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Gestational hypothyroidism-induced changes in L-type calcium channels of rat aorta smooth muscle and their impact on the responses to vasoconstrictors

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ABSTRACT

Objective(s): Thyroid hormones play an essential role in fetal growth and maternal hypothyroidism which leads to cardiovascular deficiency in their offspring. Considering this, we intended to investigate the impact of gestational hypothyroidism on offspring vascular contractibility and possible underlying mechanisms.

Materials and Methods: Hypothyroidism was induced in female rats by administration of 6-n-propyl-2-thiouracil in drinking water (0.02%) till delivery. The offspring aorta smooth muscle (without endothelium) contractile response to KCl (10-100 mM), KCl in the presence of nifedipine (10^{-4}-10^{-1} µM), phenylephrine (10^{-9}-10^{-6} M) and finally, phenylephrine and caffeine 100 mM in Ca^{2+}-free Krebs were measured.

Results: KCl and phenylephrine-induced contractions were considerably lower in gestational hypothyroid (GH) than euthyroid offspring. GH responded to nifedipine with less sensitivity than control. The GH and control groups produced almost equal contraction in respond to phenylephrine and caffeine in Ca^{2+}-free Krebs.

Conclusion: This study suggests that in hypothyroid offspring L-type Ca^{2+} channels are less functional, while intracellular Ca^{2+} handling systems are less modified by low levels of maternal thyroid hormones.

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Introduction

Physiological functioning of the body systems is highly influenced by intrauterine conditions in which the mammalian fetus develops. Suboptimal maternal environment, such as insufficient availability of nutrients, oxygen, and hormones can change the developmental regulatory planning of fetal tissue, lead to diseases, such as cardiovascular and metabolic diseases in adult life (1-3). Thyroid hormones play an essential role in fetal normal development (4, 5). T_3 reduces the vascular resistance and therefore, causes relaxation especially in arteries. Cardiovascular system response to hypothyroidism is low cardiac output, pulse pressure and increased vascular resistance, while the opposite symptoms are in effect in thyroid overactivation (5-7). Furthermore, either in hyperthyroidism or hypothyroidism, the response of the vessels is changed to the vasoconstrictors and vasodilators agents (8, 9). Most studies have focused on measuring the responses to vasoconstrictors in adult models of thyroid deficiencies, while there is not much evidence available on the maternal or gestational models of these diseases on the offspring's vascular status. In many studies, it has been demonstrated that adult models of hypothyroidism develop a defective vascular smooth muscle response to α-adrenergic agonists and KCl or barium choloride in comparison to euthyroids (10-16). However, one of the few studies that investigated the effects of prenatal hypothyroid model in rats on the density of adrenoceptors, reported a decrease in the myocardial α_1-adrenergic receptor density within 15-28 days after birth, indicating the modulation of adrenergic receptor proteins by thyroid hormones during fetal development (17). More recently a study showed that an isolated aorta smooth muscle (without endothelium) of congenitally hypothyroid adult male offspring of a female rat, who received 6-Propyl-2-thiouracil (PTU) during pregnancy, demonstrated markedly less contractile response to vasoconstrictors in comparison to euthyroids (8). The contraction response to KCl and α_1-adrenergic agonists in the vascular smooth

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muscle is produced by extracellular Ca^{2+} influx, activated by voltage gated calcium channels (VGCC) non-voltage dependent Ca^{2+} influx (15, 18), and intracellular Ca^{2+} release from internal stores, such as sarcoplasmic reticulum (SR) (12, 15, 18). The markedly lower responses of hypothyroids to KCl and α_1-agonists compared to controls, reported in previous studies suggest some impairments in function of calcium entering/releasing at the plasma membrane (through channels) (15, 19, 20) or in the intracellular systems (15, 21), respectively. There is evidence from previous studies showing that the expression and function of the VGCCs, including L-type Ca^{2+} channels, are under the control of thyroid hormones, via genomic and nongenomic mechanisms (20, 22-24). In this study we used a model of gestational hypothyroidism produced by dams who consumed PTU during pregnancy. Using PTU and methimazole as agents that induce thyroid deficiency in dams are valid models of inducing hypothyroid state in offspring, which has been used in several studies related to developmental pathophysiology of thyroid hormones (8, 25-31).

