

**Diabetes and thyroid hormones affect connexin-43 and PKC- $\epsilon$  expression in rat heart atria.**

Marcela Mitašíková, Hai Lin<sup>1</sup>, Tomáš Soukup<sup>2</sup>, Issei Imanaga<sup>1</sup>, Narcisa Tribulová

Institute for Heart Research, Slovak Academy of Sciences, Bratislava, Slovak Republic,

<sup>1</sup>Department of Physiology, School of Medicine, Fukuoka University, Fukuoka, Japan,

<sup>2</sup>Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic

Short title: diabetes and thyroid hormones affect atrial connexin-43

Corresponding author:

Narcisa Tribulová, DSc.

Institute for Heart Research, Slovak Academy of Sciences

Dubravska cesta 9, POBOx 104

840 05 Bratislava, SK

Email: [narcisa.tribulova@savba.sk](mailto:narcisa.tribulova@savba.sk)

## **Summary**

We have examined changes of the intercellular electrical coupling proteins, connexin-43 (Cx43) and PKC- $\epsilon$  in diabetes and/or after a triiodothyronine ( $T_3$ ) administration in the rat heart atria. Diabetes was induced in Wistar Kyoto rats by streptozotocin (50mg/kg, i.v.) and atria were examined 5 (acute stage) and 10 (chronic stage) weeks thereafter.  $T_3$  (10 $\mu$ g/100g/day) was applied via gastric tube during the last 10 experimental days to non-diabetic and to a part of diabetic rats. Expression and phosphorylated status of Cx43, as well as expression of PKC- $\epsilon$  have been analyzed by Western blots using mouse monoclonal anti-Cx43 and rabbit polyclonal anti-PKC- $\epsilon$  antibodies. Results have shown that the Cx43 expression was significantly increased due to  $T_3$ -treatment and acute diabetes. However, both diabetes and  $T_3$  treatment markedly suppressed phosphorylated isoforms of Cx43 when compared to non-diabetic and  $T_3$ -untreated controls and this down-regulation was less pronounced in diabetic rats after the  $T_3$ -treatment. The expression of atrial PKC- $\epsilon$  was increased due to diabetes (this increase was suppressed by  $T_3$  administration) and decreased in  $T_3$ -treated non-diabetic rats. Based on our results, we suggest that the reduced Cx43 phosphorylation in diabetic and hyperthyroid rats can deteriorate a cell-to-cell coupling and consequently facilitate a development of atrial tachyarrhythmias in diabetic or hyperthyroid animals.

## **Key words**

Connexin-43, PKC- $\epsilon$ , rat heart atria, diabetes, triiodothyronine, atrial fibrillation

## **Introduction**

Basic underlying mechanisms of an atrial fibrillation (AF), the most common cardiac arrhythmia seen in the clinical practice and one of the major causes of stroke, are still not fully elucidated. However, understanding the mechanisms behind the disease is a prerequisite for designing the best treatment. Epidemiological studies have shown that diabetes mellitus and hyperthyroidism increase a risk for AF and its prevalence in an aged population (Shimizu *et al.* 2002, Miyasaka *et al.* 2006). Both clinical and experimental studies suggest that age-related myocardial structural alterations and intercellular gap junction abnormalities can facilitate an occurrence and persistence of AF (Spach and Starmer 1995, Tribulová *et al.* 1999, Kostin *et al.* 2002). A gap junction remodeling, often associated with fibrosis and/or hypertrophy of the atrial tissue, is characterized by an altered topology and/or alterations in number and size of the gap junctions. Since cardiac gap junctions are crucial for the electrical impulse propagation throughout the myocardium and cell-to-cell synchronization, remodeling of intercellular junctions creates anatomic substrates for derangement of the electrical conduction (Spach and Heidlage 1995, Kano and Saffitz 2001). In addition to gap junction remodeling, diseased or aged heart is characterized by alterations of a gap junction channel protein expression and/or its phosphorylation (Severs 1994, Tribulová *et al.* 2002, 2005, Lin *et al.* 2006). Several types of channel proteins, connexins, are expressed in the heart. Connexin-43 (Cx43) is the major gap junction protein abundant in the atrial as well as ventricular myocardium. Potential mechanisms controlling the level of intercellular gap junctional communication in the heart include a regulation of Cx43 dynamics and its phosphorylation. The latter has been shown implicated in the regulation of the channel conductance and its degradation (Dhein *et al.* 2002).

It has been reported that the incidence of AF in humans or in experimental animals is linked with the alterations in Cx43 or Cx40 expression (Tribulová *et al.* 1999, Polontchouk *et al.*

2001, Kostin *et al.* 2002, Kanagaratnam *et al.* 2006). However, data on whether diabetes or hyperthyroidism affects the expression of Cx43 and/or its phosphorylation are missing. In the Cx43 phosphorylation, several protein kinases, such as PKA, PKG, MAPK and PKC, have been implicated. Furthermore, it was found that an acute stage of diabetes led to a PKC activation and to an increase of PKC- $\epsilon$  isoform expression in cardiovascular tissues (Inoguchi *et al.* 2001, Lin *et al.* 2006). On the other hand, the cardiac expression of PKC- $\epsilon$  has been found to be decreased due to hyperthyroidism (Rybin and Steinberg 1996, Hamasaki *et al.* 2000).

