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Effects of thyroidectomy, T_4, and DITPA replacement on brain blood vessel density in adult rats

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Schlenker EH, Hora M, Liu Y, Redetzke RA, Morkin E, Gerdes AM. Effects of thyroidectomy, T_4, and DITPA replacement on brain blood vessel density in adult rats. *Am J Physiol Regul Integr Comp Physiol* 294: R1504–R1509, 2008. First published March 19, 2008; doi:10.1152/ajpregu.00027.2008.—In hypothyroid patients, altered microvascular structure and function may affect mood and cognitive function. We hypothesized that adult male hypothyroid rats will have significantly lower forebrain blood vessel densities (BVD) than euthyroid rats and that treatment with 3,5-diiothyroprionic acid (DITPA) (a thyroid hormone analog) or thyroxine (T_4) will normalize BVDs. The euthyroid group received no thyroidectomy or treatment. The other three groups received thyroidectomies and pellets. The hypothyroid group received a placebo pellet, the DITPA group received an 80-mg DITPA-containing pellet, and the T_4 group received a 5.2-mg T_4 slow-release pellet for 6 wk. Body weights, cardiac function, and body temperatures were measured. A monoclonal antiplatelet endothelial cell adhesion antibody was used to visualize blood vessels. The euthyroid group averaged body weights of 548 ± 54 g, while the hypothyroid group averaged a body weight of 332 ± 19 g (P value < 0.001). Relative to the euthyroid group, the DITPA-treated group was significantly lighter (P value < 0.05), while the T_4-treated group was comparable in body weight to the euthyroid group. The same trends were seen with body temperature and cardiac function with the largest difference between the euthyroid and hypothyroid groups. BVD in the euthyroid group was 147 ± 12 blood vessels/mm^2 and in hypothyroid group 69 ± 5 blood vessels/mm^2 ($P = 0.013$) but similar among the euthyroid, DITPA, and T_4 groups. These results show that hypothyroidism decreased BVD in adult rat forebrain regions. Moreover, DITPA and T_4 were efficacious in preventing effects of hypothyroidism on cardiac function and BVD.

hypothyroid; angiogenesis; echocardiography; thyroid hormones

HYPOTHYROIDISM IS A RELATIVELY common disorder affecting ~5% of adults and 15% of older women (2, 10). Consequences of hypothyroidism in adults include fatigue, cold intolerance, exertion, cognitive and mood dysfunction, decreased body temperature, elevated levels of triglycerides and cholesterol, hoarseness, sleep apnea, and cardiovascular dysfunction (2, 14, 29). Although less investigated, hypothyroidism may also decrease blood vessel density, which in turn decreases delivery of oxygen and nutrients to tissues. Since hypothyroidism may depress ventilation, leading to hypoxemia (26), the consequences of decreased blood vessel density could be enhanced in hypothyroid patients who have cardiac disease or suffer from sleep apnea and exacerbate altered cognition, mood disturbances, and fatigue.

Thyroid hormone supplementation directly affects the cardiovascular system to increase cardiac contractile function and hypertrophy, as well as cause vasodilatation (17). Recent studies indicate that thyroid supplementation also may increase blood vessel density by acting on membrane receptors, (integrin $\alpha V\beta 3$) that interact with the MAPK pathway to stimulate the production of VEGF (19). Using a chick chorioallantoic membrane model, Mousa et al. (19) demonstrated that nanamolar concentrations of the thyroid hormone analog, 3,5-diiothyroprionic acid (DITPA) increased mean vessel branching to the same extent as thyroxine (T_4). Blocking the integrin receptor, MAPK, or T_4 binding to its receptor, prevented the effects of DIPTA and T_4 on blood vessel proliferation.

David and Nathaniel (8) investigated the effects of hypothyroidism on vascular density in the brain of neonatal rats. The investigators noted a significant decrease in brain blood vessel numbers in hypothyroid animals relative to controls. More recently, Gabrichidze et al. (12) reported the effects of local blood flow in the hippocampus and cerebellar cortex of rats made hypothyroid during development by feeding the dams an iodine-deficient diet. In 1-mo-old hypothyroid offspring rats, blood flow in the dorsal hippocampus was 19% lower and 14% lower in the cerebellar cortex compared with those of age-matched euthyroid rats. Both studies examined effects of prenatal hypothyroidism. Effects of hypothyroidism on blood vessel density in brains of adult rats have not been examined.