This study aimed to investigate whether gestational hypothyroidism in a rat model may modulate L-type Ca^{2+} channels functions in later adult life, determined by developing some deficiencies in aorta smooth muscle responses to the KCl and selective α_1-adrenergic agonist, phenyl-ephrine. Also, gestational thyroid hormones may induce changes in offspring intracellular Ca^{2+} handling machinery by stimulating aorta smooth muscle cells with the internal Ca^{2+} releasing vasoconstrictors, such as phenylephrine and caffeine, in a Ca^{2+}-free environment.

Materials and Methods

Animals

Female Wistar rats, 155-250 g (inbred in the Endocrine Physiology Research Center Animal Facilities) were used for mating in this study. The animals were kept in 12 hr light/dark cycle, 22±3˚C temperatures and had free access to rat chow (Pars Co., Tehran) and water. Animal handling and experiments were carried out in accordance with the local ethics committee of the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences. Maximum efforts were made to minimize the animal's possible discomfort and stress.

Induction of gestational hypothyroidism

Hypothyroidism was induced in pregnant rats by adding 200 mg PTU (Sigma Aldrich, Germany) to 1000 ml of drinking water, from the first day of pregnancy until delivery (8, 31, 32). While the control group only received tap water, upon delivery the drug was removed from drinking water.

Experimental groups

Male offspring were weighed on days 0, 15, 30, 45, 60 and 150 of birth. Five months-old (150 days) rats were placed into the control (n=9) and gestational hypothyroid (GH) (n=10) groups and assessed for the contractile force measurement of the aorta.

Thyroid hormone measurement

To confirm the PTU-induced hypothyroidism, blood samples were drawn from the dam and neonates on delivery day and from the abdominal aorta of adult male offspring, five-months after birth. All the samples were centrifuged (3000 g, for 10 min in 4˚C) and kept at -80˚C until the time of the hormonal assay. T_3 and T_4 were measured using enzyme linked immunosorbent assay kits (Pishtaz Teb Zaman Co., Iran). Intra- and interassay coefficients of variation were 3.7 and 4.3% for T_3 and 5.3 and 5.9% for T_4, respectively.

Aortic ring preparation

Adult male offspring of GH and control groups were anesthetized by IP injection of 50 mg/Kg ketamine and 10 mg/Kg xylazine (16). The thorax was cut open and a section of the thoracic aorta ring was dissected and cleaned of the connective tissue and vessels in ice cold (4˚C) Krebs-Henseleit solution (composition in mmol/l: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, Glucose 10, and $NaHCO_3$ 25, (Merck, Germany)), pH adjusted to 7.4 and temperature to 37˚C and the ring was cut into 5 mm sections in length. Endothelium was removed by gently rotating the ring around a metal wire inserted into the lumen (33). The Krebs solution inside the chamber, where the ring was hocked to an isometric sensitive force transducer (MLT0202, ADInstruments, Spain), was constantly gassed with a combination of 95% O_2 and 5% CO_2. Contraction force data were recorded by the sensitive force transducer coupled to the PowerLab data acquisition system (ML866 PowerLab 4/30, ADInstruments, Australia). Before the experiment began, the aorta ring was placed under 2.0 g resting tension and allowed to equilibrate for a period of 60 min, while the solution inside the chamber was renewed every 15 min. To determine the removal of the endothelium, acetylcholine (10^{-5} M) was added to pre-contracted aorta ring with phenylephrine (10^{-6} M). The ability of the tissue to relax in response to acetylcholine indicated the presence of endothelium (33, 34).

Isolated aorta contraction experiments

Range of KCl (10-100 mM) and phenylephrine (10^{-9}-10^{-6} M) concentrations, separated by refreshing

Table 1. Comparing the weights of male offspring in gestational hypothyroid and control groups in 0-150 days after birth

Post-natal days	GH weight (g)	Control weight (g)	*P*-value
0	6.656 ± 0.579	7.957 ± 0.425	0.1026
15	18.48 ± 1.297	24.65 ± 1.176	0.0054***
30	47.44 ± 3.761	66.26 ± 2.416	0.0002***
45	99.00 ± 9.771	128.2 ± 3.690	0.0016***
60	139.1 ± 9.525	175.6 ± 5.674	0.0021***
150	296.87 ± 3.567	311.33 ± 2.341	0.4866

