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Modulation of the c-Jun N-terminal kinase activity in the embryonic heart in response to anoxia-reoxygenation: involvement of the Ca^{2+} and mitoK_{ATP} channels

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Abstract Whether the response of the fetal heart to ischemia-reperfusion is associated with activation of the c-Jun N-terminal kinase (JNK) pathway is not known. In contrast, involvement of the sarcolemmal L-type Ca^{2+} channel (LCC) and the mitochondrial K_{ATP} (mitoK_{ATP}) channel has been established. This work aimed at investigating the profile of JNK activity during anoxia-reoxygenation and its modulation by LCC and mitoK_{ATP} channel. Hearts isolated from 4-day-old chick embryos were submitted to anoxia (30 min) and reoxygenation (60 min). Using the kinase assay method, the profile of JNK activity in the ventricle was determined every 10 min throughout anoxia-reoxygenation. Effects on JNK activity of the LCC blocker verapamil (10 nM), the mitoK_{ATP} channel opener diazoxide (50 μM) and the blocker 5-hydroxydecanoate (5-HD, 500 μM), the mitochondrial Ca^{2+} uniporter (MCU) inhibitor Ru360 (10 μM), and the antioxidant N-(2-mercaptopropionyl) glycine (MPG, 1 mM) were determined. In untreated hearts, JNK activity was increased by 40% during anoxia and peaked fivefold relative to basal level after 30–40 min reoxygenation. This peak value was reduced by half by diazoxide and was tripled by 5-HD. Furthermore, the 5-HD-mediated stimulation of JNK activity during reoxygenation was abolished by diazoxide, verapamil or Ru360. MPG had no effect on JNK activity, whatever the conditions. None of the tested pharmacological agents altered JNK activity under basal normoxic conditions. Thus, in the embryonic heart, JNK activity exhibits a characteristic pattern during anoxia and reoxygenation and the respective open-state of LCC, MCU and mitoK_{ATP} channel can be a major determinant of JNK activity in a ROS-independent manner.

Keywords JNK · mitoK_{ATP} channel · Calcium channel · Anoxia-reoxygenation · Embryo

Introduction

The signaling pathways involved in the response of the fetal heart to inadequate oxygenation, resulting from transient maternal hypoxemia, reduction in uterine or umbilical blood flow, remain to be explored. We have previously characterized in detail the electrical and contractile disturbances induced by anoxia and reoxygenation in the embryonic heart model [1, 2] and found that a moderate inhibition of the sarcolemmal L-type calcium channel (LCC) [3] or activation of the mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel can improve postanoxic recovery [4]. Opening of the mitoK_{ATP} channel is also involved in ischemic preconditioning of isolated embryonic ventricular myocytes [5] and adult heart [6].

Several studies have underlined the key role played by the mitogen-activated protein kinases (MAPKs) pathways [7] in myocardial ischemia and reperfusion, particularly the stress-activated c-Jun N-terminal kinase (JNK) [8, 9]. In

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the ventricle of the anoxic-reoxygenated embryonic chick heart, activity of the extracellular signal-regulated kinase (ERK) is not significantly altered and the profile of the p38 MAPK phosphorylation is not affected by opening of the mitoK$_{ATP}$ channel [10]. However, information regarding the JNK signaling pathway in the hypoxic fetal heart is lacking and a better understanding is especially required in the context of recent advances in developmental cardiology [11], fetal cardiac surgery [12] and research dealing with intrauterine programming [13]. Furthermore, as Ca^{2+} is one of the second messengers capable of modulating JNK activity [14], the cytosolic and mitochondrial Ca^{2+} overload induced by anoxia-reoxygenation in embryonic cardiomyocytes [15] could interfere with the JNK signaling pathway.

The aim of this work was to establish the profile of JNK activity in the ventricle of the embryonic heart during anoxia and reoxygenation and to investigate the possible link between JNK activity and the state of activation of the Ca^{2+} and mitoK$_{ATP}$ channels. The results suggest that LCC and mitochondrial Ca^{2+} and K$_{ATP}$ channels are involved in the modulation of JNK activity in the embryonic ventricle submitted to anoxia-reoxygenation.

