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Diabetes and Thyroid Hormones Affect Connexin-43 and PKC-ε Expression in Rat Heart Atria

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Summary

We have examined the changes of intercellular electrical coupling protein connexin-43 (Cx43) and of PKC-ε in heart atria of diabetic rats and/or after the treatment with triiodothyronine (T_3). Diabetes was induced in Wistar-Kyoto rats by streptozotocin (50 mg/kg, i.v.) and atria were examined after 5 (acute stage) and 10 (chronic stage) weeks. T_3 (10 μg/100 g/day) was applied *via* a gastric tube for the last 10 days prior to the end of the experiments to non-diabetic and to the half of diabetic rats. Expression and phosphorylated status of Cx43, as well as expression of PKC-ε, were analyzed by Western blots using mouse monoclonal anti-Cx43 and rabbit polyclonal anti-PKC-ε antibodies. We found that the Cx43 expression was significantly increased after the treatment with T_3 and in the acute diabetes. Both in diabetes and after T_3 treatment the phosphorylation of Cx43 isoforms was markedly suppressed compared to the non-diabetic and T_3-untreated controls. Such a down-regulation was less pronounced in diabetic rats after the T_3-treatment. The expression of atrial PKC-ε was increased in diabetic rats. This increase was suppressed after T_3 administration and the expression was decreased in T_3-treated non-diabetic rats. 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 tachyarrhythmia in diabetic or hyperthyroid animals.

Key words

Connexin-43 • PKC-ε • Rat heart atria • Diabetes • Triiodothyronine • Atrial fibrillation

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Introduction

Basic mechanisms underlying atrial fibrillation (AF), the most common cardiac arrhythmia seen in the clinical practice and one of the frequent cause of stroke, are still poorly understood. Epidemiological studies have shown that diabetes mellitus and hyperthyroidism represent a high 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 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 the 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, Kanno 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 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, the data concerning the influence of diabetes or hyperthyroidism on 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 has been found that an acute stage of diabetes led to a PKC activation and to an increase of PKC-ε isoform expression in cardiovascular tissues (Inoguchi *et al*. 2001, Lin *et al*. 2006). On the other hand, in the hyperthyroidism the expression of PKC-ε in the cardiovascular system has been reported to be decreased (Rybin and Steinberg 1996, Hamasaki *et al*. 2000).

High glucose level was shown to induce inhibition of gap junction channels permeability through excessive phosphorylation of Cx43 in cultured aortic smooth muscle cells (Kuroki *et al.* 1998). Likewise, we have recently shown that acute diabetes suppressed electrical cell-to-cell coupling and decreased conductivity in heart ventricles (Lin *et al*. 2006). Diabetes is accompanied with reduced plasma thyroid hormone levels (Ferrer *et al.* 2006) and hypothyroidism has been shown to be associated (as is diabetes) with an increase of PKC-ε expression which can be reversed by thyroid hormone replacement (Rybin and Steinberg 1996).

The aim of this study was to examine to what extent the expression and phosphorylation of Cx43 and expression of PKC-ε are altered in rat heart atria during diabetes or hyperthyroidism and by their combination.

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, SAV, Bratislava, Slovak Republic. Diabetes was induced in male Wistar-Kyoto rats (8-week-old, 300-330 g) by a single streptozotocin injection (STZ, Sigma, 50 mg/kg, i.v.). Hyperglycemia was confirmed by a blood glucose assay. The animals were housed with free access to water and standard food. Triiodothyronine (Sigma, T_3, 10 μg/100 g/day, the biologically active form of thyroid hormone) was applied by gavage via a gastric tube to non-diabetic and diabetic rats for the last 10 days prior ending the experiments. Age-matched non-diabetic rats with and without the T_3 treatment were used as well. There were two main experimental groups of rats that differed in the 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, the rats were divided into four subgroups: 1) non-diabetic rats (N, n=6); 2) diabetic rats (D, n=6); 3) non-diabetic treated with T_3 (T3, n=6) and 4) diabetic treated with T_3 (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_3 and T_4 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 and 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 100 000 g for 30 min. The pellets were used for Western blot (WB) assays. Twenty micrograms of total protein were run on a 10 % SDS-PAGE 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.

