Multifunctional Mitochondrial Epac1 Controls Myocardial Cell Death

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Rationale: **Although the second messenger cyclic AMP (cAMP) is physiologically beneficial in the heart, it largely contributes to cardiac disease progression when dysregulated. Current evidence suggests that cAMP is produced within mitochondria. However, mitochondrial cAMP signaling and its involvement in cardiac pathophysiology are far from being understood.**

Objective: **To investigate the role of MitEpac1 (mitochondrial exchange protein directly activated by cAMP 1) in ischemia/reperfusion injury.**

Methods and Results: **We show that *Epac1* (exchange protein directly activated by cAMP 1) genetic ablation (*Epac1*$^{-/-}$) protects against experimental myocardial ischemia/reperfusion injury with reduced infarct size and cardiomyocyte apoptosis. As observed in vivo, Epac1 inhibition prevents hypoxia/reoxygenation–induced adult cardiomyocyte apoptosis. Interestingly, a deleted form of *Epac1* in its mitochondrial-targeting sequence protects against hypoxia/reoxygenation–induced cell death. Mechanistically, Epac1 favors Ca^{2+} exchange between the endoplasmic reticulum and the mitochondrion, by increasing interaction with a macromolecular complex composed of the VDAC1 (voltage-dependent anion channel 1), the GRP75 (chaperone glucose-regulated protein 75), and the IP3R1 (inositol-1,4,5-triphosphate receptor 1), leading to mitochondrial Ca^{2+} overload and opening of the mitochondrial permeability transition pore. In addition, our findings demonstrate that MitEpac1 inhibits isocitrate dehydrogenase 2 via the mitochondrial recruitment of CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), which decreases nicotinamide adenine dinucleotide phosphate hydrogen synthesis, thereby, reducing the antioxidant capabilities of the cardiomyocyte.**

Conclusions: **Our results reveal the existence, within mitochondria, of different cAMP–Epac1 microdomains that control myocardial cell death. In addition, our findings suggest Epac1 as a promising target for the treatment of ischemia-induced myocardial damage.**

Key Words: calcium ■ cyclic AMP ■ ischemia reperfusion injury ■ mitochondria ■ reactive oxygen species

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Cyclic AMP (cAMP) is a ubiquitous second messenger that controls numerous physiological processes, including metabolism, Ca^{2+} homeostasis, and gene transcription. While most studies focused on the biological effects of cytosolic cAMP, its possible role in mitochondrial function and pathophysiology has been neglected until recently. Today, an increasing body of evidence strongly suggests that cAMP is also produced in the mitochondrion.[1,2] There, cAMP is generated in the mitochondrial matrix by the type 10 soluble adenylyl cyclase (sAC), which is activated in response to HCO_3^- or Ca^{2+} ions.[1,3,4] It has been proposed that cAMP produced by sAC activates a pool of the cAMP effector PKA (protein kinase A) present in the mitochondrial matrix, resulting in the phosphorylation of the cytochrome *c* oxidase subunit IV isoform 1, which consequently enhances oxidative phosphorylation and, hence, ATP synthesis.[1,3] Besides ATP production, the mitochondria play a crucial role in other important cellular processes, such as Ca^{2+} buffering, reactive

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- The second messenger cAMP is produced in the cytosol and mitochondria of cardiomyocytes.
- Dysregulation of cAMP signaling contributes to cardiac remodeling and heart failure.
- Epac1 (exchange protein directly activated by cAMP 1) induces cardiomyocyte hypertrophy in response to β-adrenergic receptor stimulation.

- *Epac1* gene deletion is cardioprotective against ischemia/reperfusion injury in vivo.
- Epac1 is activated by soluble adenylyl cyclase during hypoxia/reoxygenation to transduce cardiomyocyte death.
- Under hypoxia, the activation of mitochondrial Epac1 increases mitochondrial Ca^{2+} overload and reduces reactive oxygen species detoxification, thereby, inducing cardiomyocyte death.

cAMP is a ubiquitous second messenger, and its possible role in mitochondrial function and pathophysiology has yet to be investigated. Here we show that genetic inhibition of the cAMP-binding protein, Epac1, is cardioprotective against myocardial ischemia/reperfusion injury. Mechanistically, Epac1 is activated by soluble adenylyl cyclase and promotes cardiomyocyte death during hypoxia/reoxygenation. Furthermore, we found that mitochondrial Epac1 regulated different aspects of mitochondrial function, such as Ca^{2+} uptake, reactive oxygen species production, and mitochondrial permeability transition pore opening. Thus, the development of Epac1 pharmacological inhibitors may represent a promising therapeutic avenue for the treatment of ischemia/reperfusion injury.

Nonstandard Abbreviations and Acronyms

8-CPT-AM	8-pCPT-2′-O-Me-cAMP-AM
BRET	bioluminescence resonance energy transfer
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
GRP75	chaperone glucose-regulated protein 75
Epac1	exchange protein directly activated by cAMP 1
ER	endoplasmic reticulum
HX+R	hypoxia/reoxygenation
IDH2	isocitrate dehydrogenase 2
IP3R1	inositol-1,4,5-triphosphate receptor 1
I/R	ischemia/reperfusion
MCU	mitochondrial Ca2+ uniporter
MitEpac1	mitochondrial Epac1
MPTP	mitochondrial permeability transition pore
NADPH	nicotinamide adenine dinucleotide phosphate
NX	normoxia
PKA	protein kinase A
ROS	reactive oxygen species
sAC	soluble adenylyl cyclase
VDAC1	voltage-dependent anion channel 1
WT	wild-type

oxygen species (ROS) production, and apoptosis.[2] This is well illustrated in the context of myocardial ischemia/reperfusion (I/R), a clinical relevant form of myocardial injury in which mitochondrial Ca^{2+} overload and an excessive production of ROS trigger the opening of the mitochondrial permeability transition pore (MPTP), resulting in mitochondrial depolarization and cardiomyocyte death.[5]

In the heart, cAMP regulates many physiological processes, such as contractility, relaxation, and automaticity, and represents the strongest mechanism for increasing cardiac function in response to β-adrenergic receptor activation.[6] However, the sustained release of catecholamines from adrenergic nerves observed during myocardial ischemia overactivates myocardial β-adrenergic receptor and subsequent cAMP production that is believed to further accelerate ischemia-induced cell damage.[7,8] Therefore, cAMP production that is physiologically beneficial in the heart largely contributes to cardiac disease progression when dysregulated.[6] Because cardiomyocyte death is one of the hallmarks of myocardial I/R injury,[9] many efforts have been made to understand the signaling events involving cAMP to this process. Most studies that have demonstrated the effect of cAMP signaling on cell death have primarily focused on PKA.[8,10,11] However, cAMP also stimulates a family of proteins directly activated by cAMP, named Epac (exchange proteins directly activated by cAMP[12,13]). The Epac proteins, Epac1 and Epac2, are guanine exchange factors for the small G-proteins Rap1 and Rap2 and function in a PKA-independent manner. Compelling evidence indicate that Epac proteins induce sarcoplasmic reticulum Ca^{2+} leakage[14,15] and localize at the nuclear envelope of cardiomyocytes to promote cardiac remodeling.[16–18] However, Epac compartmentalization is still poorly described, and its involvement in cardiomyocyte death has yet to be investigated.

In this study, we showed that *Epac1* genetic ablation (*Epac1*$^{-/-}$) protected against experimental myocardial I/R injury with reduced infarct size and cardiomyocyte apoptosis. Mechanistic studies allowed us to propose a model, whereby I/R stimulated cAMP production by sAC, which in turn activated MitEpac1 (mitochondrial Epac1), leading to mitochondrial Ca^{2+} overload, a decrease in ATP production, a decrease in ROS detoxification, and eventually apoptosis. These data also suggest that the inhibition of Epac1 could reduce myocardial damage during cardiac ischemia.

Methods

Animals

All animal procedures were performed in accordance with Institutional Guidelines on Animal Experimentation and with a French Ministry of Agriculture license. Moreover, this investigation conformed to the Guide for the Care and Use of Laboratory

Animals published by the Directive 2010/63/EU of the European Parliament. Mice were housed in a pathogen-free facility, and all animal experiments were approved by the Animal Care and Use Committees of the University of Toulouse. *Epac1*-deficient mice (*Epac1*$^{-/-}$) have been engineered in our laboratory as previously described.[17] Eight-week-old male *Epac1*$^{-/-}$ mice and littermate controls (wild-type [WT]) in this study were obtained by heterozygous crossing.

Plasmid Constructs and Transfection

The Epac1–bioluminescence resonance energy transfer (BRET) sensor CAMYEL was constructed from the pQE30-CAMYEL prokaryotic expression vector (a gift from Dr L.I. Jiang) as previously described.[19] The human *Epac1* expression vector was a gift from Dr J.L. Bos. Primary neonatal rat cardiomyocytes were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

BRET Assay

Neonatal rat cardiomyocytes transfected with the Epac1-BRET sensor CAMYEL were harvested and lysed in a buffer containing 40 mmol/L HEPES, pH 7.2, 140 mmol/L KCl, 10 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.5% Triton X-100, and a cocktail of protease inhibitors (Roche Applied Science). BRET experiments were performed as previously described.[19] Emission from Renilla luciferase and citrine were measured simultaneously in a plate reader (TECAN infinite F200).

Statistical Analysis

Analyses were performed using Prism 7 (GraphPad Software). Results are expressed as mean±SEM or fold increase, as appropriate. Genotype–treatment interactions were studied by using 2-way analysis of variance. Multiple comparisons were performed with 1-way analysis of variance followed by post hoc test with Bonferroni correction or Tukey posttest. Statistical significance was set to $P<0.05$.

