Conscious Rabbits Become Tolerant to Multiple Episodes of Ischemic Preconditioning

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Abstract Although ischemic preconditioning protects myocardium from infarction in isolated hearts and in anesthetized open-chest animals, its effects have not been examined in unanesthetized animals. Furthermore, it is unknown whether animals become tolerant to multiple episodes of ischemic preconditioning. Rabbits were chronically instrumented with a balloon occluder around a major branch of the left coronary artery for reversible coronary occlusion, a left atrial catheter for radioactive microsphere injections, ECG electrodes for monitoring of myocardial ischemia, and, in some cases, a carotid artery catheter for pressure measurements and timed withdrawal of reference arterial blood samples. Eight control rabbits underwent a 30-minute coronary occlusion and then 180 minutes of reperfusion. Five of the eight rabbits developed ventricular tachycardia or fibrillation during ischemia, and infarct size averaged 37.7±2.6% of the risk area. Eight rabbits experienced a 5-minute coronary occlusion and 10 minutes of reperfusion before the 30-minute occlusion. In these preconditioned animals, potentially fatal arrhythmias during ischemia were significantly reduced (one of eight, P<.05), and infarct size was much smaller (5.6±1.1%, P<.0001). The difference could not be explained by hemodynamics or collateral blood flow, which were nearly identical in the two groups. But when the 30-minute coronary occlusion was preceded by 40 to 65 five-minute occlusions during a 3- to 4-day period in seven animals, protection was markedly attenuated. Potentially lethal arrhythmias were very common, and infarct size averaged 26.5±2.9%, substantially larger than in rabbits with only one preconditioning occlusion (P<.0001). Finally, if an interval of 2.5 to 3 days of no coronary occlusions was interposed between this period of multiple occlusions and the terminal preconditioning protocol of 5-minute occlusion and 10-minute reperfusion preceding the long 30-minute occlusion and 180-minute reperfusion, protection was again evident, and infarct size in seven rabbits averaged 10.9±1.5% of the risk zone (P<.0001 versus control and P<.001 versus multiple occlusions). It can be concluded that ischemic preconditioning significantly diminishes the incidence of ischemia-induced ventricular arrhythmias and the extent of infarction after a 30-minute coronary occlusion in unanesthetized rabbits. Unfortunately, this protection wanes after multiple 5-minute coronary occlusions have occurred but does reappear after an ischemia-free period. It is currently being hoped that ischemic preconditioning will have clinical importance, but tolerance may limit its utility in patients with recurrent angina pectoris. (Circ Res. 1994;74:998-1004.)

Key Words • coronary occlusion • myocardial infarction • preconditioning • tolerance • ventricular fibrillation

Ischemic preconditioning first demonstrated by Murry et al is the phenomenon whereby a brief episode of myocardial ischemia paradoxically diminishes the amount of necrosis occurring from a subsequent ischemic insult. We have found that myocardial protection conferred by preconditioning in rabbits is mediated by release of endogenous adenosine, which in turn occupies adenosine receptors, stimulates a pertussis toxin–sensitive G protein, and activates protein kinase C. The ability of ischemic preconditioning to salvage ischemic myocardium has been demonstrated in multiple experimental animal models including rabbit, dog, pig, and rat. However, all known studies have used open-chest anesthetized animals or isolated heart preparations. In the past, the physiological significance of data gathered in anesthetized animal models has been questioned because of the confounding effects of anesthetic agents on the autonomic nervous system, coronary dynamics, and responses to vasoactive drugs. Accordingly, it was felt necessary to determine whether the protection of ischemic preconditioning could be documented in a conscious unanesthetized animal. Furthermore, we have recently found that rabbits become tolerant to a prolonged infusion of an A1-adenosine agonist and that tolerance is accompanied by a loss of protection from preconditioning. The present study was therefore undertaken to test whether a similar tolerance might develop in rabbits exposed to multiple brief ischemic episodes, each releasing adenosine, as might occur in a patient with unstable angina pectoris. In the present study, a recently developed chronically instrumented rabbit model allowed us to demonstrate that ischemic preconditioning does result in protection of the ischemic heart in unanesthetized animals but that tolerance to this salutary effect soon develops when the hearts are exposed to multiple ischemic episodes.