High glucose was shown to induce inhibition of gap junction channels permeability through excessive phosphorylation of Cx43 in cultured aortic smooth muscles cells (Kuroki *et al.* 1998). Likewise, we have shown recently that acute diabetes suppress electrical cell-to-cell coupling and decrease conductivity in heart ventricles (Lin *et al.* 2006). Diabetes is known to be accompanied with reduced plasma thyroid hormone levels (Ferrer *et al.* 2006). Hypothyroidism itself is associated (as is diabetes) with an increase of PKC- $\epsilon$  expression (Rybin and Steinberg 1996), while hypothyroidism can be reversed by thyroid hormone replacement therapy.

Taken together, the goal of this study was to examine how are the expression and phosphorylation of Cx43 and PKC- $\epsilon$  levels altered in rat heart atria due to diabetes, hyperthyroidism and especially by the combination of both treatments.

## **Material and Methods**

All experiments were performed in accordance with the regulations of the Animal Research and Care Committees of Fukuoka University and Institute for Heart Research. Diabetes was induced in male Wistar Kyoto rats (8-week-old, 300-330g) by a single streptozotocin injection (STZ, Sigma, 50mg/kg, i.v.). Hyperglycemia was confirmed by a blood glucose assay and the animals were housed with free access to water and standard food. Triiodothyronine (Sigma, T<sub>3</sub>, 10  $\mu$ g/100g/day) was applied by *gavage* to non-diabetic and diabetic rats, for last 10 days prior

ending of the experiments. Age-matched non-diabetic rats with and without the T<sub>3</sub> treatment were used as well. There were two main experimental groups of rats that differ in stage of diabetes, i.e. we examined acute (5 weeks lasting) and chronic (10 weeks lasting) stages due to possible time-related differences in arrhythmia susceptibility. In both experimental groups, rats were divided into four subgroups: 1. non-diabetic rats (N, n=6); 2. diabetic rats (D, n=6); non-diabetic treated with T<sub>3</sub> (T3, n=6) and diabetic treated with T<sub>3</sub> (DT3, n=6). At the end of the experiment, fasting blood samples were taken to measure the glucose and thyroid hormone concentrations (total serum levels of T<sub>3</sub> and T<sub>4</sub> were measured by the RIA method using Immunotech kits for rat and human sera in order to check the thyroid status). Hearts were excised from ether anesthetized rats into ice-cold saline solution to stop beating, they were quickly weighed and atria were snap frozen in liquid nitrogen for Cx43 and PKC-ε immunoblotting.

*Western blot analysis of Cx43 and PKC-ε.* Tissue samples taken from both right and left atria were homogenized in the Tris-HCl buffer containing phenylmethane-sulphonyl fluoride on ice and centrifuged at 2000 g for 15 min at 4 °C. The supernatants were mixed with a 10% Triton X-100 followed by a centrifugation at 100 000g for 30 min. The pellets were used for WB assays. Twenty micrograms of total protein were run on a 10% SDS-PAGE electrophoresis and separated proteins were transferred onto PVDF membranes. After blocking with 5% skimmed milk in a T-PBS buffer (containing 0.1% Tween 20), the membranes were incubated with the primary monoclonal mouse anti-Cx43 antibody (Chemicon, Int., Inc., USA) at a dilution of 1: 4000 for an hour at room temperature. Secondary anti-mouse IgG antibody (Amersham Pharmacia Biotech., U.K) coupled with a fluorescent dye was used at a dilution of 1: 5000. Detection of PKC-ε was performed using a rabbit polyclonal antibody Lot. No. J1503, C-15, sc-214 (Santa Cruz Biotechnology, Inc.) at a dilution 1: 1000 and a secondary donkey anti-rabbit IgG (Chemicon, AP182P) coupled with a fluorescent dye at a dilution of 1: 2000. A low molecular weight

calibration kit (Amersham Pharmacia Biotech., U.K.) was used for detection of molecular weights of proteins.

*Statistics:* Data were expressed as mean  $\pm$  SD and unpaired Student t-test was used to analyze the statistical significance determined at  $p < 0.05$ .

## **Results.**

### *Data characterizing examined animals.*

Fasting blood glucose levels were markedly elevated and serum  $T_4$  and  $T_3$  significantly decreased in diabetic rats 5 and 10 weeks after the STZ application in comparison to non-diabetic littermates. Diabetes resulted in a decrease of both body and heart weight. Treatment of non-diabetic and diabetic rats with  $T_3$  (biologically active form of thyroid hormone) led to its elevation and suppression of  $T_4$  levels in the serum of STZ treated animals. Heart weight was significantly increased in  $T_3$ -treated compared to control rats (Tables 1 and 2).

