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A kallidin-like peptide is a protective cardiac kinin, released by ischaemic preconditioning of rat heart

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1 Bradykinin is thought to play a major role among the endogenous cardioprotective candidates of ischaemic preconditioning (IPC). Little attention has been paid to the fact that in the tissue kallidin (KAL), rather than bradykinin might be the physiological mediator of the kallikrein–kinin system. In order to evaluate the importance of one or the other peptide the release and effect of both kinins has been investigated in isolated rat hearts following IPC.

2 Bradykinin- and a KAL-like peptide were measured in the effluent of the rat isolated Langendorff heart with two different specific radioimmunoassays. The creatine kinase activity in the effluent was judged as degree of cardiac injury caused by ischaemia.

3 During IPC, which consists of three 5 min no-flow and 5 min reperfusion cycles prior to the 30 min ischaemia, the bradykinin level in the effluent did not change significantly (15.4–19.4 pg ml^{-1}). In the control group the bradykinin levels were 15.9–16.6 pg ml^{-1}.

4 During IPC KAL-like peptide (Arg^1-, instead of Lys^1-KAL), which has recently been verified by mass spectrometry, displays 5.8-fold higher levels in the effluent and significantly increases in the same time interval from 90.4 to 189 pg ml^{-1}.

5 After 30 min ischaemia the bradykinin levels in the IPC group were not significantly different to those of the control group (18.7 vs 14.4 pg ml^{-1}). The KAL-like peptide levels in the IPC group vs the control group were 105 vs 86.1 pg ml^{-1}.

6 By the 30 min ischaemia the creatine kinase activity in the IPC group increased from 0.367 to 8.93 U l^{-1} (before and 10–30 min after ischaemia). In the control group during the same time period the creatine kinase levels increased from 0.277 to 34.9 U l^{-1}. The low increase in creatine kinase activity following IPC was taken as equivalent of the cardioprotective action. A KAL antibody or HOE140 (kinin B_2-receptor antagonist) completely abolished this beneficial effect of IPC (36.6 and 53.0 U l^{-1}) when added to the perfusion medium during the reperfusion cycles of IPC prior to the 30 min ischaemia.

7 Our data suggest that in rat hearts KAL-like peptide rather than bradykinin is the physiological compound activated by IPC and acting *via* the cardiac kinin B_2-receptor. Thus, endogenously generated KAL-like peptide seems to play a major role in the cardioprotection of IPC.
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Abbreviations: Arg, arginine; BK, bradykinin; CK, creatine kinase; IPC, ischaemic preconditioning; KAL, kallidin; KALab, kallidin antibodies; KKS, kallikrein–kinin system; KLP, kallidin-like peptide; LMW, low-molecular weight; LVP, left ventricular pressure; Lys, lysine; p, plasma; RIA, radioimmunoassay; t, tissue

Introduction

The ischaemic preconditioning (IPC) of the heart is an extremely complex physiological and pathophysiological phenomenon, resulting in a protection against ischaemia–reperfusion injury (for review see Rubino & Yellon, 2000). The clinical importance of this discovery is associated with the notion that the potency of protection against cardiac injury cannot be obtained with any pharmacological strategy (Murry *et al*., 1986). During the period of IPC the heart generates and releases many different mediators. Among these opiates, catecholamines, adenosin, prostaglandines, endothelin-1 and bradykinin (BK) are supposed to be of major importance (Sanada & Kitakaze, 2004). In this context an endogenous kallikrein–kinin system (KKS) has been found in the heart, with tissue kallikrein- (Nolly *et al*., 1994), and low-molecular weight (LMW) kininogen-gene expression (Nagaoka *et al*., 1999). Therefore in the heart kallikrein may generate kinins by cleaving the substrate LMW-kininogen.

