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Enhancement of Gap Junctional Communication and Connexin43 Expression by Thyroid Hormones

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ABSTRACT. Cells in tissues coordinate their activity by sharing ions, second messengers, and small metabolites through clusters of intercellular channels called gap junctions. The thyroid hormones 3,3′,5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) are capable of modulating gap junctional communication (GJC) as are 1,25-dihydroxyvitamin D_3, retinoic acid, and other nuclear receptor ligands. T_3 and T_4 were found to stimulate GJC in WB-F344 rat liver epithelial cells dose-dependently at concentrations between 1 nM and 0.1 μM, assayed by the dye transfer method using Lucifer Yellow CH. The stimulation of cell-cell communication was preceded by an increase in connexin43 mRNA levels and was accompanied by an accumulation of connexin43 protein measurable 2 days after incubation with these compounds. These observations establish a novel role of thyroid hormones in the regulation of gap junctional intercellular communication via connexin43 gene expression. BIOCHEM PHARMACOL 55;4:475–479, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. intercellular communication; thyroid hormones; connexin43; up-regulation; epithelia; WB-F344

Thyroid, retinoid and steroid hormones play an important role in development, differentiation, and physiological responses to diverse stimuli. These molecules bind to specific intracellular receptors which belong to a superfamily of regulatory proteins activating the expression of gene networks [1]. The thyroid hormones 3,3′,5-triiodo-L-thyronine (T_3)† and L-thyroxine (T_4) are ligands of the nuclear thyroid receptor (TR) [2]. Direct interaction of T_3-liganded TRs with DNA sequences in the promoter of target genes, referred to as thyroid response elements, are commonly assumed to elicit a rapid transcriptional response even after 20 min [3]. The mRNA of rat growth hormone in rat pituitary tumour cells GH1 [4] and glucokinase mRNA in rat liver tissue [5] are both induced 3-fold within 2 hr after incubation with T_3 or T_4. Phosphoenolpyruvate carboxykinase mRNA increases 2-fold within 3 hr [6].

Ligands for nuclear receptors such as retinoic acid [7] and 1,25-dihydroxyvitamin D_3 [8, 9] are known to induce gap junctional communication (GJC). Interactions between nuclear receptors of this superfamily [10, 11] might play a role in the control of GJC in different tissues. Connexin-based intercellular channels are selectively permeable to many small molecules. Thus, GJC may influence a wide variety of cellular activities, including the regulation of growth, differentiation, and developmental signaling [12]. Pathological implications of raised GJC are, e.g., cardiac arrhythmias and heart failure [13, 14]. Similar effects have been described in context with hyperthyroidism [15]. Thus, aspects of the pathophysiology of hyperthyroidism could be associated with modulation of GJC. In the present study, we describe the influence of thyroid hormones T_3 and T_4 on GJC and their effects on connexin43 gene expression.

MATERIALS AND METHODS

Chemicals

Lucifer Yellow CH was purchased from Sigma, 3,3′,5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) from Aldrich, morpholinopropane sulfonic acid from Serva and the oligolabeling kit from Pharmacia. All other chemicals were obtained from Merck.

Cells and Culture Conditions

The WB-F344 rat liver epithelial cells were a kind gift from Dr. I.A. Cotgreave and Dr. L. Wärngard, Institute of Environmental Medicine, Karolinska Institute (Stockholm, Sweden). The human embryonal skin fibroblast cell line HFFF2 was obtained from ECACC. The cells were cultured in Dulbecco's modification of Eagle's minimal essential medium (DMEM; Gibco BRL) supplemented with fetal calf

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†*Abbreviations:* T_3, 3,3′,5-triiodo-L-thyronine; T_4, L-thyroxine; TR, thyroid hormone receptor; GJC, gap junctional communication; FCS, fetal calf serum; RA, retinoic acid.