Materials and Methods

Animal Surgery

All procedures were approved by the institutional animal care and use committee. New Zealand White rabbits weighing between 2 and 3 kg were anesthetized with intravenous sodium pentobarbital (35 mg/kg), intubated with an endotracheal tube, and mechanically ventilated with a positive pressure respirator (MD Industries, Mobile, Ala) and 100% oxygen. After intravenous administration of 100 mg Kefzol, a left thoracotomy was performed in the third intercostal space. The technique for placement of the pneumatic occluder around the coronary artery has previously been described. Briefly, a balloon occluder fashioned from 18-gauge Tygon tubing was wedged onto the blunt end of a curved taper needle, which was passed beneath a prominent branch of the left coronary artery.
The balloon occluder was pulled through the myocardium, and the end was doubled over the artery and anchored to the myocardium with 4-0 suture. Proper functioning of the occluder was confirmed by noting cyanosis of the distal myocardium and cessation of effective contraction after inflation of the balloon and hyperemia and resumption of contraction after deflation.

A 18-gauge Tygon catheter was inserted into the left atrium through the appendage and secured. The chest wound was closed in layers, and air was aspirated from the thorax. Before approximation of the skin layer, ECG wires were anchored in the subcutaneous tissue with one lead at each end of the incision. The left atrial catheter and balloon occluder were brought through the chest wall, and they, along with the ECG leads, were tunneled subcutaneously to exit between the scapulas. In a subset of rabbits, the carotid artery was exposed through an incision in the left neck. The vessel was ligated distally, and an 18-gauge Tygon catheter was inserted into the vessel and advanced retrogradely ~2 cm. The free end of the catheter was tunneled subcutaneously to exit between the scapulas near the other catheters and leads, and the neck incision was closed. Three additional rabbits were prepared expressly for quantification of coronary and collateral blood flows. In these animals, balloon occluders, left atrial catheters, and ECG leads were implanted as already described. In lieu of a carotid catheter for withdrawal of arterial blood, a PE-60 catheter was inserted directly into the descending thoracic aorta. The proximal end of the catheter was brought through the chest wall and, along with the other devices, was tunneled subcutaneously to exit between the scapulas. Left atrial, carotid, and aortic catheters were filled with a dilute solution of heparin. After surgery and again 2 days later, rabbits were given intramuscular injections of 600 000 U benzathine penicillin.

Protocol

Single-Occlusion Groups

Five to 7 days after surgery, when rabbits appeared to be fully recovered, they were brought to the laboratory and randomly divided into control and experimental groups based on the day of the week. In any given week, equal numbers of rabbits were selected for each group. The ECG leads were attached to an ECG amplifier. In those rabbits with a carotid artery catheter, the latter was connected to a disposable transducer (Cobe, Lakewood, Colo). All signals were recorded on a multichannel oscillograph.

A 5-minute period of regional ischemia was induced in the preconditioned group by manually inflating the balloon occluder and clamping the tubeing to prevent deflation. During balloon inflation and myocardial ischemia, there were never any observed behavioral changes in the rabbits that would suggest perception of a noxious stimulus. Consequently, sedation or analgesia was not felt to be required. The ECG and carotid artery pressure, when available, were recorded immediately before inflation and every 30 seconds for 2 minutes and then again at 5 minutes. Appearance of ECG ST segment elevations in addition to changes in the QRS complex confirmed the onset of myocardial ischemia. After 5 minutes, the clamp was released, and the balloon was deflated by the aspiration of air. The ECG and carotid pressure were recorded every 30 seconds for 2 minutes and then at 5 and 10 minutes after reperfusion. Normalization of the ECG documented successful reperfusion of the coronary artery. At this point, the balloon occluder was reinflated, and the tubing was clamped for 30 minutes. Recordings were made every 30 seconds for 2 minutes, at 5 minutes, and every 5 minutes thereafter.

