Apelin-induced cardioprotection against ischaemia/reperfusion injury: roles of epidermal growth factor and Src

Apelin-induced Cardioprotection Against Ischemia/Reperfusion Injury: Roles of Epidermal Growth Factor and Src

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Abstract

Aim: Apelin, the ligand of the G-protein-coupled-receptor (GPCR) APJ, exerts a postconditioning-like protection against ischemia-reperfusion injury through activation of PI3K-Akt-NO signalling. The pathway connecting APJ to PI3K is still unknown. Since other GPCR ligands act through transactivation of epidermal growth factor receptor (EGFR) *via* a matrix-metalloproteinase (MMP) or Src kinase, we investigated whether EGFR transactivation is involved in the following three features of apelin-induced cardioprotection: limitation of infarct size, suppression of contracture, and improvement of post-ischemic contractile recovery.

Method: Isolated rat hearts underwent 30-min of global ischaemia and 2-hours of reperfusion. Apelin (0.5 μM), was infused during the first 20-min of reperfusion. EGFR, MMP or Src were inhibited to study the pathway connecting APJ to PI3K. Key components of RISK pathway, namely PI3K, guanylyl-cyclase or mitochondrial K^+-ATP channels were also inhibited. Apelin-induced EGFR and PTEN phosphorylation were assessed. Left ventricular pressure and infarct size were measured.

Results: Apelin-induced reductions of infarct size and myocardial contracture were prevented by inhibition of EGFR, Src, MMP, or RISK pathway. The involvement of EGFR was confirmed by its phosphorylation. However, neither direct EGFR nor MMP inhibition affected apelin-induced improvement of early post-ischemic contractile recovery, which was suppressed by Src and RISK inhibitors only. Apelin also increased PTEN-phosphorylation, which was removed by Src inhibition.

Conclusion: While EGFR and MMP limit infarct size and contracture, Src or RISK pathway inhibition abolishes the three features of cardioprotection. Src does not only transactivate EGFR, but also inhibits PTEN by phosphorylation, thus playing a crucial role in apelin-induced cardioprotection.

Introduction

Apelin is an adipokine produced in various tissues and organs, included heart and vessel [1,2]. Different apelin isoforms have been identified and classified according to the number of amino acids and structure of the molecule. Out of the various endogenous isoforms, the predominant ones are apelin-13 and pyroglutamyl-apelin-13, which are also the most active on the cardiovascular system [3,4]. Apelin exerts inotropic [5,6] and vasodilator activities [3], and protects the heart against ischaemia-reperfusion (I/R) injury [7,8]. The enhanced susceptibility of apelin null mice to ischemic injury points at an important role of this peptide in endogenous cardioprotection [9]. Exogenous apelin is considered a pharmacological postconditioning tool as it protects only if given after, but not before, ischaemia [7].

The cardioprotection induced by endogenous and exogenous apelin against I/R injury includes limitations of the infarct size and contracture with improvement of the post-ischemic contractile recovery [7–15].

While various investigations confirm the role of apelin in counteracting post-ischemic apoptosis and reducing infarct size [7,8,10–12,14,16,17], so far only a few studies have considered the mechanism by which apelin ameliorates the post-ischemic contractile recovery [9,12,15,18]. To our knowledge only the role of NO has been studied with regard to the last of these effects [12,18]. Furthermore, it is not yet clear whether limitation of infarct size, reduction of contracture and improvement of contractile recovery are produced by apelin via the same or different signalling pathways.

Enzymes of the so-called “reperfusion injury salvage kinases” (RISK) pathway [19], namely extracellular signal-related kinase 1/2 (ERK 1/2), phosphatidylinositol 3-kinase (PI3K), protein kinase B/Akt, eNOS [8,12–14,17,18,20] are known to be part of the protective pathway activated by apelin binding to its G protein-coupled receptor (GPCR), namely APJ [21]. However, the pathway connecting APJ receptor to the activation of PI3K-Akt-NO signalling pathway by apelin has not yet been clarified. Since other GPCR ligands (e.g. acetylcholine (ACh), opioids, bradykinin (BK) and adenosine [22–26] act through the transactivation of epidermal growth factor receptor (EGFR), it may be hypothesized that this receptor is involved in apelin-triggered protection.

EGFR transactivation may occur via either a *ligand-dependent* or a *ligand-independent pathway*, the former consisting in the link with the heparin-binding epidermal growth factor (HB-EGF) shed from the membrane by a matrix-metalloproteinase (MMP), the latter in the intervention of Src, a non-receptor protein tyrosine kinase that plays a multitude of roles in cell signalling [27,28]. In the present study we wanted to assess whether apelin-induced myocardial protection requires or not the involvement of EGFR transactivation and whether and how the ligand-dependent and the ligand-independent pathways are involved in each of the three features of protection, *i.e.* reduction of infarct size, limitation of contracture and improvement of post-ischemic contractile recovery. In addition, since in a study by Kleinz and Baxter on infarct size reduction [11] it is suggested that the apelin-induced cardioprotection may be RISK pathway-independent, we also aimed to assess the possible intervention of PI3K and explore the involvement of some of the

downstream components of the RISK pathway, namely cGMP and mitochondrial-ATP sensitive K^+ channels (mito K^+_{ATP}), on the three aforementioned features of cardioprotection. Since in cancer cells Src targets phosphatase and tensing homolog (PTEN) [29] a master regulator of PI3K/Akt pathway, we aim to verify the link between Src and PTEN in cardioprotection.

In isolated perfused rat hearts, apelin was given in early reperfusion before and after EGFR was inhibited either directly or via the blockade of each of the two pathways to its transactivation: to block the ligand-dependent and ligand-independent pathway, we inhibited MMP and Src, respectively. Furthermore, we inhibited the key components of the downstream RISK pathway, i.e. PI3K, cGMP and mito K^+_{ATP}. In each of these experimental conditions we analyzed the infarct size, and the time course of contracture and contractile recovery in reperfusion.

Results

EGFR transactivation and key components of RISK pathway are involved in apelin-induced reduction of infarct size

After 2 hours of reperfusion which follow 30 min of global ischemia, in Control group (CTRL) infarct size was 60±3% of the left ventricular mass. The infusion of apelin during the first 20 min of reperfusion significantly ($p<0.001$) reduced infarct size to 30±3% (Fig. 1). To study the role of EGFR, apelin was alternatively co-infused with one of the following compounds: AG1478 (AG), a direct inhibitor of EGFR; GM6001 (GM), an inhibitor of the MMP involved in the ligand-dependent EGFR transactivation; PP2, an inhibitor of Src responsible for the ligand-independent EGFR transactivation. At the end of reperfusion, in these three inhibitor groups, infarct sizes (61±6%, 52±3%, and 63±3% of the ventricular mass, respectively) were similar to those observed in control hearts, but significantly ($p<0.001$, $p<0.01$ and $p<0.001$, respectively) higher than in Apelin group (Ap).

Apelin-induced reduction of infarct size was also abrogated by the inhibition of PI3K by LY294002 (LY) and soluble guanylyl cyclase (sGC) by ODQ, as well as by the blockade of mito K^+_{ATP} by 5-hydroxydecanoic acid (5HD). At the end of reperfusion, in these three inhibitor groups, infarct sizes (61±7%, 59±7%, and 50±5% of the ventricular mass, respectively) were similar to those observed in control hearts, but significantly ($p<0.001$, $p<0.001$, $p<0.05$, respectively) higher than in Apelin group.

In the absence of apelin none of the six inhibitors produced any effect on infarct size (data not shown).

EGFR transactivation and key components of RISK pathway are involved in apelin-induced limitation of contracture

The increase of post-ischemic left ventricular diastolic pressure (LVDP) was taken as an index of contracture. During stabilization LVDP was about 5 mmHg (Fig. 2, time -35 min). The 30 min global ischaemia and the subsequent reperfusion caused a sustained increase of LVDP in CTRL (Fig. 2). The increase started before the end of ischaemia

and continued during the first 10 min of reperfusion**,** reaching a value of about 70 mmHg. Then LVDP decreased progressively to about 45 mmHg after 2 hours of reperfusion. The increase in LVDP was significantly ($p<0.001$) reduced by apelin and remained around 30 mmHg for the entire period of reperfusion.

Similarly to the reduction of infarct size, the limitation of contracture was abolished when apelin was co-infused with the direct inhibitor of EGFR as well as with the inhibitors of MMP or Src. Also the inhibition of the key components of RISK protective pathway, namely PI3K, sGC and mito K^+_{ATP} suppressed the apelin-induced limitation of contracture.

When given alone, the six inhibitors did not produce any effect on ischemic and post-ischemic changes of LVDP (data not shown).

Apelin-induced improvement of post-ischemic contractile recovery is mainly mediated by Src and key components of RISK pathway

As indices of myocardial contractile recovery after ischaemia we analyzed the recovery of left ventricular developed pressure (LVDevP) and of the maximum rate of positive change in LVP (dP/dt_{max}) (Figs 3-4 and 6-7). As an index of lusitropic recovery, we analyzed the negative change in LVP (dP/dt_{min}) (Figs 5 and 8).

In panel 3a, the values of LVDevP in CTRL and Apelin group are compared with those of apelin plus each of the inhibitors of EGFR transactivation. In panel 3b, the values of LVDevP in CTRL and Apelin group are compared with those of Apelin+inhibitors of PI3K, cGMP and mito K^+_{ATP} located downstream EGFR. As expected, in control group ischaemia impaired the mechanical performance, as evidenced by the dramatic reduction of LVDevP immediately after ischaemia followed by an incomplete recovery during reperfusion. In fact, LVDevP fell to about 0 mmHg during ischaemia, recovered to about 20% of pre-ischaemic value after 20 min of reperfusion and was only 35% at the end of reperfusion (Fig. 3a and b).