Results

Epac1 Deficiency Is Cardioprotective Against I/R Injury

We first analyzed cardiac Epac1 expression in ischemic failing human heart samples and in mice subjected to myocardial I/R injury. In both human and mouse hearts, Epac1 protein levels were markedly increased compared with that in control hearts (Figure 1A and 1B), suggesting that Epac1 upregulation is associated with myocardial ischemic damage. To test this hypothesis, *Epac1*$^{-/-}$ mice and their WT littermates were subjected to myocardial I/R injury. Although the area at risk was similar in both genotype (Figure 1C), the infarct size-to-area at risk ratio was significantly reduced in *Epac1*$^{-/-}$ (33±4%) when compared with that in WT (53±4%; Figure 1C and 1D). Consistently, cardiomyocyte apoptosis, assayed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining, was markedly reduced in *Epac1*$^{-/-}$ mice compared with that in WT mice (Figure 1E), whereas it was similar in both genotypes at baseline (Online Figure I).

Accordingly, in WT animals, I/R injury was accompanied by an upregulation of the proapoptotic protein Bax, a downregulation of the antiapoptotic protein Bcl2 (Online Figure II), an increase in cytochrome c level, and caspase 9 and caspase 3 activation (Figure 1F through 1H). In marked contrast, this I/R-induced apoptotic response was partially inhibited by the genetic ablation of *Epac1* (Figure 1F through 1H; Online Figure II). Altogether, these results suggest Epac1 as a transducer of cardiomyocyte apoptosis after myocardial I/R injury.

Epac1 Is Activated by Soluble Adenylyl Cyclase During Hypoxia/Reoxygenation to Transduce Cardiomyocyte Death

To test whether Epac1 may directly influence cell death, we next subjected isolated cardiomyocytes from adult *Epac1*$^{-/-}$ and WT mice to either normoxia (NX) or 4-hour hypoxia followed by 2-hour reoxygenation (HX+R), which mimics in vivo I/R. Under HX+R, WT cardiomyocytes exhibited an increase in membrane permeability assayed by LDH (lactate dehydrogenase) release (Figures 2B), a decrease in cell viability assayed by trypan blue exclusion and cellular ATP level (Figures 2A; Online Figure III), and the expression of I/R-associated apoptotic markers (Online Figure IV). As observed in vivo, *Epac1* deletion prevented HX+R-induced cell damages (Figure 2A and 2B; Online Figure III). These data confirmed HX+R in isolated cardiomyocytes as an in vitro surrogate to in vivo myocardial I/R injury.

Next, to test the activation of Epac1 during HX+R, neonatal cardiomyocytes were transfected with an *Epac1* BRET sensor to monitor Epac1 activation.[19] HX+R induced a significant increase in the BRET ratio when compared with NX, which was blocked by a selective Epac1 inhibitor, CE3F4[19] (Figure 2C), confirming Epac1 activation during HX+R. Furthermore, CE3F4 prevented HX+R-induced cell damage phenocopying *Epac1* deletion (Figure 2D), and the membrane-permeant Epac1-specific agonist, 8-pCPT-2′-O-Me-cAMP-AM (8-CPT-AM),[20] mimicked the effects of HX+R in WT cardiomyocytes cultured in NX conditions (Figure 2E). Of note, 8-CPT-AM did not induce any HX+R-associated cell damages in *Epac1*$^{-/-}$ cardiomyocytes, confirming the specificity of the agonist (Figure 2E). These data indicate that Epac1 activation is sufficient to provoke cardiomyocyte cell death in HX+R conditions.

During HX+R, we observed that WT cardiomyocytes accumulated cAMP, the cognate activator of Epac1 (Figure 2F). Because sAC was recently shown to produce cAMP in hypoxic condition,[21] we investigated its possible involvement in HX+R-mediated Epac1 activation. The selective sAC inhibitor, KH7, blocked cAMP accumulation and significantly reduced HX+R-induced Epac1 activation in WT cardiomyocytes (Figure 2C and 2F), as well as HX+R-induced cell damages, similarly to CE3F4 (Figure 2D).[19] Taken together, these results provide strong evidence that Epac1 is activated by cAMP produced by sAC and directly promotes cardiomyocyte death under HX+R conditions.

MitEpac1 Promotes Cardiomyocyte Death

The protein sequence of Epac1 contains a predicted mitochondrial-targeting domain (Figure 3A). Cell fractionation followed by Western blot analysis showed that Epac1 is localized both in the cytosol and in the mitochondria (Figure 3B). To assess the specific localization of Epac1, we performed subfractionation of mitochondria (free of endoplasmic reticulum [ER]) in WT hearts (Figure 3C). The purity of the fractions was verified by immunoblotting with specific protein markers, such as monoamine oxidase-A for

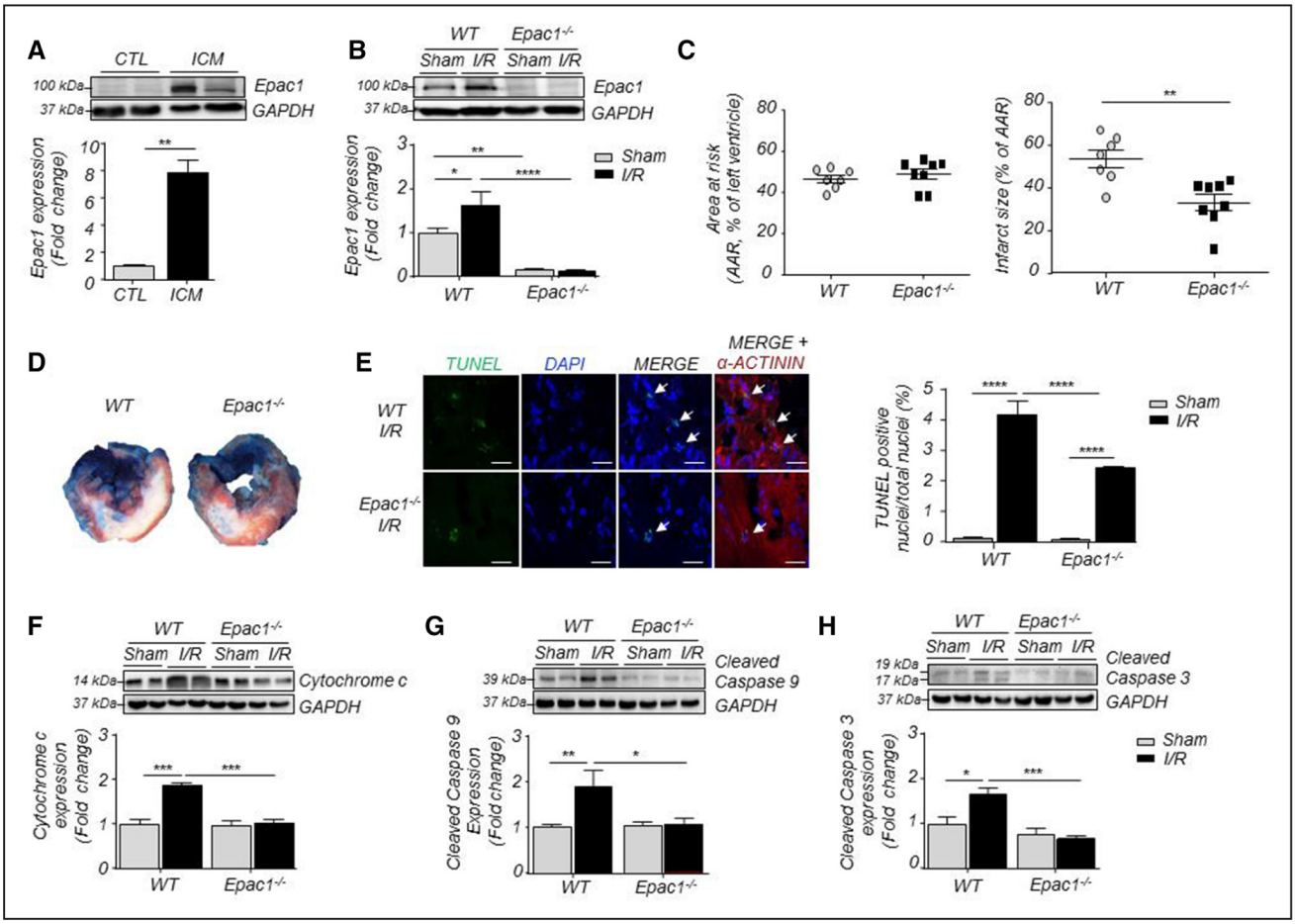

Figure 1. Epac1 deficiency is cardioprotective against ischemia/reperfusion (I/R) injury. A and **B,** Quantification of Epac1 protein in human left ventricular myocardium from nonischemic control (CTL; n=4) or patients with ischemic cardiomyopathy (ICM; n=5), and in wild-type (WT) or *Epac1*$^{-/-}$ heart mice subjected or not to I/R (n=6 per group). Representative immunoblots are shown. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), loading control. **C,** Quantification of the area at risk (AAR) expressed as percentage of left ventricle size and infarct size expressed as percentage of AAR. **D,** A representative cross-section stained with Evans blue and TTC of WT and *Epac1*$^{-/-}$ heart mice subjected to I/R. **E,** Representative images of TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining of heart sections from WT and *Epac1*$^{-/-}$ mice subjected to I/R. DAPI, nuclear marker; α-actinin, cardiomyocyte marker. Arrows indicate positive TUNEL nuclei. Scale bar, 50 μm. **Right,** Quantification of TUNEL-positive staining (n=500 in 6 independent experiments). **F-H,** Quantification of the indicated proteins (n=6 in each condition). Representative immunoblots are shown. Data are means±SEM and were analyzed with 2-way analysis of variance (ANOVA)/Bonferroni posttest. *P<0.05, **P<0.01, ***P<0.001 vs the indicated value. Epac1 indicates exchange protein directly activated by cAMP 1.

mitochondrial outer-membrane and intermembrane space, cytochrome c oxidase subunit 4 for inner membrane, and isocitrate dehydrogenase (IDH2) for the matrix, respectively. We found that Epac1 was expressed in the mitochondrial inner membrane and matrix (Figure 3C). Since the mitochondrion plays a critical role in the regulation of apoptosis, we further investigated whether MitEpac1 plays a role in HX+R-induced cell death.