A single measurement of regional myocardial blood flow was made in these groups. Five minutes after the onset of the 30-minute occlusion, 0.5 to 1 × 10^6 microspheres (NEN, Boston, Mass) labeled with either 45Sc, 99Sr, or 113Ce, suspended in saline, and mechanically agitated and ultrasonicated for 10 to 15 minutes were injected into the left atrium during 5 seconds, followed by a saline flush. For those animals with a carotid artery catheter, a reference arterial sample was collected by withdrawing arterial blood at a rate of 1.5 mL/min with a constant withdrawal pump (Harvard Apparatus) beginning a few seconds before injection of the microspheres and ending 1 minute after completion of the injection. After 30 minutes of coronary occlusion, the PE-60 catheter was removed, and the balloon was deflated to reperfuse the heart.

Reperfusion proceeded for 180 minutes. Recordings were made every 30 seconds for 2 minutes, at 5, 10, and 15 minutes, and then again at 180 minutes. The ECG was continuously displayed on an oscilloscope. If arrhythmias were observed, additional recordings were made. No antiarrhythmic agents were administered to the rabbits at any time. If ventricular fibrillation occurred, external defibrillation was attempted at an energy level of 150 W-seconds. If the animal fibrillated during a coronary occlusion, resuscitation was tried without deflating the balloon. Control rabbits underwent the same protocol except that the initial 5-minute coronary occlusion was omitted. In control hearts, collateral flow was also measured 5 minutes after the onset of the 30-minute occlusion.

Each of the three rabbits with catheters in the descending thoracic aorta also underwent a 30-minute coronary occlusion followed by immediate and 3-hour reperfusion. Coronary and collateral blood flows were measured 5 minutes after onset of the coronary occlusion. In these animals, 2 × 10^6 microspheres labeled with 153Sn were injected into the left atrium while arterial blood was withdrawn from the aortic catheter at a rate of 2.7 mL/min.

Multiple-Occlusion Groups

To determine whether protection was maintained after multiple episodes of ischemic preconditioning, animals were prepared as above, but the coronary artery was occluded for a 5-minute interval every half hour for 8 hours each day. Balloon inflation and deflation were effected with compressed air and a vacuum pump, respectively. A previously described timing circuit14 connected to a solenoid valve controlled the duration of inflation and deflation. If ventricular fibrillation occurred, attempts were always made to defibrillate the animal with external DC countershocks. After ~3 to 4 days of repetitive occlusions, most rabbits underwent a final 30-minute coronary occlusion. The 30-minute ischemic insult was produced at the end of a day when the rabbit had already undergone at least 12 five-minute occlusions. After the last 5-minute occlusion, a 10-minute period of reperfusion was allowed, as in the preconditioned rabbits described above. Then the balloon occluder was manually inflated for 30 minutes. The heart was reperfused for 180 minutes, and then the animal was killed so that risk zone and infarct sizes could be determined as described below.

To determine whether the repeated 5-minute occlusions themselves might have induced a change in collateral flow, microspheres with one radioactive label were injected 2 minutes after the onset of the second 5-minute occlusion. Microspheres with a second label were injected 2 minutes after the onset of the final 30-minute coronary occlusion.

In four rabbits, the final 30-minute coronary occlusion was omitted to test whether the 5-minute occlusions might be causing infarction. After reperfusion for 180 minutes following the final 5-minute occlusion, the animals were killed, and the hearts were removed for analysis. In these rabbits, the second injection of radioactive microspheres was made 2 minutes after the onset of the final 5-minute occlusion.

Finally, in a group of seven rabbits having successfully undergone multiple occlusions over 3 to 4 days, occlusions were suspended for the next 2.5 to 3 days. Thereafter, a single 5-minute occlusion followed by a 10-minute reperfusion period was produced, followed by the prolonged 30-minute coronary occlusion and subsequent 3-hour reperfusion. Hearts were then excised and evaluated as indicated below.

