Enhanced exercise capacity in mice with severe heart failure treated with an allosteric effector of hemoglobin, *myo*-inositol trispyrophosphate

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A major determinant of maximal exercise capacity is the delivery of oxygen to exercising muscles. *myo*-Inositol trispyrophosphate (ITPP) is a recently identified membrane-permeant molecule that causes allosteric regulation of Hb oxygen binding affinity. In normal mice, i.p. administration of ITPP (0.5–3 g/kg) caused a dose-related increase in the oxygen tension at which Hb is 50% saturated (p50), with a maximal increase of 31%. In parallel experiments, ITPP caused a dose-related increase in maximal exercise capacity, with a maximal increase of 57 ± 13% ($P = 0.002$). In transgenic mice with severe heart failure caused by cardiac-specific overexpression of Gαq, i.p. ITPP increased exercise capacity, with a maximal increase of 63 ± 7% ($P = 0.005$). Oral administration of ITPP in drinking water increased Hb p50 and maximal exercise capacity (+34 ± 10%; $P < 0.002$) in normal and failing mice. Consistent with increased tissue oxygen availability, ITPP decreased hypoxia inducible factor-1α mRNA expression in myocardium. It had no effect on myocardial contractility in isolated mouse cardiac myocytes and did not affect arterial blood pressure in vivo in mice. Thus, ITPP decreases the oxygen binding affinity of Hb, increases tissue oxygen delivery, and increases maximal exercise capacity in normal mice and mice with severe heart failure. ITPP is thus an attractive candidate for the therapy of patients with reduced exercise capacity caused by heart failure.

hypoxia | oxygen delivery

A major determinant of exercise capacity is the amount of oxygen available to the exercising muscles. Heart failure is characterized by a reduction in exercise capacity caused primarily to the inability of the cardiovascular system to increase cardiac output and hence, blood flow, to exercising muscles (1–5). Accordingly, most previous efforts to improve exercise capacity in patients with heart failure have relied on interventions that increase the pumping function of the heart and/or the distribution of blood flow to exercising muscles. Increasing the concentration of Hb also enhances the delivery of oxygen to exercising muscles, and in patients with concurrent heart failure and anemia the administration of erythropoietin to increase the red cell mass may increase maximal exercise capacity (6, 7).

The increased delivery of oxygen to exercising muscles might also be achieved at a constant Hb concentration by decreasing the oxygen binding affinity of Hb so as to release more oxygen in hypoxic tissues. The organic phosphate 2,3-bisphosphoglycerate (2,3-BPG) is the natural allosteric effector that decreases the oxygen binding affinity of human Hb, and increased levels of 2,3-BPG appear to play a compensatory role in a variety of circumstances including high altitude and chronic pulmonary disease (8, 9). It has been noted that red blood cell 2,3-BPG levels are increased in patients with low output heart failure, leading to the suggestion that interventions to further decrease Hb oxygen binding affinity might be of clinical value in such patients (10).

myo-Inositol hexakisphosphate (IP_6) is a powerful allosteric effector of Hb that increases the regulated oxygen-releasing capacity of RBC. In piglets, transfusion of RBC loaded ex vivo with IP_6 led to physiologic effects consistent with increased oxygen delivery (11, 12). Because at neutral pH, IP_6 is a partially dissociated polyanion bearing 8 negative charges (13), it is unable to cross the RBC membrane and ex vivo physical methods are required to load erythrocytes (11, 14). However, we recently showed that *myo*-inositol trispyrophosphate (ITPP) hexasodium salt is capable of crossing the RBC plasma membrane and acting as an allosteric effector of Hb, shifting the oxyhemoglobin dissociation curve to higher oxygen pressures (pO_2) (15, 16), and leading to a marked inhibition of blood vessel formation processes in vitro (16). Accordingly, we tested the hypothesis that systemic administration of ITPP would increase exercise capacity in normal mice and in mice with severe exercise limitation caused by reduced cardiac output as a result of myocardial failure.

Results and Discussion

ITPP Decreases the Oxygen Binding Affinity of Hb in Vivo. To determine whether ITPP increases the tissue availability of oxygen in vivo, ITPP was administered to normal mice by i.p. injection and the oxygen binding affinity of Hb was measured 18–24 h later. In blood from control mice, the pO_2 at which Hb is 50% saturated with oxygen (p50) averaged 37.3 ± 1.5 torr. ITPP caused a dose-related right shift in the oxygen dissociation curve, resulting in a mean 22% increase in p50 at a dose of 1 g/kg, and a mean 37% increase with the 2 g/kg dose (Fig. 1*A*). There was little or no effect of ITPP on maximal oxygen saturation at pO_2 of >100 torr (Fig. 1*B*). Thus, although ITPP had no significant effect on total oxygen carrying capacity, the right shift in the oxygen dissociation curve at O_2 tensions in the physiologic range suggests that a larger fraction of O_2 would be released at the lower oxygen tension in the tissue. After a single ITPP administration, the p50 increase was sustained for 48 h, had decreased by ≈50% in 5 days, and was no longer present after 12 days. The prolonged duration of the effect, which is consistent with the