To this end, we constructed a mutant form of *Epac1* deleted for its putative mitochondrial-targeting sequence (Epac1$^{\Delta 2-37}$; Figure 3A). Transfection experiments followed by cell fractionation and immunoblot analysis showed that Epac1$^{\Delta 2-37}$ was mainly excluded from the mitochondrial compartment of cardiomyocytes (Figure 3D through 3E). Epac1$^{\Delta 2-37}$ mutant activated Epac1 downstream effector Rap1 in a similar fashion as the WT protein (Epac1^{WT}), indicating that loss of mitochondrial targeting did not impair Epac1 function in the cytosol (Figure 3F). Importantly, cardiomyocytes transfected with Epac1$^{\Delta 2-37}$ exhibited less cell death as compared with cardiomyocytes transfected with the Epac1^{WT} when subjected to HX+R (Figure 3G). Together, these data strongly suggest that MitEpac1 participates in cardiomyocyte death during hypoxic stress.

Epac1 Promotes Mitochondrial Ca^{2+} Overload Via the Dependent Anion Channel 1/Chaperone Glucose-Regulated Protein 75/Inositol-1,4,5-Triphosphate Receptor 1 Complex and MPTP Opening During HX+R

It is well accepted that I/R injury is accompanied by an increase in mitochondrial Ca^{2+} that triggers the opening of MPTP, the subsequent depolarization of the mitochondrial membrane potential (ΔΨm), leading to cytochrome *c* release and cardiomyocyte death.[22] However, the mechanisms that control mitochondrial Ca^{2+} entry in that context are far to be understood. We, therefore, addressed whether Epac1 would play a role in such a process using a Ca^{2+} fluorescent probe

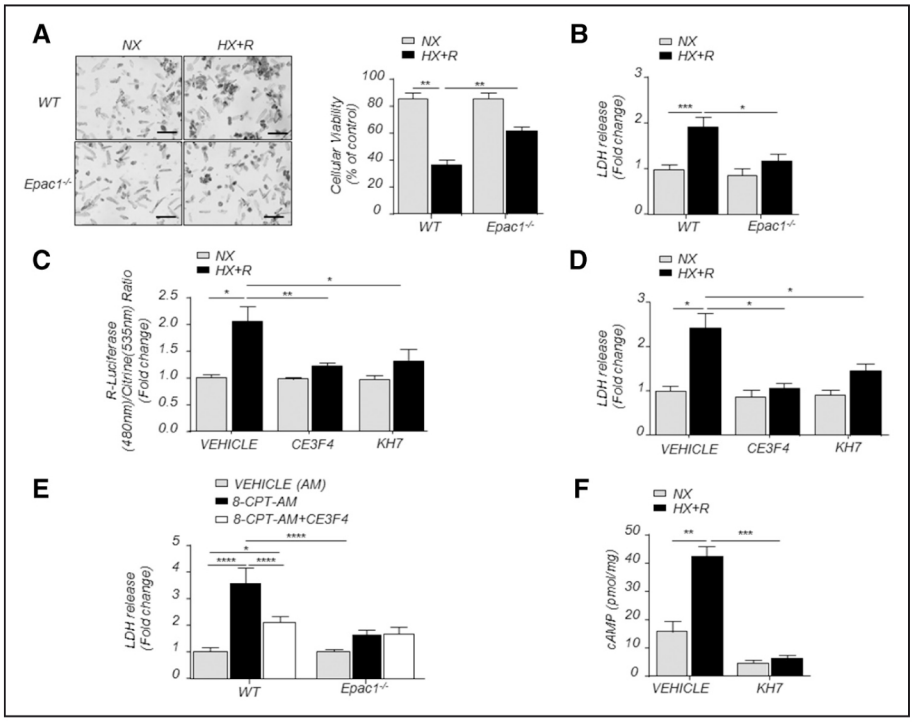

Figure 2. Epac1 is activated by soluble adenylyl cyclase (sAC) to promote cardiomyocyte death during hypoxia/reoxygenation (HX+R). A and **B,** Cell viability determined by trypan blue staining and measurement of lactate dehydrogenase (LDH) release in isolated adult WT or *Epac1*$^{-/-}$ cardiomyocytes in normoxia (NX) or HX+R condition (n=6). Representative images of trypan blue staining are shown. Scale bar, 100 μm. **C,** Analysis of Epac1 activity by bioluminescence resonance energy transfer (BRET) in neonatal rat ventricular myocytes transfected with the CAMYEL construct and pretreated with either CE3F4 (20 μmol/L, 1 hour) or KH7 (20 μmol/L, 1 hour) before to be placed in NX or HX+R condition (n=10). **D,** LDH release in adult wild-type (WT) cardiomyocytes in NX or HX+R condition (n=6–8). Cells were pretreated with either CE3F4 or KH7 as in (**C**). **E,** LDH release in WT or *Epac1*$^{-/-}$ cardiomyocytes (n=6–8). Cells were pretreated or not with CE3F4 (20 μmol/L, 30 minutes) and stimulated with 8-CPT-AM (10 μmol/L, 30 minutes). **F,** Quantification of intracellular cAMP in adult WT cardiomyocytes in NX or HX+R condition (n=7). Cells were pretreated or not with KH7 (20 μmol/L, 30 minutes). Data are means±SEM and were evaluated by 1-way analysis of variance (ANOVA)/Bonferroni posttest. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control group or the indicated value. Epac1 indicates exchange protein directly activated by cAMP 1.

Rhod-2-AM.[23] Although the disadvantage of Rhod-2-AM is its nonratiometric nature, the specific accumulation of this probe in mitochondria makes it one of the most widely used mitochondrial Ca^{2+} probe.[24] The Rhod-2-AM signal overlapped with the mitochondrial marker Mitotracker green in adult cardiomyocytes (Online Figure V). 8-CPT-AM induced a robust increase in mitochondrial Ca^{2+} in WT cardiomyocytes but not in those of *Epac1*$^{-/-}$ cells (Figure 4A; Online Figure V). Furthermore, treatment of *Epac1*$^{-/-}$ cardiomyocytes with 50 μmol/L of Ca^{2+} induced much less mitochondrial Ca^{2+} overload when compared with the WT (Figure 4B; Online Figure VI), suggesting that mitochondrial Ca^{2+} level increased in cardiomyocytes after Epac1 stimulation. Yet, we found that the mitochondrial Ca^{2+} uniporter (MCU) was involved in Epac1 effect because the MCU inhibitor, RU360, significantly reduced 8-CPT-AM-induced mitochondrial Ca^{2+} (Online Figure VIIA).

To determine how Epac1 influenced mitochondrial Ca^{2+} uptake, we next analyzed its interaction with a macromolecular complex composed of the VDAC1 (voltage-dependent anion channel 1), the GRP75 (chaperone glucose-regulated protein 75), and the IP3R1 (inositol-1,4,5-triphosphate receptor 1). This Ca^{2+}-handling protein complex of the ER (IP3R1) and the mitochondrion (VDAC1) is highly concentrated at mitochondria-associated ER membranes, where it regulates Ca^{2+} exchange between the ER and the mitochondria.[25] Immunoprecipitation assay with Epac1 antibody showed that 8-CPT-AM increased VDAC1/GRP75/IP3R1 complex formation, indicating that activated Epac1 favors the association of this complex (Online Figure VIII). We did not observe any interaction of Epac1 with the RyR2 (type 2 ryanodine receptor), a dominant sarcoplasmic reticulum Ca^{2+} channels protein. This suggests that the Epac1 interaction with the VDAC1/GRP75/IP3R1 complex is specific (Online Figure VIII). In addition, size exclusion chromatography followed by Western blot showed that VDAC1, GRP75, IP3R1, and Epac1 were eluted and detected in the same high molecular weight fraction (Online Figure IX). Altogether, these data indicate that all 4 proteins belonged to the same macromolecular complex.