Myocardial Sample Preparation

After completion of the 180-minute period of reperfusion, all experimental and control rabbits were reanesthetized with
Collateral flow was defined as flow to the nonfluorescent myocardium, whereas flow to fluorescent tissue was assumed to be normal. Raw radioactive counts per minute were summed for all fluorescent and nonfluorescent pieces and normalized for the respective collective tissue mass. To avoid contamination of ischemic myocardium by inadvertent inclusion of some tissue with normal flow, nonfluorescent risk zone pieces bordering normally perfused fluorescent myocardium were omitted. Collateral flow was expressed as a percentage of the normal flow to the same heart. In those animals with reference arterial samples, absolute flows were calculated by multiplying the raw counts per minute of the tissue sample by the withdrawal rate of the pump and then dividing by the counts per minute of the reference blood sample. Flows were normalized for mass. The volume of ischemic or risk zone tissue was calculated by multiplying planimetered areas by slice thickness. Infarct size was calculated as a percentage of the risk zone.

Statistics

Data are presented as mean±SEM. Statistical significance of comparisons between groups was assessed by ANOVA with replication, Student’s unpaired t test, and the χ² test. A value of P<.05 was considered to be significant.
As presented in Table 2, there was no significant difference in the size of risk zones between the two groups, although there was a tendency for the risk zone to be smaller in the experimental rabbits. To calculate risk zone-to-heart weight ratios, risk zone volumes were converted to mass by multiplying by 1.05, the specific gravity of myocardium. The ratios were not different in the two groups. Absolute infarct size was significantly smaller \((P<.0001)\) in preconditioned rabbits. Because of the possible influence of the size of the risk zone on the extent of infarction, infarct volume was plotted against risk zone volume for control and preconditioned rabbits in Fig 2. The relation between risk region and infarct size was strikingly different between the single preconditioned and the control hearts, again indicating profound protection.

To determine whether coronary collateral flow might be influencing infarct size, infarction as a percentage of risk area was plotted against collateral flow as a percentage of flow to normally perfused tissue (Fig 3). For control animals with only a 30-minute occlusion, there was a linear relation between the two variables \((r=.88)\). However, the range of collateral flows was small. In only the two animals with collateral flows >10% there was a possible effect on infarct size. Of note, the animals with ischemic preconditioning displayed virtually identical collateral flows, but in this group there was no relation between infarct size and flow. For the same collateral flow, infarct size was obviously much smaller in preconditioned than control rabbits.

To evaluate the effect of multiple ischemic episodes on arrhythmias and infarction, the coronary artery of 21 rabbits was subjected to repeated 5-minute periods of occlusion. Intractable ventricular fibrillation appearing after occlusions 5 and 15 of the first day and occlusion 12 of the second day resulted in the exclusion of 3 rabbits. For the remaining 18 animals completing the protocol, ventricular fibrillation also occurred in 7 of them. Interestingly, ventricular fibrillation occurred after occlusion 11 of the first day in 1 rabbit, after occlusion 13 of both the first and second days in 2 rabbits, and after occlusions 13, 14, and 15 of the second day in 3 additional rabbits, respectively. Additionally, 3 of these rabbits as well as 1 other without a prior arrhythmia also developed ventricular fibrillation during the 30-minute occlusion at the end of the protocol. In each of these instances, ventricular fibrillation was successfully converted to a sinus mechanism with DC countershocks. If the arrhythmia occurred toward the end of the 5-minute occlusion, the balloon was deflated before electrical defibrillation. However, the balloon was never deflated if ventricular fibrillation occurred during the 30-minute coronary occlusion. There was no difference in size of the risk zone in the 7 survivors developing ventricular fibrillation \((0.88\pm0.04 \text{ cm}^3)\) and those without any arrhythmias \((0.84\pm0.03 \text{ cm}^3)\).

