Osmotically-induced genes are controlled by the transcription factor TonEBP in cultured cardiomyocytes

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A B S T R A C T

Changes in cardiac osmolarity occur in myocardial infarction. Osmoregulatory mechanisms may, therefore, play a crucial role in cardiomyocyte survival. Tonicity-responsive enhancer binding protein (TonEBP) is a key transcription factor participating in the adaptation of cells to increases in tonicity. However, it is unknown whether cardiac TonEBP is activated by tonicity. Hypertonicity activated transcriptional activity of TonEBP, increased the amounts of both TonEBP mRNA and protein, and induced both the mRNA and protein of TonEBP target genes (aldose reductase and heat shock protein-70). Hypotonicity decreased the amount of TonEBP protein indicating bidirectional osmoregulation of this transcription factor. Adenoviral expression of a dominant negative TonEBP suppressed the hypertonicity-dependent increase of aldose reductase protein. These results indicated that TonEBP controls osmoregulatory mechanisms in cardiomyocytes.

Keywords:
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Heart

The composition of blood plasma is closely regulated, so that the heart is normally maintained in an osmotically stable environment [1]. Under normal physiological conditions cardiac cells do not experience transmembrane osmotic gradients that would cause them to swell or shrink [2]. Both ischemia and reperfusion are, however, associated with anisosmotic changes [3,4]. Other pathological conditions, including diabetic coma and septic shock, also induce changes in cardiac cell volume [2]. Because cardiomyocytes have a limited proliferative potential, their death leads to heart failure [5]. Given that the ability of cardiomyocytes to regulate their volume may be crucial to their survival, regulation of cell volume in the heart is emerging as an area for further investigation.

Tonicity-responsive enhancer binding protein (TonEBP) is the key transcription factor that participates in the adaptation of cells to increases in tonicity [6,7]. TonEBP is a member of the Rel family of transcriptional activators which include NFκB and NFAT [8]. Hypertonicity induces TonEBP protein and activates the transcriptional expression of target genes responsible for the metabolism of organic osmolytes, including the sodium/myo-inositol transporter (SMIT), the taurine transporter (TauT), the betaine/GABA transporter (B GT-1), the vasopressin-regulated urea transporter (UT-A) and aldose reductase (AR) [6,7]. TonEBP also induces molecular chaperones, such as heat shock protein 70-2 (Hsp70-2) and the osmotic stress protein of 94 [9,10]. This factor can also regulate processes such us embryonic development, antibody production and cell migration [11–13].

The physiological role of TonEBP in the heart is unknown. Recently, Ito et al. described that doxorubicin-induced cardiotoxicity is associated with degradation of TonEBP suggesting a cardioprotective role for this transcription factor [14]. This study did not, however, include investigation as to whether TonEBP is osmotically regulated in the heart.

Here we show that TonEBP is present in cardiomyocytes, is activated by hypertonicity, is repressed by hypotonicity and induces the expression of AR, HSP-70, and SMIT.

Materials and methods

Culture and treatment of cardiomyocytes. Isolation and culture of neonatal rat ventricular cardiomyocytes, and hyperosmotic or hyposmotic stresses induced with sorbitol or by dilution with dis-

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tilled water were described previously [15,16]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and it was approved by our Institutional Ethics Review Committee.

Preparation of protein extracts and Western blotting. Extraction of proteins and Western blots were performed as described [15]. Nuclear and cytosolic fractions were prepared by differential centrifugation [17]. Antibodies against TonEBP [18], AR [19], HSP70 (Cell Signaling Technology Inc.), β-actin (Sigma–Aldrich Inc.) and TFIIB (Sigma–Aldrich Inc.) were used at dilutions of 1:5000, 1:20,000, 1:5000, 1:1000, and 1:1000, respectively.

Immunostaining for TonEBP. Cardiomyocytes were cultured onto glass coverslips, fixed with cold methanol, blocked with 3% BSA–PBS, incubated with anti-TonEBP (1:400 in 1% BSA-PBS, 2 h) and revealed with FITC-conjugated anti-rabbit (Sigma) (1:400 in 1% BSA–PBS, 1 h). Nuclei were stained with propidium iodide. Coverslips were examined with a confocal microscope (Carl Zeiss, LSM 5 Pascal) using a 40× 1.4 NA oil immersion objective.

