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Stimulation of Rat Atrial Natriuretic Peptide (rANP) Synthesis by Triiodothyronine and Thyroxine (T_4): T_4 as a Prohormone in Synthesizing rANP

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ABSTRACT. Thyroid hormones have been reported to increase the secretion and synthesis of atrial natriuretic peptide (ANP) *in vitro*. In this study, we focused on the role of type I T_4 5′-deiodinase to investigate the stimulating effects of T_4 on ANP synthesis and secretion by measuring cellular content and secretion into the medium of immunoreactive rat ANP(IR-rANP) and rANP mRNA levels in cultured rat neonatal atrial myocytes.

Both T_3 (10^{-9}–10^{-7} M) and T_4 (10^{-8}–10^{-6} M) increased cellular content and secretion into the medium of IR-rANP in a dose-dependent manner. However, these effects of T_4 were completely inhibited by the addition of propylthiouracil, which selectively suppressed type I T_4 5′-deiodinase activity. Methimazole, which did not alter T_4 5′-deiodinase activity, had no effects on T_4-induced IR-rANP increase. In the measurements of rANP mRNA levels by dot blot analysis, T_3 (10^{-8} and 10^{-7} M) and T_4 (10^{-7} and 10^{-6} M) increased rANP mRNA levels in the same way as they increased IR-rANP content. Although T_4-increased rANP mRNA levels were also inhibited by propylthiouracil, methimazole did not alter the effect of T_4. Moreover, T_4-induced rANP mRNA accumulation in atrial myocytes was further stimulated by the addition of dithiothreitol, suggesting that the deiodinating activity was thiol sensitive.

These data suggest that the stimulating effect of T_4 on cellular IR-rANP content and rANP mRNA levels is entirely induced after it is converted to T_3 by type I T_4 5′-deiodinase in atrial myocytes and that T_4 serves as a prohormone for T_3 in synthesizing rANP. (*Endocrinology* **126:** 466–471, 1990)

IT HAS been reported that serum concentrations of α-human atrial natriuretic peptide (ANP) increased in patients with hyperthyroidism (1). We previously reported that T_3 and T_4 stimulated the synthesis of immunoreactive rat ANP(IR-rANP) in cultured rat neonatal myocytes (2). T_3 and T_4 were also reported to increase rANP mRNA accumulations in rat cardiac myocytes, suggesting the effects of thyroid hormones at the rANP gene level (3, 4).

Previous studies (5, 6) have clearly shown that most of the circulating T_3 is derived from extrathyroidal conversion of T_4 in humans. Moreover, the peripheral T_3 production is affected by type I 5′-deiodinase, located mainly in liver and kidney (7). However, there is still a paucity of information about myocardial type I 5′-deiodinase activity, which is selectively inhibited by propylthiouracil (PTU) and about its physiological significance (7).

In the present study, rANP mRNA accumulations as well as the synthesis and secretion of IR-rANP were determined in cultured neonatal rat atrial myocytes and the stimulating effect of T_4 on the synthesis of rANP was investigated in relation to type I 5′-deiodination of T_4 within atrial myocytes.

Materials and Methods

Cell culture

Neonatal atrial myocytes were prepared as described previously (8). In brief, atrias removed from neonatal rats (2- to 4-day-old) were dissociated with 0.1% collagenase (Wako Chemical, Osaka, Japan) for 10 min at 37 C and pipetted 30 times. After discarding the first supernatant, the remaining tissues were further digested with fresh enzyme and pipetted. Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, UK) supplemented with 20% fetal calf serum (FCS; Flow Laboratories, North Ryde, N.S.W., Australia) was then added to the suspended cells. The same procedure was repeated 5 times. The

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combined cell suspensions were passed through 100-μm nylon mesh and centrifuged. The dispersed cells were seeded into a gelatinized plastic dish and incubated for 30 min at 37 C. During the incubation, most of nonmyocardial cells attached to the bottom of the dish, while myocytes remained suspended. The nonattached cells were then plated in DMEM supplemented with 20% fetal calf serum (FCS). The cells were incubated at 37 C under a humidified atmosphere of 95% air-5% CO_2. After 48 h, the medium was replaced with fresh DMEM without FCS and the following agents were added to the medium according to the experimental protocol: T_4, T_3, PTU, methimazole (MMI), and dithiothreitol (DTT) (all were purchased from Sigma Chemical, St. Louis, MO).

