Differences in the Electrophysiological Response of Canine Ventricular Epicardium and Endocardium to Ischemia: Role of the Transient Outward Current

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Background. Acute ischemia is known to produce more severe electrophysiological disturbances in canine ventricular epicardium than endocardium, although the mechanism for the differential sensitivity is still unresolved. Recent studies have demonstrated the presence of a prominent transient outward current (I_o) in ventricular epicardium but not endocardium. The present study was designed to test the hypothesis that the differential sensitivity of these two tissues to ischemia results, at least in part, from a more prominent I_o in epicardium than in endocardium.

Methods and Results. Isolated canine ventricular epicardial and endocardial tissues and myocytes were studied by standard microelectrode techniques. Simulated ischemia (hyperkalemia, hypoxia, and acidosis) abolished the action potential plateau and caused a 50% to 60% shortening of action potential duration in epicardium but only a 10% to 20% shortening in endocardium. 4-Aminopyridine, an I_o inhibitor, restored the plateau in epicardium and reduced the dispersion of action potential duration between epicardium and endocardium. Stimulation protocols that minimized the contribution of I_o, such as acceleration of the stimulation rate or introduction of early premature beats, produced a paradoxical prolongation of the epicardial response caused by restoration of the action potential dome. Thus, ischemia-induced dispersion of repolarization was greatly diminished at rapid rates and after premature beats. Similar results were obtained in tissues and myocytes obtained from the same myocardial layers, suggesting that the differential sensitivities of epicardium and endocardium to ischemia are largely a result of inherent differences in cellular properties.

Conclusions. Our data suggest that the presence of a prominent I_o in epicardium but not endocardium contributes importantly to the selective electrical depression of epicardium by simulated ischemia. The repolarizing influence of I_o serves to amplify the ischemia-induced changes in inward (I_{Na} and I_{Ca}) and outward (calcium-activated) currents. By facilitating loss of the dome in epicardium, I_o contributes to the development of a marked dispersion of repolarization between normal and ischemic epicardium and between epicardium and endocardium, thereby providing the electrophysiological substrate for the genesis of reentrant arrhythmias. (Circulation. 1993;88:2903-2915.)

KEY WORDS • ventricles • myocardium • epicardium • endocardium • electrophysiology • ischemia

Numerous in vivo studies have established that acute myocardial ischemia elicits much more severe electrophysiological disturbances in epicardium than in endocardium. Epicardium exhibits more pronounced changes in monophasic action potentials\(^1\) and greater prolongation of conduction time and refractory period\(^2-6\) than does endocardium during acute ischemia. Various explanations have been offered, including contact of endocardium with cavity blood,\(^7,8\) thebesian blood flow,\(^9\) a greater capacity of endocardium for anerobic metabolism,\(^10,11\) and electrotonic interaction of endocardial muscle with subendocardial Purkinje fibers, which are much more resistant to the depressant effects of hypoxia and ischemia.\(^7,9,12\) Recent studies showing a selective depression of the epicardial response despite comparable depolarizations in resting membrane potential in epicardium and endocardium have questioned the first three proposed explanations.\(^13,14\) The hypothesis that the differential sensitivity is due to the presence of subendocardial Purkinje fibers has also been weakened by studies indicating that subendocardial Purkinje fibers are insulated from muscle and do not influence muscle electrotonically, except at the terminal Purkinje fiber–muscle junctions.\(^15,16\)

The available data suggest that the differential sensitivities of epicardium and endocardium to ischemia may reflect fundamental electrophysiological differences between the two tissue types. Notably, transmembrane and monophasic action potentials recorded from epicardium exhibit a spike-and-dome morphology that is absent in action potentials recorded from endocardium.\(^13,14,17,18\) Recent studies have shown that the differences in action potential configuration are in large part due to the presence of a prominent transient...
outward current ($I_o$) in ventricular epicardium but not endocardium.\textsuperscript{19-24} However, the precise role of $I_o$ in epicardium is not clear, and it is uncertain how conditions of ischemia influence this current. The present study was therefore designed to test the hypothesis that the presence of an additional repolarizing current ($I_o$) in epicardium contributes to its selective depression during ischemia.

**Methods**

**Studies on Syncytial Tissue Preparations**

Hearts were removed from adult, male mongrel dogs (15 to 30 kg) anesthetized with sodium pentobarbital (30 mg/kg IV). Epicardial tissues (10×15×0.6 to 1.0 mm) were shaved from the epicardial surface of the right ventricle midway along the apicobasal axis with a dermatome (Davol, Cranston, RI). Endocardial preparations consisted of trabeculae shaved from the base of the right ventricle. Only endocardial preparations that did not exhibit "Purkinje-like" action potentials (verified by roving microelectrode impalements) were used in the study to eliminate the influence of subendocardial Purkinje fibers. Epicardial and endocardial preparations from the same heart were pinned side by side in the same tissue bath (6-mL volume) and superfused with Tyrode's solution containing (in mmol/L): NaCl 129.0, KCl 4.0, Na$_2$HPO$_4$ 0.9, NaHCO$_3$ 20.0, CaCl$_2$ 1.8, MgSO$_4$ 0.5, and glucose 5.5. The solution was maintained at 37±0.5°C and had a final pH of 7.35±0.5. The Tyrode's solution was bubbled with 95% O$_2$/5% CO$_2$ to produce a PO$_2$ of 550 to 650 mm Hg in the tissue bath.