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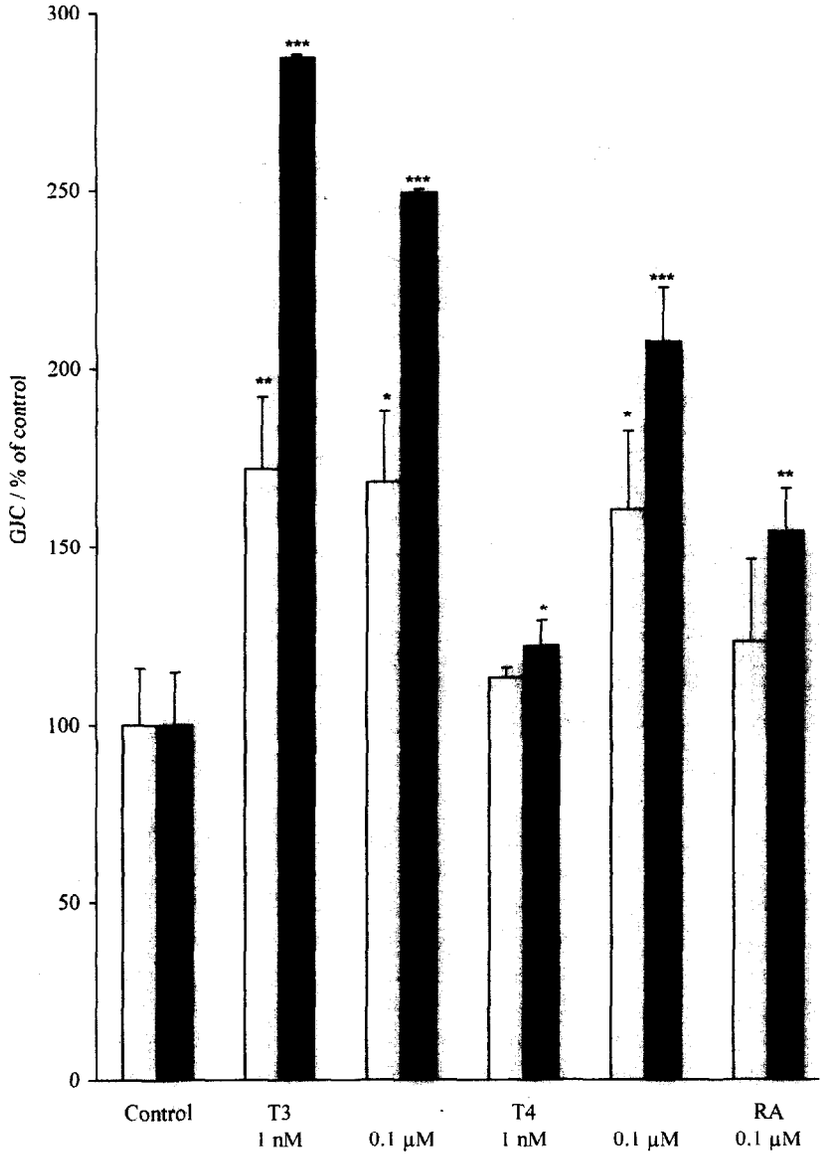

FIG. 1. Stimulation of gap junctional communication (GJC) by 3,3',5-triiodo-L-thyronine (T_3), L-thyroxine (T_4) and retinoic acid (RA) in WB-F344 cells. Confluent cells were treated as described in "Materials and Methods." At day 1 (open bars) and 2 (solid bars), GJC was determined by the microinjection/dye transfer assay. Basal level of communicating cells was 23.8 ± 3.5. Mean values of 10 individual injections were determined in four independent experiments and results standardized ($n = 4$). *$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$.

serum 10% v/v (FCS; Greiner) and 2 mM glutamine (growth medium). Cells were grown on plastic dishes (Falcon) in a humidified incubator under an atmosphere of 5% CO_2 in air.

Gap Junctional Communication

Gap junctional intercellular communication was assessed by the efficacy of diffusion of Lucifer Yellow CH (10% in 0.33 M LiCl w/v) from a single microinjected cell to its neighboring cells [16]. Confluent cells on 35 mm plates were incubated in growth medium without FCS for 24 hr, and then retinoic acid (RA) at 0.1 μM, 3,3',5-triiodo-L-thyronine (T_3) or L-thyroxine (T_4) at 0.1 μM–0.1 pM dissolved in phosphate buffer (pH 8) was added. After one and two days of incubation, microinjection was performed using microinjector 5242 and micromanipulator 5170 (Eppendorf). Dye-coupled cells were counted 5 min after injection. Mean values of 10 individual injections in cells were determined. The mean values of 4 independent experiments are given as percentage of control. Differences in GJC between controls and treated cells were calculated for significance using Student's *t*-test. Data obtained with controls were set to 100%.