Values are mean±SEM. ****P*-values <0.001vs compared with control. Number of animals per group is 15; GH: Gestational hypothyroidism

Krebs solution and single concentrations of nifedipine (10^{-4}- 10^{-} μM) prior to KCl (10-100 mM) were applied to the ring tissue inside the chamber. In the second set of experiments, the Krebs solution was replaced by Ca^{2+}-free Krebs ($CaCl_2$ was replaced by $MgCl_2$ 1.2 mmol/l). The range of phenylephrine concentrations (10^{-9}-10^{-6} M) was used to test the contractile force in Ca^{2+}-free environment. Finally, after washing with Ca^{2+}-free Krebs solution, caffeine 100 mM was added and the contractile force was recorded for 30min in response to that. Nifedipine was dissolved in ethyl-alcohol (96%) with the stock concentration of (3×10^{-3}M). Caffeine (100 mM) was dissolved, while heating and stirring, in Ca^{2+}-free Krebs (50 ml). Before applying the solution temperature was decreased to 37˚C.

At the end of experiments the length and weight of the aorta ring were measured for calculating the tension produced by each of vasoconstrictor agents in the experiment normalized to cross-sectional area with the average tension displayed as g/mm^2, using the formula below (8):

Cross-sectional area (mm^2) = weight (mg) / length (mm) × density (for the vascular smooth muscle is 1.05 mg/mm^3)

Statistical analysis

Values were presented as mean±SEM. Unpaired Student t-test was performed to analyze the differences between the tensions produced in hypothyroid vs control group. Paired Student t-test compared the differences between the data obtained from groups before and after nifedipine administration and data related to contractions recorded with Ca^{2+}-containing and Ca^{2+}-free Krebs solutions. Statistical analysis and graph plotting was performed by GraphPad Prism version 4.00 for Window's (GraphPad software, San Diego, California, USA).

Results

Weight differences

Male offspring weights in the GH and control groups were significantly different on days 15 (*P-value*<0.01), 30, 45 and 60 (*P-value*<0.001) after birth (Table 1).

Thyroid hormone measurement

serum levels of thyroid hormones, T_3 and T_4, in dams and neonate and adult offspring indicated a significant decrease in dams who had consumed PTU during pregnancy and GH neonates in comparison to their control groups (*P-value*<0.001). No differences were observed in GH adult offspring compared to controls (Table 2).

Aortic smooth muscle contraction in response to vasoconstrictors

KCl-induced contraction

KCl concentrations (10-100 mM) were used to produce a dose-response curve. In the GH group the amplitude of contractile responses to KCl at 60, 80, and 100 mM were significantly lower than controls (for 60 and 80 mM, *P-value*<0.01 and for 100 mM KCl, *P-value*<0.001). Average maximum tension was

Table 2. Levels of thyroid hormones (ng/dl) in dam, neonate (at birth) and adult offspring (at 5 months age)

Hormones	Dam		Offspring			
			Neonate		Adult	
	Control	PTU consumed	Control	GH	Control	GH
3,5,3´-Triiodothyronine (ng/dl)	93.4 ± 3.8	51.7 ± 4.9***	62.9 ± 3.4	39.5 ± 4.3***	95.7 ± 4.3	87.7 ± 5.3
Thyroxine (ng/dl)	2400 ± 0.2	520 ± 0.04***	730 ± 0.06	370 ± 0.04***	3800 ± 0.1	3400 ± 0.2

Thyroid hormones, 3,5,3´triiodothyronine (T3) and thyroxine (T4) (ng/dL) in dam and neonate and adult (5 months old) offspring. Values are mean±SEM (*** *P-value*<0.0001) compared with control; GH: Gestational hypothyroidism

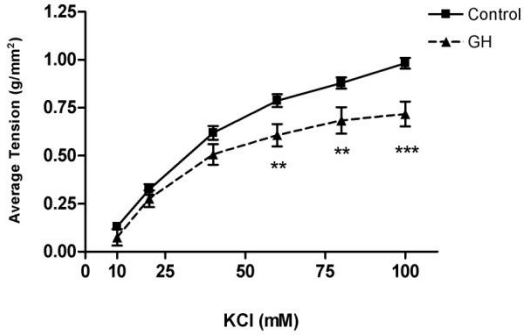


Figure 1a. Contraction-response graph showing the response of gestational hypothyroid (GH) and control groups to KCl. (**) *P-value*<0.01 and (***) *P-value* <0.001

Group compared to control (0.717±0.06 for GH vs 0.981±0.02 for control. Mean±SEM, *P-value* <0.001) (Figure 1a).