The purpose of the present study was to evaluate the effects of treating adult thyroidectomized rats with T_4 and DITPA for 6 wk on blood vessel density in forebrain regions. We hypothesized that thyroidectomy would decrease blood vessel density and that supplementation of thyroidectomized rats with DITPA or T_4 ameliorate this decrease. To test the efficacy of the treatments, body temperature, body weight, cardiac function, and serum levels of T_3 and T_4 were also evaluated.

METHODS

Male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) at ~10.5 wk of age. Some rats had undergone a thyroidectomy with parathyroid reimplantation by the supplier. Those rats that did not undergo a thyroidectomy served as euthyroid controls ($n = 7$). The thyrodectomized animals were divided into three groups. The placebo group was implanted with a

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Table 1. *Thyroid hormone levels in euthyroid and thyroidectomized rats treated with placebo, DITPA, or T_4 pellets*

	Euthyroid	Placebo	DITPA	T_4
No. of rats	7	7	5	7
T_4, μg/dl	14.7 ± 1.2^a	4.43 ± 0.9^b	5.9 ± 0.9^b	26.8 ± 9.9^c
T_3, ng/l	0.72 ± 0.23^a	0.10 ± 0.15^b	1.04 ± 0.50^a	1.19 ± 0.87^a

Data are means ± SD. [a,b]Different superscripted letters denote significant differences ($P < 0.05$) among groups for each hormone.

placebo pellet ($n = 7$). The T_4 group ($n = 7$) received a slow-release 60-day pellet containing 5.2 mg of T_4, and the DITPA group ($n = 5$) was implanted with a slow-release 60-day pellet containing 80 mg of DITPA. Pellets were prepared by Innovative Research of America (Sarasota, FL), and DITPA was kindly provided by Dr. Eugene Morkin from the University of Arizona, Tucson, AZ. The doses for DITPA and T_4 were determined from pilot studies in Sprague-Dawley rats that were based upon previous results from a hamster study using these pellets (17). All procedures on living animals were approved by the University of South Dakota Animal Care and Use Committee.

To access the effects of the treatments on body temperature, rectal temperature was measured under anesthesia before the animal was killed 6 wk after receiving the pellets (at ~18 wk of age). Trunk blood was collected from deeply anesthetized rats, separated into serum aliquots, and frozen. T_3 and T_4 levels were measured with a solid-phase competitive ELISA kit according to the manufacture's protocol (T_3 kit, Bio-Quant, San Diego, CA; T_4 kit, Diagnostic Systems Laboratories, Webster, TX).

To determine the effects of hypothyroidism and T_4 and DITPA treatments on cardiac function, rats underwent echocardiography prior to death. Rats were anesthetized with 1.5% isoflurane, their chests were shaved, and echocardiographic gel was applied to the left hemithorax. To record echocardiographic data, a Hewlett Packard Sonos 2000 echocardiographic machine with a 7.5-MHz probe was utilized. The probe was placed on the gelled area and two-dimensional M-mode echocardiograms were obtained to evaluate left ventricular dimensions, heart rate, and posterior wall thickness.

To evaluate the blood vessel density, five animals per group were perfused through the aorta with cold saline and then with 4% buffered paraformaldehyde. Brains were removed and postfixed for 5 to 6 h in the paraformaldehyde and then placed in a 30% solution of sucrose in 0.1 M phosphate buffer.

