Long-Chain Acylcarnitines Mediate the Hypoxia-Induced Increase in \( \alpha_1 \)-Adrenergic Receptors on Adult Canine Myocytes

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To elucidate the mechanisms responsible for the increase in \( \alpha_1 \)-adrenergic receptors during ischemia in vivo, we developed a procedure for measuring \( \alpha_1 \)-adrenergic receptors in isolated, calcium-tolerant adult canine myocytes. Specific \( ^{3}H \)prazosin binding was rapid, saturable, reversible, and demonstrated the expected order of potency and stereospecificity for the \( \alpha_1 \)-adrenergic receptor. Myocytes exposed to 30 minutes of hypoxia at 25°C or only 10 minutes at 37°C exhibited a twofold to threefold increase in the number of \( \alpha_1 \)-adrenergic receptors with no significant change in receptor affinity. This hypoxia-induced increase in receptor number was reversible by 10 minutes of reoxygenation at 37°C. In contrast, more prolonged hypoxia of 80 minutes or hypotonic shock actually decreased receptor number below control levels. The concentration of long-chain acylcarnitines in myocytes also increased threefold on exposure to 30 minutes of hypoxia. Sodium 2,4,6-trichlorophenol-1-oxirane-2-carboxylate (POCA, 10 μM), a potent inhibitor of carnitine acyltransferase I, not only abolished the accumulation of long-chain acylcarnitines but also the increase in \( \alpha_1 \)-adrenergic receptor number induced by 30 minutes of hypoxia. Likewise, incubation of normoxic cells with exogenous palmitoyl carnitine (1 μM) for 10 minutes also increased \( \alpha_1 \)-adrenergic receptor number in the presence or absence of POCA. Thus, hypoxia results in an increase in \( \alpha_1 \)-adrenergic receptors associated with an increase in endogenous long-chain acylcarnitines. Furthermore, inhibition of carnitine acyltransferase I prevents not only the sarcolemmal accumulation of long-chain acylcarnitines but also the exposure of the \( \alpha_1 \)-adrenergic receptor, indicating that accumulation of endogenous long-chain acylcarnitines is critical to the hypoxia-induced increase in \( \alpha_1 \)-adrenergic receptors on adult myocytes.

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Under physiologic conditions, the effects of catecholamines in the heart are mediated primarily through stimulation of \( \beta_1 \)-adrenergic receptors. Recent findings from a number of laboratories have identified an increase in \( \alpha_1 \)-adrenergic responsivity in ischemic myocardium. For example, nonspecific \( \alpha_1 \)-adrenergic blockade with phentolamine or specific, \( \alpha_1 \)-adrenergic blockade with prazosin decreases markedly the incidence of ventricular fibrillation and the frequency of premature ventricular complexes associated with coronary artery occlusion and/or reperfusion in the cat, dog, pig, and rat.\(^*\) \( \alpha_1 \)-Adrenergic receptor number has been shown to increase markedly with ischemia in the cat,\(^{10} \) dog,\(^{11} \) and guinea pig.\(^{12} \) This increase in \( \alpha_1 \)-adrenergic receptor number is reversible with sustained reperfusion\(^{10} \) and correlates closely with the increase in \( \alpha_1 \)-adrenergic responsivity during coronary occlusion and reperfusion.\(^1 \)

Several lines of evidence indicate that endogenous cardiac amphiphiles, including the structurally similar lysophosphoglycerides and long-chain acylcarnitines, increase during myocardial ischemia in vivo,\(^{15–17} \) and that comparable concentrations induce reversible electrophysiologic alterations in vitro,\(^{16–22} \) analogous to those alterations seen in the ischemic heart in vivo. We have shown recently that hypoxia in isolated rat myocytes leads to a marked, preferential increase in sarcolemmal long-chain acylcarnitines assessed using electron microscopic autoradiography.\(^{23} \) In addition, the electrophysiologic alterations resulting from hypoxia were prevented by precluding the sarcolemmal accumulation of long-chain acylcarnitines secondary to inhibition of carnitine acyltransferase I.\(^{22} \) A preliminary study has been reported in which \( \alpha_1 \)-adrenergic receptor density increased in adult rat myocytes exposed to hypoxia.\(^{24} \) Therefore, the present study was performed to assess whether accumulation of long-chain acylcarnitines contributed to the increase in \( \alpha_1 \)-adrenergic receptors during hypoxia. The first portion of the study was performed to extensively characterize the \( \alpha_1 \)-adrenergic receptors on isolated adult canine myocytes. Adult myocytes were used since responses to \( \alpha_1 \)-adrenergic stimulation have been shown to differ in neonatal compared with adult tissue.\(^{25} \) The second portion of the study was performed to assess whether hypoxia increased \( \alpha_1 \)-adrenergic receptors and whether the increase was associated with...
the accumulation of long-chain acylcarnitines measured radioenzymatically. The final series of studies were performed to elucidate whether inhibition of intracellular accumulation of long-chain acylcarnitines during hypoxia also prevented the increase in α1-adrenergic receptors and whether addition of exogenous long-chain acylcarnitines resulted in an increased number of α1-adrenergic receptors on the myocytes.

