α_1 -Adrenoceptors during simulated ischemia and reoxygenation of the human myocardium: Effect of the dose and time of administration

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Objective: We sought to investigate the effect of α_1 -adrenoceptor activity on the ischemic and reoxygenated human myocardium.

Methods: Right atrial appendages ($n = 6$ per group) obtained during elective cardiac operations were sliced and stabilized in normoxic normothermic buffer solution for 30 minutes and then subjected to 90 minutes of simulated ischemia, followed by 120 minutes of reoxygenation. In study 1 the dose responses to the α_1 -adrenoceptor agonist phenylephrine (0.01, 0.1, 1, 10, and 100 $\mu\text{mol/L}$) and to the α_1 -adrenoceptor antagonist prazosin (0.1, 1, 10, and 100 $\mu\text{mol/L}$) when administered for 10 minutes before ischemia, during ischemia, and during reoxygenation were examined. The influence of the time of administration (ie, before ischemia, during ischemia, or during reoxygenation) of phenylephrine (0.1 $\mu\text{mol/L}$) and prazosin (10 $\mu\text{mol/L}$) was then investigated in study 2. In study 3 the effect of the combined administration of phenylephrine given before ischemia and prazosin given during ischemia was investigated. In study 4 the protective effect of phenylephrine given before ischemia (for 10 minutes or for 5 minutes with a 5-minute washout period) was compared with that of ischemic preconditioning (5 minutes of ischemia and 5 minutes of reoxygenation). At the end of each protocol, the leakage of creatine kinase (in units per gram of wet weight) and the reduction of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide to insoluble formazan dye (in millimoles per gram of wet weight) were measured.

Results: Phenylephrine is maximally beneficial at 0.1 and 1 $\mu\text{mol/L}$ (creatinine kinase, 0.97 ± 0.06 and 0.95 ± 0.03 U/g, respectively; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 153.0 ± 7.8 and 156.2 ± 6.7 mmol/g, respectively) compared with ischemic control (creatinine kinase, 1.87 ± 0.03 U/g; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 108.5 ± 6.8 mmol/g; $P < .05$) but prazosin is detrimental at concentrations above 10 $\mu\text{mol/L}$ (creatinine kinase, 5.22 ± 0.29 U/g; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 69.8 ± 2.9 mmol/g; $P < .05$ vs ischemic control). In addition, phenylephrine (0.1 $\mu\text{mol/L}$) is protective when given before ischemia (creatinine kinase, 2.06 ± 0.21 U/g; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 148.5 ± 4.5 mmol/g; $P < .05$ vs ischemic control) but is detrimental when given during ischemia alone (creatinine kinase, 4.49 ± 0.98 U/g; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 70.5 ± 6.1 mmol/g; $P < .05$ vs ischemic control) and has no significant effect during reoxygenation. In contrast, prazosin (10 $\mu\text{mol/L}$) is beneficial when given during ischemia alone (creatinine kinase, 1.34 ± 0.10 U/g; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 148.5 ± 4.5 mmol/g; $P < .05$ vs ischemic control), is detrimental when given during reoxygenation alone (creatinine kinase, 1.5 ± 0.16 U/g; 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 85.0 ± 4.7 mmol/g; $P < .05$ vs ischemic control), and has no effect when given before ischemia. The use of phenylephrine before ischemia alone is as

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protective as prazosin given during ischemia alone, but the combination of the two drugs does not cause additional benefit. Interestingly, the protection afforded by phenylephrine when given before ischemia is similar to that obtained with ischemic preconditioning.

Conclusions: In the human myocardium activation of α_1 -adrenoceptors before ischemia is protective but is detrimental during ischemia, whereas blockade of α_1 -adrenoceptors is beneficial during ischemia but detrimental during reoxygenation. The degree of protection achieved by activation of the α_1 -adrenoceptors before ischemia is similar to that obtained with blockade of α_1 -adrenoceptors during ischemia and that of ischemic preconditioning.