The tissues were stimulated at a basic cycle length (BCL) of 800 milliseconds through silver bipolar electrodes inserted except at the tips. Stimuli were pulses (2 milliseconds in duration and twice diastolic threshold intensity) generated by two Pulsar 6i stimulators (Frederick Haer & Co, Brunswick, Me). Transmembrane potentials were recorded by use of glass microelectrodes filled with 2.7 mol/L KCl (15 to 30 MΩ resistances) connected to a model 750 dual microprobe amplifier (WPI, New Haven, Conn). The amplifier provides an A-B (differential) output by feeding the endocardial and epicardial channels into an internal differential amplifier. This output was used to generate a simulated ECG trace on-line. Signals were displayed on a storage oscilloscope (Tektronix, Beaverton, Ore), amplified with a programmable amplifier (model 1903A; Cambridge Electronic Design [C.E.D.], UK), and digitized with a model 1401 AD/DA system (C.E.D.). Data acquisition was controlled by a Dell System 310 personal computer (Austin, Tex), which was also used for analysis of data files with Spike2 software from C.E.D. Tissues were equilibrated with the normal Tyrode's solution until steady-state transmembrane activity was obtained (typically 3 to 4 hours). The stimulation frequency was then varied over a wide range of BCLs ranging between 250 and 2000 milliseconds, and a restitution of action potential characteristics was performed with single test pulses ($S_2$; 2 milliseconds in duration) delivered after every tenth basic beat ($S_1$). Tissues were then superfused with a solution modified to mimic conditions encountered during ischemia (hyperkalemia, hypoxia, and acidosis). The "ischemic" Tyrode's solution contained (in mmol/L): NaCl 129.0, KCl 6.0, Na$_2$HPO$_4$ 0.9, NaHCO$_3$ 20.0, CaCl$_2$ 1.8, MgSO$_4$ 0.5, and glucose 5.5. The pH was adjusted to 6.8 with 5N HCl. The glass reservoir containing the "ischemic" solution was sealed, and the solution was bubbled with 95% N$_2$/5% CO$_2$ for at least 3 hours. The PO$_2$ of the "ischemic" solution was <45 mm Hg measured in the tissue bath (ISO2 Dissolved Oxygen Meter; WPI, New Haven, Conn). Tissues were superfused with the "ischemic" solution until the action potential dome in epicardium was abolished; frequency and restitution scans were then repeated. In most instances, tissues were then exposed to "ischemic" solution containing 4-amino pyridine (4-AP; 1 to 2 mmol/L). This solution was prepared at least 15 minutes before use, since addition of 4-AP caused a transient pH change lasting 5 to 10 minutes. In some experiments, the normal and "ischemic" solutions contained propranolol (0.3 μg/mL), phenolamine (0.5 μg/mL), and atropine (1 μg/mL), since 4-AP has been reported to cause neurotransmitter release from adrenergic and cholinergic nerve endings. However, no differences in the effect of 4-AP were found in the presence or absence of these agents.

**Studies on Enzymatically Dissociated Ventricular Myocytes**

Ventricular myocytes were isolated as follows. Adult male mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg IV) containing heparin (220 IU/kg). The heart was removed, fibrillated, and submerged in bicarbonate Tyrode's solution. A large wedge of left ventricle was excised around the left anterior descending coronary artery, and the artery was cannulated and flushed with 50 mL nominally calcium-free "Krebs buffer" containing (in mmol/L): NaCl 118.5, KCl 2.8, NaHCO$_3$ 14.5, KH$_2$PO$_4$ 1.2, MgSO$_4$ 2.7, and glucose 11.1, with 0.1% bovine serum albumin (BSA). The wedge was then mounted on a recirculating pump and perfused at a rate of 12 mL/min with calcium-free Krebs buffer supplemented with 0.04% collagenase (at 37°C). After 12 to 17 minutes, the epicardial and endocardial layers (<1.5 mm thick) were shaved from the wedge with a dermatome and placed in 15 mL Krebs buffer supplemented with 1.5% BSA, 0.04% collagenase, 0.5 mmol/L MgSO$_4$, and 0.3 mmol/L CaCl$_2$. The minced tissues were bubbled with 95% O$_2$/5% CO$_2$ and agitated in a 37°C water bath. After 15 minutes, the epicardial and endocardial digests were passed through a nylon mesh (220 μm). Tissue fragments that did not pass through the mesh were returned to the water bath in fresh enzyme solution, whereas the filtrates were centrifuged at 400 rpm. Pellets were then resuspended in HEPES Tyrode's solution containing (in mmol/L): NaCl 132.0, HEPES 20, MgSO$_4$ 3.2, glucose 11.1, CaCl$_2$ 0.5, and KCl 5.0, with 1.5% BSA. The incubation was repeated three or four times at 15-minute intervals. Yields of 20% to 50% (viable to nonviable cells) were typical for each myocardial layer.

Aliquots of cells were placed in a temperature-controlled superfusion chamber (PDMI-2, Medical Systems Corp, Greenvile, NY) mounted on a Nikon Diaphot TMD inverted microscope. Cells were superfused with HEPES Tyrode's solution at a rate of 3 mL/min. The "normal" HEPES Tyrode's solution contained (in mmol/L): NaCl 132.0, KCl 4.0, CaCl$_2$ 1.8, MgSO$_4$ 1.2, HEPES 20.0, and glucose 11.1 (pH 7.4;
Fig 1. Graphs showing effects of simulated ischemia on electrophysiological responses of isolated canine ventricular tissues. In each panel, the upper and middle traces are microelectrode recordings from endocardium (Endo) and epicardium (Epi), respectively, and the lower trace is the simulated ECG obtained by differential recording of the endocardial and epicardial voltages. A, Control records obtained at a basic cycle length of 800 milliseconds. Action potentials recorded from epicardium exhibited a “spike-and-dome” configuration that was absent in those recorded from endocardium. In this example, stimulation was arranged so that endocardial activation preceded epicardial activation by 12 milliseconds to simulate transmural conduction. In most experiments, an upright T wave was obtained in the ECG. B, Action potentials recorded after 28, 29, and 30 minutes of superfusion with ischemic solution. Ischemia abolished the action potential dome in epicardium but produced only a slight shortening of action potential duration in endocardium. Endocardial to epicardial conduction increased to 17 milliseconds by 30 minutes of ischemia. C, Effects of 5 minutes of exposure to 4-aminopyridine (4-AP; 1 mmol/L), an I\textsubscript{Na} inhibitor, in the continued presence of ischemia. 4-AP typically restored the dome in epicardium within 1 to 2 minutes. Continued exposure to 4-AP resulted in a decrease in the notch in epicardium and an increase in phase 1 amplitude in both epicardium and endocardium (not shown).

