DITPA stimulates bFGF, VEGF, angiopoietin, and Tie-2 and facilitates coronary arteriolar growth

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CORONARY ANGIOGENESIS during prenatal and postnatal growth is a well-established phenomenon. However, in the nonischemic adult heart, angiogenesis may or may not occur when the heart enlarges. Although some significant vascular growth may occur in certain animal models of hypertension (reviewed in Ref. 28), in patients with long-term left ventricular (LV) hypertrophy due to aortic stenosis, coronary reserve is usually markedly depressed even in the absence of coronary artery disease (5). In contrast, when hypertrophy occurs in response to volume overload, capillary and arteriolar growth are usually proportional to the magnitude of hypertrophy, and maximal coronary perfusion is maintained (2, 28). These findings support the concept that the stimulus evoking the hypertrophy is a determinant of angiogenesis. Perhaps the best example supporting this concept is thyroid hormone-induced hypertrophy, which is associated with a rapid and marked capillary growth (1, 3, 7, 14, 24–26, 30). Moreover, we have documented a normal maximal myocardial perfusion in rat hearts in which hypertrophy was induced with thyroxine, a finding that indicates compensatory growth of resistance vessels as well (25).

In a previous study, we showed that 3,5-diodothyropropionic acid (DITPA), a thyroxine analog, stimulates modest, early growth of the capillary bed in the myocardium surviving infarction (29). This agent has been shown to attenuate symptoms of heart failure after myocardial infarction through its positive inotropic effects (13, 15, 21). The favorable changes with DITPA treatment of rats or rabbits following coronary artery ligation and infarction include increased maximal positive and negative pressure development over time (dP/dt), increased basal time constant of isovolumic relaxation, and increased circumferential shortening and restoration of repolarizing transient outward K⁺ current (8, 13, 16, 31). Unlike thyroxine, DITPA is not significantly chronotropic (13) and accordingly is a safer therapeutic agent.

Because thyroxine is a potent stimulus for microvascular growth, DITPA, via its inotropic effects, may provide an angiogenic stimulus in the noninfarcted adult heart. Our study tested the hypotheses that DITPA treatment would 1) stimulate key angiogenic growth factors and 2) facilitate microvascular growth.

METHODS

Animal model. Male Sprague-Dawley rats weighing 325–350 g were randomly assigned to either a treatment or control group. All procedures were approved by The University of Iowa Animal Care and Use Committee and were in accordance with the regulations of the Animal Welfare Act of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. One group of rats was injected daily with DITPA (3.75 mg/kg sc), and DITPA stock solution (20 mg/ml) was made with 0.01 N NaOH and stored at 4°C. Before the injection, DITPA was diluted to 5 mg/ml with 0.9% saline, and the pH was adjusted to 8.0. This dose of DITPA has previously been shown to improve LV hemodynamics and to regulate sarcoplasmic reticulum Ca2⁺-ATPase (12). The control group was injected with 0.9% saline. Hearts were

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removed from rats at various time intervals, up to 3 wk, during the treatment period. The first phase of the study addressed the effects of DITPA on growth factor protein changes. Hearts for these experiments were obtained after 1, 2, 3, and 7 days of treatment and were compared with sham (vehicle) controls injected for the same periods. Therefore, each experiment was based on five rats (1 control and 4 DITPA-treated rats). To determine the effects of chronic DITPA treatment on myocardial angiogenesis, hearts arrested in diastole were perfused fixed with a glutaraldehyde solution as previously described (2). Arteriolar and capillary growth were assessed after 21 days of treatment. In one subgroup of rats, capillary growth was assessed after 10 days of treatment.

Western blotting for growth factor proteins. To quantify the abundance of the angiogenic factors vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) (FGF-2), angiopoietin-1, and its Tie-2 receptor, we performed Western blot analyses using their specific antibodies. Heart tissues from each group were homogenized in protein extraction buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2× PMSF, 0.001% aprotinin, 0.001% leupeptin, and 0.001% pepstatin). Homogenates were centrifuged at 13,000 rpm for 10 min, and the supernatant was used for protein analysis. Proteins were measured by Bio-Rad protein assay dye (Bio-Rad). Fifty-microgram samples were run on polyacrylamide electrophoretic gels (SDS-PAGE) using 12% for VEGF and bFGF and 10% for angiopoietin-1. After separation, the proteins were transferred to polyvinylidene difluoride membranes. Protein prestain markers were run in each gel as a standard. The blots were blocked in TBS (20 mM Tris and 0.15 M NaCl, pH 7.4) containing 5% nonfat milk overnight. Blots were incubated for 2 h with specific primary antibodies (Santa Cruz Biotech; Santa Cruz, CA) and then incubated with corresponding secondary antibodies for 1 h. Immunoreactive bands were visualized with the use of the enhancement fluorochrome membranes.

