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Ischaemic Cardiac Hyperaemia: Role of Nitric Oxide and Other Mediators

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Summary

In the perfused guinea-pig heart reactive hyperaemia (RH) after occlusion of coronary flow (1–60 s) was inhibited by 100–60 % with N^G-nitro-L-arginine (100 μM) and to a lesser extent (by 35 %) after 8-phenyltheophylline (10 μM), but not by indomethacin (5 μM). Inhibition of adenosine deaminase by erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (5 μM) not only increased the concentration of adenosine in the coronary perfusate, but also prolonged the duration of RH. RH induced cardiac generation of prostacyclin, nitric oxide and adenosine as indicated by the appearance of 6-keto-$PGF_{1\alpha}$, cyclic GMP, adenosine, inosine, hypoxanthine, xanthine and urate in the perfusate. Only NO and adenosine, but not prostacyclin, were responsible for RH. RH after short-term (1–10 s) coronary occlusion was mediated by NO, whereas adenosine and NO maintained RH that followed after longer (20 s–10 min) periods of cardiac ischaemia. Prostacyclin never participated in the mediation of RH.

Key words

Cardiac reactive hyperaemia – Cardiac ischaemia – Endothelium – Prostacyclin – Nitric oxide – Adenosine

Introduction

A number of endogenous substances are claimed to regulate coronary flow or to mediate cardiac reactive hyperaemia (RH). These are endothelial nitric oxide (NO), prostacyclin (PGI_2), endothelins (ETs), "endothelium-derived contracting factor" (EDCF), neuropeptides or amines, myocardial adenosine, adenine nucleotides and hydroxy or epoxy eicosatrienoic acids. The release of these mediators is controlled by agonists of endothelial receptors, shear stress, cytokine-mediated induction of appropriate enzymes, and finally by ischaemia or hypoxia (Vane and Botting 1994).

Reopening of the occluded coronary flow is associated with RH, the intensity of which is proportional to the duration of occlusion (Katz and Lindner 1939). Myogenic (Olsson 1975) and neurogenic (Bache *et al.* 1975) mechanisms are less likely to be involved in cardiac RH than metabolic mechanisms. Adenosine was the first candidate (Berne 1980), prostanoids were also proposed (Kraemer *et al.* 1976, Hintze and Vatner 1984) and NO has recently been claimed to be a mediator of cardiac RH (Kostic and Schrader 1992, Chlopicki and Gryglewski 1993). Indeed, the removal of coronary endothelium abolishes RH (Hayashi *et al.* 1988). In addition, the basal release of NO controls basal coronary vascular tone (Amezcua *et al.* 1989, Lamontagne *et al.* 1992).

Here, we report on the interactions between NO and adenosine in mediating and maintaining reactive hyperaemia in the perfused guinea-pig heart.

Materials and Methods

Perfused guinea-pig heart

Langendorff apparatus (Hugo Sachs Elektronik-HSE) was used to study isolated hearts of guinea-pigs (body weight 200–250 g) perfused through the coronary vascular bed with Krebs-Hanseleit buffer of the following composition (mmol/l): NaCl 118, $CaCl_2$ 2.52, $MgSO_4$ 1.64, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 5.55, sodium pyruvate 2.0, equilibrated

with 95 % O_2 + 5 % CO_2 at 37 °C. Left ventricular pressure (LVP) was measured using a fluid-filled balloon inserted into the left ventricle and connected to a pressure transducer (Isotec HSE). The end-diastolic pressure was adjusted to less than 10 mm Hg. The values of dp/dt max and dp/dt min were obtained from the LVP signal by an analog differential amplifier (DIF module, HSE). The heart was paced at 273 impulses per min *via* a coaxial electrode attached to the right atrium. Coronary perfusion pressure (CPP) was monitored by a second Isotec transducer connected to a side arm of the perfusion line. In some experiments platinum electrodes were used to record the ECG from the cardiac surface. All meters and transducers were calibrated on a daily basis. CPP, LVP, dp/dt and ECG were recorded and continuously displayed using the computer programme PSC-IGEL, Poland.

