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To cite this version:
J. Fauconnier, Albano C Meli, Jérôme Thireau, Stéphanie Roberge, Jian Shan, et al.. Ryanodine receptor leak mediated by caspase-8 activation leads to left ventricular injury after myocardial ischemia-reperfusion. Proceedings of the National Academy of Sciences of the United States of America, National Academy of Sciences, 2011, 10.1073/pnas.1100286108. hal-02542114

HAL Id: hal-02542114
https://hal.umontpellier.fr/hal-02542114
Submitted on 14 Apr 2020

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Ryanodine receptor leak mediated by caspase-8 activation leads to left ventricular injury after myocardial ischemia-reperfusion

Jérémy Fauconnier, Albano C. Meli, Jérôme Thireau, Stephanie Roberge, Jian Shan, Yassine Sassi, Steven R. Reiken, Jean-Michel Rauzier, Alexandre Marchand, David Chauvier, Cécile Cassan, Christine Crozier, Patrice Bideaux, Anne-Marie Lompré, Etienne Jacotot, Andrew R. Marks, and Alain Lacampagne

Institut de la Santé et de la Recherche Médicale, U1046, F-34295 Montpellier, France; Université Montpellier 1 and 2, Unité de Formation et de Recherche en Médecine, F-34295 Montpellier, France; Russ Berrie Medical Science Pavilion, Columbia University College of Physicians & Surgeons, New York, NY 10032; Institut de la Santé et de la Recherche Médicale/Université Pierre et Marie Curie Unité Mixte de Recherche S 556, Centre Hospitalier La Pitie-Salpêtrière 91, 75634 Paris, France; Therapostis R&D Laboratories, Therapostis S.A., F-93230 Romainville, France; Institut de la Santé et de la Recherche Médicale U676, Hôpital Robert Debré, F-75019 Paris, France; and Faculté de Médecine Denis Diderot, Université Paris 7, F-75019 Paris, France

Myocardial ischemic disease is the major cause of death worldwide. After myocardial infarction, reperfusion of infarcted heart has been an important objective of strategies to improve outcomes. However, cardiac ischemia/reperfusion (I/R) is characterized by inflammation, arrhythmias, cardiomyocyte damage, and, at the cellular level, disturbance in Ca^{2+} and redox homeostasis. In this study, we sought to determine how acute inflammatory response contributes to reperfusion injury and Ca^{2+} homeostasis disturbance after acute ischemia. Using a rat model of I/R, we show that circulating levels of TNF-α and cardiac caspase-8 activity were increased within 6 h of reperfusion, leading to myocardial nitric oxide and mitochondrial ROS production. At 1 and 15 d after reperfusion, caspase-8 activation resulted in S-nitrosylation of the RyR2 and depletion of calstabin2 from the RyR2 complex, resulting in diastolic sarcoplasmic reticulum (SR) Ca^{2+} leak. Pharmacological inhibition of caspase-8 before reperfusion with Q-LETD-OPh or prevention of calstabin2 depletion from the RyR2 complex with the Ca^{2+} channel stabilizer S107 ("rycal") inhibited the SR Ca^{2+} leak, reduced ventricular arrhythmias, infarct size, and left ventricular remodeling after 15 d of reperfusion. TNF-α-induced caspase-8 activation leads to leaky RyR2 channels that contribute to myocardial remodeling after I/R. Thus, early prevention of SR Ca^{2+} leak through normalization of RyR2 function is cardioprotective.

Myocardial infarction, in the United States with >1.5 million new cases diagnosed each year, a leading cause of death. Reperfusion of infarcted heart has been the main strategy to improve outcomes (1). However, cardiac ischemia/reperfusion (I/R) is characterized by arrhythmias, cardiomyocyte damage, inflammation, and, at the cellular level, disturbance in Ca^{2+} and redox homeostasis.

