section i.e figures 1 and 2, doxorubicin caused a reduction of heart

Table 1. Influence of Doxorubicin (2mg/kg i.p) and isosorbide dinitrate (20mg/kg i.p) on urine glucose and ketone levels

No. of Rats used	Glucose level	Ketone level
Rat 1	Negative	15 mg/dL
Rat 2	Negative	5 mg/dL
Rat 3	Negative	5 mg/dL
Rat 4	Negative	15 mg/dL
Rat 5	Negative	Negative
Rat 6	Negative	Negative

Glucose - negative (below 0.1mg/dL)

Ketone - negative (below 5 mg/dL)

rate from the control levels and this was further attenuated by vasopressin treatment. While on the other hand the ET-1 antagonist, BQ 123 was able to increase the heart rate moderately. Dox-enhanced degradation of TonEBP resulting in reduced TauT expression in cardiomyocytes (Ito et al., 2009). Degradation of NFAT5, a transcriptional Regulator of Osmotic Stress-related Genes, is a critical Event for Dox induced Cytotoxicity in Cardiac myocytes (Ito et al., 2008), it appears that down regulation of TonEBP caused this reduction in heart rate. Hypertonicity has been suggested to be a major, ADH-independent factor for transcription of AQP2 in the renal medulla (Kasono et al., 2005) in which TonEBP is clearly involved (Umenishi and Schrier, 2002). Vasopressin has been known to cause bradycardia by acting on vasopressin receptors and these results are in accordance with our previous results.

ET-1 is a very potent vasoconstrictor that binds to smooth muscle endothelin receptors, of which there are two subtypes: ET_A and ET_B receptors. These receptors are coupled to a Gq-protein and receptor activation leads to the formation of IP₃, which causes the release of calcium by the sarcoplasmic reticulum (SR) and increased smooth muscle contraction and vasoconstriction. There are also ET_B receptors located on the endothelium that stimulate the formation of nitric oxide, which produces vasodilation in the absence of smooth muscle ET_A and ET_B receptor activation. This receptor distribution helps to explain the phenomenon that ET-1 administration causes transient vasodilation (initial endothelial ET_B activation) and hypotension, followed by prolong vasoconstriction (smooth muscle ET_A and ET_B activation) and hypertension. ET-1 receptors in the heart are also linked to the Gq-protein and IP₃ signal transduction pathway. Therefore, ET-1 in the heart causes SR release of calcium, which increases contractility. Our data suggests some increase in heart rate after Dox treatment and this can be attributed to vasodilatation in the peripheral blood vessels and mild reflex tachycardia.

We further experimented on modulating the TonEBP using Dox, if it may have any effect on biochemical

parameters. Electrolytes sodium and potassium are critical for cardiovascular homeostasis (Adroque and Madias, 2007). TonEBP gets decreased when there is hypokalemia in rats. Downregulation of TonEBP appeared to have also contributed to reduced expression of aquaporin-2 and UT-A urea transporters in the renal medulla. A salient feature in the hypokalemic animals was the dramatic downregulation of TonEBP at the tip of the inner medulla. The downregulation of TonEBP due to reduced medullary tonicity. We analysed the plasma potassium levels of the rats before and after treating with doxorubicin. The rationale was the activation of aldose reductase enzyme inhibition leading to glucose accumulation. A possible correlation of potassium with taurine in kidney has been implicated by earlier studies (Roysommuti et al., 2010). The results shows that the potassium levels have found to be decreased and sodium levels were increased as shown in figure 3 and 4. These electrolyte levels were analysed using electrotyte analyzer.

We also analysed the urine of rats after administering Dox for the estimation of ketone and glucose levels using glucose diastix. The result shows that the glucose levels was found to be negative, but there has been a slight change in the ketone levels. There was no effect on glucose levels but in few rats there was detection of ketone bodies in the urine. This is an interesting find suggesting the role of doxorubicin on ketone metabolism and inducing ketoacidosis.

In summary, it can be stated that doxorubicin induced degradation of TonEBP decreased the potassium levels and increased in sodium levels. So TonEBP modulation has effect on these potassium and sodium levels in the rat blood. It also can have effect on the ketone levels, because after Dox induced degradation of TonEBP the ketone levels in the urine had changed in a few animals.