RNase protection assay. Total RNA from cultured cardiomyocytes was prepared using TriZol (Invitrogen, Carlsbad, CA). RNase protection assays (RPA) were performed with radiolabeled TonEBP, AR, SMIT, and HSP-70 probes as described [20].

Transfections and luciferase reporter assays. Cardiomyocytes were cotransfected with both TonEBP-lux [8] and phRL-TK (Promega Corp.) plasmids using Lipofectamine (Invitrogen Corp.). Transfected cells were incubated with 400, 500 or 600 mOsm media. Cells were lysed and luciferase and *Renilla* activities were measured using the Dual-Luciferase® Reporter Assay System (Promega Corp.).

Adenovirus constructs and transduction. Dominant negative and wild type TonEBP genes were subcloned in pDC316 (Microbix Biosystems Inc.) [18]. Adenoviruses were produced by cotransfection of HEK293 cells with recombinant pDC316-TonEBP and pBHGloxΔE1,3Cre [21]. Cardiomyocytes were transduced with dnTonEBP, wtTonEBP or LacZ adenoviruses at a multiplicity of infection of 3000 for 24 h.

Statistical analysis. Results are shown as means ± SD. Data were evaluated by *T*-test or ANOVA analysis. Values $p < 0.05$ were considered statistically significant.

Results

Regulation of cardiac TonEBP by tonicity

TonEBP gene expression was regulated in cultured cardiomyocytes in a tonicity-dependent manner. Increases of TonEBP mRNA were observed in cardiomyocytes cultured in 500 mOsm (but not in 400 mOsm) hypertonic culture media when compared with normotonic conditions (290 mOsm) (Fig. 1A). Hypertonicity (500 mOsm) also increased TonEBP protein, this being maximal at 16 h (Fig. 1B). Translocation of TonEBP to the nucleus was ob-