Elevated plasma levels of tumor necrosis factor α (TNF-α) have been reported in cardiac reperfusion injury, myocardial infarction, and in congestive heart failure. TNF-α induces pleiotropic effects that are mediated through activation of two distinct receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Most of the deleterious effects are mediated by TNFR1 signaling (3). TNF-α also has long-term beneficial effects due to the induction of protective genes involved in cellular growth, survival, and proliferation (4). Thus, release of TNF-α after myocardial injury may activate signaling pathways that promote either cardiac adaption/protection or maladaptive responses. Multicenter trials using TNF-α antagonists in moderate to severe heart failure (HF) demonstrated adverse effects instead of benefits (5, 6). Therefore, a new therapeutic strategy specifically targeting early deleterious effects of TNF-α, without affecting its cytoprotective activity, remains a point of interest.

One of the early events in the TNF-α/TNFRI signaling pathways is activation of caspase-8 (7). This pathway is initiated by recruitment of the adaptor protein Fas-associated via a death domain (FADD), which then recruits procaspase-8 into the death-inducing signaling complex (DISC). Caspase-8 activation leads to the generation of ceramide, mitochondrial reactive oxygen species (ROS) production, Bid cleavage, followed by cytochrome c release from mitochondria, and apoptosis formation, ultimately leading to activation of effectors caspases (i.e., caspase-3) and cell death (8–10). In parallel, acute nitric oxide (NO) production through activation of the endothelial nitric oxide synthase (eNOS), or increased expression of inducible nitric oxide synthase (iNOS) inhibit key apoptotic signals triggered by TNF-α such as ceramide formation and caspase-8 (11, 12). Increased ROS and/or NO-derived reactive species (RNS) change the redox environment of Ca^{2+} transporters and channels and, thus, affect cellular Ca^{2+} cycling (13). The cardiac ryanodine receptor (RyR2) that mediates sarcoplasmic reticulum (SR) Ca^{2+} release during excitation-contraction coupling contains ÿ33 free thiol residues, rendering it highly sensitive to the cellular redox state. Cysteine oxidation facilitates RyR opening and SR Ca^{2+} leak (14, 15). Moreover, we have recently shown that S-nitrosylation of RyR1 (skeletal muscle) and RyR2 (cardiac muscle) and dissociation of their stabilizing subunit calstabin1 (FKBP12.6) or calstabin2 (FKBP12.6), respectively, induces SR Ca^{2+} leak, cardiac arrhythmia, skeletal muscle weakness, and remodeling in a Duchenne muscular dystrophy (mdx) mouse model (16, 17).

In this study, we sought to determine whether TNF-α–induced caspase-8 activation would affect RyR2 S-nitrosylation leading to diastolic SR Ca^{2+} leak and left ventricular remodeling in a rat model of I/R. Using broad-spectrum caspase inhibitors and preferential caspase-8 inhibitors, and stabilization of the RyR2 macromolecular complex with a rycal (S107), we showed that early caspase-8 activation increases mitochondrial ROS and NO production, resulting in S-nitrosylation of RyR2 and depletion of calstabin2 from the channel complex causing a diastolic SR Ca^{2+} leak that leads to acute pathological left ventricular remodeling.


Conflict of interest statement: A.R.M. is on the scientific advisory board and own shares in ARMGO Pharma, Inc., a start-up company developing RyR targeted drugs for clinical use in the treatment of heart failure and sudden death.

This article is a PNAS Direct Submission.

To whom correspondence should be addressed. E-mail: alain.lacampagne@inserm.fr.
Results

Effect of TNF-α and Caspase-8 Activation on RyR2 Function in Vitro.