RIA for rANP

ANP extraction from cultured cells and RIA were performed as previously described (9). Briefly, atrial myocytes obtained were immediately added into 1 M acetic acid/20 mM HCl and heated at 100 C for 10 min. The samples were then homogenized and centrifuged for 25 min at 12,000 × *g*. The resulting supernatant was stored at −20 C until assayed. Cellular protein was measured by Lowry's method using BSA as standard (10).

Preparation and analysis of rANP mRNA

Total RNA was isolated from cultured cells by the guanidine isothiocyanate-cesium chloride procedure (11). RNA was quantified by UV absorption at 260 nm. To measure the size of hybridizing RNA band, Northern blot analysis was performed as described previously (12) with some modifications (9). Denatured total cellular RNA sample was size fractionated on a 1.2% agarose gel containing 18.5% formaldehyde and transferred to nitrocellulose filter by the method of Thomas (13). The filter was baked for 3 h at 80 C and prehybridized for 4 h at 60 C in a solution containing 50% formamide, 5× standard saline citrate (SSC, 1× = 0.15 M NaCl-15 mM sodium citrate), 50 μg/ml sonicated denatured salmon sperm DNA, 0.1% sodium dodecyl sulfate (SDS) and 1× Denhardt's solution (1× = 0.02% BSA, Ficoll, and polyvinyl pyrrolidone). Hybridization was performed for 12 h at 45 C in the same solution in the presence of ^{32}P-labeled rANP cDNA probe. The filter was washed in 0.5× SSC-0.1% SDS at 60 C and autoradiographed for 24 h at −80 C.

To quantify the differences in rANP mRNA accumulations, we performed dot blot analysis according to the method of White and Bancroft (14). For each sample, five dilutions of denatured total cellular RNA were dotted in duplicate onto each half of a nitrocellulose filter. After baking, the filter was divided for probing with rANP cDNA and the reference β-actin. Prehybridization, hybridization, and autoradiography were carried out as described for Northern blot analysis. The spots on the autoradiograph were quantified by densitometric scanning. Each value was plotted as a function of the amounts of dotted total RNA and the slope was determined using linear regression analysis. Relative rANP mRNA levels were normalized for the slope of the corresponding β-actin mRNA (*i.e.* individual results of rANP mRNA accumulations were expressed as a ratio of rANP/β-actin mRNA). We have found that dot-blotting density was linear up to 2 μg total cellular RNA blot and that the level of β-actin mRNA was not altered significantly by treating with T_3 and T_4, as previously described (4).

The rANP probe used in this study was a 782-base pair *Pst*I fragment of rANP cDNA; prANP10 (15) and β-actin probe was 760-base pair *Bal*I fragment of chicken β-actin cDNA (Oncor Inc., Gaithersburg, MD) (16). Both probes were labeled by multipriming DNA labeling system (Amersham, UK) using deoxycytidine 5′-[α-^{32}P]triphosphate (3000 Ci/mmol; Amersham, Japan) to obtain a specific activity of 5–10 × 10^8 cpm/μg DNA template.

Statistical analysis

Data are presented as the mean ± SEM. Statistical testing was performed by Schéff's multiple comparison after analysis of variance.

Results

Measurements of IR-rANP

In cultured neonatal rat atrial myocytes in serum-free DMEM, T_4 (10^{-6} M) as well as T_3 (10^{-7} M) increased cellular content and secretion into the medium of IR-rANP as a function of time up to the second day and made a plateau before it decreased after the sixth day. Therefore, in the following studies, the effects of various agents were assessed after the incubation in serum-free DMEM for 2 days.

