Basal Ornithine Decarboxylase Activity Modifies Apoptotic and Hypertrophic Marker Expression in Post-Ischemic Hearts

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Abstract: Polyamines play a role in ischemia-reperfusion injury of brain, kidney and probably heart. Primary data on cardiac myoblasts suggested that the induction of polyamine metabolism induces a hypertrophy like effect in normoxic hearts but apoptosis in reperfused hearts. The aim of this study was to investigate the relevance of these findings for post-ischemic hearts. Rat hearts were exposed to 45 min global normothermic flow arrest followed by 120 min of reperfusion. Controls were constitutively perfused for 165 min under normoxic conditions. Ornithine decarboxylase (ODC) activity was inhibited by administration of difluoromethylornithine (DFMO, 100 μM) starting 30 min after the onset of reperfusion and lasting for 10 min. Calcium receptor activation was induced by administration of putrescine (100 μM) and its inhibition by administration of NPS (10 μM).Left ventricular mRNA expression of bcl-2, bax, and BNP were determined by real time RT-PCR. Results: BNP was induced by putrescine *via* activation of calcium receptors in normoxic and post-ischemic hearts. Inhibition of ODC had a strong effect on bcl-2 expression whereby putrescine induced bax in post-ischemic but not normoxic hearts. Inhibition of ODC increased the bcl-2/bax ratio but putrescine worsened it. In conclusion, induction of polyamine metabolism induced a pro-apoptotic profile in left ventricles *via* calcium receptor activation in post-ischemic hearts but not in normoxic hearts and induced BNP expression under both conditions.

Keywords: Calcium receptor, polyamines, putrescine.

1. INTRODUCTION

Acute coronary occlusion resulting in myocardial infarction is the main mechanism by which coronary artery disease reduces survival [1]. Restoration of blood flow is the most successful intervention that improves survival and functional recovery. It stops ischemia-induced cell damage and allows cells which are still alive to re-start contractile activity but it adds new stress to the heart at the same time. This phenomenon is known as reperfusion injury. At the time of reperfusion a couple of quite different processes are initiated such as recovery of pump activity and induction of a complex process of tissue regeneration termed remodelling. An induction of hypertrophic growth of survived myocytes may compensate for damaged myocytes as well as an induction of pro-apoptotic pathways [2]. The molecular mechanisms determining apoptotic cell death in the post-infarcted myocardium are still not fully understood but changes in the expression of pro- and anti-apoptotic genes like bax and bcl-2 are part of this process. The expression of pro- and anti-apoptotic genes starts to change during the first two hours of reperfusion as analyzed in Tyrode-perfused Langendorff hearts [3-5].

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to putrescine. Since ODC activation is part of pro-hypertrophic signalling in non-infarcted hearts, one may predict that an activation of the polyamine metabolism by ODC activation in reperfused hearts bridges the polyamine metabolism with key events of remodelling such as hypertrophy, apoptosis, and probably inotropy. Indeed administration of the polyamine spermidine prior to ischemia improved the functional recovery of the heart [6]. Spermidine also attenuates apoptosis in neonatal cardiomyocytes [7]. Most recently a study on H9c2 cells has challenged the hypothesis that ODC activation is protective in the post-ischemic heart [8]. H9c2 cells are embryonic rat-heart derived cardiomyoblasts. These cells were exposed to simulated ischemia resulting in an induction of ODC and of the expression of the pro-apoptotic molecule bax. Moreover, the authors supported direct evidence that both findings are causally related to each other. However, ODC activation is normally coupled to hypertrophy and most pro-hypertrophic signals have anti-apoptotic properties [9]. In contrast, ODC activation was linked to pro-apoptotic effects in other cases [10, 11]. Therefore, ODC activity may contribute to either pro- or anti-apoptotic pathways depending on cell types and environmental signals. This leads to the following question: Does ODC activity influence post-infarct recovery in functional aspects or in respect to the expression of either pro- or anti-apoptotic genes in the post ischemic heart?