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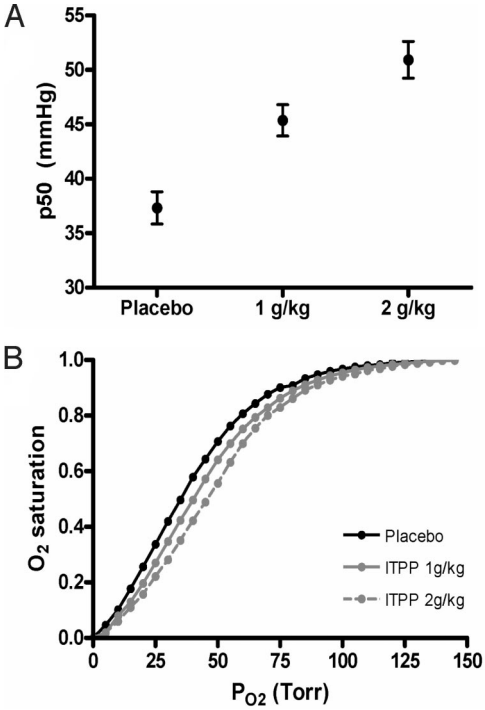

Fig. 1. ITPP increases Hb oxygen dissociation in vivo. ITPP was administrated by i.p. injection to normal mice, and Hb p50 (the pO_2 at which Hb is 50% saturated with oxygen) was measured 24 h later by using a HEMOX analyzer. (*A*) Mean p50 values measured in blood samples from mice receiving placebo ($n = 5$), 1 g/kg ITPP ($n = 5$), or 2 g/kg ITPP ($n = 4$), demonstrating a dose-related increase in p50. (*B*) Representative Hb oxygen dissociation curves for the 3 treatment groups. ITPP administration caused a parallel right-shift in the dissociation curve, thus increasing the O_2-releasing capacity at physiologic pO_2 levels while having little or no effect on total O_2 carrying capacity.

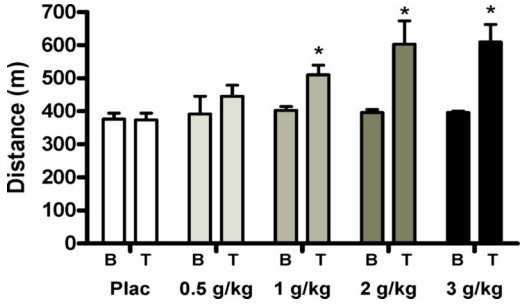

Fig. 2. ITPP increases exercise capacity in normal mice. Maximal exercise capacity was determined as the maximal distance run (meters) until exhaustion on a motorized treadmill by using air-puff stimulation. Bars represent distance run at baseline (B) and 16–24 h after i.p. administration of ITPP (T) in doses ranging from 0.5 to 3 g/kg or placebo. *, $P < 0.05$ vs. baseline; $n = 22$ in baseline group, 4–7 in each treatment group.

half-life of RBC in mice, suggests that the effect of ITTP on Hb is relatively long-lived.

ITPP Suppresses Hypoxia-Inducible Factor (HIF) 1α in Myocardium in Vivo. To further test the ability of ITPP to increase oxygen delivery to tissues in vivo, we measured the expression of HIF 1α mRNA in myocardium from normal mice. In normal mice, ITPP administration (2 g/kg, i.p.) decreased the level of myocardial HIF mRNA, measured 3 days after administration, from 5.5 ± 1.4 to 1.7 ± 0.6 arbitrary units ($P = 0.06$; $n = 3$ per group). These data are consistent with the thesis that the ITPP-induced decrease in Hb oxygen affinity, as reflected by the increase in p50, results in increased availability of oxygen at the tissue level.

ITPP Increases Exercise Capacity in Normal Mice. Because ITPP should increase the availability of oxygen at the tissue level, we hypothesized that ITPP would increase exercise capacity in vivo in mice. We therefore measured the maximal exercise capacity of normal mice by using a progressive workload motorized treadmill with air puff motivation to ensure maximal effort. Maximal exercise capacity was measured in a blinded manner at baseline and again 24 h after the i.p. injection of ITPP or placebo. The exercise capacity was similar in all groups at baseline (Fig. 2). Whereas placebo had no effect on maximal exercise capacity, ITPP caused a dose-related increase of up to ≈50%, with a plateau in effect between the 2 highest doses of 2 and 3 g/kg.