Results

Effects of Simulated Ischemia on Ventricular Action Potentials

Fig 1 shows representative tracings of transmembrane action potentials and simulated transmural ECGs recorded from canine endocardial and epicardial tissues under control conditions and after exposure of the preparations to simulated ischemic conditions. Under control conditions (panel A), action potentials recorded from epicardium display smaller phase 0 and phase 1 amplitudes but a larger notch than those recorded from endocardium. Typically, action potential duration (APD) was longer in endocardium than epicardium (see Table 1), resulting in a positive T wave in the simulated ECG. The ECG was obtained by differential recording of the endocardial and epicardial transmembrane activity. Exposure of the tissues to ischemic Tyrode’s solution (panel B) caused a decrease in the amplitude of phase 0 and marked changes in the plateau (dome) of the action potential recorded from epicardium. After 28 minutes of ischemia, the spike-and-dome morphology of the epicardial response was accentuated. One minute later, marked depression of the dome occurred and was followed by...
TABLE 1. Effects of Simulated Ischemia and 4-Aminopyridine on Action Potential Parameters in Canine Ventricular Epicardial and Endocardial Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Intervention</th>
<th>Phase 0 Amp, mV</th>
<th>Phase 1 Amp, mV</th>
<th>Notch, mV</th>
<th>Phase 2 Amp, mV</th>
<th>APD$_{90}$, ms</th>
<th>MDP, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicardium</td>
<td>20</td>
<td>Control</td>
<td>98.4±5.2</td>
<td>62.6±5.4</td>
<td>35.7±7.2</td>
<td>107.7±3.3</td>
<td>204.2±14.2</td>
<td>-84.8±1.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>“Ischemia”</td>
<td>76.4±7.9*</td>
<td>73.3±3.8*</td>
<td>6.9±3.3*</td>
<td>13.2±6.1</td>
<td>133.2±11.7*</td>
<td>-67.8±1.0*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>“I’” + 4-AP</td>
<td>78.1±4.9*</td>
<td>73.3±3.8*</td>
<td>7.4±3.1*</td>
<td>20.7±4.6</td>
<td>231.4±16.1</td>
<td>-82.4±1.6</td>
</tr>
<tr>
<td>Endocardium</td>
<td>20</td>
<td>Control</td>
<td>115.5±3.4</td>
<td>102.2±4.1</td>
<td>8.7±3.9</td>
<td>10.7±4.6</td>
<td>231.4±16.1</td>
<td>-82.4±1.6</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>“Ischemia”</td>
<td>97.8±3.4*</td>
<td>89.9±2.2*</td>
<td>3.6±2.8*</td>
<td>88.9±2.3*</td>
<td>174.5±14.6*</td>
<td>-67.2±1.2*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>“I’” + 4-AP</td>
<td>97.4±4.8*</td>
<td>91.3±3.7*</td>
<td>2.2±2.2*</td>
<td>89.2±3.7*</td>
<td>191.7±21.2*</td>
<td>-67.1±1.7*</td>
</tr>
</tbody>
</table>

*P<.05 compared with same tissue control; †P<.05 compared with same tissue “ischemia” value.

Amp indicates amplitude; Notch, phase 2 amplitude minus phase 1 amplitude; APD$_{90}$, action potential duration at 90% repolarization; MDP, maximum diastolic potential; “I”, “ischemia”; and 4-AP, 4-aminopyridine (1 to 2 mmol/L, 15 minutes of exposure). All values are mean±SD obtained at a basic cycle length of 800 milliseconds.

complete abolition of the dome after a total of 30 minutes of ischemia. As a result, APD at 90% repolarization (APD$_{90}$) was markedly abbreviated in epicardium. APD$_{90}$ decreased from 204.2±14.2 milliseconds in control to 90.4±23.3 milliseconds during ischemia (Table 1). In contrast, ischemia produced only a 10% to 20% abbreviation of APD$_{90}$ in endocardium, despite a similar decrease in resting membrane potential in the two tissue types. The marked dispersion of repolarization that developed was manifested as an elevated ST segment in the ECG trace. As an initial test of the hypothesis that the presence of a prominent I$_m$ in epicardium but not endocardium contributes to the differential sensitivities of the two tissues to “ischemia,” we exposed the preparations to the I$_m$ inhibitor 4-AP. In panel C, 1 mmol/L 4-AP was introduced 10 minutes after the dome was abolished in epicardium. 4-AP restored the plateau phase of the epicardial action potential within 1 minute, and the records shown were obtained after 5 minutes of ischemia plus 4-AP. The ST segment and T wave also returned to near normal in the simulated ECG. With continued exposure to 4-AP, phase 0 and phase 1 amplitudes increased further as the size of the notch decreased (time course, ~10 minutes; data not shown, but see Table 1). Thus, 4-AP greatly reduced the marked dispersion of repolarization between epicardium and endocardium caused by ischemia (Table 1).

Ischemia-Induced Changes in Action Potential Duration

The complete time course of changes in APD$_{90}$ recorded in endocardium and epicardium during the experimental protocol is summarized in Fig 2. In epicardium, simulated ischemia produced a triphasic response characterized by an initial abbreviation of APD$_{90}$, followed by a slight prolongation caused by accentuation of the notch, and finally a marked abbreviation caused by loss of the action potential dome. Introduction of 4-AP (1 mmol/L) resulted in restoration of the dome and prolongation of APD$_{90}$. With continued exposure to 4-AP, APD$_{90}$ decreased progressively as the spike-and-dome morphology (notch) of the epicardial response attenuated. The effects of ischemia and 4-AP were rapidly reversed upon washout (reperfusion).

In contrast, endocardium exhibited only slight changes in APD$_{90}$ throughout the experimental protocol. Action potentials in endocardium were much more resistant to the depressant effects of ischemia. Major depression of action potentials in endocardium occurred only after several hours of ischemia. Similar results were obtained in 20 epicardial and endocardial preparations, as summarized in Table 1.