Next, we investigated whether such an effect of Epac1 on VDAC1/GRP75/IP3R1 complex formation would also occur in hypoxic condition. As expected, we observed by in situ proximity ligation assay that HX+R significantly increased IP3R1/GRP75, IP3R1/VDAC1, and GRP75/VDAC1 interactions in WT cardiomyocytes (Figure 4C through 4E). The absence of *Epac1* did not modify VDAC1/GRP75/IP3R1 interaction in NX condition. However, in HX+R condition, the lack of *Epac1* reduced VDAC1/GRP75/IP3R1 interaction compared with that in WT cardiomyocytes (Figure 4C

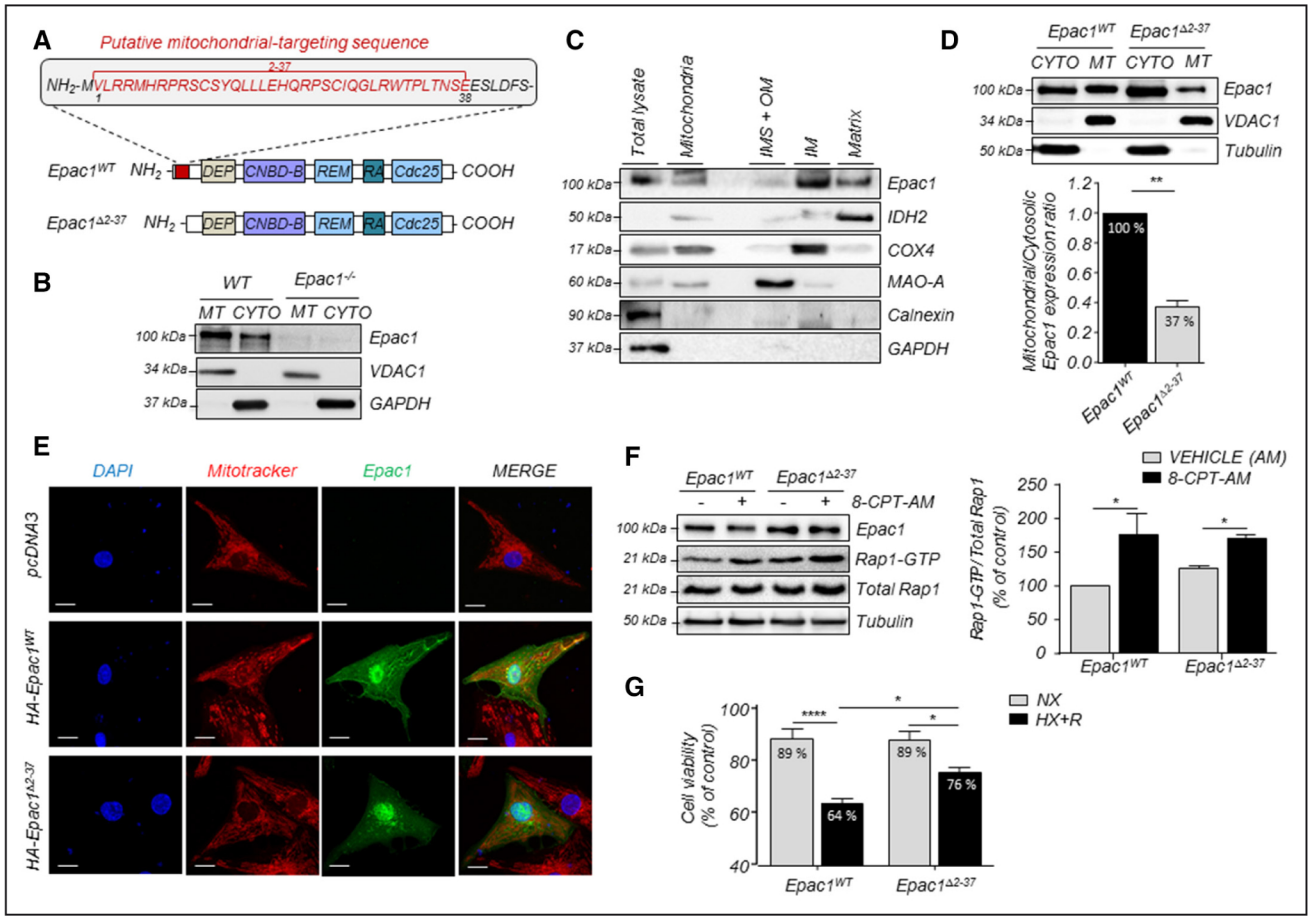

Figure 3. MitEpac1 promotes cardiomyocyte death. A, Schematic representation of the different domains of Epac1^{WT}. Epac1$^{\Delta 2-37}$ mutant bears a deletion in Epac1 mitochondrial-targeting sequence. **B,** Representative immunoblot of cytosolic (CYTO) and mitochondrial (MT) Epac1 expression in cardiac samples of WT or *Epac1*$^{-/-}$ mice. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and voltage-dependent anion channel 1 (VDAC1) expression were used as loading control of cytosolic and mitochondrial fractions, respectively. **C,** Localization of Epac1 in the inner membrane and matrix of mouse cardiac mitochondria. Monoamine oxidase-A (MAO-A), cytochrome c oxidase (subunit 4 [COX4]) and IDH2 (isocitrate dehydrogenase) are markers for mitochondrial outer-membrane (OM) and intermembrane space (IMS), inner membrane (IM) and matrix, respectively. Calnexin, endoplasmic reticulum (ER) marker. **D,** Mitochondrial Epac1/VDAC1 to cytosolic Epac1/tubulin expression ratio in neonatal rat ventricular myocytes transfected with the indicated plasmids (n=4). Tubulin and VDAC1 expression were used as loading control of cytosolic and mitochondrial fractions, respectively. Representative immunoblots are shown. **E,** Immunofluorescence staining of HA-Epac1^{WT} and HA-Epac1$^{\Delta 2-37}$ transfected in primary cardiomyocytes. Epac1 was visualized with an anti-HA. DAPI and Mitotracker stains mark the position of nuclei and mitochondria, respectively. Scale bar, 10 μm. **F,** Amounts of Rap1-GTP (n=3 in each group) in primary cardiomyocytes transfected with the indicated plasmids. Cells were stimulated or not with 8-CPT-AM (10 μmol/L, 10 minutes). Representative immunoblots are shown. **G,** Cell viability of primary cardiomyocytes transfected with the indicated plasmids (n=8). Data are means±SEM and were evaluated by 1-way analysis of variance (ANOVA)/Bonferroni or Tukey posttest. **P*<0.05, ****P*<0.001, *****P*<0.0001 vs control group or the indicated value. Epac1 indicates exchange protein directly activated by cAMP 1; HA, human influenza hemagglutinin; and MitEpac, mitochondrial exchange protein directly activated by cAMP 1.

through 4E). Altogether, these results suggest that Epac1 is involved in mitochondrial Ca^{2+} overload by increasing the interaction with the VDAC1/GRP75/IP3R1 complex, hence, promoting Ca^{2+} transfer from the ER to the mitochondria. Our finding that Epac1 activation enhanced the effect of histamine on mitochondrial Ca^{2+} uptake further support this conclusion (Figure 4F; Online Figure X). Indeed, histamine is known to stimulate IP3R-mediated Ca^{2+} transfer from ER to mitochondria.[26] Figure 4F through 4G and Online Figure X showed that 8-CPT-AM potentiated the effect of histamine on mitochondrial Ca^{2+} uptake in WT cardiomyocytes. This Ca^{2+} transfer was significantly reduced in the presence of CE3F4 (Figure 4F). Consistently, an IP3R-specific inhibitor, xestospongin C, also reduced Epac1-induced mitochondrial Ca^{2+} signal in WT cardiomyocytes (Online Figure VIIB). These data provide evidence that Epac1 activation facilitates ER to mitochondrial Ca^{2+} transfer.

Because mitochondrial Ca^{2+} overload promotes MPTP opening and subsequently triggers cell death, we next investigated the role of Epac1 on MPTP opening in mitochondria isolated from the heart of WT or *Epac1*$^{-/-}$ mice. MPTP opening was determined in response to supraphysiological mitochondrial Ca^{2+} increase as previously described.[27] Although 8-CPT-AM potentiated Ca^{2+}-induced MPTP opening in WT mitochondria, isolated mitochondria from *Epac1*$^{-/-}$ mice were resistant to Ca^{2+}-induced MPTP opening (Figure 5A). Using the calcein acetoxymethyl ester loading/$CoCl_2$ quenching technique to visualize the open/closed status of MPTP, we observed that the lack of *Epac1* delayed MPTP opening in HX+R conditions compared with