The heart was equilibrated for 15 min at a constant perfusion pressure of 55 mm Hg and afterwards perfused at a constant flow rate to obtain CPP of 60–70 mm Hg. The hearts were included in experiments when firstly, CPP could be adjusted to a level ≥ 60 mm Hg, secondly, a bolus injection of sodium nitroprusside (3 nmoles) decreased CPP by more than 20 mm Hg, and finally an occlusion of coronary flow for 1 s produced detectable reactive hyperaemia (RH). RH was evoked by interrupting coronary inflow for periods from 1 s to 10 min. RH was expressed as an area of vasodilatation and calculated by weighing appropriate cuttings of tracing paper or alternatively its duration was measured in minutes.

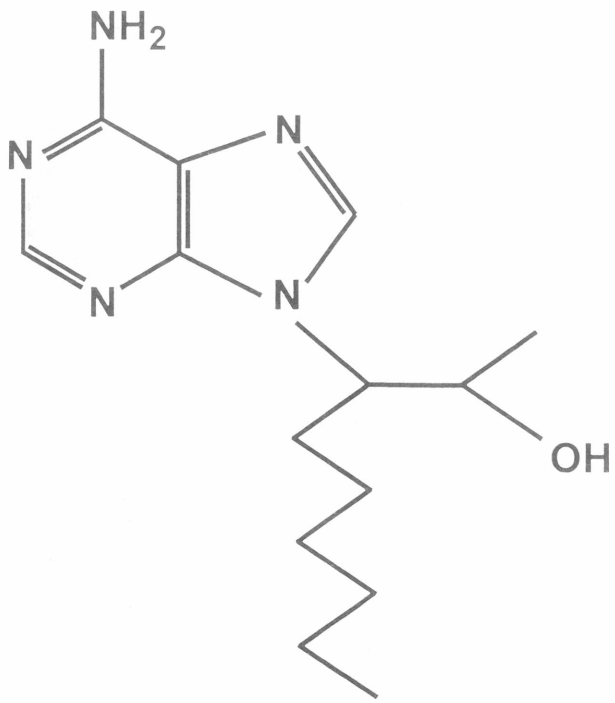

Fig. 1
Chemical structure of EHNA – an adenosine deaminase inhibitor.

Bolus injections of investigated vasodilators (e.g. bradykinin or sodium nitroprusside) were administered proximally to the aortic cannula. The inhibitors or antagonists used in this study (N^G-nitro-L-arginine, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), 8-phenyltheophylline, indomethacin (Figs 1 and 2), had been infused into the perfusion line (0.1 ml/min) for a minimum period of 20 min before vasodilatation was evoked by either RH or an exogenous vasodilator and infusion was continued to the end of the observation period.

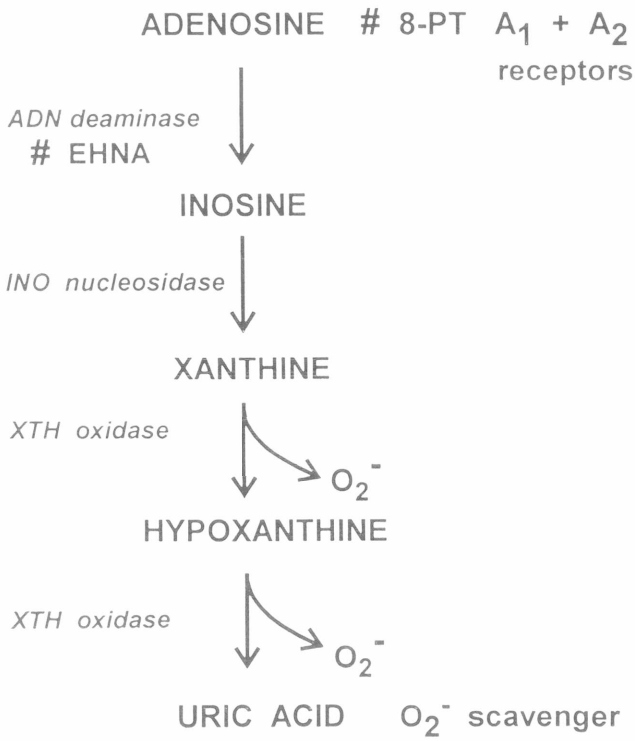

Fig. 2
Cardiac metabolism through the adenosine deaminase (ADN), inosine nucleotidase (INO) and xanthine oxidase route (XTH). The site of its enzymatic blockade by EHNA and the site of receptor antagonism of adenosine by 8-PT are indicated.

