INVESTIGACIÓN BÁSICA

Activation of mitochondrial oxidative phosphorylation during (+/-)-isoproterenol-induced cell injury of myocardium

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Summary

Hydrolytic and synthetic activities of mitochondrial ATPase were studied during (+/-)-isoproterenol-induced cell injury of the myocardium (67 mg/kg body weight). This research was a long-term study (72 h) in which rat heart homogenates, and a potentiometric method were used. Hydrolytic activities in homogenates from (+/-)-isoproterenol-treated rats were not statistically different, during the whole long-term study, from the hydrolytic activity in normal homogenates. The synthetic activity (mitochondrial oxidative phosphorylation) of mitochondrial ATPase increased at 3, 6, and 18 h (35, 48 and 23% respectively) after (+/-)-isoproterenol administration with regard to the control group. At 12 h and 21-72 h after drug administration, the data revealed no differences between synthetic activity of mitochondrial ATPase in control *vs* (+/-)-isoproterenol treated homogenates. The facts that synthetic and hydrolytic activities in homogenates from (+/-)-isoproterenol treated rats were never lower than the synthetic and hydrolytic activities in normal homogenates, and that activation of mitochondrial oxidative phosphorylation occurred at some times after (+/-)-isoproterenol treatment, suggest that no considerable and "negative" modifications occur in the active configuration of mitochondrial ATPase during (+/-)-isoproterenol-induced injury of the myocardium (67 mg/kg body weight).

Resumen

ACTIVACIÓN DE LA FOSFORILACIÓN OXIDATIVA
MITOCONDRIAL DURANTE UNA LESIÓN CELULAR DEL
MIOCARDIO INDUCIDA POR EL (+/−)-ISOPROTERENOL

En el presente trabajo se investigaron las actividades hidrolítica y sintetizadora de la ATPasa mitocondrial durante una lesión celular del miocardio inducida por el isoproterenol (67 mg/kg de peso corporal). La investigación fue un estudio secuencial (72 h), en la cual fueron usados homogenados de corazón de rata y una técnica potenciométrica. Las actividades hidrolíticas en homogenados de ratas tratadas con (+/-)-isoproterenol fueron estadísticamente iguales, durante todo el estudio secuencial, a la actividad hidrolítica en homogenados normales. La actividad sintetizadora de la ATPasa mitocondrial (fosforilación oxidativa mitocondrial) aumentó a las 3, 6 y 18 h (35, 48 y 23%, respectivamente) con respecto al control después de la administración de (+/-)-isoproterenol. A las 12 h y 21-72 h, después de la administración de la droga, los datos no muestran diferencia entre la actividad sintetizadora de la ATPasa mitocondrial en homogenados de ratas tratadas con (+/-)-isoproterenol y la actividad sintetizadora en homogenados normales. Los hechos de que las actividades sintetizadoras e hidrolíticas de la ATPasa mitocondrial, en homogenados de ratas tratadas con (+/-)-isoproterenol, nunca fueron más bajas que las actividades sintetizadora e hidrolítica en homogenados normales, y de que una activación de la fosforilación oxidativa mitocondrial ocurre a algunos tiempos después del tratamiento con (+/-)-isoproterenol indican que es posible que no tengan lugar modificaciones considerables y "negativas" en la configuración activa de la ATPasa mitocondrial durante una lesión celular del miocardio inducida por el (+/-)-isoproterenol (67 mg/kg de peso corporal).

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Introduction

Isoproterenol is a synthetic b-adrenergic agonist. In the case of b-adrenergic agonist action,[1] the circulating hormone or drug is the first "messenger", interacting with the b-adrenergic receptor on the external surface of the target cells. The drug hormone-receptor complex activates the enzyme adenyl cyclase on the internal surface of plasma membrane of the target cell, which accelerates the intracellular formation of cyclic adenosine monophosphate (cyclic AMP)-the second- "messenger", which then stimulates or inhibits various metabolic or physiological processes.[2-4] Isoproterenol exists as a pair of optical isomers: (-)-isoproterenol and (+)-isoproterenol. (+/-)-Isoproterenol is a racemic mixture of the two above mentioned stereoisomers. In regard to the ability to activate b-adrenergic receptors, (-)-isoproterenol and (+)-isoproterenol are the active and inactive isomers, respectively.