Effect of Nifedipine on KCl-induced contraction

To investigate the effect of thyroid hormones on Ca^{2+} conductance through the membrane channels, nifedipine was used within the range of 10^{-10}-10^{-7}M, as separate doses against KCl (10-100 mM). Nifedipine, in a concentration dependent manner, reduced the contractile force of aorta smooth muscle in both GH and control groups (Figure 1b-e).

Generally, in control group, nifedipine with concentrations 10^{-9} M (*P-value*<0.05) and 10^{-8}-10^{-7} M (*P-value*<0.001) inhibited the response to KCl 100mM. In GH group, nifedipine with concentrations 10^{-8}-10^{-7} M, inhibited the response to KCl 100 mM (*P -value*<0.001) (Figure 1f).

Phenylephrine-induced contraction

Phenylephrine (10^{-9}-10^{-6}M)-induced contractions were used to produce a dose-response curve. GH responses to phenylephrine (10^{-7}, 10^{-6}M) were noticeably lower than control (*P-value*<0.05 and *P-value*<0.01, respectively) (Figure 2a). However, in Ca^{2+}-free Krebs solution, the contractile responses of both groups to phenylephrine were almost the same (Figure 2b). The responses of aorta smooth muscle to phenylephrine was significantly reduced, when

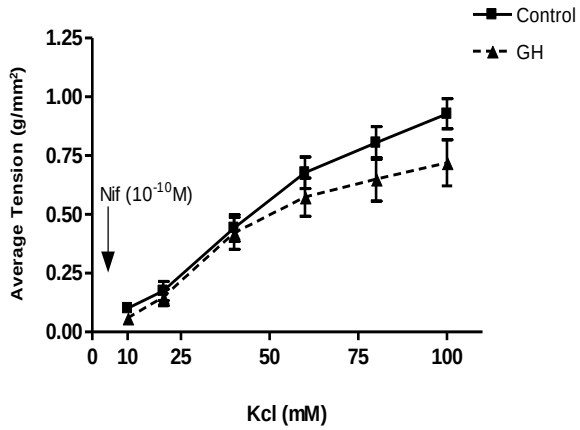


Figure 1b

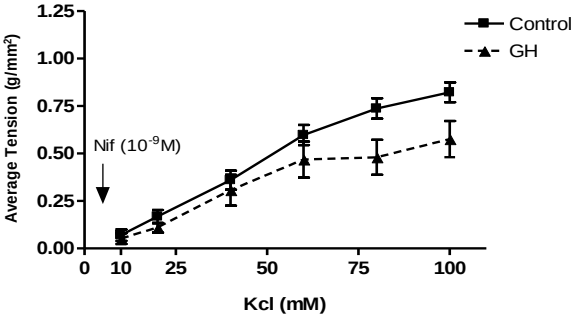
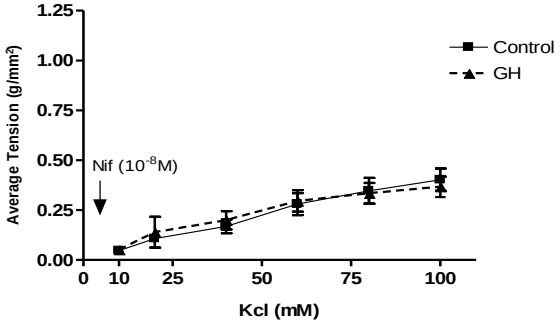


Figure 1c



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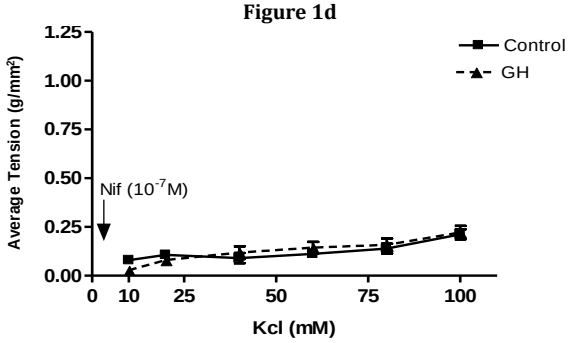