The physiological importance of kinins in IPC has been studied *in vivo* in pigs (Schulz *et al*., 1998), dogs (Murry *et al*., 1986; Vegh *et al*., 1994), and cats (Pan *et al*., 2000). In humans the kinin-induced preconditioning effect has been supported in patients undergoing angioplasty (Leesar *et al*., 1999). There are

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numerous experimental data on the cardioprotective effect of IPC in isolated hearts of rabbits (Wall *et al*., 1994; Feng & Rosenkranz, 1999), rats (Bouchard *et al*., 1998) and guinea pigs (Rubin & Levi, 1995). These studies revealed that the kinin B_2-receptor is involved in the protection against ischaemic injury and reperfusion-induced arrhythmia. Recent papers point to the clinical importance of the kinin B_2-receptor as a potential therapeutic target (Fryer *et al*., 2002). Lately data suggests that the kinin B_1-receptor is also present in myocardium and contributes to the beneficial effect of kinins (Bouchard *et al*., 1998). Moreover, kinins seem to have a favourable influence on the cardiac energy metabolism (Linz *et al*., 1997). In this context the role of protective mediators and intracellular signalling systems has been investigated.

For biochemical and physiological reasons the KKS is divided in a plasma (p) and a glandular-, or tissue (t)-KKS. The KKS_p consists of plasma kallikrein, which is generating BK by cleavage of high-molecular weight kininogen. Kallidin (KAL) is the biologically active peptide of the KKS_t, released from LMW-kininogen by tissue kallikrein. Recent data of our group suggests that both systems are differently regulated in humans (Hilgenfeldt *et al*., 1998). Until now a clear distinction of physiological functions between both peptides, BK and KAL, has not been investigated. Although BK has been pointed as the only mediator on kinin B_2-receptors in rats by most investigators, we have shown in a previous paper that in the rat a KAL-like peptide (KLP) is generated from LMW-kininogen by rat tissue kallikrein (Hilgenfeldt *et al*., 2005). Due to structural similarity between KAL and KLP (a N-terminal Lys- vs a N-terminal Arg-residue) it can be assumed that both are more or less physiologically equivalent. Moreover, we have developed a specific radioimmunoassay (RIA) for both, BK and KLP, respectively. Therefore the study analyses the type of kinin released from the isolated perfused rat heart and follows its concentration under the experimental conditions of IPC and reperfusion.

Methods

Animals

Male SPF Sprague–Dawley rats (Charles River, Germany), 260–320 g were kept under standard conditions with free access to food and water. All experiments were performed in accordance with the Federation of European Laboratory Animal Science Association (FELASA) guidelines for animal experimentation.

Langendorff heart perfusion

We anaesthetised rats with pentobarbital sodium, i.p., 60 mg kg^{-1} body weight and injected 500 U heparin i.v. to prevent blood clotting. After opening of the chest we rapidly chilled the hearts with ice-cold saline to stop the contraction and to reduce oxygen consumption. We cannulated the ascending aorta and immediately perfused the coronary arteries with prechilled medium. Then we quickly excised the hearts and mounted them on the thermostatted Langendorff apparatus (whole procedure within 1 min). Spontaneously, beating started within a few seconds of retrograde perfusion with nonrecirculating modified Krebs–Henseleit buffer containing (mM): 118 NaCl; 24 $NaHCO_3$; 4 KCl; 1.2 KH_2PO_4; 1 $MgSO_4$; 5 D-glucose; 2 pyruvate sodium and 2.5 $CaCl_2$. The medium was aerated with a 95% O_2 + 5% CO_2 mixture at 37°C (pH 7.4) and passed through a 3 μm filter before entering the heart in order to remove any particular contamination.

We conducted the experiments under conditions of constant perfusate pressure (coronary perfusion pressure) of 70 mmHg. We measured coronary flow volumetrically. Left ventricular pressure (LVP) and heart rate were monitored using the latex balloon catheter placed in the left ventricle and connected to a pressure transducer and bridge amplifier (WPI, Germany). We recorded and analysed all data using the software developed by Shandong Pharmacy Institute (China).

Sample collection and sample preparation

We cannulated the pulmonary artery with a 14-gauge catheter and collected the coronary venous effluent continuously during the experiment, except during the no-flow periods.