It has been shown that isoproterenol induces myocardial cell injury similar to that reported for myocardial infarction, myocardial ischemia, cardiac stress, and Chagasic cardiomyopathy.[5-8] Several studies have investigated the molecular-cellular mechanisms of isoproterenol-induced cell injury of the myocardium[9-12] (and the references in[10]). Among these, the study by Chagoya de Sanchez and co-workers[9] is a thorough study, which establishes a long-term, integrated model of isoproterenol-induced myocardial cell damage (encompassing structural, biochemical and physiological aspects). In these studies different constituents of the mitochondrial structure have been implicated in isoproterenol-induced cell injury of the myocardium, including mitochondrial ATPase, which is directly responsible for ATP synthesis supported by respiration, which supplies, under normal conditions, more than 80% of the energy demand of mammalian cells (for reviews see[13,14]). The mitochondrial ATPase is an oligomenic protein of the inner mitochondrial membrane constituted by a hydrophylic complex (F_1) that contains the catalytic site(s) for ATP synthesis or hydrolysis, connected to a hydrophobic protein complex (F_0), inserted in the lipid membrane, whose function is the coupling of catalytic activities of F_1 to transmembrane proton translocation. The behavior of the hydrolytic activity of mitochondrial ATPase during isoproterenol-induced myocardial cell injury has been studied.[10-12] The results of our previous study[12] show that (+/-)-isoproterenol does not induce alterations in the hydrolytic activity of mitochondrial ATPase, and suggest the possibility that kinetic alterations in the hydrolytic activity of mitochondrial ATPase, during isoproterenol treatment, observed by Curti et al[10] and Capozza et al[11] would be of artifactitious origin.

Extrapolating findings from the studies on the behavior of hydrolytic activity of mitochondrial ATPase during isoproterenol-induced cell injury of myocardium[10-12] to the behavior of synthetic activity of this enzyme during the above mentioned drug administration is not possible, because the sites for ATP hydrolysis and synthesis, in mitochondrial ATPase, and their mechanisms are known to be distinct.[15] In this sense, the present investigation was aimed at studying the synthetic activity of mitochondrial ATPase (mitochondrial oxidative phosphorylation) during (+/-)-isoproterenol-induced cell injury of the myocardium.

To know the mechanism(s) by which isoproterenol-injury of the myocardium occurs, an easy to manipulate and reproduce experimental model is necessary. Mitochondrial parameters during isoproterenol-induced cell injury of the myocardium have been studied using different experimental conditions, e.g. isoproterenol dose, type of isoproterenol ((-)-isoproterenol or (+/-)-isoproterenol), administration schedule, and type of heart preparation (homogenates, mitochondria or submitochondrial particles). Most of these studies have been short-term experiments, hindering an evaluation of the temporal behavior of these mitochondrial parameters during isoproterenol treatment. An experimental long-term model (distinguishing the main stages of cardiotoxicity: preinfarction, infarction, and postinfarction) of isoproterenol-induced myocardial cell damage in rats has been reported.[9] In addition, kinetic parameters of hydrolytic activity of mitochondrial ATPase measured in isolated mitochondria or submitochondrial particles from isoproterenol-ischemic cardiac tissues could potentially display alterations that are due to experimental artifacts rather than to physiological alterations taking place *"in vivo"* during isoproterenol treatment.[12] For these reasons, in the present investigation, synthetic and hydrolytic activities of mitochondrial ATPase were assayed during a long-term study (72 h) using rat heart homogenates.

