

Neonatal rat hearts cannot be protected by ischemic postconditioning

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Short title: Neonatal rat hearts cannot be protected by postconditioning

Summary

Although there are abundant data on ischemic postconditioning (IPoC) in the adult myocardium, this phenomenon has not yet been investigated in neonatal hearts. To examine possible protective effects of IPoC, rat hearts isolated on days 1, 4, 7 and 10 of postnatal life were perfused according to Langendorff. Developed force (DF) of contraction was measured by an isometric force transducer. Hearts were exposed to 40 or 60 min of global ischemia followed by reperfusion up to the maximum recovery of DF. IPoC was induced by three cycles of 10, 30 or 60 second periods of global ischemia/reperfusion. To further determine the extent of ischemic injury, lactate dehydrogenase (LDH) release was measured in the coronary effluent. Tolerance to ischemia did not change from day 1 to day 4 but decreased to days 7 and 10. None of the postconditioning protocols tested led to significant protection on the day 10. Prolonging the period of sustained ischemia to 60 min on day 10 did not lead to better protection. The 3x30s protocol was then evaluated on days 1, 4 and 7 without any significant effects. There were no significant differences in LDH release between postconditioned and

control groups. It can be concluded that neonatal hearts cannot be protected by ischemic postconditioning during first 10 days of postnatal life.

Key words

Neonatal rats, ischemic postconditioning, tolerance to ischemia, contractile function, lactate dehydrogenase

Introduction

Myocardial ischemia undoubtedly belongs to the most frequent (and hence the most widely studied) cardiovascular diseases of modern times. Ischemia originates as the disproportion between the amount of oxygen supplied to the cardiac cell and the amount actually required by the cell. The extent of ischemic injury depends, however, not only on the intensity and duration of ischemia but also on the cardiac tolerance to oxygen deprivation. Cardiac sensitivity to hypoxia changes significantly during ontogenetic development: tolerance of the immature heart to acute oxygen deprivation is significantly higher as compared with the adult myocardium (Riva and Hearse 1993, Ošťádalová *et al.* 1998). Thus, the question arises whether we can further increase the already high resistance of the immature mammalian heart. To the most effective experimental protective mechanisms belong long-lasting adaptation to chronic hypoxia (for a review see Ošťádal and Kolář 2007) and brief adaptation, so called ischemic preconditioning (for a review see Bolli 2007). Whereas abundant data are available on the two phenomena in adults, the information on the immature heart are only sporadic. We have shown previously (Ošťádalová *et al.* 1998) that classical ischemic preconditioning, at least in rats, is absent at birth and the enhanced postischemic recovery of contractile function can be observed only at the end of the first postnatal week; similar results were obtained after adaptation to chronic hypoxia (Ošťádalová *et al.* 2002).

The protective effect of another phenomenon - ischemic postconditioning (Zhao *et al.* 2003) - is comparable with ischemic preconditioning and was observed in many species, including humans (Laskey 2005). Data on the possible protective effect of postconditioning on the immature heart are still lacking (for a review see Ošťádal *et al.* 2009a). The aim of our study was, therefore, to evaluate the effect of ischemic postconditioning in rats during the first ten days of postnatal development. Since different protocols of ischemic postconditioning were described for adult animals (for a review see Skyschally *et al.* 2009), several of them

were preliminary tested in 10-day-old animals and then employed on the 1st, 4th and the 7th day of postnatal life.

Methods

All the investigations conform to the European Community and NIH guidelines for using experimental animals (National Academy Press, 1996). All procedures were approved by Animal Studies Committee of the 2nd Faculty of Medicine, Charles University in Prague.

Animal model

A total of 113 neonatal Wistar rats at the ages of 1, 4, 7 and 10 days of both sexes were used throughout the experiments. Experimental and control groups were composed from at least three different litters. All mothers had free access to water and a standard laboratory diet *ad libitum*.