Experimental protocol

The experiments started after an equilibration time of 20 min. A 30 min treatment period was followed by a 30 min of global ischaemia (no-flow). Then we reperfused the hearts for another 30 min.

The 30 min treatment period prior to no-flow ischaemia consisted of: (1) normoxic perfusion (control group); (2) preconditioning with three ischaemic cycles of 5-min duration (no-flow ischaemia) separated by three reperfusion cycles of 5 min duration (IPC group); (3) preconditioning with three ischaemic cycles of 5 min duration (no-flow ischaemia) separated by three reperfusion cycles of 5 min duration with a medium containing KAL antiserum at a dilution of 1 : 1000 (IPC-KALab group); (4) preconditioning with three ischaemic cycles of 5 min duration (no-flow ischaemia) separated by three reperfusion cycles of 5 min duration with a medium containing the kinin B_2-receptor antagonist, HOE140, 100 nM, (IPC-HOE group). The schematic of the four different protocols is shown in Figure 1.

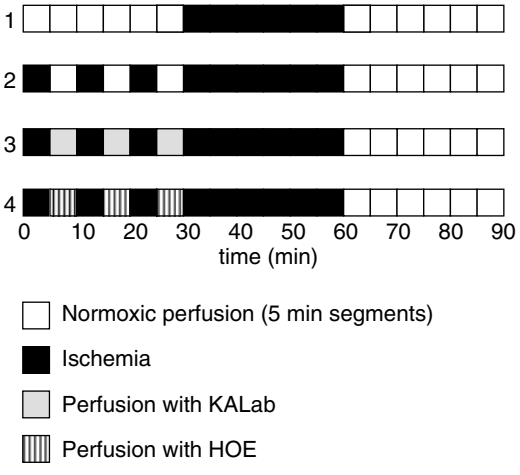

Figure 1 Schematic of the four different protocols: (1) control group; (2) IPC group; (3) IPC + KALab group; and (4): IPC + HOE group.

Exclusion criteria

During equilibration, we excluded hearts if they met one of the following criteria: (1) unstable contractile function, (2) coronary flow outside the range of 9–15 ml min^{-1}, (3) heart rate below 240 beats min^{-1} or appearance of severe arrhythmia, (4) LVP below 70 mmHg, (5) creatine kinase (CK) activities in the effluent >1 mU ml^{-1} at the end of equilibration.

Kinins immunoassay

We analysed BK and KLP using a specific BK- and a specific KAL-RIA, respectively (Hilgenfeldt *et al*., 1995). In short: 1 ml coronary effluents were collected into chilled RIA tubes containing an inhibitor cocktail (ratio of sample to inhibitors, 9 : 1). In all, 1 ml of the inhibitor cocktail contained aprotinin, 10 kIU, soybean trypsin inhibitor, 800 μg, hexadimethrine bromide, 4 mg, 1,10-phenanthroline, 10 mg, and EDTA, 20 mg. The samples were mixed with 1 ml ethanol absolute, centrifuged for 15 min at 4°C at $6200 \times g$. The supernatants were evaporated to dryness under N_2 at room temperature and stored frozen at -70°C until they were assayed. We reconstituted the samples with RIA buffer and incubated them with rabbit anti-BK or anti-KAL antiserum in presence of ^{125}I-BK tracer (^{125}I-Tyr^8-BK) and ^{125}I-KAL tracer (^{125}I-Tyr^9-KAL), respectively. After 24 h incubation at 4°C we separated the antibody bound peptide with a charcoal suspension. After centrifugation, the supernatant was aspirated and the charcoal pellet was counted in a gamma counter (LB 2111, Berthold, Bad Wildbad, Germany). KLP differing from KAL by the exchange of the N-terminal Lys- vs a N-terminal Arg-residue displays a crossreactivity with the KAL antiserum in the order of 80% (Figure 2). Therefore, the KLP data were obtained with a KLP standard curve.

