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Effect of Apelin-Apelin Receptor System in Postischaemic Myocardial Protection: A Pharmacological Postconditioning Tool?

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Abstract

In the heart, a great part of ischaemia and reperfusion injuries occurs mainly during the first minutes of reperfusion. The opening of the mitochondrial permeability transition pores is the end point of the cascade to myocardial damage. Also, oxidative stress contributes to cell death. Postconditioning is a protective maneuver that can be selectively timed at the beginning of reperfusion. It is hypothesized that it acts *via* the reperfusion injury salvage kinase pathway, which includes nitric oxide–dependent and nitric oxide–independent cascades. Apelin is an endogenous peptide that can protect the heart from reperfusion injury if given at the beginning of reperfusion but not before ischaemia. It is hypothesized that it may trigger the reperfusion injury salvage kinase pathway *via* a specific apelin receptor. Apelin can also limit the oxidative stress by the activation of superoxide dismutase. Apelin and apelin receptor expression increase early after ischaemia and at the beginning of an ischaemic heart failure. These observations suggest that the endogenous release of the peptide can limit the severity of an infarction and ameliorate myocardial contractility compromised by the appearance of the failure. Due to its protective activities, apelin could be a therapeutic tool if administered with the same catheter used for angioplasty or after the maneuvers aimed at bypassing a coronary occlusion. *Antioxid. Redox Signal.* 14, 909–922.

Introduction

Ischaemia-reperfusion injury

ALTHOUGH THE MYOCARDIAL INJURY that follows ischaemia and reperfusion (I/R) is related to the duration of the ischaemia, it has also been seen that a large component of the damage takes place during the first minutes of reperfusion when oxygen overflow amplifies ischaemic injury or causes additional damage (6, 56, 63, 114, 120, 122). This timing of the injury occurrence explains the efficacy of the postconditioning maneuvers in limiting the infarct size and in ameliorating the mechanical recovery of the heart (74).

Inadequate resynthesis of ATP, loss of membrane phospholipids, oxidative stress by reactive oxygen species (ROS), and intracellular Ca^{2+} overload have been suggested as contributing to reperfusion injury (18, 56, 58, 76). The most important pathophysiological derangements of reperfusion injury are reperfusion arrhythmias, transient mechanical dysfunction of the heart or "myocardial stunning," and cell death (41).

In injured areas, the hypercontracture of cardiomyocytes plays a critical role in sarcolemmal rupture and cell death (4, 35, 58, 65, 74, 81, 86). In particular, it has been demonstrated that oxidative stress and calcium overload can induce the abrupt opening of the mitochondrial permeability transition pores (MPTP), which in the presence of Ca^{2+} overload strongly contributes to hypercontracture (81).

The opening of MPTP has been proposed as the almost key event in the transition from reversible to irreversible reperfusion injury, that is, cell death, during the early minutes of reperfusion (28). In fact, the opening of these channels causes a further production of ROS as well as mitochondrial swelling and uncoupling of mitochondrial oxidative phosphorylation, which result in myocardial cell necrosis and apoptosis (5, 18). The importance of MPTP as a target for myocardial protection has been described in several investigations (27, 32, 40, 87).

Beside their role in causing the opening of MPTP, other interplays exist between oxidative stress and Ca^{2+} overload. It has been reported that *in vitro* lipid peroxidation caused by ROS can result in an intense cellular damage when coupled with Ca^{2+} overload (12, 94). Moreover, 4-hydroxy-2-nonenal, a product of lipid peroxidation, has been seen to induce Ca^{2+} overload *via* a further generation of ROS in cardiac myocytes (66). These findings suggest that, in addition to synergistic

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damaging effect of free radicals and Ca^{2+} on membrane function, there is the possibility of a positive feedback between intracellular Ca^{2+} accumulation and ROS. This hypothesis is reinforced by the observation that oxidative stress resulting from ROS generation can lead to oxidative modification of contractile proteins directly or indirectly by causing Ca^{2+} overload (10).

In a recent review, Inserte *et al.* (35) focused their attention on the relationship between Ca^{2+} overload and MPTP opening. Two pathways are indicated as responsible for alterations of Ca^{2+} handling, that is, a hypoxic impairment of Na^+/K^+-ATPase and a reduced intracellular pH. In both cases, the result is an activation of Na^+/H^+ exchanger (NHE) and, consequently, of Na^+/Ca^{2+} exchanger (NCE) (74, 75). This altered Ca^{2+} handling is responsible for hypercontracture and contributes to the opening of MPTP, which, in turn, worsens hypercontracture, by preventing the mitochondrial energy production needed for the reactivation of Na^+/K^+-ATPase (32, 81). Cellular Ca^{2+} overload, which persists until the restoration of the activity of Na^+/K^+-ATPase, contributes to cell apoptosis also by activating calpain that hydrolyzes cytoskeleton proteins (36).

Free radicals and reperfusion injury

ROS play an important role in I/R injury. They can modify and inactivate proteins, lipids, DNA, and RNA and induce cellular dysfunctions (1, 11, 15, 21, 79, 124).

Superoxide anion ($O_2^{-}\bullet$) is produced in reperfusion in response to the activity of xantine oxidase and NADPH oxidase (NADPH OX). Xantine oxidase, which is present in both neutrophils and vascular endothelium (44, 123), causes the release of $O_2^{-}\bullet$ *via* its activity on adenosine-derived hypoxanthine, whereas neutrophil NADHP OX transforms molecular O_2 into $O_2^{-}\bullet$. ROS are reported to originate also from cardiomyocytes (22, 96, 100). The activation of neutrophils requires their adhesion to the vascular endothelium, which occurs rather late during reperfusion (24) (Fig. 1), a phenomenon normally inhibited by a physiological endothelial release of nitric oxide (NO) (53, 78). Moreover, the production of $O_2^{-}\bullet$ can be greatly enhanced when mitochondrial respiratory chain is stimulated under conditions of altered redox state, as occurs in I/R (56).

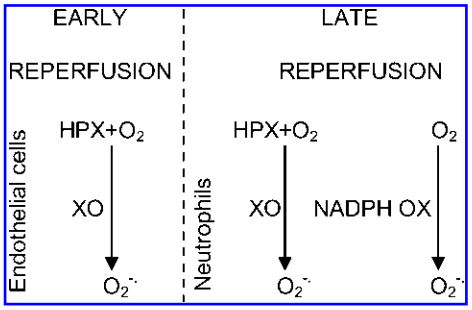

FIG. 1. Production of superoxide anion in early (immediately after the end of ischaemia) and late (after adhesion of neutrophils to the endothelium) reperfusion. During the first minutes of reperfusion, endothelial xantine oxidase causes the production of superoxide anion ($O_2^{-}\bullet$) by acting on hypoxanthine (HPX). Later, after adhesion to the vascular endothelium, neutrophils also release xantine oxidase and induce the same reaction. Neutrophils also release NADPH oxidase (NADPH OX) that transforms molecular oxygen (O_2) into $O_2^{-}\bullet$.