Heart function

The animals were weighted and killed by cervical dislocation. The chest was quickly opened and stainless steel cannula (with an external diameter of 0.45 mm for day 1 and 4 or 0.8 mm for day 7 and 10) was inserted into the aorta. The heart was rapidly excised, the atria were removed and were perfused in the Langendorff mode under constant pressure corresponding to the mean arterial blood pressure for the given developmental stage (Litchfield 1958; Zicha *et al.* 1986) i.e. 25, 42, 57 and 73 cm H₂O on d 1, 4, 7 and 10 respectively. The hearts were perfused with a Krebs-Henseleit solution containing (in mmol/l): NaCl 118.0; KCl 4.7; CaCl₂ 1.25; MgSO₄ 1.2; NaHCO₃ 25.0; KH₂PO₄ 1.2; glucose 7.0 and mannitol 1.1. The solution was saturated by a mixture of 95% O₂ and 5% of CO₂ (pH 7.4) and temperature was maintained at 37°C. The hearts were electrically stimulated at a rate of 200 beats/min using silver electrodes attached to the base of the heart. The stimulation was performed with pulses of alternating polarity, 1ms duration and voltage set at 50% above the threshold level. The resting force was gradually increased by means of micromanipulator to the level at which the developed force (DF) was approximately 80% of the maximum force reached at the optimum preload. The contractile function of this isolated heart was measured using an isometric force transducer connected by a glass fiber, two-arm titanium lever and silk suture (0.7 metric) to the apex of the heart. The DF (g) and +df/dt max (g/min) were evaluated automatically from the force signal using an online computer according to Ošťádalová *et al.* (1998).

Experimental protocol

After a period of stabilization, baseline values of DF were recorded. The hearts from each experimental group were exposed to 40 min of global ischemia, part of hearts was exposed also to prolonged ischemia (60 min). At the beginning of reperfusion, one-half of the hearts were postconditioned by subjecting them to three 30s (10s or 60s respectively) periods of global ischemia, each separated by the period of reperfusion of the same duration (Fig. 1). The remaining hearts were simply reperfused up to the maximum recovery of DF (the last value of DF before its decay). DF was measured in all hearts in 3-min intervals during the reperfusion period. Tolerance to ischemia was expressed as a recovery of DF (percentage of baseline values). After the experiment, the hearts were weighed.

LDH measurement

To further determine the extent of the ischemic injury, the measurement of LDH in the coronary effluent was performed. The samples of effluent were collected at the 1st, 3rd, 6th and 12th min of reperfusion and from the rest of the effluent after the end of reperfusion. The amount of effluent was measured by weighing the test-tube before and after the sample was taken. Samples for LDH were kept at +4°C until the measurement using the method of Mukherjee (2010): 100 µl of the sample was incubated with 150 µl of freshly prepared NAD⁺ solution (3mM) and 100 µl of lactate (0.9 M) in TRIS-HCl buffer (0.1M; pH = 9.0) at 37°C for 15 min, followed by addition of 250 µl of 2,4-dinitrophenylhydrazine (0.1% w/v in 2M HCl). The reaction was stopped by the addition of 2.5 ml NaOH (0.4M) and the absorbance was measured at 505 nm after 20 min. After quantitative estimation of pyruvate, LDH activity was calculated. Calibration was performed with standard pyruvate solution and evaluated by linear regression.

Statistical analysis

The results are expressed as means \pm S.E.M. Each observation of ischemic postconditioning was obtained from at least seven heart preparations in each group. Body weight, heart weight and baseline and recovery of DF values were evaluated by one-way Anova as well as data for the 10 days 40 min ischemia group. Data from other postconditioned groups as well as the results of LDH release were evaluated by unpaired t-tests. The statistical analyses were performed using StatView 5.0 (SAS Institute, Cary, N.C., USA). The results were considered statistically significant when $p < 0.05$.