Relation Between Phase 1 Amplitude and Susceptibility to Simulated Ischemia

Ischemia led to loss of the dome in 20 of the 29 epicardial preparations studied. The duration of expo-

![Fig 2. Graph of time course of changes in action potential duration at 90% repolarization (APD$_{90}$) during ischemia and endocardium plus 4-aminopyridine (4-AP) in a representative experiment. Changes in APD$_{90}$ are plotted for endocardium (○) and epicardium (●). Simulated ischemia was associated with an initial abbreviation, then prolongation, of APD$_{90}$ in epicardium until elimination of the dome occurred, at which time a marked abbreviation of APD$_{90}$ was obtained. Switching the bath solution to an ischemic solution containing 1 mmol/L 4-AP reversed the depression of action potentials in epicardium and markedly prolonged APD$_{90}$. In contrast, endocardium exhibited only small changes in APD$_{90}$ throughout the experimental protocol. Thus, a large dispersion of APD$_{90}$ was created between epicardium and endocardium during ischemia. The dispersion in APD$_{90}$ between epicardium and endocardium was greatly reduced in the presence of 4-AP. In all cases, APD$_{90}$ was measured at a basic cycle length of 800 milliseconds.](http://circ.ahajournals.org/Downloaded from http://circ.ahajournals.org/ by guest on November 13, 2017)
to ischemia (Fig 4). Under control conditions (panel A), the spike-and-dome configuration and size of the notch in epicardium are much more exaggerated at slow stimulation rates (BCL, 2000 milliseconds) compared with fast rates (BCL, 400 milliseconds). The rate-dependence of $I_o$ in dog is such that the current increases in amplitude with deceleration of the stimulation rate because of its slow reactivation kinetics.\textsuperscript{19,22,27,28} In contrast, $I_{Ca}$ decreases in amplitude with deceleration.\textsuperscript{29,30} Thus, the accentuated spike-and-dome configuration in epicardium at slower rates probably reflects a larger $I_o$ opposing a smaller $I_{Ca}$. At fast stimulation rates, this relation would be reversed, with a smaller $I_o$ opposing a larger $I_{Ca}$. This shift in the balance of current is thought to account for attenuation and accentuation of the notch at fast and slow stimulation rates, respectively. The relation between these two opposing currents would be dramatically altered if ischemia inhibited $I_{Ca}$ to a greater extent than $I_o$. Under such conditions, action potential configuration would become more sensitive to rate-dependent changes in $I_o$.

Indeed, acceleration of the stimulation rate under “ischemic” conditions led to restoration of the dome and thus to a paradoxical prolongation of the action potential in epicardium, as shown in Fig 4B. In this case, the ischemia-induced loss of the dome was reversed by acceleration of the stimulation rate from a BCL of 2000 milliseconds to 400 milliseconds. The reduced availability of $I_o$ at the faster rate allows for reappearance of the action potential dome that was lost because of ischemia-induced changes in $I_{Ca}$ and other currents.

Fig 5 plots the complete APD/rate relations obtained in the experiment illustrated in Fig 4. Under control conditions (panel A), progressive acceleration of the stimulation rate from a BCL of 4000 to 200 milliseconds was attended by a progressive shortening of APD\textsubscript{90} in endocardium and epicardium. During ischemia (panel B), APD\textsubscript{90} in epicardium was markedly abbreviated at BCLs longer than 600 milliseconds because of absence of the action potential dome. In contrast, the rate dependence of APD\textsubscript{90} in endocardium during ischemia was similar to that of control. Ischemia thus gave rise to a 110- to 130-millisecond dispersion of APD\textsubscript{90} between epicardium and endocardium at BCLs ranging from 1000 to 4000 milliseconds. At faster BCLs (<600 milliseconds), the dispersion of APD was nearly eliminated because of restoration of the dome in the epicardial action potential.

**Restitution of Action Potential Characteristics During Ischemia**

To further characterize the role of $I_o$, we examined the restitution of action potential parameters in epicardium (Fig 6) and endocardium (Fig 7) under control and simulated ischemic conditions. Each panel is composed of 9 or 10 superimposed traces recorded from an epicardial or endocardial preparation. The first response in each panel is the last of a train of 10 basic beats. Subsequent beats are the responses elicited by premature stimuli delivered progressively later in diastole. Under control conditions in epicardium (Fig 6A), the spike-and-dome morphology, absent in early premature beats, develops and becomes progressively more pronounced as the S\textsubscript{1}-S\textsubscript{2} interval prolongs. Also, the restitution of phase 0, phase 1, and phase 2 amplitude

**Rate-Dependence of Action Potential Configuration During Ischemia**

As a second test of the hypothesis that the presence of an additional outward current renders epicardium more susceptible to the effects of ischemia, we examined the rate-dependence of transmembrane activity in endocardium and epicardium before and after exposure

![Diagram](http://circ.ahajournals.org/)

**Fig 3.** Scatterplots showing correlation between phase 1 amplitude (A) or phase 1 voltage (B) in epicardial tissues and duration of ischemia required for loss of the dome. For each plot, phase 1 amplitude or the voltage attained at the end of phase 1 was measured in 20 epicardial preparations under control conditions at a basic cycle length of 800 milliseconds. These values were then plotted against the duration of ischemia elapsed until complete abolition of the dome was observed in these preparations. The solid line in each plot is a linear fit of the data.

Sure to ischemia before loss of the dome ranged between 15 and 95 minutes and was directly related to the amplitude of phase 1 (measured from the resting membrane potential to the nadir of the notch) or to the voltage at the end of phase 1 recorded under control conditions (Fig 3, panels A and B). Epicardial tissues with smaller phase 1 amplitudes (large notch) depressed sooner than tissues exhibiting a small notch. Epicardial tissues with a phase 1 amplitude >75 mV under control conditions (BCL, 800 milliseconds) did not show loss of the action potential dome even after 120 to 150 minutes of “ischemia.” The voltage at the end of phase 1 was more positive than −10 mV in all cases in which the dome was maintained after 2 hours of ischemia. These epicardial tissues were usually isolated from young dogs in which the $I_o$-mediated action potential notch is known to be less fully developed.\textsuperscript{20,26}
was biphasic in epicardium, since the amplitude of premature beats elicited early in diastole was greater than that of the basic beat. In contrast, the restitution of action potential parameters was monotonic in endocardium (Fig 7A); the amplitude of early premature beats was never greater than that of the basic beats.

In epicardial tissues exposed to ischemia (Fig 6B), premature beats elicited early in diastole exhibit an almost normal response in which the dome is restored. Responses elicited later in diastole exhibit a progressively more pronounced phase 1; the spike-and-dome morphology becomes more prominent until, at an S1-S2 interval of 450 milliseconds, the dome is abolished. Also noteworthy is the postpolarization refractoriness that develops during ischemia in both epicardium (Fig 6B) and endocardium (Fig 7B). Fig 6C illustrates the effect of the I\textsubscript{Na} inhibitor 4-AP (1 mmol/L) to restore the dome of the basic beat and to greatly diminish time-dependent changes in the early phases of the action potential in epicardium. The restitution of action potential parameters in endocardium during "ischemia" in the presence of 4-AP is illustrated in Fig 7C.