RIA of cyclic GMP and 6-keto-$PGF_{1\alpha}$

Samples of the coronary effluent were collected for 30 s under control conditions as well as after 1, 2, 3, 5 and 10 min of the vasodilator response. These samples were stored at −20 °C before the RIA estimation.

Concentrations of cyclic GMP or 6-keto-$PGF_{1\alpha}$ in 100 μl samples of the effluent were radioimmunoassayed (RIA) using commercially available kits (Amersham) and expressed in pmoles/min.

HPLC assay of purines

The presence of adenosine, inosine, hypoxanthine, xanthine and urate (Fig. 2) in the coronary perfusate was analyzed by reverse-phase high performance liquid chromatography using chromatographic purity standards (Sigma). The HPLC system (Beckman Instruments) included two pumps (model 110B), analog interface AI 406, system organizer with a sample loop injector and mixer as well as a UV detector (model 166). The system was controlled by the Gold 7.12 chromatographic software. Samples of the coronary effluent were injected into a 100x4.6 mm I.D. stainless steel column packed with 7 μM ODS material (Kontron Instruments). The mobile phase consisted of 50 mM $NH_4H_2PO_4$ adjusted to pH 6.0 and 14 % methanol (v/v). Isocratic conditions were used with a flow rate of 1 ml/min. The absorbance was measured at 254 nm. Inhibitor of adenosine deaminase (EHNA) (Fig. 1) was infused into the coronary bed at three concentrations of 1.5, 5 and 15 μM (n=6 for each concentration of EHNA) and the concentrations of adenosine and its metabolites (Fig. 2) were compared in the control and EHNA-treated hearts in the basic situation as well as 15, 30, 45, 60, 120, 180, 300 and 600 s which followed the 10 min period of occlusion of the coronary vascular bed (Fig. 3a,b).

Statistics

The results were presented as means ± standard errors for n determinations. Differences between means were evaluated by the unpaired Student's test at a level of significance of $P \leq 0.05$.

Results

Haemodynamics and ECG

CPP was 65±0.5 mm Hg (n=45), LVP was 102±4 mm Hg (n=45) and dp/dt max was 924±53 mm Hg/s (n=12). Coronary occlusion (1–60 s) produced time-dependent reactive hyperaemia (RH). Short periods of occlusion (1–5 s) hardly affected dp/dt. Following the 15 s occlusion, only a transient suppression of dp/dt occurred while an ischaemic ECG pattern (ST segment elevation and T wave reversal) was seen and disappeared within seconds after reopening of coronary flow. The occlusion of 60 s produced a drop of dp/dt close to zero, yet recovery was prompt. Inhibition of adenosine deaminase by EHNA (5 μM) produced little effect on LVP or dp/dt max, however, it nearly doubled the duration of RH.

Inhibition of nitric oxide synthesis

A 20-min lasting infusion of N^G-nitro-L-arginine (L-NNA, an inhibitor of nitric oxide synthase, 100 μM) (Vargas *et al.* 1991) had no effect on LVP, dp/dt, ECG and heart rate, but it elevated CPP by 7.9±5 mm Hg (n=12). The pretreatment with L-NNA abolished RH which was induced by one second coronary occlusion and reduced RH after 3, 5, 10 and 15 s occlusion periods by 95±2, 91±1, 89±4 and 84±1 % (n=8), respectively. RH which followed occlusion periods of 20–60 s was reduced by L-NNA by 80–60 %. A thromboxane A_2 analogue, U 46619, (30 pM) produced a rise in CPP by 6.7±1.1 mm Hg, n=6, similar to that evoked by L-NNA (100 μM). However, unlike L-NNA, U 46619 did not influence hyperaemic responses to coronary occlusions. After the cessation of L-NNA treatment, infusions of L-arginine (1 mM) but not of D-arginine partially reversed (by 72.2±5 %, n=5) the blockade by L-NNA of RH responses (data for 15 s of coronary occlusion).