Materials and methods

Preparation of cardiac homogenates

Female Sprague/Dawley rats weighing 150-160 g were killed by cervical dislocation, their hearts were rapidly excised, placed in cold sucrose (0.29 M) during three minutes, squeezed with a manual press (0.8 mm hole-diameter), and gently homogenized with 12 strokes using a Potter homogenizer, in 3.5 ml homogenization medium/1 g heart (0.17 M KCl, 10 mM EDTA, 0.1% bovine serum albumin (BSA), and 10 mM Tris-HCl, pH 7.45; HCl or KOH). Cardiac homogenates from (+/-)-isoproterenol-treated rats were prepared as described above using rats killed 3, 6, 12, 18, 21, 48, and 72 h after receiving a subcutaneous injection of (+/-)-isoproterenol-HCl (67 mg/kg body weight).

Determination of hydrolytic and synthetic activities of mitochondrial ATPase

The hydrolytic activity of mitochondrial ATPase was assayed in the (Ca^{2+}, Na^+, Mg^{2+})-free buffer, 100 mM sucrose, 75 mM KCl, and 3 mM Tris-HCl (pH 7.5), according to.[12] The synthetic activity of mitochondrial ATPase was estimated using a potentiometric method,[16] with some modifications. Briefly, inorganic phosphate $(H_2PO_4^-$ or $P_i)$, which disappeared from the assay medium due to the synthesis of ATP by mitochondrial ATPase, was calculated from the change in pH (DpH), according to the reactions: $ADP^{3-} + H_2PO_4^- \circledR ATP^{4-}$; $H_2PO_4^- \ll HPO_4^{2-} + H^+$, and following calibration of the system with KH_2PO_4. The assay buffer for the determination of the synthetic activity of mitochondrial ATPase was 250 mM sucrose, 5 mM KH_2PO_4, 2 mM Tris-HCl, and 0.1 mM EGTA, pH 7.4 (HCl or KOH), at 37°C. Incubations were carried out in thermostated glass vessels with magnetic stirring. The rate of pH change was measured at 37ºC using a pH-meter connected to a computer providing an online display of rate values. Velocities of ATP synthesis were obtained in nmol P_i/mg of protein per minute. Optimal oligomycin concentration was determined in control experiments. To obtain the optimal oligomycin concentration, the rate of ATP synthesis was determined in heart homogenates pre-incubated during 2 minutes with different oligomycin concentrations. The optimal oligomycin concentration was the minimal concentration that abolished further ATP synthesis by mitochondrial ATPase. The final concentration of ethanol, which was added with oligomycin or rotenone, never exceeded 0.23% (v/v). To estimate the synthetic activity of mitochondrial ATPase, rat heart homogenates were incubated during 2 min with 2.0 mM rotenone and 5 mM succinate, and then the velocities of ATP synthesis, after two consecutive additions, were calculated *(Fig. 1)*: (i) with ADP; (ii) with oligomycin. The synthetic activity of mitochondrial ATPase was the velocity of ATP synthesis after ADP addition (the reason for this consideration is given in the results section below). Protein was determined by the biuret method,[17] using bovine serum albumin (BSA) as standard.

All reagents were obtained from Sigma. (+/-)-isoproterenol was a 50/50 racemic mixture of the two isoproterenol stereoisomers (data from Sigma's technical service). Reagents were selected to contain the smallest contamination by Na^+, Mg^{2+} and Ca^{2+}. The reaction mixture contained small amounts of sodium, calcium, and magnesium, which originated from heart homogenates and from some impurities of the reagents. These amounts were determined by atomic absorption spectroscopy and they were 1.1 x 10^{-4}M, 1.6 x 10^{-5} M and 2.0 x 10^{-5} M for Na^+, Mg^{2+} and Ca^{2+}, respectively.
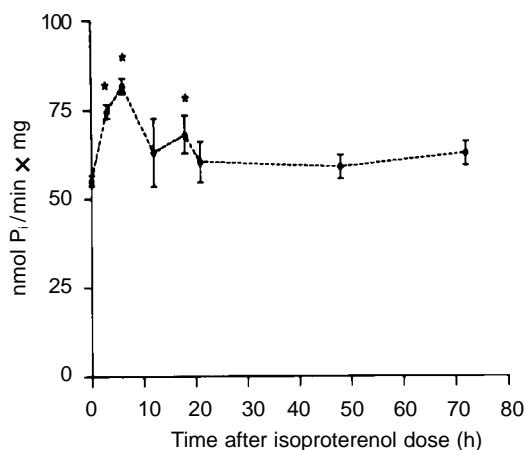
Results