Apelin swiftly improved the recovery of LVDevP, which reached about 70% of the pre-ischemic value at the end of the 20 min of apelin administration and remained in the range of a 70-80% for the entire period of reperfusion (Fig. 3a and b). Surprisingly, the inhibition of either EGFR or MMP, with AG or GM respectively, affected only partially the apelin-induced improvement of LVDevP in the late phase of reperfusion (Fig. 3a). Indeed, when apelin was co-infused with each of these two inhibitors (Fig. 3a) a significant ($p<0.05$ for Ap+AG and $p<0.01$ for Ap+GM *vs* CTRL) increase in LVDevP at the time points from 10 to 30 min was observed. This increase was almost identical to that of Apelin group during the first 30 min of reperfusion. Then, LVDevP declined slowly to a value that at the end of reperfusion was intermediate between CTRL and Apelin group. Thus, the LVDevP for Ap+AG and Ap+GM groups at the time points from 40 to 120 min of reperfusion did not result significantly different from either CTRL or Apelin group.

By comparing each other the patterns of the continuous recovery of LVDevP throughout the reperfusion period, in

Ap+AG and Ap+GM groups the recovery resulted significantly lower ($p<0.001$) than in Apelin group and higher ($p<0.001$) than in CTRL (Fig. 3a).

Of note, unlike what observed with AG and GM, the inhibition of Src, removed the effect of apelin on the continuous LVDevP recovery during the reperfusion ($p<0.001$ *vs* Apelin, Ap+AG and Ap+GM groups; p =ns *vs* CTRL).

Co-infusion of apelin with each of the antagonists of the downstream components of RISK pathway (*i.e.*, PI3K. sGC, or mito K^+_{ATP}; Fig. 3b) abolished the apelin-induced improvement of LVDevP recovery for the entire period of reperfusion ($p<0.001$ *vs* Apelin, and p= ns *vs* CTRL, for these three groups).

As it may be seen in Fig. 4, in all groups the recovery of dP/dt_{max} was similar to the recovery of LVDevP reported in Fig. 3. In particular, in Apelin group and in Ap+AG or Ap+GM groups, after 20 min of reperfusion dP/dt_{max} recovery was about 60% of pre-ischemic value ($p<0.01$ *vs* CTRL for all three groups). Thereafter, dP/dt_{max} reached about 70% and remained unchanged until the end of reperfusion in Apelin group, while it declined to 45-55% in Ap+AG or Ap+GM groups (Fig. 4a). As observed for LVDevP, when apelin was co-infused with each of these inhibitors the continuous recovery of dP/dt_{max} throughout the reperfusion period was significantly lower ($p<0.001$) than in Apelin group and higher ($p<0.001$) than in CTRL. The post-ischaemic recovery of dP/dt_{min} (Fig. 5) was similar to that of dP/dt_{max}.

When administrated alone, no inhibitor modified the recovery of LVDevP (Fig. 6), dP/dt_{max} (Fig. 7) and dP/dt_{min} (Fig. 8). These results allowed us to rule out the opinion that the observed recovery of LVDevP (Fig. 3a), was due to an inotropic effect of inhibitors in Ap+AG or Ap+GM groups.

Apelin increases EGFR and PTEN phosphorylation when it is given in early reperfusion.

Western blot analysis showed that apelin administration induced an increase in EGFR phosphorylation in myocardial tissue with respect to the I/R control group (Fig. 9a).

As it may be seen in Fig 9b, apelin infusion increased PTEN phosphorylation while the inhibition of Src by PP2 reduced the amount of phosphorylated PTEN to a value similar to the I/R control.

Discussion

In the present study, we demonstrate for the first time the role of EGFR and its activators (Src and MMPs) and confirm the involvement of downstream components of RISK pathway, namely PI3K-Akt-NO-cGMP-mito K^+_{ATP}, in apelin-induced limitation of I/R injury.

As regards the connection of APJ to PI3K, our data suggest that the involvement of EGFR pathway varies depending on which of the three features of protection, *i.e.* reduction of infarct size, limitation of contracture and improvement of post-

ischemic mechanical recovery, is considered. In particular, apelin effect on infarct size and contracture is abolished by PP2, AG and GM, inhibitors of Src, EGFR and MMP respectively, whereas the improvement of post-ischemic contractile recovery was suppressed only by the inhibition of Src. Therefore, among the inhibitors of EGFR pathway, only PP2 abolishes all the studied features of apelin-induced protection.

Various studies report that the reduction of cell death in response to cardioprotective agents, such as adenosine, ACh, BK and opioids, takes place *via* either a ligand-dependent [22–26,30] or a ligand-independent [31] EGFR transactivation. However, these investigations did not consider the post-ischemic cardiac mechanical function. To the best of our knowledge, the role of EGFR in mechanical recovery was reported only in a study by Williams-Pritchard group [26], which however considered only the role of ligand-dependent pathway in the adenosine-induced improvement of cardiac recovery, without investigating the reduction of infarct size. These authors concluded that EGFR plays a pivotal role in cardioprotection and signalling responses to A1 adenosine receptor stimulation.

In the present investigation, either the direct inhibition of EGFR or the blockade of each of the two pathways to its transactivation, completely suppressed the apelin-induced reduction of infarct size and removed contracture, showing that these two features of cardioprotection are EGFR-dependent, as reported for other cardioprotective compounds [22,23,25]. The hypothesis of the involvement of EGFR is corroborated by the apelin-induced increase in EGFR phosphorylation demonstrated with Western Blot. The novelty in our study is that a joint activity of the ligand-dependent and ligand-independent pathway is required in apelin limitation of infarct size and contracture, which in ischaemia-reperfusion are both characterized by intracellular Ca^{2+} overload [32].

Importantly, the direct inhibition of EGFR fails to remove the improvement of post-ischemic mechanical recovery, which is abolished only with the inhibition of Src. The recovery of myocardial contractility during the time-course of reperfusion is strongly improved by apelin, whose effect is differently affected depending on which inhibitor was co-infused. Apelin produces a better and faster recovery of LVDevP which, if compared to the control, is significantly increased after 10 min of reperfusion and reaches the maximum value after 30 min, to remain almost unchanged until the end of the observation period. In the initial 20 min of reperfusion, the improvement of LVDevP recovery could be totally removed only by inhibiting the ligand-independent pathway with Src inhibition, while it is unaffected by MMP and EGFR inhibition, as it may be seen in Fig. 3a, where the curves of LVDevPs of Ap+GM and Ap+AG groups are initially superimposed to that of Apelin group. It may then be argued that the recovery of contractility during reperfusion is dependent on Src, whereas MMPs and EGFR do not show any role, because their inhibition does not affect the initial recovery of LVDevP by apelin. Similar changes were observed in the recovery of both dP/dt_{max} and dP/dt_{min}.

In reperfusion the dying cells and the hypocontractility (stunning) of the viable tissue cause a reduction of developed

pressure. Indeed, the number of dying cells increases during reperfusion when the lethal injury can be prevented by a postconditioning intervention immediately after the end of ischaemia [33]. Apelin given in reperfusion attenuates the reduction of LVDevP by reducing cell death and limiting hypocontractility. Thus, in Apelin group at the beginning of reperfusion it is not surprising to see that LVDevP increases suddenly to a value (70% of the pre-ischemic one) which persists until the end of the 2 hours of reperfusion. Surprisingly, when apelin is co-infused with AG or GM, the swift recovery of contractility in early reperfusion is preserved, suggesting that in the presence of these inhibitors apelin still exerts an inotropic effect, thus causing a limitation of the stunning. In the late phase of reperfusion, in the presence of these two inhibitors, *i.e* in Ap+AG or Ap+GM groups, LVDevP is intermediate between the values observed in Apelin and in control groups (Fig. 3). This late reduction of developed pressure in Ap+AG and Ap+GM can be explained by the increasing number of dead cells, as evidenced by a greater extent of the infarction in these two groups with respect to Apelin group (Fig. 1). Since in Ap+AG, Ap+GM groups the infarcted area has a similar extent to that of CTRL, and given that in these two groups LVDevP is intermediate between the values observed in Apelin and CTRL groups, we suggest that apelin-induced improvement of post-ischemic contractile recovery is, at least in part, independent of the reduction of infarct size. Since it has been reported that MMP inhibitors may improve contractility [34], it might be argued that the improvement in contractile recovery observed in Ap+GM group in reperfusion is due to the *per se* effect of MMP inhibition. However, we demonstrated that the recovery of LVDevP, dP/dt_{max} and dP/dt_{min} is not improved either when GM, the MMP inhibitor, or AG, the direct EGFR inhibitor, are administrated in the absence of apelin (Figs 6a, 7a and 8a). The lack of inotropic effect of these two inhibitors is in line with what previously observed by Williams-Pritchard group [26].

Inasmuch as neither the direct EGFR inhibition with AG nor the inhibition of MMP with GM affect the activity of apelin on the early post-ischemic contractile recovery, it is likely that Src kinase activation can ameliorate the contractile recovery in reperfusion *via* a pathway which neglects EGFR.

Src kinase does not only activate EGFR, but it can also play several additional roles, including the inhibition of PTEN as observed in cancer cells [29]. In the past years, PTEN was mainly studied in tumors [35]. Recently, it has been observed that PTEN and PI3K/Akt play crucial roles in myocardial hypertrophy, fibrosis, remodeling and I/R damage [36–38]. Indeed, PTEN activity counteracts PI3K/Akt signalling activation. Moreover, it has been suggested that PI3K itself is inadequate for the activation of Akt signalling in heart preconditioning without the key contribution of the inactivation of PTEN [39], induced by its oxidation or phosphorylation [37]. Therefore, in additional experiments we investigated whether PTEN phosphorylation is induced by apelin *via* Src intervention. The results indicate that apelin increases PTEN phosphorylation, an effect that is strongly limited by the Src inhibitor PP2. These data confirm that in addition to EGFR, Src kinase has multiple targets, thus explaining Src pivotal role in apelin-induced cardioprotection. Although

here we demonstrate, for the first time, the existence of a link between Src and PTEN in cardioprotective context, whether and how PTEN affects apelin-induced improvement of contractile recovery remains to be ascertained.