### *Cx43 and PKC- $\epsilon$ Western blot analysis.*

The treatment of WKY rats with  $T_3$  led to a significant increase of total the Cx43 expression in atrial tissues of both used experimental groups (Fig. 1B, 2B). An increase of Cx43 was detected also in diabetic rat heart atria and it was significant in the acute (Fig. 1B), but not in the chronic stage of diabetes (Fig. 2B). However, both diabetes (regardless the stage) and  $T_3$  treatment resulted in a dramatic decrease of phosphorylated isoforms of Cx43 (Fig.1A, 2A) compared to non-diabetic or  $T_3$ -untreated rats. Interestingly, the decline of the phosphorylated status of Cx43 was less pronounced in the atria of diabetic rats treated with  $T_3$ . The atrial expression of PKC- $\epsilon$  (Fig. 3A,B) was significantly increased in the acute as well as in the chronic stages of diabetes, but suppressed in diabetic rats treated with  $T_3$ . In contrast to diabetes,  $T_3$  administration markedly decreased PKC- $\epsilon$  expression in atria of non-diabetic rats.

## **Discussion.**

In our study we have focused on changes in the expression of phosphokinase C- $\epsilon$  isoform and phosphorylation of the gap junction channel protein Cx43 in the rat heart atria. We have found that experimental diabetes and/or the T<sub>3</sub> treatment significantly affect the expression of Cx43 and PKC- $\epsilon$ , as well as the phosphorylation status of Cx43 isoforms.

It has been shown that cardiovascular risk factors associated with a development of AF include diabetes and the hyperthyroidism. While the initiation of AF is usually associated with an ectopic pulse formation (i.e. a triggered electrical activity often originating in the cardiomyocytes in the area of the pulmonary veins), sustaining of this arrhythmia is linked to a presence of proarrhythmia substrates facilitating re-entry mechanism (Wang *et al.* 1996, Olson 2001). The former has been shown promoted by thyroid hormones (Chen *et al.* 2002). The latter was likely associated with the structural and gap junction remodeling (Tribulová *et al.* 1999, Kostin *et al.* 2002) that has been correlated to an electrical remodeling, i.e. changes in an intra-atrial and inter-atrial conduction (Spach and Starmer 1995, Spach and Heidlage 1995). Once established, AF is not only self-perpetuating but also self-destructive, thus prompting a rapid treatment. Using ventricular tissues of the same animals as in the present study (Lin *et al.*, to be published) we have found that the T<sub>3</sub> administration led to a hypertrophy of ventricular cardiomyocytes, while chronic diabetes was accompanied with a focal fibrosis of ventricular tissue. Since early (acute) and late (chronic) stage of diabetes differ in the degree of cardiomyopathy and susceptibility to ventricular arrhythmias (Lin *et al.*, to be published) we examined atria in both stages. It can be expected that both interventions diabetes and T<sub>3</sub> administration affect the structure of atrial tissues as well.

A comparison of the Cx43 expression in the atria and ventricles supports this expectation. It shows the decrease of phosphorylated isoforms of Cx43 in both atria and ventricles of T<sub>3</sub>-treated

rats, as well as in the atria of diabetic rats, in which ventricular Cx43 was, in contrast, hyperphosphorylated (i.e. excessively phosphorylated). An increase of phosphorylation status of Cx43 in the ventricles due to acute diabetes has been shown previously (Lin *et al* 2006).

Cx43 is phosphoprotein (as recognized using alkaline phosphatase treatment) and changes in its phosphorylation status can modulate function of connexin channels (Lampe *et al.* 2000). Western blot analysis revealed two phosphorylated forms of Cx43 and one nonphosphorylated in atria, similarly as in ventricles (Lin *et al* to be published, Lin *et al* 2006). However, the T<sub>3</sub> administration to diabetic rats suppressed the Cx43 phosphorylation in the ventricles while not in the atria (see representative western blots showing phosphorylated and nonphosphorylated Cx43 forms in Fig. 4).

Diabetes also affected differently a total level of Cx43 that was significantly increased only in the atria, but it remained either unchanged (Lin *et al.*, to be published) or decreased (Lin *et al.* 2006) in the ventricles of diabetic rats. It suggests a chamber-specific regulation of Cx43 expression. Deterioration of the Cx43 mediated cell-to-cell communication and a reduced signal transduction was thus found in diabetic cardiac (Lin *et al.* 2006) and vascular tissues (Inoguchi *et al.* 2001). Also a mild hyperthyroidism, induced by the administration of T<sub>3</sub>, was accompanied by an increased expression of Cx43 only in the atria, but not in the ventricles (Lin *et al.*, to be published). Exposure of neonatal cultured cardiomyocytes to T<sub>3</sub> for 24-48 hrs resulted in an elevation of total Cx43 (Tribulová *et al.* 2004). However, the administration of thyroid hormone to old male and female rats did not affect significantly the Cx43 expression (Tribulová *et al.* 2005). It has been reported that thyroid hormone receptors bind to an element in a connexin 43 promoter in a tissue specific manner (Stock and Sies 2000). Perhaps this process can be influenced or can interfere with age-related alterations in intracellular signaling pathways.