Between 40 and 65 (average 58±1) 5-minute coronary occlusions were performed over 3 to 4 days in 18 rabbits. To document any possible change in coronary collateral flow, radioactive microspheres were injected during both the second and final balloon occlusion. In these animals, initial collateral flow averaged 3.8±0.5% of normal tissue flow, which was unchanged \((4.2\pm0.8\%)\) at the end of the protocol. In 4 control rabbits with only multiple 5-minute occlusions, no infarction was detectable with TTC staining (Fig 1). In 7 animals that additionally underwent a final 30-minute balloon inflation 10 minutes after the last 5-minute occlusion of the 3- to 4-day multiple-occlusion protocol, infarct size averaged 26.5±2.9% of the risk zone. As seen in Fig 1, these infarcts were significantly larger than those observed in rabbits preconditioned with a single 5-minute ischemic episode \((P<.0001)\). Infarcts in this group approached the size seen in the nonpreconditioned control hearts, although they were still significantly smaller \((P<.025)\). Although 6 of the 7 rabbits in this group had infarcts comparable in size to those in the control group, 1 rabbit had a very small infarct (8.8% of the risk zone). Yet the risk zone in this animal was large \((1.04 \text{ cm}^3)\), and the rabbit fibrillated twice during the repetitive 5-minute occlusion protocol and again during the final 30-minute coronary occlusion. Therefore, significant myocardial ischemia was evident during the coronary occlusions. Except for this 1 rabbit, animals with multiple 5-minute preconditioning cycles were nearly indistinguishable from control nonpreconditioned rabbits (Fig 2).

Finally, seven rabbits with multiple episodes of myocardial ischemia allowed to rest in their cages for 2.5 to 3 days without any coronary occlusions had a single 5-minute ischemic preconditioning stimulus before the standard 30-minute occlusion and reperfusion. In these animals, infarct size averaged 10.9±1.5% of the risk zone (Fig 1), significantly smaller than that in rabbits with no ischemic preconditioning \((P<.0001)\) as well as in those with multiple episodes of ischemic preconditioning without a prolonged ischemia-free interval \((P<.001)\). There was no difference in infarct size between this group and that with only a single ischemic preconditioning period.

In the rabbits studied above, collateral blood flow to ischemic myocardium consistently averaged 4% to 6.5%
Fig 1. Infarct size plotted as a percentage of volume of risk zone for control rabbits with a 30-minute coronary occlusion (OCCL), rabbits with ischemic preconditioning (PC) from a single 5-minute coronary occlusion before the 30-minute occlusion, rabbits with only multiple 5-minute occlusions, rabbits with repetitive 5-minute coronary occlusions preceding the 30-minute occlusion, and finally rabbits with a single 5-minute ischemic preconditioning period after repetitive occlusions and an ischemia-free interval. Open circles represent individual data points; filled circles represent means. Vertical bars depict SEM. One cycle of ischemic PC significantly decreased infarct size after a 30-minute coronary occlusion (P<.0001). This protection was markedly attenuated if animals had multiple 5-minute coronary occlusions before the 30-minute occlusion. Infarct size in this latter group was still less than that observed in control rabbits (P<.025) but greater than that in rabbits with only one preconditioning stimulus (P<.0001). However, protection from a 5-minute coronary occlusion was restored in rabbits with multiple occlusions and an interposed ischemia-free period (P<.0001 vs control rabbits and P<.001 vs rabbits with multiple occlusions).

