Insulin-induced improvement of postischemic recovery is abolished by inhibition of protein kinase C in rat heart

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Objective: We demonstrated earlier that postischemic addition of insulin improves recovery of function in isolated rat heart by phosphatidylinositol 3-kinase. Activation of phosphatidylinositol 3-kinase before ischemia improves recovery of the heart after ischemia through protein kinase C. We tested whether protein kinase C activation is required for the positive inotropic effect of insulin during reperfusion.

Methods: Isolated working rat hearts were perfused with Krebs-Henseleit buffer containing [2-³H]glucose (5 mmol/L, 0.05 μ Ci/mL) plus oleate (0.4 mmol/L) and were subjected to 15 minutes of global ischemia followed by 35 minutes of reperfusion with or without insulin (1 mU/mL). We measured cardiac power, glucose uptake, and tissue metabolites. The protein kinase C inhibitor chelerythrine (5 μ mol/L) was added either at the beginning of the experiment or together with insulin. Experiments were repeated under normoxic conditions.

Results: Cardiac power before ischemia was 9.63 to 12.4 mW. Insulin improved recovery of power after ischemia ($96.3\% \pm 10.8\%$ versus $65.7\% \pm 3.79\%$, $P < .05$). This effect was abolished by chelerythrine ($55.3\% \pm 6.49\%$). However, chelerythrine given at reperfusion did not block insulin's effect on recovery ($101.0\% \pm 4.25\%$, $P < .05$). Postischemic glucose uptake was not increased by insulin (3.07 ± 0.32 before, 3.45 ± 0.34 μ mol/min/gdw after ischemia, not significant) and was not affected by chelerythrine (3.01 ± 0.26 before, 3.29 ± 0.32 μ mol/min/gdw after ischemia, not significant). Under normoxic conditions, chelerythrine did not influence insulin's effects on glucose uptake or power.

Conclusion: The results suggest that (1) insulin's effect on recovery is dependent on ischemia-induced protein kinase C activation, (2) the activity of protein kinase C during reperfusion may not be important for this effect of insulin, and (3) protein kinase C plays no role in insulin's effect on glucose uptake under normoxic or postischemic conditions.

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Insulin improves postischemic contractile function of the heart in both experimental and clinical situations.^{1,2} The mechanisms discussed for this effect include modulation of postischemic substrate utilization,³ systemic vasodilation,⁴ and direct effects of insulin on the heart.^{1,5} We demonstrated previously that insulin directly improves postischemic cardiac power of the isolated working rat heart without affecting glucose uptake or glucose or fatty acid oxidation.¹ We recently found that the positive inotropic effect of insulin is mediated through phosphatidylinositol 3-kinase (PI3K), which is not associated with an increase in glucose uptake.⁶

PI3K also plays a role in the mechanism of ischemic preconditioning where it is activated preischemically, which in turn leads to activation of protein kinase C (PKC).⁷ Activation of PKC ϵ , 1 of the 2 major isoforms in adult rat hearts, was shown to be required and sufficient to improve postischemic cardiac function of transgenic mice and to reduce ischemic cell damage in isolated rat hearts.^{8,9} In

analogy to ischemic preconditioning, we speculated that the postischemic positive inotropic effect of insulin may also be mediated by PKC.

PKC has also been demonstrated to interfere with insulin signaling, affecting metabolic pathways.¹⁰ We therefore wanted to assess whether the lack of insulin's ability to stimulate glucose uptake after ischemia could be attributed to PKC activation.

Finally, the timing of PKC activation appears to be important for its mode of action.¹¹ To obtain information about potential differential effects of PKC activation at different time points, we blocked PKC either throughout the experiments or only during reperfusion.

We found that the postischemic positive inotropic effect of insulin is dependent on ischemia-induced PKC activation but that the activity of PKC during reperfusion may not be important for this effect of insulin. We also found that PKC does not play a role in insulin's effect on glucose uptake either under normoxic or under postischemic conditions.

Methods

Animals

Male Sprague-Dawley rats (260-370 g, *n* = 51) were obtained from Charles River (Sulzfeld, Germany) with free access to food and water. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, and the use of animals and the experimental protocols were approved by the Animal Welfare Committee of the University of Freiburg, Germany.