Figure 1e

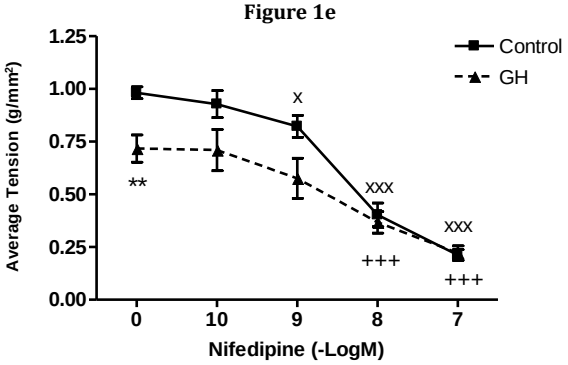

Figure 1f

Figures 1. Contraction-response graph shows the responses of gestational hypothyroid (GH) and control groups to KCl after adding (b) nifedipine (10^{-10} M), (c) nifedipine (10^{-9} M), (d) nifedipine (10^{-8} M) and (e) nifedipine (10^{-7} M). (f) Comparing the responses of both groups to KCl in presence of different concentrations of nifedipine (0-10^{-7} M). (**) *P-value*<0.01; indicates difference between control and GH. (+++) *P-value*<0.001; indicates difference between different concentrations of nifedipine in GH. (x, xxx) *P-value*<0.05, *P-value* <0.001, respectively; indicates difference between different concentrations of nifedipine in control

shifting from the Ca^{2+}-containing to Ca^{2+}-free Krebs solutions (*P-value*<0.001). However, responses were the same for the control and GH groups in Ca^{2+}-free Krebs (Figure 2c).

Caffeine-induced contraction

Caffeine (100 mM) was added in a Ca^{2+}-free Krebs environment. Recording contraction from the aorta smooth muscle of control and GH groups for 30 min revealed no specific difference in contractile force between the two groups (0.317 ± 0.04, 0.271 ± 0.05; mean±SEM for control and GH groups, respectively; *P-value*=0.514)(Figure 3).

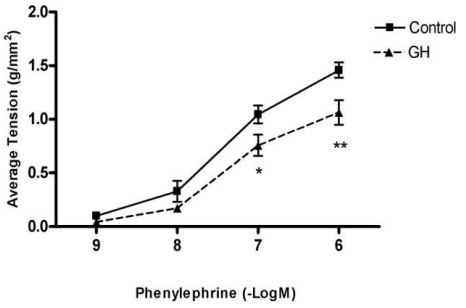

Figure 2a

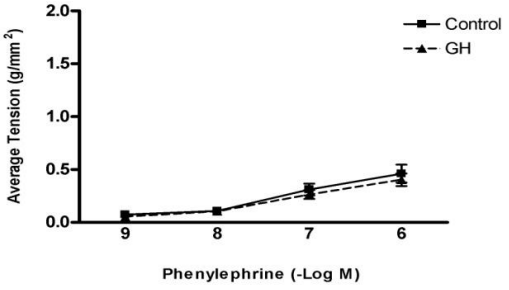

Figure 2b

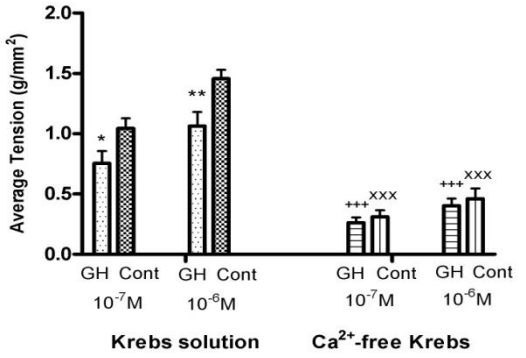

Figure 2c

Figures 2. Average contractile force of gestational hypothyroid gestational hypothyroid (GH) groups control in response to phenylephrine (10^{-9}-10^{-6}M) in (a) Ca^{2+}-containing (b) Ca^{2+}-free Krebs solutions. (c) Differences in contraction between the two groups regarding the Ca^{2+} content of Krebs solution. (****P-value**<0.01) indicates the difference between the GH and control groups, (**+++P-value**<0.001) between GH responses in normal and Ca^{2+}-free Krebs and (**XXXP-value**<0.001) between control responses in normal and Ca^{2+}-free Krebs