Table 1. Main characteristics of the 13-week-old non-diabetic (N), non-diabetic treated with T_3 for 10 days (NT3), 5-week diabetic (D) and 5-week diabetic rats treated with T_3 (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)	4.7±0.4	5.5±0.5*	26.8±4.5*	29.8±3.9*
Serum T_3 (nmol/l)	1.46±0.15	1.77±0.10*	1.08±0.21*	1.85±0.01*#
Serum T_4 (nmol/l)	56.93±11.84	15.44±2.19*	34.11±2.14*	14.16±1.51*#

Values are means ± S.D. of 6 rats in each group. * Significantly different from N ($P<0.05$), # 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_3 for 10 days (NT3), 10-week diabetic (D) and 10-week diabetic rats treated with T_3 (DT3).

	N	NT3	D	DT3
Body weight (g)	497±42.8	463±16.6	375±25.2*	321±50*#
Heart weight (g)	1.22±0.05	1.59±0.11*	1.18±0.99	1.38±0.15*#
BG (mmol/l)	5.5±0.6	4.9±0.6	28.4±5.5*	30.0±4.7*
Serum T_3 (nmol/l)	1.46±0.08	1.89±0.12*	1.08±0.14*	1.32±0.06*#
Serum T_4 (nmol/l)	67.18±6.44	19.05±3.22*	37.58±10.89*	13.64±1.61*#

Values are means ± S.D. of 6 rats in each group. *Significantly different from N ($P<0.05$), # significantly different from D ($P<0.05$), BG - fasting blood glucose.

Statistics

Data were expressed as mean ± S.D. and unpaired Student t-test was used to analyze the statistical significance determined at $p<0.05$.

Results

General characteristics of experimental animals

Fasting blood glucose levels were markedly elevated, while serum levels of thyroxine (T_4) and triodothyronine (T_3) were significantly decreased in diabetic rats 5 and 10 weeks after the STZ application in comparison to non-diabetic littermates. Diabetes was accompanied by a decrease in body and heart weights. Treatment of non-diabetic and diabetic rats with T_3 led to its elevation and suppression of T_4 levels in the serum of STZ-treated animals. Heart weight was significantly increased in non-diabetic T_3-treated groups, as well as in "chronic" diabetic T_3-treated group compared to control rats (Tables 1 and 2).

Cx43 and PKC-ε analysis

The treatment of WKY rats with T_3 led to a significant increase of the total Cx43 expression in atrial tissues of both T_3-treated experimental groups (Figs 1B and 2B). A significant increase of Cx43 expression was detected also in diabetic rat heart atria 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 (Figs 1A and 2A) compared to non-diabetic 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 phosphokinase PKC-ε (Figs 3A and 3B) was significantly increased in the acute as well as in the chronic stages of diabetes. On the other hand, T_3 administration markedly decreased PKC-ε expression in atria of diabetic (Fig. 3A) and non-diabetic (Fig. 3B) rats.

Discussion

In our study we have focused on changes in the expression and phosphorylation of the gap junction channel protein Cx43, as well as on expression of PKC-ε isoform in the rat heart atria. The main finding of our