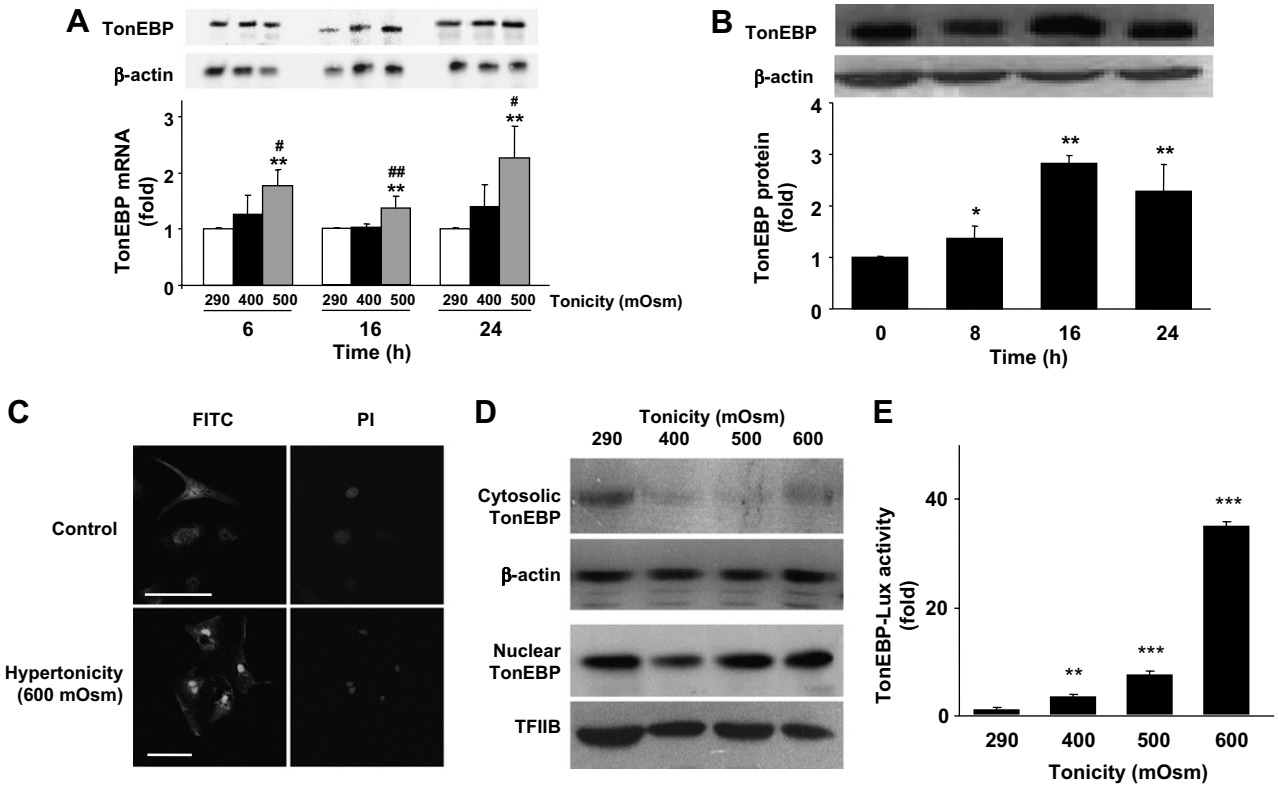

Fig. 1. Effect of hypertonicity on TonEBP mRNA and protein levels. (A) RNase protection assay of total RNA from cardiomyocytes incubated with 290 (white bars), 400 (black bars) or 500 (gray bars) mOsm culture media for 6, 16, and 24 h. A representative experiment showing protected bands for TonEBP and β-actin is presented. TonEBP mRNA abundance is expressed as percentage of control at 290 mOsm. (B) Cells were incubated with 600 mOsm media for 8, 16, and 24 h. Western blot was performed using anti-TonEBP. Data correspond to the average of five independent experiments; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs 0 h; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs 290 mOsm. (C) Cardiomyocytes were grown on coverslips, incubated with or without 600 mOsm culture media for 16 h, and immunofluorescences were performed with anti-TonEBP (FITC). Nuclei were stained with PI. Scale bar 50 μm. (D) Cardiomyocytes were exposed to 290, 400, 500, and 600 mOsm for 16 h, and total protein from nucleus and cytosol fractions were obtained. Western blots for TonEBP, β-actin and TFIIB were performed as described in Materials and methods. (E) Cardiomyocytes were cotransfected with TonEBP-Lux and phRL-TK plasmids, incubated 24 h, exposed to 290, 400, 500, and 600 mOsm for 16 h and total protein extracts were obtained. Luciferase and *Renilla* activities were measured using the Dual-Luciferase® Reporter Assay System (Promega Corp.). Values are expressed as luciferase/*Renilla* activities and correspond to the average of three independent experiments. $^{**}p < 0.01$ vs 290 mOsm, $^{***}p < 0.001$ vs 290 mOsm.

served after 16 h of incubation in 500 mOsm medium (Fig. 1C). Western blot of nuclear and cytosolic fractions also showed translocation of TonEBP to the nucleus in a tonicity-dependent manner (Fig. 1D). A tonicity-dependent transcriptional activation of TonEBP was also determined (Fig. 1E). These results showed that hypertonicity increased in cardiomyocytes TonEBP mRNA and protein, induced TonEBP nuclear translocation and activated TonEBP transcriptional activity.

Hypotonicity (202 mOsm) decreased TonEBP protein (Fig. 2A). Immunocytochemical detection of TonEBP showed no nuclear translocation at 16 h of exposure at 202 mOsm medium (Fig. 2B). These data show that cardiac TonEBP is bidirectionally regulated by tonicity, suggesting an osmoregulatory action for this transcription factor in cardiac tissue.

Regulation of TonEBP target genes by tonicity

Increases of mRNAs for AR, HSP-70, and SMIT were detected as early as 6 h after exposure to 400 and 500 mOsm media (Fig. 3A). An increase in AR protein was seen after exposure of cells to 600 mOsm medium for 16 and 24 h (Fig. 3B). The increase in AR protein was also tonicity-dependent (Fig. 3C). AR activity also showed increases with exposure to 600 mOsm at 16 and 24 h. Therewere, however, changes in neither AR protein nor enzymatic activity after incubation with 202 mOsm medium for 8, 16, and 24 h.