Different from the up-regulation of Cx43 expression in the rat atria, its phosphorylation was markedly decreased by both diabetes and the T<sub>3</sub> treatment. It is likely that dramatic abnormalities in the phosphorylation status may affect the Cx43 channel properties and function (Stagg and Flechter 1995, Lampe *et al.* 2000) and consequently influence cardiac arrhythmia susceptibility. It should be noted that a decline of Cx43 phosphorylation found in ventricular tissue was indeed associated with an increased susceptibility of T<sub>3</sub>-treated rats to a ventricular fibrillation (Lin *et al.*, to be published). On the other hand, the diabetes induced increase of the Cx43 phosphorylation in rat heart ventricles, which resulted in a decreased susceptibility to life-threatening cardiac arrhythmia. Since there was the significantly decreased phosphorylation of Cx43 in the atria of diabetic or T<sub>3</sub>-treated rats, we can assume that it likely promotes the appearance and sustaining of AF in such rats. It remains, however, not clear, how the treatment of diabetic rats with T<sub>3</sub> prevents a dramatic decline in Cx43 phosphorylation observed after the STZ administration. Accordingly, further studies are also needed to examine how the modulation of Cx43 phosphorylation affects the vulnerability of diabetic and/or T<sub>3</sub>-treated rats to AF.

It has been previously shown (Doble *et al.* 2000, Lampe *et al.* 2000, Bowling *et al.* 2002, Lin *et al.* 2006) that PKC- $\epsilon$  is one of the protein kinases, which directly phosphorylates Cx43. We have found its increased expression both in the atria (this study) and in the ventricles (Lin *et al.* 2006) of diabetic rats. However, the phosphorylation of Cx43 (supposed to be most likely PKC- $\epsilon$ -related) was increased only in the ventricles (Lin *et al.* 2006), but surprisingly not in the atria of diabetic rats (cf. Fig. 3). This difference remains also to be elucidated. Different from diabetes, the T<sub>3</sub> administration resulted in a decrease of PKC- $\epsilon$  in both the atria (this study) and the ventricles (Lin *et al.*, to be published). It was associated with the decrease of the Cx43 phosphorylation in both tissues and this could affect the gap junction channel mediated intercellular communication (Stagg and Flechter 1990).

In conclusion, our findings indicate that diabetes and mild hyperthyroidism in rats up-regulate the Cx43 atrial expression, but they significantly decrease the Cx43 phosphorylation. Decline in the phosphorylation status of Cx43 may contribute to the deterioration of the cell-to-cell electrical coupling promoting occurrence of AF.

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Address for reprints: Narcisa Tribulová, DSc., Institute for Heart Research, Slovak Academy of Sciences, Dubravská cesta 9, POBox 104, 840 05 Bratislava, Slovakia. E-mail: [narcisa.tribulova@savba.sk](mailto:narcisa.tribulova@savba.sk)

## Text to Figures

**Fig. 1.** A representative Western blot (upper panel) and densitometric quantification of Cx43 immunoblots (bottom panel) in the atria of non-diabetic (N), T<sub>3</sub> treated non diabetic rats (T3), 5 weeks diabetic (D) and 5 weeks diabetic T<sub>3</sub>-treated (DT3) rats. Note the significant decrease of the Cx43 phosphorylated (P1+P2) to unphosphorylated (P0) ratio in T<sub>3</sub>-treated and especially in diabetic rat heart atria. Interestingly, the Cx43 phosphorylation is not suppressed in T<sub>3</sub> treated diabetic rats. The total Cx43 levels are increased in T<sub>3</sub>-treated as well as diabetic rat heart atria and partly in diabetic T<sub>3</sub> treated rats. Data (n=6) are means  $\pm$ SD and \*p<0.05 vs N, #p<0.05 vs D

**Fig. 2.** A representative Western blot (upper panel) and densitometric quantification of Cx43 immunoblots (bottom panel) in the atria of non-diabetic (N), T<sub>3</sub>-treated non-diabetic (T3), 10 weeks diabetic (D) and 10 weeks diabetic T<sub>3</sub>-treated (DT3) rats. Note a dramatic decrease of the phosphorylated (P1+P2) to unphosphorylated (P0) Cx43 ratio in T3 and D groups, while the administration of T<sub>3</sub> to diabetic rats (DT3) partly attenuates the effect of diabetes. The total Cx43 levels are significantly increased due to the T<sub>3</sub> treatment. Data (n=6) are means  $\pm$ SD and \*p<0.05 vs N, #p<0.05 vs D

**Fig. 3.** Representative Western blots of PKC- $\epsilon$  and their quantitative densitometric analysis in the atria of non-diabetic (N), T<sub>3</sub>-treated non-diabetic (T3), diabetic (D) and diabetic T<sub>3</sub> treated (DT3) rats. The upper panel represents the acute diabetic stage (5 weeks after the STZ administration) and bottom panel the chronic diabetic stage (10 weeks after the STZ administration). Note that the expression of PKC- $\epsilon$  was significantly suppressed in T<sub>3</sub>-treated rats (bottom panel), but increased in both groups with diabetes. The T<sub>3</sub> treatment in acute diabetic rats almost attenuated PKC- $\epsilon$  level

increased due to diabetes, but in chronic diabetic rats it suppressed only partially the PKC- $\epsilon$  increase observed in diabetic rats. Data (n=6) are means  $\pm$ SD and \*p<0.05 vs N, #p<0.05 vs D

**Fig. 4.** Representative immunoblots showing the Cx43 expression in the atria (upper panel) and for comparison in the ventricles (bottom panel) of non-diabetic (N), T<sub>3</sub>-treated non-diabetic (T3), diabetic (D) and diabetic T<sub>3</sub>-treated (DT3) rats. Note the decrease of phosphorylated P1 and P2 isoforms of Cx43 both in the atria and ventricles of T<sub>3</sub>-treated rats, as well as in the atria of diabetic rats in which, in contrast, ventricular Cx43 isoforms are hyper-phosphorylated. The T<sub>3</sub> administration to diabetic rats suppressed the Cx43 phosphorylation in the ventricles, but not in the atria. P0 – non-phosphorylated isoform of the Cx43.