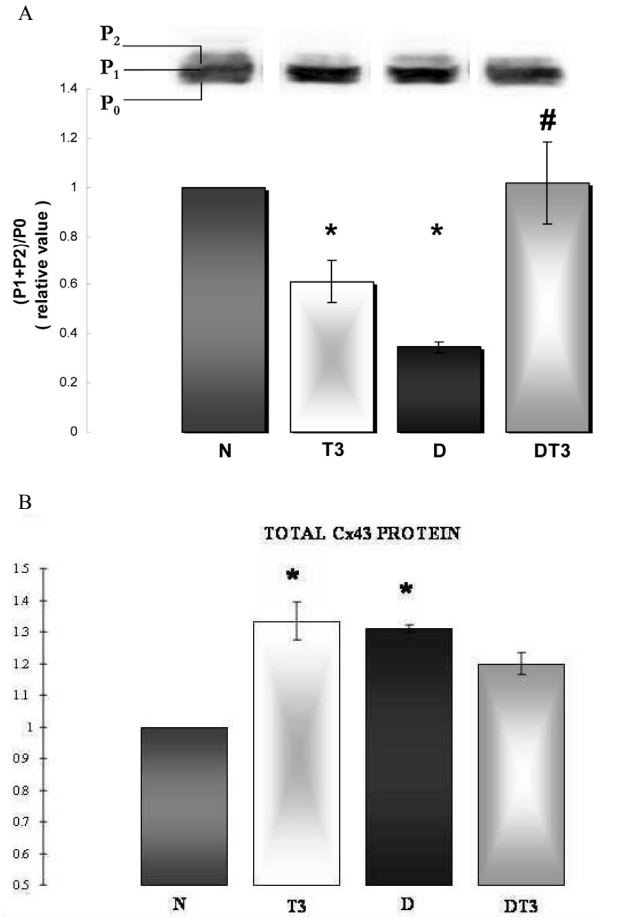

Fig. 1. A representative Western blot (upper panel) and densitometric quantification of Cx43 immunoblots (bottom panel) in the atria of non-diabetic (N), T_3-treated non-diabetic rats (T3), 5-week diabetic (D) and 5-week diabetic T_3-treated (DT3) rats. Note a significant decrease of the Cx43 phosphorylated (P1+P2) to non-phosphorylated (P0) ratio in T_3-treated and especially in diabetic rat heart atria. Interestingly, the Cx43 phosphorylation is not suppressed in T_3-treated diabetic rats. The total Cx43 levels are increased in T_3-treated as well as diabetic rat heart atria and partly in diabetic T_3-treated rats. Data (n=6) are means ± S.D. and * $p<0.05$ *vs.* N, # $p<005$ *vs.* D.

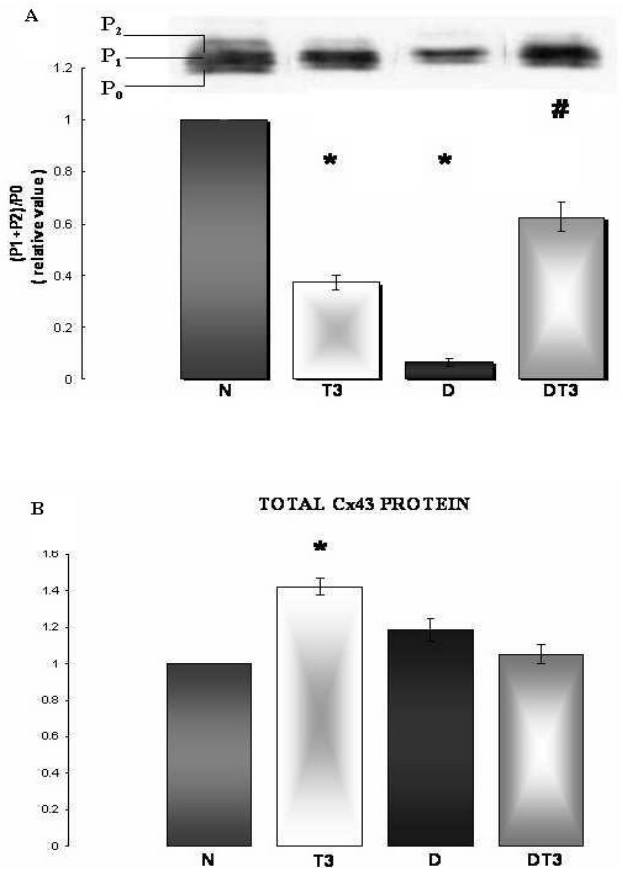

Fig. 2. A representative Western blot (upper panel) and densitometric quantification of Cx43 immunoblots (bottom panel) in the atria of non-diabetic (N), T_3-treated non-diabetic (T3), 10-week diabetic (D) and 10-week diabetic T_3-treated (DT3) rats. Note a dramatic decrease of the phosphorylated (P1+P2) to non-phosphorylated (P0) Cx43 ratio in T3 and D groups, while the administration of T_3 to diabetic rats (DT3) partly attenuates the influence of diabetes. The total Cx43 levels are significantly increased due to the T_3 treatment. Data (n=6) are means ± S.D. and * $p<0.05$ *vs.* N, # $p<005$ *vs.* D.

study demonstrates that experimental diabetes and/or T_3 treatment significantly affect the expression of Cx43 and PKC-ε, as well as the phosphorylation status of Cx43 isoforms.