Materials and Methods

Preparation of Myocytes

Adult canine myocytes were isolated by a newly developed procedure to obtain consistent myocyte preparations with approximately 80% viability. Dogs were anesthetized with thiopental (30 mg/kg), the hearts excised rapidly, and small (2 cm × 1 cm × 0.2 cm) pieces of epicardial tissue from the left ventricle were removed and placed in Krebs-Henseleit buffer, gassed with 95% O2-5% CO2 at pH 7.4. The tissue was rinsed once in Krebs-Henseleit buffer and twice in 15 μM Ca2+/HEPES buffer (115 mM NaCl, 5 mM KCl, 35 mM sucrose, 10 mM glucose, 10 mM HEPES, and 4 mM taurine, pH 6.95 with 1 N NaOH bubbled with 100% O2.). Each piece of tissue was placed in a 125-ml polyethylene wide-mouth bottle with 22 ml of 15 μM Ca2+/HEPES buffer with 0.05% collagenase (Worthington, type CLS II), and the bottle was placed in a 35° C water bath. A Harvard respirator (model 607) was connected to the needle end of a 10-ml glass syringe (without plunger) with the wide end placed in the polyethylene bottle such that the solution was drawn into the syringe with each inspiratory cycle of the respirator. The respirator was adjusted to permit the HEPES/collagenase solution plus tissue to be drawn up to ¾ of the syringe height, 25 times a minute. A stream of O2 (100%) was applied continuously to each bottle during the isolation procedure. The tissue was triturated for 30 minutes after which the HEPES/collagenase solution was decanted and replaced with fresh HEPES/collagenase solution. A second 30-minute incubation was followed by 6 additional 15-minute incubation periods. Fresh HEPES/collagenase solution was used for each of the 8 incubation steps. The two initial 30-minute harvests contained primarily red blood cells and contracted myocytes and were therefore discarded. The HEPES/collagenase solution from each subsequent 15-minute harvest was centrifuged at 50g for 6 minutes and the myocyte pellet resuspended in 2 ml of 50 μM Ca2+/HEPES, pH 7.2.

The percentage of elongated myocytes was improved considerably by separating elongated myocytes from contracted and rounded cells in a percoll gradient followed by a bovine serum albumin (BSA) gradient. The myocytes were suspended initially in 5.3 ml of 50 μM Ca2+/HEPES with 1.4% BSA (pH 7.2), mixed with 4.7 ml isotonic percoll solution (4.23 ml percoll, 470 μl 9% NaCl, 1 drop of 1 N acetic acid, pH 7.3), and centrifuged at 700g for 8 minutes. The bottom layer containing the elongated myocytes was placed on a BSA gradient (60 mg/ml, Sigma A4503 fraction V, 30 ml of 50 μM Ca2+/HEPES) and centrifuged at 50g for 5 minutes. The pellet was resuspended in 50 μM Ca2+/HEPES and washed once in the same medium. The Ca2+ concentration of the myocyte preparation was brought up slowly by steps to 100 μM, 200 μM, 350 μM, and finally 0.5 mM over 2 hours by addition of appropriate amounts of 10 mM Ca2+/HEPES every 30 minutes. The overall recovery of the myocyte preparation was 30–40% of the wet weight of the tissue. The myocytes were kept in the HEPES buffer at 0.5 mM [Ca2+] at 25° or 37° C before and during experimental procedures.

Assessment of Myocyte Viability

Myocyte viability was assessed by a number of procedures. Morphologic analysis using light microscopy was routinely performed, viable myocytes being those that are elongated, with clear striations, sharp edges, and no blebs on the cell surface. Exclusion of trypan blue (0.05%) was also used as an indicator of cell viability. Biochemical analysis of cell viability included measurement of cytosolic enzyme release (lactate dehydrogenase, creatine kinase) and levels of high-energy phosphates (adenosine 5'-triphosphate [ATP], creatine phosphate). Lactate dehydrogenase was assayed by following the decrease in absorbance at 340 nm as pyruvate was reduced to lactate with coupled oxidation of nicotinamide adenine dinucleotide (reduced form, NADH). Creatine kinase was assayed by the method of Oliver and involves the production of ATP from creatine phosphate and adenosine 5'-diphosphate (ADP). The ATP generated by the creatine kinase reaction was utilized in a hexokinase/glucose 6-phosphate dehydrogenase coupled enzyme system, which ultimately yields an amount of reduced nicotinamide adenine dinucleotide phosphate (NADP) proportional to the activity of creatine kinase activity. The formation of reduced NADP was followed by absorbance at 340 nm. ATP was assayed by a modification of the method of Buch. Phosphoglycerate kinase was used to catalyze the formation of ADP and 1,3-diphosphoglycerate from ATP and 3-phosphoglycerate. Glyceraldehyde phosphate dehydrogenase then catalyzes the formation of glyceraldehyde 3-phosphate and NAD from 1,3-diphosphoglycerate and NADH. The decrease in absorbance at 340 nm as NADH was oxidized to NAD is proportional to the ATP originally present in the sample. Creatine phosphate was assayed by forming ATP from creatine phosphate and ADP via creatine kinase. The ATP was subsequently assayed as outlined above, a method based on that of Fawaz and colleagues. All assays were evaluated for linearity over the range used for this study and for recovery by the addition of exogenous ATP, creatine phosphate, lactate dehydrogenase, and creatine kinase. All recoveries are consistent and exceed 90% of the amount added. Assessments of myocyte viability were performed during the experimental time period as outlined in "Results." Morphologic assessments were performed on aliquots before and after the radioligand binding assay of normoxic and hypoxic cells.
Action potentials from viable myocytes were obtained using intracellular microelectrodes (resistance 20 to 50 MΩ and filled with 3 M KCl) with current injection of 2 to 8 nA and stimulus duration of 5 to 7 msec. The cells were perfused with Krebs buffer ([K+] 4 mM, pH 7.3) at 35°C, flow rate of 2 to 4 ml/min, and action potentials were recorded from single cells over a period of 1 hour.