To address these important questions a previously established ex vivo model of ischemia/reperfusion was used in this study. ODC was inhibited by administration of α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, given during reperfusion. Putrescine, the product of the enzymatic activity of ODC, was used as a control to bypass ODC inhibition. In order to address the question whether putrescine acts in an autocrine fashion, NPS 2390 was used to antagonize calcium receptor stimulation. Noteworthy, all drugs were administered 30 min after the onset of reperfusion to exclude any influence on infarct sizes. As a matter of fact, this study is intended to describe the effect of

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polyamines on post-ischemic hearts rather than on reperfusion injury.

2. MATERIALS AND METHODS

2.1. Isolated Rat Heart Preparation

Experiments were performed on isolated hearts from male Wistar rats as previously described [12]. Hearts were rapidly excised and the aorta was cannulated for retrograde perfusion with a 16-gauge needle connected to a Langendorff perfusion system. The perfusion system consisted of a warmed storage vat for perfusate solution, a speed rotary pump and a temperature controlled chamber in which hearts were mounted. Hearts were perfused with a modified Tyrode solution as described before [12]. After attachment to the Langendorff system, all hearts were allowed to stabilize for at least 20 min.

2.2. Experimental Protocols

In total 12 different groups were analyzed (each n=8 rat hearts). The following experiments were performed: Ischemia and Reperfusion (I/R): 45 min flow arrest and 120 min reperfusion; I/R + DFMO in which DFMO (100 μM) was given 30 min after the onset of reperfusion and washed out again after 10 min. I/R + putrescine in which putrescine (100 μM) was given 30 min after the onset of reperfusion and washed out again after 10 min; I/R + DFMO + putrescine in which DFMO and putrescine were given 30 min after the onset of reperfusion and washed out again after 10 min; I/R + NPS 2390 (NPS) in which NPS (10 μM) was given 30 min after the onset of reperfusion; I/R + NPS + putrescine in which NPS and putrescine were administered 30 min after the onset of reperfusion. All drugs were administered 30 min after the onset of reperfusion because at that time reperfusion-induced cell damage has been established and any drug administration does not interfere with reperfusion-induced damage any more. Therefore, any interventions performed in this study focus on the influence on the post-ischemic hearts and were not intended to reduce the reperfusion-induced cell damage. For all four groups normoxic controls were performed in which the 45 min flow arrest was replaced by normoxic perfusion. Perfusion flow was held constant and during the experiments hearts were allowed to beat free.

2.3. Real Time RT-PCR

At the end of all experiments left ventricles including the septum were separated from atria and right ventricles. Samples were immediately frozen with fluid nitrogen and stored at -80°C until use. Total RNA from left ventricles was extracted with Trizol (Invitrogen) as described by the manufacturer. RT reactions were performed for 1 h at 37°C in a final volume of 10 μl using 1 μg RNA, 100 ng of oligo$(dT)_{15}$, 1 mM dNTPs, 8 units of RNasin, and 60 units of Moloney murine leukemia virus reverse transcriptase. Aliquots were used for real-time PCR using the I-cycler (Biorad, Germany) and SYBR-green fluorescence for quantification. HPRT was used as a housekeeping gene to normalize sample contents. Primers used for determination had the following sequences: HPRT forward: CCA GCG TCG TGA TTA GTG AS, HPRT reverse: CAA GTC TTT CAG TCC TGT CC, bax forward: ACT AAA GTG CCC GAG CTG ATC, bax reverse: CAC TGT CTG CCA TGT GGG G, bcl-2 forward ATG GCG CAA GCC GGG AGA AC, bcl-2 reverse: CTT GTG GCC CAG GTA TGC AC, BNP forward: ATG ATT CTG CTC CTG CTT TTC CC, BNP reverse: TCT GCA TCG TGG ATT GTT CTG. The calculations of the results were carried out according to the $2^{-\Delta\Delta Ct}$ methods as described [13]. After amplification reaction, products were controlled and separated on 2 % agarose gels, stained with SYBR Safe, and photographed under UV illumination.