Blood vessel density was determined using immunohistochemistry for the endothelial maker endothelial cell adhesion molecule-1 or CD31 using modifications of methods described by Newton et al. (21). Briefly, 20-mm slices from forebrain regions [0.48 to −0.28 mm relative to Bregma according to Paxinos and Watson (23)] were cut using a Leica cryostat. Slices were placed on ProbeOn Plus microscopic slides (Fischer Scientific). Five slices were collected from each animal. To contain the chemicals used during the immunohistochemical procedures, each slice was encircled using an ImmEdge pen (Vector Laboratories). First slides containing the slices were placed into a DAKO cytomation target retrieval solution and steamed for 20 min. Subsequently the slices were washed for 5 min in PBS and were then incubated in a solution of 0.075% hydrogen peroxide (Sigma). After two rinses for 5 min each with PBS, the slices were blocked with 5.0% BSA in PBS for 30 min at 4°C. The slices were then washed two times for 5 min each with PBS and incubated in a 1:500 dilution of mouse anti-rat monoclonal antibody against CD31 (Serotec) in PBS containing 2.5% BSA overnight at 4°C. At the same time control slices did not receive the primary antibody but were treated with BSA-PBS.

After an overnight incubation, slices were washed three times for 5 min and incubated at room temperature with biotinylated goat anti-mouse secondary antibody, rat adsorbed (1:400, Vector) for 1 h. Following three rinses for 5 min each in PBS, the slices were incubated in Avidin/Biotinylated enzyme complex for 1 h at room temperature. After being rinsed three times for 5 min in phosphate buffer, the slices were stained with 3.3′-diaminobenzidine containing 0.01% hydrogen peroxide for 10 min. The slices were then rinsed in phosphate buffer, dried, dehydrated in solutions containing increasing concentrations of alcohol, dipped in Histochoice for 15 s, and then coverslipped with Permount.

To determine blood vessel density, four sections with areas of 3.44 mm^2 for each slice were selected in forebrain regions corresponding to barrel field, fore limb and jaw region, and primary motor cortex and hindlimb regions according to the rat brain atlas of Paxinos and Watson (23). Image J (25) was used to count the number of blood vessels in each of these sections. The density was calculated as the number of vessels per area. There was no difference in density in the four regions, and an average value per animal was used to represent the vessel density. Animal identity was coded and only disclosed after all animals had been evaluated.

Data were analyzed to determine the effects of thyroidectomy by comparing thyroid hormone levels, body temperatures, weights, and vessel densities between the euthyroid and placebo-treated thyrodectomized animals using an unpaired Student's *t*-test. To compare the effects of DITPA or T_4 to the euthyroid data, a one-way ANOVA was used with post hoc Dunnett's test with corrections for multiple comparisons. StatMost (Dataxiom Software, Los Angeles, CA) was used to conduct the statistical analyses. Significance was accepted at $P < 0.05$.

RESULTS

Thyroid hormone levels. T_3 levels were significantly lower in placebo relative to euthyroid group ($P < 0.0001$; Table 1). In contrast, the ANOVA showed no differences between the euthyroid, T_4, and DITPA groups. In placebo-treated rats, T_4 levels were significantly lower than those of euthyroid rats ($P < 0.0001$). The ANOVA indicated group differences in T_4

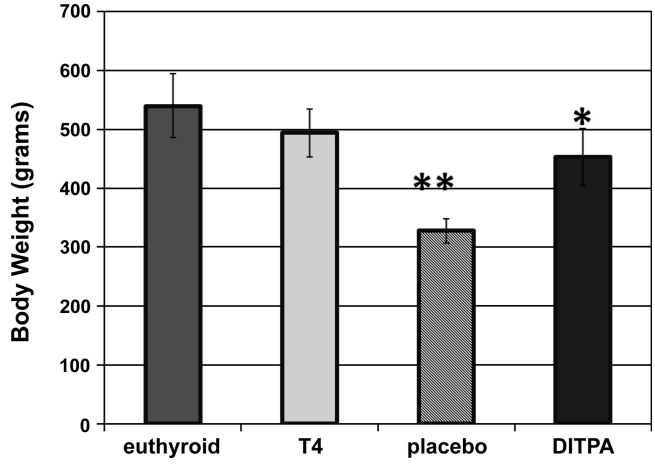


Fig. 1. Body weights of euthyroid ($n = 7$) and thyroidectomized rats treated with placebo pellets ($n = 5$), thyroxine (T_4; $n = 7$), or 3,5-diiothyroprionic acid (DITPA; $n = 5$). Values are means ± SD. Asterisks indicate significant differences ($P < 0.05$) relative to the euthyroid rats.