Stereological image analysis of angiogenesis. Hearts from rats treated with DITPA for 3 wk and those from saline-injected controls were perfused fixed (at 120 mm Hg) as previously described, and specimens were excised (2). The specimens were dissected with reference to the orientation of muscle fibers. One-micrometer-thick sections of LV samples embedded in Spurr’s plastic were cut perpendicular to the long axis of muscle bundles, placed on glass slides, and stained with Richardson’s solution (Azure II and methylene blue). Images from these sections were captured and capillary and arteriolar parameters measured using Image Pro software (23, 27). Capillary analysis, based on 300–400 profiles from each region of the heart, included length density (total capillary length in 1 mm3 tissue), volume density, and diameter. Length density (Lv) was calculated as follows: \( L_v = (a/b)N_a \) length density (number of profiles per unit area). Lv is the best indicator of vascular growth because it represents the aggregate vessel length in a unit volume of tissue and is not affected by plane of sectioning. Volume density (Vv) was calculated as the product of \( \pi r_1^2 \times r_2^2 N_a \) where \( r_1 \) and \( r_2 \) are the long and short radii of the capillary. Arteriolar analyses were conducted as described above. Tissue sections were systematically scanned, and arterioles were identified and captured with the digital image analysis system. From each region of a rat heart, 100–125 arterioles were measured. The areas of tissue fields per heart averaged about 5–9 mm2.

Statistical analyses. For a one-way comparison between control and DITPA-treated groups, we utilized a Student’s t-test. For capillary data where two comparisons were made, we used ANOVA and the Bonferroni procedure for multiple comparisons. Analysis of growth factor changes over time was based on the Helmert Contrast t-values test. The latter tests the hypothesis that treatment affected a change by considering all of the time points together and comparing them to the nontreated control.

RESULTS

DITPA and heart mass and hemodynamics. Chronic administration of DITPA for 3 wk did not affect body mass, heart mass, or hemodynamics as documented by the data in Table 1. Arterial and ventricular pressures (systolic, diastolic, and mean) were virtually identical in the two groups. Because some growth occurred during the 3-wk period, as indicated by body weight increases of ~12%, the data suggest that the drug treatment did not interfere with this process. Moreover, because ventricular weights were similar in the two groups, the inotropic effects of DITPA did not cause cardiac hypertrophy. Thus DITPA did not alter any of the hemodynamics measured or growth of the heart.

DITPA and key growth factor protein. As seen in Figs. 1 and 2, VEGF and bFGF proteins increased in response to DITPA treatment. The increases in the septum and the LV free wall were comparable. We examined the increases in both VEGF164 and VEGF188, because they constitute the two major VEGF family members in the heart. In fact, VEGF188 protein levels in the heart are higher than VEGF164. The increases in VEGF164 tended to be slightly higher than those for VEGF188 (Fig. 1). Protein increases were already evident 24 h after treatment and were generally sustained over the 1-wk period studied. Increases in bFGF were also documented (Fig. 2). On the basis of the Helmert Contrasts t-values test, the overall increases in these proteins in response to DITPA treatment are statistically significant: VEGF164, \( P < 0.001 \); VEGF188, \( P < 0.002 \); bFGF, \( P < 0.001 \). We also conducted Western blot analyses for angiopoietin-1 and its Tie-2 receptor (Fig. 3). A modest, but significant (\( P < 0.002 \)), increase
in angiopoietin-1 was noted with DITPA treatment. Tie-2 increased by approximately twofold ($P < 0.001$) during the first week of treatment. We did not find significant differences between the increases in the LV free wall and the interventricular septum.