2.4. Materials

DFMO (Calbiochem; Merck Bioscience Ltd., USA) and putrescine (Sigma/RBI (Sigma-Aldrich Chemie, Taufkirchen, Germany) were dissolved as a stock solution of 100 mM dissolved in either dimethyl sulfoxide (DMSO) or sterile water (putrescine) and stored at -20°C. NPS 2390 (NPS) was obtained from Sigma/RBI and dissolved in DMSO as well. Working solutions were prepared by dilution with perfusion buffer. Control hearts received an equal volume of the vehicle solutions.

2.5. Statistics

Data are expressed as box and whiskers plots or means±s.e.m. as indicated in the legends of the figures. One-way ANOVA was used to compare different groups and if appreciable a Student-Newman-Keuls test was performed for *post hoc* analysis. In cases in which two groups were compared, Mann-Whitney-U-tests for paired samples were employed. $P<0.05$ was regarded as significant.

3. RESULTS

At first we investigated the effect of intracellular and extracellular polyamines on ODC expression. Neither inhibition of ODC nor stimulation of CaRs by putrescine caused any difference in ODC mRNA expression in normoxic hearts (Fig. **1A**). However, in post-ischemic hearts both experiments increased basal ODC expression (Fig. **1A**). In order to address the question whether a modification of polyamine metabolism in the normoxic or I/R heart causes an induction of molecular markers of hypertrophy mRNA expression levels of ANP were determined next. I/R increased ANP mRNA expression and this was not influenced by DFMO (Fig. **1B**). Putrescine decreased ischemia-induced ANF expression but not *via* stimulation of CaRs (Fig. **1B**). Finally, expression of BNP way determined. I/R alone did not influence BNP mRNA expression and DFMO did not modify this in any way although it increased BNP expression in normoxic hearts (Fig. **1C**). However, putrescine caused an increase of BNP mRNA expression in normoxic and I/R hearts that was attenuated by NPS in all cases (Fig. **1C**).

Next, mRNA expressions of the anti-apoptotic bcl-2, of the pro-apoptotic bax, and the ratio of bcl-2 to bax expression were determined. I/R did not modify the expression of bcl-2, but reduced the expression of bax resulting in a slight increase of the bcl-2/bax ratio (Fig. **2**). However, DFMO caused a significant increase in the expression of the anti-apoptotic bcl-2 without any effect on bax expression leading to a significant increase in the bcl-2/bax ratio (Fig. **2**). Putrescine induced the pro-apoptotic bax and normalized DFMO induced bcl-2/bax ratio (Fig. **2**). The effect of putrescine on bax expression was dependent on calcium receptor stimulation because NPS attenuated this effect (Fig. **2B**, **C**). In normoxic hearts putrescine caused a reduction of bax and thereby improved bcl2/bax ratios (Fig. **2**).