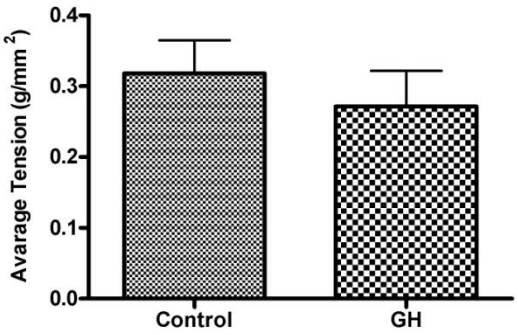

Figure 3. Average tension produced by control and gestational hypothyroid (GH) groups in response to caffeine 100mM in Ca^{2+}-free Krebs solution (*P-value*=0.514); GH: Gestational hypothyroidism

Discussion

In this study we tried to determine the role of thyroid hormones in developing functional calcium handling system in vasculature during fetal development to produce suitable physiological responses in adulthood. The aortic rings from the offspring of PTU consumed dams with denuded endothelial layer were prepared and their responses to vasoconstrictors were examined against those from euthyroid mothers. Our results from the initial GH and control groups responses to KCl and selective α_1-adrenoceptor agonist, phenylephrine, were almost similar to those reported in previous studies of hypothyroid adult rats who received anti-thyroid agent or thyroidectomy against euthyroids (14, 16, 35, 36). In all the studies, the hypothyroid group (preparations with denuded endothelium) reaction to KCl and α_1-adrenoceptor agonists was markedly reduced in comparison to euthyroids (8, 14, 35). In our study, the marked lower responses of GH to KCl and phenylephrine, relative to controls, suggests some defects in the functioning of Ca^{2+} entering through Ca^{2+} channels or release from intracellular resources.

In an attempt to determine the possible sites of Ca^{2+} handling dysfunction in the GH aorta smooth muscle cells, we first used nifedipine, as a VGCC (L-type Ca^{2+} channel) blocker. Before nifedipine administration, the response of the control group to KCl 100mM was markedly higher than the GH response. Adding increasing concentrations of nifedipine made the contractile force induced by KCl in control group closer to GH, until both groups reached to almost equal contractile forces at the nifedipine dose of 10^{-7}M. The stronger response to KCl and nifedipine inhibitory effect in euthyroids compared to GH suggest some displacement or dysfunction in the plasma membrane L-type Ca^{2+} channels of the latter group, which may possibly be related to the reduced thyroid hormone levels in fetal life. Many previous studies have shown that the

expression and function of the VGCCs are controlled by thyroid hormones, via genomic and nongenomic mechanisms (20, 22-24). Investigating the effect of T_3 on the slow Ca^{2+} channel function in cultured chick ventricular cells demonstrated an increase in slow channel Ca^{2+} influx and Ca^{2+} channel antagonist binding sites under the influence of T_3 in comparison to cells grown in environment without T_3 (22). There are some other reports indicating the increase in the Ca^{2+} currents by VGCC in aortic ring tissues (12) and rat ventricular cells along with reduction in expression of α_1c-subunit of VGCC (genomic regulation) (20), or increase in L-type Ca^{2+} channel current and mRNA expression in hyperthryroid rabbit myocytes (24), while not affecting the Ca^{2+} release from SR in all of them. In contrast to previous reports, another study conducted on rat cardiac and vascular tissue demonstrated a decrease in the Ca^{2+} channel density in the hyperthyroid versus hypothyroid rat model (23). Most of the previous results related to the smooth muscle of cardiac vessels indicated that increase in thyroid hormones above the physiological levels would increase influx through the L-type Ca^{2+} channels, possibly by increasing the number of protein channels or the Ca^{2+} current; our data revealed the same event, through a reverse mechanism. Gestational hypothyroidism caused markedly weaker KCl-induced contractile force compared to euthyroid offspring and lower response to KCl in the presence of maximum concentration of nifedipine, indicating that it is likely a scarcity of thyroid hormones during fetal life causing the reverse mechanistic profile to hyperthyroidism, most likely through the genomic regulatory mechanism, affecting the Ca^{2+} channel number or conductivity.