Fig 8 graphically illustrates the restitution data obtained in the experiment pictured in Figs 6 and 7. Under control conditions (Fig 8A), APD\textsubscript{90} values in epicardium and endocardium were similar and relatively constant over the range of S1-S2 intervals tested. The effective refractory period (ERP) measured under control conditions at a BCL of 800 milliseconds was 185 milliseconds in epicardium and 195 milliseconds in endocardium (\(\Delta\text{ERP}_{\text{endo-epi}}=10\) milliseconds). During ischemia (Fig 8B), the dispersion of APD\textsubscript{90} between epicardium and endocardium was small at short coupling intervals but increased dramatically at S1-S2 intervals \(>400\) milliseconds because of loss of the dome in epicardium. The latter would be expected if I\textsubscript{Na} availability is greater later in diastole. The ERP measured under ischemic conditions was 125 milliseconds in epicardium and 190 milliseconds in endocardium. Thus, \(\Delta\text{ERP}_{\text{endo-epi}}\) during ischemia increased to 65 milliseconds versus 10 milliseconds in control, whereas \(\Delta\text{APD}_{\text{endo-epi}}\) increased from 10 milliseconds in control to 100 milliseconds during ischemia (at a BCL of 800 milliseconds).

The effects of 4-AP (1 mmol/L) on the restitution of APD\textsubscript{90} in epicardial and endocardial preparations exposed to ischemia are shown in Fig 8C. The addition of 4-AP led to restoration of the dome at all S1-S2 intervals, but APD\textsubscript{90} remained considerably briefer in epicardial responses compared with those recorded from endocardium. The ERPs measured during ischemia plus 4-AP were considerably longer than those measured during ischemia alone because of the effect of 4-AP to dramatically prolong APD in epicardium. The ERP was 165 milliseconds in epicardium and 195 milliseconds in endocardium (\(\Delta\text{ERP}_{\text{endo-epi}}=30\) milliseconds
Loss of the Plateau in Epicardium as a Mechanism for Electrical Alternans

Under ischemic conditions, complete suppression of the dome in most epicardial preparations was preceded by a period of electrical alternans of varying duration. Fig 9 illustrates the two most common patterns of alternans observed. Each panel shows microelectrode recordings from an endocardial and epicardial preparation and the simulated ECG obtained by differential recording of the two voltage traces. Panel A illustrates a 2:1 pattern in which the action potential dome was alternately present and absent, resulting in alternating long and short action potentials. Since the endocardial APD remained constant during this period, the T-wave alternans seen in this example was due entirely to changes in APD of epicardium. This particular example of 2:1 alternans was stable for a period of approximately 2 minutes at a BCL of 800 milliseconds. Thereafter, all epicardial beats were devoid of a dome at a BCL of 800 milliseconds, but alternans could be observed at progressively faster stimulation rates.

Fig 9B illustrates another example of electrical alternans recorded in a different set of preparations. The 3:1 pattern shown is another one commonly encountered during ischemia. In this example, an epicardial action potential with a dome was followed by two beats lacking a dome. Alternans of the T-wave amplitude and polarity were a direct result of the changes in APD of epicardium.

In all cases, the pattern of alternans was found to be a sensitive function of the stimulation rate. At any
Fig 7. Tracings showing restitution of action potential characteristics in an endocardial preparation under control conditions, during ischemia, and during ischemia plus 4-aminopyridine (4-AP). Traces are arranged as in Fig 6. Under control conditions (A), the restitution of action potential characteristics is monophasic in endocardium. During ischemia (B), the restitution is qualitatively similar to the control, although postpolarization refractoriness is evident in the endocardial traces. Addition of 4-AP to the ischemic solution (C) did not appreciably alter the restitution pattern elicited in endocardium. The traces in C were recorded after 15 minutes of exposure to 1 mmol/L 4-AP.

Fig 8. Plots showing effects of ischemia or ischemia plus 4-aminopyridine (4-AP) on the restitution of action potential duration at 90% repolarization (APD90) in canine endocardium (Endo) and epicardium (Epi). A, restitution of APD90 in epicardium (○) and endocardium (□) under control conditions. The restitution protocol is the same as described in Fig 6. Under control conditions, progressive shortening of the S1-S2 interval resulted in premature responses with progressively shorter APD90 in both epicardium and endocardium. B, restitution of APD90 obtained in the same preparations during ischemia. During ischemia, the restitution of APD90 in endocardium was qualitatively similar to control. In epicardium, however, the APD90 of premature beats introduced at S1-S2 intervals <350 milliseconds was greater than that of the basic beats because of restitution of the dome. Indeed, a premature beat introduced at an S1-S2 interval of 350 milliseconds elicited a response in epicardium that was longer than that obtained in endocardium. The dome was not present in premature beats introduced at S1-S2 intervals >400 milliseconds, and this is reflected by the very short APD90. C, restitution of APD90 obtained after 15 minutes of exposure to 4-AP (1 mmol/L) in the presence of ischemia. All restitution scans were performed at a basic cycle length of 800 milliseconds.

Instant in time during ischemia, small changes in rate were often sufficient to induce alternans, shift the pattern of alternans, or abolish alternans entirely. The window of rates at which alternans was observed varied from preparation to preparation (range, 600 to 1800 milliseconds).

Effects of Ischemia on Electrical Activity in Single Cardiac Myocytes

In another experimental series, we tested the hypothesis that the differential sensitivity of epicardial and endocardial to ischemia was a result of differences in intrinsic electrophysiological properties of the cells and not of extracellular properties of the two tissue types or electrotonic influences of neighboring cells. Myocytes isolated from discrete epicardial and endocardial sites were studied before and after exposure to ischemia.