Apelin has been considered one of the most potent endogenous inotropic substances [6]. However, while apelin-induced enhancement in contractility in failing heart is well demonstrated [40–42], in normal conditions it is still a matter of controversy [5–7,9,42,43]. After I/R, apelin has always been seen to induce an improvement in post-ischemic mechanical recovery, mediated by an increase in NO production [7,12]. More recently, it has been suggested that the improvement in mechanical recovery could be due to the apelin-induced preservation of SERCA activity with suppression of myocardial contracture brought about by Ca^{2+} overload [9]. It is possible that the preservation of SERCA is due to the NO-induced removal of the inhibitory effect of phospholamban [44]. Indeed, the preservation of SERCA activity may be responsible for the improvement of inotropy and lusitropy, as well as of the limitation of contracture. Inasmuch as both contracture and infarct size are Ca^{2+} overload-dependent, SERCA activity preservation may be responsible, at least in part, for the infarct size reduction that we observed.

The diagram reported in figure 10 describes the relationship of APJ receptor with EGFR transactivation and with Src-PTEN in response to apelin administration in reperfusion. Indeed, our data clearly show that the three features of cardioprotection can be achieved only if PI3K and Src are both activated by apelin. Since Src inhibits PTEN by phosphorylation, our results are in line with the opinion that in regulating Akt signalling both PI3K activation and PTEN inhibition are required to achieve cardioprotection [39,45]. The suppression of all features of apelin-induced cardioprotection by either Src or PI3K inhibition or by the inhibition of downstream components of RISK pathway (GC and mito K^{+}_{ATP} channels), suggests that the RISK pathway is essential for the apelin-induced cardioprotection and that the Src kinase activity converges on components of RISK pathway, at least *via* PI3K/Akt signalling. Whether and how other targets of Src are involved in determining apelin-cardioprotection remains to be investigated.

In conclusion, different is the role of EGFR and Src in apelin-induced three features of cardiac protection against I/R injury. While apelin effect on infarct size and myocardial contracture is abolished by direct EGFR inhibition, as well as by the inhibition of Src and MMP, the improvement of post-ischemic contractile recovery is only suppressed by the inhibition of Src. We can assert that EGFR is not involved in improving contractility because its inhibition does not affect the initial recovery of LVDevP induced by apelin. The pivotal role of Src might be attributed to the fact that this kinase does not only transactivate EGFR, but also affects the function of PTEN by phosphorylation. Although we do not know how Src is involved in contractile recovery, we suggest that Src-activated cell signalling converges on components of RISK pathway, because our data clearly indicate that these components are essential in all the three features of apelin-induced cardioprotection.

Materials and methods

Animals

Adult male Wistar rats (Harlan-Italy, S. Pietro al Natisone, Italy) weighing 300-400 g were housed three per cage in a ventilated cage rack system under standard conditions. The animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The purposes and the protocols of our studies have been approved by the Ministero della Salute, Rome, Italy and by the Ethical Committee of the University of Turin.

Chemicals

All the reagents required for the assessment of infarct size, as well as those for the perfusion buffer, were purchased from Sigma-Aldrich (Milan, Italy).

The other compounds were purchased as follows: apelin 13 was purchased from American Peptide (American Peptide, Vista, Ca, USA); GM6001 (2R)-N'-hydroxy-N-[(2S)-3-(5H-indol-3-yl)-1-methylamino-1-oxopropan-2-yl]-2-(2-methylpropyl) butanediamide) from Calbiochem (Merck Millipore, Darmstadt, Germany); LY294002 (2-Morpholin-4-yl-8-phenylchromen-4-one) from Cayman Chemical Company (Ann Arbor, Michigan. USA); AG14782 (Morpholin-4-yl-8-phenylchromen-4-one,4-(3- chloroanilino)-6,7-dimethoxyquinazoline), PP2 (l-tert-Butyl-3-(4-chlorophenyl)-1H-pyrazolo[3,4- d]pyrimidin-4-amine), 5-HD (5-hydroxydecanoic acid) and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1- one) were obtained from Sigma-Aldrich.

Isolated Heart Preparation

Ten minutes after intramuscular injection of heparin (1000 IU/kg body weight)[46], the animals were anaesthetized as previously reported [18,47]. The absence of blink and paw withdrawal reflex was checked before the rats were sacrificed. After thoracotomy, the hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer (127 mM NaCl, 17.7 1.26 mM $MgCl_2$, mM $NaHCO_3$, 1.5 mM $CaCl_2$, 5.1 mM KCl, 11 mM D-glucose and 10 μg/ml xylocaine).

Then the hearts were attached to a Langendorff apparatus, so that the coronary arteries were perfused through the aorta at constant flow with the above Krebs-Henseleit buffer in a non-recirculating way. The buffer was saturated with a 95% O2 and 5% CO2 gas mixture and infused at 37°C as previously described [18,37,48].

The coronary flow was adjusted at 9±1 ml/min/g with a constant-flow perfusion pump (Watson-Marlow 505DU, Falmouth, Cornwall, UK) which kept the coronary perfusion pressure (CPP) at 80 - 85 mmHg during the stabilization

period. The same flow level was maintained throughout the entire time-course of the experiments. CPP was continuously monitored with an electronic pressure transducer (Monitoring kit mk5- 02 DTNVF, Abbott, Milan, Italy) connected to the perfusion line [18,37,47]. A small hole in the left ventricular wall allowed the drainage of the thebesian flow.

A polyvinyl chloride balloon was placed in the left ventricle through the left atrium, filled with saline, and connected via a catheter to a pressure transducer to record the left ventricular pressure (LVP). The saline in the balloon was kept at a volume that produced an end diastolic LVP of about 5 mmHg. No change of ventricular volume was allowed during the experiments [18,37,47]. LVP and CPP were continuously recorded with a data acquisition system (Lab-View software, National Instrument Corporation, Austin, Texas, USA). To keep the heart rate constant throughout the experimental protocol, the hearts were paced at 280 bpm with a Grass S11 stimulator (Grass Instruments, Quincy, Mass, USA). At the beginning of the ischaemia pacing was stopped and it was restarted after the 3rd min of reperfusion [18,37,47].

Experimental Protocols

Hearts were explanted from 70 rats. Out of these hearts, only 66 were used because 4 were discarded due to the very low, if any, developed pressure after connection to the perfusion line.

After 20 min of stabilization, all the hearts underwent I/R which consists in 30 min of global ischaemia followed by 120 min of reperfusion [16,18,48–51] . Global ischaemia was obtained by arresting the perfusion pump. During ischaemia the hearts were kept at 37°C by the surrounding buffer. Each heart was randomly assigned to one of the following experimental protocols (Fig. 11):

Control group (CTRL, n = 9): these hearts underwent I/R without any treatment.

Apelin group (Ap, n = 9): after ischaemia, these hearts were perfused with 0.5 μM apelin-13 during the initial 20 min of reperfusion to mimic ischemic postconditioning. The dose of Apelin was chosen on the basis of our previous dose-response study, in which the dose sufficient to reduce reperfusion injury resulted 0.5 μM [18]. The apelin-13 fragment was chosen because it is reported to exhibit the most potent myocardial protective effect [3].

Apelin+inhibitors groups (n = 39): to study the involvement of EGFR in apelin-induced cardioprotection we used: AG1478 (AG, 0.3 μM; n = 7) to inhibit the EGFR tyrosine kinase activity [30]; GM6001 (GM, 0.5 μM; n =8) to inhibit MMPs involved in the activation of EGFR in a ligand-dependent manner through the cleavage of the HB-EGF [30] and PP2 (2 μM; n = 7) to block Src kinase involved in the ligand-independent EGFR activation [52]. To study the pathway downstream EGFR, we blocked the key components PI3K, cGMP and mito K^+_{ATP} of RISK pathway using the following compounds: LY294002 (LY, 50 μM; n = 5), a potent PI3K blocker ODQ (10 μM; n = 6), a selective and irreversible inhibitor of the sGC [51]; 5-HD (100 μM; n = 6), a selective mito K^+_{ATP} antagonist [48,53].

Each of the above inhibitors was given starting 5 minutes before ischaemia and during the first 25 min of reperfusion,

thus bracketing the 20 min infusion of apelin. Inhibitors were also tested alone at the same concentration and for the same periods. Doses and schedules of administration of apelin-13 and inhibitors were chosen on the basis of previous results of our and other groups in similar experiments [18,30,48,51,52,54]. When necessary the doses were adjusted on the basis of the absence of their influence on cardiac contractility in basal conditions, as observed in pilot experiments. The inhibitors AG, PP2 and ODQ were dissolved in dimethyl sulphoxide (DMSO) at the final concentration < 0.01%. As previously reported [47,55], the administration of DMSO alone at this concentration did not modify infarct size and post-ischemic cardiac function.

Assessment of infarct size

Infarct size was assessed with the nitro-blue-tetrazolium staining technique as previously described [18,37,47,51,56,57]. In brief, at the end of the experiments each heart was rapidly removed from the perfusion apparatus and the left ventricle was cut into 1-2 mm thick short-axis slices. After 20 minutes of incubation in 0.1% solution of nitro-blue-tetrazolium in phosphate buffer at 37°C, the unstained necrotic tissue was carefully separated from the stained viable tissue and then weighed by an independent observer in a blinded manner. Since ischaemia was global, the total left ventricle corresponded to the risk area. Thus, the necrotic mass was expressed as a percentage of the left ventricle.