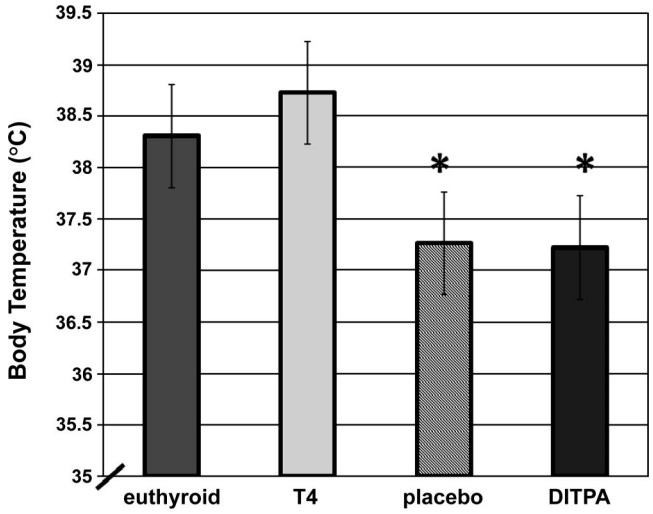


Fig. 2. Body temperatures of euthyroid ($n = 7$) and thyroidectomized rats treated with placebo pellets ($n = 5$), T_4 ($n = 7$), or DITPA ($n = 5$). Values are means ± SD. *Significant differences ($P < 0.05$) relative to the euthyroid rats.

levels among the DITPA, T_4, and the euthyroid groups. Supplementation of thyroidectomized rats with T_4 caused a significantly higher level of plasma T_4 compared with that in the euthyroid group ($P = 0.02$). By contrast, T_4 levels were significantly lower in the DIPTA group relative to the euthyroid group ($P < 0.0001$).

Body weights. Body weights of the thyroidectomized placebo-treated rats were significantly lower than those of the euthyroid controls ($P < 0.001$; Fig. 1). One-way ANOVA indicated that there were significant differences among the euthyroid, DITPA, and T_4 groups ($P = 0.02$). Specifically, the DITPA group had lower body weights relative to the euthyroid group ($P < 0.05$), but there was no difference in body weight between the euthyroid-and T_4-treated groups.

Body temperatures. Body temperatures were significantly lower in the placebo group relative to the euthyroid group ($P = 0.0013$; Fig. 2). The one-way ANOVA indicated that there was a significant effect of treatment among the euthyroid, T_4, and DITPA groups ($F_{2,16} = 10.73$; $P = 0.0011$). Specifically, the DITPA group exhibited significantly lower body temperatures than either the euthyroid or the T_4-treated group ($P < 0.02$ for each).

Echocardiographic data. Relative to euthyroid control rats, placebo-treated thyroidectomized rats showed lower heart rates ($P = 0.01$), smaller left ventricular diastolic diameters ($P = 0.008$), and smaller posterior wall thicknesses during systole and diastole (PWTs, $P = 0.0009$; PWTd, $P = 0.035$; Table 2). Moreover, heart rates of the DITPA-treated rats were significantly lower than those of the T_4-treated animals ($P = 0.007$). By contrast, there was no significant difference of left ventricular systolic diameters (LVIDs) or PWTd in thyroidectomized rats treated with T_4 or DITPA relative to euthyroid controls. PWTs were still less in these two groups relative to control ($P < 0.04$). There were no significant differences between placebo-treated thyroidectomized and euthyroid rats or thyroidectomized T_4 or DITPA-treated rats in left ventricular ejection fraction or LVIDs.

Blood vessel density. Figure 3 illustrates representative slides from rats with the different treatments. Clearly, the placebo sections show lower numbers of CD31-positive stained blood vessels. Many blood vessels appear to be cut transversely, although there are also examples of longitudinally cut blood vessels. Figure 4 summarizes that blood vessel data. There were significantly fewer blood vessels found in the placebo group relative to the euthyroid group ($P = 0.0126$). Moreover, there were no significant differences in blood vessel density among the euthyroid, DITPA, and T_4 groups. Thus, treatments with either T_4 or DITPA prevented the decrease of forebrain blood vessel density noted in thyroidectomized rats that received placebo pellets.