Fig 10 shows the effects of ischemia on the electrical activity of myocytes dissociated from endocardial and epicardial layers of the left ventricular free wall. Under control conditions, transmembrane action potentials recorded from endocardial and epicardial myocytes showed distinctions similar to those observed in the respective syncytial tissue preparations. Epicardial cells exhibited a lower phase 0 and phase 1 amplitude but a larger notch than did cells isolated from endocardium. When the cells were exposed to simulated ischemia (panel B), the action potential dome in the epicardial
Selective Depression of Epicardium by Ischemia

Fig 9. Typical patterns of electrical alternans obtained during simulated ischemia. In each panel, the upper and middle traces are microelectrode recordings from endocardium (Endo) and epicardium (Epi), respectively, and the lower trace is the simulated ECG obtained by differential recording of the endocardial and epicardial voltages. A, A 2:1 pattern of alternans obtained at a basic cycle length (BCL) of 800 milliseconds. The action potentials in epicardium show an alternating sequence of long and short durations caused by the alternate presence and absence of the dome. Action potential duration is relatively constant in endocardium, so that the changes in the T wave are due entirely to the changes occurring in epicardium. B, A 3:1 pattern of alternans obtained during ischemia in a different set of epicardial and endocardial preparations at a BCL of 800 milliseconds. In this example, an action potential with a dome in epicardium is followed by two action potentials where the dome is suppressed. Changes in both the amplitude and polarity of the T wave were obtained in the ECG under these conditions. Action potential duration remained constant in endocardium during the period over which the record was taken.

cell was abolished within 15 minutes, resulting in a marked abbreviation of APD. Ischemia-induced abolition of the dome occurred more rapidly in epicardial myocytes (10 to 15 minutes) than in the syncytial tissue preparations (15 to 60 minutes). Endocardial myocytes exhibited only a slight shortening of APD and depolarization during exposure to ischemia, paralleling the changes observed in syncytial preparations.

Addition of 4-AP (1 mmol/L) to the ischemic solution resulted in restoration of the dome in the epicardial cell within 1 minute (Fig 10C). The records shown in panel C were obtained after 5 minutes of exposure to ischemia plus 4-AP. As in the syncytial preparations, continued exposure to 4-AP caused a progressive decrease in the size of the notch, resulting in a progressive abbreviation of APD (time course of ≈5 to 8 minutes). In contrast, 4-AP prolonged APD in the endocardial cell.

Table 2 summarizes the effects of ischemia and of ischemia plus 4-AP on action potential parameters in seven epicardial and six endocardial cells. The responses obtained in dissociated myocytes were very similar to those recorded in syncytial tissue preparations from the corresponding ventricular layers.

Discussion

The present study demonstrates that tissues as well as dissociated myocytes isolated from canine ventricular epicardium are more sensitive to electrical depression by ischemic conditions than are tissues and myocytes obtained from ventricular endocardium. Simulated ischemia was found to produce electrophysiological changes in multicellular preparations nearly identical to those in single cardiac myocytes enzymatically dissociated from corresponding layers of the ventricular wall. These results suggest that the differential responsiveness of epicardium and endocardium to ischemia is in large part a result of intrinsic electrophysiological differences between the two cell types, since extracellular ionic influences and electronic interactions can be discounted in the experiments involving single myocytes.

Electrophysiological Differences Between Canine Epicardium and Endocardium

Several electrophysiological distinctions between the epicardial and endocardial layers of the canine ventricle have been described. Epicardial action potentials recorded in vitro and monophasic action potentials recorded in situ display a characteristic spike-and-dome morphology that is absent in recordings from endocardium. 

Evidence from microelectrode studies on isolated tissues and voltage clamp studies on disso-
TABLE 2. Effects of Simulated Ischemia and 4-Aminopyridine on Action Potential Parameters in Canine Ventricular Myocytes Isolated From the Epicardial or Endocardial Layers

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>n</th>
<th>Intervention</th>
<th>Phase 0</th>
<th>Phase 1</th>
<th>Notch, nV</th>
<th>Phase 2</th>
<th>APD90, ms</th>
<th>MDP, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicardial</td>
<td>7</td>
<td>Control</td>
<td>114.9±6.5</td>
<td>69.2±5.0</td>
<td>45.5±7.1</td>
<td>100.4±4.1</td>
<td>225.5±32.0</td>
<td>−84.8±3.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&quot;Ischemia&quot;</td>
<td>97.9±9.2*</td>
<td>. . . . .</td>
<td>. . . . .</td>
<td>59.2±13.4*</td>
<td>−69.0±2.6*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&quot;I+4-AP&quot;</td>
<td>109.6±5.7</td>
<td>87.0±3.2*</td>
<td>18.2±8.2*</td>
<td>87.9±2.9*</td>
<td>138.3±22.1*</td>
<td>†−70.5±1.8*</td>
</tr>
<tr>
<td>Endocardial</td>
<td>6</td>
<td>Control</td>
<td>125.1±5.9</td>
<td>109.3±4.3</td>
<td>8.4±4.5</td>
<td>107.5±5.5</td>
<td>244.0±41.0</td>
<td>−86.6±1.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&quot;Ischemia&quot;</td>
<td>112.3±7.8*</td>
<td>97.5±5.9*</td>
<td>5.5±4.0</td>
<td>94.9±5.8*</td>
<td>222.7±35.5</td>
<td>−72.8±2.6*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&quot;I+4-AP&quot;</td>
<td>111.9±7.6*</td>
<td>96.0±3.3</td>
<td>3.8±3.4</td>
<td>94.7±3.5*</td>
<td>237.4±30.3</td>
<td>−72.2±2.5*</td>
</tr>
</tbody>
</table>

Amp indicates amplitude; Notch, phase 2 amplitude minus phase 1 amplitude; APD90, action potential duration at 90% repolarization; MDP, maximum diastolic potential; "I," "ischemia"; and 4-AP, 4-aminopyridine (1 to 2 mmol/L, 15 minutes of exposure). All values are mean±SD obtained at a basic cycle length of 800 milliseconds.