Results

Tolerance to ischemia and effect of postconditioning

Body and heart weights and baseline contractile parameters are summarized in Table 1. The body and heart weights as well as DF increased during the whole investigated period; there were no differences between control and postconditioned groups. Tolerance to ischemia, expressed as the postischemic recovery of DF, changed significantly during the first ten days of postnatal life. There was no significant difference between 1-day-old and 4-day-old animals; tolerance to ischemia then declined on days 7 and 10 (Fig. 2, gray columns). Three different postconditioning protocols were examined in 10-day-old rats. All three failed to significantly improve the recovery of DF (Fig. 3). The increased duration of ischemia (60 min) and the most powerful protocol of postconditioning found in the previous experiment (3x30s) was applied. However, it completely failed to protect the heart (Fig. 4). The same protocol revealed no protective effect on days 1, 4 and 7 (Fig. 2).

LDH release

Concentrations of LDH in samples expressed significant negative correlation between the total LDH release and recovery of DF (Fig. 5). There was no difference between postconditioned and control animals on days 1, 4, 7 and 10.

Discussion

The major result of this study is the observation that different protocols of ischemic postconditioning were unable to increase ischemic tolerance to oxygen deprivation during the first ten days of postnatal life. The data on LDH release confirm the inefficiency of ischemic postconditioning during early period of ontogenetic development. This finding slightly differs from the above mentioned developmental studies on the protective effect of ischemic preconditioning and adaptation to chronic hypoxia (Ošřádalová *et al.* 1998, 2002), where the first signs of protection were observed already by the end of the first postnatal week. It suggests possible different timing of the development of the protective effect of ischemic postconditioning, repeatedly described in adults (for a review see Ovize *et al.* 2009); we can, therefore, suppose that the protection will occur somewhere between day 10 and adulthood. Unfortunately, our experimental setup of the immature isolated heart does not allow studying the older animals. Nevertheless, this question needs further analysis, particularly as far as the

possible ontogenetic differences in the efficiency of the different protocols of ischemic postconditioning are concerned.

Ischemic postconditioning in the adult hearts is well described phenomenon. It has already been found in various organs other than heart (e.g. brain (Zhao *et al.* 2006)) and has been successfully tested in clinical medicine (Staat *et al.* 2005). Possible mechanisms responsible for its protective effects include mitochondrial permeability transition pore (Argaud *et al.* 2005), mitochondrial K-ATP channels (Yang *et al.* 2004) or various protective kinases such as PI3K-Akt pathway (Tsang *et al.* 2004). To answer the question whether the mechanisms of the protective effect of ischemic postconditioning differs during ontogenetic development remains to be clarified.

Neonatal rat hearts are more resistant to ischemia; the comorbidities that adversely affect the adult heart, such as hypertension (Bešík *et al.* 2007), do not abolish this high neonatal resistance (Charvátová *et al.* 2012). Historically, many mechanisms were suggested to be responsible for high neonatal tolerance to ischemia. It is known that neonatal heart differs from adult heart in metabolic factors such as glycolytic capabilities (Hoerter 1976), amino acid metabolism (Julia *et al.* 1990), calcium metabolism (Boucek *et al.* 1984) or vascularization (Rakušan 1999). Moreover, the involvement of mitochondrial permeability transition pore has been suggested (Milerová *et al.* 2010) similarly as in the mechanisms of cardioprotection. Recently, Liaw *et al.* (2013) demonstrated that neonatal hearts exhibit greater Akt reserves available for phospho-activation compared with mature heart. Downstream a tone of the major effectors, neonatal hearts show the greatest degree of phospho-inhibition of glycogen synthase kinase 3 β . In terms of postnatal changes of myocardial tolerance to oxygen deprivation, these data reveal a complex series of changes in pro-survival and pro-death proteins with maturation.

The unsuccessful attempts to increase cardiac tolerance to oxygen deprivation in neonatal rats suggest that we might be dealing with the more general biological phenomenon: the already high resistance of the cardiac muscle cannot be further increased by another protective mechanism. A similar situation as in the immature mammalian heart can also be observed in the highly tolerant hearts of poikilotherms (Overgaard *et al.* 2004) or in the myocardium of young females (for a review see Ošťádal *et al.* 2009b).