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REFERENCES

- Adrogue HJ, Madias NE (2007). Sodium and potassium in the pathogenesis of hypertension. *N Engl. J. Med.* 356 :1966-1978.
- Burg MB, Ferraris JD, Dmitrieva NI (2007). Cellular response to hyperosmotic stresses. *Physiol. Rev.* 87:1441-1474
- Cha JH, Woo SK, Han KH, Kim YH, Handler JS, Kim J, Kwon HM (2001). Hydration status affects nuclear distribution of transcription factor tonicity responsive enhancer binding protein in rat kidney. *J. Am. Soc. Nephrol.* 12: 2221-2230,
- Favale NO, Sterin Speziale NB, Fernandez, Tome MC (2007). Hypertonic-induced lamin A/C synthesis and distribution to nucleoplasmic speckles is mediated by TonEBP/NFAT5 transcriptional activator. *Biochem. Biophys. Res. Commun.* 364:443-449
- Hasler U, Jeon US, Kim JA, Mordasini D, Kwon HM, Feraille E, Martin PY (2006). Tonicity-responsive enhancer binding protein is an essential regulator of aquaporin-2 expression in renal collecting duct principal cells. *J. Am. Soc. Nephrol.* 17: 1521-1531,
- Han KH, Woo SK, Kim WY, Park SH, Cha JH, Kim J, Kwon HM (2004). Maturation of TonEBP expression in developing rat kidney. *Am. J. Physiol. Renal. Physiol.* 287: F878-F885
- Hasler U, Vinciguerra M, Vandervalle A, Martin PY, Feraille E (2005). Dual effects of hypertonicity on aquaporin-2 expression in cultured renal collecting duct epithelial cells. *J. Am. Soc. Nephrol.* 16: 1571-1582
- Irrarazabal CE, Williams CK, Ely MA, Birrer MJ, Garcia-Perez A, Burg MB, Ferraris JD (2008). Activator protein-1 contributes to high NaCl-induced increase in tonicity-responsive enhancer/osmotic response element-binding protein transactivating activity. *J. Biol. Chem.* 283: 2554-2563,
- Jeon US, Kim JA, Sheen MR, Kwon HM (2006). How tonicity regulates genes: story of TonEBP transcriptional activator. *Acta. Physiol.* 187: 241-247,
- Keizo Kasano, Tomoyuki Saito, Takako Saito, Hiroyuki Tamemoto, Chieko Yanagidate, Shinichi Uchida, Masanobu Kawakami, Sei Sasaki, San-e Ishikawa (2005). Hypertonicity regulates the aquaporin-2 promoter independently of arginine vasopressin. *Nephrol Dial Transplant.* 20: 509-515
- Küper C, D Steinert, M L Fraek, F X Beck, and W Neuhofer. EGF receptor signaling is involved in expression of osmoprotective TonEBP target gene aldose reductase under hypertonic conditions. *AJP - Renal Physiol* May 2009 vol. 296 no. 5 F1100-F1108
- Lam AKM, Ko BCB, Tam S, Morris R, Yang JY, Chung SK, Chung SSM. Osmotic response element-binding protein (OREBP) is an essential regulator of the urine concentrating mechanism. *J Biol Chem.* 279: 48048-48054, 2004.
- Mather KJ, AA Lteif, E Veeneman, R Fain, S Giger, K Perry, GD Hutchins (2010). Role of endogenous ET-1 in the regulation of myocardial blood flow in lean and obese humans. *Obesity*, 181-63-70
- Nakayama Y, Pang T, Sands JM, Bagnasco SM (2000). The TonE/TonEBP pathway mediates tonicity-responsive regulation of UT-A urea transporter expression. *J. Biol. Chem.* 275: 38275-38280
- Navarro P, Chiong M, Volkwein K, Moraga F, Ocaranza MP, Jalil JE, Lim SW, Kim JA, Kwon HM, Lavandero S (2008). Osmotically-induced genes are controlled by the transcription factor TonEBP in cultured cardiomyocytes. *Biochem Biophys Res Commun.* Jul 25; 372(2):326-30
- Qi Cai, Joan D Ferraris, Maurice B Burg (2005). High NaCl increases TonEBP/OREBP mRNA and protein by stabilizing its mRNA. *Am. J. Physiol. Renal Physiol.* 289: F803-F807
- Roysommuti S, P Malila, W Lerdweeraphon, D Jirakulsomchok, JM Wyss (2010). Perinatal taurine exposure alters renal potassium excretion mechanisms in adult conscious rats. *J. Biomed. Sci.* 17(Suppl 1):S29
- Storm R, Klussmann E, Geelhaar A, Rosenthal W, Maric K (2003). Osmolality and solute composition are strong regulators of AQP2 expression in renal principal cells. *Am. J. Physiol. Renal. Physiol.* 284: F189-F198
- T Ito, Y Fujio, SW. Schaffer J Azuma (2009). Involvement of transcriptional factor TonEBP in the regulation of the taurine transporter in the cardiomyocyte. Takashi Ito, Yasushi Fujio, Stephen W. Schaffer and Junichi Azuma. *Advances in Experimental Medicine and Biology*, Volume 643, Part IX, 523-532
- Takashy Ito, Yasushi Fujio, Junichi Azuma (2008). Enhancement of proteasome-linked TonEBP/NFAT5 degradation in cardiomyocytes exposed to doxorubicin. *The FASEB J.*
- Tyagi MG, Thomas M (1999). Enhanced cardiovascular reactivity to desmopressin in water-restricted rats: facilitatory role of immunosuppression. *Methods Find Exp. Clin. Pharmacol.* Nov; 21(9):619-24.
- Umenishi F, Schrier RW (2002). Identification and characterization of a novel hypertonicity-responsive element in the human aquaporin-1 gene. *Biochem Biophys Res Commun* 292: 771-775