*P<.05 compared with same cell control; †P<.05 compared with same cell "ischemia" value.

diated myocytes22,27,28 have suggested that the spike-and-dome configuration is caused by the presence of an INa in canine epicardium and that differences in the early phases of the action potential are in large part attributable to the prominence of this current in epicardium but not endocardium.19,22 The 4-AP sensitive transient outward current (Ito) was shown to be four to five times larger in epicardial myocytes than in endocardial cells.22 In contrast, the inward rectifier, IK1, was comparable in magnitude in the two cell types.22 Similar regional differences in the contribution of INa to epicardial versus endocardial action potentials have also been described in the cat23 and rabbit ventricle.24

**Effects of Ischemic Conditions on Transmembrane Currents**

Components of ischemia have been shown to influence one or more ionic currents when tested in vitro. The combination of hypoxia, acidosis, and zero glucose was found to decrease the inward Na+ current (INa) in canine ventricular myocardiun as measured by changes in phase 0 amplitude and the maximal rate of rise of phase 0 (Vmax).31,32 It is still unknown whether the decrease in INa during ischemia is due to a direct effect on the Na+ channel or an indirect effect of the ischemia-induced membrane depolarization to decrease INa through voltage-dependent inactivation of Na+ channels. In the present study, "ischemia" caused 22±9% and 15±3% decreases in phase 0 amplitude in epicardium and endocardium, respectively (see Table 1). A decrease in INa and secondary changes in excitability may account for the slight postrepolarization refractoriness seen in both tissues during "ischemia." The mild ischemic conditions used in this and corollary studies (in which multiple impalements were used) did not produce prominent slowing of conduction in epicardium or endocardium.

Another current known to be affected by conditions of ischemia is the inward calcium current, ICa. Acidosis directly inhibits ICa in many cardiac tissues.33-35 Kimura et al36 reported that metabolic inhibition, a major component of ischemia, caused a greater reduction of peak ICa in epicardial versus endocardial cells from the feline ventricle (37% versus 21%). Under control conditions, however, they found no difference in ICa between the two cell types. No data are currently available for the characteristics of ICa in canine epicardial versus endocardial cells or how ICa is altered by conditions of ischemia in each cell type. However, the feline data suggest that the contribution of ICa to the action potential plateau may be quite different in epicardium versus endocardium under ischemic conditions. The rise in intracellular Ca2+ activity ([Ca2+]i) that typically occurs during ischemia37-39 may reduce ICa even further.

The effects of ischemia on outward repolarizing currents in ventricular tissues are not well established. Increased K+ permeability has been reported during hypoxia in voltage-clamped feline ventricular muscle.40 Also, a Ca2+-activated transient outward K+ current (Ito) that is insensitive to 4-AP has been reported in canine myocytes.27,28 However, recent evidence suggests that Ito may be a Ca2+-activated chloride current41 rather than a Ca2+-activated K+ current, but this point remains to be settled. Moreover, the extent to which an increase in [Ca2+]i during ischemia37-39 alters these outward currents is still not fully appreciated, although some increase in the Ca2+-activated currents would be expected.

**Role of INa in the Selective Depression of Epicardium During 'Ischemia'**

As discussed above, ischemic conditions, through direct or indirect interaction with ionic channels, can cause a decrease in inward currents (INa, ICa) but an increase in outward repolarizing currents, especially those that contribute to the early phases of the action potential. These changes, coupled with the marked preexisting differences in INa in epicardium versus endocardium, may provide a fundamental basis for understanding the differential sensitivities of the two tissues to the depressant effects of ischemia.

Our data suggest that the opposite effects of ischemia on inward versus outward currents (ie, inhibition versus accentuation) result in a progressively greater imbalance between the opposing inward and outward currents flowing at the end of phase 1 in epicardium. This imbalance would explain why the notch is initially accentuated and the dome subsequently abolished as ischemia progresses. In the early phases of ischemia, phase 1 of the epicardial action potential begins at less negative potentials and proceeds to more negative potentials at which the availability of ICa is diminished. As a consequence, the second upstroke giving rise to phase 2 (plateau) of the action potential and the start of phase
3 are delayed, resulting in a transient prolongation of APD (Fig 2, 15 to 25 minutes of ischemia). With longer exposure to ischemia, a further shift in the balance of currents (caused by greater inhibition of I\(\text{Ca}\) and activation of I\(\text{Ca}(\text{Ca})\)) would lead to the termination of phase 1 at still more negative potentials. Eventually, the greater net outward current flow during phase 1 would overwhelm I\(\text{Ca}\) and any slowly inactivating (or window) sodium current, thus causing an all-or-none repolarization. The failure of the inward currents to overtake the outward currents (mainly I\(\text{o}\) and I\(\text{c}\)) as they normally do at the end of phase 1 would result in abolition of the dome and thus in a marked abbreviation of APD in epicardium.

The presence of a large and early repolarizing current such as I\(\text{o}\) is essential to produce all-or-none repolarization. The relatively weak I\(\text{o}\) in endocardium may explain why electrical activity in this tissue is less sensitive to ischemia, since a large imbalance of currents would not be generated. In support of this hypothesis, block of I\(\text{o}\) by 4-AP readily reversed the ischemia-induced depression of action potentials in epicardium (see Figs 1, 2, and 9). It should be noted that 4-AP is not a selective blocker of I\(\text{o}\), but at the concentration used (1 mMol/L), its actions to inhibit the inward rectifier (I\(\text{k}\)) and delayed rectifier (I\(\text{d}\)) currents are minor if present at all. Moreover, the magnitude of I\(\text{k}\) would be negligible at plateau membrane potentials due to the inward rectification properties of this current.

Rate Dependence of Action Potential Configuration During Ischemia

Acceleration of the stimulation rate or the introduction of premature beats early in diastole also reversed the ischemia-induced depression of the plateau in epicardium (Figs 4 through 7). The epicardial action potentials elicited at faster rates and after premature stimuli show a paradoxical prolongation caused by restoration of the dome. These results can be explained on the basis of the rate dependence of I\(\text{o}\) and I\(\text{c}\), which are usually opposite. The availability of I\(\text{o}\) is less at fast stimulation rates and after premature stimuli because full reactivation of I\(\text{o}\) requires >1500 milliseconds in canine ventricular tissue.21-24,42 Under normal conditions, the process is slowed further in depolarized tissues, since I\(\text{o}\) reactivation is voltage dependent.28 In contrast to I\(\text{o}\), I\(\text{c}\) generally increases in amplitude with acceleration of the stimulation rate. Thus, the repolarizing influence of I\(\text{o}\) (phase 1) grows larger at slow rates, whereas the depolarizing influence of I\(\text{c}\) (phase 2) increases at fast rates. Under normal conditions, this results in accentuation of the epicardial notch at slow rates or attenuation of the notch at fast rates. If ischemia inhibits I\(\text{c}\) but not I\(\text{o}\), as our data suggest, the balance between I\(\text{c}\) and I\(\text{o}\) would shift overwhelmingly in favor of I\(\text{o}\) at all but the fastest stimulation rates. This overwhelming repolarizing influence would prevent the development of a plateau in the epicardial response during “ischemia” (ie, all-or-none repolarization). Only at fast stimulation rates would I\(\text{c}\) be able to overtake I\(\text{o}\) and thus generate a plateau. Consequently, a decrease in the availability of I\(\text{o}\), whether through acceleration of the stimulation rate or addition of an inhibitor such as 4-AP, is effective in reversing the ischemia-induced depression of action potentials in epicardium.