(Fig. 1) shows a typical trace in the determination of synthetic activity of mitochondrial ATPase. After addition of ADP, heart mitochondrial ATPase, which is located on the inner mitochondrial membrane, begins to synthetize ATP. Before ADP and after oligomycin there is no change in buffer pH, which means that, in our assay conditions, the change in pH after ADP addition is caused only by mitochondrial ATP synthesis: the synthetic activity of mitochondrial ATPase is the velocity after ADP addition.

(Fig. 2) shows the synthetic activity of mitochondrial ATPase during (+/-)-isoproterenol-induced cell injury of the myocardium, measured in heart homogenates and during a long-term study (72 h). The synthetic activity of mitochondrial ATPase increased at 3, 6, and 18 h (35, 48, and 23%, respectively) after (+/-)-isoproterenol administration with regard to the control. At 12 h and 21-72 h after drug administration, data reveal no difference between synthetic activity of mitochondrial ATPase in controls *vs* (+/-)-isoproterenol treated homogenates.

(Fig. 3) shows the hydrolytic activity of mitochondrial ATPase during (+/-)-isoproterenol-induced cell injury of the myocardium, measured
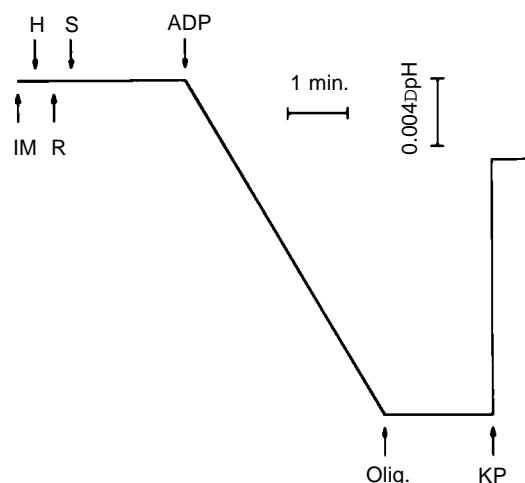
Fig. 1. A typical trace in the determination of the synthetic activity of mitochondrial ATPase (mitochondrial oxidative phosphorylation) by a potentiometric method. Adenosine 5' -diphosphate (ADP, 250 μM), rat heart homogenates (H, 1 mg/3.5 ml incubation medium), incubation, medium (IM, 3.5 ml), KH_2PO_4 (KP, 200 nmol/3.5 ml incubation medium), oligomycin (OLIG, 0.8 μg/mg protein), rotenone (R, 2.0 μM), and succinate (S, 5 mM) were added where indicated. Rat heart homogenates were incubated during 2 minutes, with 2.0 μM rotenone and 5 mM succinate, before adding ADP. KH_2PO_4 was added as trace calibration.

Fig. 2. Synthetic activity of mitochondrial ATPase (mitochondrial oxidative phosphorylation) during (+/-)-isoproterenol treatment (67 mg/kg body weight). The control is when the time after isoproterenol dose is 0 h. The results are given in nmol P_i/mg of protein per minute. All data are averages $\pm$ SEM (n = 5; excepting 3, 48, and 72 h after treatment, in which n = 4). *Significant statistical difference against the control group (P < 0.05).

in heart homogenates and during a long-term study (72 h). The hydrolytic activities of mitochondrial ATPase in homogenates from (+/-)-isoproterenol-treated rats, during the whole long-term study (72 h), were not sta-

tistically different from the activity in homogenates from normal rats. This observation indicates that during (+/-)-isoproterenol treatment (67 mg/kg body weight) no important physiological alterations affecting hydrolytic activity take place in mitochondrial ATPase.