Superoxide anion can be transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), which is significantly reduced in cardiomyocytes that have undergone hypoxia and reoxygenation (H/R) (118). In the presence of Fe^{2+} or Cu^{+}, hydrogen peroxide is transformed into the highly toxic hydroxyl radical (OH•) (45) (Fig. 2).

Superoxide anion can also be scavenged by NO with production of the highly reactive peroxynitrite ($ONOO^{-}$) (84). The toxicity of $ONOO^{-}$ is attributed to its split-off generating the highly reactive nitrogen dioxide radical (NO_2•) and OH• (Fig. 2). It is likely that also the inhibition exerted by $ONOO^{-}$ on the mitochondrial respiratory chain is mediated by NO_2• and $O_2^{-}\bullet$ (7).

It has been speculated that in reperfusion, a burst of $ONOO^{-}$ takes place during the first minute after the end of ischaemia (23). Since neutrophil can produce $O_2^{-}\bullet$ only after the adhesion to the vascular endothelium, only vascular endothelial cells are responsible for the early production of $ONOO^{-}$. Later in reperfusion, when the adhesion occurs, neutrophils also start to produce $O_2^{-}\bullet$, which results in $ONOO^{-}$ formation (24). In rat hearts that have previously undergone regional ischaemia, $O_2^{-}\bullet$ production has been seen after 5 h of reperfusion together with NO production by inducible NO synthase, confirming that NO, superoxide anion, and peroxynitrite may be involved in I/R injury (60). Although it is accepted that the first minutes of reperfusion are determinant for irreversible cell damage, Liu *et al.* (60) suggest that oxidative stress can also occur late after the end of ischaemia.

Physiological protective systems against oxidative stress have been developed by almost all animal species. They

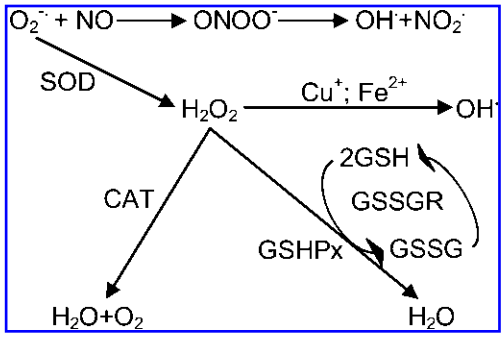

FIG. 2. Removal of superoxide anion ($O_2^{-}\bullet$) by nitric oxide (NO) and superoxide dismutase (SOD). In the presence of NO, $O_2^{-}\bullet$ is scavenged to form peroxynitrite ($ONOO^{-}$), which splits off into highly active hydroxyl radical (OH) and nitrogen dioxide radical (NO_2). The presence of $O_2^{-}\bullet$, $ONOO^{-}$, OH, NO_2 is responsible for cell dysfunction and death. In addition to this pathway, a protective role can be exerted by SOD. In the presence of SOD, $O_2^{-}\bullet$ can be converted into the less toxic hydrogen peroxide (H_2O_2), which can be transformed into water (H_2O) as a result of the oxidization of reduced glutathione (GSH) into glutathione disulfide (GSSG) by glutathione peroxidase (GSHPx). The reaction can be reverted by glutathione-reductase (GSSGR). Also, catalase may have a protective effect by transforming H_2O_2 into H_2O and O_2.

mainly consist of various enzymes such as SOD, catalase (CAT), and glutathione peroxidase-glutathione reductase system. Also, glutathione and plasma proteins exert an antioxidant function (115). However, this endogenous antioxidant system is not sufficient to exert a protection when a large amount of ROS is produced in reperfusion (Fig. 2).

Pre- and Postconditioning against I/R injury

On the bases of the above described pathways to reperfusion injury, the main targets for the procedures aimed at limiting reperfusion injury are represented by the prevention of Ca^{2+} overload, oxidative stress, and the MPTP opening. Both ischaemic preconditioning (IP) and ischaemic postconditioning (PostC) have been demonstrated to significantly attenuate I/R injury. Although IP can be obtained with one or more brief coronary occlusions of 2–3 min each before the infarcting ischaemia, PostC may be performed with one or more brief occlusions of a few seconds, starting very early (a few seconds) after the end of the ischaemia (90). The main advantage of PostC with regard to IP consists in the real possibility of clinical application. In fact, due to the unpredictability of an ischaemic event, IP is mainly a theoretical approach, whereas PostC can be utilized with angioplasty procedures (99). As a matter of fact, the protective role of PostC has emphasized the importance of reperfusion in inducing I/R injury. Since the postC maneuvers are not protective if they are carried out after the first minutes of reperfusion (49), this period seems to be confirmed as the interval when most damage takes place.

Initially, the opening of mitochondrial ATP-sensitive potassium ($mitoK^+_{ATP}$) channels by protein kinase C (PKC) was considered the almost final step to myocardial protection (30, 85, 105). The cascade leading to the activation of PKC was supposed to be triggered by both adenosine released by the hypoxic cardiomyocytes and NO produced in response to the release of bradykinin by a kininogenase activated by the hypoxia-induced acidosis (71). Recently, the common pathway to hinder the activation of MPTP has been attributed to the so-called reperfusion injury salvage kinase (RISK) pathway (33, 89, 91), which includes NO-dependent and NO-independent cascades.

In the RISK pathway, both adenosine and bradykinin, released in response to either IP or PostC, bind to membrane G-protein coupled receptors, thus activating a cascade involving phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB, commonly called Akt). Akt can lead to the activation of endothelial NO synthase (eNOS) with production of NO, which is responsible for the opening of $mitoK^+_{ATP}$ channels *via* guanylyl cyclase-cGMP cascade and the activation of protein kinases G and C_ε (PKC_ε) (33). The flux of K^+ into mitochondria results in the production of ROS, which prevents the opening of MPTP through the activation of various survival kinases, such as, p38-mitogen activated protein kinase, c-Jun NH2 terminal, PKC, Akt, and tyrosine kinase (72, 89). It is noteworthy that ROS, which are usually responsible for cell injury, exert a protective role against I/R injury when they are released secondary to the opening of $mitoK^+_{ATP}$ (33) (Fig. 3). This cascade can be defined as NO-dependent.

It is likely that the protective effect of ROS after $mitoK^+_{ATP}$ opening depends on the timing and limited extent of their release. The importance of ROS in myocardial protection seems to be confirmed by the observation that pretreatment of isolated rat heart with H_2O_2 improves contractile recovery and metabolic function after reperfusion (111).