Radioligand Binding Assay for Characterization of α,-Adrenergic Receptors

For characterization of α,-adrenergic receptors, a modified method of the assay of Karliner and colleagues was used. Myocyte preparations (400 μl, approximately 2 mg protein) were incubated in duplicate for 15 minutes with selected concentrations (0.025–1.0 nM) of [3H]prazosin (100 μl, specific activity 82 Ci/mmol) to obtain total binding. Nonspecific binding was performed in duplicate in the presence of phentolamine (10−3 M). After incubation in a shaking water bath at 25°C, the binding reaction was terminated by rapid filtration on a Brandel M48 cell harvester through glass-fiber filter paper (Whatman GF/B presoaked in 0.1% PEI to reduce nonspecific binding to the filters). The filters were then washed with 50 ml of 50 mM Tris, 10 mM MgCl2 (pH 7.4, 4°C) before being placed in scintillation vials and allowed to dry. Scintillation cocktail was added to each vial and radioactivity determined with a Beckman LS 3801 liquid scintillation counter. A 14C-acetyl carnitine contained in the acid soluble fraction was added to [3H]acetyl CoA (New England Nuclear, Boston, Mass.) to react with NEM. Each tube received 50 μl of 70% perchloric acid, were vortexed, and 200 μl of KOH (5 M) were added to hydrolyze the acid soluble and acid insoluble fractions. All samples received 50 μl of HEPES (1 M) were added to the filters and 800 μl of KOH (5 M) were added to hydrolyze the acid soluble fraction containing free and short-chain acylcarnitine. Both sets of vials were incubated at 80°C for 2 hours to hydrolyze acyl groups on the carnitine moiety. The vials were cooled on ice, and 200 μl of HEPES (1 M) were added to the filters. All samples received 50 μl of 70% perchloric acid, were vortexed, and an additional 70% perchloric acid added to attain a pH of 7.0. After cooling on ice for 30 minutes, the vial contents were filtered through Gelman A/E filters (0.3 μm, 13 mm) into preweighed vials, and the vials were reweighed.

The assay involves the conversion of all carnitine present in the acid soluble and acid insoluble fractions to [14C]acetyl carnitine via carnitine acetyl transferase using [14C]acetyl CoA (New England Nuclear, Boston, Mass.). The reaction was driven to completion by the presence of excess N-ethyl maleimide (NEM). Three blanks (600 μl distilled water), a standard curve (10 to 1,000 pmol l-carnitine in 600 μl distilled water), and 200 μl myocyte extract sample (with 400 μl distilled water) were added to successive tubes. To each tube, 400 μl of the following solution was added: HEPES 300 mM, EDTA 3 mM, NEM 5 mM, [14C]acetyl CoA (specific activity 6.71 μCi/mol) 12.43 μM, pH 7.4. The tubes were vortexed and allowed to stand for 10 minutes to permit any free reduced coenzyme A (CoASH) to react with NEM. Each tube received 50 μl of a solution containing 100 μl carnitine acetyl transferase (Sigma Chemical Co., St. Louis, Mo.) and 75 μl HEPES (0.83 M)/ml, pH 7.4. After 60 minutes incubation at room temperature, 750 μl of each assay tube was placed on an acid-washed Dowex resin column in a 13 mmx4 cm serum filter tube (Clay-Adams, Scientific Products, St. Louis, Mo.). Excess [14C]acetyl CoA bound to the resin while the eluate contained [14C]acetyl carnitine. Aquasol (New England Nuclear, 10 ml) was added to the resin eluate, and the vial was counted with quench correction using a Beckman LS 3801 liquid scintillation counter. A standard curve was constructed and linear regression analysis performed. The carnitine content of the acid...
soluble and insoluble fractions from myocyte preparations were determined from the calculated regression equation and expressed per milligrams protein. We and others have shown that the acid insoluble fraction contains exclusively acylcarnitines of chain lengths greater than 12 carbons, whereas the acid soluble fraction contains free carnitine and short-chain acylcarnitine with carbon chain lengths less than 12. 

Determination of the Influence of Exogenous Long-Chain Acylcarnitines on \( \alpha_{1} \)-Adrenergic Receptors

Myocytes (approximately 2–3 \times 10^6 cells) were incubated with palmitoyl-f-carnitine (1 \muM) in HEPES buffer (0.5 mM \[Ca^{2+}\], pH 7.2, total volume 8 ml) for 10 minutes at room temperature. When present, POCA (sodium 2-[5-(4-chlorophenyl)-pentyl]-oxiran-2-carboxylate) was added at a final concentration of 10 \muM. Immediately after 10 minutes incubation, the cells were assayed for specific \[^{3}H\]prazosin binding as described above.

Statistics

Significant differences in ATP and creatine phosphate content, lactate dehydrogenase (LDH) and creatine kinase (CK) activity were determined at both 25° and 37° C (Figure 2). There were no significant differences in ATP or creatine phosphate content or LDH or CK release are shown by the asterisk (\( p<0.05 \) versus values at time 0). For the statistical determination of difference between those values at 25° C were seen, the corresponding values for ATP being 63 ± 3 at time 0 and 61 ± 5 nmol/mg protein 2 hours later and for creatine phosphate, 63 ± 5 and 52 ± 4 nmol/mg protein at time 0 and 2 hours, respectively. There were no significant changes in the release of LDH or CK over the 2-hour normoxic time interval (Figure 1).