DITPA and vascular growth. Table 2 lists the data regarding capillary parameters, i.e., length density, volume density, and diameters, that we use to assess angiogenesis. Twenty-one days of DITPA treatment did not affect any of these parameters in either the LV free wall or the interventricular septum. To determine whether these capillary parameters increased transiently, we obtained data from four rats treated with DITPA for only 10 days. As seen in Table 2, length density in this group is higher than in the nontreated controls. However, the differences do not reach statistical significance due to the smaller mean diameters in the DITPA group. The frequency distributions for arteriolar diameters are illustrated in Fig. 5. The major difference between the DITPA and control groups is that the former has a higher percentage of arterioles with diameters $<10\mu m$ than the latter. This difference is seen in both the LV free wall and interventricular septum. These data are consistent with the increase in length density (Fig. 4) associated with DITPA treatment, i.e., neoformation of arterioles. Length density of arterioles $<16\mu m$ is 80 $\mu m$ for the DITPA group compared with 52 for the control group.

DISCUSSION

The salient finding of this study is that DITPA, a thyroxine analog, stimulates coronary arteriolar growth. This growth is associated with elevations of VEGF$_{164}$, VEGF$_{188}$, bFGF, angiopoietin-1, and Tie-2 proteins and occurred in the absence of any alterations in arterial and ventricular pressures or heart mass. Although the arteriolar growth can be accounted for by the elevations in these growth factors, which together influence tube formation and smooth muscle recruitment (27), the mechanisms by which they are recruited
The possible stimuli are addressed in the subsequent discussion. Thyroid hormones and DITPA as modulators of angiogenesis. Several studies have shown that exogenous thyroid hormones lead to both cardiac hypertrophy and coronary angiogenesis (1, 3, 24–26, 30). In contrast, the thyroxine analog DITPA does not induce cardiac hypertrophy. We (24) previously showed that cardiac hypertrophy is not a prerequisite for angiogenesis in the thyroxine model. Accordingly, we hypothesized that chronic DITPA treatment would, by upregulating key growth factors, stimulate coronary angiogenesis in the absence of ischemia and hypertrophy. Interest in DITPA originated because of its potential for the treatment of heart failure (reviewed in Ref. 21). The data indicate that in the postinfarcted heart, DITPA improves systolic and diastolic function and that its effects are intrinsic to cardiac muscle. However, less is known about the effects of this agent in the normal, noninfarcted heart, although treatment in rabbits resulted in an increased circumferential shortening and enhanced velocity of shortening (−dP/dt) (13). Having previously documented capillary growth in infarcted hearts in rats treated with DITPA (29), we anticipated that this agent would also evoke capillary growth in noninfarcted hearts. However, although capillary growth is not supported by our data, we have documented growth of the arteriolar bed in response to chronic DITPA treatment. Length density is a key parameter for the quantification of vessel growth because it provides a measure of the aggregate length of a particular vessel in a given volume of tissue. Our finding that the DITPA group had more arterioles of the smallest diameter (<10 μm) fits with the finding that length density was higher in this group. These vessels represent neovascularization involving the arteriolar bed. That capillary length density was not elevated after 3 wk of treatment does not rule out the possibility of capillary formation at earlier time points followed by smooth muscle recruitment and the formation of arterioles. Our earlier finding that DITPA treatment stimulates capillary growth in the myocardium 10 days after infarction may have been influenced by ischemia, compensatory cardiac hypertrophy, and ventricular remodeling.

DITPA and growth factor enhancement. We (26) previously showed that proliferation of the capillary bed in response to thyroxine occurs in the venous ends of capillaries and coincides with upregulation of bFGF mRNA and protein. In the current study we have documented increases in several angiogenic growth factors in response to DITPA: bFGF, the two dominant VEGF splice variants (VEGF164 and VEGF188), and angiopoietin-1. Moreover, the increase in angiopoietin-1 was accompanied by an increase in its receptor Tie-2. Thus, in the nonischemic, nonhypertrophic myocardium, this thyroxine analog stimulates key growth factors that facilitate angiogenesis. VEGF is effective in stimulating all of the

Table 2. Myocardial capillary morphometry data

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>DITPA Treated, 10 days (n = 4)</th>
<th>DITPA Treated, 21 days (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LV</td>
<td>Septum</td>
<td>LV</td>
</tr>
<tr>
<td>Length density, mm/mm³</td>
<td>8.887 ± 354</td>
<td>8.939 ± 276</td>
<td>9.894 ± 795</td>
</tr>
<tr>
<td>Volume density, mm³/mm³</td>
<td>10.79 ± 0.68</td>
<td>10.39 ± 0.64</td>
<td>10.01 ± 0.68</td>
</tr>
<tr>
<td>Diameter, μm</td>
<td>3.69 ± 0.11</td>
<td>3.59 ± 0.13</td>
<td>3.40 ± 0.16</td>
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Values are means ± SE; n, number of rats. LV, left ventricular free wall.
major events in the angiogenic cascade, i.e., endothelial cell proliferation, migration, and tube formation (10).