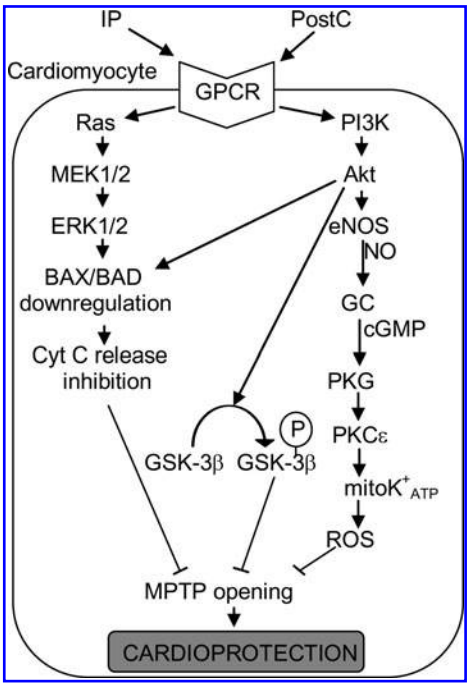

FIG. 3. The reperfusion injury salvage kinase pathway leading to myocardial protection by preventing the opening of mitochondrial permeability transition pores (MPTP). Ischaemic preconditioning (IP) and postconditioning (PostC) induce the myocardial protection by production of different mediators such as, for example, adenosine and bradykinin that bind to a G-protein coupled receptor (GPCR), which initiates an NO-independent protective cascade, by activating Ras, a protein leading to cell growth, differentiation, and survival and an NO-dependent cascade, by activating phosphoinositide 3-kinase (PI3K). In the NO-independent cascade, Ras downregulates BAX/BAD proapototic proteins *via* a mitogen activated extracellular regulated kinase 1/2 (MEK1/2) and an extracellular signal regulated kinase 1/2 (ERK1/2). BAX/BAD inhibit the release of cytochrome C (Cyt C), thus preventing the opening of MPTP. BAX/BAD can also be directly downregulated by protein kinase B (Akt). In the NO-dependent cascade, PI3K activates endothelial NO synthase *via* Akt. The resultant NO activates guanylyl cyclase (GC), which leads to the production of cyclic guanosin monophosphate (cGMP). The subsequent activation of protein kinase G (PKG) and ε isoforms of protein kinase C ($PKC\varepsilon$) causes the opening of mitochondrial ATP-dependent potassium ($mitoK^+_{ATP}$) channels followed by the production of reactive oxygen species (ROS) responsible for the inhibition of MPTP. An inhibition of MPTP is also induced by another NO-independent cascade triggered by the phosphorylation of glycogen synthase kinase 3β (GSK3β) by Akt.

Since the opening of MPTP is responsible for the release of a further amount of ROS (5), it is likely that the activation of $mitoK^+_{ATP}$ channels by NO-cGMP cascade contributes to the limitation of the oxidative stress by preventing MPTP opening.

The protective role of NO also includes the reduction of ROS production by neutrophils due to the inhibition of their adherence to vascular endothelia (24).

The activation of Akt can also prevent the opening of MPTP, in an NO-independent cascade, *via* the phosphorylation of glycogen synthase kinase-3β as well as *via* the downregulation of BAX/BAD proapoptotic proteins of β-cell lymphoma-2 (Bcl2) family with inhibition of cytochrome C release. BAX/BAD downregulation can also be the result of another NO-independent cascade starting from Ras protein activation by G-protein coupled receptor and including mitogen-activated extracellular regulated kinase 1/2 (MEK 1/2) and extracellular signal regulated kinase 1/2 (Fig. 3). Thus, if we consider the activation of G proteins as triggering the RISK pathway, we can assume that the latter includes an NO-dependent and two NO-independent cascades.

In isolated cardiomyocytes, Sun *et al.* (92) have shown that PostC limits postischaemic oxidative stress by an improved activation of SOD, which has been seen to be responsible for the reduction of superoxide anion as detected with lucigenin chemiluminescence. This increase in SOD activity can also explain the attenuation of lipid peroxidation as revealed by the reduction of malonilaldehyde (MDA) levels.

In vivo, Fang *et al.* (23) demonstrated that when remote PostC maneuvers are performed after the end of a 30 min occlusion of left coronary artery, a significant increase in SOD activity associated with a decrease in MDA level was measured in rat hearts at the end of 2 h of reperfusion (23). It is obvious that this antioxidant activity characterizes PostC rather than IP. On the basis of these findings, it can be argued that the reduction of lipid peroxidation observed *in vivo* is likely to be due not only to an attenuation of the inflammatory response but also to a replacement of the highly oxidant superoxide anion by the less toxic H_2O_2 in response to the activity of SOD.

Also, the Janus kinases-signal transducers and activators of transcription (STAT) pathway have been seen to lead to myocardial protection. It has been suggested (8, 112) that the protection by PostC is dependent on STAT-3 protein level, although it is a common opinion that the Janus kinases-STAT pathway to protection is mainly involved in late protection by IP (9, 55, 110).

Possible detrimental role of reactive nitrogen species in reperfusion

In spite of the general opinion about the beneficial effect of NO in IP and PostC, we have seen that the mechanism leading to reperfusion injury also suggests the possibility of a detrimental role in reperfusion, when the reaction with $O_2^{-}\bullet$ originates $ONOO^{-}$, which splits off into $NO_2\bullet$ and $OH\bullet$ (24) (Fig. 2). It has been suggested that the beneficial or detrimental activity of NO is mediated by the amount and timing of its release. In fact, a physiological (about 10 nM) endothelial concentration of NO does not prevent the transformation of $O_2^{-}\bullet$ into H_2O_2 by SOD leading a very little $ONOO^{-}$ production in early reperfusion, whereas a large concentration ($>1\,\mu$M) may result in the prevalence of the synthesis of $ONOO^{-}$ over the dismutation of $O_2^{-}\bullet$ in late reperfusion. This hypothesis is consistent with the observation that either NO-donors or NOS-inhibitors can reduce I/R, if given at appropriate concentrations (112).

Although NO can contribute to myocardial injury with the mediation of $ONOO^{-}$, the possibility of a beneficial effect of $ONOO^{-}$ mediated by NO has also been suggested (103). This effect, however, is likely to occur only with the administration of exogenous peroxynitrite (24). The authors hypothesized that $ONOO^{-}$ combines with thiol groups to form NO, which, in this case, is responsible for protection.

Apelin and Myocardial Protection

Apelin as an endogenous peptide

Apelin is an endogenous peptide initially isolated from bovine stomach (97). It has been seen to act as the ligand for the orphan G-protein-coupled apelin receptor (APJ). The expression of apelin mRNA has been found not only in the gastrointestinal tract but also in adipose tissue, brain, liver, kidney, skeletal muscle, heart, vessels, and lung (51).