ITPP Improves Exercise Function in Mice with Severe Heart Failure. To test the hypothesis that ITPP would increase exercise capacity in animals with heart failure, we used transgenic mice with dilated cardiomyopathy caused by cardiac-specific overexpression of Gαq, as described (17). These mice have severe left ventricular (LV) dilation (LV end-diastolic dimension = 4.3 ± 0.4 mm vs. 3.0 ± 0.1 mm in normal mice; $P < 0.001$; $n = 10$) and a markedly reduced LV fractional shortening (29 ± 4% vs. 62 ± 2% in normal mice; $P < 0.001$; $n = 10$). Baseline maximal exercise capacity is consequently severely depressed to ≈60% of that in normal mice (Fig. 3*A*). ITPP administration caused striking dose-related increases in maximal exercise capacity of 34% and 71% for the 1 and 2 g/kg doses, respectively, whereas placebo had no effect (Fig. 3*A*). The magnitude of the ITPP-induced increase in exercise capacity was related to the increase in Hb p50 (Fig. 3*B*).

It has recently been reported that, in mice, bone marrow transplantation with a Hb variant having a low oxygen affinity or

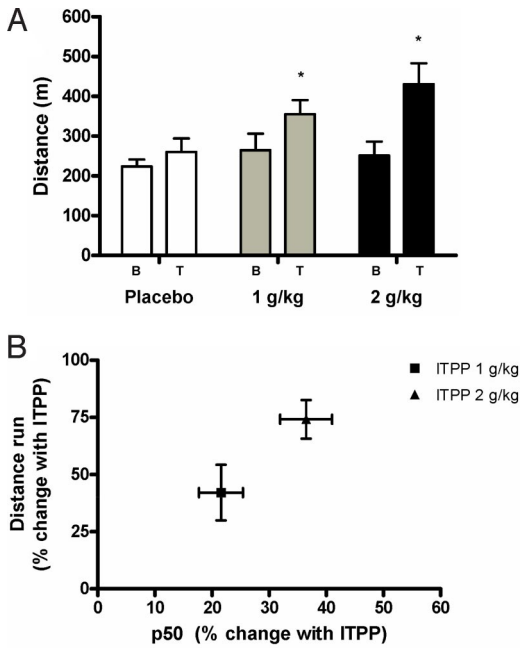

Fig. 3. ITPP restores exercise capacity in mice with severe heart failure. Transgenic mice with cardiac-specific overexpression of Gαq have an ≈40% reduction in maximal exercise capacity compared with normal mice. (*A*) Distance run at baseline (B) and 16–24 h after administration of ITPP (T) or placebo. *, $P < 0.05$ vs. baseline distance; $n = 5$–6 per group. (*B*) Relationship between the ITPP-induced changes in p50 and maximal exercise capacity, as displayed in *A*.

i.v. administration of another type of allosteric Hb modifier increased the voluntary running time (19). Whereas measurement of voluntary running time precludes conclusions about possible effects on maximal exercise capacity, we used here an exercise protocol designed to measure maximal exercise capacity, which is directly related to the maximal oxygen delivery.

Our findings suggest that increasing the availability of oxygen with ITPP may be a means of improving maximal exercise in patients with heart failure who have impaired exercise capacity caused by reduced cardiac output. However, it should be noted that the causes of reduced exercise in patients with heart failure are incompletely understood and may involve multiple factors other than oxygen delivery, including deconditioning, atrophy, and/or metabolic dysfunction of the skeletal (18). It is also possible that the Gαq mouse model of dilated heart failure may not fully reflect these and other causes of exercise impairment in humans.

Oral ITPP Increases p50 and Exercise Capacity. Previous efforts to modify RBC Hb affinity pharmacologically have been limited by the failure of effector molecules to cross RBC membranes (11, 14), short duration of effects (19), and poor solubility in water (20). Because ITPP is both membrane permeant and readily soluble in water (15, 16), we considered the possibility that ITPP would be absorbed after oral ingestion.

To determine whether oral ingestion of ITPP causes a shift in Hb oxygen affinity, normal mice were allowed to drink ad libitum water in which ITPP or IP_6 was dissolved at a concentration of 20 mg/mL. Hb p50 increased by ≈16% in mice that drank ITPP, but was unchanged in mice that drank water (+3%) or water with IP_6 (+3%), which is membrane impermeant. To determine whether oral ingestion would also improve exercise function, maximal exercise capacity was determined in normal mice ($n = 4$) and mice with heart failure caused by Gq overexpression ($n = 2$) before and after drinking water containing ITPP (20 mg/mL, ad libitum) for 4–8 days. Oral ingestion of ITPP increased maximal exercise capacity by an average of 34 ± 10% ($P < 0.002$; $n = 6$).