Ten minutes after the myocytes were suspended in hypoxic HEPES buffer (Po_2 10 to 15 mm Hg) at 25° C, the content of ATP and creatine phosphate decreased markedly to 35 ± 5 nmol/mg protein and 29 ± 6 nmol/mg protein, respectively. Further significant decreases in ATP and creatine phosphate content occurred after 30 and 80 minutes of hypoxia (Figure 1). Increased release of LDH and CK indicating irreversible cellular disruption was not evident after 30 minutes of hypoxia at 25° C but was evident after 80 minutes of hypoxia when LDH release was 42 ± 4% and CK release was 54 ± 9% of total enzyme activity. 

In separate parallel experiments, the extent of release of LDH and CK over time during the hypoxic interval was determined at both 25° and 37° C (Figure 2). There

![Figure 1. Assessment of viability in adult canine myocytes at 25° C. The release of lactate dehydrogenase (LDH) and creatine kinase (CK) activity is shown in the upper panel. The percentage of total intracellular enzyme activity appearing in the supernatant (ordinate) is expressed over time during normoxia (0 to 120 minutes) and hypoxia (120 to 200 minutes). Each value represents mean ± SEM of 3 experiments. Increased release of enzyme activity indicating irreversible cell damage is only evident after 80 minutes of hypoxia (*\( p<0.05 \) versus values at 130 minutes). The cellular content of adenosine triphosphate (ATP) and creatine phosphate are shown in the lower panel over time during normoxia (0 to 120 minutes) and hypoxia (120 to 200 minutes). Each value represents the mean ± SEM of 3 experiments. Significant decreases in ATP or creatine phosphate content or LDH or CK release are shown by the asterisk (\( p<0.05 \) versus values at time 0).](image-url)
was an initial modest decrease in enzyme release between the initiation of hypoxia and the 10-minute time point. This is the result of the normoxic HEPES buffer being replaced with hypoxic buffer under a nitrogen atmosphere to initiate the hypoxic interval. Therefore, statistical analysis to determine differences in enzyme release over the hypoxic time interval was performed using the 10-minute time point as the control value. At 25°C a significant increase in LDH activity in the extracellular buffer occurred after 80 minutes of hypoxia whereas at 37°C increased LDH release occurs more rapidly with a significant increase after only 40 minutes of hypoxia. Similar, although not identical, time intervals of hypoxia were required for the release of CK activity into the buffer. Thus, marked irreversible membrane damage is evident after 70 minutes of hypoxia at 25°C or 30 minutes of hypoxia at 37°C (Figure 2).

Morphologic assessment of the myocytes also revealed that 30 minutes of hypoxia at 25°C was associated with a modest fall in viability (67-71% elongated and excluding trypan blue), whereas a marked decline in viability was evident at 80 minutes of hypoxia (30-45% elongated and excluding trypan blue). In contrast, hypoxia for only 30 minutes at 37°C resulted in a marked decline in viability (25-36%), whereas no decrease in viability was seen after 10 minutes of hypoxia at 37°C (70-79%).

Cellular ATP and creatine phosphate fell more sharply during hypoxia at 37°C than at 25°C such that by 10 minutes, ATP had fallen to 22 ± 6 nmol/mg protein at 37°C compared to 35 ± 5 nmol/mg protein at 25°C (Figure 3). Similar changes were seen in cellular creatine phosphate content. Indeed, ATP and creatine phosphate after 30 minutes of hypoxia at 37°C had fallen to levels similar to that seen after 80 minutes of hypoxia at 25°C (Figure 3).

Thus, irreversible cell damage induced by hypoxia as indicated by release of cytosolic enzymes, very low high-energy phosphate levels, and morphologic derangements occurred after 70 to 80 minutes at 25°C and after 30 to 40 minutes at 37°C. Myocytes that had been exposed to a hypotonic medium (HEPES buffer without sodium chloride or sucrose) for 30 minutes exhibited similar characteristics to those which had undergone prolonged hypoxia. Morphologic assessment revealed no elongated striated myocytes (all rounded cells); LDH activity in the supernatant was 70% of total; CK release was 76%; ATP content was 36 ± 4 nmol/mg protein and creatine phosphate content was 28 ± 7 nmol/mg protein.