Materials and methods

Reagents

Dimethylsulfoxide (DMSO), mitoK$_{ATP}$ channel opener diazoxide and blocker 5-hydroxydecanoate (5-HD), radical scavenger N-(2-mercaptopropionyl)glycine (MPG), were purchased from Sigma (Sigma–Aldrich, Buchs, Switzerland). L-Type Ca^{2+} channel inhibitor verapamil (Isoptin®) was from Abbott and mitochondrial Ca^{2+} uniporter (MCU) inhibitor Ru360 was purchased from Calbiochem (JURO Supply, Lucerne, Switzerland).

[γ-33P]ATP was from Amersham Biosciences and inhibitors of proteases from Roche Biosciences.

Preparation and in vitro mounting of the heart

Fertilized eggs from Lohman Brown hens were incubated during 96 h at 38°C and 95% relative humidity to obtain stage 24 HH embryo (according to Hamburger and Hamilton [16]). Spontaneously beating hearts were carefully excised and placed in the culture compartment of an airtight stainless steel chamber. The chamber was equipped with two windows for observation and maintained under controlled conditions on the thermostabilized stage (37.5°C) of an inverted microscope (IMT2 Olympus, Tokyo, Japan) as previously detailed [4]. Briefly, the culture compartment (300 µl) was separated from the gas compartment by a 15 µm transparent and gas-permeable silicone membrane (RTV 141, Rhône-Poulenc, Lyon, France). Thus, pO_2 at the tissue level could be strictly controlled and rapidly modified (within less than 5 s) by flushing high-grade gas of selected composition through the gas compartment. At this developmental stage, the heart lacks vascularization and the myocardial oxygen requirement is met exclusively by diffusion.

The standard HCO_3/CO_2 buffered medium was composed of (in mmol/l): 99.25 NaCl; 0.3 NaH_2PO_4; 10 $NaHCO_3$; 4 KCl; 0.79 $MgCl_2$; 0.75 $CaCl_2$; 8 D+glucose. This culture medium was equilibrated in the chamber with 2.31% CO_2 in air (normoxia and reoxygenation) or in N2 (anoxia) yielding a pH of 7.4. All reagents were diluted in this medium containing 0.5% DMSO (vehicle).

Anoxia-reoxygenation protocol

After 45 min of in vitro stabilization under normoxia at 37.5°C (stab), the hearts were submitted to strict anoxia during 30 min and then reoxygenated during 60 min. The pharmacological agents were present throughout anoxia-reoxygenation. The hearts were collected every 10 min and the ventricle was carefully dissected on ice and stored at −80°C for subsequent determinations. As control, in a separate set of experiments, hearts were maintained under steady normoxia for 60 and 90 min after stabilization, corresponding to the time points of 30 and 60 min of postanoxic reoxygenation, respectively. Ventricles of these untreated hearts were also dissected and stored at −80°C.

Kinase assay

Ventricular JNK activity was determined using a published method [17] with minor modifications. Ventricles were homogenized in ice-cold lysis buffer (in mmol/l: 20 Tris-acetate (pH 7), 270 sucrose, 1 EGTA, 1 EDTA, 50 NaF, 10 β-glycerophosphate, 10 dithiothreitol (DTT), 10 4-nitrophenyl phosphate disodium salt hexahydrate (PNPP), 1% Triton X-100 and inhibitors of proteases). Insoluble material was removed by a 5 min centrifugation at 10,000g and protein contents were measured by the method of Lowry [18] with bovine serum albumin as standard.

Soluble ventricular protein extract (30 µg) were incubated for 3 h at 4°C in the presence of 1 µg GST-c-Jun$_{(1–219)}$ bound to gluthatione-agarose beads as both JNK-specific ligand and substrate. The beads were washed three times in washing buffer (same as lysis buffer but with 0.1% Triton X-100) and twice in kinase buffer (in mmol/l, 20 HEPES pH 7.5, 10 $MgCl_2$, 20 β-glycerophosphate, 10 DTT, 10 PNPP and inhibitors of proteases). Kinase reaction was carried out for 30 min at 30°C in 20 µl of kinase buffer containing 5 µCi

[γ-33P]ATP. Reaction products were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), gels were dried, and phosphorylation signals were visualized by autoradiography, quantified by PhosphoImager (Quantity-one 1.4.0, Biorad) and expressed as fold increase relative to the respective preanoxic value (stab).