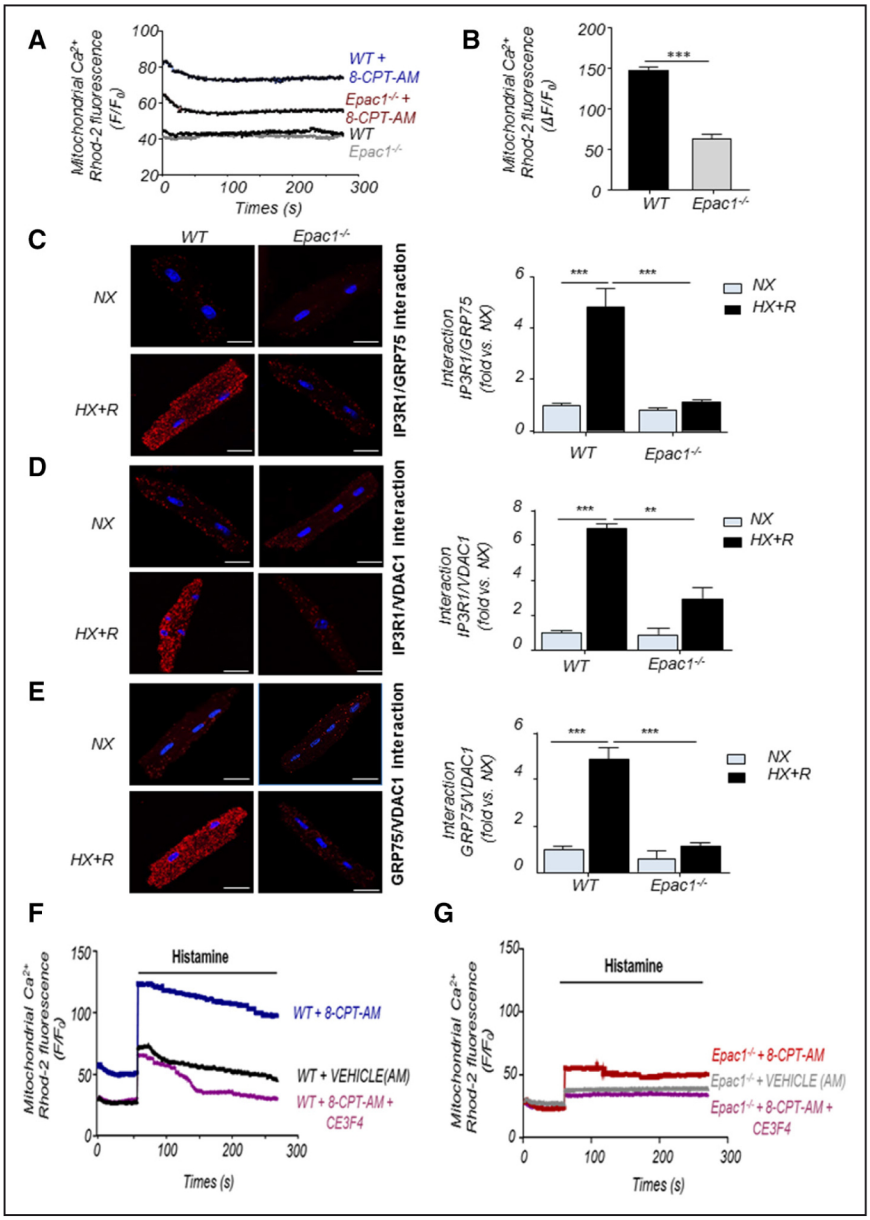

Figure 4. Epac1 increases mitochondrial Ca^{2+} uptake via the VDAC1/GRP75/IP3R1 Ca^{2+} handling protein complex. A, Mitochondrial Ca^{2+} accumulation in adult wild-type (WT) or *Epac1*$^{-/-}$ cardiomyocytes stimulated or not with 8-CPT-AM (10 μmol/L). Representative traces of the averaged values of mitochondrial Ca^{2+} accumulation (measured as $\Delta F/F_0$, where F is the Rhod-2-AM fluorescence signal at 270 s, and F_0 is the signal at time 0; n=6 in each group). **B,** *Epac1* deletion prevents mitochondrial Ca^{2+} uptake. Experiments were performed in WT or *Epac1*$^{-/-}$ cardiomyocytes, which were incubated with 50 μmol/L external Ca^{2+}. Averaged values of mitochondrial Ca^{2+} accumulation measured as $\Delta F/F_0$, where F is the Rhod-2 fluorescence signal at 270 s, and F_0 is the signal at time 0 of 50 μmol/L Ca^{2+} addition (n=6 each experiment). **C–E,** Typical images of in situ interactions (red fluorescent dot) between IP3R1 and GRP75 or VDAC1 and GRP75 with VDAC1 in WT or *Epac1*$^{-/-}$ cardiomyocytes in normoxia (NX) or hypoxia/reoxygenation (HX+R) conditions. Nuclei were stained in blue with DAPI. Scale bar, 20 μm. Graphs show the quantification of the proximity ligation assay. Data are means±SEM of 4 independent experiments, and statistical analysis was performed by a 2-way analysis of varinace (ANOVA) with a Bonferroni posttest. $^{**}P<0.01$, $^{***}P<0.001$ vs control group or the indicated value. **F and G,** Representative traces of mitochondrial Ca^{2+} accumulation in WT **(F)** or *Epac1*$^{-/-}$ **(G)** cardiomyocytes treated or not with 8-CPT-AM (10 μmol/L) or CE3F4 (20 μmol/L) and challenged with histamine (100 μmol/L). Epac1 indicates exchange protein directly activated by cAMP 1; GRP75, glucose-regulated protein 75; IP3R1, inositol-1,4,5-triphosphate receptor 1; and VDAC1, voltage-dependent anion channel 1.

NX in WT cardiomyocytes (Figures 5B). In addition, JC-1 staining and Western blot analysis revealed that 8-CPT-AM or HX+R led to depolarization of $\Delta\Psi m$ (Figure 5C) and increased cytochrome *c* release (Online Figure XI) in WT cardiomyocytes but not in *Epac1*$^{-/-}$ cardiomyocytes or CE3F4-treated WT cells, highlighting the ability of Epac1 to promote MPTP opening. It is worth mentioning that cells cotreated with 8-CPT-AM and NIM-811 (Figure 5C), a nonimmunosuppressive-specific inhibitor of cyclophilin D that also interacts with the VDAC1/GRP75/IP3R1 complex,[26] had similar effect as CE3F4. Altogether, these results strongly suggest MPTP as a major downstream effector in the Epac1 cardiac cell death signaling cascade.

MitEpac1 Negatively Regulates IDH2 Activity Through a CaMKII-Dependent Pathway

Besides Ca^{2+}, ROS play an important role in I/R injury, so we examined the effects of *Epac1* genetic ablation in this process. Dihydroethidium fluorescence staining showed that I/R-induced ROS generation was decreased in the heart of *Epac1*$^{-/-}$ mice after I/R when compared with WT animals (Figure 6A). As the mitochondrial generation of ROS is considered the principal mechanism that initiates cellular oxidative stress in I/R injury,[28] we then tested whether Epac1 was involved in mitochondrial ROS accumulation. 8-CPT-AM treatment of WT cardiomyocytes provoked an increase in ROS content assayed using the superoxide-sensitive probe MitoSOX Red; superoxide production remained low and similar to NX condition in cells devoid of *Epac1* treated with 8-CPT-AM or in WT cardiomyocytes treated with 8-CPT-AM and CE3F4 (Figure 6B).

To elucidate the molecular mechanisms whereby Epac1 influenced the redox state of cardiomyocytes, we performed a phosphoproteomic analysis using 2-dimensional electrophoresis/ProQ Diamond staining coupled to reverse-phase liquid chromatography and tandem mass spectrometry. Using this approach, we identified several phosphopeptides

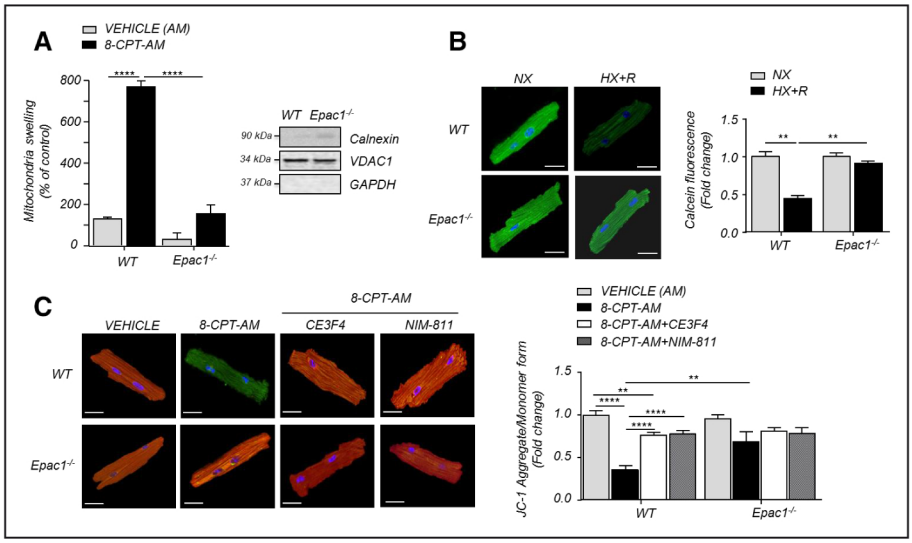

Figure 5. MitEpac1 induces mitochondrial permeability transition pore (MPTP) opening during hypoxia/reoxygenation (HX+R). **A,** Determination of mitochondrial swelling induced by 50 μmol/L Ca^{2+} in wild-type (WT) or *Epac1*$^{-/-}$ cardiomyocytes treated or not with 10 μmol/L 8-pCPT-2′-O-Me-cAMP-AM (8-CPT-AM; n=6). **Right**, Representative immunoblot showing that the mitochondrial fractions used for mitochondrial swelling are free of endoplasmic reticulum (ER). GAPDH (glyceraldehyde-3-phosphate dehydrogenase), voltage-dependent anion channel 1 (VDAC1), and calnexin expression are markers for the cytosol, mitochondria, and ER, respectively. **B,** Representative images of mitochondrial green fluorescence of calcein and its quantification in WT or *Epac1*$^{-/-}$ cardiomyocytes in normoxia (NX) or hypoxia/reoxygenation (HX+R) condition (n=6–8 for each group). DAPI, nuclear marker (blue). Scale bar, 20 μm. **C,** Quantification of fluorescence of JC-1. Representative images of JC-1 staining in WT or *Epac1*$^{-/-}$ cardiomyocytes pretreated with CE3F4 (20 μmol/L, 30 minutes) or NIM-811 (5 μmol/L, 30 minutes) and stimulated with 8-CPT-AM (10 μmol/L, 30 minutes; n=6) are shown. Red fluorescence indicates hyperpolarized (aggregate) mitochondria; green fluorescence indicates depolarized (monomer) mitochondria. DAPI, nuclear marker (blue). Scale bar, 20 μm. Data are means±SEM and were analyzed by 1-way analysis of variance (ANOVA)/Bonferroni posttest. *P<0.05, **P<0.01, ****P<0.0001 vs control group or the indicated value. Epac1 indicates exchange protein directly activated by cAMP 1.