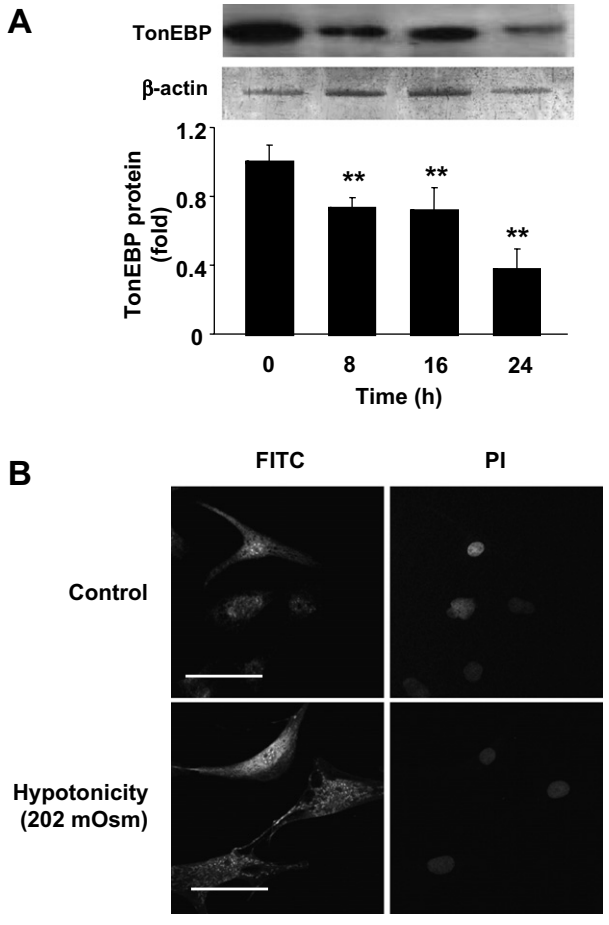

Fig. 2. Effect of hypotonicity on TonEBP protein level. (A) Cells were incubated with 202 mOsm culture media for 8, 16, and 24 h. Western blot was performed using anti-TonEBP. Data correspond to the average of three independent experiments; $^{**}p < 0.01$ vs 0 h. (B) Cardiomyocytes were grown on coverslips, incubated with or without 202 mOsm culture media for 16 h and immunofluorescences were performed with anti-TonEBP (FITC). Nuclei were stained with PI. Scale bar 50 μm.

Incubation with hypertonic medium increased the amount of HSP-70 protein earlier than it did that of AR. At 16 h of exposure to 600 mOsm there was a maximum of 4.12 (SD 0.5) fold over control (Fig. 3D). Increasing tonicity also increased the amount of HSP-70 protein (Fig. 3E).

These data indicate that hypertonicity induces expression of TonEBP target genes. We did not, however, see any decrease in the amount of AR protein under hypotonic conditions (data not shown).

Effect of dominant negative and wild type TonEBP overexpression on aldose reductase

In order to demonstrate that AR gene expression was controlled by TonEBP, cardiomyocytes were transduced with adenovirus overexpressing either wtTonEBP (Ad wtTonEBP) or dnTonEBP (Ad dnTonEBP) and AR protein levels were assessed by Western blot. In isotonic conditions, Ad wtTonEBP increased basal AR protein level, but Ad dnTonEBP did not reduced AR level (Fig. 4A). However, Ad dnTonEBP blocked hypertonic-dependent increase of AR (Fig. 4B). Moreover, Ad wtTonEBP further increased AR protein level (Fig. 4B). These results suggest that TonEBP control hypertonic-dependent expression of AR in cardiomyocytes.

Discussion

Our main findings were that: (a) cardiac TonEBP is bidirectionally regulated by tonicity, and (b) TonEBP controls tonicity-induced genes such as AR. Taken together, these data suggest an osmoregulatory role of TonEBP in the heart.

TonEBP was first described in the heart by immunohistochemistry [22]. Although there was a high level of expression in the heart, no physiological function was described [22]. Zhang et al. investigated the osmoregulatory action of TonEBP in non-renal tissues by evaluating the decrease of TonEBP mRNA and protein using the rat "vasopressin escape" model of hyposmolality [23]. They found a decrease in both TonEBP protein and mRNA in the liver with no change in TonEBP mRNA in the heart. Moreover, they found no TonEBP protein in cardiac tissue [23]. These authors suggest that in liver, tonicity bidirectionally control TonEBP activity, but that, in other tissues, because TonEBP does not respond to hypotonicity, this transcription factor may have another function [23]. Our results show that hypertonicity induced both TonEBP mRNA and protein in cardiomyocytes; and increases in mRNA and protein for TonEBP target genes were also found. These data suggest that cardiac TonEBP was activated by hypertonicity. Moreover, hypotonicity decreased the amount of TonEBP protein and no nuclear translocation was observed. Our data show that cardiac TonEBP is bidirectionally controlled by tonicity, suggesting an osmoregulatory action in the heart.