Characteristics of [3H]Prazosin Binding to Intact Myocytes

Total, specific, and nonspecific binding of [3H]prazosin to adult canine myocytes is shown in Figure 4. Specific binding was 70% of total binding at or below Kd and 60% above Kd. Specific binding demonstrated saturation at 0.6 nM [3H]prazosin. Binding analysis was performed at a [3H]prazosin concentration close to Kd (0.30 nM) and was found to be rapid, with half maximal binding occurring at 4 minutes and maximal binding at 15 minutes at 25°C (Figure 5).
Dissociation of bound [\textsuperscript{3}H]prazosin by phentolamine (10 \textmu M) was rapid with half maximal dissociation at 2.5 minutes (Figure 5). The equilibrium dissociation constant \(K_d\), a ratio of the reverse and forward binding rate constants \(K_{\text{on}}/K_{\text{off}}\), was calculated as 0.37 nM, similar to the \(K_d\) calculated from the saturation binding experiments (Figure 4). Kinetic analysis performed at a low [\textsuperscript{3}H]prazosin concentration (0.025 nM) was found to be essentially the same with maximal binding occurring at 15 minutes and the equilibrium dissociation constant, \(K_d\), calculated as 0.25 nM. In addition, kinetic analysis was also performed with hypoxic myocytes and with myocytes exposed to a hypotonic buffer for 30 minutes. In both instances, maximal binding was exhibited at 15 minutes, and the equilibrium binding dissociation constant was similar to the \(K_d\) calculated previously (0.36 nM for hypoxic myocytes and 0.23 nM for hypotonic myocytes). Association and dissociation curves were analyzed by a kinetics computer program by McPherson\textsuperscript{40} written for the IBM-AT. In all cases, a two-site fit was not significantly better than a one-site fit. Thus, specific binding of [\textsuperscript{3}H]prazosin appeared to associate and dissociate from only one site. Hill plots of [\textsuperscript{3}H]prazosin binding and displacement by phentolamine yielded coefficients of 0.98 to 1.05 indicating the absence of any cooperative interaction. The displacement of specific [\textsuperscript{3}H]prazosin binding by selected adrenergic agonists and antagonists is shown in Figure 6. The displacement paralleled the expected order of potency and stereospecificity for the \(\alpha_1\)-adrenergic receptor. The displacement curves were analyzed by EBDA, a program written for computer analysis of competition studies.\textsuperscript{41} The concentration of agonists or antagonists producing 50% displacement (EC\textsubscript{50}), the calculated binding constant \(K_d\), and the Hill coefficient (\(n_H\)) are shown in Table 1. The specific \(\alpha_1\)-adrenergic antagonists prazosin (EC\textsubscript{50} 0.75 nM) and hydroxyethylaminomethyl tetralone (HEAT or BE-2254, EC\textsubscript{50} 23 nM) were considerably more potent at displacing specifically bound [\textsuperscript{3}H]prazosin than the \(\alpha_2\)-adrenergic antagonist yohimbine (EC\textsubscript{50} 1,010 nM). The nonspecific \(\alpha\)-adrenergic antagonist phentolamine (EC\textsubscript{50} 344 nM) was more potent than yohimbine, but less potent than the \(\alpha_1\)-adrenergic antagonists (Table 1). \textsuperscript{[3]H}Norepinephrine (EC\textsubscript{50} 1.63 \textmu M) was more potent than its d-isomer (EC\textsubscript{50} 1,070 \textmu M), demonstrating the stereospecificity of [\textsuperscript{3}H]prazosin binding. The specific \(\alpha\)-agonist l-phenylephrine (EC\textsubscript{50} 0.92 \textmu M) was at least two orders
of magnitude more potent than the \( \beta \)-adrenergic agonist \( l \)-isoproterenol (\( EC_{50} \) was assessed from the displacement curve as the Eadie-Hofstee program could not calculate an \( EC_{50} \) that was approximately 1.2 mM). All Hill coefficients were not significantly different from unity, indicating the absence of cooperative interactions.

**Influence of Hypoxia on \( \alpha \)-Adrenergic Receptors**

Hypoxia was produced by suspending the myocyte preparation in 1 mM glucose/HEPES buffer (pH 7.2) with a \( Po_2 \) between 10 and 15 mm Hg. This was achieved by gassing the HEPES buffer with 100% purified \( N_2 \) for 2 hours and transferring an appropriate volume of this solution, in an air-tight apparatus, to the myocyte preparation under a \( N_2 \) atmosphere.

Typical Scatchard plots of specific \([H]prazosin binding to normoxic myocytes and those exposed to hypoxic conditions for 30 minutes at 25° C are shown in the inset in Figure 4, and mean values (\( n = 8 \)) are given in Table 2. Hypoxia of 30 minutes duration at 25° C led to a marked increase in \( B_{max} \), from 1.93 ± 0.18 to 5.42 ± 0.68 fmol/mg protein (\( p<0.01 \)), with no significant change in receptor affinity. The extent of myocyte viability after 30 minutes of hypoxia at 25° C was similar to that seen in normoxic myocytes as assessed by morphologic and biochemical criteria (see above). In contrast, hypoxia of 80 minutes duration at 25° C, a time interval that led to a marked reduction in the number of elongated myocytes and increased LDH and CK release (Figures 1 and 2), resulted in a modest decrease in receptor number to 1.35 ± 0.21 fmol/mg protein (\( p<0.01 \) compared with control myocytes, Table 2). A similar decrease in receptor number and viability occurred after exposure of myocytes to a hypotonic medium (Table 2).

The data presented in Figure 2 suggested that irreversible cell damage during hypoxia, assessed by enzyme release into the buffer surrounding the cells, began to occur more rapidly at 37° C than at 25° C. Accordingly, \( \alpha \)-adrenergic receptors were measured

| Table 1. \( EC_{50} \) and Binding Affinity Constant (K\(_d\)) for Adrenergic Antagonists (A) and Agonists (B) in Normoxic Canine Myocytes |
|----------------|----------------|----------------|
| Agonist | \( EC_{50} \) (nM)\( ^* \) | \( K_d \) (nM) | \( n_H \) |
| Prazosin | 0.75 ± 0.91 | 0.54 ± 0.59 | 1.0 ± 0.1 |
| BE-2254 | 23.0 ± 12.8 | 3.56 ± 1.43 | 0.9 ± 0.1 |
| Phentolamine | 344 ± 290 | 172 ± 155 | 0.9 ± 0.1 |
| Yohimbine | 1,010 ± 170 | 886 ± 182 | 1.0 ± 0.2 |
| B. Adrenergic agonists |
| Agonist | \( EC_{50} \) (\( \mu \)M) | \( K_d \) (\( \mu \)M) | \( n_H \) |
| \( l \)-phenylephrine | 0.92 ± 0.26 | 0.37 ± 0.98 | 0.9 ± 0.1 |
| \( l \)-norepinephrine | 1.63 ± 0.71 | 0.62 ± 0.34 | 0.9 ± 0.2 |
| \( d \)-norepinephrine | 1,070 ± 8,040 | 431 ± 320 | 1.0 ± 0.1 |