Statistical analysis

All values are reported as mean ± standard error of the mean (S.E.). The significance of any difference between the groups was assessed using Student *t*-test. The statistical significance was defined by a value of $P < 0.05$.

Results

Profile of JNK activity during anoxia-reoxygenation

In control conditions (vehicle), JNK activity increased by 40% after 10–20 min anoxia ($P < 0.05$). During reoxygenation, JNK activity progressively increased, peaking after 30–40 min and further declined (Fig. 1). This culmination of JNK activity was specifically related to reoxygenation, since it was not attributable to the conditions and the duration (135 min) of culture in the chamber (Fig. 2).

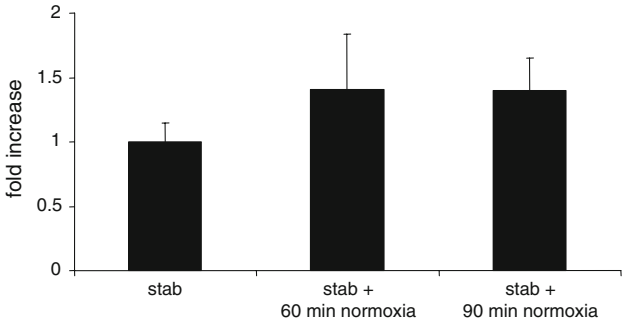

Fig. 2 Stability of JNK activity under normoxia. In vitro culture under steady normoxia did not affect ventricular JNK activity assessed by kinase assay. Fold increase: JNK activity is given relative to the mean of stab values. Stab: 45 min preanoxic stabilization; stab + 60 min and stab + 90 min correspond to 30 and 60 min of reoxygenation in the anoxia-reoxygenation protocol, respectively (see Fig. 1). Mean ± S.E.; $n = 6$–8 determinations of JNK activity

Modulation of JNK activity by the opening state of the mitoK$_{ATP}$ channel

The mitoK$_{ATP}$ channel opener diazoxide (50 µmol/l) decreased JNK activity after 30 min anoxia and throughout reoxygenation (Fig. 1). As the diazoxide-induced inhibition was the strongest at the peak of JNK activity ($P < 0.02$), we selected this time point to pharmacologically explore the possible mechanisms linking the open state of the mitoK$_{ATP}$ channel with the JNK activity. Under preanoxic conditions, however, none of