Basic FGF also regulates endothelial cell growth (9) but in addition acts on smooth muscle cells (19). A role for bFGF in the growth of arterioles and arteries is well documented. First, arteriolar growth is dependent on bFGF as indicated by our previous work (27), which demonstrated inhibition of coronary arteriolar growth in neonatal rats treated with anti-bFGF-neutralizing antibodies. Second, bFGF protein administration to dogs with Aneroid occlusion of a coronary artery enhanced collateral development (11) and collateral perfusion (18). Third, arterial enlargement in response to high flow is preceded by increased bFGF levels in arterial smooth muscle cells (20). Thus our documentation of enhanced arteriolar length density with chronic DITPA treatment is consistent with the enhancement of bFGF and VEGF. Accordingly, our data suggest that DITPA may provide for better myocardial perfusion in situations where cardiac enlargement has occurred, e.g., cardiomyopathy and hypertension.

Our observation that angiopoietin-1 and its Tie-2 receptor proteins are elevated with DITPA treatment is not surprising because a role for angiopoietin-1/Tie-2 signaling in neovascularization is considered to be essential for both angiogenesis and endothelial cell survival (6). Moreover, angiopoietin-1 is upregulated by VEGF (4). This ligand and its receptor appear to function in vessel maturation because pericytes, the cells that are recruited to endothelial tubes late in the angiogenic cascade, express angiopoietin-1 both in vitro and in vivo (22). The fact that angiopoietin-1 expression is induced in healing skin wounds and in VEGF-induced angiogenesis indicates that it functions in adults.

How does DITPA increase angiogenic growth factors? Two major stimuli for coronary angiogenesis in the adult are enhanced metabolism or mechanical factors, i.e., shear stress or stretch. The former leads to increased myocardial perfusion, which may in turn enhance shear stress. Growth factor upregulation and/or angiogenesis in response to stretch has been documented in models of volume overload, including arteriovenous shunt (2) and bradycardia (32), and in in vitro experiments in which endothelial cells were subjected to cyclic stretch (33). In the thyroxine model, metabolism, and therefore O2, are elevated leading to increased myocardial perfusion and shear stress. Although DITPA is similar to thyroxine in that it is inotropic and lusitropic (8), it is not chronotropic nor does it significantly enhance O2 (21). The analog has been shown to affect a marked (44%) increase in LV circumferential shortening and a shortening of LV isovolumetric relaxation in baboons (8). In isolated ventricular myocytes from rabbits treated with DITPA, Ca2+ uptake in microsomal preparations was higher than in the nontreated group (17). These data taken together suggest more efficient ventricular function after treatment with this thyroxine analog, but they are contrasted with the metabolic effects of thyroxine, which would be likely to stimulate angiogenesis, namely increased O2 consumption and enhanced myocardial perfusion. How-

Fig. 4. Arteriolar length and volume densities. Data are means ± SE and number of rats is indicated in parentheses. *Significant intergroup difference (P = 0.02).

Fig. 5. Frequency distribution histogram of arteriolar diameters. In both the interventricular septum (A) and LV free wall (B), a higher proportion of small, terminal arterioles (<10 μm) occurs in the DITPA-treated groups. This finding is indicative of arteriolar neo-

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ever, the major effects of DITPA, increased circumferen-
tial shortening and enhanced velocity of shortening
(−dP/dt), may jointly provide for a decrease in extravas-
tular compressive forces. This would then decrease the
time period of diminished microvessel diameter and in-
crease the time period in which the vascular dimensions
are characteristic of diastole.

In summary, this study has documented an angio-
genic effect of DITPA in the normal nonischemic, non-
infarcted heart. Although the usefulness of this thyrox-
ine analog in improving ventricular function of the
postinfarcted heart study is well documented, this
study is the first to show growth of arterioles, the major
resistance vessels, with chronic DITPA treatment.

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