Inhibition of synthesis of prostanoids

The presence of indomethacin (an inhibitor of prostaglandin H synthase, 5 μM) in the perfusing buffer slightly increased coronary tone by 3.7±0.4 mm Hg (n=8), and did not influence either haemodynamic parameters or hyperaemic responses (RH) to the interruption of coronary inflow (1–60 s), whereas indomethacin at a concentration of 5 μM blocked the postischaemic release of PGI_2.

Cyclic GMP and 6-keto-$PGF_{1\alpha}$ in coronary effluent

The basal level of cyclic GMP in the coronary effluent was 0.76±0.10 pmoles/min. The extrusion of cyclic GMP increased to 208±30 % (n=5) of the basal value in the course of the second minute of RH which followed a 15 s interruption of coronary inflow. The kinetics of the cyclic GMP response during RH was similar to that induced by a bolus injection of bradykinin (10 pmoles).

The basal level of 6-keto-$PGF_{1\alpha}$ in the coronary effluent was 1.26±0.11 pmoles/min (n=4). It increased to 243±25 % of the basal value following a 30 s period of cardiac ischaemia. The bradykinin (3 pmoles)-induced release of 6-keto-$PGF_{1\alpha}$ was similar to that which occurred in the course of RH, but slightly higher. The release of 6-keto-$PGF_{1\alpha}$ by both procedures (RH or bradykinin) was blocked in the presence of indomethacin (5 μM).

Blockade of adenosine receptors

8-phenyltheophylline (8-PT, a non-selective antagonist of adenosine A_1 and A_2 receptors, Fig. 2) at

a concentration of 10 μM shifted to the right vasodilator effects of exogenous adenosine, but it had no effect on LVP, dp/dt, basal CPP and RH induced by short coronary occlusion for periods of 1–10 s. However, 8-PT (10 μM) attenuated RH which was induced by 15 or 60 s occlusion periods by 20.2±7 and 35.3±2 % (n=6), respectively. This last inhibitory effect of 8-PT on RH was reversed spontaneously (up to 81.4±5 % of the control value) within 30 min after washout of the drug.