High concentrations of selective α_1-agonist, phenylephrine (10^{-7}-10^{-6} M), produced significantly weaker contractions in the GH compared to control. This was similar to previous results obtained from comparable data of hypothyroid vs euthyroid adult rats that received α-agonist agents (14, 16, 35, 36). In our study, while the contraction responses to higher concentrations of phenylephrine were considerably higher for controls compared to GH, both groups' responses significantly reduced in Ca^{2+} -free Krebs solution and became almost equal.

Experimentally, the activation of a_1-adrenoceptors produces an increase in the intracellular $[Ca^{2+}]$, via two routes; opening the plasma membrane Ca^{2+} channels and releasing Ca^{2+} from internal sources. Plasma membrane Ca^{2+} channels are either VGCCs or non-voltage dependent Ca^{2+} channels, including; receptor-operated calcium channels (ROCCs), such as G-protein-coupled receptors GPCRs, like, α_1-adrenoceptor-operated calcium channels and store-operated channels (SOCs) (34, 36, 37) (both are usually blocked by Ni^{2+}) (15, 19). It has been shown that aorta smooth muscle contraction induced by α_1-adrenoceptors is highly dependent on Ca^{2+} entry from extracellular environment. A study showed that nimodipine, strongly inhibit contraction force produced by noradrenaline, almost similar to the inhibitory effect of prazocine on aorta smooth muscle contraction (15), implying the joint effect of α_1-adrenoceptor to voltage and non-voltage dependent Ca^{2+} channels for replenishing intracellular $[Ca^{2+}]$ and producing contraction.

In the scarcity of extracellular Ca^{2+}, an α_1-agonist, such as phenylephrine depletes the internal Ca^{2+} sources, which the contractile force would be entirely dependent on. In our study, while there was a significant difference in control and GH responses to phenylephrine in the presence of ample extracellular Ca^{2+}, their responses to the same stimulant became similar in Ca^{2+}-free Krebs. The change in the responses relative to the presence and the absence of extracellular $[Ca^{2+}]$, suggests that low thyroid hormone levels during fetal life does not modify the intracellular Ca^{2+} releasing systems in GH, while strongly affects gene expression, protein structure or function of L-type Ca^{2+} channels. Therefore, while GH aorta cells are still dependent on normal extracellular $[Ca^{2+}]$ to produce contraction in response to α_1-adrenergic agonist vasoconstrictors, they are less sensitive (35, 36) in their responses to them in comparison to euthyroids. Furthermore, in Ca^{2+}-free Krebs, the caffeine-induced contraction force was almost the same for both groups. In Ca^{2+}-free environment, caffeine acts on ryanodine receptors to release Ca^{2+} from internal sources (11, 15, 38), a result which likely supports those mentioned above in the way that internal Ca^{2+} -releasing machinery is least changed by low thyroid hormones during fetal life.

Another point to consider in this study was the normal levels of thyroid hormones (T_3 and T_4) in the GH adult rats compared to controls. Therefore, the differences in the contractile responses observed between adult GH and euthyroids may have been stemmed from genetic modifications in the structure and function of the Ca^{2+} channels. These modifications are initiated in the prenatal or early postnatal periods of life and developed in adulthood, during which thyroid hormones began to reach to their normal physiological levels.

This study provides new insights towards deeper understanding of the changes in the vascular reactions in gestational hypothyroidism, by emphasizing on the role of modified calcium channels in producing contractions. However, it still lacks the mechanistic view of the underlying reasons, which can be gained through precise measurements of changes at the genes or/and protein levels of the Ca^{2+} channels, which warrants to be considered in the future.

Conclusion

The contractile responses to vasoconstrictors in adult hypothyroid rats as well as fetal or GHs are considerably reduced in comparison to euthyroids. Our results suggest that lower levels of thyroid hormones during pregnancy affects Ca^{2+} handling system by reducing the Ca^{2+} conductance through plasma membrane with least modification in internal Ca^{2+} releasing/storing systems. Obtained results which may have some valuable clinical relevance in the future on treatment options for cardiovascular problems of offspring born to hypothyroid mothers.

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Conflict of interest

The authors declare that they have no conflict of interest related to this manuscript.

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