Human, mouse, rat, and cow apelin cDNAs encode a 77 aminoacid preproprotein with the active sequence in the C-terminal region (48, 97). From this region, various isoforms of apelin have been obtained and classified according to the number of aminoacids. Although apelin-13 and -36 are the most frequently studied, C-terminal fragments also of apelin-36, that is, apelin-12, -16, -17, and -19, have been investigated (13, 20, 34, 98). Although it has been ascertained that fragments shorter than 12 amino acids are biologically inert, little information is available on the mechanisms regulating the proteolytic cleavage from preproapelin-77 to the various active isoforms and to shorter inactive C-terminal fragments. In an *in vitro* study, Vickers *et al.* (102) found that angiotensin-converting enzyme type 2 hydrolyzes both apelin-13 and apelin-36 with high catalytic efficiency. In particular, the activity on apelin-36 involves the C-terminal 13 aminoacids that are identical to those of apelin-13. Angiotensin-converting enzyme type 2 is a carboxypeptidase that negatively regulates the renin-angiotensin-aldosterone system by cleaving the active angiotensin II to biologically active (vasodilator) and inactive peptides (70). Out of the various isoforms, apelin-13 has been found to be the most active isoform on the cardiovascular system (62, 89).

A few data are available on the source of circulating apelin. Foldes *et al.* (25) suggest that the heart is the predominant source of plasma apelin. In particular, the apelin level is 200-fold higher in the atria than in the ventricles. Moreover, a positive correlation exists between atrial and plasma apelin level.

It has also been suggested that in cardiovascular apparatus, apelin is produced by endothelial cells, whereas in cardiomyocytes it is absent or below the level of detection (51, 52). In particular, according to Sheikh *et al.* (88), cardiovascular apelin is produced *in vivo* only by endothelium even in response to cardiac injury or hypoxia, as showed using an apelin-LacZ gene-target mice model. It can be argued that the high level of apelin present in the heart is due to high vascularization of the organs. However, Ronkainen *et al.* (80) observed that small isoforms (from apelin-12 to apelin-16) are secreted in the medium of neonatal cardiomyocytes after hypoxia. It is noteworthy that the isoforms present in the cells are longer than apelin-36 and that their concentrations during hypoxia do not decrease in parallel with the increase of shorter isoforms. The authors suggest that in hypoxic conditions, the release of apelin from neonatal cardiomyocytes depends on a *de novo* synthesis rather than on the depletion of a cellular store.

It has been demonstrated that the synthesis of apelin in hypoxia is mediated by the hypoxia inducible factor-1 (HIF-1)

(80, 88), a transcription factor composed of α and β subunits. Under normoxic conditions, HIF-1α protein is subject to ubiquitination and proteosomal degradation (93). On the contrary, during hypoxia, HIF-1α proteins accumulate and translocate into the nuclei where they combine with HIF-1β forming HIF-1.

The role of HIF-1 has been confirmed by the observation that in normoxic condition, desferrioxamine, a pharmacological compound able to activate HIF-1 *in vitro*, is able to increase apelin synthase and secretion. Thus, it has been argued that in normoxic condition, the low level of apelin secreted by cardiomyocytes is due to a low level of active HIF-1 (80). It is then likely that HIF-1 is a trigger of an endogenous mechanism of protection against ischaemia injury.

Apelin receptors

APJ, which possesses seven transmembrane domains and is formed by 377 aminoacids, has been found to be similar to angiotensin II receptor AT1 (59, 69). In particular, 31% of the aminoacids forming the APJ is present in the AT1 receptor (38). O'Dowd *et al.* (69) showed that the shared aminoacids are mainly located in the hydrophobic transmembrane regions. This similarity is not sufficient to allow angiotensin II to bind to APJs, suggesting a strict specificity between ligand and receptor (69, 97). Moreover, the specificity of APJ can be confirmed by the observation that apelin activity antagonizes angiotensin II effect exerted on pressure *via* AT1 receptors (2, 37).

Cardiovascular apelin is believed to be produced mainly by the endothelial cells, whereas APJs are reported to be present on both endothelial and smooth muscle cells of the vessels as well as on cardiomyocytes (48, 51, 52, 77).

Similar to apelin, APJ level also increases after hypoxia (88). However, although it is known that apelin synthesis is regulated by HIF-1, the regulating mechanism of APJ gene expression has not yet been clarified.

Apelin on cardiovascular system

Apelin is reported to improve myocardial contractility and to induce vasodilatation (2, 95).

In normal isolated rat hearts, Szokodi *et al.* (95) observed that apelin-16 induces a dose-dependent increase in contractility, which, if compared with the classical β-adrenergic response, develops more slowly and lasts 30 min or longer. In ongoing experiments carried on in our laboratory, we have studied the inotropic effect of apelin-13 on isolated rat hearts. The changes in inotropy have been assessed by the increase in left ventricular developed pressure (LVDevP). We have observed (unpublished observation) that an increase of LVDevP starts immediately at the beginning of a 20 min perfusion of apelin at the concentration of 500 nM. A 20–40% increase in LVDevP was reached within the first minute, then it declined, and was completely over 6–10 min later, even if the perfusion continued. It is possible that the difference between our data and those of Szokodi *et al.* (95) may depend on the difference in the isoforms used.

It has been reported that apelin does not change the activity of calcium and potassium voltage-dependent channels (19). The positive inotropic effect of apelin has been attributed to the activation of phospholipase C (PLC) with production of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Although IP_3 is responsible for the release of Ca^{2+} from sarcoplasmic reticulum (SR) *via* IP_3 receptor (IP_3R) opening, DAG activates NHE *via* PKC phosphorylation. In turn, the resultant increase in intracellular Na^+ concentration brought about by NHE leads to a further increase in intracellular Ca^{2+} concentration *via* the activation of NCE (39, 57, 95) (Fig. 4). The increase in intracellular Ca^{2+} concentration by apelin can also be due to the Ca^{2+}-induced opening of ryanodine channel receptors of SR (104).

Karmazyn *et al.* (47) support the hypothesis that the alcalinization of the intracellular environment due to NHE increases myofilament sensitivity to calcium, whereas other authors are of the opinion that the increase of calcium concentration alone is the main factor leading to the inotropic effect (19, 95). It is noteworthy that PKC, at least in isoforms α, δ, and ε, is also involved in cardiac protection (113).

The vasodilator effect of apelin is attributed to the release of NO from endothelial cells (38, 39, 57). As illustrated in Figure 5, the activation of eNOS is mainly attributed to G-protein-PI3K-Akt cascade (57, 121), although the PLC-DAG-IP_3 signaling pathway with increase of intracellular Ca^{2+} concentration has also been suggested (Fig. 6A) (39).