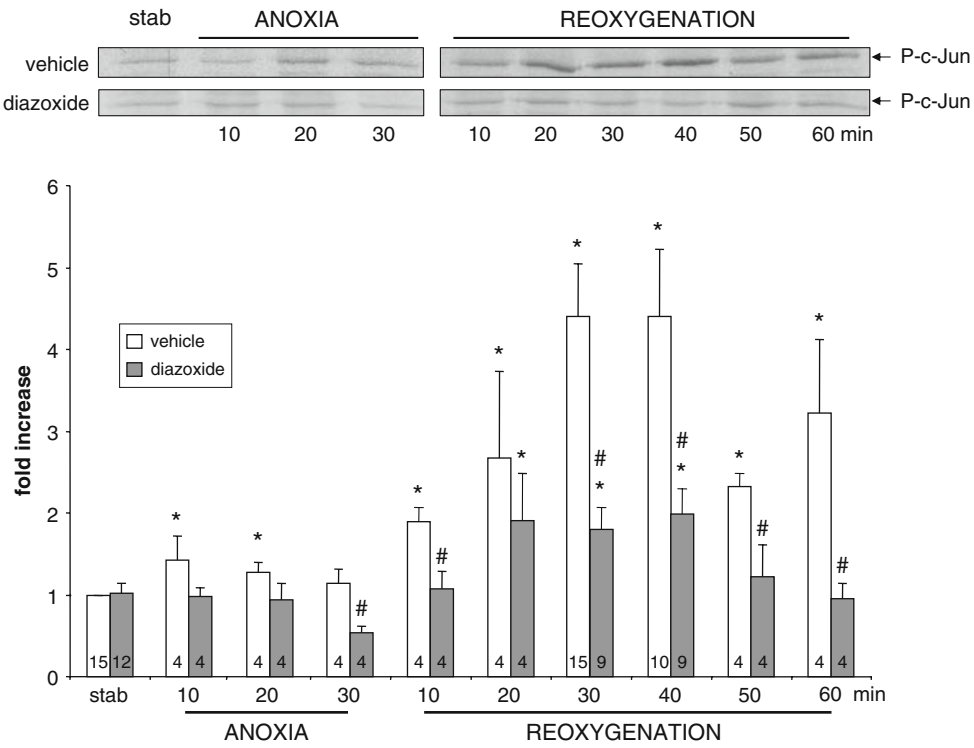

Fig. 1 Profile of JNK activity in the embryonic ventricle during anoxia-reoxygenation. Activation of the mitoK$_{ATP}$ channel by diazoxide decreased JNK activity. Upper panels show representative autoradiogram of JNK activity during anoxia and reoxygenation. Fold increase: JNK activity is given relative to the preanoxic stab value of vehicle. Stab: 45 min preanoxic stabilization; mean ± S.E. of number of determinations indicated in columns; $^{*}P < 0.05$ vs. stab; $^{\#}P < 0.05$ vs. vehicle

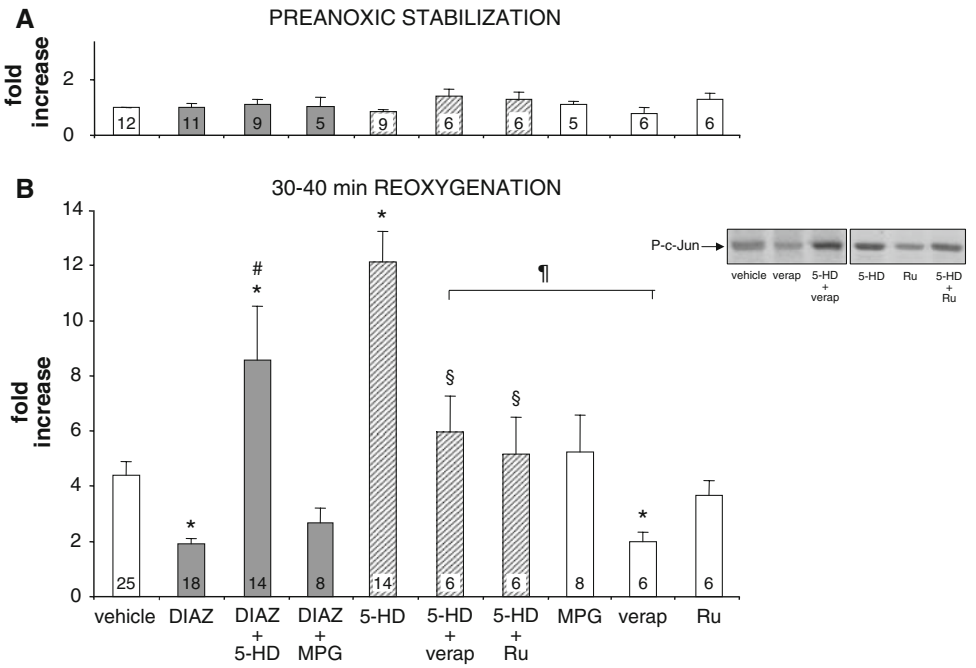

Fig. 3 Pharmacological modulation of JNK activity in the embryonic ventricle during preanoxic stabilization (**a**) and after 30–40 min reoxygenation (**b**). JNK activity was dependent on the open-state of the mitoK$_{ATP}$ channel through Ca^{2+}-dependent mechanisms during reoxygenation exclusively. In panel (**a**), JNK activity is given relative to the value of vehicle. In panel (**b**), JNK activity is given relative to the respective preanoxic stabilization value shown in panel (**a**). Insets show representative autoradiograms of JNK activity during reoxygenation in relation to Ca^{2+} handling. DIAZ: diazoxide; verap: verapamil; Ru: Ru360; Mean ± S.E. of number of determinations indicated in columns; $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. DIAZ; $^{\S}P < 0.05$ vs. 5-HD; $^{\P}P < 0.05$ 5-HD + verap vs. verap