The effects of T_3 and T_4 on cellular content of IR-rANP are shown in Fig. 1. Neonatal rat atrial myocytes which were cultured in serum-free DMEM for 2 days

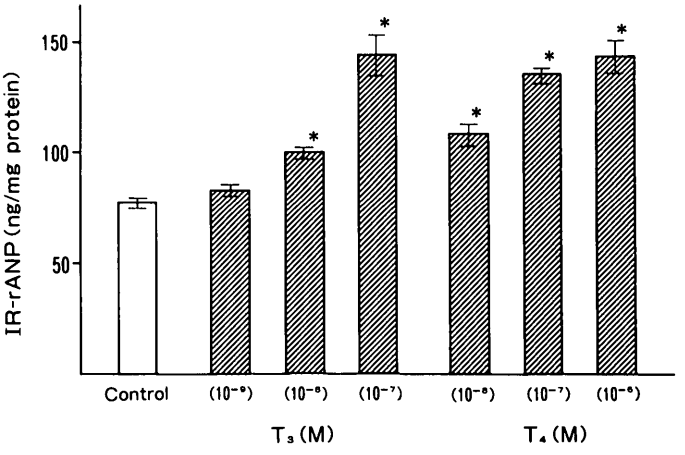


FIG. 1. Effects of T_3 and T_4 on IR-rANP accumulation in cultured rat neonatal atrial myocytes. Collagenase-dispersed atrial myocytes (10^5 cells per dish) were cultured in the medium supplemented with 20% FCS at 37 C. After 2 days, the medium was replaced with serum-free medium containing the indicated additives (control-no additives), and then the cells were incubated at 37 C for an additional 2 days. Data are expressed as the mean ± SE of the experiments repeated a minimum of three times. Each experiment was done in triplicate. *, $P < 0.01$ compared to control.

(control cells) contained 76.2 ± 3.5 ng IR-rANP/mg protein. Treatment with 10^{-8} M T_3 significantly increased cellular IR-rANP content up to 99.1 ± 1.5 ng/mg protein. T_3, at the concentrations ranging from 10^{-9}–10^{-7} M, produced a dose-responsive increase in IR-rANP content (Fig. 1). With 10^{-8} M T_4, cellular IR-rANP content was significantly increased to 109 ± 6 ng/mg protein. The effect of T_4 was also observed in a dose-dependent manner at the concentrations ranging from 10^{-8}–10^{-6} M (Fig. 1).

However, as shown in Fig. 2, by the addition of 10^{-6} and 10^{-5} M PTU to 10^{-6} M T_4, T_4-stimulated increase in IR-rANP content was completely inhibited (143 ± 7 to 81.2 ± 2.3 or 80.0 ± 4.6 ng/mg protein, respectively). To examine the effect of PTU *per se* on myocardial IR-rANP content, the cells were treated with 10^{-5} M PTU alone. These cells contained 82.1 ± 5.7 ng IR-rANP/mg protein, which did not differ significantly from that in control cells (Fig. 2). We did not detect any lactate dehydrogenase activity as an intracellular marker of cell damage in culture medium to which PTU was added. The addition of 10^{-7} and 10^{-6} M MMI did not alter 10^{-6} M T_4-stimulated IR-rANP content (143 ± 7 to 127 ± 8 or 139 ± 6 ng/mg protein, respectively) (Fig. 2).

IR-rANP secreted into culture medium was 7.0 ± 0.1 ng/ml after atrial myocytes were incubated in serum-free DMEM for 2 days. When 10^{-8} M T_3 was added to serum-free DMEM, IR-rANP secretion was increased to 14.0 ± 0.3 ng/ml ($P < 0.05$). The addition of 10^{-8}–10^{-6} M T_4 also increased IR-rANP secretion to the maximum of 15.5 ± 0.6 ng/ml ($P < 0.05$). However, when 10^{-6} and 10^{-5} M PTU was added to 10^{-6} M T_4, T_4-stimulated increase in IR-rANP concentration was completely abolished (15.5 ± 0.6 ng/ml to 7.1 ± 0.4 or 6.7 ± 0.7 ng/ml, respectively).