Connexin43 Protein

Confluent cells grown on 60 mm plates were incubated in growth medium without FCS for 24 hr, and T_3 or T_4 dissolved in phosphate buffer (pH 8) was added. Twenty-five μg cellular protein, isolated after a two-day incubation with the test compounds (0.1 μM), was separated by SDS-PAGE and electrotransferred onto a nylon membrane. After incubation with mouse monoclonal antibodies generated to a peptide containing amino acids 252–270 of rat connexin43 (Affinity) at a dilution of 1:2000 in blocking buffer (ICN), detection was with the AURORA Western blotting kit (ICN).

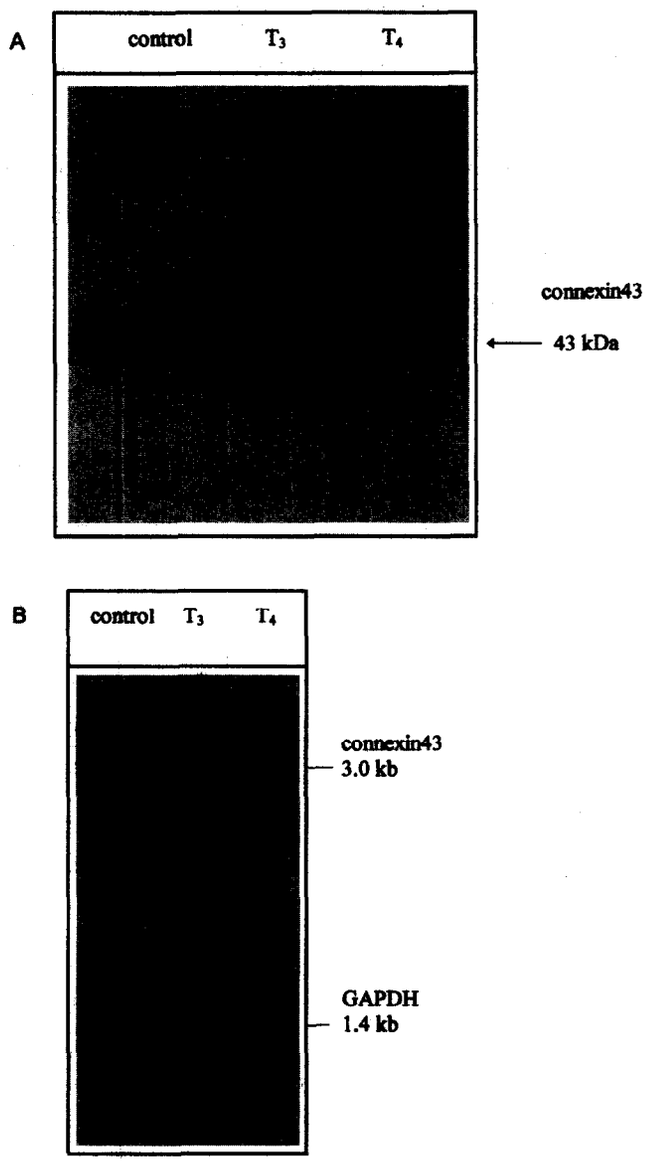

FIG. 2. Induction of connexin43 protein (A) and mRNA (B) by 3,3',5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) in WB-F344 rat liver epithelial cells. (A) Cells were incubated for 2 days with the thyroid hormones at 0.1 μM, and Western blot analysis was performed with 25 μg cellular protein in each lane. (B) Cells were incubated for three hr with the thyroid hormones at 0.1 μM, and Northern blot analysis was performed. The figure shows representative results of experiments repeated at least three times each.

Connexin43 mRNA

Confluent cells grown on 100 mm plates were incubated in growth medium without FCS for 24 hr. Total RNA was isolated according to the acid phenol method [17]. Equal amounts of RNA (25 μg), isolated after a 3, 6, and 9 hr incubation of confluent cells with T_3 or T_4 at 0.1 μM, were separated by agarose gel electrophoresis and transferred onto a nylon membrane. Connexin43 mRNA was measured by hybridization with a radioactively labeled 0.7 kb AccI fragment of mouse connexin43, kindly provided by Dr. K. Willecke (Bonn, Germany) [18]. Glyceraldehydephosphate dehydrogenase (GAPDH), used for standardization, was measured using a radioactively labeled 1.3 kb *Eco*RI fragment of the plasmid pRLCGAP, kindly provided by Dr. R. Wu (Ithaca, NY, USA) [19]. Densitometric analysis was performed using a gel scanner with GSXL software (Pharmacia). Contents of connexin43 mRNA related to GAPDH mRNA are expressed as a percentage of solvent controls; mean values ± SEM of three independent experiments are given.