TonEBP activation has been described also in other non-renal tissues. In thymus and lymphocytes, during the activation of an immune response, there are increases in both TonEBP protein and activity [24]. Ho et al. hypothesized that during an immune response, immature thymocytes and activated lymphocytes undergo rapid cell proliferation, associated with a high rate of metabolic activity, leading to a decrease in the intracellular volume [6,25]. Increased macromolecular synthesis consumes amino acids and inositol, which function as intracellular osmolytes, decreasing the intracellular water and thus represent an osmotic stress that is functionally identical to exposing a cell to extracellular hypertonicity [6]. Thus, the osmotic stress response pathway defined by TonEBP appears to mediate cellular adaptation not only to overt extracellular osmotic stress, but also rather to any process that affects intracellular water homeostasis [6].

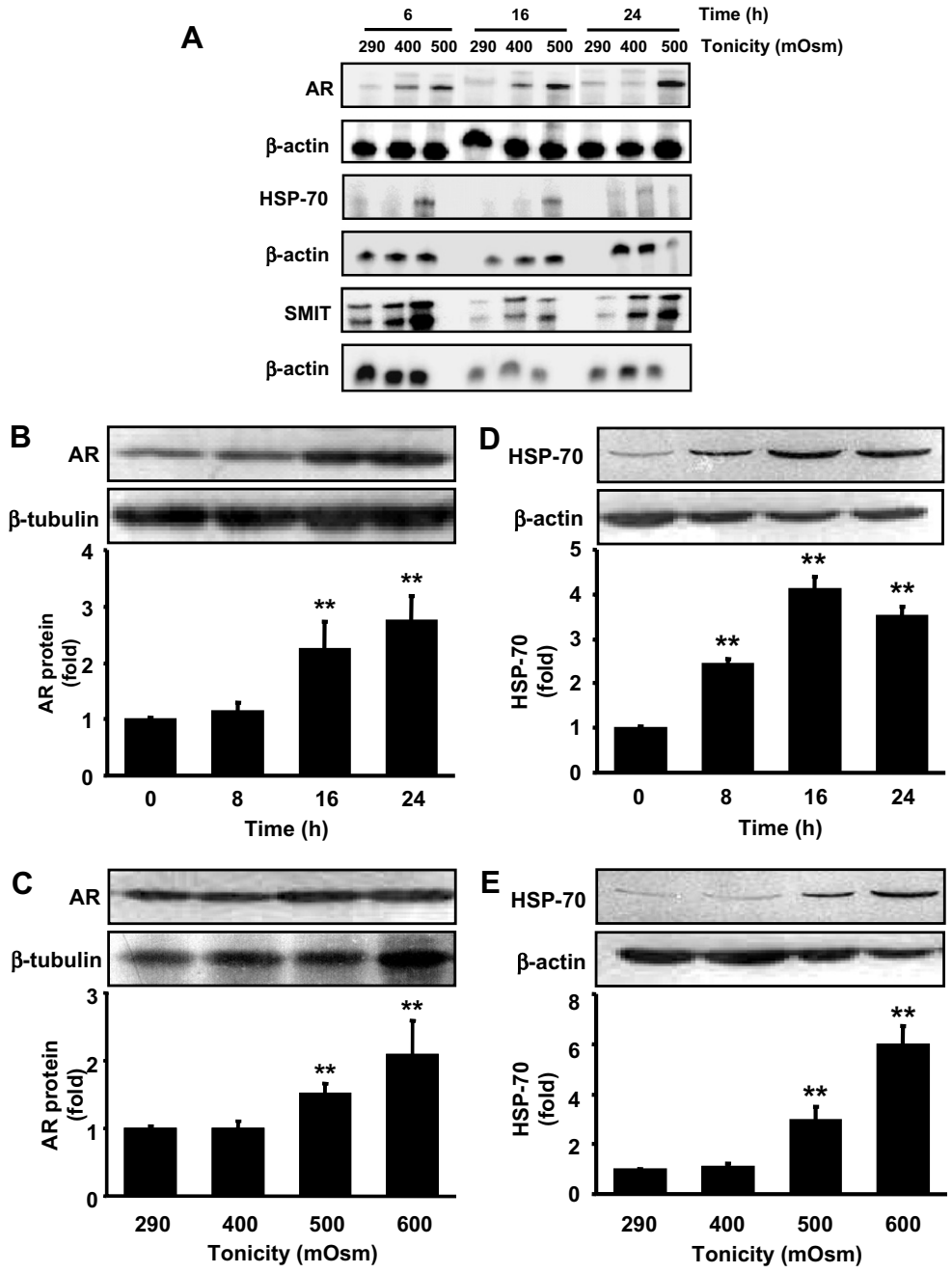

Fig. 3. Effect of hypertonicity on TonEBP target genes. (A) RNase protection assay of total RNA from cardiomyocytes incubated with 290, 400 or 500 mOsm culture media for 6, 16, and 24 h. Protected bands for AR, HSP-70, SMIT, and β-actin are shown. (B,D) cardiomyocytes were incubated with 600 mOsm culture media for 8, 16, and 24 h. (C,E) cardiomyocytes were incubated with 290, 400, 500, and 600 mOsm for 16 h. Western blot was performed using anti-AR (B,C) or anti-HSP-70 (D,E). Data are the mean (SD) of four independent experiments. $^{**}p < 0.01$ vs 0 h or 290 mOsm.

Because uninterrupted contraction is a unique feature of the cardiomyocytes, these cells have a high rate of metabolic activity. In conditions that heart rate and contraction are increased metabolic activity is further enhanced. Although cardiomyocytes do not proliferate, increased protein biosynthesis