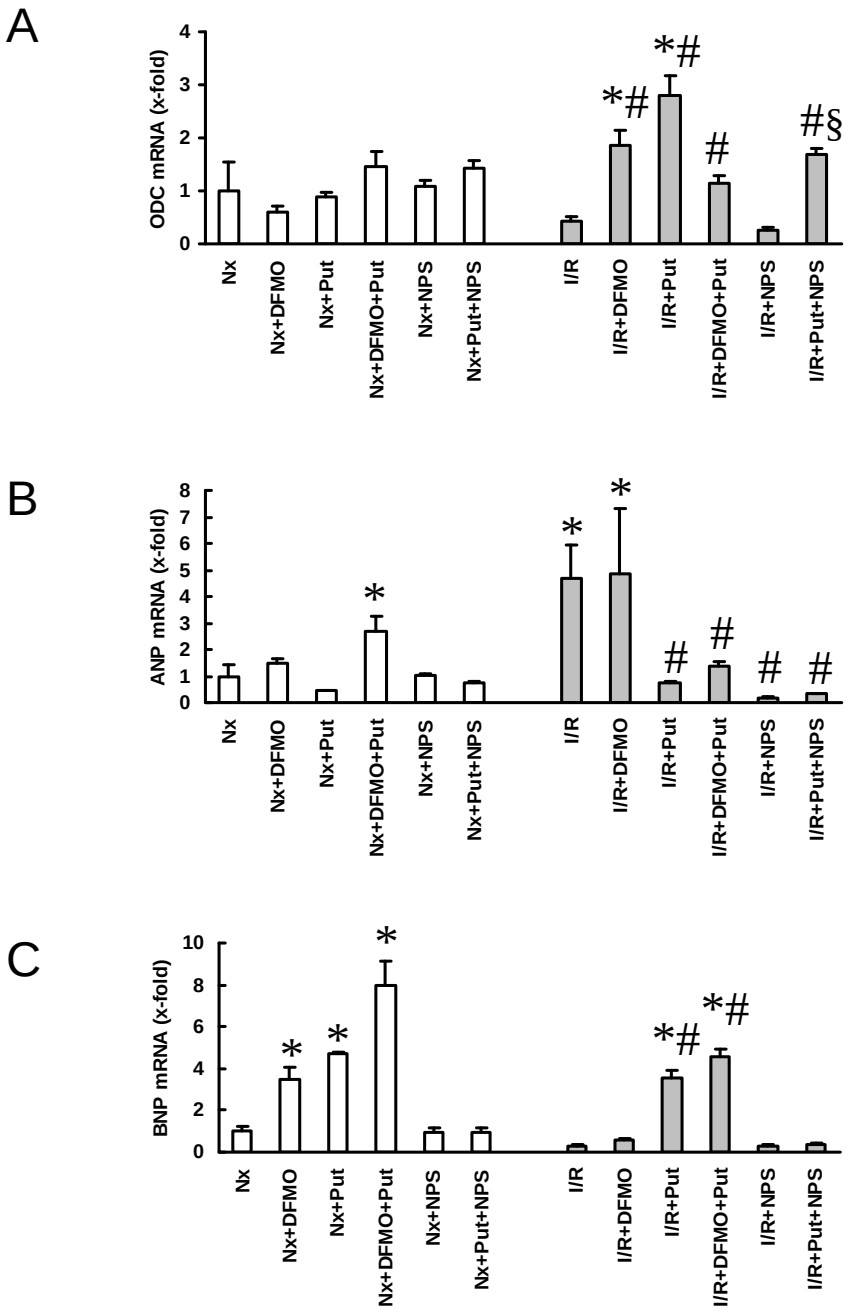

Fig. (1). Effect of ischemia/reperfusion (I/R) on the mRNA expression of brain natriuretic peptide (BNP) and the effect of polyamine metabolism on its expression. Each group n=8 hearts. Nx, normoxic controls; I/R, 45 min flow arrest and 2 h reperfusion; DFMO, difluoromethylornithine (100 μM) used as an irreversible inhibitor of ODC; Put, putrescine (100 μM); NPS 2390, NPS (10 μM) used as a specific antagonist of the calcium receptor. *, $p<0.05$ vs. Nx; #, $p<0.05$ vs. I/R; §, $p<0.05$ vs. I/R+putrescine.

DISCUSSION

In this study we investigated effects of ODC activity and putrescine on early regulation of mRNA expression levels of factors linked to hypertrophy or apoptosis. The main findings of this study are first that the product of ODC enzymatic activity, putrescine, increases the expression of the pro-apoptotic bax in post-ischemic hearts but not in normoxic hearts as predicted from studies on H9c2 cells and second that inhibition of ODC activity improves the bcl-2/bax ratio at least on the mRNA expression level. Furthermore, the data of this study that the effect of putrescine on left ventricular BNP expression is not modified at all by ischemia/reperfusion.