DISCUSSION

This study indicated that although rats treated with DITPA had lower body weights and body temperatures than those of euthyroid rats, blood vessels densities were comparable to those of euthyroid animals. Cardiac function, for the most part, was normalized in both T_4 and DITPA-treated thyroidectomized rats. Moreover, in the T_4-treated rats, T_4 plasma levels were higher than in the euthyroid group, but body temperature, body weights, and blood vessel density were similar to those in the euthyroid group. DITPA and T_4 treatments restored blood vessel densities in forebrain regions to levels noted in euthyroid rats.

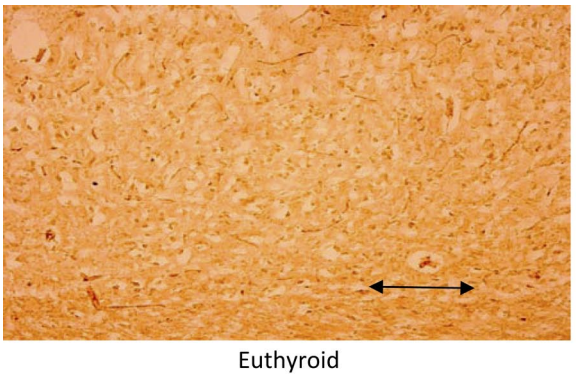
The decrease of body weight observed in the thyroidectomized rats has been reported previously (3) and may be the result of decreased levels of growth hormone (22) that were reversed by T_4 replacement (5). Interestingly, DITPA-treated thyroidectomized rats in the present study exhibited lower body weights and body temperatures, suggesting that DITPA did not act through the same mechanisms as thyroid hormones that bind to thyroid receptors (13).

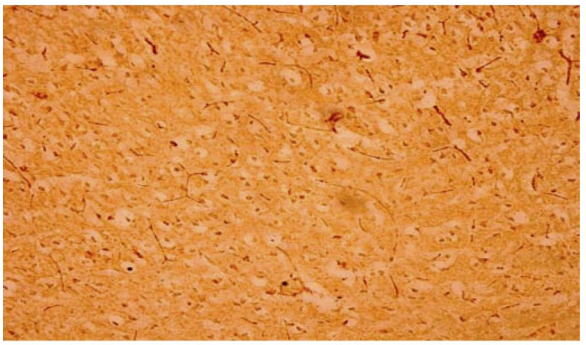
Hypothyroidism is a risk factor for the development of cardiovascular disease (29). In an animal model of dilated cardiomyopathy, the TO-2 hamster, which also exhibits subclinical hypothyroidism, Kuzman et al. (17) recently reported that DITPA and T_4 treatments improved, but did not normalize, cardiac function. DITPA did normalize the myocyte cross-

Table 2. *Echocardiographic data*

Treatment Groups, *n*	Heart Rate, beats/min	LVEF, %	LVIDs, mm	LVIDd, mm	PWTs, mm	PWTd, mm
Control ($n = 7$)	342±29	72.0±12.9	4.57±1.02	8.20±0.6	3.33±0.63	2.21±0.67
Placebo ($n = 5$)	265±29*	60.2±10.0	4.71±0.7	7.06±0.6*	1.80±0.49*	1.41±0.48*
T_4 ($n = 4$)	385±44	74.0±8.9	4.30±1.0	7.77±−0.7	2.60±0.53*	1.66±0.39
DITPA ($n = 5$)	317±26†	68.7±4.5	4.47±0.3	7.45±0.5	2.58±0.41*	1.67±0.19

Values are means ± SD for the 4 groups. LVEF%, left ventricular ejection fraction; LVIDs, left ventricular systolic diameter; LVIDd, left ventricular diastolic diameter; PWTs, systolic posterior wall thickness; PWTd, diastolic posterior wall thickness. *Differences ($P < 0.05$) relative to the control (euthyroid) group values; †significant difference ($P < 0.05$) between DITPA and T_4 groups in heart rate.