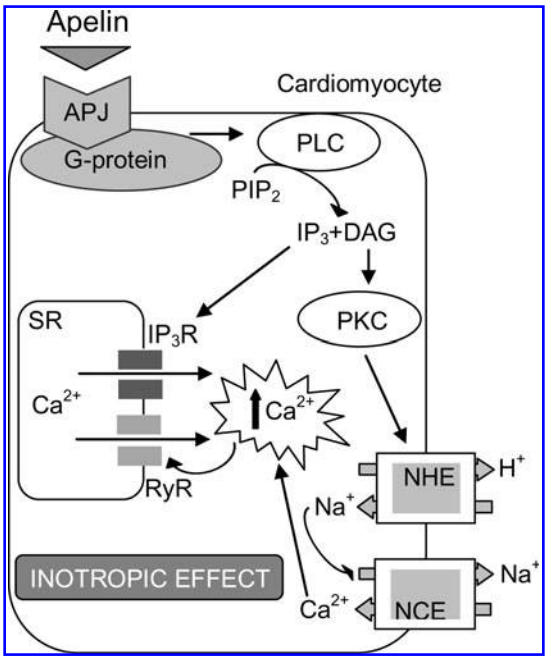

FIG. 4. Inotropic effect of apelin. In cardiomyocytes, the binding of the peptide with its specific apelin membrane receptor (APJ) activates phospholipase C (PLC) *via* a G-protein. PLC transforms phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Although IP_3 recruits Ca^{2+} by opening IP_3 receptors (IP_3R) of the sarcoplasmic reticulum (SR), DAG phosphorylates PKC. Phosphorylated PKC activates Na^+/H^+ exchanger (NHE), causing an increase in cellular Na^+ concentration, which, in turn, activates Na^+/Ca^{2+} exchanger (NCE) resulting in an increase in cardiomyocyte Ca^{2+} concentration. This initial increase in cytosolic Ca^{2+} concentration is responsible for the opening of ryanodine channel receptors (RyR) with a further release of Ca^{2+} from sarcoplasmic reticulum (SR). The overall increase in cellular Ca^{2+} concentration ameliorates myocardial inotropy.

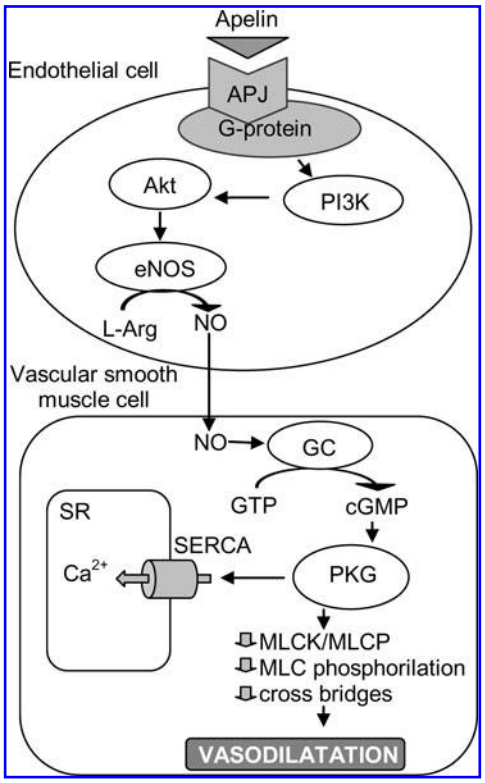

FIG. 5. Apelin-induced vasodilatation by NO released from vascular endothelial cells in a Ca^{2+}-independent manner. In the endothelial cell, the link of the peptide with APJ activates PI3K, which leads to the activation of endothelial NO synthase (eNOS) *via* Akt. The diffusion of NO into the smooth muscle cell induces a reduction of intracellular Ca^{2+} level. In fact, the activation of guanylyl cyclase (GC) transforms GTP into cGMP, which activates sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) *via* protein kinase G (PKG). Ca^{2+} is then forced into SR from cytosol. PKG also reduces the ratio between myosin light chain kinase and myosin light chain phosphatase (MLCK/MLCP). MLCP, thus, leads to dephosphorylation of myosin light chains (MLC), thereby reducing the number of cross bridges. The final result of these processes is a decrease in vascular smooth muscle tone, that is, vasodilatation.

Unlike that observed with normal endothelium, in the case of endothelial dysfunction, apelin causes vasoconstriction. In fact, if endothelial cells cannot produce NO, the binding of apelin to the APJs of smooth muscle fibres leads to their contraction. This is mediated by an intracellular Ca^{2+} increase and phosphorylation of myosin light chains brought about by PLC-DAG-IP_3 pathway (39, 57). The intracellular Ca^{2+} concentration increase seems to be operated by the opening of IP_3R of SR and then by Ca^{2+}-induced Ca^{2+} released from ryanodine channel receptors (Fig. 6B).

Apelin and myocardial protection

Apelin has been seen to protect the heart against I/R injury both *in vitro* and *in vivo* (50, 89, 91, 118). The effective concentration required for protection seems to depend on the extension of the ischaemic area. Thus, in the case of regional ischaemia in isolated rat hearts, a concentration of 10 nM significantly reduced the infarct size (50); whereas in the case of global ischaemia, significant limitations have been obtained with concentrations of 1000 nM (89, 91).

To induce myocardial protection against the damage by I/R, the proper time of apelin administration has not yet been attentively considered. Recently, in isolated rat hearts, Zheng *et al.* (118) administered apelin 20 min before and 30 min after 40 min of global ischaemia, a procedure that does not give any information whether it is better to give the peptide before or after ischaemia. Moreover, the point studied in these experiments was not the limitation of infarct size but the improvement of the mechanical recovery related to the limitation of oxidative stress and apoptosis.

In the experiments performed by Kleinz and Baxter (50) on isolated rat hearts, apelin was ineffective if given 5 min before and 15 min during a 35 min of regional ischaemia, whereas it reduced the infarct size if given 5 min during and 15 min after a similar regional ischaemia. In ongoing experiments performed in our laboratory on isolated rat hearts (unpublished observations), the administration of apelin-13 at the concentration of 500 nM was seen to significantly reduce the infarct size and to induce a better recovery of the mechanical performance only if given during the first 20 min of reperfusion after a global ischaemia, whereas no effect was obtained if the compound was infused at the same concentration before ischaemia (unpublished observation). In other words, from the above findings, it appears that apelin can mimic PostC but not IP.

In our laboratory, as reported earlier, the infusion of apelin-13 at the same concentration for a 20 min period in the absence of I/R caused only a transient increase of LVDevP, showing that the mechanical recovery which persisted for the 2 h of postischaemic reperfusion was not due to the positive inotropic effect of the compound but that it was an aspect of cardiac protection.

It has been observed that the administration of apelin during the first 10 min of reperfusion increases the phosphorylation of the kinases involved in the RISK pathway, that is, Akt and ERK1/2 (89, 91). Moreover, Simpink *et al.* (89) have demonstrated that apelin-triggered RISK pathway inhibits MPTP opening and that this protective effect is abolished by the inhibition of either PI3K or ERK 1/2, suggesting that the protection is mediated by an NO-dependent and an NO-independent cascade, respectively (Fig. 7).

According to the NO-dependent cascade, it can be expected that the inhibition of MPTP by apelin may be obtained with the opening of mitoK^+_{ATP} channels followed by the mitochondrial release of ROS and PKC activation. In our laboratory, the reduction of infarct size and the improvement of LVDevP brought about by apelin-13 were abolished by the NOS-inhibitor L-NNA (unpublished observation), showing that NO is involved as a mediator of the effects of apelin-13 on the overall protection consisting in a limitation of infarct size and improvement of postischaemic mechanical recovery. Interestingly, the recovery of LVDevP is mainly the effect of a reduction of the ventricular diastolic pressure, that is, of the hypercontracture which characterizes reperfusion injury and is attributed to Ca^{2+} overload.