that were differentially represented in WT cardiomyocytes stimulated or not with 8-CPT-AM (Figure 6C), among which was spot 155, which corresponded to the IDH2 (isoelectric point 8.88, molecular weight 50,90 kDa). IDH2 is an enzyme located in the mitochondrial matrix that is mainly and abundantly expressed in the heart. IDH2 plays a key role in the cellular defense against oxidative damage by supplying nicotinamide adenine dinucleotide phosphate (NADPH[29]) to regenerate 2 major antioxidant molecules that are the mitochondrial glutathione and thioredoxin.[30] In line with the phosphoproteome data, coimmunoprecipitation experiments showed that Epac1 activation increased IDH2 serine-specific phosphorylation that leads to the decrease in IDH2 activity (Figure 6D). As expected, IDH2 activity was significantly decreased in the mitochondria isolated from the hearts of WT mice and treated with 8-CPT-AM or placed in HX+R condition (Figure 6E). In contrast, Epac1 inhibition or genetic ablation prevented the decrease in mitochondrial IDH2 activity and the subsequent decline in NADPH production (Figure 6E and 6F). Furthermore, there was no decrease in IDH2 activity in cardiomyocytes transfected with Epac1$^{\Delta 2-37}$ compared with those transfected with Epac1^{WT}, suggesting that MitEpac1 was selectively involved in the regulation of IDH2 phosphorylation, hence, activity (Figure 6G). Finally, IDH2 knockdown decreased cell viability compared with siRNA control in HX+R conditions (Online Figure XIIB, bar 5 compared with bar 7). 8-CPT-AM failed to potentiate the effect of IDH2 knockdown compared with siRNA control in HX+R conditions, indicating that Epac1-induced cell death involved IDH2 inhibition (Online Figure XIIB, bar 6 compared with bar 8). Taken together, these data suggest that MitEpac1 regulates the phosphorylation of IDH2 and, therefore, decreases the ability of the mitochondria to counteract the increase in ROS production during HX+R.

Because Epac1 is not a phosphotransferase, we next sought to identify the missing link between Epac1 and IDH2 phosphorylation. CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) is a key downstream effector of Epac1 and is localized both in the cytosol and in the mitochondria.[31] Surprisingly, *Epac1* deletion specifically decreased mitochondrial CaMKII protein level while having no effect on the total amount of CaMKII protein (Figure 7A and 7B). Consistent with previous findings showing that ≈10% of CaMKII is localized to mitochondria,[32] we did not observe any significant increase in cytosolic CaMKII expression in *Epac1*$^{-/-}$ cardiomyocytes compared with WT (Figure 7C). Similar to the results obtained in *Epac1*$^{-/-}$ cells, transfection of Epac1$^{\Delta 2-37}$ in WT cardiomyocytes specifically reduced mitochondrial CaMKII protein level (Figure 7D and 7E). Furthermore, coimmunoprecipitation experiments showed that Epac1, CaMKII, and IDH2 were involved in the same macromolecular complex (Figure 7F). Consistently, the CaMKII inhibitor KN93 prevented Epac1-mediated IDH2 phosphorylation (Figure 6D) and decrease in activity in the mitochondria isolated from the hearts of WT mice and treated with 8-CPT-AM but had no effect in *Epac1*$^{-/-}$ mitochondria (Figure 7G). As expected, this effect also translated on NADPH content (Figure 7H). Interestingly, bioinformatic

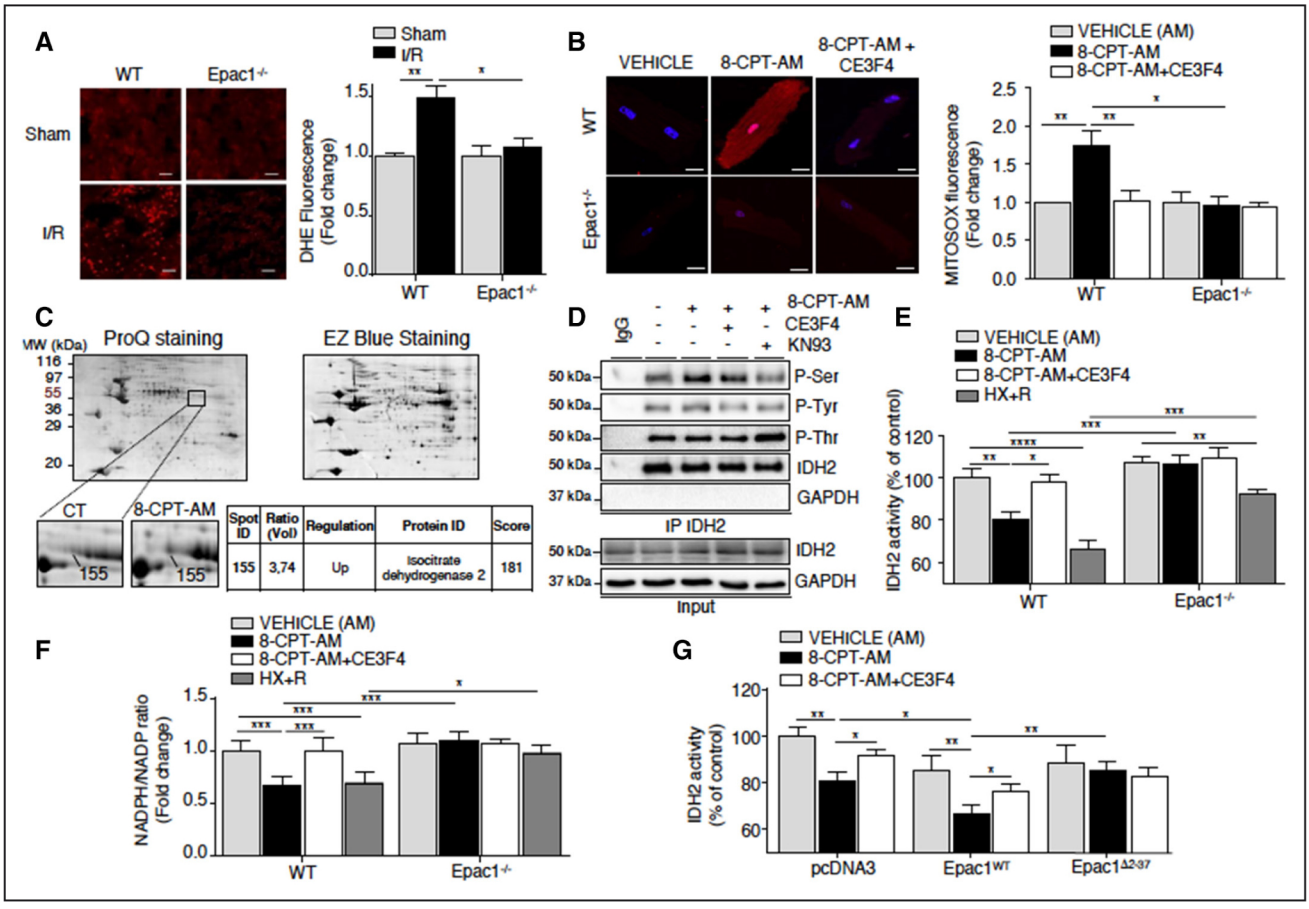

Figure 6. MitEpac1 (mitochondrial exchange protein directly activated by cAMP 1) phosphorylates isocitrate dehydrogenase 2 (IDH2) and negatively regulates its activity. A, Representative images of dihydroethidium (DHE) fluorescence staining of heart cryosections from WT and *Epac1*$^{-/-}$ mice subjected or not to ischemia/reperfusion (I/R). Scale bar, 20 μm. Quantification of DHE fluorescence staining (n=4). **B,** Representative images of MitoSOX red fluorescence staining and its quantification (n=6) in wild-type (WT) or *Epac1*$^{-/-}$ cardiomyocytes pretreated or not with CE3F4 (20 μmol/L, 30 minutes) and stimulated or not with 8-pCPT-2′-O-Me-cAMP-AM (8-CPT-AM; 10 μmol/L, 30 minutes). DAPI (blue), nuclear marker. Scale bar, 20 μm. **C,** ProQ Diamond and EZ Coomassie Blue staining of a representative 2D gel of cardiomyocyte protein extracts. A representative enlargement of the gel showing the marked region is illustrated. The ratio of volume value is indicated in the table. The change in the intensity of the protein spot (155) is indicated as increased (up) in the stimulated 8-CPT-AM (10 μmol/L, 10 minutes) vs control (CT). **D,** Representative immunoblots of 3 independent experiments showing IDH2 phosphorylation at Ser, Tyr, and Thr residues. Cardiomyocytes were preincubated or not with either CE3F4 (20 μmol/L, 30 minutes) or KN-93 (5 μmol/L, 30 minutes) and were stimulated or not with 8-CPT-AM (10 μmol/L, 30 minutes) before immunoprecipitation (IP) experiments against IDH2. IgG and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used as control for IP. Input is a control of cell lysates. **E,** IDH2 activity; and **F,** NADPH/NADP ratio in isolated mitochondria from WT and *Epac1*$^{-/-}$ mice hearts. Mitochondria were either pretreated or not with CE3F4 (20 μmol/L, 30 minutes) and stimulated or not with 8-CPT-AM (10 μmol/L, 30 minutes), or incubated 30 minutes in hypoxia (HX) followed by 15 minutes of reoxygenation (HX+R; n=7). **G,** Determination of IDH2 activity in cardiomyocytes transfected with the indicated plasmids. Cells were preincubated or not (VEHICLE) with CE3F4 (20 μmol/L, 30 minutes) and stimulated with 8-CPT-AM (10 μmol/L, 30 minutes) (n=6). Data are means±SEM and were evaluated by 2-way analysis of variance (ANOVA)/Bonferroni posttest. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 vs control group or the indicated value. Epac1 indicates exchange protein directly activated by cAMP 1; ID, identity; and NADPH, nicotinamide adenine dinucleotide phosphate hydrogen.

analysis of IDH2 sequence led us to identify a potential phosphorylation site for CaMKIIδ isoform (analysis with Group-based Prediction System v3.0), which is present in the mitochondrial matrix of cardiomyocytes (Online Figure XIII). Knock down of CaMKIIδ with specific siRNA (Si-CaMKIIδ) prevented 8-CPT-AM-induced IDH2 phosphorylation, suggesting that this CaMKIIδ isoform specifically targets IDH2 (Online Figure XIV). Altogether, these data strongly suggest that MitEpac1 is involved in the mitochondrial localization of CaMKII, which phosphorylates IDH2 when Epac1 is activated and, therefore, leaves the cells vulnerable to oxidative damage during HX+R and I/R injury (Figure 8).