Acute application of TNF-α (1 h, 10 ng/mL) to freshly isolated control cardiomyocytes induced caspase-8-like and caspase-3-like activities sequentially (Fig. S1A). In addition, application of TNF-α resulted in a progressive and significant increase in the MitoSOX Red fluorescence within 1 h in control cells or in myocytes preincubated with a preferential caspase-3/7 inhibitor (Z-DEVD-FMK; 10 μM) (103 ± 10%, n = 30 and 90.5 ± 12%, n ≥ 29, respectively), whereas there was no change in fluorescence in control cells preincubated either with preferential caspase-8 inhibitors [Q-LETD-OPh (18), 10 μM; or Z-IETD-FMK, 10 μM] or with broad spectrum caspase inhibitor (Q-VD-OPh; 10 μM) (Fig. 1A and B). Of note, this mitochondrial ROS production was associated with a significant ΔΨm depolarization that was inhibited by caspase-8 inhibitors (Fig. S1B). Several studies have reported that TNF-α increases NO production either acutely through ceramide production and eNOS activation (12) or after induction of iNOS expression (19). Thus, we tested the effect of TNF-α on NO production by using DAF-FM, which increases its fluorescence when oxidized by NO (20). TNF-α application caused a progressive increase in the DAF-FM fluorescence, which was inhibited by caspase-8 inhibitor (32 ± 5%, n = 20 vs. 0.2 ± 0.8%; n = 20; P < 0.05, Fig. 1C and D). TNF-α-induced NO production was also significantly decreased in the presence of a ceramide catabolism inhibitor (NOE; 10 μM), suggesting that caspase-8–induced ceramide production is essential for TNF-induced NO production. Moreover, TNF-α failed to increase NO level when eNOS was inhibited (L-NIO; 1 μM) (Fig. 1D). These results suggest that TNF-α induced caspase-8 activation is an upstream event in the signaling pathways involving ceramide production (21) and eNOS activation (12).

Under these proinflammatory conditions, simultaneous production of superoxide anion (O2·−) and NO can generate peroxynitrite formation (22). Among proteins involved in excitation-contraction coupling, RyR2 is highly sensitive to peroxynitrite and subsequent S-nitrosylation (13). In the present study, acute incubation of cardiomyocytes with TNF-α (10 ng/mL) for 1 h was sufficient to cause RyR2 S-nitrosylation and calstabin2 depletion from RyR2 complexes (Fig. 1E). TNF-α also increases open probability (Po) of RyR2 channels incorporated into planar lipid bilayers (Fig. 1F) and Ca2+-spark frequency in intact ventricular cardiomyocytes (Fig. 1G). In the presence of the caspase-8 inhibitor, Q-LETD-OPh, the TNF-α–induced changes in RyR2