The reduction of hypercontracture by apelin seems to be in contrast with the inotropic effect of the peptide mediated by the opening of the Ca^{2+} channels of SR as well as by the activation of sarcolemmal NHE-NCE system, both

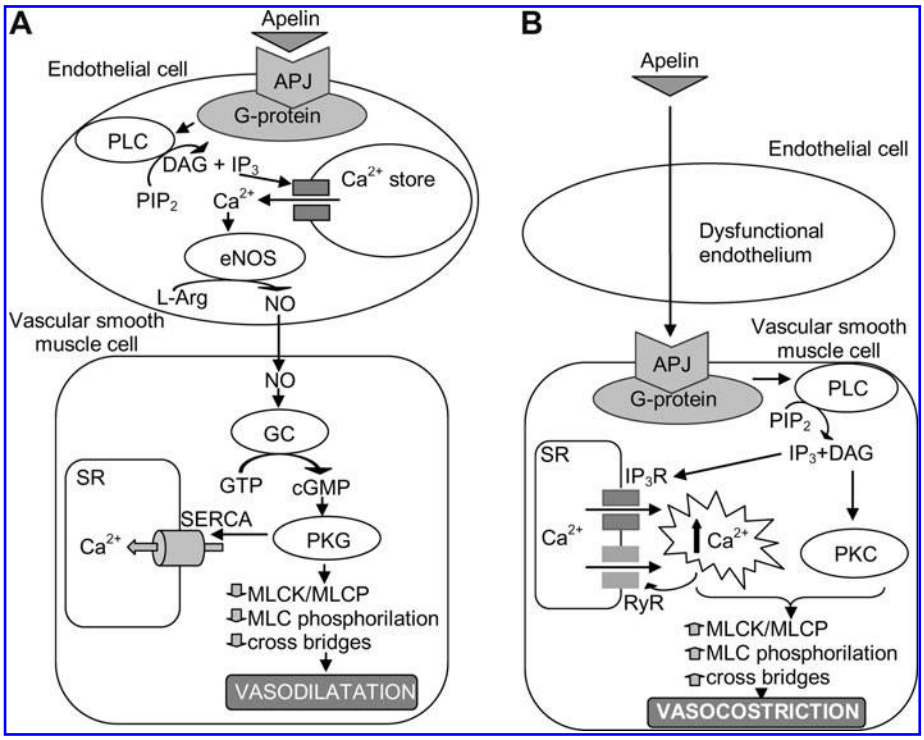

FIG. 6. Apelin-induced vasodilatation and vasoconstriction. (A) In the endothelial cell, the link of the peptide with APJ activates PLC, which transforms phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and DAG. IP_3 induces the release of Ca^{2+} from endothelial calcium stores. The increase in cytosolic concentration of calcium activates the eNOS to produce NO from L-arginine (L-Arg). The diffusion of NO into the smooth muscle cell induces a reduction of intracellular Ca^{2+} level. In fact, the activation of guanylyl cyclase (GC) transforms GTP into cGMP, which activates sarco/endoplasic reticulum Ca^{2+}-ATPase *via* PKG. Ca^{2+} is then forced into SR from cytosol. PKG also reduces the ratio between MLCK and MLCP. MLCP, thus, leads to dephosphorylation of myosin light chain (MLC), thereby reducing the number of cross bridges. The final result of these processes is a decrease in vascular smooth muscle tone, that is, vasodilatation. **(B)** When the endothelium is dysfunctional, the peptide directly binds to APJs present on smooth muscle cells. The binding activates PLC, which transforms phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and DAG. Although IP_3 induces the release of Ca^{2+} from SR, DAG phosphorylates a PKC. Enhancement of cytosolic Ca^{2+} concentration and PKC activation increases the ratio between MLCK and MLCP. MLCK, thus, leads to phosphorylation of myosin light chains (MLC), thereby raising the number of cross bridges. The final result of these processes is an increase in vascular smooth muscle tone, that is, vasoconstriction.

mechanisms resulting in an increase of intracellular Ca^{2+} concentration (95). If the hypercontracture is due to Ca^{2+} overload, its attenuation should depend on a reduction of this overload. This hypothesis is consistent with the observation that apelin can increase sarco/endoplasmic reticulum Ca^{2+}-ATPase activity, thus forcing Ca^{2+} into SR and improving myocardial relaxation (102). The result of the above complex effects of apelin on Ca^{2+} movements should be a faster rate of SR Ca^{2+} uptake and release, which explains the improvement of postischaemic mechanical recovery (104). The failure to suppress hypercontracture when apelin is given with L-NNA suggests that also the activity of apelin on sarco/endoplasmic reticulum Ca^{2+}-ATPase is mediated by NO.

The release of NO in RISK pathway may be attributed to the activation of eNOS by Akt. However, apelin induces enhancement of NO production not only by the activation of eNOS but also by an increase of eNOS protein level. In incubated aortic tissues of the rat, Jia *et al.* (42) observed that the addition of apelin increased NO-release in a concentration-dependent manner. Then, semiquantitative RT-PCR allowed them to see a 53% increase in vascular eNOS mRNA level, whereas inducible NO synthase mRNA was unaffected. The increase of eNOS mRNA level was accompanied by a more remarkable increase of eNOS protein up to 319%. Although these results indicate that apelin upregulates eNOS gene expression in vessels, it cannot be excluded that a similar effect occurs in myocardium. So far, it has been found that in neonatal rat cardiomyocytes exposed to H/R, apelin increases eNOS protein level by 40% (118). Unfortunately, to our knowledge, no data are available on increases in eNOS level when apelin is given in normal hearts in the absence of hypoxia.

The presence of L-arginine (L-Arg) is required to allow eNOS to catalyze the production of NO. An L-Arg/eNOS/NO pathway has also been indentified in vascular adventitia (67). The transport of L-Arg into the cells is mediated by cationic aminoacid transporters, which were found to increase in aortic tissue in response to apelin (42). The transport of L-Arg into the cells and the increased eNOS protein expression seem to combine with the RISK pathway in underlining the role of NO in apelin-induced myocardial protection.

The phosphorylation of ERK by apelin and the effect of its blockade (89) suggest that also the relevant NO-independent cascade elicited by the activation of G-protein is responsible for an apelin protective activity obtained *via* the downregulation of BAD/BAX proapoptotic proteins. On the contrary, in apelin protection, the involvement of glycogen synthase kinase-3β in BAD/BAX downregulation is not taken into consideration due to the lack of experimental data.