Upon reperfusion the heart is exposed to various pro-apoptotic events such as oxidative burst, mechanical load, excessive release of catecholamine and other factors [2]. As a result of this caspase 3 is getting activated, an enzyme closely linked to apoptosis. However, post-ischemic hearts

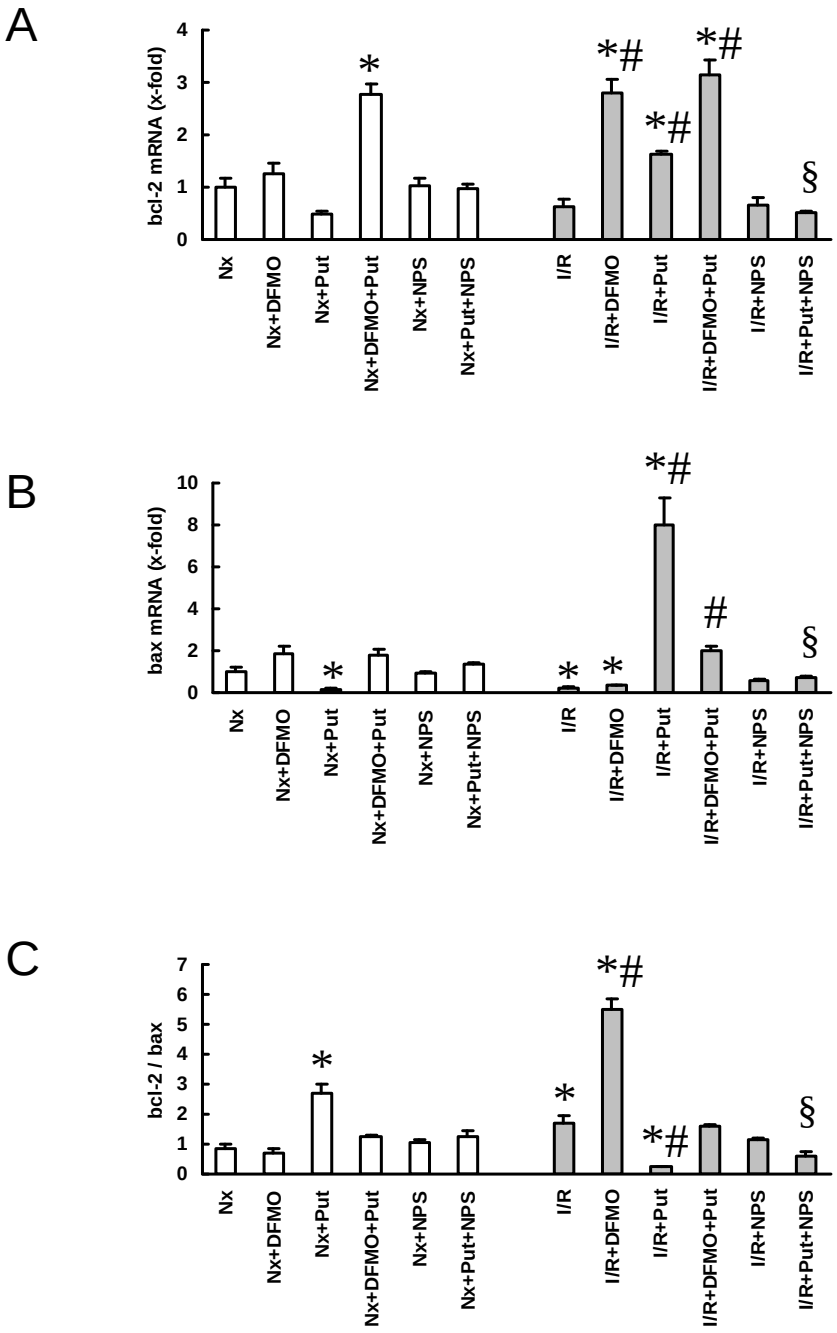

Fig. (2). Effect of ischemia/reperfusion (I/R) on the mRNA expression of the anti-apoptotic bcl-2 (**A**), the pro-apoptotic bax (**B**),and bcl-2 to bax ratio (**C**). Each group n=8 hearts. Nx, normoxic controls; I/R, 45 min flow arrest and 2 h reperfusion; DFMO, difluoromethylornithine (100 μM) used as an irreversible inhibitor of ODC; Put, putrescine (100 μM); NPS 2390, NPS (10 μM) used as an antagonist of the calcium receptor. *, $p<0.05$ vs. Nx; #, $p<0.05$ vs. I/R; §, $p<0.05$ vs. I/R+putrescine.

are quite resistant against pro-apoptotic events at the same time. It was shown before that an altered expression of bcl-2 and bax, controlling the release of cytochrome C from mitochondria, is involved in this protection [2, 14]. A previous study on cardiac-like cells suggested that an activation of the polyamine metabolism increases the expression of the pro-apoptotic gene bax and favours the susceptibility against apoptosis in post-ischemic heart cells but not in normoxic cells [8]. Basically, we confirmed this relationship in an established ischemia/reperfusion model because putrescine, the end product of ODC activity caused a marked increase in bax expression in post-ischemic hearts but not in non-ischemic hearts. As a result of this the bcl-2-to-bax ratio shifts into the direction of bax and an increased susceptibility to apoptosis must be predicted. Noteworthy, putrescine caused this effect *via* a stimulation of calcium receptors. An endogenous induction of polyamine metabolism by ischemia alone seems not be sufficient to induce bax expression. Ad-