Cardiac function assessment

Left ventricular pressure was measured as previously described [18,37,47,51]. LVDevP was calculated as the difference between systolic and diastolic pressure. Also dP/dt_{max} and dP/dt_{min} were calculated. During reperfusion these three parameters were expressed as percent of the baseline values before ischaemia. The changes in LVDevP and dP/dt_{max} were taken as indices of contractility (i.e. inotropy), while dP/dt_{min} was taken as index of diastolic function (i.e. lusitropy). Also LVDP increase was compared to the corresponding baseline value. Since the volume of the intraventricular balloon was kept constant throughout the experiment, LVDP increase was taken as an index of myocardial contracture [48,58]. Assessments of contractility and contracture were made throughout the entire time course of reperfusion.

Western Blot

Three groups (n=3 per each group) of additional experiments were performed. As a control the first group underwent I/R only. The other two groups received apelin with or without PP2. In all groups, after 10 minutes of reperfusion, the left ventricle was isolated and freeze-clamped in liquid nitrogen and then stored at −80°C. Myocardial tissue was processed as previously described [18]. 40 μg of total lysates were size-fractionated by SDS–PAGE in 4–12% gels (Invitrogen), and electroblotted onto polyvinylidene difluoride membranes (PVDF) (Amersham-GE Healthcare,

Buckinghamshire, UK). Membranes were blocked with TBS-T/BSA 1% or 5% w/v for 1 h at room temperature, followed by overnight incubation at 4°C with the following antibodies: anti-p-EGFR(Tyr1173) (1:200) and anti-EGFR (1:500) (from SantaCruz); anti-p-PTEN(Ser380/Thr382/383) (1:1000) and anti-PTEN (1:1000) (from Cell Signaling); GAPDH (1:1000) (SantaCruz). Immunoreactive bands were detected by incubating with a secondary antibody conjugated with horseradish peroxidase and enhanced chemiluminescence reagent (Pierce). Protein amounts were analyzed using Image J analysis software version 1.50i and normalized to their respective control. For EGFR, the phosphorylated protein was normalized on the total EGFR; for PTEN, since the analyzed phosphorylation of Ser/Thr residues within the C-terminal region could modulate the amount of total PTEN protein [59,60], GAPDH was first used to ensure equal loading of the samples and then both p-PTEN and total PTEN were normalized on the control I/R.

Statistical analysis

Data are expressed as means ± S.E.M. One-way ANOVA with Tukey post-test was performed to evaluate the significance of the differences among groups in infarct size and in continuous cardiac mechanical function throughout the reperfusion period. Two-way repeated measure ANOVA with Bonferroni post-test was used to evaluate the statistical significance of the differences in contractile function among groups at each time point selected every 10 min starting from the end of ischaemia.

All analyses were performed with GraphPad Prism version 5.00 (GraphPad Software, San Diego California, USA), with $p<0.05$ as the significant cut-off.

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Author contributions

Conception and design of the experiments: PP and RR. Performing the experiments: LA, AF and RR. Analysis and interpretation of the data: AF, LA, CG, PP, GL, PGM and RR. Drafting the article: GL, PP and RR. Revising the manuscript critically for important intellectual content: AF, LA, CG, PP, GL, PGM and RR.

Conflict of interest

The authors declare that they have no conflict of interest.

Declaration

The material submitted is conform with Good Publishing Practice in Physiology.

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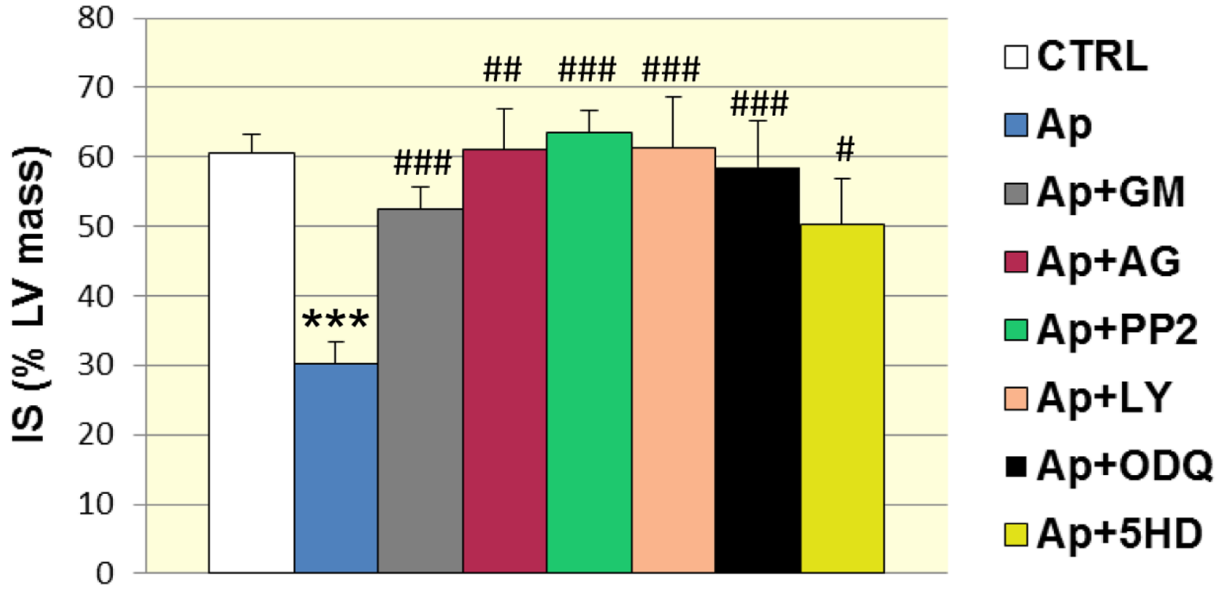

Figure 1. Infarct size (IS) at the end of 120 min of reperfusion, expressed as percent of the left ventricle (% LV) taken as the risk area. The reduction of infarct size induced by apelin (Ap) is removed by is removed by each inhibitor. Data are expressed as means±SE. *** $p<0.001$ *vs* CTRL; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ and $^{\#\#\#}$ $p<0.001$ *vs* Ap.

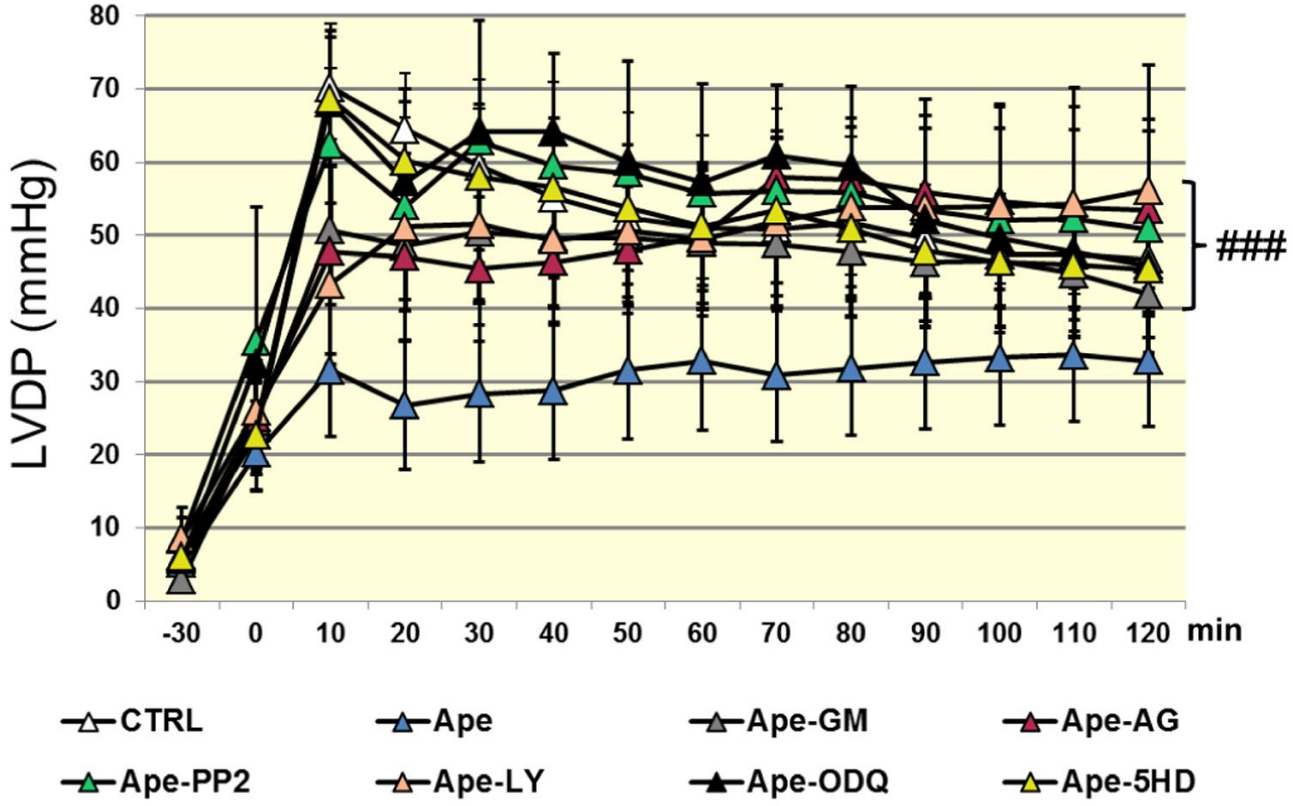

Figure 2. Changes in left ventricular diastolic pressure (LVDP) during the 30 min of ischaemia and the 120 min of reperfusion. In CTRL the increase in LVDP (contracture) begins before the end of ischaemia, and continues during the first 10 min of reperfusion. Then it decreases slightly until the end of the observation period. The limitation of contracture by apelin (Ap) is totally removed by by each inhibitor. Measurements were made at the time points (in min) indicated on *x*-axis, i.e immediately before ischaemia (-30), at the end of ischaemia (0) and every 10 min during reperfusion. Data are expressed as means±SE. Significance is related to the patterns of the continuous recovery of LVDP: $^{###}$ $p<0.001$ *vs* Ap.