may be associated with cardiac hypertrophy. A physiological role for cardiac TonEBP, may, therefore, be associated, in a way similar to that in thymocytes and lymphocytes, with the regulation of intracellular water homeostasis—especially in conditions with increased metabolic activity. This feature could be particularly important during heart development, because a substantial proportion of TonEBP knock-out mice died by mid-gestation [24,26]. The basis for this embryonic lethality was not clarified, but kidney dysfunction seems unlikely to be responsible, because maintenance of the extracellular milieu of the fetus depends on the placenta, not the fetal kidney [26]. Rat fetal hearts display an increased heart rate as compared to adult hearts [27], and also exhibit proliferation of cardiomyocytes. Regulation of intracellular osmolarity must, therefore, be critical and the absence of TonEBP could be lethal. Therefore, we propose that the physiological function of cardiac TonEBP may, in normal conditions, relate to the regulation of intracellular water homeostasis. However, further experiment will be required to verify this hypothesis.

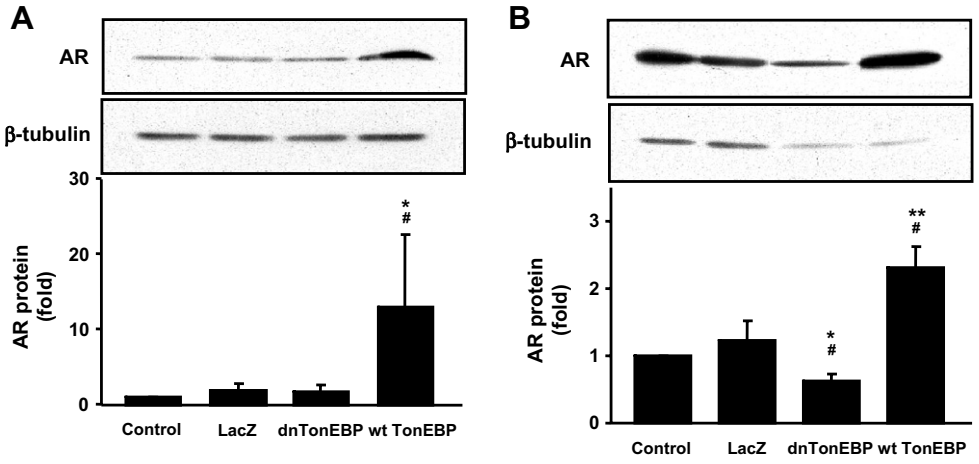

Fig. 4. Effect of overexpression of wild type and dominant negative TonEBP on aldose reductase levels in cardiomyocytes. Cells were transduced (MOI 3000) for 24 h with wtTonEBP, dnTonEBP or LacZ adenoviruses, and then incubated for 16 h with an isotonic culture medium (290 mOsm, A) or a sorbitol hypertonic culture medium (600 mOsm, B). Protein extracts were prepared and equal amounts of protein were submitted to Western blot using anti-AR and anti β-tubulin antibodies. Results represent the mean (SD) of three independent experiments. $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, $^{\#}p < 0.05$ vs LacZ.

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