ministration of putrescine was required to change bax expression. Indeed, we found no induction of ODC in post-ischemic hearts (data not shown). ODC activation is mediated by an increase in ODC mRNA expression within minutes when cardiomyocytes are exposed to isoprenaline as a pro-hypertrophic stimulus [9]. Polyamine metabolism itself is likely to be activated in post-ischemic hearts, because arginase, which metabolizes arginine to ornithine and urea, is up-regulated during myocardial ischemia [15]. However, if ischemia-dependent arginase activation requires a co-activation of ODC to shift arginine metabolism into the direction of polyamine metabolism, one would have seen this in these hearts because ODC activity is regulated on the transcriptional level. While putrescine, the natural product of ODC enzyme activity, induced bax and reduces bcl-2/bax ratio, inhibition of ODC increased bcl-2 expression and improved bcl-2/bax ratio.

On the other hand, polyamines as products of ODC enzymatic activity improve cardiac function [6]. This can be observed in these hearts in parallel to the induction of bax because as long as cells are not damaged by apoptosis they maintain functional activity [16]. Most likely, putrescine acts in a paracrine way to improve the functional recovery. In principle, polyamines are extruded from cells and act *via* binding to and activation of calcium sensing receptors [17]. It is in line with these suggestions that spermidine, another polycationic product of the polyamine metabolism, is also sufficient to improve post-ischemic function even if administered before ischemia [6].

Despite the effect of ODC activity on pro- and antiapoptotic genes, polyamine metabolism is normally considered as a pro-hypertrophic pathway [9]. Therefore, the expression of hypertrophy-associated gene like BNP was also analyzed. Endogenous ODC activity seems not to be involved in the regulation of BNP expression in post-ischemic hearts. Putrescine induced the expression of BNP. The effect was slightly more improved in co-presence of DFMO. The finding may indicate a difference between endogenous polyamines, required to stabilize nucleic acids, and exogenous polyamines, acting by binding to the calcium-sensing receptor. In post-ischemic hearts inhibition of ODC by DFMO was no more able to increase BNP mRNA expression indicating that BNP expression cannot influenced by intracellular polyamine effects under these conditions.

