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DITPA, A Thyroid Hormone Analog, Reduces Infarct Size and Attenuates the Inflammatory Response Following Myocardial Ischemia

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Background. **Thyroid hormone can have positive effects on the cardiovascular system but its therapeutic potential is limited secondary to its adverse effects. DITPA (3,5-diiodothyroproprionic acid) is a synthetic thyroid hormone analog with positive inotropic effects similar to thyroid hormone but with minimal systemic effects. DITPA has previously been shown to reduce pathologic remodeling and improve cardiac output following myocardial infarction; however, few studies have examined the role of DITPA in determining infarct size or the early inflammatory response following myocardial ischemia. We examined the role of DITPA in the acute phase following infarction.**

Materials and Methods. **Mice were subjected to surgical induction of myocardial infarction and were then randomized to receive daily injections of DITPA or vehicle control. After 3 d, animals were sacrificed and infarct size was determined by H and E staining. Myocardial macrophage and neutrophil accumulation was determined by immunofluorescent staining. Immunoblotting and enzyme-linked immunosorbent assay (ELISA) were used to examine the levels of intercellular adhesion molecule-1 (ICAM-1), keratinocyte-derived chemokine (KC), monocyte chemoattractant protein (MCP-1), and interleukin 6 (IL-6) in homogenates from the ischemic tissue.**

Results. **Compared with vehicle control, DITPA treated animals had smaller infarcts (52.1% ± 5.7%** ***versus*** **37.3% ± 3.6%, $P < 0.05$) and decreased macrophage (32 ± 4** ***versus*** **14 ± 1 cells/HPF, $P < 0.05$, and neutrophil (14 ± 2** ***versus*** **7 ± 1 cells/HPF, $P < 0.05$) accumulation. Myocardial ICAM-1, (2.37 ± 0.4** ***versus*** **1.1 ± 0.2, $P < 0.05$), KC levels (33.32 ± 12.4 pg/mg,** ***versus*** **21.24 ± 8.9 pg/mg, $P < 0.05$), and IL-6 levels (112.3 ± 78 pg/mg** ***versus*** **37.3 ± 25.9 pg/mg, $P < 0.05$) were also reduced in the DITPA treated group, while MCP-1 levels were equivalent between groups.**

Conclusions. **Treatment with DITPA attenuates the acute inflammatory response and reduces myocardial infarct size. The reduction in myocardial ICAM-1, KC, and IL-6 levels in the DITPA group was associated with a decrease in macrophage and neutrophil accumulation.**

Key Words: **thyroid hormone analogs; myocardial ischemia; inflammatory cells; animal model.**

INTRODUCTION

Ischemic heart disease remains the leading cause of mortality in all developed countries [1]. Despite advances in therapeutic modalities, the 1-y mortality for patients with severe heart failure is nearly 50% [1]; hence, new therapies are necessary to improve outcome. One potential therapy that has been proposed is the use of thyroid hormone [2, 3]. Thyroid hormone is well known to exert positive inotropic and chronotropic effects on the heart [4, 5]; however, its use in euthyroid patients is limited due to the potentially dangerous side-effects associated with iatrogenic hyperthyroidism [6], namely tachyarrhythmias, weight loss, and anxiety. Thus, synthetic thyroid hormone analogs that bind preferentially to certain thyroid hormone receptor types have been developed in order to take advantage of the beneficial effects of thyroid hormone in specific organs, while limiting the potentially harmful systemic effects [7–9].

The synthetic thyroid hormone analog 3,5-diiodothyropropionic acid (DITPA) is one such compound with positive inotropic activity but minimal effect on heart rate or metabolic rate compared with thyroid

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hormone itself [8]. Studies in rats and rabbits have demonstrated that DITPA improves left ventricular performance in post-infarction models of heart failure when administrated alone or in combination with an angiotensin-1 converting enzyme inhibitor [9]. Most animal studies have focused on the role of DITPA in attenuating post-infarction pathologic remodeling; the mechanism of which may involve induction of angiogenesis and arteriolar growth in the ischemic myocardium [10–12]. DITPA has also been studied in the clinical setting, and a phase I study reported encouraging results in patients with chronic heart failure [13], however, the recently published results of a larger, phase II study of DITPA failed to demonstrate a clinical benefit [14].

It is well recognized that in the early stage after myocardial ischemia there is an up-regulation in the expression levels of different proinflammatory mediators (e.g., cytokines and chemokines) in the blood and/or myocardium, as well as an increase in inflammatory cell (e.g., neutrophils and macrophages) infiltration into the infarcted area of the myocardium [15]. While this inflammatory response is a prerequisite for healing and scar formation [16], it may also contribute to post-infarction myocardial injury and pathologic remodeling [15–18]. Thus, attenuation of the inflammatory response may represent a therapeutic target for myocardial protection.