Discussion

In this work, we provided new insights into understanding the role and compartmentalization of cAMP and Epac1. The novel contributions include the following: (1) Epac1 was upregulated in human ischemic failing hearts, and its genetic deletion significantly reduced infarct size in the setting of I/R injury, providing the first direct evidence that Epac1 activation contributes to I/R injury; (2) either genetic ablation or pharmacological inhibition of Epac1 protected against I/R- or HX+R-induced cardiomyocyte apoptosis; (3) mechanistic studies demonstrated that Epac1 was activated by sAC and promoted MPTP opening and cell death during HX+R; (4) Epac1 regulated different

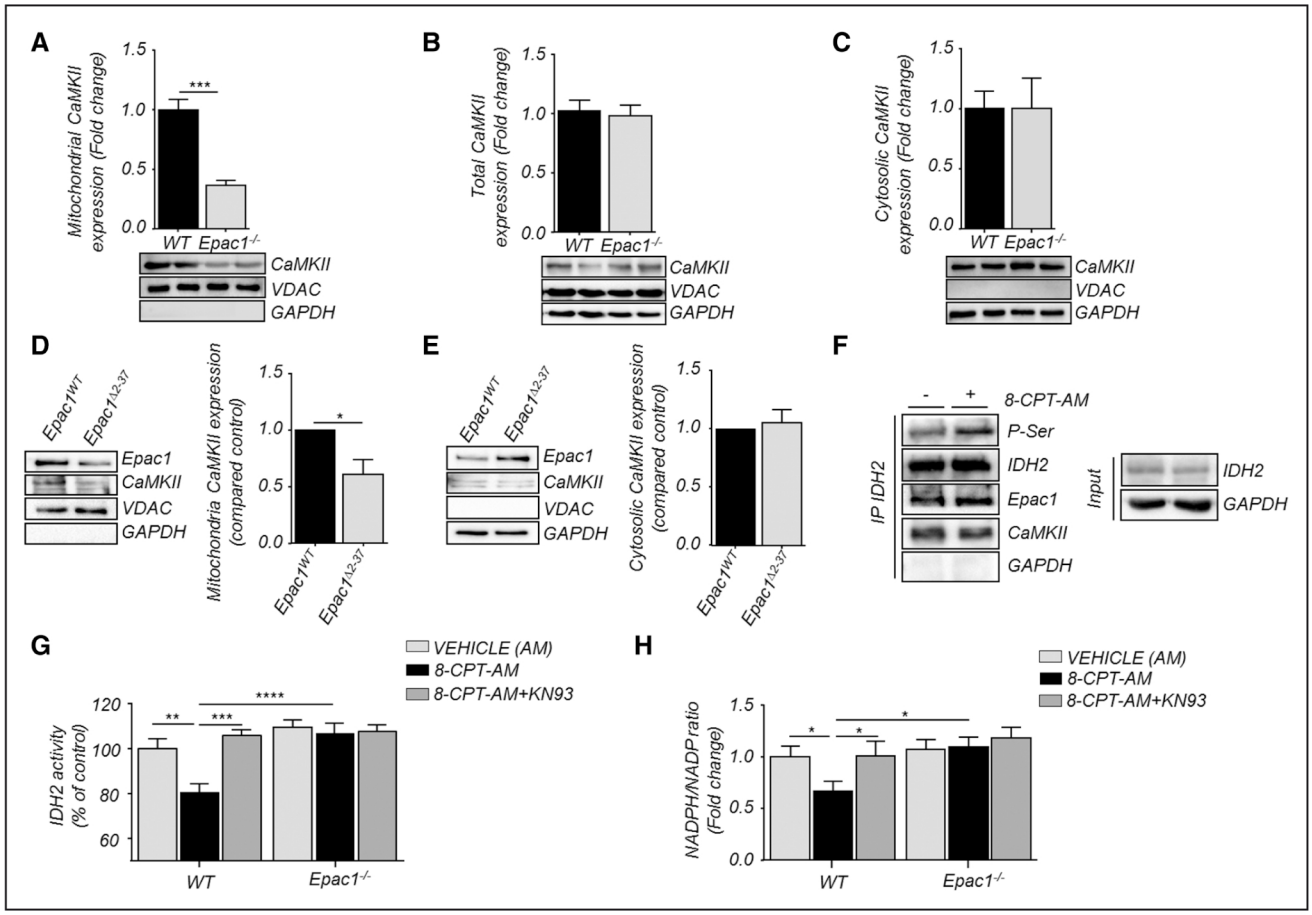

Figure 7. Epac1 regulates the mitochondrial localization of CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), which inhibits isocitrate dehydrogenase 2 (IDH2) activity. A, Quantification of mitochondrial CaMKII; **B,** total CaMKII; and **C,** cytosolic CaMKII protein expression in the hearts of wild-type (WT) or *Epac1*$^{-/-}$ mice. Representative immunoblots of 4 independent experiments are shown. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and voltage-dependent anion channel 1 (VDAC1) expression were used as loading control of cytosolic and mitochondrial fractions, respectively. **D,** Quantification of mitochondrial CaMKII; and **E,** cytosolic CaMKII in cardiomyocytes transfected with the indicated plasmids (n=6). Representative immunoblots are shown. **F,** Representative immunoblots showing the interaction of IDH2 with CaMKII and Epac1 in cardiomyocytes stimulated or not with 8-pCPT-2′-O-Me-cAMP-AM (8-CPT-AM; 10 μmol/L, 30 minutes) before immunoprecipitation (IP) experiments against IDH2. IgG and GAPDH were used as control for IP. Input is a control of cell lysates. **G,** IDH2 activity; and **H,** NADPH/NADP ratio in isolated-mitochondria from the hearts of WT or *Epac1*$^{-/-}$ mice. Mitochondria were preincubated or not with KN93 (5 μmol/L, 30 minutes) and stimulated or not (VEHICLE) with 8-CPT-AM (10 μmol/L, 30 minutes; n=7). Data are means±SEM and were evaluated by 2-way analysis of variance (ANOVA)/Bonferroni posttest. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control group or the indicated value. Epac1 indicates exchange protein directly activated by cAMP 1; and NADPH, nicotinamide adenine dinucleotide phosphate hydrogen.

aspects of mitochondrial function, such as Ca^{2+} uptake, ROS production, and MPTP opening. Our results further add credence that Epac1 is highly compartmentalized and functions as an important stress response switch in the heart.

Our in vivo and in vitro data showed that the genetic ablation or the inhibition of Epac1 prevented cardiomyocyte apoptosis during I/R injury or HX+R stress, respectively. Recent studies have shown that the various biological actions of Epac1 depend on its subcellular localization. Indeed, the perinuclear localization of Epac1 in cardiomyocyte is consistent with its role in the regulation of gene transcription during cardiac hypertrophic remodeling.[16–18] Furthermore, independently of its effect on sarcoplasmic reticulum function, Epac1 was reported to act on the myofilament compartment where it regulates the phosphorylation of sarcomeric proteins to increase myofilament Ca^{2+} sensitivity.[33] Finally, Epac1 was previously found in the mitochondrion of Epac1-transfected COS-7 cells in a cell cycle–dependent manner,[34] although it was not linked to any biological function. Here we found that Epac1 is indeed localized in the mitochondrion of cardiomyocytes, where it exerts its regulatory role on cardiomyocyte cell death via its activation by cAMP produced by sAC. These findings allow to provide a mechanistic confirmation to the previously described data, showing that sAC-dependent cAMP signaling modulated the mitochondrial pathway of apoptosis in adult cardiomyocytes.[21] These data also reinforce the importance of Epac1 localization because mainly MitEpac1 is involved in inducing apoptosis in HX+R-cultured cells. During the course of our study, Wang et al[35] demonstrated that a modest increase in mitochondrial cAMP levels, through direct activation of sAC with HCO_3^-, prevented Ca^{2+}-induced MPTP opening through Epac1, which they assumed that Epac1 might protect from cardiomyocyte death. Based on our observations, this pathway is not operational in the setting of HX+R because MitEpac1