**Table 1.**

Main characteristics of the 13-week-old non-diabetic (N), non-diabetic treated with T<sub>3</sub> for 10 days (NT3), 5 weeks diabetic (D) and 5 weeks diabetic rats treated with T<sub>3</sub> (DT3).

	N	NT3	D	DT3
Body weight (g)	450±33.7	420±23.6	345±45.9*	307±43.5*
Heart weight (g)	1.18±0.06	1.53±0.10*	1.26±0.28	1.17±0.23
BG (mmol.l <sup>-1</sup> )	4.73±0.4	5.53±0.5*	26.8±4.5*	29.76±3.9*
Serum T <sub>3</sub> (nmol/L)	1.46±0.15	1.77±0.10*	1.08±0.21*	1.85±0.00* <sup>#</sup>
Serum T <sub>4</sub> (nmol/L)	56.93±11.84	15.44±0.17*	34.11±2.14*	14.16±1.51* <sup>#</sup>

Values are means ± SD of 6 rats in each group. \*Significantly different from N (P<0.05),  
<sup>#</sup> significantly different from D (P<0.05), BG – fasting blood glucose

**Table 2.**

Main characteristics of the 18- week-old non-diabetic (N), non-diabetic treated with T<sub>3</sub> for 10 days (NT3), 10 weeks diabetic (D) and 10 weeks diabetic rats treated with T<sub>3</sub> (DT3).

	N	NT3	D	DT3
Body weight (g)	497±42.8	463±16.6	375±25.2*	321±50* <sup>#</sup>
Heart weight (g)	1.22±0.05	1.59±0.11*	1.18±0.99	1.38±0.15* <sup>#</sup>
BG (mmol.l <sup>-1</sup> )	5.47±0.6	4.94±0.6	28.44±5.5*	30.00±4.7*
Serum T <sub>3</sub> (nmol/L)	1.46±0.08	1.89±0.12*	1.08±0.14*	1.32±0.06* <sup>#</sup>
Serum T <sub>4</sub> (nmol/L)	67.18±6.44	19.05±3.22*	37.58±10.89*	13.64±1.61* <sup>#</sup>

Values are means ± SD of 6 rats in each group. \*Significantly different from N (P<0.05),  
<sup>#</sup> significantly different from D (P<0.05), BG – fasting blood glucose



Fig. 1. A, B.