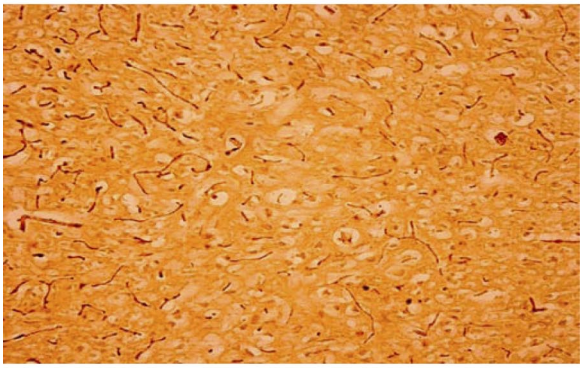


Fig. 3. Representative pictures of CD31-positive blood vessels from euthyroid control and thyroidectomized rats treated with placebo pellets, T_4, or DITPA. The double arrow is equivalent to 100 μm.

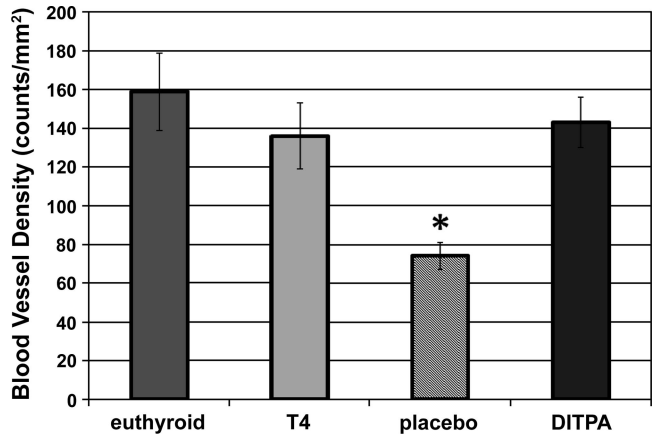


Fig. 4. Summary statistics of blood vessel densities from euthyroid and thyroidectomized rats treated with placebo pellets, T_4, or DITPA ($n = 5$). Values are means ± SD. *Significant differences ($P < 0.05$) relative to the euthyroid rats.

sectional area. Both DITPA and T_4 treatments, however, normalized cardiac blood flow at baseline conditions and after administration of adenosine. The mechanism responsible for the increased blood flow following T_4 and DITPA treatment may be associated with increased levels of fibroblast growth factor 2. In another study, Wang et al. (30) noted that in normal Sprague-Dawley rats short-term DITPA administration increased vascular endothelial growth factor, basic fibroblast growth factor, angiopoietin-1, and Tie-2, but an increase in capillary density was not noted. However, work done by the same group (32) in a postinfacrtion rat model showed that DITPA treatment was able to increase arteriolar length density in the septum when the infarct size was greater than 50%. Thus, DITPA treatment may improve myocardial blood vessel development in animal models with cardiovascular impairments.

Dagre et al. (7) found abnormal endothelial function in patients exhibiting either overt hypothyroidism or subclinical hypothyroidism. The duration of reactive hyperemia was decreased with increasing levels of thyroid-stimulating hormone levels. In a hypothyroid group treated with T_4, reactive hyperemia durations were comparable to those of euthyroid control subjects. The authors suggested that a decreased production of nitric oxide in hypothyroid patients may be responsible for their diminished vascular responses. Moreover, increased nitric oxide is associated with angiogenesis (4).

Another factor associated with angiogenesis is increased levels of VEGF. In a recent study, Dedecjus et al. (9) evaluated poor platelet plasma VEGF levels in hypothyroid patients before and after 2 mo of treatment with T_4. Prior to treatment, VEGF levels were significantly reduced in the hypothyroid patients relative to that in the controls. Following treatment with T_4, VEGF levels in the hypothyroid patients were comparable to those of euthyroid controls. Although the authors did not measure blood vessel density, they suggested that the increase in VEGF levels may improve blood vessel density in patients.

Constant et al. (6) measured cerebral blood flow (CBF) in patients who underwent a thyroidectomy for thyroid cancer and were hypothyroid for 4–5 wk and euthyroid then following thyroid hormone replacement. In addition, psychometric and

cerebral glucose metabolism was measured in these patients. During hypothyroidism, CBF was decreased 23.4%, whereas glucose metabolism was decreased 12.1% relative to patients in the euthythroid state. In addition, hypothyroid patients showed more anxiety, depression, and psychomotor retardation.