CK assay

CK activity in the coronary effluent was measured with a colourimetric enzyme assay provided by Sigma-Aldrich, Germany. Briefly: 100 μl effluent was incubated with 200 μl CK reagent. The kinetic was measured every 30 s for 3 min at 340 nm in a spectral photometer (Spectramax 250, Molecular Device, München, Germany). The calculation of the enzymatic activity (CK activity at 30°C in $U\,l^{-1}$) occurred according to the following equation:

$$\mathrm{CK}\ (U \times l^{-1}) = \frac{\delta A/\min \times V_t \times 1000}{6.22 \times d \times V_s}$$

where V_t is the total volume (ml), V_s the sample volume, d the cuvette diameter, δA/min the change in absorbance per min, 6.22 the absorption of 1 μmol NADH at 340 nm. One unit CK is defined as the amount of enzyme in the reaction mixture which is linked with the conversion of 1 μmol of NAD to NADH min^{-1} at 30°C.

Statistical analysis

The data are expressed as mean values with standard error of the mean (s.e.m.); n = number of hearts in every group is 10. Statistical significance between the groups is verified by using one-way analysis of variance with subsequent Student–Newman–Keuls *post hoc* tests. Differences between mean responses comparing two groups were determined by *t*-test. Values of $P<0.05$ (two-tailed test) were regarded as significant.

Results

In order to analyse BK-binding to the KAL antiserum (lot no. 4H79.6) we used a BK standard curve as competition for a KAL tracer. As shown in Figure 2 there was no binding of the BK to the KAL antiserum at all. This suggests that the antiserum may not interfere with the BK levels as determined by RIA. However, KLP showed a marked crossreactivity with the KAL antiserum. This point was already addressed in the previous article (Hilgenfeldt *et al*., 2005).

Figure 3a shows the BK levels in the coronary effluent of the IPC group vs the control group. Prior IPC the BK level in the effluent of the IPC group was 15.6 ± 4.96 vs 15.9 ± 1.52 pg ml^{-1} in the control group. During the three reperfusion cycles of IPC the BK levels did not change significantly (19.4 ± 4.68 vs 16.6 ± 2.53 pg ml^{-1}, IPC group vs control group, mean ± s.e.m., t = 5–30 min). After the 30 min ischaemia the BK levels in the medium of the IPC group and of the control group were unchanged (18.7 ± 1.32 vs 14.4 ± 0.55 pg $\times$ ml^{-1}, mean ± s.e.m., t = 65–90 min).

As shown in Figure 3b the KLP levels are approximately five- to six-fold higher than those of BK. The KLP level in the medium of the IPC group vs the control group prior IPC was 90.4 ± 6.67 vs 84.3 ± 6.27 pg ml^{-1}. During the three reperfusion cycles of IPC the KLP levels significantly increased (189 ± 76.4 vs 77.5 ± 15.3 pg ml^{-1}, IPC group vs control group, mean ± s.e.m., t = 5–30 min, $P<0.05$). After 30 min ischaemia the KLP levels in the IPC group declined to 105 ± 45.4 vs

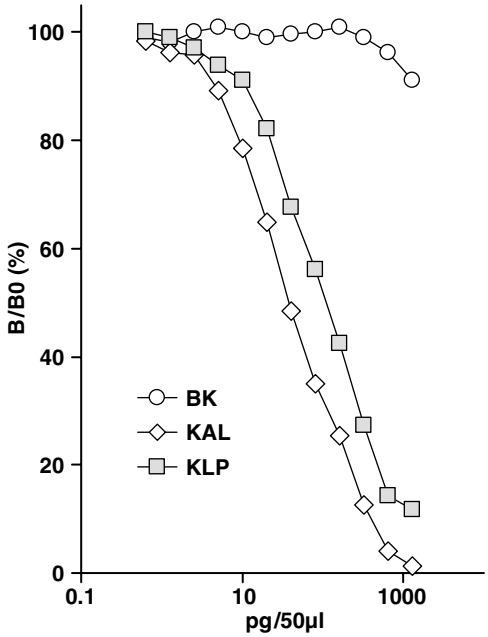

Figure 2 Binding characteristic of a KAL antiserum at a dilution of 1 : 50000. ^{125}I-Tyr^9-KAL was used as tracer molecule (5000 counts min^{-1}). The binding of bradykinin (BK: open circles; kallidin (KAL: white squares) and kallidin-like peptide (KLP: grey rhombus) is shown. B/B_0 (%): bound tracer in the presence and absence of cold antigen, respectively.