Clinical studies have shown that thyroid hormone levels decrease acutely following myocardial infarction (MI) and that this decrease is inversely correlated with an increase in proinflammatory cytokines [19, 20]. Furthermore, the magnitude of decrease in thyroid hormone levels and associated increase in proinflammatory cytokines closely correlates with the severity of cardiac dysfunction and mortality risk in patients with heart failure and myocardial infarction [21–23].

The main purpose of this study is to investigate the role of DITPA in the early phase of MI and to determine whether DITPA exerts a protective effect against the inflammatory response following permanent ischemia.

MATERIAL AND METHODS

Animals and Surgical Procedures

Male wild type C57BL/6 mice (body weight, 25–30 g) were used for all experiments ($n = 24$). All procedures were approved by the University of Colorado Denver Animal Care and Use Committee and were in accordance with the regulations of the Animal Welfare Act of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996).

Animals were anesthetized using a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) through intraperitoneal injection and ventilated using small animal ventilator (Harvard Apparatus, Holliston, MA, USA). Using a lateral thoracotomy approach, heart was exposed and left anterior descending branch of the left coronary artery was ligated. The heart was internalized and the chest was closed after making sure that lungs were completely inflated. Animals were allowed to recover for 24 h and at postoperative day 1, they were randomized to receive daily doses of either DITPA (3.75 mg/ kg body weight) or vehicle control (VC) for 3 d. The dose and timing of DITPA were chosen based on previous studies investigating the role of DITPA in myocardial ischemia [10, 24]. The survival rate following the surgery was around 60%–70% and was equivalent between groups.

Histologic Analysis of Area at Risk and Infarct Size

On postoperative d 3, animals were anesthetized and 0.5 mL of Evans blue dye was injected through the left ventricular free wall after clamping the ascending aorta to demarcate the left ventricular area at risk (AAR). Hearts were excised, sliced into 1 mm thick slices below the ligature from apex towards atrioventricular groove. Apical and basal views of the slices were photographed and planimetric calculation of AAR was performed using Image J image-analysis software (National Institute of Health, Bethesda, MD). AAR was calculated and expressed as a percentage of the total left ventricular area.

In a different set of animals, hearts were harvested, embedded in freezing medium, and snap-frozen in a mix of dry ice and isopentane, and cut into 6 μm sections. Serial cryosections were cut below the ligation site. To evaluate infarct area, hematoxylin and eosin staining was performed and infarct size was measured using Image J software and expressed as a infarct % [(infarct area/LV area) $\times$100%].

Immunofluorescent Staining of Neutrophils and Macrophages

Immunofluorescent staining was performed as described previously [25]. Myocardial cryosections (5 μm thick) were treated with a mixture of 30% methanol and 70% acetone and fixed in 4% paraformaldehyde. Sections were incubated with blocking solution (10% BSA in PBS) for 45 min. Sections were then incubated with a mixture of rat monoclonal antibody against mouse neutrophil marker protein (Clone 7/4; ABD Serotec, Oxford, UK) and rabbit polyclonal antibody against mouse macrophage marker protein CD68+ cells (Santa Cruz Biotechnology, Santa Cruz, CA), 5 μg/mL each in antibody buffer, for 90 min. Sections were then incubated with a rabbit polyclonal antibody against PMNs or CD68+ cells, and then incubated with Cy3-tagged secondary goat anti-rabbit IgG (imaged in the red channel). Nuclei were stained with *bis*-benzimide (DAPI, imaged in the blue channel), and glycoproteins on cell surfaces with Alexa 488-tagged wheat germ agglutinin (imaged in the green channel). Microscopic observation and photography were performed with a Leica DMRXA digital microscope. Images were analyzed using Slide Book 2.6 software (I. I. I., Denver, CO) to obtain quantitative estimates of area. Numbers of neutrophils and macrophages were counted in five random fields by a blinded observer.

Western Blot Analysis of ICAM-1

Intercellular adhesion molecule 1 (ICAM-1) levels in myocardial homogenate were analyzed by immunoblotting as previously described [26]. Myocardial homogenate was mixed with an equal volume of sample buffer [100 mM Tris–HCl (pH 6.8), 2% SDS, 0.02% bromophenol blue and 10% glycerol]. Crude protein (10 μg) was fractioned on 4%–20% acrylamide gradient gels and transferred onto nitrocellulose membranes. Membranes were incubated in PBS containing 5% nonfat dry milk for 1 h to block nonspecific binding. Then, membranes were incubated with the appropriate primary antibodies (ICAM-1 antibody was diluted 1:200, HSP 25 antibody 1:1000) overnight at 4°C. After washing with TPBS, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:5000 dilution with TPBS containing 5% dry milk) at room temperature for 1 h. Bands were developed using enhanced chemiluminescence (ECL; Thermo Scientific, Rockford, IL) and exposed on Fuji X-ray films (LIEGT-LABS, Dallas, TX, USA). Band density was analyzed using the NIH