Fig 2. Relation between infarct and risk zone sizes plotted for control rabbits with only a 30-minute coronary occlusion (OCCL), rabbits with a single cycle of ischemic preconditioning (PC) before the 30-minute coronary occlusion, rabbits with multiple cycles of ischemic preconditioning before the final prolonged occlusion, and rabbits with a single cycle of ischemic preconditioning after multiple cycles and then an interposed ischemia-free interval. Although risk zones were comparable in the four groups, infarcts were considerably smaller in rabbits with isolated single preconditioning ischemic periods and those with single preconditioning ischemic periods after multiple occlusions and ischemia-free intervals than in the other two groups. 38% of the risk zone, respectively) attest to the inadequacy of the flow. However, in those animals with carotid catheters in which flow was quantified, flows to normal (5.1 mL·min⁻¹·g⁻¹) as well as ischemic (0.4 mL·min⁻¹·g⁻¹) tissues were surprisingly high. Because flow quantification is very dependent on the adequacy of the arterial withdrawal sample and because it could not be determined whether transient neck flexion or
rotation in these conscious animals might have interfered with withdrawal from the carotid catheter, three animals were prepared with catheters in the descending thoracic aorta. Arterial withdrawal would therefore not be influenced by animal movement. To further maximize the accuracy of the flow measurements, the number of microspheres injected was increased, only the radioactive label with the highest energy was selected, and a faster arterial withdrawal rate was used. In these three rabbits, infarction averaged 39.0±1.7% of the risk zone. Blood flow to normal myocardium was 4.3±0.6 mL·min⁻¹·g⁻¹; flow to ischemic tissue averaged 0.2±0.1 mL·min⁻¹·g⁻¹. Again, the ratio of collateral to normal tissue blood flow was 5.4±1.6%.

Discussion

Ischemic preconditioning is gaining increasing attention as a potentially useful clinical tool to limit the extent of myocardial infarction. Paradoxically, those animals exposed to a brief ischemic episode before a more prolonged ischemic insult have less infarction than animals experiencing only the longer ischemic insult despite the additional ischemia.¹⁻⁵,⁷⁻¹¹ The present study expands these observations to conscious unanesthetized animals without the confounding difficulties engendered by the effects of anesthesia on reflex pathways and the autonomic nervous system. Previous studies by Vatner¹² have clearly demonstrated the potential limitations of an anesthetized animal model in the study of the cardiovascular system. Virtually all previous studies of ischemic and pharmacologic preconditioning have used either Langendorff isolated-heart preparations or animals with hearts exposed through a thoracotomy. Although these animal models have provided important information, it was unclear whether the preparation itself might have influenced the outcome. The present experiments document the effectiveness of ischemic preconditioning in yet another animal model and suggest that ischemic preconditioning is not merely a function of the method of investigation.

As noted in Table 2, control and experimental groups had comparable head weights and volumes of risk region. Furthermore, heart rates in the two groups were also comparable throughout the protocol. Because of attempts to simplify the surgical procedure, indwelling arterial catheters were introduced into only a subset of the rabbits studied. As noted in Table 1, blood pressures were also not different in control and preconditioned rabbits. Collateral flow averaged 6% of normal flow in these animals, and these observations are consistent with previous observations by us¹⁴ and others.¹⁶ Again, there was no difference in relative or absolute collateral flows between the two groups.

Fig 1 demonstrates a striking difference in infarct size between rabbits with an isolated 30-minute occlusion and those with one preceding 5-minute period of ischemic preconditioning. To further document a significant difference in infarct size between the two groups, infarct size as a function of risk area was plotted for these rabbits. As depicted in Fig 2, two distinctly different populations of points are apparent. The difference in infarct size between the two groups was also not explicable by variations in collateral flow to the ischemic tissue. As demonstrated in Fig 3, individual collateral flows were comparable in the two groups, yet infarcts were obviously much smaller in the preconditioned rabbits. There was no correlation between infarct size and collateral flow in the latter. Although a linear relation was apparent in the control group, only the two animals with collateral flows exceeding 10% seemed to have a small benefit. The range of collateral flows is indeed narrow compared with the much greater flow variability in dogs,¹⁶ and the ultimate effect on infarct size is small.

Additionally, preconditioning appears to attenuate serious and frequently life-threatening ventricular arrhythmias. Whereas five of eight control rabbits developed either ventricular tachycardia or fibrillation, only one preconditioned rabbit was similarly affected. Studies in anesthetized rats have concluded that preconditioning ischemia can attenuate serious ventricular arrhythmias during both ischemia and early reperfusion,¹⁷⁻²⁰ although observations in dogs have yielded less consistent results.¹²⁻²³ However, rat and canine myocardium, unlike rabbit and human cardiac tissue, contains xanthine oxidase, which in these models may be largely responsible for these arrhythmias by generating free radicals after the reintroduction of oxygen. The present study may be the first to clearly demonstrate that ischemic preconditioning also has a salutary effect on the occurrence of potentially lethal arrhythmias in xanthine-deficient hearts of conscious rabbits. It is interesting to note that arrhythmias of this type are rare in the anesthetized rabbit and have been too few to quantify. Clearly, the unanesthetized rabbit should provide a useful small-animal xanthine oxidase-deficient model for ischemia-induced arrhythmias.

