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Ramiprilat attenuates hypoxia/reoxygenation injury to cardiac myocytes via a bradykinin-dependent mechanism

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Abstract

Isolated rat neonatal cardiac myocytes were subjected to immersion in hypoxic ($PO_2 < 2$ mm Hg), glucose-free Tyrode's solution for 5 h followed by concomitant reoxygenation and staining with the membrane-impermeant fluorophore, propidium iodide, in normoxic ($PO_2 > 150$ mm Hg), serum-free culture media for 15 min in order to assess sarcolemmal damage indicative of myocyte viability due to hypoxia/reoxygenation injury. Prior to hypoxic exposure, cells were pretreated for 90 min with the angiotensin-converting enzyme inhibitor cyclopenta[*b*]pyrrole-2-carboxylic acid, 1-[2-[(1-carboxy-3-phenylpropyl)amino]-1-oxopropyl]octahydro-[2*S*-[1[*R**(*R**)]2α, 3aβ,6aβ]] (ramiprilat), concomitantly with ramiprilat and H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH (bradykinin B_2 receptor antagonist HOE 140), the bioactive peptide Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin) or concomitantly with bradykinin and HOE 140. Hypoxia/reoxygenation injury to untreated control cardiac myocytes was characterized by a significant loss of sarcolemmal integrity measured at 75 ± 4% of total cell fluorescence (mean ± S.E., $n = 42$ cultures). Compared to propidium iodide staining of the above untreated control myocytes, those pretreated with 30 or 100 μM ramiprilat showed a significant reduction of propidium iodide staining to 45 ± 9% and 40 ± 8% ($n = 9$, $P < 0.05$) of untreated controls, respectively. Pretreatment with the protective concentrations of ramiprilat concomitant with 10 μM HOE 140 abolished the significant reduction in propidium iodide staining observed with ramiprilat alone. Similarly, pretreatment with 10 or 100 nM bradykinin significantly reduced propidium iodide staining to 35 ± 5% and 60 ± 10% ($n = 6$, $P < 0.05$) of the untreated hypoxic controls, respectively. In addition, concomitant pretreatment with protective concentrations of bradykinin and 10 μM HOE 140 also abolished the significant reduction in propidium iodide staining observed with bradykinin alone. The results indicate that the angiotensin-converting enzyme inhibitor ramiprilat has a protective effect on isolated cardiac myocytes exposed to hypoxia/reoxygenation and that this effect is most likely related to a local action of bradykinin on the cardiac myocyte via the activation of the kinin B_2 receptor.

Keywords: Ramiprilat; Angiotensin-converting enzyme inhibitor; Bradykinin; Bradykinin B_2 receptor antagonism; HOE 140 (H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH); Cardiac myocyte; Hypoxia/reoxygenation injury

1. Introduction

Angiotensin-converting enzyme inhibitors have been shown to provide cardioprotection following ischemic injury in whole animal models (Westlin and Mullane, 1988; Martorana et al., 1990). Recent investigations have documented that the angiotensin-converting enzyme inhibitor, ramiprilat, reduces myocardial infarct size when administered at a subhypotensive dose in a rabbit model of ischemia/reperfusion (Hartman et al., 1993a). These results suggest that the renin-angiotensin system may modulate the myocardial response to injury at the cellular level rather than by systemic effects.

Cardiac myocytes have been shown to express genes for angiotensinogen and renin (Dzau and Re, 1987; Lindpaintner et al., 1990) and also to contain angiotensin-converting enzyme (Hial et al., 1979; Dostal et al., 1992), the enzyme responsible for converting angiotensin I to angiotensin II. The myocardial renin-angiotensin system is upregulated in response to ischemia, as has been shown by increased angiotensin-converting enzyme activity and production of angiotensin II during and following an ischemic event

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(Tian et al., 1991). The increased local production of angiotensin II is postulated to negatively affect the survival of cardiac tissue following injury due to ischemia/reperfusion. However, a recent investigation has shown that increased circulating angiotensin II levels, or conversely, angiotensin II receptor antagonism with losartan, do not exacerbate or reduce experimental infarct size, respectively, in a rabbit model of myocardial ischemia/reperfusion injury (Hartman et al., 1993b).

In addition to angiotensin I, angiotensin-converting enzyme also has a high binding affinity for the endogenous peptide Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin), which, when bound to the enzyme is degraded to inactive peptide fragments (Bhoola et al., 1992). Angiotensin-converting enzyme inhibitors should therefore block the inactivation of bradykinin and preserve local levels of the endogenously generated peptide. This has been shown in an isolated working rat heart model, in which the venous effluent from the isolated heart had a low concentration of bradykinin, and upon addition of ramiprilat to the arterial perfusate, the concentration of bradykinin was significantly increased (Baumgarten et al., 1993). The investigators not only demonstrated that ischemia itself also stimulated the output of bradykinin by the coronary circulation, but they also reported that cyclopenta[*b*]pyrrole-2-carboxylic acid, 1-[2-[(1-carboxy-3-phenylpropyl)amino]-1-oxopropyl]octahydro-[2*S*-[1[*R**-(*R**)], 2α,3aβ,6aβ]] (ramiprilat) administration prior to ischemia produced highly significant increases in bradykinin outflow from the isolated heart. Hartman et al. (1993a) have recently shown that pretreatment with H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH (HOE 140), a specific bradykinin B_2 receptor antagonist reversed the cardioprotective effects (infarct size reduction) of ramiprilat given during occlusion in an open-chest rabbit model of ischemia/reperfusion. Collectively, the above evidence suggests that the cardioprotective actions of the angiotensin-converting enzyme inhibitor ramiprilat may be mechanistically based on the preservation of endogenously generated bradykinin and the activation of cellular pathways stimulated by bradykinin binding to the B_2 receptor. The anatomical tissue location of the B_2 receptors involved in the cardioprotective mechanism mediated by bradykinin is unclear. The responsible receptors could be located on coronary vascular endothelial cells and/or within the cardiac myocytes themselves (McEachern et al., 1991), thereby exerting cardioprotective effects via paracrine- or autocrine-based pathways. Thus, one objective of this investigation was to determine if ramiprilat could reduce injury to isolated rat neonatal cardiac myocytes caused by prolonged hypoxic exposure followed by reoxygenation. Further, we assessed the mechanistic basis of this protection in relation to bradykinin by attempting to block putative bradykinin B_2 receptors on the cardiac myocytes with HOE 140 and also by testing for direct protective effects of exogenously added bradykinin.