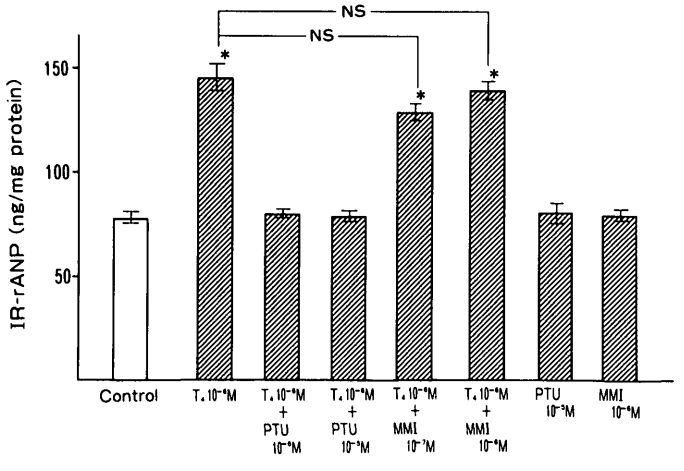
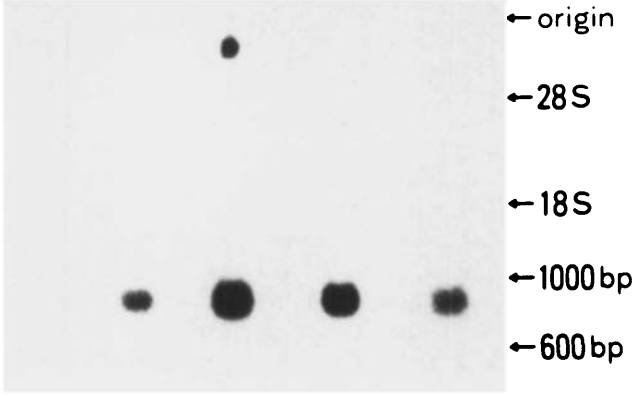


FIG. 2. Effects of PTU and MMI on T_4-stimulated IR-rANP accumulation in cultured rat neonatal atrial myocytes. Atrial myocytes (10^5 cells per dish) after 2-day culture (see Fig. 1 and *Materials and Methods*) were incubated for an additional 2 days in serum-free medium containing the indicated additives (control-no additives). Data are expressed as the mean ± SE of the experiments repeated a minimum of three times. Each experiment was done in triplicate. *, $P < 0.01$ compared to control. NS, Not significant.

Analysis of rANP mRNA

The addition of either T_4 or T_3 to serum-free medium significantly increased the relative ANP mRNA levels on the second or third day and then, gradually declined. Therefore, in the following studies, we assessed the effects of various agents on rANP mRNA levels by adding them into serum-free medium for 2 days.

Figure 3 shows the result of Northern blot analysis by the use of ^{32}P-labeled rANP cDNA probe. Total RNA extracted from control cells contained single hybridizing RNA band. This band migrated to occupy approximately 950 base pair location. T_3 (10^{-8} M), T_4 (10^{-6} M) and PTU (10^{-5} M) did not affect the overall size of the ANP transcripts. Total RNA extracted from MMI or DTT-treated cells also contained the same RNA band (data



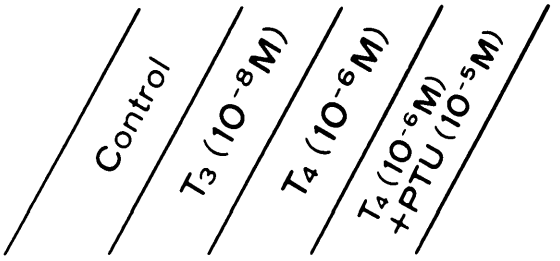


FIG. 3. Northern blot analysis of rANP mRNA in cultured rat neonatal atrial myocytes. Atrial myocytes after 2-day culture were incubated in serum-free medium containing the indicated additives (control-no additives) for an additional 2 days in the same conditions as in Fig. 1. Total cellular RNA (10 μg in each lane) extracted from atrial myocytes were electrophoresed on a 1.2% agarose-formaldehyde gel. rANP mRNA was visualized by hybridization at 45 C for 12 h with ^{32}P-labeled rANP cDNA probe.

not shown).