*Statistical significance of agonists at \( p<0.05 \); 1 vs. 3, 2 vs. 3.
†Statistical significance of agonists at \( p<0.05 \); 1 vs. 3, 2 vs. 3.
**Statistical significance of antagonists at \( p<0.01 \); 1 vs. 2.
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\( n_H \) was similar to that seen in normoxic myocytes as

| Table 2. Alterations in \( \alpha \)-Adrenergic Receptor Density (\( B_{max} \)) and Affinity (\( K_d \)) in Isolated Adult Canine Myocytes at 25° C |
|----------------|----------------|----------------|
| Conditions at 25° C | \( n \) (fmol/mg protein) | \( K_d \) (nM) |
| Control (normoxia) | 8 | 1.93 ± 0.18 0.30 ± 0.05 |
| Hypoxia (30 min) | 8 | 5.42 ± 0.68** 0.57 ± 0.10 |
| Hypoxia (30 min) + POCA | 8 | 2.73 ± 0.53†† 0.43 ± 0.10 |
| B. Binding to irreversibly injured myocytes |
| Control (normoxia) | 6 | 2.34 ± 0.25 0.28 ± 0.07 |
| Hypoxia (80 min) | 6 | 1.35 ± 0.21** 0.27 ± 0.06 |
| Hypotonic shock (30 min) | 6 | 1.05 ± 0.17** 0.10 ± 0.02† |
| POCA | sodium 2-[5-(4-chlorophenyl)-pentyl]oxirane-2-carboxylate. |
| Values are mean ± SEM. Statistical significance by analysis of variance and the Tukey-Kramer multiple comparisons test. **\( p<0.01 \) and †\( p<0.05 \) compared with control; ††\( p<0.01 \) and †††\( p<0.05 \) compared with hypoxic values.
after 10 minutes of hypoxia at 37°C to assess the effects of hypoxia at this more physiologic temperature (Table 3). At 10 minutes, receptor number increased over twofold from control, normoxic values of 1.90 ± 0.25 fmol/mg protein to hypoxic values of 4.15 ± 0.55 (p < 0.01), with no change in receptor affinity (Table 3). In addition, 10 minutes of reoxygenation at 37°C after 10 minutes of hypoxia resulted in a return of the receptor number to control normoxic values (1.99 ± 0.32 fmol/mg protein, p < 0.01 compared with hypoxic values, Table 3).

The concentration of long-chain acylcarnitines in the myocyte preparation increased threefold during 30 minutes of hypoxia at 25°C, from normoxic levels of 21.3 ± 2.5 to 66.2 ± 13.3 pmol/mg protein (p < 0.01, Table 4). Although the level of short-chain acylcarnitine and free carnitine decreased modestly, 951 ± 113 to 866 ± 190 pmol/mg protein, this change did not achieve statistical significance (Table 4). Pretreatment of cells with POCA (10 μM), a specific and potent inhibitor of carnitine acyltransferase I, completely abolished not only the increase in long-chain acylcarnitines induced by hypoxia (Table 4), but also prevented the increase in α₁-adrenergic receptor number, seen after 30 minutes of hypoxia at 25°C (Table 2 and Figure 7). Pretreatment with POCA also prevented the increase in α₁-adrenergic receptor number seen after 10 minutes of hypoxia at 37°C (Table 3). Thus, inhibition of carnitine acyltransferase I with POCA blocked both the increase in α₁-adrenergic receptor number and the increase in long-chain acylcarnitine levels induced by hypoxia. Indeed, the magnitude of the increase in α₁-adrenergic receptor density under hypoxic conditions parallels closely the magnitude of the change in the cellular concentration of long-chain acylcarnitines (Figure 7).

Influence of Exogenous Long-Chain Acylcarnitines on α₁-Adrenergic Receptors

To exclude an effect of POCA per se on [H]prazosin binding to hypoxic myocytes, independent of inhibition of carnitine acyltransferase I, an additional series of studies were performed. These studies consisted of assessing whether exogenous long-chain acylcarnitines, in the presence or absence of POCA (10 μM), would lead to an increase in α₁-adrenergic receptor density in normoxic myocytes. Incubation of myocytes under normoxic conditions with palmitoyl carnitine (1 μM) for 10 minutes led to a significant increase in receptor density in the presence or absence of POCA (Table 5). The Kᵦ also increased in the presence of exogenous long-chain acylcarnitine (Table 5). Thus,
Exogenous acylcarnitines led to an increased exposure of α₁-adrenergic receptors in intact normoxic myocytes, despite pretreatment with POCA.

Discussion

Recent results from our laboratory and others have demonstrated that the arrhythmogenic effects of catecholamines in the ischemic and reperfused heart can be mediated through stimulation of not only β-adrenergic, but also α₁-adrenergic receptors. We have reported previously that ischemia in vivo results in an increase in α₁-adrenergic receptor number in membrane fractions derived from the ischemic region, an effect that is reversible with sustained reperfusion. Similar findings have been reported in the dog and guinea pig in response to ischemia. However, the mechanisms responsible for the increase in α₁-adrenergic receptor number during ischemia are unknown. The findings of the present study indicate that sarcolemmal accumulation of long-chain acylcarnitines during hypoxia can mediate an increase in α₁-adrenergic receptor number on adult canine myocytes. This mechanism may contribute to the increase in α₁-adrenergic receptors and, in turn, enhanced α₁-adrenergic responsiveness in ischemic myocardium in vivo. The major findings that support this conclusion include: 1) hypoxia leads to a marked increase in α₁-adrenergic receptor density; 2) the increase in receptor density is associated with a threefold increase after short periods of reoxygenation; 3) the increase in receptor density is reversible after short periods of reoxygenation; 4) inhibition of carnitine acyltransferase I with POCA prevented not only the increase in long-chain acylcarnitines but also the increase in α₁-adrenergic receptor number; and 5) exogenous delivery of long-chain acylcarnitine, in the presence or absence of POCA, in normoxic cells led to an analogous increase in α₁-adrenergic receptor number on isolated adult myocytes. This latter finding indicates that the effect of POCA to prevent the increase in α₁-adrenergic receptor density during hypoxia is not mediated by a nonspecific effect of POCA to interfere with binding of [H]prazosin to the surface α₁-adrenergic receptor.