2. Materials and methods

All procedures in this study were performed in compliance with the Declaration of Helsinki, the Animal Welfare Act Regulations, 9 CFR parts 1, 2 and 3 and with the *Guide for the Care and Use of Laboratory Animals*, DHEW Publication (NIH) 85-23, 1985.

2.1. Materials

Ramiprilat was obtained from Hoechst AG, Frankfurt, Germany. Propidium iodide was obtained from Molecular Probes, Eugene, OR, USA. All cell culture solutions, assay kits, and supplies were obtained from Sigma Chemical Company, St. Louis, MI, USA, unless otherwise noted. Hanks balanced salt solution (Ca^{2+}/Mg^{2+}-free; HBSS) was supplemented with ethylenediamine tetraacetic acid (0.5 mM), Hepes (*N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid) buffer (20 mM), $NaHCO_3$ (4.2 mM), glucose (5.6 mM), and gentamicin sulfate (50 mg/l). Tissue digestion solution was made up of 98.4 ml HBSS, 1.6 ml of a 5% bacto-trypsin solution in HBSS (Difco, Detroit, MI, USA), and 20 mg of collagenase (CLS2, 200 U/mg, Worthington Biochemical Corporation, Freehold, NJ, USA). Culture media was a mixture (1 : 1) of Dulbecco's Modified Eagles Medium and Ham's Nutrient Mixture F-12 supplemented with 10% fetal bovine serum, glucose (25 mM final concentration), Hepes buffer (20 mM), $NaHCO_3$ (14.3 mM), insulin (1.4 U/l), and gentamicin sulfate (40 mg/l). Glucose-free, modified Tyrode's solution consisted of $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), KCl (5.4 mM), NaCl (138 mM), KH_2PO_4 (0.4 mM), Na_2HPO_4 (0.3 mM), and Hepes buffer (20 mM), pH adjusted to 7.4 with alkali. Laminin (Engelbreth-Holm-Swarm lathrytic mouse tumor source) precoated 6-well culture plates were purchased from Collaborative Biomedical Products, Bedford, MA, USA.

2.2. Cardiac myocyte isolation and culture

The isolation procedure used was similar to a previously published technique (Linseman et al., 1994). Twenty neonatal (1–2 day old) Sprague-Dawley rat pups were used for each isolation. The animals were killed by decapitation and the hearts aseptically removed and placed in a 60 mm petri dish containing 10 ml of HBSS and subsequently subjected to washing in three more dishes of HBSS. Hearts were then cut into quarters with fine surgical scissors and washed twice more in HBSS. The tissue was then transferred into a sterile 50 ml trypsinization flask containing a micro stir bar and 20 ml of digestion solution (preheated to 37°C). The flask was loosely capped and placed on a stir plate in a 37°C, humidified incubator (5% CO_2-95% room air) for 20 min. The solution was decanted from the trypsinization flask and discarded as the final wash. Fresh pre-warmed digestion solution was added

to the tissue suspension and stirred for 20 min in the incubator. The solution was decanted into 25 ml of culture media, inverted several times to quench the trypsin reaction, and stored at room temperature, inverting every 10 min to avoid sedimentation. The digestion procedure was repeated three more times and the cell suspensions were centrifuged at 400 × g for 5 min at 4°C. Supernatants were discarded and the cell pellets were resuspended in 100 ml of culture media which was equally dispersed into four uncoated 75 cm^2 culture flasks and placed in the incubator for 90 min. This step was performed to remove unwanted nonmuscle cells (e.g., fibroblasts and endothelial cells) (Kasten, 1973). The nonadherent cells (myocytes) were drawn off into four 50 ml centrifuge tubes and the flasks were rinsed with media (20 ml each) which was added to the cell suspensions and centrifuged as above. The resulting cell pellets were combined and resuspended in culture media.

Viability was assessed by trypan blue exclusion and the number of viable cells was determined by counting on a hemocytometer. Total yields generally averaged from 4×10^7 to 7×10^7 myocytes per isolation. Myocytes were seeded at a density of 10^6 cells per well in 35 mm, six-well laminin precoated plates. Cells were allowed to attach for 24 h and were then washed with media. Media was changed every second day. The average purity (assessed visually by phase contrast microscopy) was 80–90% cardiac myocytes, 5–15% fibroblasts and 3–5% endothelial cells after 5 days in culture. Cultures were used for experimentation on day 5 post-isolation at which time the myocytes had formed a confluent monolayer displaying spontaneous synchronous contractions.