The expression of ODC was obviously not modified by ischemia/reperfusion itself. Furthermore, under normoxic conditions neither inhibition of ODC nor activation of calcium receptors influenced the basal expression of ODC mRNA. However, regulation of ODC steady state mRNA levels seems to be altered in post-ischemic hearts as both DFMO and putrescine increased ODC mRNA expression. The mechanism behind this altered regulation of ODC mRNA in post-ischemic hearts is less clear and requires further analysis. Putrescine increased ODC mRNA expression at least in part *via* interaction with calcium receptors as the effect of putrescine could be reduced by NPS. However, it was not completely normalized. Furthermore, DFMO also increased ODC mRNA expression indicating a feedback

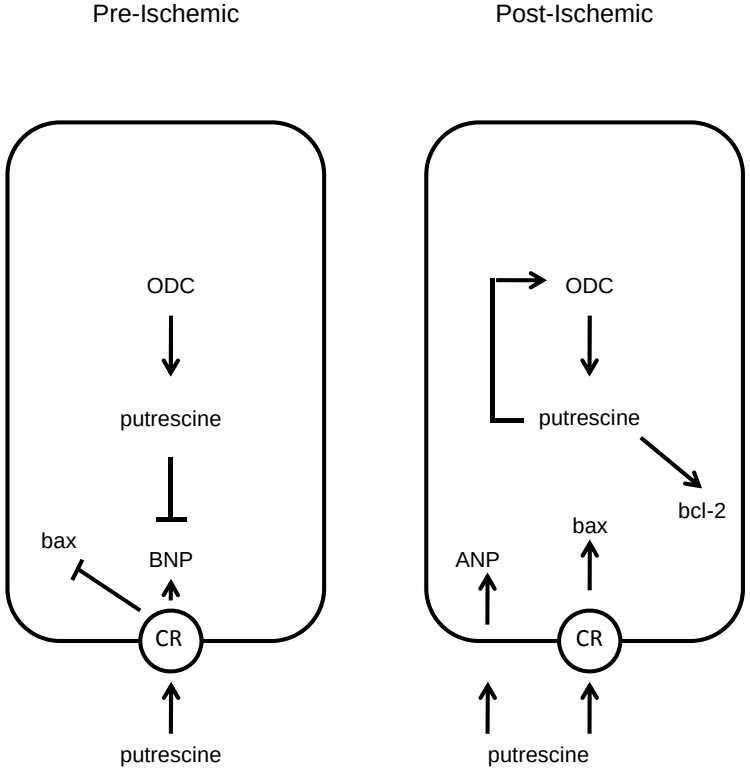

Fig. (3). Conclusive summary of the findings. Left: In pre-ischemic hearts endogenous ODC activity controls BNP expression and calcium receptor (CR)-dependent effects counterbalance the effect of ODC on BNP and attenuate bax expression. Right: In post-ischemic hearts endogenous ODC activity forms a feedback loop to its own regulation and induces bcl-2 expression whereas stimulation of calcium receptors antagonizes this by activating bax expression and reducing ANP expression.

mechanism in post-ischemic hearts but the effects of DFMO and putrescine were not additive, indicating that a cross-talk between the endogenous feedback inhibition and calcium receptor-dependent signalling.

In summary this study provides strong evidence for a significant difference in post-ischemic coupling of endogenous and exogenous polyamine metabolism. The data of the study are summarized in Fig. (**3**) where the effects of ODC inhibition and calcium receptor activation in normoxic and post-ischemic hearts were compared. Ischemia/reperfusion shifts arginine metabolism from NO to polyamine metabolism by induction of arginase [1]. In principle this leads to an elevated release of putrescine that acts in an autocrine way on heart cells by modifying bax and BNP expression. As BNP is both a cardioprotective natriuretic peptide and a cardiac risk marker these observations are of relevance for the understanding of the early post-infarct remodelling.

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