To quantify the relative changes in rANP mRNA levels, dot blot analysis was performed. The stimulating effects of T_3 and T_4 on rANP mRNA levels, the inhibition of the T_4-stimulation by PTU and the enhancement of the T_4-stimulation by DTT were evident by the inspection of Figs. 4 and 5. As presented in Fig. 6, 10^{-8} and 10^{-7} M T_3 treatment of atrial myocytes resulted in ap-

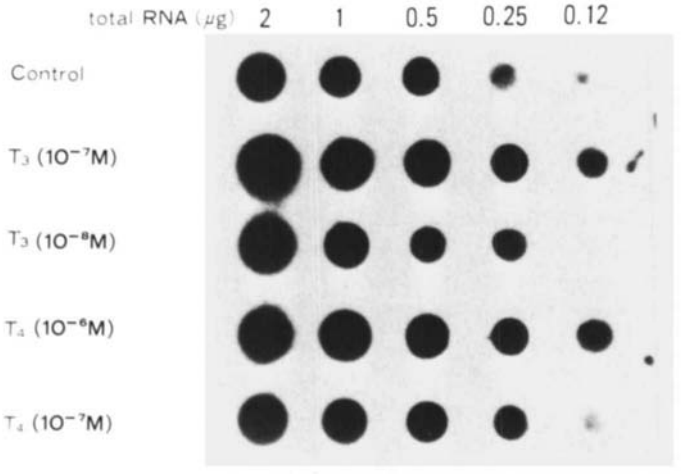


FIG. 4. Dot blot analysis of rANP mRNA in cultured rat neonatal atrial myocytes after the treatments with T_3 and T_4. Atrial myocytes after 2-day culture were incubated in serum-free medium containing the indicated additives (control-no additives) for an additional 2 days. Total cellular RNA extracted from each group of the cells was applied to nitrocellulose filter at five different dilutions and hybridized with ^{32}P-labeled rANP cDNA probe.

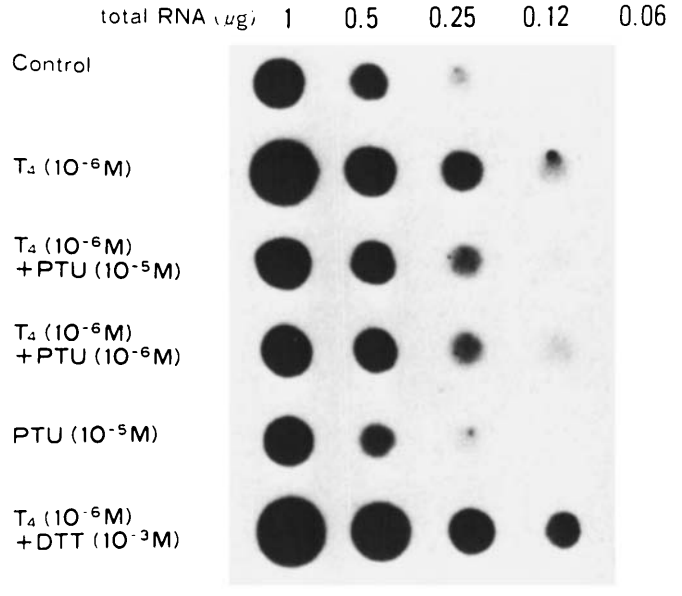


FIG. 5. Dot blot analysis of rANP mRNA in cultured rat neonatal atrial myocytes after the treatments with T_4, PTU, and DTT. Atrial myocytes after 2-day culture were incubated in serum-free medium containing the indicated additives (control-no additives) in the same condition as in Fig. 3. Dot blotting and hybridization were performed in the same procedure as in Fig. 4.