In contrast with the suggested role of the RISK pathway in myocardial apelin-induced protection, Kleinz and Baxter (50) could not suppress the protection with PI3K inhibitor wortmannin, so they concluded that the activity of apelin in reducing the infarct size is exerted *via* an unknown mechanism. It is noteworthy that the suppression obtained by Simpkin *et al.* (89) was observed with the use of PI3K inhibitor

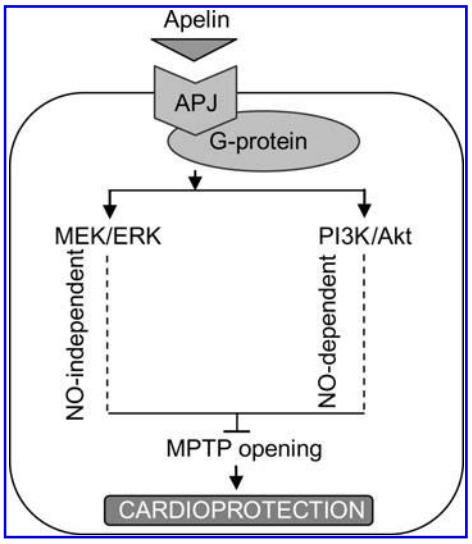

FIG. 7. Apelin and myocardial protection induced through NO-independent and NO-dependent cascades. Apelin binds to a G-protein that triggers mitogen-activated extracellular regulated kinase/extracellular signal-regulated kinase (MEK/ERK) and PI3K/Akt cascades. Both these cascades lead to the inhibition of MPTP resulting in cardioprotection. In the figure, the two cascades are simply indicated as NO-independent and NO-dependent. In the case of MEK/ERK cascade, the inhibition of MPTP is due to the inhibition of cytochrome C that follows the down-regulation of BAD/BAX antiapoptotic proteins. This cascade is NO-independent. In the case of PI3K/Akt cascade, the inhibition of MPTP is mediated by NO produced by the activity of endothelial NO synthase. In this NO-dependent cascade, NO activates gunylyl cyclase that transforms GTP into cGMP. The resultant activation of protein kinase G causes the opening of mitochondrial ATP-dependent K^+ channels, which leads to the inhibition of MPTP *via* the production of reactive oxygen species.

LY294002 instead of wortmannin. It is likely that the different effect depends on the different specificity of the two inhibitors.

Apelin and oxidative stress

The role of apelin in limiting the oxidative stress has been studied in rat myocardium after I/R and in isolated cardiomyocytes after H/R. In isolated cardiomyocytes after H/R, Zeng *et al.* (118) observed an increased proportion of cells positive for dihydroethidium fluorescence, an index of superoxide concentration. The presence of apelin in the culture medium starting 30 min before the hypoxic insult significantly reduced dihydroethidium fluorescence. Apelin also reduced the production of MDA in both types of experimental preparations. It is note-worthy that apelin was seen to improve SOD activity, which was otherwise reduced by the hypoxic and ischaemic maneuvers (118). A reduction of MDA by apelin had been previously described by Jia *et al.* (43).

According to the RISK pathway hypothesis, apelin leads to the activation of eNOS and, consequently, to NO release (89). Since NO prevents mitochondrial oxygen damage and lipid peroxidation (68, 109), we can argue that apelin can also protect the heart against oxidative stress due to the antioxidant properties of NO.

During reperfusion, large amounts of $O_2^-\bullet$ are released by both endothelial cells and neutrophils as well as by cardiomyocytes (96). The simultaneous presence of $O_2^-\bullet$ and NO can result in the production of peroxynitrite, which decomposes into highly oxidant free radicals. Since there is a competition between NO and SOD for superoxide anion, the increased activity of SOD by apelin can remove a large fraction of $O_2^-\bullet$ and reduce the production of $ONOO^-$, thus limiting the oxidative stress.

It is possible that in a late phase of reperfusion, the protection exerted by NO is also related to its ability to inhibit the production of $O_2^-\bullet$ through the inactivation of neutrophil NADPH OX (26). Also, a rapid conversion of peroxynitrite into nitrite and nitrate cannot be excluded (73). Unfortunately, no investigation has so far been performed to see whether the protection may be achieved with the administration of apelin in a late phase of reperfusion due to its ability to enhance SOD activity.

The enhancement of SOD activity by apelin leads to the production of H_2O_2 (118). This compound is usually believed to be less toxic than $O_2^-\bullet$. If we accept this opinion, this activity of apelin also must be considered as an aspect of its protective role. However, Mitchell *et al.* (64) found that the exposure of lung fibroblasts from Chinese hamster to $O_2^-\bullet$ and H_2O_2 resulted in a dose-dependent cytotoxicity, which was suppressed by CAT but not by SOD, showing that the predominant toxic radical was hydrogen peroxide and not superoxide anion. Similar results were obtained by Wink *et al.* (107). These findings seem to be in contrast with the hypothesis that the upregulation of SOD by apelin is a component of the pathway to myocardial protection. It is important to underline that no report indicates whether apelin can also regulate the activity of CAT.

In spite of the suggested antioxidant effect of the peptide on myocardium, a controversy exists about the effect of the apelin-APJ system on the vessel wall. In fact, although it has been reported that it favors oxidative stress-linked atherosclerosis *via* activation of NADPH OX (31), a beneficial effect of apelin in angiotensin II model of atherosclerosis has also been reported (16). Although there is no doubt that coronary atherosclerosis is harmful to myocardium, the pathway to the production of plaques is a long-term process, which is unlikely to alter *per se* the acute antioxidant effect of apelin on myocardium.

As previously ascribed to I/R injury, the opening of MPTP induces a further production of ROS, which contributes to the oxidative stress (5). It may also be speculated that, if ROS are released by the opening of MPTP (5), apelin may still protect against oxidative stress *via* the activation of SOD, also at this stage of the injury pathway.

Antiapoptotic effect of apelin

One of the aspects of myocardial ischaemic insult is cell death by apoptosis. The death may occurs several hours after the apoptotic process has been triggered (46). In various studies, apelin has been found to prevent the apoptosis of mouse (96) and human (108) osteoblasts, rat cardiomyocytes (119), and rat neurons (117). Interestingly, in all these investigations, it was seen that this antiapoptotic effect was mediated by PI3K-Akt pathway and, in rat cardiomyocytes and mouse neurons, also by ERK1/2. Both these pathways lead to

the inhibition of the BAD/BAX proapoptotic proteins, thus confirming the importance of the RISK pathway in myocardial protection.

Why does apelin protect only if given after ischaemia?

In the literature, various substances (*e.g.*, opioids, adenosine, sildenafil, sphingosine, and bradykinin) are reported to protect the myocardium against I/R injury if administrated either before or after a prolonged ischaemia (15, 29, 33, 54, 61, 82, 83, 101, and 116). Unlike these compounds, apelin seems to be effective only if given after ischaemia, thus mimicking PostC but not IP. Since the RISK pathway is triggered by both IP and PostC, it does not provide any reliable explanation of this issue.