Conflict of Interest

There are no conflicts of interests.

Acknowledgements

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Table 1. Body weight, heart weight, HW/BW ratio, pre-ischemic DF and df/dt max; means \pm S.E.M.

Day	Protocol		n	Body weight (g)	Heart weight (mg)	HW/BW (mg/g)	DF (g)	Df/dt max (g/min)
1	Controls		10	6.73 \pm 0.19	31.97 \pm 1.52	0.211	1.99 \pm 0.11	51.57 \pm 3.32
	IPoC 3x30 s		12	6.89 \pm 0.16	30.88 \pm 1.16	0.223	1.81 \pm 0.12	46.86 \pm 3.00
4	Controls		7	9.93 \pm 0.48	50.24 \pm 1.26	0.198	2.63 \pm 0.18	65.19 \pm 3.78
	IPoC 3x30 s		7	10.03 \pm 0.44	53.01 \pm 1.99	0.189	2.76 \pm 0.16	66.86 \pm 7.73
7	Controls		8	14.80 \pm 0.90	76.90 \pm 2.43	0.192	3.85 \pm 0.20	91.54 \pm 4.74
	IPoC 3x30 s		10	14.69 \pm 0.61	75.15 \pm 2.62	0.195	3.56 \pm 0.24	85.97 \pm 4.11
10	40 min isch.	Controls	10	21.02 \pm 0.91	102.80 \pm 4.20	0.204	6.16 \pm 0.16	148.10 \pm 5.27
		IPoC 3x10 s	8	22.69 \pm 1.04	105.80 \pm 5.29	0.214	6.21 \pm 0.31	146.46 \pm 9.27
		IPoC 3x30 s	11	20.90 \pm 0.76	99.50 \pm 2.98	0.210	6.15 \pm 0.27	146.43 \pm 6.96
		IPoc 3x60 s	9	20.86 \pm 0.83	103.08 \pm 5.24	0.202	5.91 \pm 0.14	143.07 \pm 3.61
	60 min isch.	Controls	11	20.41 \pm 0.87	99.63 \pm 3.07	0.205	5.42 \pm 0.21	133.21 \pm 7.2
		IPoC 3x30 s	11	21.95 \pm 0.62	103.30 \pm 3.28	0.212	5.79 \pm 0.19	141.42 \pm 5.06

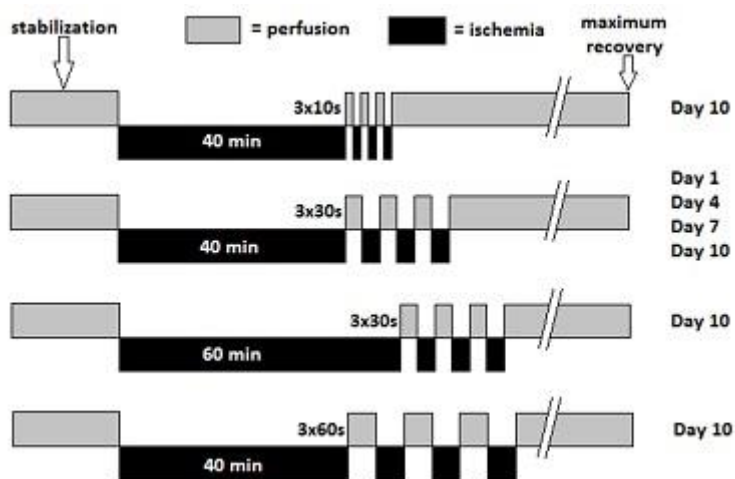


Fig. 1. Scheme summarizing experimental groups and protocols of ischemic postconditioning.