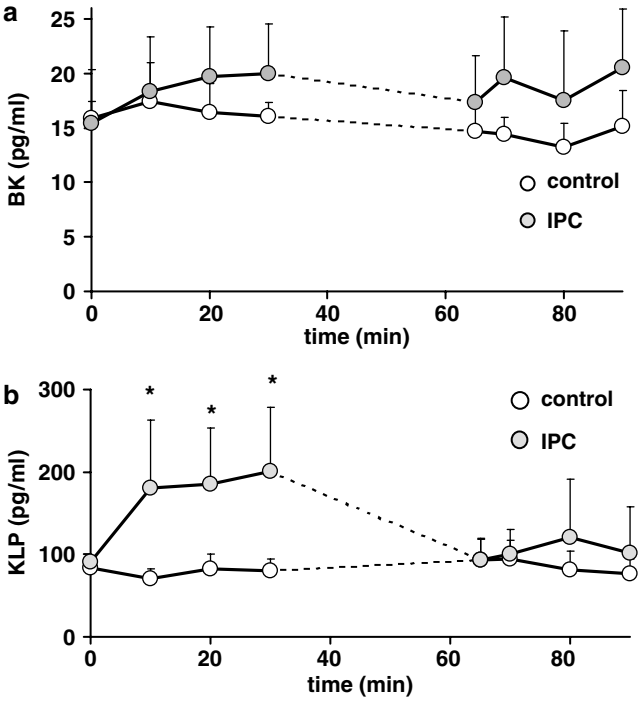

Figure 3 Release of (a) BK and (b) KLP from isolated rat heart of control group (open symbols) and of IPC group (closed symbols) before (0–30 min) and after (60–90 min) 30 min ischaemia. The dotted line represents the 30 min ischaemia period. $N=10$, mean values with s.e.m. $^{*}P<0.05$, IPC vs control group.

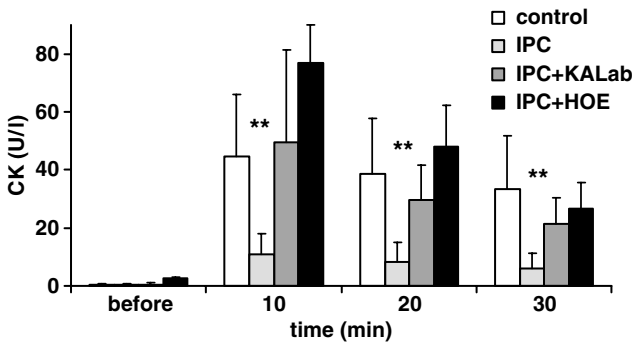

Figure 4 CK activity ($U\,l^{-1}$) in coronary effluent before and after 30 min ischaemia of control group (white columns), of IPC group (light grey columns), of the IPC + KALab group (dark grey columns), and of the IPC + HOE group (black columns) before and 10, 20, and 30 min after 30 min ischaemia. $N=10$, mean values with s.e.m., $^{**}P<0.01$, IPC vs control.