the reagents, alone or in combination, affected JNK activity relative to vehicle (Fig. 3a) or disturbed the regular contractile activity of the isolated hearts (not shown). After 30–40 min reoxygenation, inhibition of JNK activity by diazoxide was suppressed by the mitoK$_{ATP}$ channel blocker 5-HD (500 µmol/l) and, importantly, 5-HD alone tripled JNK activity with respect to vehicle ($P < 0.01$) (Fig. 3b). The radical scavenger MPG (1 mmol/l), known otherwise to abolish the diazoxide-induced ROS production and cardioprotection at reoxygenation [4], affected neither JNK activity nor the diazoxide-induced JNK inhibition.

Modulation of JNK activity by sarcolemmal (LCC) and mitochondrial (MCU) Ca^{2+} channels

Relative to vehicle, LCC inhibitor verapamil (10 nmol/l) decreased JNK activity by 55%, whereas the mitochondrial Ca^{2+} uniporter inhibitor Ru360 (10 µmol/l) had no significant effect (Fig. 3b). However, the 5-HD-mediated JNK activity during reoxygenation was abolished by verapamil and also by Ru360. These observations indicate that Ca^{2+} entry is a prerequisite for JNK stimulation and that MCU is involved in JNK activation induced by the mitoK$_{ATP}$ channel blocker 5-HD.

Discussion

To the best of our knowledge, this is the first time that JNK activity is explored in the embryonic myocardium submitted to an anoxic episode. Our main findings indicate that JNK activity in the ventricle of the isolated embryonic heart (1) is increased by anoxia and reoxygenation, (2) is modulated by the open-state of the mitoK$_{ATP}$ channel, and (3) is dependent on Ca^{2+} flux through both LCC and MCU.

In the adult heart the effects of ischemia on JNK activation remain controversial whereas all studies show an enhanced JNK activity during reperfusion [8, 9, 19, 20]. Our data indicate that JNK pathway contributes to the short-term response of the heart to oxygen lack and reintroduction also during early embryogenesis, although the metabolic consequences of anoxia-reoxygenation differ to a certain extent from those of ischemia-reperfusion.

Although 5-HD has been shown to abolish ROS production induced by the mitoK$_{ATP}$ channel opener diazoxide during reoxygenation [4], it markedly increased JNK activity (Fig. 3b). Furthermore, the membrane permeable antioxidant MPG which significantly reduces ROS production at reoxygenation [4] did not suppress JNK activation. Taken together, these observations clearly indicate that endogenous ROS are not prerequisite for JNK

activation in the embryonic myocardium by contrast with neonatal cardiomyocytes [21] and adult heart [8]. Such a developmental discrepancy implies that JNK pathway can be modulated differently in prenatal and postnatal myocardium since, for example, the physiological oxygen level, the oxidative metabolism and the capacity to generate ROS are lower in embryonic tissues [22–24]. The facts that diazoxide improves recovery of atrio-ventricular conduction and E-C coupling during the first 20 min of reoxygenation [4], and reduces JNK activity from the end of anoxia onward (Fig. 1), suggest that this protection might be indirectly related to a reduction of JNK activity.

It has been shown that after 30 min reoxygenation, that is, when JNK activity reached its highest value (Fig. 1), Ca^{2+} uptake is maximally increased in embryonic ventricular cells [15] and contractility of the ventricle is transiently above its basal level [25], reflecting a rise of intracellular Ca^{2+}. The present data support the concept that extracellular Ca^{2+} entry through LCC is a prerequisite for JNK activation in the reoxygenated embryonic myocardium (Fig. 3b) alike in neonatal [21] and adult cardiomyocytes [8].

Subcellular fractionation studies have shown that JNK can also be localized within or associated with mitochondrial structures [26, 27]. The facts that mitochondria are capable of taking up some of the cytosolic Ca^{2+} through the MCU in case of Ca^{2+} overload [28, 29] and that $mitoK_{ATP}$ channel opening can decrease the mitochondrial inner membrane potential (Ψm) and Ca^{2+} content in the matrix during hypoxia [30] could partly explain that MCU inhibition suppressed the 5-HD-induced JNK activation. Our results, indeed, show that blocking moderately Ca^{2+} entry through sarcolemmal LCC or blocking Ca^{2+} flux into mitochondrion through MCU reduces JNK activity during reoxygenation, especially when $mitoK_{ATP}$ channel are closed by 5-HD.