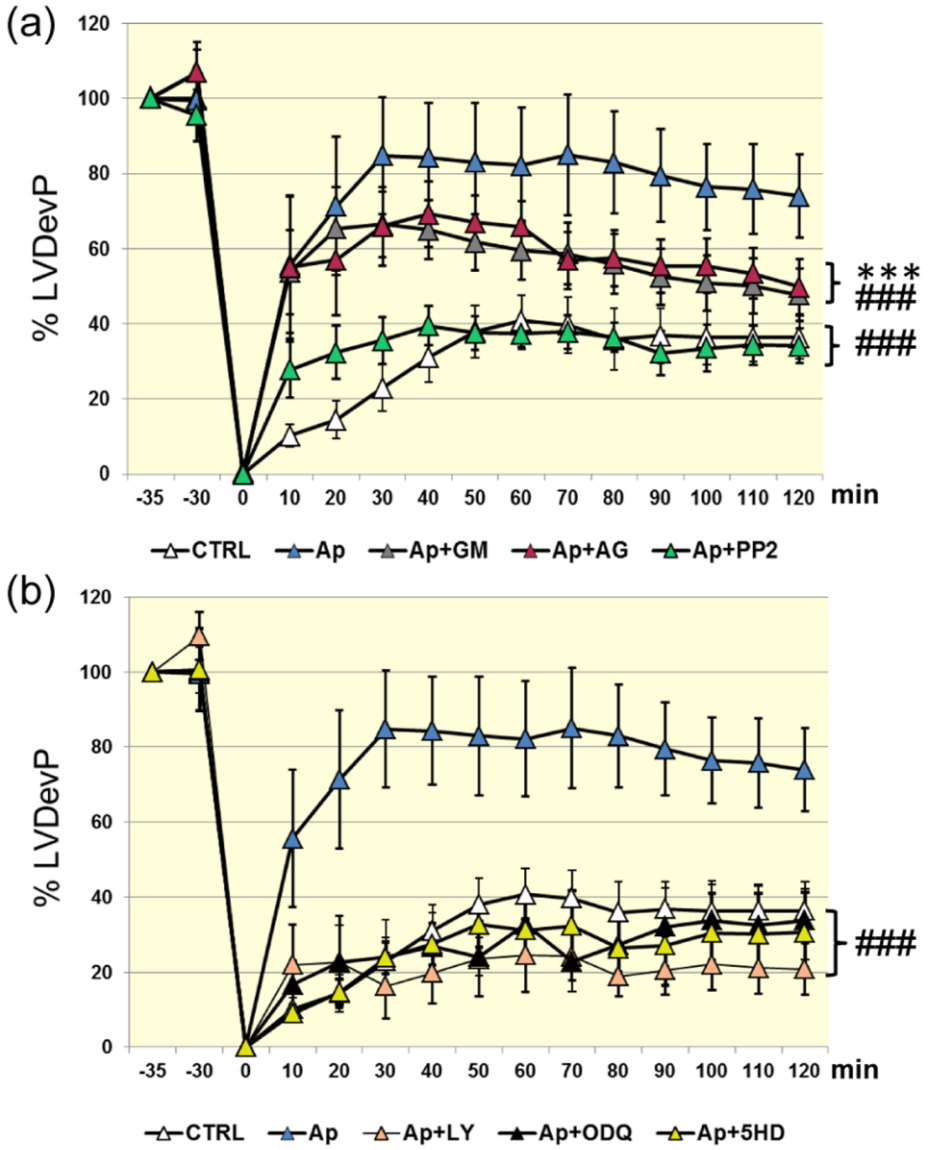

Figure 3. Percent changes of left ventricular developed pressure (LVDevP) during ischaemia and reperfusion with respect to baseline. Panel (a): the apelin-induced improvement of post-ischaemic LVDevP recovery was totally removed by Src inhibition (Ap+PP2), but only partially affected in the late phase of reperfusion after MMP and EGFR inhibition (Ap+GM and Ap+AG, respectively). Panel (b): the apelin-induced improvement of post-ischaemic LVDevP recovery was completely removed by the inhibition of the key components, of the RISK pathway by LY, ODQ and 5HD. In both panels (a) and (b), the time points (in min) on *x*-axis, are: stabilization (-35), immediately before ischaemia (-30), at the end of ischaemia (0) and every 10 min during reperfusion. Data are expressed as means±SE.Significance is related to the patterns of the continuous recovery of LVDevP: *** $p<0.001$ *vs* CTRL; ### $p<0.001$ *vs* Ap. A point to point comparison revealed that, at 10, 20 and 30 min of reperfusion, LVDevP in Ap+GM and Ap+AG groups was not statistically different with respect to Apelin group, but significantly different with respect to CTRL ($p<0.01$ for Ap+GM *vs* CTRL; and $p<0.05$ for Ap+AG *vs* CTRL; these significances are not reported in the figure).

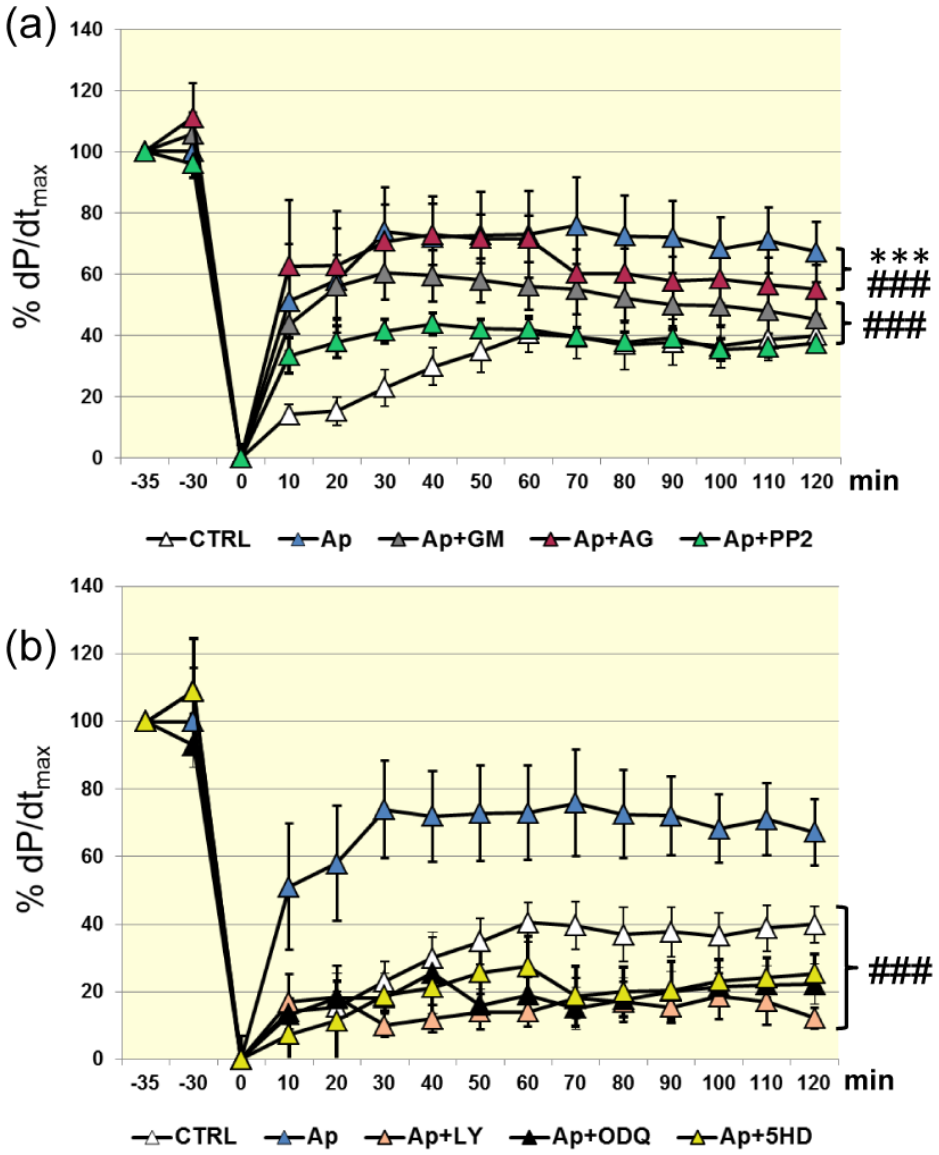

Figure 4. Percent changes of dP/dt_{max} during ischaemia and reperfusion period with respect to normalized baseline. Panel (a): the apelin-induced improvement of post-ischaemic dP/dt_{max} recovery was completely removed by Src inhibition (Ap+PP2), but only partially affected in the late phase of reperfusion after MMP and EGFR inhibition (Ap+GM and Ap+AG, respectively). Panel (b): the apelin-induced improvement of post-ischaemic dP/dt_{max} recovery was completely removed by the inhibition of the key components PI3K, sGC and mito K^{+}_{ATP} of the RISK pathway by LY, ODQ and 5HD respectively. In both panels (a) and (b), measurements were made at the time points (in min) indicated on *x*-axis, are: stabilization (-35), immediately before ischaemia (-30), at the end of ischaemia (0) and every 10 min during reperfusion. Data are expressed as means±SE. Significance is related to the patterns of the continuous recovery of dP/dt_{max}: *** $p<0.001$ *vs* CTRL; $^{###}$ $p<0.001$ *vs* Ap. A point to point comparison revealed that, at 10, 20 and 30 min of reperfusion, dP/dt_{max} recovery in Ap+GM and Ap+AG groups was not statistically different with respect to Apelin group, but significantly different with respect to CTRL ($p<0.01$ for Ap+AG *vs* CTRL; and $p<0.05$ for Ap+GM *vs* CTRL; these significance are not reported in the figure).