2.3. Hypoxia / reoxygenation protocol and assessment of injury

A rectangular glass tank containing 3 liters of glucose-free, modified Tyrode's solution was placed in a 37°C water bath. The pH and osmolality of the solution were maintained within physiological range (7.2–7.4 and 310–330 mOsm) throughout the experiment. The solution was equilibrated with pure nitrogen for 90 min via an air stone mounted on the bottom of the tank to produce a hypoxic environment. Oxygen partial pressure (PO_2) of the solution was measured with a polarographic dissolved oxygen electrode (Cole-Parmer). The PO_2 of the solution was < 2 mm Hg throughout the 5 h hypoxic exposure (normoxic $PO_2 = 150–180$ mm Hg). During the 90 min nitrogen equilibration period, cells were separately pretreated in the incubator with media alone or media containing either ramiprilat, HOE 140 or bradykinin. The media was then withdrawn and the plates were submerged in the hypoxic solution for 5 h. Nitrogen perfusion of the hypoxic solution was continued for the entire experimental period during which temperature (37°C) and PO_2 (< 2 mm Hg) were monitored continuously.

After 5 h of submersion in the nitrogen-perfused bath the culture plates were removed from the hypoxic solution and the excess liquid was removed by aspiration. One milliliter of propidium iodide (40 μg/ml in normoxic, serum-free, culture media) was added to each well and incubated at 37°C for 15 min to serve as the reoxygenation period. Propidium iodide is a membrane-impermeant nuclear stain which has been used as a fluorescent indicator for loss of membrane integrity (Lemaster et al., 1987). After 15 min of reoxygenation, the propidium iodide solution was removed and each well was washed with 2 ml of normoxic Tyrode's solution and a final 1 ml of Tyrode's was added. The propidium iodide fluorescence was quantitated on a Cytofluor 2300 fluorescence plate reader using a 530 nm excitation filter and a 645 nm emission filter. A fluorescence value indicative of a loss of membrane integrity in 100% of the cells was obtained by lysing untreated media control cultures with digitonin (100 μM) prior to propidium iodide staining. Background autofluorescence was measured and subtracted by staining intact, untreated media control cultures. Total cellular protein was measured spectrophotometrically by the Bradford method (coomassie blue) after solubilizing the cells in alkali (Freshney, 1987). Propidium iodide values for all cultures were calculated as fluorescence units/mg of total cellular protein in order to control for variability in cell number per culture. Propidium iodide values for drug-pretreated cultures are expressed as a percentage of the hypoxic control fluorescence. In addition to untreated media control cultures which were exposed to normoxic culture media in the incubator for the entire experimental period, other cultures were also maintained in normoxic, glucose-free Tyrode's solution for 5 h at 37°C to determine if glucose deprivation alone, in the absence of hypoxia, was detrimental to the cells.

2.4. Validation of hypoxia / reoxygenation model

Neonatal rat cardiac myocytes were shown previously (Linseman et al., 1994) to undergo a $76 \pm 12\%$ loss of sarcolemmal integrity (positive propidium iodide nuclear stain) after 5 h of hypoxic exposure. Therefore, all experiments in this investigation used a 5 h period of hypoxia to induce cellular damage. However, in the Linseman et al. (1994) investigation, myocytes were stained with propidium iodide in a normoxic solution for 10 min in ambient atmospheric conditions making reoxygenation injury a possible complicating factor when interpreting drug effects on cardiac myocyte viability. Therefore, a preliminary experiment was performed for this investigation to assess cell injury associated with various reoxygenation times related to propidium iodide staining duration. One group of myocytes was stained with propidium iodide dissolved in the hypoxic Tyrode's solution (15 min exposure time) and fluorescence was quantitated immediately following the 5 h of hypoxia. Several other groups of myocytes were

subjected to 5 h of hypoxia and then stained with propidium iodide dissolved in normoxic, serum-free, culture media for 5, 10, 15, 30, 60 or 120 min at 37°C before quantitation of fluorescence. This experiment was performed in order to determine if reoxygenation injury occurred during the propidium iodide staining procedure, and at which duration of normoxic exposure was the reoxygenation injury maximal. The injury observed could then be validly termed hypoxia/reoxygenation injury.

2.5. Treatment groups

Cells were pretreated for 90 min (effective ramiprilat pretreatment time was determined in a preliminary set of experiments) at 37°C prior to hypoxia/reoxygenation with culture media alone (CONH/R) or with ramiprilat dissolved in culture media at concentrations of 10, 30 and 100 μM. Parallel groups run in separate experiments were pretreated with media alone, 30 and 100 μM ramiprilat alone, 10 μM HOE 140 alone dissolved in culture media, or concomitantly with 10 μM HOE 140 and 30 or 100 μM ramiprilat before being exposed to hypoxia/reoxygenation. In a separate series of experiments cells were pretreated for 90 min at 37°C prior to hypoxia/reoxygenation with culture media alone or bradykinin dissolved in culture media at concentrations of 1, 10, 100 and 1000 nM. Parallel groups run in separate experiments were pretreated with culture media alone, 10 and 100 nM bradykinin alone, 10 μM HOE 140 alone, or concomitantly with 10 μM HOE 140 and 10 or 100 nM bradykinin before exposure to hypoxia/reoxygenation. In each of the above experiments a group of cardiac myocytes were run in parallel where culture media was replaced with glucose-free Tyrode's and the cells were incubated under normoxic conditions at 37°C for 5 h to ascertain if substrate (glucose) deprivation alone caused detectable injury to the cardiac myocytes.