Discussion

It is important to note that the different experimental conditions used in studies on molecular-cellular mechanisms of isoproterenol-induced cell injury of the miyocardium, related to type of animals, isoproterenol dose, type of isoproterenol ((-)-isoproterenol or (+/-)-isoproterenol), administration schedule, and type of heart preparation (homogenates, mitochondria or submitochondrial particles) have made it difficult to integrate the whole body of valuable information that has been obtained in different investigations. To this regard, in the present investigation, a long-term study (72 h) was performed, similar to that reported by Chagoya de Sanchez and co-workers,[9] in which it is possible to evaluate the time course of the isoproterenol-induced pathology. In addition, cardiac homogenates were used since mitochondria or submitochondrial particles isolated from isoproterenol-ischemic tissues may undergo additional alterations.[12]

In the present investigation, Mg^{2+} was not used in the assay buffer to determine hydrolytic and synthetic activities of mitochondrial ATPase (see Materials and Methods). Experimental conditions without magnesium ions represent more physiological conditions for assaying the activity of this enzyme. Normal total magnesium content in heart mitochondria is very high,[18] and a part of this magnesium is tightly bound to mitochondrial ATPase.[19]

Data from *(Fig. 2)* show that synthetic activities in homogenates from (+/-)-isoproterenol-treated rats are never lower than the synthetic activity in normal homogenates, indicating that probably no considerable and "negative" modifications in the active configuration of the mitochondrial ATPase take place during (+/-)-isoproterenol-induced cell injury of the myocardium (67 mg/kg body weight). Additionally, these data display an increase in synthetic activity at 3, 6, and 18 h (35, 48, and 23%, respectively) after (+/-)-isoproterenol treatment. If mitochondria do not suffer considerable and "negative" functional modifications during the above mentioned drug
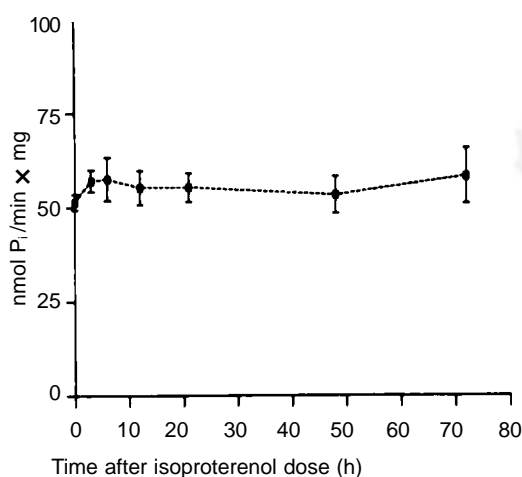
Fig. 3. Hydrolytic activity of mitochondrial ATPase during (+/-)-isoproterenol treatment (67 mg/kg body weight). The control is when the time after isoproterenol dose is 0 h. The results are given in nmol P_i/mg of protein per minute. All data are averages ± SEM (n = 5; excepting 48 and 72 h after treatment, in which n = 4).

administration, the activation of mitochondrial oxidative phosphorylation at some times after (+/-)-isoproterenol treatment is expected. This interpretation is based on the fact that the effects of isoproterenol have been classically associated to an excessive energy demand caused by a large increase in cardiac work,[20] and the myocardium has the ability to balance the rate of energy conversion (i.e., ATP production by mitochondria) and work (i.e., controlled ATP hydrolysis by myosin adenosinetriphosphatase) along a wide range of cardiac work loads.[21] It is important to note that in the long-term study (96 h) of Chagoya de Sanchez and co-workers,[9] a decrease, with respect to the control, was observed in the synthetic activity of mitochondrial ATPase at 12-96 h after (-)-isoproterenol treatment. Additionally, in that study,[9] activation of mitochondrial oxidative phosphorylation was not observed at any time after the above mentioned drug administration. It is possible that the type of isoproterenol (67 mg (-)-isoproterenol/kg body weight) used by these investigators injured the myocardium, at such a degree that mitochondria suffered considerable and "negative" functional modifications and/or mitochondria from isoproterenol-ischemic tissues could have been altered during isolation procedures.[12]