It has been shown that the diabetes and the hyperthyroidism represent the risk factors associated with a development of AF. While the initiation of AF is usually associated with a pulse formation 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 to be promoted by thyroid hormones (Chen *et al*. 2002). The latter is 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, we have found that the T_3 administration led to a hypertrophy of ventricular cardiomyocytes, while chronic diabetes was accompanied with a focal fibrosis of ventricular tissue (Lin *et al*. to be published). Since early (acute) and late (chronic) stage of diabetes differed in the degree of cardiomyopathy and susceptibility to ventricular arrhythmias (Lin *et al*. to be published), in the present study we examined atria in both acute and chronic stages of diabetes and in their combination with T_3 administration.

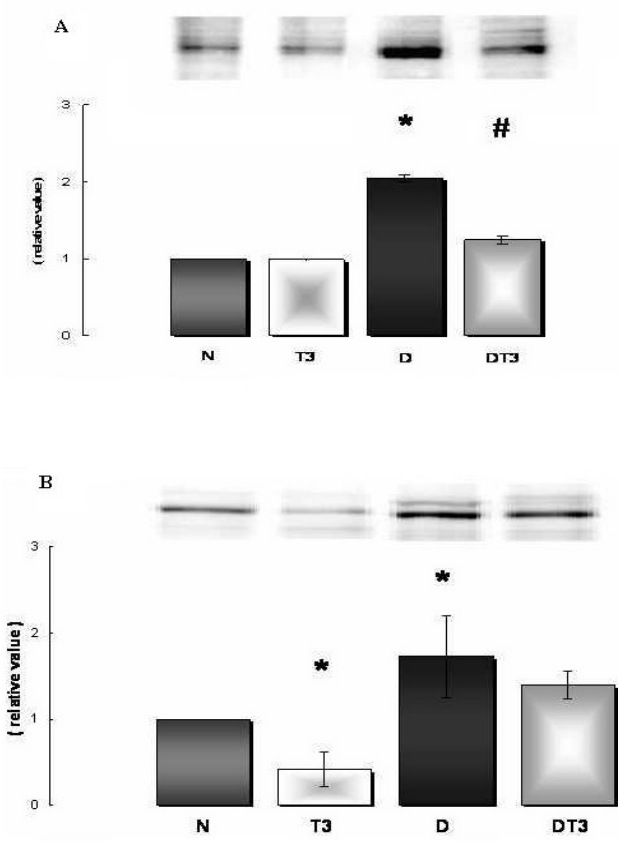

Fig. 3. Representative Western blots of PKC-ε and their quantitative densitometric analysis in the atria of non-diabetic (N), T_3-treated non-diabetic (T3), diabetic (D) and diabetic T_3-treated (DT3) rats. The upper panel represents the acute diabetic stage (5 weeks after the STZ administration) and bottom panel the chronic diabetic stage (10 weeks after the STZ administration). Note that the expression of PKC-ε was significantly suppressed in T_3-treated rats (bottom panel), but increased in both groups with diabetes. The T_3 treatment in acute diabetic rats almost abolished PKC-ε increase due to diabetes, but in chronic diabetic rats it suppressed only partially the PKC-ε increase observed in diabetic rats. Data (n=6) are means ± S.D. and * $p<0.05$ *vs.* N, # $p<005$ *vs.* D.