2.6. Angiotensin-converting enzyme activity in isolated cardiac myocytes

Cells were incubated at 37°C with 0, 10, 30 or 100 μM ramiprilat dissolved in Tyrode's solution (glucose 25 mM, pH 7.4) containing 30 ng/ml angiotensin I for 90 min. Following the 90 min incubation, the Tyrode's solution covering the cells was removed and assayed for angiotensin I content by radioimmunoassay (Incstar, Stillwater, MN, USA. GammaCoat ^{125}I Plasma Renin Activity Radioimmunoassay Kit # CA-1533, 1553). The cells were solubilized in alkali and cellular protein was measured spectrophotometrically by the Bradford method. Angiotensin I concentration was calculated as $ng^{-1} \cdot ml^{-1} \cdot mg^{-1}$ cellular protein and expressed as a percentage of angiotensin I concentration in Tyrode's solution not exposed to the cultured cardiac myocytes. In this way, activity of the angiotensin-converting enzyme could be quantitated in the cultured cardiac myocyte system by determining the amount of angiotensin I converted to angiotensin II. When angiotensin-converting enzyme activiy is uninhibited by ramiprilat more angiotensin I will be converted and less will be detected in the Tyrode's solution.

2.7. Statistical analysis

Statistical differences between the untreated media control, hypoxia/reoxygenation control, HOE 140 control and ramiprilat-, ramiprilat + HOE 140-, bradykinin- and bradykinin + HOE 140-treated cultures were analyzed by ANOVA (one way analysis of variance) and comparisons between treatment groups were made using Tukey's HSD (honest significant difference) post-hoc test (Statistica/W Ver. 4.5 software). Mean values were considered statistically significantly different at $P < 0.05$. Results are graphically expressed as the means ± S.E. Values for n indicate the number of individual cultures (plate wells) assayed. Each mean value is based on results obtained from two to five independent isolations.

3. Results

Normoxic incubation of cardiac myocytes at 37°C in glucose-free Tyrode's solution for 5 h did not cause any positive staining with propidium iodide ($n = 12$). The mean value for sarcolemmal damage due to hypoxia/reoxygenation exposure for all control hypoxia/reoxygenation groups in this investigation as a percentage of total possible damage (digitonin lysis) was 75 ± 4% ($n = 42$).

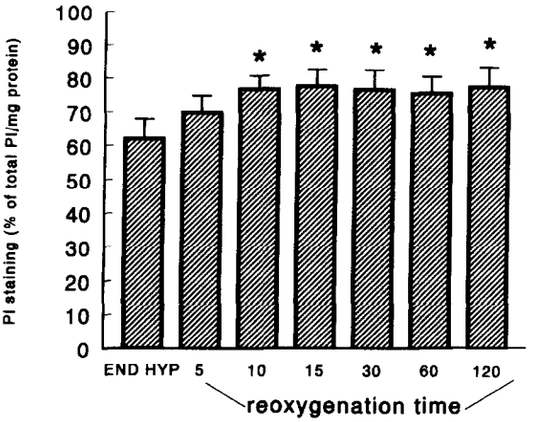

Fig. 1. Propidium iodide staining as a percentage of total possible staining (100% represents degree of propidium iodide staining in control cells lysed with 100 μM digitonin) assessed without reoxygenation at the end of 5 h of hypoxic exposure (END HYP) and assessed with reoxygenation during staining with propidium iodide at different durations (5, 10, 15, 30, 60, 120 min) following 5 h of hypoxic exposure. Data are means ± S.E., $n = 6$ individual cultures for each time point. * Significantly different from END HYP at $P < 0.05$.

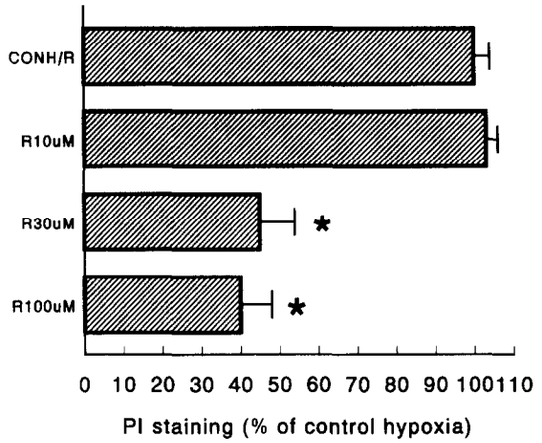

Fig. 2. Propidium iodide staining of cardiac myocytes expressed as a percentage of propidium iodide staining observed in the untreated hypoxia/reoxygenation control group (CONH/R, set at 100% propidium iodide staining) pretreated for 90 min with ramiprilat (R10, R30 and R100 μM) in culture media prior to 5 h of hypoxia and 15 min of reoxygenation. Data are means ± S.E., $n = 9$ individual cultures for each time point. * Significantly different from CONH/R at $P < 0.05$.