Lack of Direct Vascular and Cardiac Effects of ITPP. Pharmacologic agents that exert vasodilator and/or positive inotropic effects may increase cardiac output, blood flow to skeletal muscle, and exercise capacity in heart failure (21). To determine whether ITPP exerts vasodilator effects, blood pressure was measured in 12 normal mice at baseline and 24 h after i.p. injection of ITPP (2 g/kg) or placebo. Neither ITPP nor placebo affected systolic blood pressure (before ITPP = 134 ± 6 mm Hg; after ITPP = 135 ± 4 mm Hg; P not significant), diastolic blood pressure (before ITPP = 76 ± 12 mm Hg; after ITPP = 73 ± 4 mm Hg; P not significant), or heart rate (before ITPP = 706 ± 12; after ITPP = 720 ± 17 beats per min; P not significant).

To determine whether ITPP exerts a direct positive inotropic effect, contractile properties were measured in freshly-isolated adult rat ventricular myocytes, as described (22). ITPP (500 μM; 10 min) had no effect on baseline myocyte sarcomere shortening (−9 ± 29%; P not significant) or on the rate of sarcomere shortening (−17 ± 27%; P not significant), whereas the positive control norepinephrine caused 4- to 6-fold increases in sarcomere shortening (+405 ± 155%, $P < 0.01$) and the rate of shortening +670 ± 170%; $P < 0.01$).

Conclusion

The present results show that i.p. and oral administration of ITPP cause a decrease in Hb-oxygen affinity that is associated with a parallel, dose-related increase in maximal exercise capacity in both normal mice and mice with reduced exercise capacity caused by severe myocardial failure. These observations demonstrate that allosteric modulation of Hb oxygen binding affinity can exert clinically meaningful effects on maximal exercise capacity. Accordingly, ITPP is an attractive therapeutic candidate to alleviate symptoms in patients with reduced exercise capacity caused by low cardiac output heart failure. ITPP may also enhance physical performance of otherwise healthy individuals, in particular, under extreme conditions such as high attitude or intense physical exercise.

Methods

Synthesis of ITPP. This membrane-permeant allosteric effector of Hb was synthesized as reported (15), by using an improved procedure. Briefly, IP_6 dodecasodium salt (Sigma) was converted via its perprotonated form, obtained by passage over Dowex 50 resin (Sigma–Aldrich) in its H^+ form, into its triethylammonium salt and then, upon heating with dicyclohexylcarbodiimide (Sigma–Aldrich) in acetonitrile/water 2/1 solution, triply cyclized to give the triethylammonium salt of ITPP. Thereafter, an aqueous solution of the latter was passed over Dowex Marathon C Na^+ resin (Sigma–Aldrich) until cation exchange of triethylammonium against sodium was complete. Evaporation of the aqueous solution gave the sodium salt of ITPP in high purity (≈90% yield).

p50 Measurements. The p50 value (pO_2 at which 50% of Hb is saturated with O_2), a measure of the affinity of Hb for oxygen, was determined by using a HEMOX Analyzer (TCS Scientific), by constructing Hb dissociation curves based on dual wavelength spectrophotometry as described (16).

RT-PCR for HIF-1α mRNA. Total RNA was extracted from mouse hearts by using a Total RNA Purification System (Invitrogen). Quantitative RT-PCR was performed by using cybergreen and the I-cycler iQ RT-PCR (Bio-Rad). The primers for HIF-1α were: 5′-TCAAGTCAGCAACGTGGAAG-3′ and 5′-TATCGAGGCTGTGTCGACTG-3′.

Exercise Testing. Maximum exercise capacity was measured by using a rodent treadmill equipped with an air puff motivator (Columbus Instruments) as described (23). Animals were familiarized with running on the treadmill. For tests, the treadmill was set at a constant incline of 15°, and the initial speed of 15 m/min was increased by 3 m/min every 2 min. Total exercise time was recorded as the elapsed time to exhaustion and then converted to distance. Exhaustion was determined by an observer blinded to treatment group and was defined as the point at which the animals could not keep pace with the treadmill and had no response to the air puff stimulus. All exercise evaluations were performed twice and the results were averaged.

Gαq Transgenic Mice. Gαq transgenic mice were kindly provided by Gerald W. Dorn II (University of Cincinnati, Cincinnati) (17) and subsequently bred at Boston University Medical Center. This mouse overexpresses Gαq exclusively in the myocardium under the myosin heavy chain promoter. The present study used the Gαq line that overexpresses 40 (Gαq40) copies of the transgene on a FVB background, which leads to a 5-fold increase in the transgene protein levels. Age-matched controls were bred by using heterozygote Gαq male mice with WT females obtained from Charles River. Animals were studied at 12–14 weeks of age. The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all study procedures and use of animals.

Statistical Analysis. Data are shown as mean ± SEM. Group comparisons were performed by using Student's t test (2 groups) or 1-way ANOVA (multiple groups). Baseline versus treatment comparisons were performed by paired t test. All tests were 2-tailed, and $P < 0.05$ was considered significant.

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