Direct evidence for the effects of I\(\text{c}\) inhibition on action potential configuration is provided by studies of Ca\(^{2+}\) channel blockade. Exposure to Ca\(^{2+}\) channel blockers or Ca\(^{2+}\)-free solutions results in abolition of the plateau and abbreviation of the action potential in canine epicardium but only a slight abbreviation of the action potential in endocardium (A. Lukas and C. Antzelevitch, unpublished observation). The effects of Ca\(^{2+}\) channel blockade (under nonischemic conditions) were very similar to those of ischemia in the present study but without the membrane depolarization. These findings provide support for the hypothesis that small or moderate changes in I\(\text{c}\) can cause marked changes in action potential configuration in canine epicardium but relatively minor changes in endocardium.

Role of the ATP-Regulated K\(^{+}\) Current in the Responses to ‘Ischemia’

Another outward current that may play a role in the ischemia-induced loss of the plateau and marked abbreviation of action potentials in epicardium is the ATP-regulated potassium current, I\(\text{KATP}\).46 Activation of this time-independent outward current by reduced ATP levels would provide an additional repolarizing influence. We tested this hypothesis in four experiments but found that glybenclamide (20 to 50 \(\mu\)mol/L), an inhibitor of I\(\text{KATP}\), was unable to restore the plateau in epicardium under ischemic conditions (A. Lukas and C. Antzelevitch, unpublished observation). Possibly, the severity of “ischemia” was too mild in our study to cause significant activation of I\(\text{KATP}\).

Differential Sensitivities of Canine Epicardium and Endocardium to Ischemia

Our results point to the difference in I\(\text{o}\) between canine ventricular epicardium and endocardium as a major factor responsible for the differential responsiveness to ischemia but do not rule out the participation of other factors. The briefer action potentials observed in epicardium after 4-AP reversal of ischemia-induced depression (Figs 1, 2, 7, and 9 and Table 2) suggest that factors other than I\(\text{o}\) also sensitize the epicardium to ischemia. The persistence of postrepolarization refractoriness in the presence of 4-AP during ischemia supports this suggestion. Differences in activation of I\(\text{KATP}\) or depression of I\(\text{c}\) in epicardium versus endocardium as described by Furukawa et al47 and Kimura et al46 in feline ventricular cells may constitute two additional factors.

Physiological Implications

The greater sensitivity of ventricular epicardium to ischemia has several important consequences. Chief among these is the development of a marked dispersion of repolarization and refractoriness between epicardium and endocardium in the ischemic zone as well as between normal and ischemic myocardium. This heterogeneity provides the electrophysiological substrate for the initiation of reentrant arrhythmias, as we have demonstrated in a corollary study.48 Reentry induced under these conditions is readily abolished after introduction of inhibitors of I\(\text{o}\), consistent with the effects of these agents to diminish ischemia-induced electrical heterogeneity (see Figs 1, 2, 7, and 9). It is noteworthy that some traditional antiarrhythmics have I\(\text{o}\) blocking
actions. Imaizumi and Giles\(^8\) suggested that the inhibition of \(I_N\) by quinidine may be concurrent with depression of \(I_N\) in the antiarrhythmic actions of the drug.

The biphasic restitution of action potential characteristics in epicardium suggests that supernormal conduction may be possible in epicardium under ischemic conditions. Supernormal conduction or excitability has been demonstrated in vivo with extracellular stimulating electrodes usually applied to the ventricular epicardium.\(^{50,51}\) A supernormal phase of conduction has also been reported in epicardial strips mounted in a three-chamber bath in which a central inexcitable zone was created by superfusion with an “ion-free” isotonic sucrose solution.\(^{20,21}\) Under these conditions, premature beats elicited early in diastole conducted successfully across the inexcitable zone, whereas the basic beats failed to conduct. Supernormal conduction in epicardium would be expected to be more accentuated during ischemia, since the biphasic restitution of action potential characteristics is much more pronounced once depression of the dome occurs in epicardium (Fig 6). As a result, early premature beats (in which the dome is restored) would have a higher margin of safety for conduction in ischemic epicardium than would basic beats (in which the dome is abolished) because the greater amplitude and much longer duration of premature beats would generate more local circuit current for activation of adjacent tissues.

Finally, our data suggest that loss of the action potential dome in epicardium may contribute to ischemia-induced ST segment elevation as well as T-wave alternans.\(^{52-55}\)

**Limitations of the Study**

A major criticism of in vitro models of ischemia has been that they cannot mimic many of the components of true ischemia (buildup of metabolites, K\(^+\) accumulation, low O\(_2\) tension, transmural gradients, etc.). The advantage of the in vitro models is that transmembrane electrical activity and changes in ionic currents are more easily recorded during “ischemia.” Also, the modulating effects of extracellular and electrotonic influences on the ischemic responses in tissues can be precluded in studies of single cardiac cells.

Before selecting the “ischemic” solution used in the present study, we first tested many different test solutions in which we varied [K\(^+\)], added lactate, included cyanide, etc. Also, individual components of ischemia (hypoxia, high [K\(^+\)], acidosis) were tested alone and in combination. Severe “ischemic” solutions caused abolition of the dome and eventually inexcitability in epicardium with a time course similar to that reported in vivo (several minutes). However, the onset of depression was too rapid to allow testing of stimulation protocols or drugs. Therefore, we selected an “ischemic” solution with a composition that produced depression in epicardium with a time course slow enough to provide us with a window to test drugs or interventions and thereby help elucidate the mechanism underlying the differential sensitivity to “ischemia.” Thus, the results presented in this study apply to relatively mild ischemic insults. To what extent these data apply to more severe “ischemic” conditions remains to be established.

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