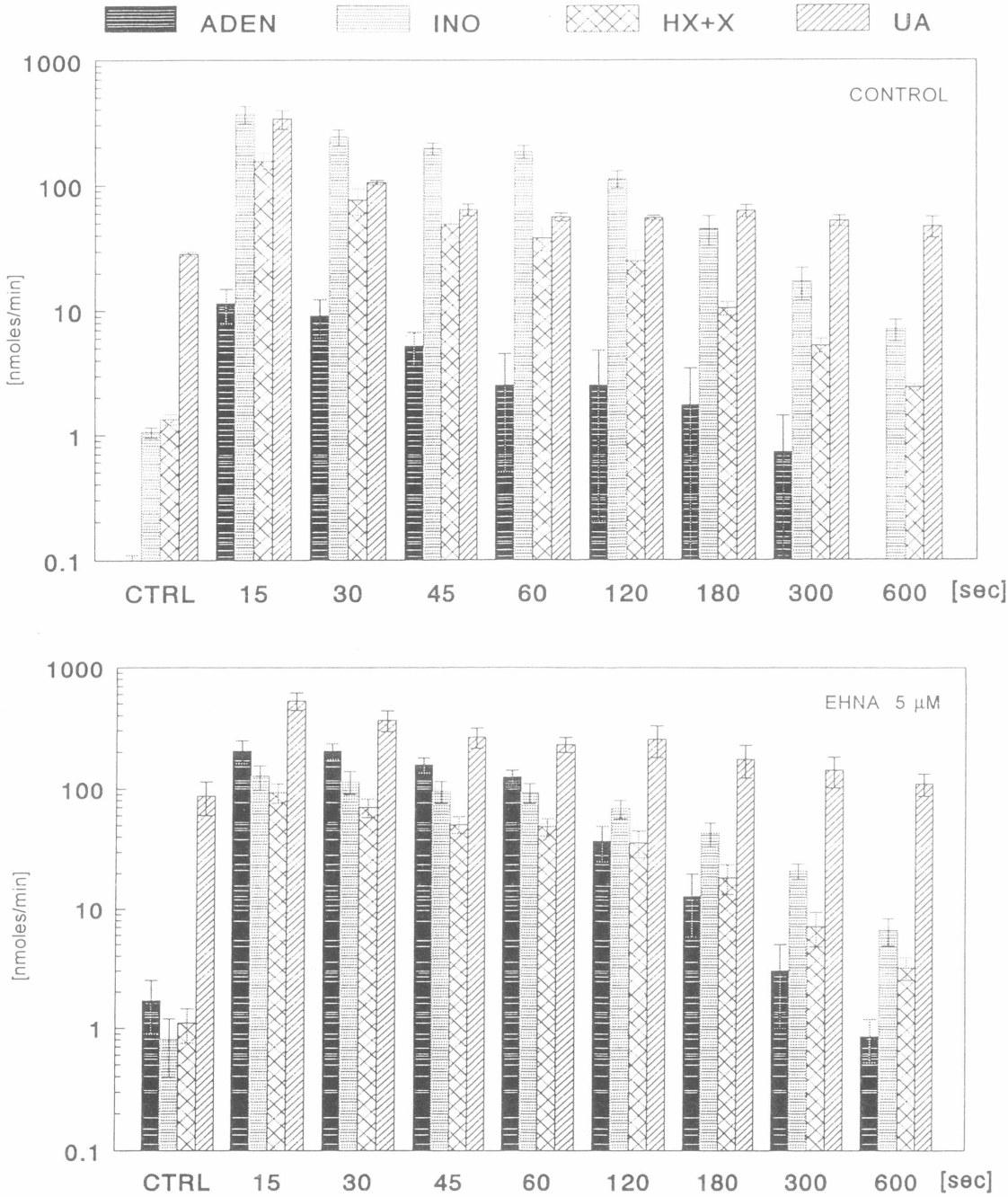

Fig. 3

Release of adenosine (ADEN) and its metabolites inosine (INO), hypoxanthine and xanthine (HX + X) and uric acid (UA) during cardiac reactive hyperaemia (RH) which followed 10 min occlusion of coronary flow, as compared to their basal release (CTRL). The height of columns with bars represent (log scale 0.1–1000 nmoles/min) concentrations ± S.E.M. of adenosine and its metabolites in coronary effluent at various time intervals (15–600 s) after reopening of coronary flow in the absence (Fig. 3a, CONTROL) and in the presence (Fig. 3b) of an inhibitor of adenosine deaminase (EHNA, 5 μM).

Purines in coronary effluent

Under basal conditions, adenosine was hardly detected in coronary effluent (0.1 nmoles/min) while its metabolites were found at the following concentrations: urate 23.8±2.5, inosine 1.3±0.15 and hypoxanthine + xanthine 1.2±0.09 nmoles/min (n=5). The coronary occlusion for a period of 10 s did not influence the extent of release of adenosine metabolites. A distinct change occurred following the occlusion lasting 10 min. Then, even adenosine appeared in the effluent at concentrations ranging from 1 to 10 nmoles/min (depending on the time of collection, Fig. 3a), whereas concentrations of adenosine metabolites increased to 10–400 nmoles/min (Fig. 3a). The inhibition of adenosine deaminase was observed only after infusion of EHNA at concentrations of 5 μM or 15 μM but not 1.5 μM. There was no significant difference between the effectiveness of EHNA at concentrations of 5 or 15 μM. EHNA at 5 μM resulted in the appearance of adenosine in the effluent under basal conditions (1.9±0.3 nmoles/min) and, what is even more important, following the 10 min occlusion of coronary flow adenosine concentrations increased at various time intervals from 1 to 200 nmoles/min (Fig. 3 b).