Activation of α_1 -adrenoceptors induces positive inotropic, chronotropic, and dromotropic actions in the heart and vascular effects¹⁻⁴ that are clinically exploited to optimize hemodynamic conditions. However, it is generally believed that activation of α_1 -adrenoceptors is detrimental to the ischemic heart, a thesis that would be supported by the increased release of cardiac and plasma catecholamines⁵ and the enhanced density of cardiac α_1 -adrenoceptors during ischemia.⁶⁻⁸ However, it has recently been shown that α_1 -adrenoceptors mediate the protection induced by ischemic preconditioning in animals and in the human myocardium⁹⁻¹⁴ by means of phospholipase C and protein kinase C activation,¹³ and this has given rise to the possibility that the effect of α_1 -adrenoceptors in ischemic injury may depend on the dose and the time of their activation (ie, before, during, or after ischemia). To investigate this, right atrial appendages were obtained from patients undergoing elective coronary bypass operations, and the muscles were subjected to various protocols in a validated in vitro model of simulated ischemia and reoxygenation.¹⁵

Methods

Experimental Preparation

Experiments were performed on myocardium obtained from the right atrial appendage of patients undergoing elective coronary artery operations or aortic valve replacement in a cell necrosis model that was developed in our laboratory and described previously.¹⁵ Patients were excluded if they had enlarged atriums, atrial arrhythmias, poor left ventricular function (ejection fraction, <30%), and right ventricular failure or were taking oral hypoglycemic agents, opioid analgesics, K_{ATP} channel openers, or catecholamines. Local ethical committee approval was obtained for the harvesting technique. The specimens were collected in oxygenated N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid-buffered solution at 4°C to 5°C and immediately sectioned and prepared for study. The appendage was mounted onto a ground glass plate with the epicardial surface face down and then sliced freehand with surgical skin graft blades (Shwann-Morton) to a

thickness of between 300 and 500 μ m. The specimen and the slide were always kept moist throughout the procedure. The muscles (weight 30-50 mg) were then transferred to conical flasks (25-mL Erlenmeyer flasks, Schott Glaswerk) containing 10 mL of oxygenated buffered solution, and the flasks were placed in a shaking water bath maintained at 37°C. The oxygenation of the incubation medium was maintained by a continuous flow of 95% O₂/5% CO₂ gas mixture to obtain a PO₂ of between 185 and 225 mm Hg and a PCO₂ of between 45 and 50 mm Hg. The PO₂, PCO₂, and pH in the incubation medium were monitored by means of intermittent analyses of the effluent with an automated blood gas analyzer (model 855 Blood Gas System, Chiron Diagnostics), and the pH was kept between 7.36 and 7.45. For the induction of simulated ischemia, the medium was bubbled with 95% N₂/5% CO₂ (pH 6.80-7.00), and D-glucose was replaced with 2-deoxyglucose. In this preparation creatine kinase (CK) leakage (in units per gram of wet weight) was taken as a measure of tissue injury, and the reduction of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) to an insoluble formazan dye (in millimoles per gram of wet weight) was used to assess tissue viability. The atrial tissue was not paced, and the force developed was not measured in these studies.

Solutions and Drugs

The incubation medium was prepared daily with deionized distilled water and contained the following: NaCl₂, 118 mmol/L; KCl, 4.8 mmol/L; NaHCO₃, 27.2 mmol/L; MgCl₂, 1.2 mmol/L; KH₂PO₄, 1.0 mmol/L; CaCl₂, 1.25 mmol/L; D-glucose, 10 mmol/L; and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 20 mmol/L. As mentioned above, during simulated ischemia, D-glucose was removed and substituted with 2-deoxyglucose (10 mmol/L) to maintain a constant osmolarity. Phenylephrine and prazosin were dissolved in deionized distilled water immediately before their use. All reagents were obtained from Sigma.