Materials

Chemicals, enzymes, and cofactors were obtained from Sigma Chemical Co (Deisenhofen, Germany), MERCK (Darmstadt, Germany), or Roche (Mannheim, Germany). Regular human insulin (Actrapid) was obtained from Novo Nordisk (Mainz, Germany). Bovine serum albumin (BSA), Cohn fraction V, fatty acid free was obtained from Intergen Company (Purchase, NY).

Radioisotopes and Glucose Uptake

High-performance liquid chromatography-purified [2-³H]glucose was obtained from NEN Life Science Products (Cologne, Germany). The purity of the tracer was verified by measuring the intrinsic ³H₂O content. The tracer was discarded if the intrinsic water content was >1% of the total activity. Glucose uptake was determined by the rate of ³H₂O production from [2-³H]glucose as described previously.¹²

Working Heart Preparation and Contractile Performance

The preparation has been described elsewhere in detail.¹³ Hearts were perfused as working hearts at 37°C with recirculating Krebs-Henseleit buffer (200 mL) containing glucose (5 mmol/L) and Na-oleate (0.4 mmol/L) bound to 1% BSA and equilibrated with 95% O₂-5% CO₂. Perfusate [Ca²⁺] was 2.5 mmol/L. All experiments were carried out with a preload of 15 cm H₂O and an afterload of 100 cm H₂O. All hearts were beating spontaneously.

Aortic flow and coronary flow were measured every 5 minutes and cardiac output was calculated as the sum of both values. Heart rate as well as systolic and diastolic aortic pressures were measured continuously with a pressure transducer connected to a physiologic recording device (Hugo Sachs Elektronik, Umkirch, Germany). Cardiac power was determined from the product of cardiac output and mean aortic pressure:

Cardiac power

$$= \frac{\text{Mean aortic pressure (cm H}_2\text{O)} \cdot \text{cardiac output (mL/min)}}{620}$$

Recovery was assessed by calculating average cardiac power during reperfusion and comparing that to average cardiac power before ischemia, which corrects for potential intraindividual differences in preischemic cardiac power of the hearts.

Perfusion Protocol

Hearts were perfused according to 1 of 11 perfusion protocols. Figure 1 shows a schematic of the 5 normoxic and 6 ischemic protocols. Isolated working rat hearts were perfused with Krebs-Henseleit buffer containing glucose (5 mmol/L, [2-³H]glucose 0.05 μ Ci/mL) plus oleate (0.4 mmol/L bound to 1% BSA). In the ischemia protocols (Figure 1, B), 20 minutes of normoxic perfusion were followed by 15 minutes of total, global, normothermic ischemia and 35 minutes of reperfusion with or without insulin (1 mU/mL). Chelerythrine was dissolved in a 20 mmol/L dimethylsulfoxide stock solution and was given either at the beginning of the experiment or after ischemia alone or together with insulin at an end concentration of 5 μ mol/L. At this concentration, chelerythrine fully inhibits all forms of PKC without affecting other protein kinases.¹⁴ At the end of the experiments, hearts were freeze-clamped to determine levels of glycogen, glucose 6-phosphate, and lactate. The experimental protocols were repeated under normoxic conditions (Figure 1, A).

Statistical Analysis

All data are presented as mean $\pm$ SEM. Statistical comparison was made using repeated measures analysis of variance or unpaired analysis of variance with post hoc comparison by Newman-Keuls test as appropriate.¹⁵ Differences were considered statistically significant when *P* < .05. As the statistical power of the main differences was high, the number of experiments per group was limited to 3 to 6.

Results

Normoxia

Table 1 shows cardiac power of hearts under normoxic conditions. During the first 30 minutes of the experiments cardiac power ranged from 7.94 $\pm$ 0.82 to 10.8 $\pm$ 1.32 mW. When chelerythrine was present in the perfusate from the beginning of the experiments, cardiac power was higher than in the other groups (*P* < .01). However, in all experimental groups cardiac power remained stable throughout the experiments and was not affected by any of the interventions.

A Normoxia **B Ischemia**

Figure 1. Experimental protocols for hearts perfused under normoxic conditions (A) or subjected to 15 minutes of total, global, normothermic ischemia (B). All hearts were perfused at physiological workload with Krebs-Henseleit buffer containing glucose (5 mmol/L) and oleate (0.4 mmol/L) as substrates and were freeze-clamped at the end of experiments. Chelerythrine (5 μ mol/L) or insulin (1 mU/mL) or both were present in the perfusate for the indicated time periods.