Although Ψm and mitochondrial Ca^{2+} concentration have not been measured because of the thickness of the tissue and interferences due to contractions, our findings support the hypothesis that during reoxygenation Ca^{2+} entry through LCC and/or influx through MCU can activate cytosolic JNK and/or JNK associated with mitochondria (see model proposed in Fig. 4).

When $mitoK_{ATP}$ channels are opened by diazoxide, Ψm is known to drop [30, 31], thereby limiting mitochondrial Ca^{2+} entry through MCU and consequently Ca^{2+} overload, reducing JNK activation. Conversely, when $mitoK_{ATP}$ channels are blocked by 5-HD, Ψm should be maintained and act as a driving force for Ca^{2+} transport through MCU, which also could contribute to activate JNK.

Thus, in the embryonic heart, JNK activity exhibits a characteristic pattern during anoxia and reoxygenation and the respective open-state of LCC, MCU and $mitoK_{ATP}$ channels can be a major determinant of JNK activity in a ROS-independent manner. This work provides a first step in understanding the regulation of the JNK signaling pathway in the fetal heart transiently exposed to hypoxia. In particular, the cellular targets as well as the long-term functional consequences of an acute activation of this pathway during cardiogenesis deserve further investigations.

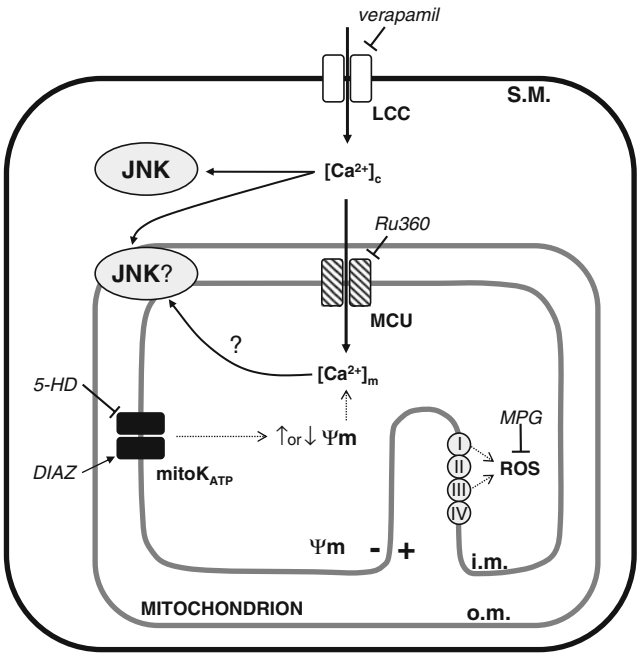

Fig. 4 Schematic representation based on the present findings and illustrating the possible modulation of JNK activity by open-state of L-type Ca^{2+} channel (LCC), mitochondrial Ca^{2+} uniporter (MCU) and $mitoK_{ATP}$ channel during reoxygenation of the embryonic ventricle. Inactivation of LCC (verapamil) decreased the reoxygenation-induced peak of JNK activity. Inhibition of MCU (Ru360) or LCC suppressed the 5-HD-induced JNK activity (see Fig. 3). Opening (DIAZ) and blockade (5-HD) of the $mitoK_{ATP}$ channel could respectively decrease and increase Ψm, $[Ca^{2+}]m$ and JNK activity. Intracellular antioxidant (MPG) had no effect on JNK activity. S.M.: sarcolemmal membrane; i.m. and o.m.: inner and outer mitochondrial membrane; Ψm: i.m. potential; $[Ca^{2+}]c$ and $[Ca^{2+}]m$: cytosolic and mitochondrial Ca^{2+} concentrations; MCU: mitochondrial Ca^{2+} uniporter; I–IV: respiratory chain complexes; ROS: reactive oxygen species

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