Experimental Protocols

After sectioning the atrium, the preparations were allowed to stabilize for 30 minutes and then randomly allocated to various protocols. In all the studies, simulated ischemia was induced for a period of 90 minutes, followed by 120 minutes of reoxygenation. Some of the preparations were not made ischemic and instead were

Figure 1. CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90 minutes of ischemia and 120 minutes of reoxygenation and incubated with various concentrations of phenylephrine. The drug was present in the incubation media for 10 minutes before the induction of ischemia, during ischemia, and during reoxygenation. Columns represent the mean of 6 experiments, and bars represent the SEM. * $P < .05$ versus phenylephrine-free group.

aerobically perfused for 240 minutes to serve as aerobic matched controls.

Study 1: Dose response to phenylephrine and prazosin. In this study various concentrations of the α_1 -adrenoceptor agonist phenylephrine (0.01, 0.1, 1, 10, and 100 $\mu\text{mol/L}$) and the α_1 -adrenoceptor antagonist prazosin (0.1, 1, 10, and 100 $\mu\text{mol/L}$) were added to the incubation media for 10 minutes before ischemia, during ischemia, and during reoxygenation ($n = 6$ preparations per group).

Study 2: Influence of the time of administration of phenylephrine and prazosin. Influence of phenylephrine (0.1 $\mu\text{mol/L}$) and prazosin (10 $\mu\text{mol/L}$) was investigated by the exposure of the myocardial tissue to the optimal concentrations of the drugs shown in study 1 for 10 minutes before ischemia, during ischemia, and during reoxygenation alone and in combination ($n = 6$ preparations per group).

Study 3: Potency of phenylephrine- and prazosin-induced protection. The most beneficial dose and time of administration for phenylephrine and prazosin seen in the 2 previous studies were used alone and in combination ($n = 6$ preparations per group) to study the potency of the protection offered by the drugs.

Study 4: Phenylephrine and ischemic preconditioning. To investigate the potency of protection of the α_1 -agonist phenylephrine compared with that of ischemic preconditioning, right atrial specimens ($n = 6$ per group) were subjected to a protocol of ischemia-reoxygenation identical to the one used in the previous studies. Phenylephrine at a concentration of 0.1 $\mu\text{mol/L}$ was used before ischemia for 10 minutes or for 5 minutes followed by 5-minute washout period. Ischemic preconditioning was induced by 5 minutes of ischemia and 5 minutes of reoxygenation immediately before the 90-minute ischemia, a protocol shown to afford maximal protection in this preparation.¹⁶

Figure 2. CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90 minutes of ischemia and 120 minutes of reoxygenation and incubated with various concentrations of prazosin. The drug was present in the incubation media for 10 minutes before the induction of ischemia, during ischemia, and during reoxygenation. Columns represent the mean of 6 experiments, and bars represent the SEM. * $P < .05$ versus prazosin-free group.

Assessment of Tissue Injury and Viability

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120-minute reoxygenation period. This was assayed by means of a kinetic UV method based on the formation of NAD (Sigma Catalogue No. 1340-K), and the results were expressed as units per gram of wet weight.

Tissue viability was assessed by the reduction of MTT to a blue formazan product at the end of the experimental period. The tissue was loaded into a Falcon conical tube (15 mL, Becton Dickinson Labware) into which 2 mL of phosphate buffer solution (0.05 mol/L) containing MTT (1.25 mg/mL; 3 mmol/L at final concentration) was added and then incubated for 30 minutes at 37°C. After this, the tissue was homogenized in 2 mL of dimethylsulfoxide (Homogenizer Ultra-Turrax T25, dispersing tool G8, IKA-Labortechnik) at 9500 rpm for 1 minute. The homogenate was then centrifuged at 1000g for 10 minutes, and 0.2 mL of the supernatant was dispensed into a

98-well flat-bottom microtiter plate (Nunc Brand Products). After this, the absorbance of the blue formazan formed was measured on a plate reader (Benchmark, Bio-Rad Laboratories) at 550 nm, and the results were expressed as millimoles per gram of wet weight.