Results shown in *(Fig. 3)* clearly evidence that (+/-)-isoproterenol treatment (67 mg/kg body weight) does not alter the hydrolytic activity during

the whole long-term study (72 h). Taken together, these results *(Figs. 2 and 3)* indicate that probably no considerable and "negative" alterations of mitochondrial ATPase occur during (+/-)-isoproterenol-induced cell injury of the myocardium (67 mg/kg body weight). Hence, it is possible that the observed alterations in kinetic parameters of hydrolytic activity of isoproterenol-treated mitochondrial ATPase reported in other studies[10,11] might be interpreted as experimental artifacts. The main reason for this interpretation is the fact that isoproterenol-treatment induces ischemic alterations in the myocardium[6,22,23] and mitochondria from ischemic tissues could be altered during isolation procedures.[24] To this regard, kinetic parameters of mitochondrial ATPase measured in isolated mitochondria or sub-mitochondrial particles from isoproterenol-treated tissues could potentially display alterations that are due to experimental artifacts rather than to physiological alterations that take place *"in vivo"* during isoproterenol treatment. Our results *(Fig. 3)* show that during the whole long-term study (72 h) the hydrolytic activities of mitochondrial ATPase were identical, within statistical limits, in the presence and absence of (+/-)-isoproterenol treatment of the myocardium. These results do not mean that (+/-)-isoproterenol-activation of heart mitochondrial oxidative phosphorylation could not take place at the level of mitochondrial ATPase. This interpretation is based on the fact that the sites for ATP hydrolysis and synthesis, in mitochondrial ATPase, and their mechanisms are known to be distinct.[15]

Finally, it is important to say something about the possible cytosolic "transducer", which activates mitochondrial oxidative phosphorylation during (+/-)-isoproterenol treatment. The property of cardiac ATP hydrolysis/synthesis rates, which can change several fold without a change in cytosolic [ADP] or $[P_i]$,[25] suggests that the cytosolic network controlling work and ATP hydrolysis contains elements that modulate mitochondrial ATP production in parallel.[26] An example of a single cytosolic transducer that could activate both work and biochemical energy conversion in the heart is Ca^{2+}. Calcium is intimately related to the contractile processes ocurring in the myocardium,[27-29] and there are evidences suggesting that the intracellular calcium concentration could also influence the supply of energy, ATP, by modulating several mitochondrial enzymes.[30,31] It is possible that Ca^{2+}

activates mitochondrial oxidative phosphorylation during isoproterenol-induced cell injury of the myocardium since an increase in intracellular calcium[30] and mitochondrial free calcium transients have been observed (with the isoproterenol-increase in work) during excitation-contraction coupling (potentiated by isoproterenol) in cardiac myocytes.[32]

Conclusions

The hydrolytic activities of mitochondrial ATPase, during the whole long-term study (72 h), in homogenates from (+/-)-isoproterenol-treated rats were statisitically equal to the hydrolytic activity in normal homogenates. The synthetic activities (mitochondrial oxidative phosphorylation) in homogenates from (+/-)-isoproterenol-treated rats were never lower than the synthetic activity in normal homogenates. Additionally, the synthetic activity of mitochondrial ATPase increased at 3, 6, and 18 h (35, 48, and 23%, respectively) after (+/-)-isoproterenol administration with regard to the control. Taken together, these results indicate that probably no considerable and "negative" modifications in the active configuration of the mitochondrial ATPase take place during (+/-)-isoproterenol-induced injury of the myocardium (67 mg/kg body weight). The fact that during the whole long-term study (72 h) the hydrolytic activities of mitochondrial ATPase were identical, within statistical limits, in the presence/absence of (+/-)-isoproterenol treatment does not mean that (+/-)- isoproterenol-activation of heart mitochondrial oxidative phosphorylation could not take place at the level of mitochondrial ATPase, because the sites for ATP synthesis and hydrolysis, in the mitochondrial ATPase, and their mechanisms are known to be distinct.

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