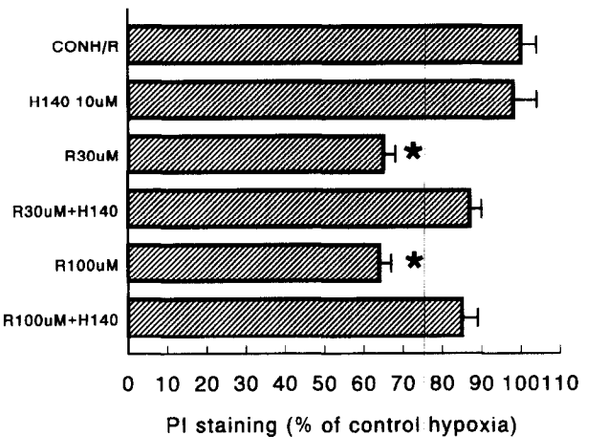

Fig. 3. Propidium iodide staining of cardiac myocytes expressed as a percentage of propidium iodide staining observed in the untreated hypoxia/reoxygenation control group (CONH/R, set at 100% propidium iodide staining) pretreated for 90 min with 10 μM HOE 140 alone (H140-10 μM), ramiprilat (R30 and R100 μM) or 10 μM HOE 140 and ramiprilat concomitantly (R30 μM + H140 and R100 μM + H140) in culture media prior to 5 h of hypoxia and 15 min of reoxygenation. Data are means ± S.E., $n = 15$ individual cultures for each time point. * Significantly different from CONH/R at $P < 0.05$.

3.1. Hypoxia / reoxygenation model validation

Fig. 1 illustrates that 5 h of hypoxia and glucose deprivation caused a positive propidium iodide staining value of 62 ± 6% ($n = 6$, all groups) of total when untreated myocytes were not exposed to normoxic conditions during the propidium iodide staining procedure. However, untreated cardiac myocytes which were stained with propidium iodide dissolved in serum-free culture media under normoxic conditions for various durations showed an increase in positive propidium iodide staining to 77 ± 4% of total. This increase was small but statistically significant, and reached a maximal value at 10 min of normoxic propidium iodide staining or reoxygenation time.

3.2. Ramiprilat pretreatment

Pretreatment of cardiac myocytes with 30 or 100 μM ramiprilat dissolved in culture media ($n = 9$ each group) significantly reduced the amount of positive propidium iodide staining to 45 ± 9% and 40 ± 8% of the hypoxia/reoxygenation control myocytes, respectively (Fig. 2). However, pretreatment with 10 μM ramiprilat did not reduce positive propidium iodide staining. Concomitant pretreatment of myocytes with 10 μM HOE 140 and 30 or 100 μM ramiprilat (Fig. 3, $n = 15$ each group) reversed the significant reductions in positive propidium iodide staining of cardiac myocytes exposed to hypoxia/reoxygenation from 65 ± 3% to 87 ± 3% and from 64 ± 3% to 85 ± 4% of the hypoxia/reoxygenation control myocytes, respectively. Whereas pretreatment with 10 μM HOE 140 alone did not increase the positive propidium iodide staining value above that of the hypoxia/reoxygenation control myocytes.

3.3. Bradykinin pretreatment

Pretreatment of cardiac myocytes with 10 or 100 nM bradykinin dissolved in culture media ($n = 6$ each group) significantly reduced the amount of positive propidium iodide staining to 35 ± 5% and 60 ± 10% of the hypoxia/reoxygenation control myocytes, respectively (Fig. 4). However, pretreatment with either 1 nM or 1 μM bradykinin did not significantly reduce positive propidium

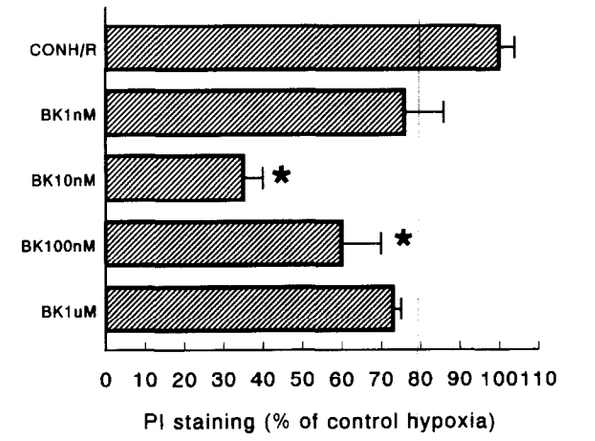

Fig. 4. Propidium iodide staining of cardiac myocytes expressed as a percentage of propidium iodide staining observed in the untreated hypoxia/reoxygenation control group (CONH/R, set at 100% propidium iodide staining) pretreated for 90 min with bradykinin (BK1 nM, BK10 nM, BK100 nM and BK1 μM) in culture media prior to 5 h of hypoxia and 15 min of reoxygenation. Data are means ± S.E., $n = 6$ individual cultures for each time point. * Significantly different from CONH/R at $P < 0.05$.