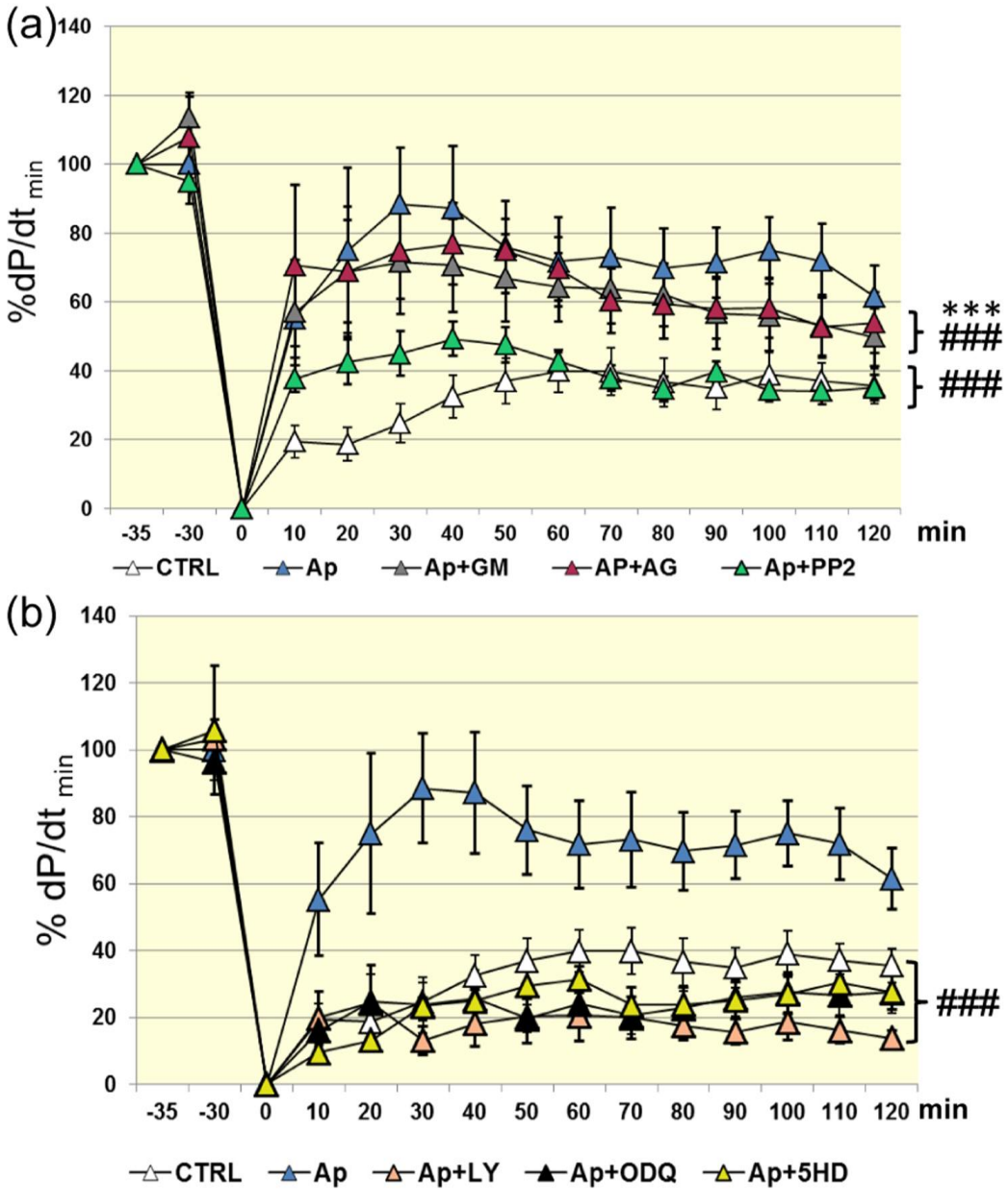

Figure 5. Percent changes of dP/dt_{min} during ischaemia and reperfusion period with respect to normalized baseline. Panel (a): the apelin-induced improvement of post-ischaemic dP/dt_{min} recovery was completely removed by Src inhibition (Ap+PP2), but only partially affected in the late phase of reperfusion after MMP and EGFR inhibition (Ap+GM and Ap+AG, respectively). Panel (b): the apelin-induced improvement of post-ischaemic dP/dt_{min} recovery was completely removed by the inhibition of the key components PI3K, sGC and mito K^{+}_{ATP} of the RISK pathway by LY, ODQ and 5HD respectively. In both panels a and b, measurements were made at the time points (in min) indicated on *x*-axis, are: stabilization (-35), immediately before ischaemia (-30), at the end of ischaemia (0) and every 10 min during reperfusion. Data are expressed as means±SE. Significance is related to the patterns of the continuous recovery of dP/dt_{min}: *** $p<0.001$ *vs* CTRL; $^{###}$ $p<0.001$ *vs* Ap.

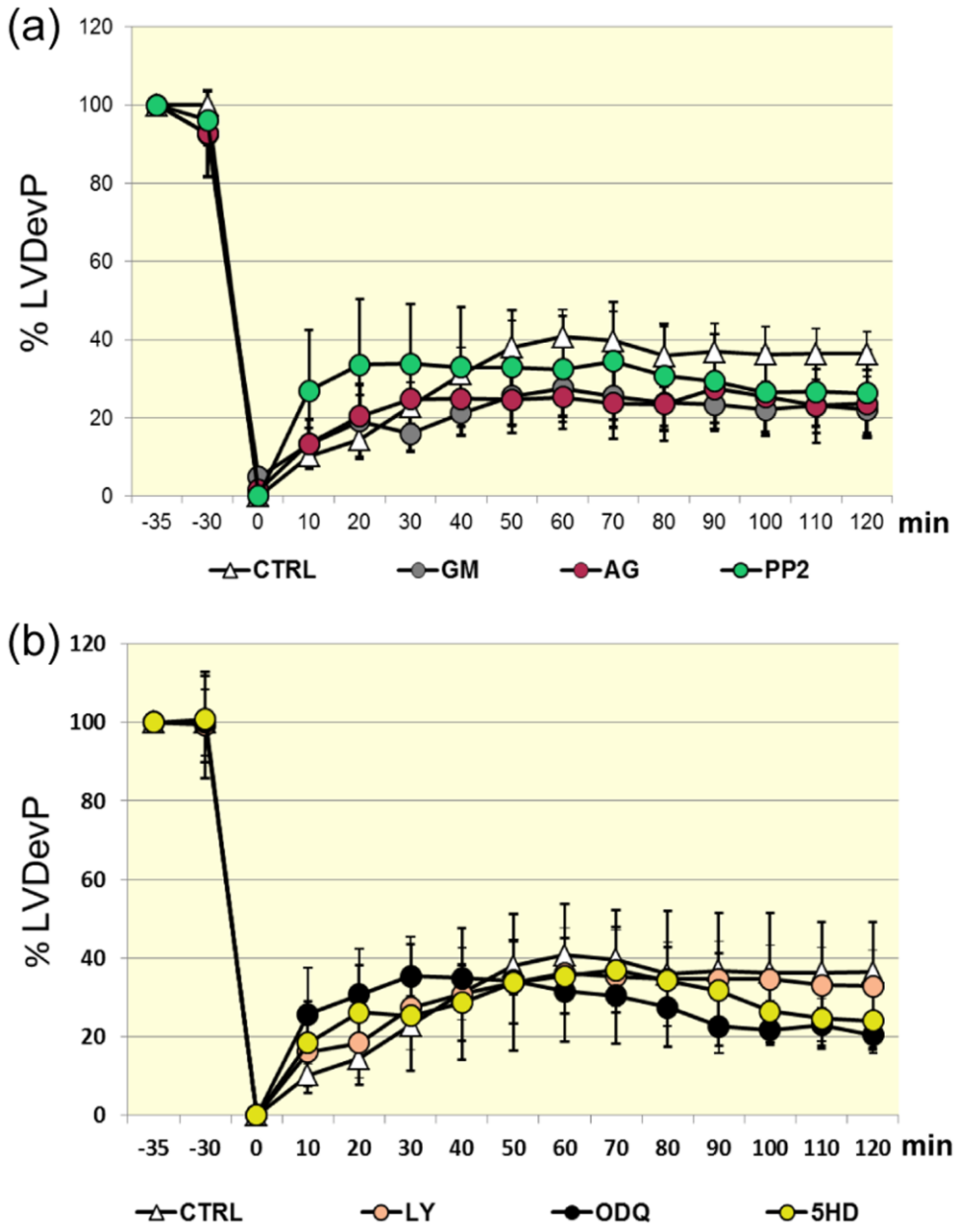

Figure 6. Percent changes of left ventricular developed pressure (LVDevP) during ischaemia and reperfusion with respect to normalized baseline. The post-ischaemic recovery of LVDevP was not affected by by each inhibitor alone. Panel (a): inhibition of epidermal growth factor receptor with AG and of its activators MMP and Src with GM and PP2 respectively; Panel (b): inhibition of the key components PI3K, sGC and mito K^{+}_{ATP} of the RISK pathway by LY, ODQ and 5HD respectively. In both panels, the traces after inhibitors are superimposed to the trace of CTRL. In panels a and b, measurements were made at the time points (in min) indicated on *x*-axis are: stabilization (-35), immediately before ischaemia (-30), at the end of ischaemia (0) and every 10 min during reperfusion. Data are expressed as means±SE.