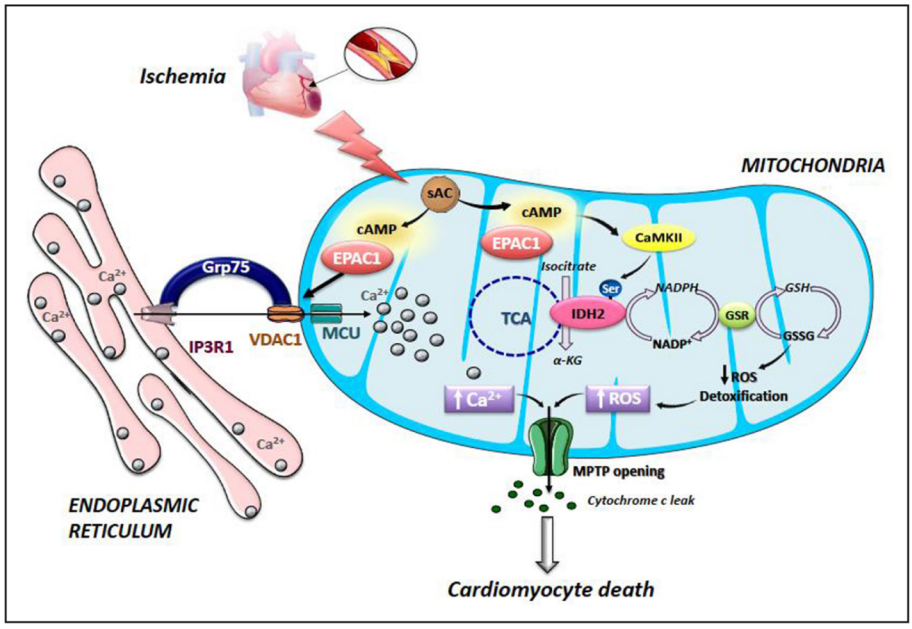

Figure 8. Schema illustrating a working hypothesis of Epac1 signaling in ischemia/reperfusion (I/R) injury. In the setting of I/R, soluble adenylyl cyclase type 10 (sAC) is activated to promote cAMP accumulation, thereby, activating mitochondrial Epac1 (MitEpac1). MitEpac1 increases Ca^{2+} transfer from the endoplasmic reticulum (ER) to mitochondria via the VDAC1/GRP75/IP3R1 complex. In addition, MitEpac1-CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) forms a multiprotein complex with isocitrate dehydrogenase 2 (IDH2), a critical enzyme of the tricarboxylic acid (TCA) cycle involved in reactive oxygen species (ROS) detoxification. CaMKII inhibits IDH2 activity by Serine phosphorylation, thereby, decreasing nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) synthesis in the matrix. MitEpac1-induced mitochondrial Ca^{2+} overload and ROS accumulation promotes mitochondrial permeability transition pore (MPTP) opening and myocardial cell death. α-KG indicates α-ketoglutarate; Epac1, exchange protein directly activated by cAMP 1; GRP75, chaperone glucose-regulated protein 75; GSH, reduced glutathion; GSR, glutathione reductase; GSSG, Oxidized glutathion; IP3R1, inositol-1,4,5-triphosphate receptor 1; MCU, mitochondrial Ca^{2+} uniporter; $NADP^+$, nicotinamide adenine dinucleotide phosphate; and VDAC1, voltage-dependent anion channel 1.

shows opposite effect, such as stimulation of mitochondrial Ca^{2+} entry, MPTP opening, and cell death. One explanation for this discrepancy could be the higher levels of cAMP production observed in the model of HX+R together with the activation of other actors, such as ROS and CaMKII signaling.

Altogether, these data strongly suggest a major role of the mitochondrial sAC-Epac1 axis in the physiology and pathophysiology of the cardiomyocyte. These findings add up to the accumulating evidence linking Epac protein signaling to cardiomyocyte functions. Indeed, Epac proteins exert their biological function in combination with scaffolding proteins, such as β-arrestin, cAMP phosphodiesterases that regulates the duration and intensity of cAMP signaling, and PKA.[36,37] Because various mitochondrial compartments contain these proteins that are able to sense or respond to cAMP and may have antagonistic outputs,[2] further studies are needed to identify the multimolecular complexes that affect cAMP-Epac1 signaling and Epac1 mitochondrial function in cardiomyocytes.

In this study, we also identified at least 2 different mechanisms that contribute to the deleterious activation of Epac1 during HX+R in vitro and possibly by extension to myocardial I/R injury in vivo. First, we identified Epac1 as a regulator of Ca^{2+} entry in the mitochondria. Indeed, the lack of *Epac1* prevented Ca^{2+} accumulation during HX+R. The MCU was involved in this effect of Epac1 because RU360 reduced 8-CPT-AM-induced mitochondrial Ca^{2+} (Online Figure VIIA). Because CaMKII increases MCU current[31] and is a downstream effector of MitEpac1, we speculate that Epac1 regulates Ca^{2+} entry into the mitochondria via CaMKII-dependent MCU activation. In addition, based on a previous finding that Epac1 also induced cytosolic Ca^{2+} overload via the production of IP3 and IP3R activation,[38] we hypothesize that Epac1 could induce IP3R channel opening from the ER and subsequent Ca^{2+} transfer from the ER to the mitochondria. Consistently, Epac1 controlled the assembly of the ER/mitochondrial VDAC1/GRP75/IP3R1 protein complex that has been previously shown to influence Ca^{2+} exchange between the ER and the mitochondria.[25] Finally, Epac1-mediated mitochondrial Ca^{2+} overload subsequently provoked MPTP opening, cytochrome *c* release, and eventually cell death. Interestingly, cyclophilin D was also shown to interact with the VDAC1/GRP75/IP3R1 complex at the mitochondria-associated ER membrane interface,[26] and its inhibition prevented mitochondrial Ca^{2+} overload by depressing ER/mitochondria interactions and protected cells against lethal reperfusion injury.[26] Therefore, our data show that the proper association of the VDAC1/GRP75/IP3R1 complex is paramount to mitochondrial Ca^{2+} overload during HX+R, and that MitEpac1 plays a crucial role in that process. Whether this Epac1-dependent sarcoplasmic reticulum–mitochondria Ca^{2+} coupling may also contribute to modulate the global Ca^{2+} homeostasis and excitation–contraction coupling in cardiomyocytes under physiological conditions has yet to be investigated.

Second, our study revealed a key role for Epac1 in the accumulation of mitochondrial ROS production on HX+R. Recently, Mukai et al[39] reported that high glucose–induced

mitochondrial ROS production in pancreatic β-cells was decreased by stimulation with the glucagon-like peptide-1 receptor ligand exendin-4 in an Epac-dependent manner. Here we further showed that MitEpac1 inhibited IDH2 via its phosphorylation on serine residues. This mitochondrial enzyme is primarily expressed in oxidative tissues, including the heart and skeletal muscle, where it plays an essential role in maintaining mitochondrial redox homeostasis.[29] In cardiac mitochondria, IDH2 is the major source of the mitochondrial NADPH required for glutathione production and thioredoxin recycling.[40] Therefore, Epac1-mediated inhibition of IDH2 impaired NADPH production, hence, decreased the antioxidant capabilities of the cardiomyocytes during HX+R. We extended our work to identify the signaling pathway that accounted for Epac1-inhibited IDH2. Interestingly, we found that Epac1 promoted the mitochondrial matrix import of CaMKII, where it interacted with and phosphorylated IDH2. Importantly, these data could explain how CaMKII, which does not harbor any N-terminal mitochondrial targeting sequence, transfers to the mitochondria,[31] although the precise mechanism remains to be identified. Also, previous work has indicated that mitochondrial CaMKII directly controlled MCU activity by phosphorylation, thereby, regulating the rate of Ca^{2+} influx across the inner mitochondrial membrane to influence MPTP and subsequent cell death during myocardial I/R.[31] However, the cardiomyocytes of knockout mice for the MCU channel were not protected against cardiomyocyte death after I/R injury,[41] suggesting other CaMKII to be involved. Therefore, our identification of IDH2 as a CaMKII critical target in HX+R contributes to better understand the multifacet role of CaMKII in this context. In contrast to what is observed in the heart, Epac1 activation prevented mitochondrial ROS accumulation by decreasing superoxide anion formation during renal I/R injury, thus, limiting the degree of oxidative stress.[42] Although these findings seem contradictory, Epac signaling is spatially and temporally regulated by diverse anchoring mechanisms that control specific functions of this cAMP-sensitive guanine exchange factor by recruitment to distinct subcellular localizations.[37] We, therefore, speculate that cell type–specific signals contribute to differential effect of Epac on mitochondrial ROS production.

To conclude, our finding provides a novel mechanism in which different cAMP–Epac1 microdomains control the mitochondrial cell death pathway (Figure 8). We propose a model whereby, in the setting of I/R, Epac1 localized at the mitochondria-associated ER membrane is activated by sAC to induce mitochondrial Ca^{2+} overload via the ER/mitochondrial interaction and subsequent MPTP opening. In addition, we unveil an unsuspected role report for Epac1 in recruiting CaMKII at the mitochondria. MitEpac1–CaMKII pathway also inhibited IDH2 activity via serine-dependent phosphorylation to reduce ROS detoxification, thereby, promoting cardiomyocyte death during I/R. Finally, our study highlights on the therapeutic potential of Epac1 inhibition and the development of Epac1 pharmacological inhibitors as new drugs to treat I/R injury. Because both Epac1 and IDH2 proteins have been implicated in cancer, such as melanoma, they might have more general clinical implications in other systems and diseases.

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Disclosures

None.

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