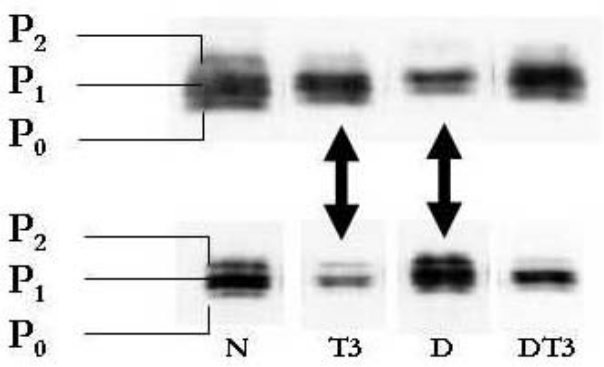

Fig. 4. Representative immunoblots showing the Cx43 expression in the atria (upper panel) and for comparison in the ventricles (bottom panel) of non-diabetic (N), T_3-treated non-diabetic (T3), diabetic (D) and diabetic T_3-treated (DT3) rats. Note the decrease of phosphorylated P1 and P2 isoforms of Cx43 in both the atria and ventricles of T_3-treated rats, as well as in the atria of diabetic rats in which, in contrast, ventricular Cx43 isoforms are hyperphosphorylated. The T_3 administration to diabetic rats suppressed the Cx43 phosphorylation in the ventricles, but not in the atria. P_0 – non-phosphorylated isoform of the Cx43.

As expected, a comparison of the Cx43 expression in the atria and ventricles showed the decrease of phosphorylated isoforms of Cx43 in both atria and ventricles of T_3-treated rats, as well as in the atria of diabetic rats, in which ventricular Cx43 was, in contrast, hyperphosphorylated. An increase of phosphorylating status of Cx43 in the ventricles due to acute diabetes has been reported previously (Lin *et al.* 2006).

Cx43 is a phosphoprotein and changes in its phosphorylating status can modulate function of connexin channels (Lampe *et al.* 2000). Western blot analysis revealed one non-phosphorylated and two phosphorylated forms of Cx43 in atria, similarly as in ventricles (Lin *et al.* 2006, Lin *et al.* to be published). However, the T_3 administration to diabetic rats suppressed the Cx43 phosphorylation in the ventricles, but not in the atria (see representative Western blots showing phosphorylated and non-phosphorylated Cx43 isoforms in Fig. 4).

Diabetes also affected differently a total level of Cx43 that was significantly increased 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). Hyperthyroidism also induced by the administration of T_3, was accompanied by an increased expression of Cx43 only in the atria, but not in the ventricles (Lin *et al*. to be published). Interestingly, an exposure of neonatal cultured cardiomyocytes to T_3 for 24-48 h resulted in an elevation of the total Cx43 level as well (Tribulová *et al*. 2004b). On the other hand, the administration of thyroid hormone to old male and female rats did not affect significantly the Cx43 expression (Tribulová *et al*. 2005) or arrhythmia susceptibility (Tribulová *et al*. 2004a). 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), but it remains in realm of speculation whether a similar process can interfere with age-related alterations in intracellular signaling pathways.

In contrast to the up-regulation of Cx43 expression in the rat atria, its phosphorylation was

markedly decreased by both diabetes and the T_3 treatment. It is likely that dramatic abnormalities in the phosphorylation status may affect the Cx43 channel properties and function (Stagg and Flechter 1990, 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_3-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 resulted in a decreased susceptibility to life-threatening cardiac arrhythmia. We can assume that the significantly decreased phosphorylation of Cx43 in the atria of diabetic or T_3-treated rats likely promotes the appearance and sustaining of AF in such rats. It remains, however, not clear how the treatment of diabetic rats with T_3 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_3-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-ε is one of the protein kinases, which directly phosphorylates Cx43. We have found its increased expression in both the atria (this study) and the ventricles (Lin *et al*. 2006) of diabetic rats. However, the phosphorylation of Cx43 (supposed to be most likely PKC-ε-related) was increased only in the ventricles (Lin *et al*. 2006), but not in the atria of diabetic rats (Fig. 3). Administration of T_3 resulted in a decrease of PKC-ε associated with the decrease of the Cx43 phosphorylation in both the atria (this study) and the ventricles (Lin *et al*. to be published), which could affect the gap junction channel mediated intercellular communication (Stagg and Flechter 1990).

In conclusion, our findings suggest that diabetes and mild hyperthyroidism in rats upregulate the Cx43 atrial expression and significantly decrease the Cx43 phosphorylation. The decline in the phosphorylation of Cx43 is likely to contribute to the deterioration of the cell-to-cell electrical coupling promoting the occurrence of AF.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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