But perhaps more important than the observation that the preconditioning phenomenon applies to conscious unanesthetized animals is the realization that the protection of ischemic preconditioning is markedly attenuated in the face of recurring preconditioning stimuli but can be reinstated after an ischemia-free interval. Although it has been reported that the protection after one episode of ischemic preconditioning in the pig could not be renewed after several hours during which the protective effect naturally waned,²⁴ we have found that in the rabbit full protection can be achieved with a second episode of ischemic preconditioning 2 hours after the first.²⁵ Therefore, it appears that many episodes of ischemic preconditioning are required before tachyphylaxis develops.

Previous work from this laboratory has already demonstrated that acute pretreatment with the A₁-adenosine agonist 2-chloro-N⁶-cyclo-pentyladenosine (CCPA) can mimic ischemic preconditioning.⁴ The protection after adenosine agonist administration was not maintained, however, when CCPA was infused over a 72-hour period.¹³ Chronic exposure to the A₁-adenosine agonist presumably resulted in downregulation of the myocardial receptor or its signal transduction pathway. Liang and Donovan²⁶ and Longabaugh et al²⁷ have already demonstrated downregulation of adenosine receptors and regulatory G proteins, respectively, after chronic exposure of cells to A₁-adenosine agonists. It is also possible that downregulation may occur at the level of protein kinase C. These CCPA-tolerant animals also could not be protected with ischemic preconditioning. CCPA-induced downregulation of adenosine receptors or their signaling pathways apparently also made the heart insensitive to the endogenously released adenosine responsible for triggering the protection after a 5-minute period of ischemia. The present data suggest
that this same type of tolerance can be induced physiologically by repeated bursts of endogenous adenosine production during multiple 5-minute coronary occlusions. This suggestion is further supported by the additional observation that the protective effect is reinstated after an ischemia-free interval, during which upregulation would be expected to restore baseline sensitivity of receptors and/or the signal transduction pathway.

In addition to the larger infarcts observed in these rabbits with multiple prior 5-minute coronary occlusions, ventricular fibrillation was very common. Ten of 21 rabbits developed ventricular fibrillation, and 4 had multiple episodes. This is in striking contrast to the apparent diminution in potentially lethal arrhythmias seen in animals preconditioned with a single 5-minute ischemic period. Of note, when ventricular fibrillation occurred, it appeared quite consistently after 11 to 15 ischemia/reperfusion cycles, or 5.5 to 7.5 hours after initiation of the protocol on a given day. This interval for day 1 may be too short for significant receptor downregulation to have occurred since in the study reported by Tsuchida et al.\textsuperscript{13} protection of ischemic myocardium and bradycardia (an A\(_1\)-mediated effect on atrial tissue) were still apparent after 6 hours of CCPA infusion. One possible explanation is that suppression of arrhythmias and protection from infarction may be controlled by separate and distinct mechanisms. Prior observations in the rat also suggest that arrhythmias and infarction may have separate controlling pathways, since a single 5-minute coronary occlusion attenuated ventricular arrhythmias during a subsequent coronary occlusion despite the lack of any salutary effect on infarct size.\textsuperscript{11}

It is not yet known how many repeated ischemic episodes can be tolerated before the protective effect of ischemic preconditioning is lost, but the clinical implications are striking. Whereas the individual with only one episode of angina pectoris may be protected if a coronary thrombosis occurs shortly thereafter, the patient with unstable angina or one with a history of stable but frequent angina may lose this protective effect. Furthermore, such a patient may not be amenable to protection from an adenosine-based drug either. Additional investigations are needed to determine the precise mechanism of this tolerance.

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