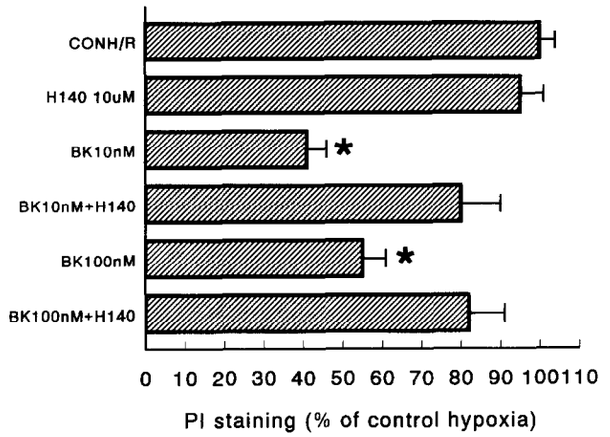

Fig. 5. Propidium iodide staining of cardiac myocytes expressed as a percentage of propidium iodide staining observed in the untreated hypoxia/reoxygenation control group (CONH/R, set at 100% propidium iodide staining) pretreated for 90 min with 10 μM HOE 140 alone (H140-10 μM), bradykinin (BK10 and BK100 nM) or 10 μM HOE 140 and bradykinin concomitantly (BK10 nM + H140 and BK100 nM + H140) in culture media prior to 5 h of hypoxia and 15 min of reoxygenation. Data are means ± S.E., $n = 12$ individual cultures for each time point. * Significantly different from CONH/R at $P < 0.05$.

iodide staining. Concomitant pretreatment of myocytes with 10 μM HOE 140 and 10 or 100 nM bradykinin (Fig. 5, $n = 12$ each group) reversed the significant reduction in positive propidium iodide staining of cardiac myocytes exposed to hypoxia/reoxygenation from 41 ± 5% to 80 ± 10% and from 55 ± 6% to 82 ± 9% of the hypoxia/reoxygenation control myocytes, respectively. Whereas pretreatment with 10 μM HOE 140 alone did not increase the positive propidium iodide staining value above that of the hypoxia/reoxygenation control myocytes.

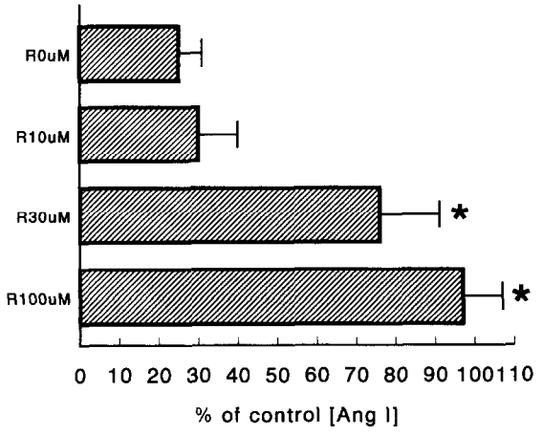

Fig. 6. Angiotensin I concentration expressed as a percentage of control (solution not exposed to cardiac myocytes) in Tyrode's solution also containing 0 (R0 μM), 10 (R10 μM), 30 (R30 μM) or 100 μM (R100 μM) ramiprilat which was incubated with cardiac myocytes for 90 min. Data are means ± S.E., $n = 4$ individual cultures for each concentration of ramiprilat. * Significantly different from 0 μM ramiprilat group at $P < 0.05$.

3.4. Verification of angiotensin-converting enzyme inhibition

Fig. 6 documents that only the two highest concentrations of ramiprilat significantly reduced conversion of angiotensin I to angiotensin II.

4. Discussion

This investigation illustrates that neonatal rat cardiac myocytes can be consistently injured by a combination of glucose deprivation and hypoxic exposure for a 5 h time period. Glucose deprivation alone for 5 h does not cause injury to the cardiac myocytes. The 5 h time course of hypoxia and glucose deprivation is similar to other studies that employ neonatal cardiac myocytes in similar models of hypoxia-induced injury (Thandroyen et al., 1992; Linseman et al., 1994). Positive propidium iodide staining as a marker of membrane damage is also well documented (Lemaster et al., 1987), and in this model of injury correlates well with leakage of the cellular enzymes lactate dehydrogenase and creatine kinase reported in a previous study as independent indicators of cellular viability (Linseman et al., 1994).

In a previous study using the same model of hypoxic cardiac myocyte injury, Linseman et al. (1994) showed that a small, but statistically significant, amount of reoxygenation injury occurred following hypoxia when cells were re-exposed to normoxic conditions for an 18 h period. An additional 14% of total cellular lactate dehydrogenase was lost during this time period and was proposed to be related to reoxygenation injury. In the present investigation, reoxygenation injury additional to hypoxic damage was quantitated using positive propidium iodide staining (Fig. 1) instead of lactate dehydrogenase leakage. Propidium iodide staining increased significantly by approximately 16% over a 2 h period of subsequent normoxia and was maximal by 10 min of normoxic exposure. This indicates that reoxygenation increases damage to the sarcolemma of the cardiac myocytes after hypoxic exposure and that the model of injury used in this investigation incorporates both phases of injury in parallel with in vivo studies of ischemia/reperfusion. The protective drug effects observed in this investigation are being assessed after maximal reoxygenation injury (indicated by propidium iodide exclusion) should have occurred thereby rendering the results increasingly relevant to in vivo evaluations of myocardial ischemia/reperfusion injury.