Statistical Analysis

All data are presented as means $\pm$ SEM. Analysis of variance was used for multiple comparisons, with application of a post hoc Tukey test.

Results

Study 1: Dose Response to Phenylephrine and Prazosin

The results shown in Figure 1, A and B, on CK leakage and MTT reduction demonstrate that phenylephrine induces a dose-response curve with maximal protection at 0.1 and

Figure 3. CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90 minutes of ischemia and 120 minutes of reoxygenation and incubated with 0.1 μmol/L phenylephrine for different times. Columns represent the mean of 6 experiments, and bars represent the SEM. * $P < .05$ versus the phenylephrine-free group.

1 μmol/L and a loss of protection at doses of 10 μmol/L and greater. It is important to note that although protection was lost at the highest concentrations of phenylephrine, higher doses did not exert a detrimental effect, and therefore CK leakage and MTT mean values were similar to those seen in the phenylephrine-free group.

As seen in Figure 2, A and B, CK leakage and MTT reduction were not significantly affected by prazosin when present in the incubation media throughout the experiment at the lowest concentrations of 0.1 and 1 μmol/L. However, in contrast with the results for phenylephrine, prazosin was detrimental at the highest concentrations (10 and 100 μmol/L), as shown by the significant increase in CK leakage and a decrease in MTT reduction.

Study 2: Influence of the Time of Administration of Phenylephrine and Prazosin

Figure 3, A and B, shows the results of phenylephrine when given at different times at a chosen concentration of 0.1 μmol/L, which was shown to be the most effective dose in study 1. These results demonstrate, in terms of both CK leakage and MTT reduction, that maximal protection was obtained when phenylephrine was administered before ischemia alone, an effect that was equivalent to the one obtained with phenylephrine throughout the entire experimental period. Phenylephrine given during the reoxygenation period alone was less protective than when given before ischemia, and this was reflected by a modest but significant decrease in CK leakage without affecting MTT reduction.

Figure 4. CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90 minutes of ischemia and 120 minutes of reoxygenation and incubated with 10 $\mu\text{mol/L}$ prazosin for different times. Columns represent the mean of 6 experiments, and bars represent the SEM. * $P < .05$ versus the prazosin-free group.

Interestingly, the administration of phenylephrine during ischemia alone was detrimental, as seen by the significant decrease in MTT reduction. It is worth noting that the adverse effect of phenylephrine during ischemia was completely counteracted when the drug was also present before ischemia and during reoxygenation.

Figure 4, A and B, shows the results on CK leakage and MTT reduction for prazosin given at various times at a concentration of 10 $\mu\text{mol/L}$. These results show that prazosin did not have a significant effect when given before ischemia alone, that it was detrimental when given during reoxygenation alone to a degree similar to the administration of prazosin during the entire experimental period, and

that it was protective when given during ischemia alone. Importantly, the beneficial action of prazosin during ischemia was abolished when the drug was present before ischemia.

Study 3: Potency of Phenylephrine- and Prazosin-Induced Protection

As indicated by the results on CK leakage and MTT reduction shown in Figure 5, A and B, phenylephrine given before ischemia alone and prazosin given during ischemia alone afforded similar protection, and the combination of the two did not result in greater protection than that obtained with each drug given alone.

Figure 5. CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90 minutes of ischemia and 120 minutes of reoxygenation and incubated with phenylephrine ($0.1 \mu\text{mol/L}$) for 10 minutes before ischemia, with prazosin ($10 \mu\text{mol/L}$) during ischemia, and with the 2 drugs in combination. Columns represent the mean of 6 experiments, and bars represent the SEM. * $P < .05$ versus the group with ischemia-reoxygenation (I/R) alone.