Glucose uptake (Table 2) in the first 30 minutes ranged from 2.60 ± 0.18 to 3.11 ± 0.48 μ mol/min/gdw and was the same in all groups. Chelerythrine did not affect glucose uptake. Insulin caused a significant increase in glucose uptake in all groups. Chelerythrine had no influence on this effect either. In the groups where chelerythrine was given alone either at the beginning or after 30 minutes, glucose uptake was lower during 30 to 60 minutes than during the first 30 minutes. This is unlikely to be chelerythrine's effect because addition of chelerythrine at the beginning (N-Chel[0]) did not affect glucose uptake during the first 30 minutes.

Ischemia

Table 3 shows cardiac power of hearts before ischemia and during reperfusion. Figure 2 shows the recovery of contractile function in percent of preischemic power. Hearts generated between 9.48 ± 0.59 and 12.4 ± 0.72 mW before ischemia. Again, cardiac power was higher compared with the control group when chelerythrine was present from the beginning of the experiment. Contractile function remained significantly suppressed in the control group during reperfusion. Insulin given at the beginning of reperfusion improved postischemic function to near normal values. Addition of chelerythrine alone either at

TABLE 1. Cardiac power in the first and second half of the experiments under normoxic conditions*

Group	Cardiac power (mW)	
	0-30 minutes	30-60 minutes
N-Ins	7.94 ± 0.82	8.06 ± 0.81
N-Chel(0)	10.6 ± 1.28†	9.85 ± 1.58†
N-Chel(30)	8.72 ± 0.68	8.57 ± 0.57
N-Chel(0)+Ins	10.8 ± 1.32†	10.8 ± 1.15†
N-Chel(30)+Ins	8.87 ± 1.21	8.77 ± 1.28

*Values are mean ± SEM and represent the average contractile performance in the respective time period (n = 3 to 5 per group). Hearts were perfused for 60 minutes under normoxic conditions. Insulin (Ins, 1 mU/mL) was added after 30 minutes. Chelerythrine (Chel, 5 μmol/L) was added at the beginning of the experiment (0) or after 30 minutes (30).

†P < .01 versus all other groups.

the beginning of the experiments or after ischemia did not influence recovery of function. Chelerythrine fully inhibited the effects of insulin on recovery when given at the beginning of the experiments. Chelerythrine did not block insulin's effect on recovery when given at the end of ischemia.

Glucose uptake before ischemia ranged from 2.74 ± 0.13 to 3.29 ± 0.27 μmol/min/gdw with no differences among groups and did not differ from baseline glucose uptake in the groups under normoxic conditions. After ischemia, the glucose uptake measured in each group did not differ from the corresponding glucose uptake measured before ischemia.

Tissue Metabolites

Table 4 shows tissue contents of glycogen, glucose-6-phosphate (G6P), and lactate at the end of the experiments. Glycogen content was higher in hearts perfused under normoxic conditions than in hearts subjected to ischemia/reperfusion. Glycogen content was lowest in postischemic hearts where chelerythrine was present from the beginning of the experiment. Under normoxic conditions G6P content was lowest when chelerythrine was given after 30 minutes. There were no differences in G6P content among groups after ischemia. Lactate content was highest in the control group's postischemic hearts.

Discussion

We have shown that (1) the effect of insulin on recovery of contractile function is dependent on ischemia-induced PKC activation, (2) the activity of PKC during reperfusion may not be important for this effect of insulin, and (3) PKC plays no role in insulin's effect on glucose uptake either under normoxic or under postischemic conditions.

Inhibition of PKC before but not after ischemia abolished the postischemic, positive inotropic effect of insulin

TABLE 2. Glucose uptake in the first and second half of the experiments under normoxic conditions*

Group	Glucose uptake (μmol/min/g dry wt)	
	0-30 minutes	30-60 minutes
N-Ins	2.60 ± 0.18	3.55 ± 0.38†
N-Chel(0)	2.66 ± 0.25	1.89 ± 0.29‡
N-Chel(30)	2.88 ± 0.41	2.25 ± 0.26‡
N-Chel(0)+Ins	3.11 ± 0.48	4.16 ± 0.62†
N-Chel(30)+Ins	2.72 ± 0.52	4.07 ± 0.72†

*Values are mean ± SEM and represent the average glucose uptake in the respective time period (n = 3 to 5 per group). Hearts were perfused for 60 minutes under normoxic conditions. Insulin (Ins, 1 mU/mL) was added after 30 minutes. Chelerythrine (Chel, 5 μmol/L) was added at the beginning of the experiment (0) or after 30 minutes (30).