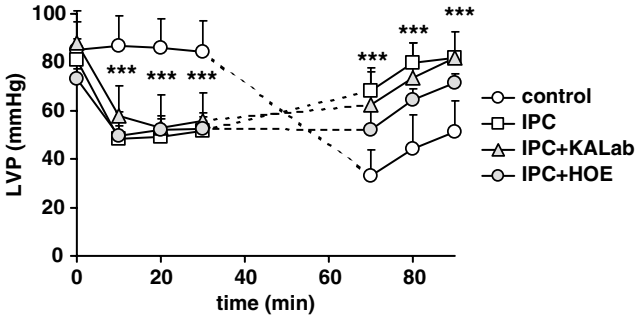

Figure 5 Change in LVP (mmHg) before and after 30 min ischaemia. Control group: open circles; IPC: grey squares; IPC + KALab: grey triangles; IPC + HOE: grey circles. $N=10$, mean values with s.e.m., $n=10$; $^{***}P<0.0001$. Asterisk show statistical significance of IPC group vs control group. Not shown: $^{**}P<0.005$: IPC + KALab group vs control group (time 10–30 and 70–90 min), $^{**}P<0.005$: IPC + HOE group vs control group (time 10–30 min) and $^{*}P<0.05$: IPC + HOE group vs control group (time 70–90 min).

$86.1\pm22.2\,pg\,ml^{-1}$ (IPC group vs control group, mean ± s.e.m., $t=65$–90 min). Thus even after 30 min ischaemia of IPC group and of the control group in the coronary effluent the KLP levels were approximately five- to six-fold higher than those of BK.

The CK activity in the perfusate of the hearts before and after 30 min ischaemia is shown in Figure 4. We judged a decline in CK activity to be a correlate of the cardioprotective effect. When the hearts were reperfused after 30 min ischaemia the CK activity in the IPC group vs the control group increased from $0.367\pm0.425\,U\,l^{-1}$ vs 0.277 ± 0.373 up to 8.93 ± 6.79 vs $34.9\pm18.5\,U\,l^{-1}$ (mean ± s.e.m., mean valued, $t=0$–30 and 65–90 min). At 10 min after the end of the 30 min ischaemia the CK activity was at the highest level and thereafter moderately declined. The increase in CK following IPC was significantly smaller than in the control group ($P<0.01$). In order to show that KLP contributes to this cardioprotective effect, we treated the IPC + KALab group in the three reperfusion cycles with the specific KALab, which is able to bind specifically KLP, but not BK. Furthermore we investigated the importance of the kinin B_2-receptor for the cardioprotective effect of IPC by addition of the kinin B_2-receptor antagonist HOE140 to the perfusion medium during the reperfusion cycles in IPC. As shown in Figure 4 the cardioprotective effect of IPC is completely abolished by both treatments. After the 30 min ischaemia there was no significant difference between the CK levels in the perfusate of the IPC + KALab group ($36.6\pm23.7\,U\,l^{-1}$), of the IPC-HOE group ($53.0\pm12.9\,U\,l^{-1}$), and of the control group.

Figure 5 displays the change in LVP following IPC. An immediate and significant decline in LVP occurred already after the first 5 min ischaemia cycle of IPC (80.9 ± 8.9 vs 48.5 ± 10.8 mmHg) and maintains over the whole period of IPC. In the IPC + KALab group and IPC + HOE group the decline in LVP was similar and also maintains over the whole period of IPC. The 30 min ischaemia caused a moderate recovery of LVP in the IPC-, IPC + KALab, and the IPC + HOE-group. In contrast in the control group 30 min ischaemia caused a decline in the LVP (84.5 ± 12.5 vs 35.7 ± 14.7 mmHg, $t=30$ vs 65 min). This suggests that the pre- and post-ischaemic change in the LVP in the IPC group is not influenced by the action of kinins.

Discussion

It is generally accepted that the KKS is divided in a KKS_p and a KKS_t with their biologically active peptides, BK and KAL, respectively. They may not be physiologically equally important and regulated (Linz *et al*., 1997). Most investigations do not clearly discriminate between BK and KAL. There is

a yet unproved assumption that finally after enzymatic conversion of KAL BK is the major mediator of most kinin actions. There are two reasons for this blurred vision. First: most measurements of endogenous kinins have been performed with highly sensitive antibodies, which, however, do not discriminate between BK and KAL. Second: due to differences in the sequence between human and rat kininogen, KAL = Lys^0-BK cannot be released from rat kininogen. In addition, it has been shown that BK is the only kinin generated from high-molecular weight kininogen by rat tissue kallikrein (Kato *et al*., 1985).