Study 4: Phenylephrine and Ischemic Preconditioning

Figure 6, A and B, shows that ischemic preconditioning induces a significant decrease in CK leakage and better preserves MTT reduction when compared with the effects seen in muscles subjected to ischemia-reoxygenation alone. The results confirm previous findings from our laboratory using an identical preparation and protocol.¹⁶ They also show that the α_1 -agonist phenylephrine given for 10 minutes before ischemia or for 5 minutes with a 5-minute washout period before ischemia, thus mimicking the ischemic precondition-

ing protocol, results in similar protection to that seen with ischemic preconditioning.

Discussion

The present study has demonstrated that α_1 -adrenoceptors play an important role in the ischemia-reoxygenation-induced injury of the human atrial myocardium. Thus they show that stimulation of α_1 -adrenoceptors with phenylephrine protects against injury, whereas α_1 -adrenoceptor blockade with prazosin is detrimental; both effects are

Figure 6. CK leakage (A) and MTT reduction (B) of human right atrial myocardium subjected to 90 minutes of ischemia and 120 minutes of reoxygenation after ischemic preconditioning (5 minutes of ischemia and 5 minutes of reoxygenation) or incubation with phenylephrine (0.1 μ mol/L) for 10 minutes or for 5 minutes with a 5-minute washout period. *Columns* represent the mean of 6 experiments, and *bars* represent the SEM. * $P < .05$ versus the group with ischemia-reoxygenation (I/R) alone.

obtained in a dose-dependent manner. They have also shown that the effect of the stimulation or blockade of α_1 -adrenoceptors depends on the time of administration so that α_1 -adrenoceptor stimulation is protective when given before ischemia but detrimental when given during ischemia. On the contrary, α_1 -adrenoceptor blockade is beneficial during ischemia and detrimental during reoxygenation and has no significant effect before ischemia. It appears that similar maximal protection can be obtained with α_1 -adrenoceptor stimulation before ischemia and with α_1 -adrenoceptor blockade during ischemia, although the combination of the two does not induce additional protection. Furthermore, the

protective effect of α_1 -adrenoceptor stimulation before ischemia is as potent as ischemic preconditioning. These studies are the first in dissecting the role of α_1 -adrenoceptors during ischemia and reoxygenation of the human myocardium, and the results have important mechanistic and clinical implications that warrant further discussion.

Our results showing that α_1 -adrenoceptor activation before ischemia is cardioprotective are in agreement with the observation of other investigators that α_1 -adrenoceptors participate in the protection induced by ischemic preconditioning in the rat heart^{9,17} and in the human myocardium.¹³ However, it is worth noting that although α_1 -adrenoceptor

stimulation mimicked the protection of ischemic preconditioning in our studies, protection was less and did not replicate that obtained with ischemic preconditioning in the study of Cleveland and colleagues,¹³ who also used the human myocardium. Certainly, there are differences in the experimental model and the doses of the α_1 -adrenoceptor agonist phenylephrine used in the 2 studies that may explain the differing results. Thus our studies were carried out in a model of necrosis in which muscles were not electrically stimulated and were subjected to 90 minutes of simulated ischemia, whereas in the study of Cleveland and colleagues,¹³ the muscles were stimulated, and functional recovery, as opposed to necrosis, was assessed after only 45 minutes of ischemia. Furthermore, our dose-response study with phenylephrine showed that there is a bell-shaped response, with maximal protection at concentrations of 0.1 and 1 $\mu\text{mol/L}$ and loss of protection at concentrations of 10 $\mu\text{mol/L}$ or greater, as used in their study.¹³ It is of interest that similar protection was obtained with phenylephrine administered for 10 minutes immediately before ischemia or for only 5 minutes with a 5-minute washout period before ischemia, thus suggesting that a short period of stimulation of α_1 -adrenoceptors is sufficient to attain maximal benefit.