Discussion

The duration and intensity of reactive hyperaemia (RH) in perfused guinea-pig hearts was proportional to the duration of coronary occlusion (1–60 s). Even the most intensive RH was transient in character (3–5 min), and so was the suppression in myocardial contractility or the ECG signs of myocardial ischaemia. RH evoked by 15 s interruption of coronary inflow was comparable in size to vasodilatation produced by bradykinin (10 pmoles) or by acetylcholine (100 pmoles). Vasodilatation by these "endothelium-dependent" vasodilators and by short-lasting myocardial ischaemia, were both suppressed (by 60 to 100 %) by a NO synthase inhibitor, L-NNA, thus pointed to NO as a major mediator of RH. This was already proposed by Kostic and Schrader (1992). Reversal of the inhibitory effect of L-NNA by L-arginine and comparable increments of cyclic GMP in the coronary effluent by either RH or bradykinin provided additional substantial evidence. It has been reported that activation of soluble guanylate cyclase by NO leads to the intracellular accumulation of cyclic GMP which eventually is removed either by phosphodiesterases or *via* its efflux from the cell (Wu *et al.* 1993). We have found that this last route of inactivation of cGMP might be a useful tool for demonstrating the role of NO in RH.

NO alone is a sufficiently strong vasodilator to deal with small cardiac RH, however, a more pronounced RH requires additional enhancement of NO by adenosine. The role of adenosine in cardiac RH was thoroughly studied by Berne (1980). In our experiments, the participation of adenosine in RH was proved by a partial inhibition (up to 35 %) of an intensive RH by an adenosine receptor antagonist (8-PT) and by a massive release of adenosine metabolites during RH, however, only when it ensued from coronary obturation which lasted longer than 20 s. The origin of adenosine or its metabolites in the coronary effluent is a complex issue (Borst and Schrader 1991) and it requires further studies. RH which was induced by a 10 min lasting occlusion of coronary flow was associated with a genuine outburst of adenosine generation, however, even then adenosine appeared in the coronary effluent at concentrations lower than 10 nmoles/ml. The tenfold increase in adenosine during RH could be achieved by the pharmacological inhibition of adenosine deaminase with EHNA.

Indomethacin at a concentration which was sufficient to abolish the ischaemic or bradykinin-induced release of 6-keto-$PGF_{1\alpha}$ showed no effect on vasodilatation produced by either of these procedures. In both instances PGI_2, though released into the coronary perfusate, hardly affected vascular tone of the coronary bed in contrast with other reports which pointed to the mediation of RH by prostanoids (Hintze and Vatner 1984, Kraemer *et al.* 1976). A feasible explanation is that, during an ischaemic crisis, PGI_2 appears in cardiac circulation to combat the activation of platelets and to prevent the formation of intracoronary thrombi, rather than to dilate coronary arteries (Gryglewski *et al.* 1995). Nevertheless, exogenous PGI_2 and NO may synergize as cardioprotective agents in ischaemic myocardium (Gryglewski and Swies 1992), and as fibrinolytic agents in the plasma of atherosclerotic subjects (Bieron *et al.* 1993).

In conclusion, short-term occlusion (1–10 s) of the coronary vascular bed of the guinea-pig heart is associated with reactive hyperaemia which seems to be mediated exclusively by NO. The long-term interruption of coronary inflow (15–60 s) is followed by more pronounced reactive hyperaemia owing to the combined action of NO and adenosine. Really long occlusion of coronary flow (10 min) may release as much as 10 nmoles/min of adenosine. This release can be increased ten times by pharmacological inhibition of adenosine deaminase, and then the duration of RH is nearly doubled. Out of the disputed triad of mediations (nitric oxide, adenosine and prostacyclin) which are abundantly released during reactive hyperaemia, nitric oxide and adenosine share the responsibility for maintaining increased coronary flow after reopening of

the coronary bed, whereas prostacyclin seems to maintain the thromboresistance of arterial walls. The triad *in toto* is responsible for cardioprotection in various patterns of cardiac ischaemic injury.

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Reprint Requests

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