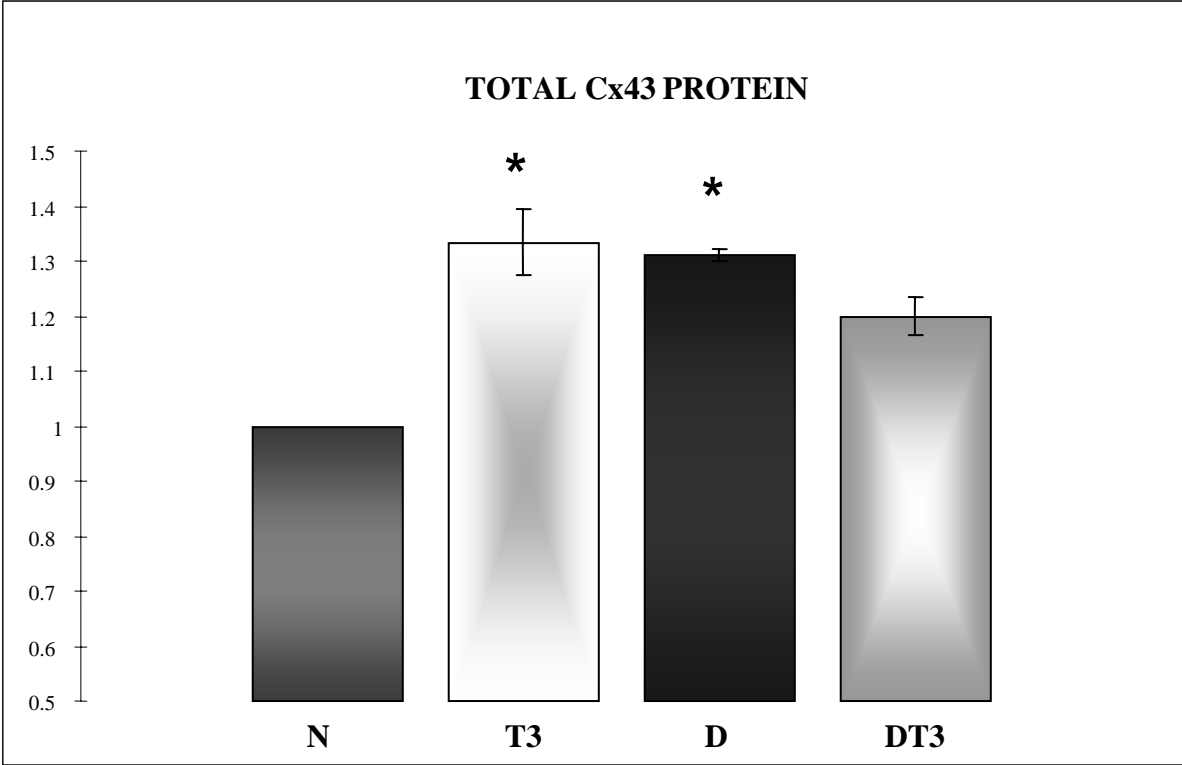
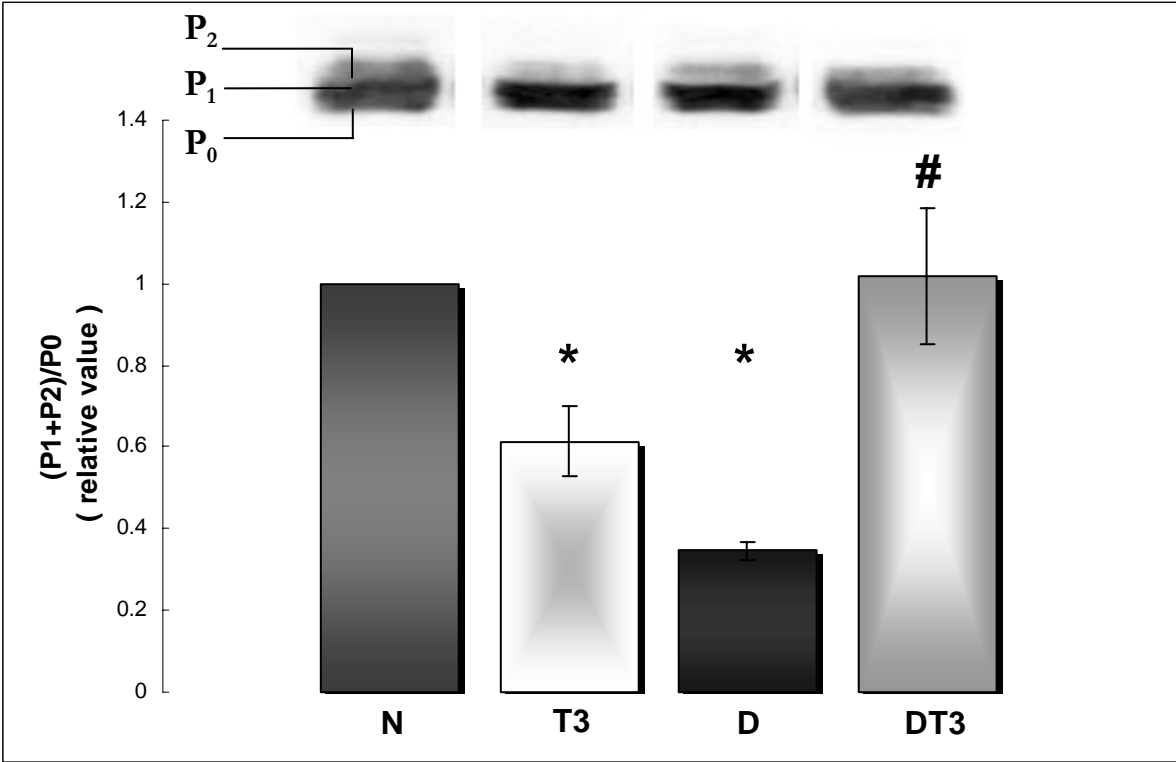


Fig. 2. A, B.