Previous studies designed to characterize the potential alterations in α₁-adrenergic receptors in response to ischemia have been performed using membrane preparations derived from ischemic tissue. These procedures, by necessity, involve homogenization of myocardial and nonmyocardial tissue and subsequent separation of membrane-enriched fractions. This approach does not permit discrimination between myocardial and nonmyocardial cellular elements. To overcome these difficulties, we developed an adult canine myocyte preparation with intact sarcolemma, devoid of nonmyocardial tissue, to investigate the mechanisms responsible for the increase in α₁-adrenergic receptors. The procedures developed for isolation of adult canine myocytes gave consistently good yields and viability as determined by morphologic, biochemical, and electrophysiologic criteria. Intracellular recordings from elongated myocytes revealed resting membrane potentials of −87 mV and normal transmembrane action potentials, with V_m of phase 0 in the order of 150 V/sec, values similar to those reported by others in intact epicardial tissue slices from the dog.

The cellular contents of ATP and creatine phosphate measured during control incubation for two hours at 25° or 37° C were comparable to or slightly higher than those reported by others in myocyte preparations and comparable to that in perfused hearts. In addition, the release of lactate dehydrogenase and creatine kinase over time during normoxia is similar to the values reported by others and below 15% of total enzyme activity. The onset of irreversible cell injury occurred considerably earlier at 37° C and correlated with a more marked fall in the levels of ATP and creatine phosphate than was seen at 25° C. Piper and colleagues, using adult rat myocytes, found that ATP and creatine phosphate levels decreased rapidly after the onset of hypoxia (20 minutes), although not quite as rapidly as in the present study, and increased release of LDH activity occurred at 30 minutes of hypoxia at 37° C, observations comparable to those reported in this study. In addition, Smith and colleagues found that a fall in the PO2 of the solution bathing chick myocytes to below 12 mm Hg resulted in a rapid decline in the spontaneous beating rate after 10 minutes at 37° C. Evidence was provided to suggest that at this PO2, the rapid decline in contractions resulted from a rapid decrease in high-energy phosphates. Reoxygenation after 10 minutes of hypoxia at 37° C was accompanied by a rapid return to control rates of beating, suggesting no irreversible membrane damage occurred, comparable to our findings in the present study.

Specific [H]prazosin binding to myocytes was found to be rapid, saturable, and reversible. Adrenergic agonists and antagonists displaced in an order of potency and stereospecificity expected for binding to the α₁-adrenergic receptor. The specific α₁-adrenergic antagonists prazosin and BE-2254 showed a greater degree of competitive inhibition of specific [H]prazosin binding than the specific α₁-adrenergic antagonist yohimbine, indicating that the binding was specific to the α₁-adrenergic receptor.

Scatchard plots of specific [H]prazosin binding to canine myocytes under normoxic conditions yielded a...
mean $B_{\text{max}}$ value of 1.93 ± 0.2 fmol/mg protein and an affinity constant ($K_d$) of 0.30 ± 0.1 nM. Sarcolemmal membrane preparations from canine hearts contain an $\alpha_1$-adrenergic receptor number reported to be 54.6 ± 8.2 fmol/mg protein with a $K_d$ of 7.02 ± 3.5 nM. However, these results were obtained with very low specific binding (20 to 30%), which may explain the relatively low affinity for the receptor compared with that in the present study. In addition, the purified sarcolemmal preparation will result in a large increase in $B_{\text{max}}$ due to the large increase in amount of receptor protein per total cellular protein compared with that found in intact, functional myocytes. Intact myocytes from adult rats have a higher density of $\alpha_1$-adrenergic receptors, 76.7 ± 11.1 fmol/mg protein, and a higher affinity, $K_d$ 0.16 ± 0.08 nM, than in the present study. The greater affinity of the receptor may be due to either species differences or secondary to cell lysis. In the present study, cell lysis induced by hypotonic shock resulted in an increase in receptor affinity. This suggests that disruption of the cell results in changes in receptor conformation and ability to interact with ligands. Indeed, Skomedal and colleagues reported that in a rat myocyte preparation, two populations of $\alpha_1$-adrenergic receptors are present with different binding affinities. Muntz and colleagues found a $B_{\text{max}}$ value of 59.1 fmol/mg protein and $K_d$ of 0.26 nM using [$^3H$]prazosin binding in tissue slices from rat hearts, an affinity constant nearly identical to that found in the present study. Specific [$^3H$]prazosin binding to myocytes after a period of hypoxia of 30 minutes at 25°C, or only 10 minutes at 37°C, resulted in a twofold to threefold increase in receptor number compared with control, normoxic values. Thus, at the physiologic temperature of 37°C, a very rapid increase in receptor number was seen. These time intervals were associated with low ATP and creatine phosphate levels but there was no increased release of LDH or CK activities and no discernible morphologic derangements, indicating the absence of any sarcolemmal disruption. The rapid reversibility of the increase in $\alpha_1$-adrenergic receptor number induced by hypoxia was demonstrated by the return to control $B_{\text{max}}$ values after reoxygenation at 37°C.