The expression of both APJs and apelin levels are reported to vary at the end of ischaemia and during reperfusion (50, 118). Kleinz and Baxter (50) studied APJ and apelin mRNA in isolated rat hearts after 35 min of coronary occlusion and after 30 min of reperfusion. Immediately after the end of the ischaemic period, they observed a 2.43-fold increase in apelin mRNA level, which was however back to the control value at the end of reperfusion. On the contrary, in *in vivo* experiments carried out in rat hearts, a significant upregulation of apelin mRNA was observed after 24 h of local ischaemia (80). In these experiments, however, no reperfusion was performed; and it is not clear whether the upregulation is really due to the ischaemia or to the subsequent heart failure.

The changes of APJ mRNA level were slightly different. In fact, Kleinz and Baxter (50) observed only a tendency to the increase, which did not reach statistical significance either at the end of ischaemia or after 30 min of reperfusion. In rat hearts, after 30 min of reperfusion preceded by 40 min of global ischaemia, Zeng *et al.* (118) saw an eightfold significant increase of APJ-mRNA expression with a 41% up-regulation of APJ protein expression. In neonatal cardiomyocytes, after a period consisting of 3 h of hypoxia followed by 2 h of normoxia, the same group found a sevenfold increase of APJ mRNA level and 35% increase of the receptor protein.

At a first sight, the upregulation of APJs after ischaemia might explain why the peptide exerts the protective effect only if it is given after ischaemia. However, the time course of APJ protein expression seems to limit this possibility. In fact, although apelin is effective if infused at the onset of reperfusion, that is, when the largest part of injury takes place, a significant increase in APJ protein expression was observed 30 min later (118). Although data about APJ protein expression during the first 30 min of reperfusion are not available, an increase in APJ mRNA in the heart is not exclusive of cardiomyocytes as it may occur also in coronary endothelial cells, thus leading to an overestimation of the receptors in myocardium (14).

It may be argued that apelin given in early reperfusion is effective due to the priming exerted on the receptors by the endogenous postischaemic release of the peptide. At the moment, no experimental study is available to accept or to refute such a hypothesis. An increase in plasma apelin after myocardial ischaemia is not commonly found. In fact, in humans, 2 days after acute myocardial infarction, plasma apelin was seen to be significantly decreased with regard to a control population and not restored after 24 weeks (106). In brief, the reason that apelin can mimic PostC rather than IP has not yet been fully elucidated.

Possible protective role of endogenous apelin

So far, the protective activity of endogenous apelin has been described only in ischaemic heart failure model, where both apelin and APJs have been seen to increase (3, 14). In particular, it has been seen that apelin concentration increases in the early stage of failure but falls in the late stage of the disease (19, 39). It may be argued that apelin upregulation may represent an adaptive mechanism to maintain the contractile function of the heart in the initial phase of ischaemic failure. In agreement with this point of view, the progression of heart failure has been associated with the progressive reduction of the peptide expression (39). The upregulation of apelin after ischaemia suggests that it could have a limiting effect against the severity of a myocardial infarction in the sense that it cannot be excluded that in the absence of apelin the extension of the injured area would be greater. Unfortunately, at the moment, no data are available to ascertain this point.

As just mentioned, after ischaemia, apelin level declines before APJ expression becomes significant. Such a time course seems to weaken the hypothesis of a beneficial effect attenuating the severity of an infarction. However, no definitive statement can be made in the absence of a precise knowledge of the kinetics of the ligand-receptor system.

Apelin is reported to act also as a antimitogenic, chemotactic, and antiapoptotic agent for endothelial cells of frog embryo (17) and to take part in the induction of cardiac differentiation in Xenopus embryo (37). These findings suggest that, in addition to cardiac protection, apelin might also favor heart repair.

Conclusion

Apelin protects the myocardium against I/R injury when administrated during the early minutes of reperfusion, that is, before the reversible damage becomes irreversible. In contrast, it has not been seen to be effective if given before ischaemia. The protection by apelin has been attributed to the RISK pathway with the two cascades mediated by PI3K/Akt and MEK/ERK system, respectively. PI3K/Akt cascade is NO-dependent. In fact, Akt induces the production of NO through the activation of eNOS. As in the RISK pathway triggered by PostC, NO is expected to lead to the opening of $mitoK^{+}_{ATP}$ channels *via* the activation of protein kinases G by cGMP. Then, the opening of $mitoK^{+}_{ATP}$ channels inhibits MPTP *via* a mechanism that includes the production of ROS. Unlike PI3K/Akt cascade, MEK/ERK cascade is NO-independent. In fact, it prevents the opening of MPTP by the inhibition of cytochrome C operated by the downregulation of BAD/BAX proapoptotic proteins. Also, the activation of SOD, which transforms superoxide anion into the less toxic hydrogen peroxide, may contribute to the protective effect of apelin.

In summary, apelin is a compound able to mimic PostC by triggering the RISK pathway. Thus, we propose apelin to be an agent capable of limiting the reperfusion injury if given in the early minutes of reperfusion during percutaneous transluminal coronary angioplasty or other maneuvers aimed at reopening occluded coronary arteries. The release of apelin

from medicated stents should also be taken into consideration.

Acknowledgments

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Abbreviations Used

APJ = apelin receptor
AT1 = angiotensin II receptors
CAT = catalase
Cyt C = cytochrome C
DAG = diacylglycerol
eNOS = endothelial NO synthase
GPCR = G-protein coupled receptor
GSHPx = glutathione peroxidase
GSSGR = glutathione reductase
H/R = hypoxia and reoxigenation
H_2O_2 = hydrogen peroxide
HIF-1 = hypoxia inducible factor-1
I/R = ischaemia and reperfusion
IP = preconditioning
IP_3 = inositol 1,4,5-trisphosphate
IP_3R = inositol 1,4,5-trisphosphate channel receptor
L-Arg = L-arginine
LVDevP = left ventricular developed pressure
MDA = malonilaldehyde
MEK1/2 = mitogen-activated extracellular regulated kinase 1/2
$mitoK^+_{ATP}$ = mitochondrial ATP-sensitive potassium (channels)
MPTP = mitochondrial permeability transition pores
NADPH OX = NADPH oxidase
NCE = Na^+/Ca^{2+} exchanger
NHE = Na^+/H^+ exchanger
NO = nitric oxide
$NO_2\bullet$ = nitrogen dioxide radical
$O_2^-\bullet$ = superoxide anion
OH• = hydroxyl radical
$ONOO^-$ = peroxynitrite
PI3K = phosphoinositide 3-kinase
PKB = protein kinase B commonly called Akt
PKC = protein kinase C
PKG = protein kinases G
PLC = phospholipase C
PostC = postconditioning
RISK = reperfusion injury salvage kinase
ROS = reactive oxygen species
RyR = ryanodine channel receptor
SERCA = sarco/endoplasmic reticulum Ca^{2+}-ATPase
SOD = superoxide dismutase
SR = sarcoplasmic reticulum
STAT = signal transducers and activators of transcription

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