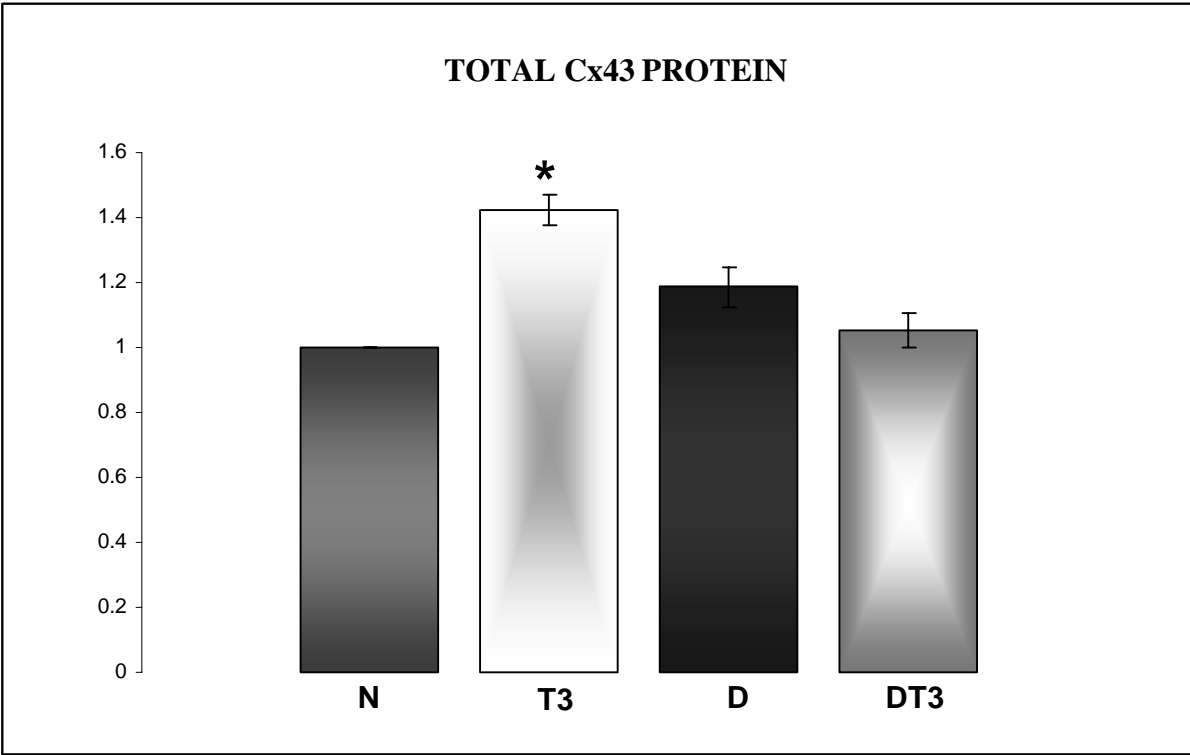
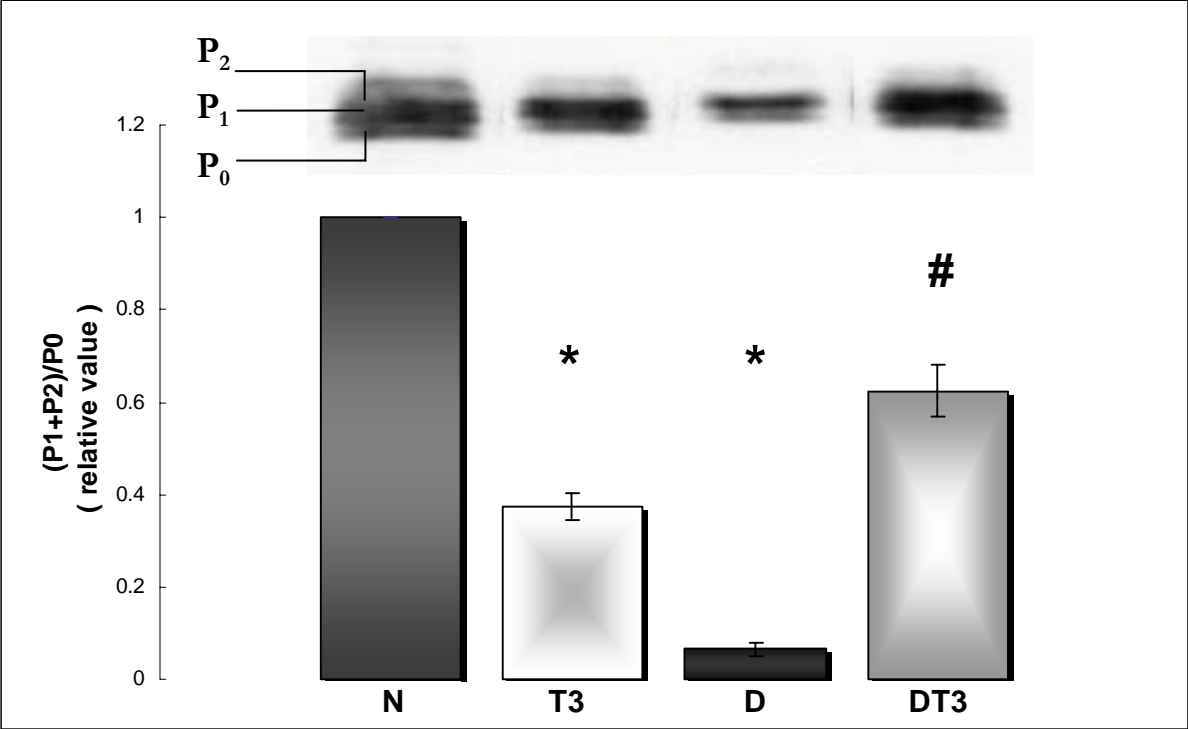
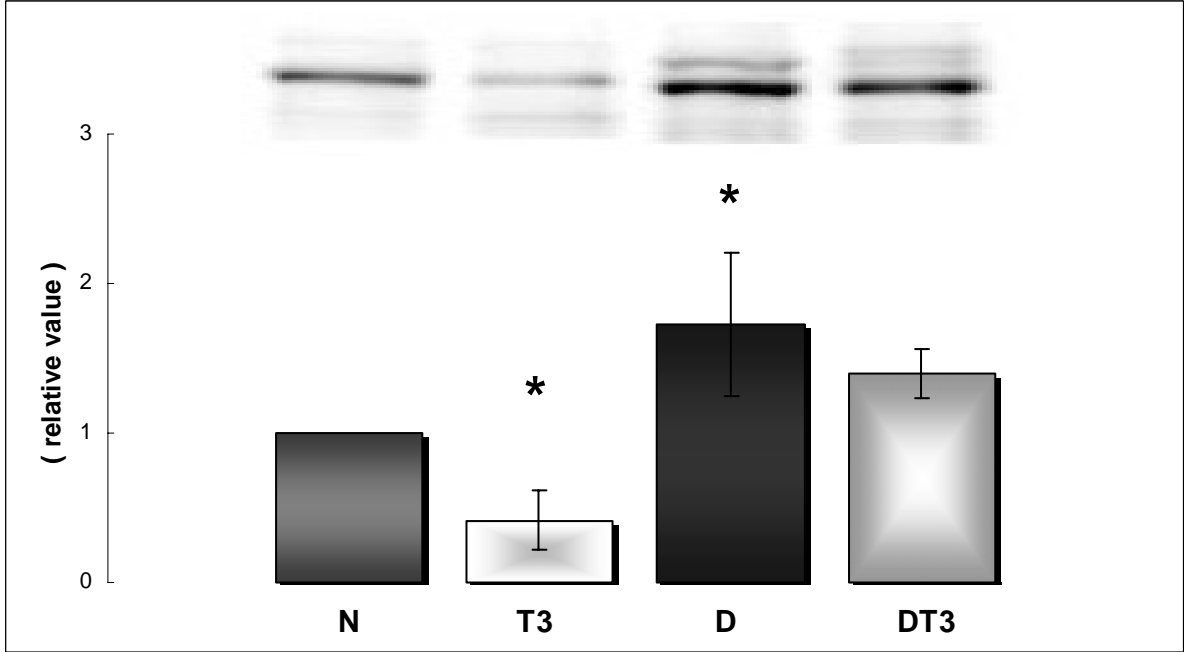