†P < .01 versus 0 to 30 minutes.

‡P < .05.

TABLE 3. Cardiac power before ischemia and during reperfusion*

Group	Cardiac power (mW)	
	Before ischemia (0-20 minutes)	During reperfusion (35-70 minutes)
I-Control	9.63 ± 0.51	6.42 ± 0.64‡
I-Ins	10.4 ± 1.08	9.64 ± 0.60
I-Chel(0)	12.4 ± 0.72†	7.99 ± 1.43‡
I-Chel(35)	9.48 ± 0.59	7.04 ± 1.02§
I-Chel(0)+Ins	12.0 ± 0.54†	6.66 ± 0.90‡
I-Chel(35)+Ins	9.56 ± 1.16	9.69 ± 1.38

*Values are mean ± SEM and represent the average contractile performance in the respective time period (n = 4 to 6 per group). Insulin (Ins, 1 mU/mL) was added at the beginning of reperfusion. Chelerythrine (Chel, 5 μmol/L) was added at the beginning of the experiment (0) or during reperfusion (35). See Figure 1 and text for details.

†P < .05 versus I-Control and I-Chel(35).

‡P < .01 versus before ischemia.

§P < .05.

on recovery of contractile function. This was in contrast to our initial hypothesis that insulin affects postischemic contractile function by PI3K-activated PKC. Our observations could be due to at least 2 different scenarios. First, it is conceivable that the time required for PKC inhibition by chelerythrine is longer than the time insulin takes to activate PKC. Though we did not measure the PKC activity in this study, this explanation seems rather unlikely because the mechanism of PKC inhibition by insulin suggests a rather fast onset of action¹⁴ (see below). The second and more likely scenario is that PKC is not part of the postischemic insulin pathway, although PKC activation is required for insulin's effect on recovery of function. This speculation is supported by observations of PKC activation during ischemia. PKC isoforms α, β_{1/2}, δ, ε, and ξ have been identified in rat heart.¹⁶ Ischemia results in the activation of the major

Figure 2. Postischemic recovery of contractile function in percent of preischemic power of hearts subjected to the ischemic protocols (see Figure 1, $n = 4$ to 6 per group). Insulin (*Ins*, 1 mU/mL) was added at the beginning of reperfusion. Chelerythrine (*Chel*, 5 μ mol/L) was added either at the beginning of the experiment (*I-Chel(0)*) or at the beginning of reperfusion (*I-Chel(35)*). See text for details. Values are mean $\pm$ SEM. * $P < .05$ compared with control.

isoforms α , δ , ϵ , and ξ during short episodes of ischemia and specific activation of PKC δ and ϵ after prolonged episodes in isolated perfused rat hearts.¹⁷ One could speculate that the ischemic activation of PKC ϵ is crucial in insulin's postischemic effect, because (1) PKC ϵ is activated during short and long episodes of ischemia,¹⁷ (2) the number of atypical isoforms in adult rat heart is negligible,¹⁸ and (3) the ϵ -isoform of PKC is a key mediator of protection of infarct size and recovery of function afforded by ischemic preconditioning.^{8,19,20} The observation that PKC inhibition during ischemia but not during reperfusion abolishes the insulin effect is striking because it suggests that PKC may modulate a component in the insulin signaling cascade, which then mediates the positive inotropic effect. The PKC activity during reperfusion does not seem to play a role in this effect. This conclusion receives support from a recent study by Simonis and colleagues,¹¹ who show PKC playing a comparable role in ischemic preconditioning. Brief ischemic episodes lead to PKC activation and translocation, which were reversed during the reperfusion periods and which were undetectable during a third ischemic period. The authors conclude that sustained activation of PKC is no prerequisite for preconditioning.