This investigation illustrates that the angiotensin-converting enzyme inhibitor ramiprilat had a direct protective effect on isolated cardiac myocytes subjected to insult by hypoxia/reoxygenation. The protective action of ramiprilat appears related to preservation of the endogenous peptide, bradykinin, since the bradykinin B_2 receptor antago-

nist HOE 140 blocked protection by ramiprilat. In addition, exogenously added bradykinin also reversibly (by HOE 140) protected the cardiac myocytes from hypoxia/reoxygenation injury. There are however, several characteristics of the results obtained in our investigation that require explanation. First, the effective (protective) concentration of ramiprilat observed in the present investigation is much higher than that observed to provide protection in whole animal and isolated heart models of ischemia/reperfusion injury. This could be due to a difference in cellular location of the angiotensin-converting enzyme and accessibility to the converting enzyme by ramiprilat. There is evidence that the angiotensin converting enzyme is located on the luminal surface of the membrane of endothelial cells (Linz et al., 1992) whereas it is located perinuclearly within the cytoplasm of the cardiac myocyte (Dostal et al., 1992). In experimental models with an intact functional coronary endothelium ramiprilat would have clear access to the angiotensin-converting enzyme. Also, since the endothelial cell to cardiac myocyte ratio is much greater in the whole heart the angiotensin-converting enzyme blockade may alter the physiology of the endothelial cell and indirectly afford protection of the cardiac myocytes at much lower doses of ramiprilat by a regional paracrine effect. A recent investigation by De Mello et al. (1992) illustrates that enalaprilat (an angiotensin-converting enzyme similar in structure to ramiprilat) does not easily cross the membrane of the cardiac myocyte. The prodrug, enalapril, easily penetrates the cell membrane and is converted to the active angiotensin-converting enzyme inhibitor within the cardiac myocyte. Assuming there is a similar relationship between ramipril, ramiprilat and membrane penetration, then a higher extracellular concentration of ramiprilat would be needed in order to obtain an effective level of intracellular angiotensin-converting enzyme inhibition. The possibility also exists that the angiotensin-converting enzyme expressed in cardiac myocytes is a different isoform than that expressed on endothelial cells (Schunkert et al., 1993). Ramiprilat may have a lower binding affinity for the cardiac isoform and a higher concentration of ramiprilat would again be required for an effective level of enzyme inhibition. In addition, an investigation by Dostal et al. (1992) suggests that angiotensin-converting enzyme density and/or activity may be much higher in cardiac myocytes than endothelial cells which would also require higher concentrations of ramiprilat in order to inhibit angiotensin-converting enzyme to effective levels.

The lack of concentration-dependent protection with either ramiprilat or exogenous bradykinin is also problematic. Angiotensin-converting enzyme activity in the experimental cultured cardiac myocyte system was not inhibited by 10 μM ramiprilat but did trend toward a concentration-dependent inhibition of activity at 30 and 100 μM concentrations of ramiprilat (Fig. 6). This illustrates the correlation between angiotensin-converting enzyme inhibition and protection of the cardiac myocytes against hypoxia/reoxygenation injury in the isolated cell model used in the present investigation. The lack of a strong concentration-protection relationship may be due to the type of mechanisms triggered by bradykinin B_2 receptor activation that are responsible for preservation of sarcolemmal integrity. The study by Linz et al. (1992), which determined the response of nitric oxide production and prostacyclin synthesis to different concentrations of both exogenous ramiprilat and bradykinin in endothelial cells, illustrates two observations relevant to the present investigation. First, nitric oxide production and prostacyclin synthesis did not show a strong concentration-related response to either ramiprilat or bradykinin, rather, the response was all or nothing in nature. Second, the effective concentrations of bradykinin which produced maximal nitric oxide and prostacyclin synthesis were identical to those observed in the present investigation, however, our effective concentrations of ramiprilat were 10-fold higher. This observation provides more evidence to the argument that ramiprilat has restricted access to or limited affinity for the angiotensin-converting enzyme in cardiac myocytes. In addition, there may also be variability of angiotensin-converting enzyme expression and activity between different cardiac myocyte preparations which would affect the basal concentrations of bradykinin intra- and extra-cellularly. Furthermore, the protective effect elicited by increased bradykinin concentration may be activated only above a specific threshold amount of bradykinin B_2 receptor activation or a threshold level of receptor-stimulated processes within the cell, also possibly in an all or none fashion. Supra-threshold concentrations of bradykinin may be deleterious to the myocytes by possibly causing bradykinin B_2 receptor downregulation or membrane damage due to highly increased phospholipase activity and large increases in intracellular Ca^{2+} concentration (Hall, 1992).

Recent work by Rabkin (1993), using a similar model of hypoxia/reoxygenation injury in neonatal chick cardiac myocytes, has shown that the angiotensin-converting enzyme inhibitors captopril and lisinopril enhance the recovery of spontaneous contraction rates during reoxygenation following a 3 h hypoxic exposure. In that study, lisinopril additionaly preserved myocyte viability during exogenous oxidative challenges even though the lisinopril molecule contains no thiol group. Other thiol-containing angiotensin-converting enzyme inhibitors have previously been suggested to be cardioprotective due to their anti-oxidant potential (Grover et al., 1991). The above results would collectively suggest that the protective effects of ramiprilat are not related to antioxidant properties since it also contains no obvious anti-oxidant moieties.