RESULTS AND DISCUSSION

The present study demonstrates a novel role of thyroid hormones 3,3',5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) in the modulation of gap junctional communication (GJC). Both compounds are capable of inducing GJC, as measured in the dye-transfer assay (Fig. 1), in WB-F344 rat liver epithelial cells which express connexin43 [20]; no morphological changes in the cells were apparent. After incubation of WB-F344 cells with 0.1 μM or 1 nM of T_3 (physiological concentration in human serum: 1.5 nM T_3), GJC increased *ca.* 1.7-fold at day 1 over solvent control. A further increase to approximately 2.7-fold was observed at day 2. At lower concentrations, the effects of T_3 on GJC were less pronounced; no induction was observed at 0.1 pM (data not shown). The influence of T_4 (physiological concentration in human serum: 0.1 μM T_4) on GJC was less than that of T_3. Hardly any induction was observed with 1 nM T_4 on days 1 and 2, while a 2-fold increase was detected with 0.1 μM T_4 at days 1 and 2. Inductory effects of T_3 were also found in the embryonal human skin fibroblasts HFFF2. There was a 1.8-fold increase in GJC after a 6-day incubation with 0.1 μM T_3, but no significant increase was observed after incubation with T_4 (data not shown). The response of WB-F344-cells to treatment with retinoic acid (0.1 μM), a compound which stimulates GJC in fibroblasts and various other cell lines, was lower, only 1.5-fold at day 2 (Fig. 1). With retinoic acid, increases were observed after 1 hr in the human amniotic cell line FL [21], but only after several days in murine fibroblasts C3H/10T1/2 [22, 23] or human dermal fibroblasts [24].

The up-regulation of GJC after incubation with T_3 and T_4 is associated with a 4- to 5-fold increase in connexin43 protein as shown by Western blot analysis after two days of incubation with both compounds (Fig. 2A). The two bands corresponding to 45 kDa and 47 kDa represent phosphorylated connexin43 [25]. Rises in connexin43 protein levels are preceded by increases in connexin43 mRNA levels. The levels of connexin43 mRNA were already elevated ca. 1.6- to 2-fold at 3 hr (Fig. 2B). The effect of T_3 or T_4 on mRNA levels was similar and remained constant over a period of 9 hr (Table 1). No increase in connexin43 mRNA was observed with *all-trans* retinoic acid within this period (data not shown). In the human embryonal skin fibroblast cell line HFFF2, similar effects of T_3 on connexin43 mRNA levels were observed. After 5 to 9 hr of incubation, a 1.6–2 fold increase in mRNA levels was detected.

TABLE 1. Induction of connexin43 mRNA by 3,3'-5-triiodo-L-thyronine and L-thyroxine at 0.1 μM concentration in WB-F344 rat liver epithelial cells

Incubation time (hr)	Connexin43 mRNA Level (fold increase)	
	3,3',5-triiodo-L-thyronine	L-thyroxine
3	1.8 ± 0.1	1.8 ± 0.2
5	1.4 ± 0.03	1.6 ± 0.5
9	1.7 ± 0.4	1.8 ± 0.6

Data are given as means ± SEM for three independent experiments. Similar results were obtained with the human fibroblasts HFFF2.

The mechanism underlying stimulation of GJC seems to be different in different cell types. An increased level of connexin43 mRNA and protein has been described after treatment with *all-trans*-retinoic acid in murine C3H/10T1/2 fibroblasts [26], while in the rat liver epithelial cell line IAR203, retinoic acid enhances connexin43 protein levels without increasing mRNA [27]. Accumulated mRNA might result either from elevated transcription rates or from stabilization of mRNA. The stimulation of GJC by *all-trans* retinoic acid in mouse F9 teratocarcinoma cells has been ascribed to stabilization of connexin43 mRNA [28]. The promoter region of the rat connexin43 gene [29] contains a direct repeat (DR3) consensus sequence at −480 bp to −464 bp, a possible binding site, for example, for nuclear receptor heterodimers containing TR [30]. Thus, the relatively rapid transcriptional response of T_3 and T_4 may be due to direct regulation of the connexin43 gene.

Hyperthyroidism causes, among other adverse effects, atrial arrhythmias and congestive heart failure [15]. In addition, raised GJC has been related to pathological conditions such as cardiac arrhythmias and heart failure [13, 14]. Here we show for the first time that thyroid hormones influence GJC *in vitro*. It might be speculated that this effect is involved in the biochemical mechanism underlying the pathological effects of hyperthyroidism.

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