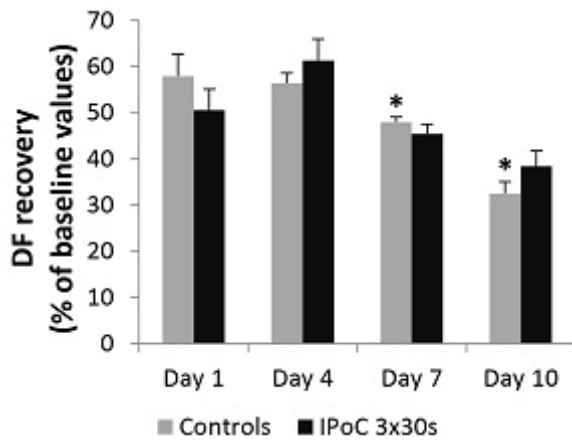


Fig. 2. Tolerance to ischemia (DF, expressed as the percentage of baseline values) and postconditioning (IPoC) during postnatal development. *significantly different ($p < 0.05$) as compared to 1-day-old controls.

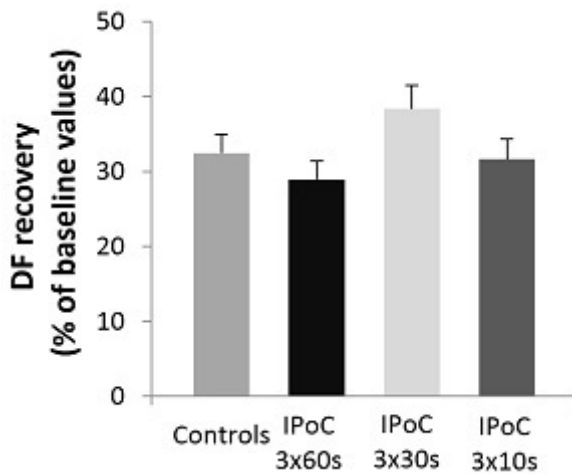


Fig. 3. Tolerance to ischemia (DF, expressed as the percentage of baseline values) and three postconditioning (IPoC) protocols in 10-day-old animals.

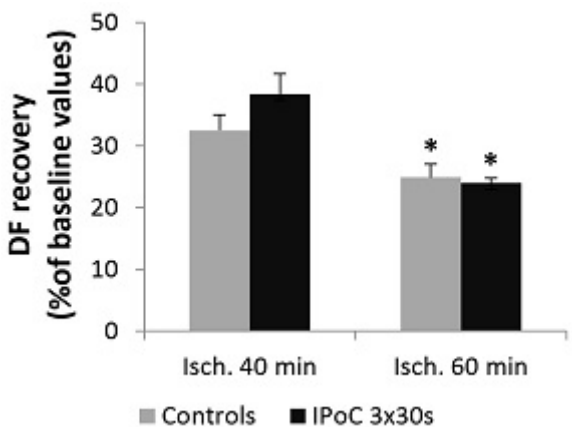


Fig. 4. Tolerance to ischemia (DF, expressed as the percentage of baseline values) and postconditioning (IPoC) after 40 and 60 min ischemia. *significantly different ($p < 0.05$) as compared to correspondent 40 min ischemia group.

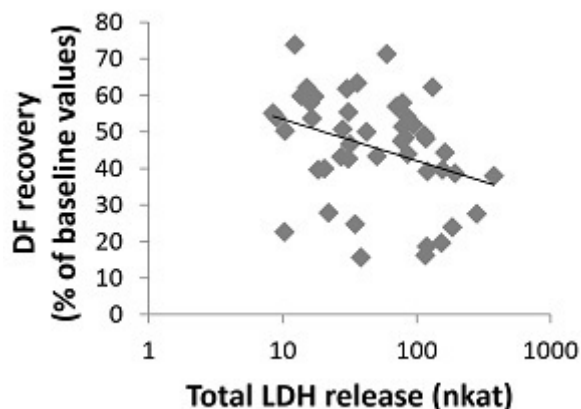


Fig. 5. Statistically significant correlation ($r=-0.36$) of total LDH release (nkat) and DF recovery (percentage of baseline values), $n=46$.

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