In contrast, more prolonged hypoxia of 80 minutes at 25°C or cell lysis by incubation in a hypotonic medium resulted in a decrease, rather than an increase, in $\alpha_2$-adrenergic receptor number compared with control values. With these interventions, the percentage of elongated, striated cells, assessed morphologically and by release of cytoplasmic enzymes, was below 40%. Thus, irreversible disruption of the cell does not lead to increased exposure of latent $\alpha_1$-adrenergic receptors.

Maisel and colleagues have shown a translocation, during ischemia in the guinea pig heart, of $\beta$-adrenergic receptors from an intracellular light vesicle fraction to the sarcolemmal fraction. They have reported preliminary data suggesting that this translocation does not occur for the $\alpha_1$-adrenergic receptor, despite the fact that there was a marked increase in $\alpha_1$-adrenergic number in sarcolemmal fractions from ischemic tissue. These findings suggest that the increase in $\alpha_1$-adrenergic receptors seen with short periods of hypoxia in the present study are due to exposure of latent $\alpha_2$-adrenergic receptors within, or closely associated with, the sarcolemma. The fact that our previous study demonstrated, using electron microscopic autoradiography, a preferential increase in sarcolemmal long-chain acylcarnitines during hypoxia in isolated myocytes, indicates that the insertion of this amphiphile into the sarcolemma of an intact cell may be the critical mechanism responsible for the exposure of the $\alpha_1$-adrenergic receptor. This conclusion is also supported by the finding that exogenous palmitoyl carnitine, which would readily incorporate into the sarcolemma, also resulted in an increased density of the $\alpha_1$-adrenergic receptor in normoxic cells.

Fluidity is an inherent property of membranes, the alteration of which affects the accessibility of surface receptors to ligand binding. For example, methylation of phosphatidylethanolamine to phosphatidylcholine increases membrane fluidity and concurrently increases the number of binding sites for the $\beta$-adrenergic ligand [$^3H$]dihydroalprenolol in rat reticulocytes. Conversely, phospholipase $A_2$, which results in the production of lysophosphoglycerides, reduces [$^3H$]dihydroalprenolol binding. During these experiments de novo synthesis of new receptors was not possible; the appearance of new receptors was probably due to alteration in membrane charge or microenvironment of the membrane surrounding the receptor. Thus, accumulation of long-chain acylcarnitines in the sarcolemma with subsequent alterations in sarcolemmal fluidity and the microenvironment surrounding the receptor is the likely mechanism responsible for the increased exposure of $\alpha_1$-adrenergic receptors, by unmasking latent receptors closely associated with the sarcolemma.

Based on data derived from multiple cell systems, cell surface receptors to hormones, including catecholamines, are regulated through synthesis, internalization, externalization, recycling, and degradation. Since protein synthesis of new receptors and subsequent transfer and externalization to the surface of the sarcolemma obviously requires an extended period of time, the exposure of $\alpha_1$-adrenergic receptors in response to 10 minutes of hypoxia at 37°C is probably not mediated by this process. This conclusion is also supported by the data demonstrating that exogenous exposure to long-chain acylcarnitines for only 10 minutes leads to a comparable increase in $\alpha_1$-adrenergic receptors. The increase in long-chain acylcarnitines in hypoxic myocytes occurs to a significant extent in the sarcolemma based on our recent findings using electron microscopic autoradiography. The resultant membrane perturbations induced by insertion of this amphiphile into the sarcolemma could lead to increased exposure or externalization of the $\alpha_1$-adrenergic receptor protein. The question of whether this exposed $\alpha_1$-adrenergic receptor is actually coupled to intracellular events by production of inositol trisphosphate.
from phosphatidylinositol 4,5-bisphosphate and capable of mediating a physiologic response in response to catecholamines is presently under investigation.

The possibility that stimulation of this externalized receptor during myocardial ischemia and early reperfusion leads to an increase in cytosolic calcium is supported by our findings demonstrating that α1-adrenergic blockade attenuates markedly the increase in cytosolic calcium during reperfusion of reversible injured tissue. Likewise, attenuation of the α1-adrenergic mediated increase in cytosolic Ca++ during reperfusion leads to a marked reduction in the extent of ultimate cellular necrosis. Thus, under pathophysiologic conditions, such as acute myocardial ischemia, stimulation of the externalized α1-adrenergic receptor may lead to a mobilization and net increase in cytosolic calcium.

The recently demonstrated antiarrhythmic effect of inhibition of carnitine acetyltransferase I with POCA in vivo during ischemia is likely mediated not only by prevention of the direct electrophysiologic effects of accumulating long-chain acylcarnitines, but possibly by blockade of the increase in α1-adrenergic receptors during the time course of ischemia. In summary, the present findings indicate, for the first time, one mechanism responsible for the increase in α1-adrenergic receptors during hypoxia in intact myocytes. The question of whether this mechanism is also operative during ischemia in vivo and whether the newly exposed receptor is coupled to breakdown of phosphatidylinositol requires investigation. Whether stimulation of the β-adrenergic receptor under ischemic conditions, leading to intracellular lipolysis, increased free fatty acids and in turn, enhanced production of long-chain acylcarnitines and enhanced externalization of the α1-adrenergic receptor should also be a focus of investigation.

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