We used the nonselective PKC inhibitor chelerythrine in this study. The IC_{50} of chelerythrine for PKC inhibition is 0.66 μ mol/L. The IC_{50} for other protein kinases (PKA, TPK, Ca^{++} /CM-PK) is >0.1 mmol/L.¹⁴ Thus, at the concentration of 5 μ mol/L we used, PKC should be fully

inhibited, with no significant inhibition of other protein kinases. This conclusion is supported by the work of Davies and coworkers.²¹ Chelerythrine interferes with the catalytic site of the enzyme, which is present in all isoforms. From a kinetic standpoint, we cannot exclude that chelerythrine, when given together with insulin after ischemia, takes longer to inhibit PKC than insulin does to activate PKC. However, delayed PKC inhibition by chelerythrine seems unlikely because the mechanism of PKC inhibition suggests a rather fast onset of action.¹⁴ We also experimented with staurosporine initially, a different type of PKC inhibitor. However, staurosporine exhibited a significant negative inotropic effect that prevented the hearts to generate the required afterload.

Addition of chelerythrine right at the beginning of the experiments unexpectedly resulted in the hearts' best performances. In these experiments cardiac performance was stable throughout the entire experiment under normoxic conditions. Under postischemic conditions, hearts showed a decrease in cardiac power comparable to the control group. The significance of this observation is not clear at this time. It is conceivable that PKC-isoforms whose inhibition increases cardiac power are activated during preparation of the hearts in ice-cold KH-buffer. Thus, the increase in cardiac power by chelerythrine may therefore be a feature of the isolated working rat heart. However, no changes in cardiac function were observed when chelerythrine was given later during the experiments. Therefore it is unlikely

that postischemic cardiac power was influenced by unspecific effects of chelerythrine.

In preliminary studies, we attempted to perfuse hearts with the phorbol ester phorbol 12-myristate 13-acetate (PMA) to mimic ischemia-induced PKC-activation. However, as with staurosporine, the PKC activation by PMA in effective concentrations was also associated with a significant negative inotropic effect, which did not allow reliable perfusions of the hearts (data not shown). We believe that the inability to use the drug in our model may be associated with our isolated working rat heart preparation. This has also been observed by others who demonstrated a negative inotropic effect of PMA or other PKC activators such as 1,2-dioctanoyl-sn-glycerol (DOG) in the isolated rat heart.^{22,23} The full elucidation of the underlying mechanism may therefore require the use of a different experimental model.

To assess whether metabolic effects of insulin may contribute to the postischemic effects of insulin on contractile function, we measured glucose uptake and determined the tissue content of those metabolites affected by insulin (ie, glycogen, glucose-6-phosphate, and lactate). The effect of insulin on recovery of contractile function was independent of its effects on glucose uptake and of the measured metabolites. Although the addition of insulin resulted in recovery of function to near basal values, insulin was not able to increase glucose uptake. We have demonstrated already a postischemic positive inotropic effect of insulin where insulin did not affect glucose or fatty acid oxidation.¹ Here we provide further evidence for our hypothesis, namely that the effect of insulin on contractile function is direct rather than secondary to modulations of substrate metabolisms. This conclusion is supported by a lack of major changes in tissue metabolites by insulin measured at the end of the experiments. Under normoxic conditions, insulin increases glucose uptake and does not affect contractile function in the isolated working rat heart. It is interesting to note in this context that administration of glucose-insulin-potassium infusion to patients after cardiac surgery improves cardiac function and reduces mortality at a time where insulin responsiveness is severely diminished.²⁴ Thus, it appears that ischemia induces a shift in insulin responsiveness from mainly metabolic to contractile effectors. Such an effector may be the L-type Ca-channel. It has recently been demonstrated that the L-type Ca-channel is directly activated by insulin in a dose-dependent manner.²⁵ This mechanism may be potentially responsible for insulin's positive inotropic effect.