Recently, we have developed specific antisera which are selective for BK and KAL, respectively (Hilgenfeldt *et al*., 1995). With the KAL-specific RIA we have found KLP (Arg^1, instead of Lys^1-KAL), which is released from rat LMW-kininogen by rat tissue kallikrein. The sequence of KLP has been verified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Hilgenfeldt *et al*., 2005). With both specific RIAs we were able to follow the release of kinins from isolated perfused hearts in more detail.

Sicuteri *et al*. (1967) was the first to discuss the release of kinin from heart following myocardial ischaemia in connection with angina pectoris and myocardial pain. In this context a significant increase in BK release was observed following occlusion of the left anterior descending coronary artery suggesting a contribution of BK to the generation of cardiac pain following ischaemia (Kimura *et al*., 1973). These data were confirmed in dogs by using a kinin RIA (Matsuki *et al*., 1987).

In the meantime the initial release of kinins and other protective mediators in the early stage of ischaemia has been extensively investigated. Most investigators conclude that the early BK release is one of the essential protective mediators of cardiac IPC (Goto *et al*., 1995; Parratt *et al*., 1997). In this context, a local tissue KKS had been identified in rat heart (Xiong *et al*., 1990; Nolly *et al*., 1994).

Now, with a specific RIA for BK and KAL, our data clearly show for the first time, that both BK and KLP are released from the isolated perfused rat heart. In the control group, the release of KLP is approximately 5.5-fold higher than that of BK. During IPC the KLP levels in the coronary effluent significantly increased already during the first ischaemic cycle and reached approximately 10-fold higher levels during preconditioning compared to the BK levels.

Despite our highly specific findings, the importance of kinins in the protection against infarction mediated by IPC is still controversial (Bugge & Ytrehus, 1996). In their study, they treated the hearts with external BK prior to the 3 min ischaemia, but HOE140 did not interfere with IPC. In contrast to these findings our data clearly show that there is a significant stimulation of the tissue KKS in the heart connected with IPC. KLP seems to be of major importance for the kinin action in the rat heart. We judged the cardioprotective effect by the CK activity measured in the effluent after the 30 min ischaemia period. There is a significantly lower CK activity after IPC which was linked with the high release of KLP. This effect raised the notion, that KLP *via* the kinin B_2-receptor is mainly responsible for the cardioprotective effect of the kinins in IPC and might be more important than BK. This was confirmed by the observation that the cardioprotective effect of IPC can be abolished by perfusion with the specific KALab as well as with the kinin B_2-receptor antagonist HOE140 prior to the 30 min ischaemia. This effect was specific, as no difference in CK activity occurred prior and after 30 min ischaemia in hearts perfused with an unspecific antiserum vs the control group (data not shown). The increase in CK after 30 min ischaemia in the IPC + HOE group is substantially, but not significantly higher, than in the control group, which indicates, that a basal cardiac kinin activity was blocked by the B_2-antagonist causing an increase in cardiac damage. The lower deleterious effect of the KALab may be due to the limitation of the local action of the antiserum. We believe this idea should be confirmed in more detailed experiments.

One specific cardiac function changed already during the first cycle of IPC. There is a subsequent decline in LVP already during the first cycle of IPC. This effect could neither be altered by the KALab nor by the action of the B_2-receptor antagonist, HOE140. After 30 min ischaemia the LVP was significantly higher in the IPC groups vs the control group. This indicates a beneficial effect of IPC on the cardiac energy metabolism according to the report of Linz *et al*. (1997).

In conclusion, in the rat heart the endogenous tissue KKS expresses a KLP which is stimulated by IPC. This peptide rather than BK seems to mediate the cardioprotective effect following IPC. The cardioprotective effect displays a significantly lower increase in CK after a 30 min ischaemia, which can be blocked with a KAL antibody and the kinin B_2-receptor antagonist, HOE140, respectively.

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