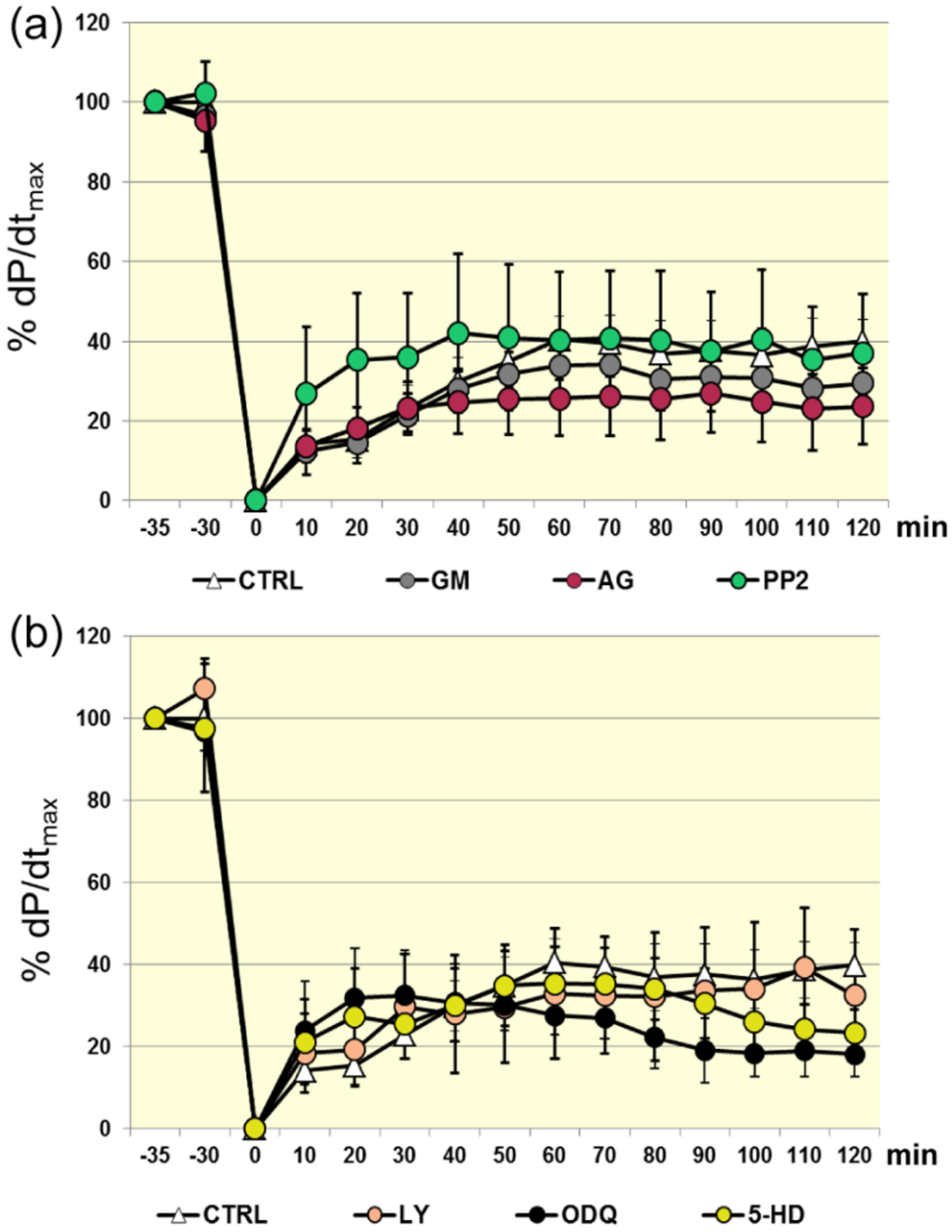

Figure 7. Percent changes of dP/dt_{max} during ischaemia and reperfusion with respect to normalized baseline. The post-ischaemic recovery of dP/dt_{max} was not affected by each inhibitor alone. Panel (a): inhibition of EGFR with AG and of its activators MMP and Src with GM and PP2 respectively; Panel (b): inhibition of the key components PI3K, sGC and mito K^{+}_{ATP} of the RISK pathway by LY, ODQ and, 5HD respectively. In both panels, the traces after inhibitors are superimposed to the of CTRL. In panels a and b, measurements were made at the time points (in min) indicated on *x*-axis are: stabilization (-35), immediately before ischaemia (-30), at the end of ischaemia (0) and every 10 min during reperfusion. Data are expressed as means±SE.

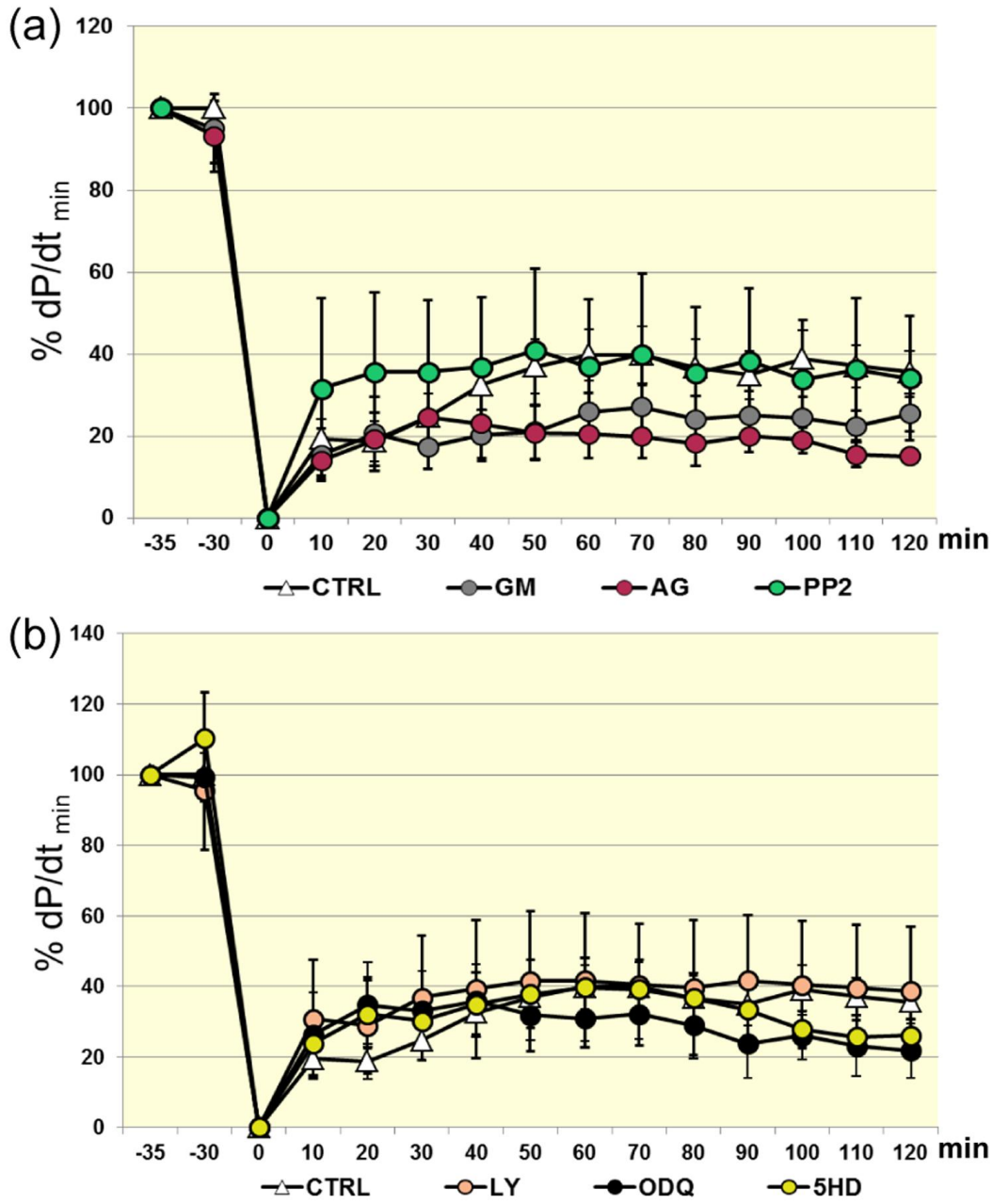

Figure 8. Percent changes of dP/dt_{min} during ischaemia and reperfusion with respect to normalized baseline. The post-ischaemic recovery of dP/dt_{min} was not affected by each inhibitor alone. Panel (a): inhibition of EGFR with AG and of its activators MMP and Src with GM and PP2 respectively; Panel (b): inhibition of the key components PI3K, sGC and mito K^{+}_{ATP} of the RISK pathway by LY, ODQ and, 5HD respectively. In both panels, the traces after inhibitors are superimposed to the trace of CTRL. In panels a and b, measurements were made at the time points (in min) indicated on *x*-axis are: stabilization (-35), immediately before ischaemia (-30), at the end of ischaemia (0) and every 10 min during reperfusion. Data are expressed as means±SE.