In another study in mildly hypothyroid patients before and after treatment, Krausz et al. (16) noted lower CBF in specific brain regions, such as the right parietooccipital gyri, cuneus, and insula. Interestingly, in this patient population, CBF did not normalize when they became euthyroid, suggesting a persistent effect.

Using a methylimidaze-induced model of hypothyroidism in neonatal rats, Berkowitz et al. (1) investigated thyroid hormone status on retinal blood vessel density. Compared with euthyroid control rat pups, the hypothyroid animals had decreased blood vessel densities that were dependent upon the dose of methylimidaze.

These studies support our findings that decreasing thyroid hormone levels could result in decreased blood vessel densities. Thyroid hormones can act on both membrane-bound or cytoplasmic receptors to affect blood vessel densities. Mousa et al. (19) using a chick chorioallantoic membrane model indicated that administration of both T_4 unbound and bound to agarose (which prevented transport of T_4 into the cell) increased blood vessel growth relative to a PBS control. Moreover, they determined that DITPA had the same effect as the two preparations of T_4 used in that experimental design. Combining DITPA and an integrin $\alpha v\beta 3$ inhibitor or DITPA and tetrac, an inhibitor of T4 binding to membrane receptors, prevented the angiogenic effects of DITPA. These studies and the ones presented in this paper suggest that both T_4 and DITPA may promote angiogenesis.

Induction of angiogenesis may be beneficial in a number of disorders. For example, in conditions of ischemia, stroke, and heart disease, and subclinical hypothyroidism, increased blood vessel density may improve perfusion of brain and systemic tissues, improving cognitive and functional capabilities (4, 7, 11, 17). Administration of T_4, aside from its angiogenic effect, may have some potentially adverse effects, such as increased metabolic rate and increased sympathetic nervous system stimulation (27, 28). In contrast, DITPA has been shown to have beneficial effects on cardiovascular function, improve adverse chamber and myocyte remodeling, and improve myocardial blood flow in hamsters with dilated cardiomyopathy (15).

Another mechanism by which hypothyroidism may affect cardiovascular function and angiogenesis is by increasing angiotensin II receptor levels. Recently, Carneiro-Ramos et al. (3) evaluated angiotensin receptors (AT_1 and AT_2) levels in hypothyroid rat hearts. Although serum levels of angiotensin II were not elevated in the hypothyroid rats, cardiac AT_1 receptor expression was. Moreover, Munzenmaier and Greene (20) showed that rat brain blood vessel density was increased by blocking AT_1 receptors, but not by blocking angiotensin converting enzyme. Whether AT_1 receptor levels are increased in the brains of hypothyroid rats needs to be determined.

Recent studies in the brain also indicate that factors promoting angiogenesis may also enhance neurogenesis (24). Hypothyroidism can reduce normal neurogenesis in rat dentate gyrus by 30% (18). Thyroid hormone supplementation not only increased the number of neurons, but also improved the depressive behavior exhibited by the hypothyroid animals. At this time, it is not clear whether these thyroid-related changes in neurogenesis are directly related to thyroid hormone activity or are secondary to microvascular changes affected by thyroid hormones. Thus, cognitive and psychological improvements noted when treating hypothyroid patients with thyroid hormone are most likely due to a number of factors including improved blood flow and increased number of neurons.

Perspectives and Significance

This study indicated that hypothyroidism in rats induces a decrease in brain blood vessel density that can be modulated by DITPA and T_4 replacement. Future studies are needed to determine the underlying mechanisms responsible for these effects. Since thyroid hormone status affects not only blood vessels but also other cell types on the brain, including neurons, astrocytes and oligodendrocytes, more inclusive studies are required to understand how this relatively common disorder can affect the quality of life of patients who suffer not only from outright hypothyroidism but also from subclinical hypothyroidism. Finally, since not all treated hypothyroid patients show full recovery (16, 31), the efficacy of hormone replacement in reversing these dysfunctions needs to be clarified.

GRANTS

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