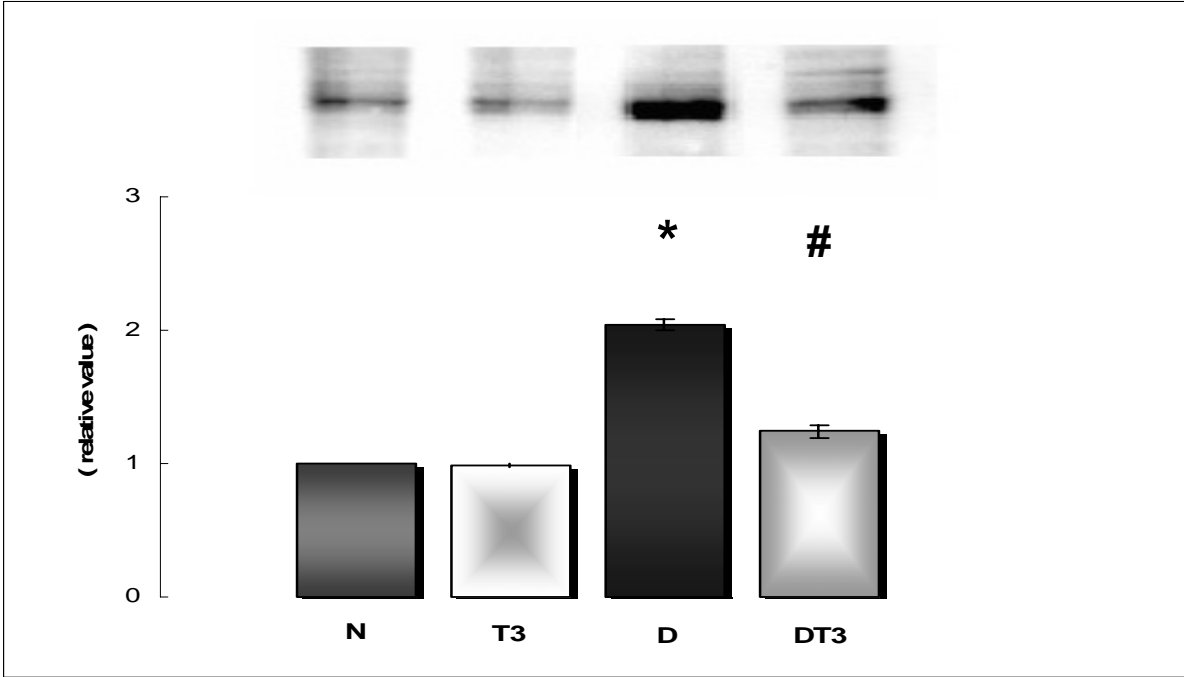


Fig. 3 A, B



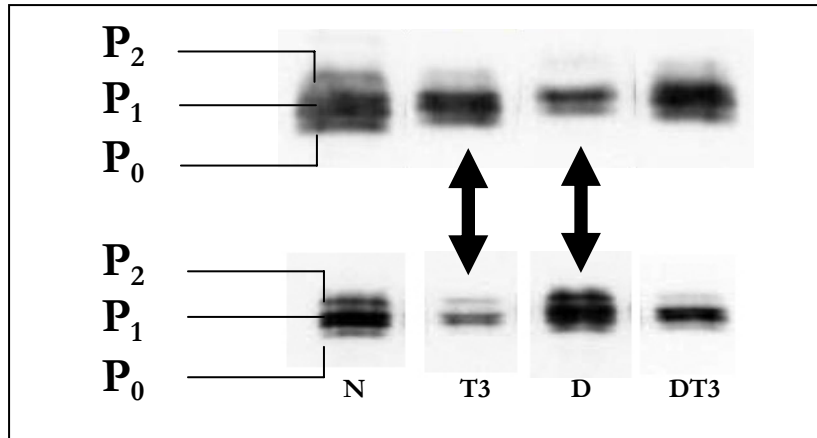


Fig. 4