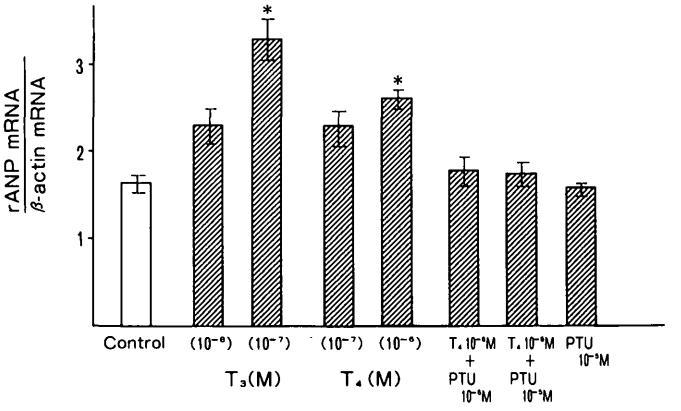


FIG. 6. Effects of T_3, T_4, and PTU on rANP mRNA accumulation in cultured rat neonatal atrial myocytes. Relative rANP mRNA levels were quantified by densitometric scanning of the autoradiographic signals. The levels of rANP mRNA levels were normalized to the level of β-actin mRNA in the duplicated sample to correct for variations in sample loading. Results are expressed as the mean ± SE of three samples. *, $P < 0.05$ compared to control.

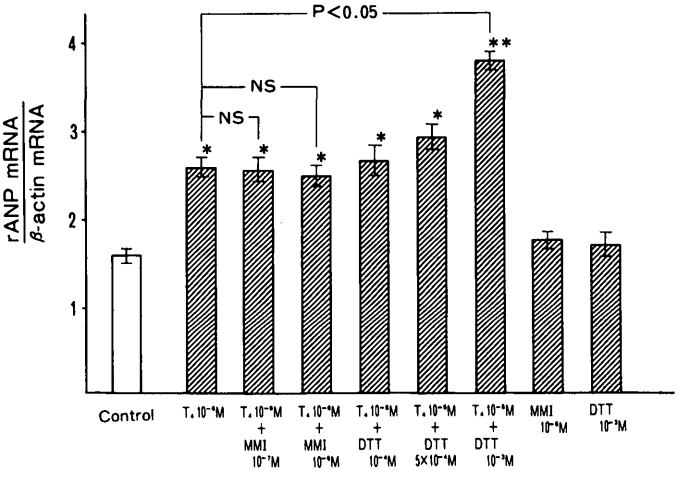


FIG. 7. Effects of MMI and DTT on T_4-stimulated rANP mRNA accumulation in cultured rat neonatal atrial myocytes. The quantification method of rANP mRNA levels was the same as in Fig. 6. Results are expressed as the mean ± SE of three samples. *, $P < 0.05$ compared to control. **, $P < 0.01$ compared to control. NS, Not significant.

proximately 1.4- and 2.0-fold increase, respectively, in rANP mRNA levels above the control value. The addition of 10^{-7} and 10^{-6} M T_4 also revealed 1.4- and 1.6-fold increase, respectively. The treatment with 10^{-5} M PTU alone had no effect on rANP mRNA levels. However, with the addition of 10^{-6} and 10^{-5} M PTU to 10^{-6} M T_4, the effect of T_4 on rANP mRNA was completely inhibited (Fig. 6). As shown in Fig. 7, with the addition of 10^{-7} and 10^{-6} M MMI, there was no observed inhibition of T_4-induced increase in rANP mRNA levels. In addition, the stimulating effect of 10^{-4}–10^{-3} DTT on T_4-induced rANP mRNA accumulation was apparent (Fig. 7).