Studies using isolated rat hearts have shown that bradykinin outflow increases in response to global hypoxia (Baumgarten et al., 1993). This would suggest that the enzyme responsible for formation of bradykinin from kininogen may be expressed and active within the cardiac

myocytes (Parratt, 1994). Alternatively, kininogen may be bound to the membrane of the myocytes and cleaved by kallikrein to liberate bradykinin (Schmaier, 1992) or stored in vesicles within the cardiac myocytes (Zanzinger et al., 1994). Upon exposure to hypoxic conditions the bradykinin forming enzyme may be upregulated by a decrease in pH (Moshi et al., 1992) or bradykinin may be released from cellular stores in order to act in an autocrine or paracrine fashion thereby modulating protective physiological mechanisms within cardiac tissue. Assuming that angiotensin-converting enzyme activity is always present at some basal level within the cardiac myocytes, inhibition of angiotensin-converting enzyme by ramiprilat would result in an increasing extracellular concentration of bradykinin available for binding to the bradykinin B_2 receptor, receptors which have recently been shown to be present on cultured neonatal rat cardiac myocytes (Minshall et al., 1994).

The mechanisms by which bradykinin directly protects neonatal cardiac myocytes from sarcolemmal damage induced by hypoxia/reoxygenation injury is unclear. A current interpretation of events distal to initial bradykinin B_2 receptor binding by bradykinin have been described by a subsequent association with a sarcolemmal G protein which activates phospholipase C and A_2 (possibly other isoforms as well) finally resulting in the intracellular generation of inositol triphosphate, diacylglycerol and arachidonic acid, respectively (Hall, 1992). Increased intracellular levels of inositol triphosphate would facilitate the release of internally stored Ca^{2+} which may serve to activate Ca^{2+}-dependent enzymes such as nitric oxide synthase (Hall, 1992). Generation of diacylglycerol results in activation of protein kinase C which has also been shown to be involved in the cardioprotective mechanism associated with the ischemic preconditioning phenomenon (Armstrong et al., 1994 and Yongge et al., 1994). Protein kinase C may selectively inhibit or activate certain ion channels (Higashida and Brown, 1986) in the cardiac myocyte and impart a protective effect by decreasing the electrical excitability of the cell which may result in the uncoupling of excitation-contraction and decrease the use of cellular high energy phosphate stores. Increased levels of arachadonic acid within the myocytes caused by phospholipase A_2 activation could result in the generation of prostacyclin within the myocyte (Church et al., 1993), a paracrine mediator which has been shown to exert beneficial effects in whole animal models of ischemia/reperfusion injury (Van Gilst et al., 1986; Pi and Chen, 1989). Prostacyclin is also a putative K_{ATP} channel opener (Siegel et al., 1990; Jackson et al., 1993) and may exert its protective effects by opening K_{ATP} channels on the cardiac myocytes. Recent work has shown that opening of the K_{ATP} channel in neonatal cardiac myocytes using the K_{ATP} channel opener, cromakalim, prior to hypoxia/reoxygenation injury reduced the amount of propidium iodide staining observed (unpublished data). Openers of K_{ATP} channels have also been shown to reduce infarct size when given prior to ischemia/reperfusion in isolated hearts and intact canine or rabbit models (Grover et al., 1990; Toombs et al., 1992).

Generation of nitric oxide by stimulation of the enzyme nitric oxide synthase has been linked to B_2 receptor agonism (Wiemer et al., 1991). Furthermore, nitric oxide generation via either inducible or constitutive nitric oxide synthase expressed by cardiac myocytes has been shown to reduce the contractility of the myocytes in an autocrine fashion (Balligand et al., 1993, 1995). The nitric oxide-related contractile inhibition may reduce ATP consumption during hypoxic exposure thereby attenuating sarcolemmal damage associated with decreased ATP availability for essential membrane functions. A recent investigation using a rabbit model of ischemia/reperfusion has shown that blocking nitric oxide synthase enzyme prior to administration of ramiprilat reverses the reduction in infarct size observed with ramiprilat treatment alone (Hartman et al., 1994). This suggests that nitric oxide generation due to ramiprilat's effect to increase local levels of bradykinin which mediates the upregulation of nitric oxide synthase activity during ischemia/reperfusion may be linked to the beneficial effects on the survival of cardiac myocytes reported in the present investigation.

In summary, this investigation has shown that the angiotensin-converting enzyme inhibitor, ramiprilat, exerts a protective effect on isolated rat neonatal cardiac myocytes exposed to 5 h of hypoxia followed by 15 min of reoxygenation [1]. Furthermore, antagonism of bradykinin B_2 receptors with HOE 140 during pretreatment with ramiprilat reverses the protective effects of the angiotensin-converting enzyme inhibitor suggesting that bradykinin participates in the protective mechanism of ramiprilat. In addition, pretreatment of cardiac myocytes with bradykinin also resulted in reduced damage to myocyte membranes. The protection afforded by bradykinin was similarly reversed by pretreatment with HOE 140 suggesting that bradykinin binding to the bradykinin B_2 receptor on the cardiac myocyte membrane initiates a cascade of intracellular events which may individually or synergistically impart an increased resistance to cellular damage resulting from the hypoxia/reoxygenation insult. The results obtained in this investigation parallel those observed in many whole animal models of myocardial ischemia/reperfusion injury which show angiotensin-converting enzyme inhibitors to be effective at reducing infarct size (myocardial cellular necrosis). Most significantly, this investigation illustrates that the angiotensin-converting enzyme inhibitor ramiprilat and the bioactive peptide bradykinin have protective effects on the cellular physiology of the isolated cardiac myocyte independent of complicating effects from

[1] These results were previously presented in part at the symposium 'Myocardial Protection from Surgical Ischemic-Reperfusion Injury', Asheville, NC, USA, 1994.

the coronary vasculature, myocardial work load or the autonomic nervous system.

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