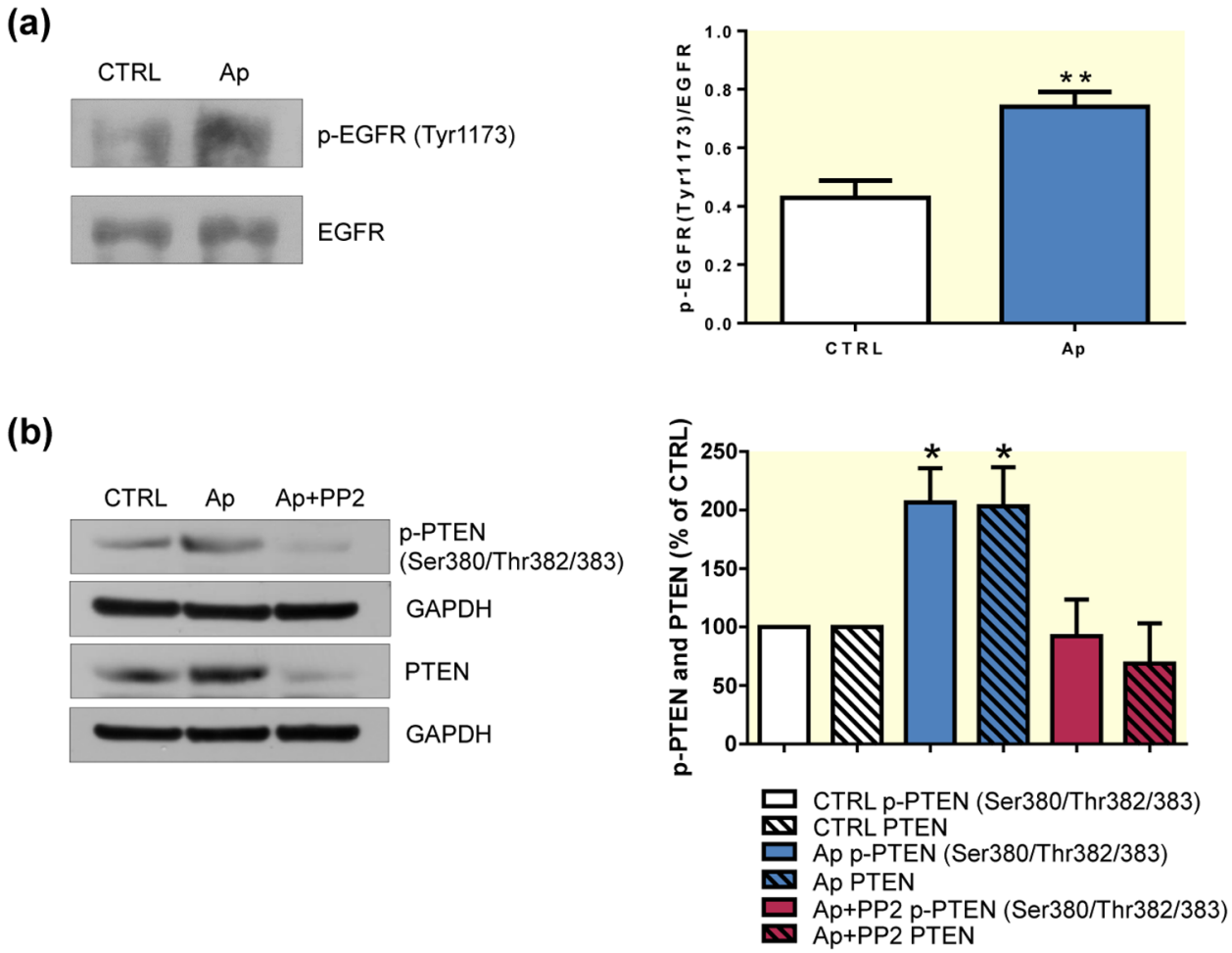

Figure 9. Phosphorylation of EGFR and PTEN in left ventricular lysates from control (CTRL), Apelin (Ap) or Ap+PP2 groups, after 10 min of reperfusion. Panel (a): Representative WB of EGFR phosphorylation (Tyr1173) and total EGFR of protein extract from CTRL and Ap groups. Histograms represent the ratio of phosphorylated over total EGFR protein expression which reveals that apelin induces an increase in EGFR phosphorylation with respect to CTRL. Data are expressed as arbitrary unit ± SE. ** $p<0.01$ *vs* CTRL. Panel (b): Representative WB of PTEN phosphorylation (Ser380/Thr382/383) and total PTEN of protein extract from CTRL, Ap and Ap+PP2 groups and their relative GAPDH. Histograms represent the variation in PTEN protein phosphorylation and total expression in Ap or Ap+PP2 groups compared to CTRL. The analysis reveals that apelin increases PTEN phosphorylation which return to a value similar to CTRL in the presence of Src inhibition by PP2. Data are reported as percent variation ± SE *vs* CTRL. * $p<0.05$ *vs* CTRL.

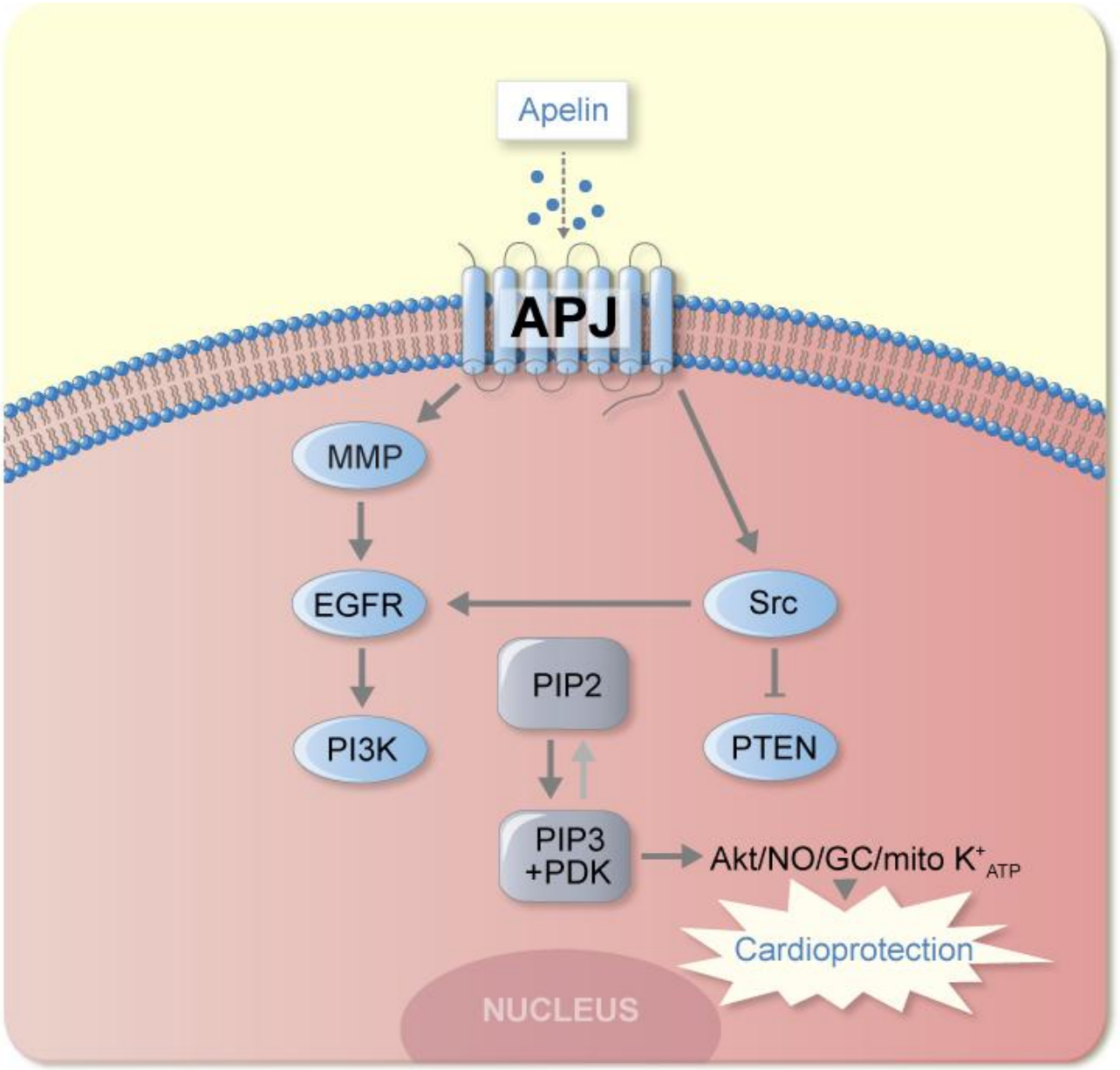

Figure 10. Apelin signalling to protection. The binding of apelin to APJ induces cardioprotection via MMP-EGFR and Src pathways. EGFR transactivation by MMP and Src activates PI3K. Src does not only transactivate EGFR, but also inhibits PTEN. PI3K activation and PTEN inhibition lead to conversion of phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) resulting in Akt activation, opening of mito K^{+}_{ATP} channels and protection, i.e. reduction of infarct size and contracture plus improvement of post-ischemic contractile recovery.

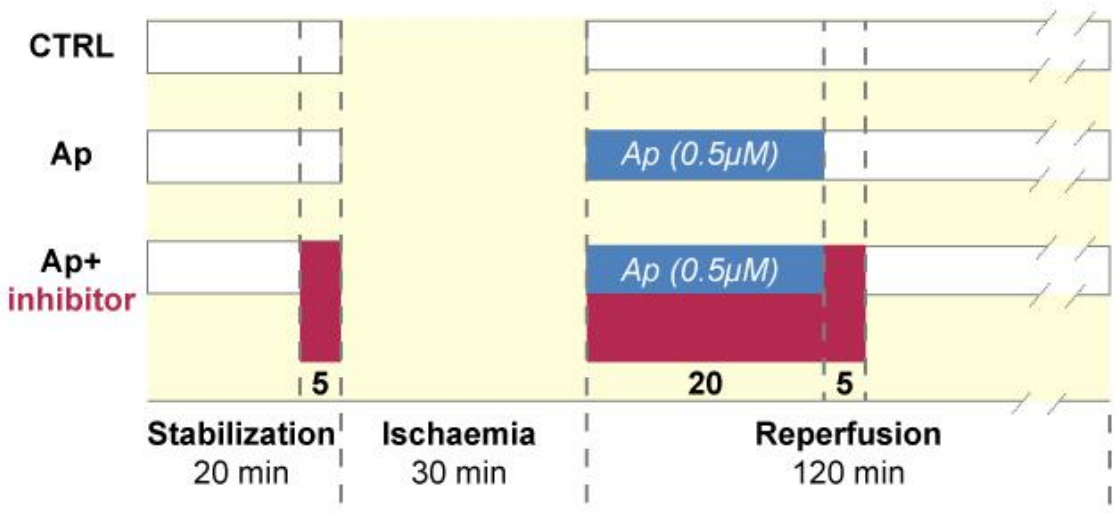

Figure. 11. Experimental protocols. CTRL: the isolated Langendorff-perfused hearts after stabilization underwent 30 min of global ischaemia followed by 120 min of reperfusion; Ap: apelin-13 (0.5 μM) was infused during the initial 20 min of reperfusion; Ap + inhibitor groups: apelin was infused during the first 20 min of reperfusion while the infusion of each inhibitor started 5 min before ischaemia and continued for the first 25 min of reperfusion.