Discussion

Thyroid hormone exerts many of its physiological actions at the nuclear level in various tissues by promoting

transcription of discrete genetic information from DNA (17), and the nuclear receptors for T_3 are now generally believed to be the sites of initiation of thyroid hormone action (18). It was previously reported that T_3 enhanced the specific mRNA levels for GH in GH1 cells (19) and α_2 microglobulin in rat liver (20), as well as the synthesis of the corresponding proteins. The heart is also known to be one of thyroid hormone responsive organs as it has substantial numbers of nuclear T_3 receptor (21). For example, thyroid hormone influences the contractile state of cardiac muscle by altering the expression of cardiac myosin heavy chain gene (22). As concerns the stimulating effect of T_3 on atrial rANP mRNA levels, it was suggested that the effect also took place through a classical nuclear receptor-mediated mechanism (3, 4).

On the other hand, Braverman *et al.* (5) clearly showed extrathyroidal conversion of T_4 to T_3 in human, and it has been assumed that thyroid hormone action may depend on the conversion of T_4 to T_3 by T_4 5′-deiodinase before the interaction of T_3 with the nuclear receptor. But, it is still controversial at the present time whether or not T_4 5′-monodeiodination occurs in myocardium. Chopra (23) previously reported that T_3 converted from T_4 in rat myocardium did exist, although its amount was only approximately one-twentyfifth that in liver. However, Kaplan (24) did not detect T_3 production in rat heart muscle, in which iodothyronine metabolism was extensively investigated in rat. Therefore, in myocardium, the contribution of the local T_3 generation to the saturation of nuclear T_3 receptors is not clear at the present time (7). At this point, it is of interest to investigate the changes in T_4-stimulated synthesis and secretion of ANP in relation to the changes in 5′-monodeiodination of T_4.

In the present study, we found the stimulating effects of T_4 as well as T_3 on rANP mRNA accumulation and the synthesis and secretion of IR-rANP in cultured neonatal rat atrial myocytes. These effects of T_4 and T_3 on rANP are consistent with the other reports (3, 4). Moreover, it was remarkable that T_4-induced synthesis and secretion of IR-rANP and rANP mRNA accumulation were completely inhibited by the addition of PTU. It was unlikely that this inhibitory effect of PTU resulted from the progressive cell morbidity or cell toxicity, because we did not detect any lactate dehydrogenase activity as an intracellular marker of cell damage in culture medium to which PTU was added. Furthermore, MMI, which does not inhibit the peripheral deiodination of T_4 (25), had no effects on both IR-rANP and rANP mRNA accumulations and DTT, a thiol-protecting agent, stimulated T_4-induced accumulation of rANP mRNA. These findings strongly suggest that the effect of T_4 on rANP synthesis in atrial myocytes is initiated after the conversion of T_4 to T_3 by 5′-monodeiodinase of T_4. Moreover, since it is well known that PTU inhibits selectively type I 5′-deiodinase activity and that it has little or no effects on type II and III deiodinases (7), the finding that PTU had inhibitory effect on T_4-induced ANP synthesis supports the existence of type I 5′-deiodinase of T_4 in atrial myocytes as in rat liver, kidney, and thyroid (7, 26, 27).

To our knowledge, there have been no reports about the physiological importance of local T_3 generation from T_4 in myocardium. We have demonstrated in this study that type I T_4 5′-deiodinase activity exists and plays an essential role in synthesizing and secreting ANP by generating T_3 from T_4 within rat neonatal atrial myocytes. It seems possible that intracellular T_3 generated from T_4 by 5′-deiodinase contributes to the saturation of nuclear T_3 receptors and to the exertion of thyroid hormone action in the heart. Moreover, it is conceivable that T_4 *per se* has little biological activity in physiological concentrations to stimulate synthesis and secretion of ANP and the conversion of T_4 to T_3 is the obligatory step for displaying the biological effects. The present observations support the previous postulation (28) that T_4 serves as a prohormone for T_3, at least in synthesizing and secreting rANP in rat neonatal atrial myocytes.

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