The harmful effect seen when α_1 -adrenoceptors were activated only during ischemia and the protection obtained with their blockade were not unexpected and are supported by the reported literature.^{18,19} However, an important contribution of the present study is that the activation of α_1 -adrenoceptors during ischemia does not diminish the protection induced by the activation of these receptors before ischemia. It was also important that the protection seen with α_1 -adrenoceptor blockade during ischemia is lost when the blockade is continued during reoxygenation. These results contradict the never-confirmed assumption that activation of α_1 -adrenoceptors during reoxygenation may extend reperfusion injury.²⁰ In fact, they show that activation of α_1 -adrenoceptors during reoxygenation does not significantly influence myocardial injury and that, on the contrary, α_1 -adrenoceptor blockade augments injury.

Although the signal transduction pathways that follow the activation of α_1 -adrenoceptors are well described,²¹⁻³³ the precise mechanisms responsible for their opposing actions in ischemia-reoxygenation remain unclear. It is possible that the increase in cytosolic calcium levels induced by α_1 -adrenoceptor agonists through cyclic adenosine monophosphate¹⁹ may mediate the effects discussed above. Indeed, calcium overload can be harmful when occurring during ischemia,³⁴ and it may precondition the heart and be protective^{35,36} when it occurs before ischemia. The demonstration by Miyawaki and Ashraf³⁷ that a transient increase in cytosolic calcium levels during ischemic preconditioning is an important trigger for the activation and translocation of the protein kinase C isoforms α and δ further supports this hypothesis.

The binding of agonists to α_1 -adrenoceptors also causes the activation of phospholipase C, and this hydrolyzes phosphatidylinositol-4,5-bisphosphate, resulting in the production of inositol-1,4,5-trisphosphate and 1,2-diacylglycerol.^{21,22} Inositol-1,4,5-trisphosphate acts on the sarcoplasmic reticulum, increasing intracellular calcium levels,^{23,24} whereas 1,2-diacylglycerol activates protein kinase C,²⁵ which in turn activates the transsarcolemmal voltage-dependent calcium channels²⁶⁻²⁹ and the Na^+/H^+ channels³⁰ and the opening of mitochondrial K_{ATP} channels.³¹ There is evidence that stimulation of α_1 -adrenoceptors through activation of protein kinase C also enhances 5'-nucleotidase activity and hence adenosine formation,³² which has been shown to influence the outcome of myocardial ischemia-reperfusion in a number of experimental models and species.^{33,38,39} To what extent each of these mechanisms is participating in the action of α_1 -adrenoceptors during ischemia and reoxygenation is unclear; however, from our studies, it is evident that the result of the use of agents that activate or blockade these receptors may vary widely depending on time of initiation and termination of administration, and it is possible that more than one mechanism may be involved. Clearly, more studies are needed to elucidate the underlying mechanism of these actions.

A possible limitation of the present study is the use of atrial tissue as opposed to ventricular tissue, and therefore any extrapolation should be made with caution. Another possible limitation might be that right atrial appendages were obtained from patients taking antianginal medication, and this potentially may have had some influence on the ischemia-reoxygenation injury. Furthermore, our studies were performed in an in vitro preparation, and therefore the clinical application of the results should also be made with caution.

Despite the above potential shortcomings, the findings of our study may have important therapeutic implications for myocardial protection during cardiac operations, cardiac transplantation, angioplasty, and acute myocardial infarction, for which agents acting on α_1 -adrenoceptors are frequently used. Particular attention must be paid during cardiac operations in which phenylephrine is used routinely to elevate the mean arterial blood pressure during cardiopulmonary bypass, and in doing so, one may be unwittingly exacerbating myocardial injury during cardiac ischemia, when collateral blood flow enters the ischemic myocardium. However, these studies have shown that such undesirable effects can be fully counteracted by the administration of phenylephrine before the induction of cardiac ischemia.

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