PKC has been demonstrated to inhibit the insulin receptor.^{10,26} PKC activation in this context may occur through ischemia-induced accumulation of free fatty acids²⁷ and may be responsible for the clinically observed state of insulin resistance. We observed a state of insulin resistance

TABLE 4. Tissue contents of glycogen, glucose-6-phosphate, and lactate of hearts at the end of the experiments*

Group	Metabolites ($\mu\text{mol/g dry wt}$)		
	Glycogen	Glucose-6-phosphate	Lactate
Normoxia			
N-Ins	128.2 $\pm$ 3.10	1.53 $\pm$ 0.11	3.88 $\pm$ 0.70
N-Chel(0)	126.6 $\pm$ 13.3	1.20 $\pm$ 0.18	2.35 $\pm$ 0.35
N-Chel(30)	114.4 $\pm$ 5.55	0.96 $\pm$ 0.17†	3.05 $\pm$ 0.81
N-Chel(0) + Ins	137.0 $\pm$ 10.6	1.55 $\pm$ 0.05	3.69 $\pm$ 0.55
N-Chel(30) + Ins	125.7 $\pm$ 13.4	1.71 $\pm$ 0.02	3.21 $\pm$ 0.05
Ischemia			
I-Control	90.8 $\pm$ 7.57	1.65 $\pm$ 0.14	4.81 $\pm$ 0.56§
I-Ins	97.5 $\pm$ 6.81	1.40 $\pm$ 0.16	3.47 $\pm$ 0.36
I-Chel(0)	73.3 $\pm$ 7.73	1.19 $\pm$ 0.16	2.76 $\pm$ 0.31
I-Chel(35)	91.3 $\pm$ 5.13	1.21 $\pm$ 0.15	2.88 $\pm$ 0.14
I-Chel(0) + Ins	67.4 $\pm$ 3.18†	1.23 $\pm$ 0.12	4.12 $\pm$ 0.27
I-Chel(35) + Ins	115.9 $\pm$ 13.1	1.68 $\pm$ 0.08	3.04 $\pm$ 0.49

*Values are mean $\pm$ SEM and represent the average tissue content of the metabolites at the end of the experiments under normoxic and ischemic conditions.

† $P < .05$ versus I-Ins and I-Chel(35)+Ins.

‡ $P < .05$ versus N-Ins, N-Chel(0)+Ins, and N-Chel(30)+Ins.

§ $P < .05$ versus other groups except I-Chel(0)+Ins.

with respect to glucose uptake in hearts after ischemia. However, it is unlikely that inhibition of the insulin receptor by PKC is responsible for this effect for 2 reasons. First, inhibition of PI3K in previous experiments abolished insulin's effect on contractile function after ischemia,⁶ suggesting that the proximal part of the insulin signaling cascade is still intact under these conditions. Second, the PKC inhibition did not reinstate insulin's effect on postischemic glucose uptake, so that PKC is unlikely to mediate mechanisms inhibiting insulin-stimulated glucose uptake. Unexpectedly, chelerythrine was not able to block insulin-induced glucose uptake under normoxic conditions, which may reflect the minor expression of atypical isoforms in adult rat hearts. We found the lowest glycogen content in hearts where chelerythrine had been given from the beginning of the experiments. However, none of the groups differed significantly from the control group. Interpreting these differences is difficult because we did not determine glycogen content before and at the end of ischemia in our experiments. It is important to realize in this context that glycogen turnover is more important with respect to contractile function than glycogen content at the end of the experiments.^{28,29}

We have recently shown that insulin improves recovery of function through PI3K,⁶ and we demonstrate here that PKC activation is also required for this effect. Both enzymes are also involved in the mechanism of ischemic preconditioning, which is known as the most powerful

method to protect hearts from subsequent ischemia. The greatest handicap in the use of ischemic preconditioning is the need for pretreatment, excluding its clinical use in situations of unexpected acute ischemia (myocardial infarction). In these situations insulin can be given postischemically. Our results suggest a similar link between ischemic preconditioning and the insulin-induced improvement of postischemic cardiac power. It is conceivable that observations made by us and others⁷ may differ because of the involvement of different isoforms of PKC and/or PI3K. However, if the observations should describe the same mechanisms, this conclusion would give rise to the intriguing speculation that the protective mechanism of ischemic preconditioning may be exploited by activating the insulin signaling cascade at the end of ischemia.

We conclude that the postischemic positive inotropic effect of insulin is dependent on ischemia-induced PKC activation and that the activity of PKC during reperfusion may not be important for this effect of insulin. We found that PKC plays no role in insulin's effect on glucose uptake either under normoxic or under postischemic conditions. This means that insulin has a direct effect on contractile function after ischemia, which is mediated through a pathway that differs from the conventional signaling cascade by requiring ischemic PKC activation.

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