Fig. 1. (A) Representative MitoSOX red fluorescence recorded at 0, 30, and 60 min of TNF-α (10 ng/mL) or TNF-α + Q-LETD-OPh (10 μM) application in single ventricular rat cardiomyocytes. (B) Mean data ± SEM of normalized MitoSOX red fluorescence after 60 min of TNF-α application. *, statistical difference compared with control conditions (P < 0.05; n ≥ 20 cells in each conditions). Each caspase inhibitor (10 μM) was preincubated 15 min before TNF-α application. For S107 experiments, the animals were orally treated with S107 (25 mg/100 mL, in drinking water) 1 wk before cells isolation. Note that caspase-8 inhibitors (Q-LETD-OPh and Z-IETD-FMK) and broad spectrum caspase inhibitor (Q-VD-OPh) prevents TNF-α–induced mitochondrial ROS production, whereas caspase-3/7 inhibitor (Z-DEVD-FMK) and S107 did not. (C Left) Typical images of TNF-α–induced NO production measured with DAF-FM using confocal microscope. (C Right) Time change of normalized DAF-FM fluorescence in presence of TNF-α (10 ng/mL) or TNF-α + OLETD-OPh (10 μM). (D) Mean data ± SEM of normalized DAF-FM fluorescence after 60 min of TNF-α application. *, statistical difference compared with control conditions (P < 0.05; n ≥ 20 cells in each conditions). Note that caspase-8 inhibitor (Q-LETD-OPh), broad spectrum caspase inhibitor (Q-VD-OPh), and eNOS inhibitor (L-NIO) prevent TNF-α–induced NO production, whereas ceramide inhibitor (NOE; 10 μM) or S107 did not. (E) Representative cardiac RyR2 immunoprecipitation and immunoblot and bar graphs showing Cys nitrosylation of cardiac RyR2 and the amount of calstabin2 in the cardiac RyR2 complex. The bar graph shows the relative amount of calstabin2 associated with the RyR2 channel complex for each group determined by dividing the calstabin2 signals by the total amount of RyR2 that was immunoprecipitated (a.u.). The bar graphs, depicting the relative amount of RyR2 S-nitrosylation for each group, were determined by dividing the Cys-NO signals by the total amount of RyR2 immunoprecipitated (a.u.). Data presented as mean ± SEM (F) RyR2 single channels isolated from left ventricular cardiomyocytes treated 1 h with TNF-α (10 ng/mL) or with Q-LETD-OPh (10 μM) followed by 1 h with TNF-α (10 ng/mL). (F) Representative RyR2 single-channel traces from control, TNF-α treated, and Q-LETD-OPh+TNF-α–treated samples. Single channel activities were recorded at 150 mM free cytosolic (c) Ca2+ concentration and 53 mM Ca(OH)2 medium (trans) at 0 mM. Channel openings are shown as upward deflections from the closed level (c). Example of channel activity is shown at two different time scales (10 s for one upper trace and 1 s for two lower traces in each block) as indicated by dimension bars. Summary data of relative values of RyR2 Po of control, TNF-α treated and Q-LETD-OPh+TNF-α treated samples. *P < 0.05 vs. control (G) Spontaneous SR Ca2+ release events were recorded in fluo-4-AM–loaded intact cardiomyocytes by laser scanning confocal microscopy. Representative ΔF/F lines can images (1.54 ms per line) were recorded in the absence of (control) or after 1 h of TNF-α incubation. Ca2+ sparks frequency is used as an index of diastolic SR Ca2+ leak. Caspases inhibitors are indicated as follows: Q-LETD, Q-LETD-OPh; Z-IETD, Z-IETD-FMK; Z-DEVD, Z-DEVD-FMK; Q-VD, Q-VD-OPh. Data are expressed as mean ± SEM (P < 0.05 vs. control; n ≥ 30 cells in each condition).
S-nitrosylation, calstabin2 binding to the RyR2 complex, RyR2 Po, and Ca\(^{2+}\) sparks frequency were prevented (Fig. 1 E–G). Of note, RyR2 PKA-dependent phosphorylation site (ser2808) was unchanged (Fig. S2). The increase in Ca\(^{2+}\) sparks frequency was also prevented by the RyR Ca\(^{2+}\) release channel stabilizer “rcyal” S107 (Fig. 1G). Q-LETGD-OPh or S107 did not change basal Ca\(^{2+}\) sparks frequency, and a combined treatment similarly decreased TNF-α–increased sparks frequency compared with a single treatment with Q-LETGD-OPh or S107 (Fig. S1C). However, S107 treatment did not prevent ΔΨ\(_{m}\) depolarization (Fig. S1B), mitochondrial ROS production (Fig. 1 A and B), or NO production (Fig. 1 C and D), suggesting that RyR2 leak does not affect caspase-8–mediated mitochondrial dysfunction. The antioxidant N-acetyl cysteine (NAC) also normalized Ca\(^{2+}\) spark frequency (Fig. 1G). Moreover, TNF-α decreased the Ca\(^{2+}\) transient amplitude, Ca\(^{2+}\) release kinetics, SR Ca\(^{2+}\) load, and cell shortening (Fig. S3). Q-LETGD-OPh prevented the TNF-α-induced decrease in the Ca\(^{2+}\) transient amplitude, Ca\(^{2+}\) release kinetics, SR Ca\(^{2+}\) load, and cell shortening (Fig. S3). S107 or NAC did not affect basal Ca\(^{2+}\) transient characteristics (Fig. S3 B–E). In such conditions and in agreement with a previous report (2), TNF-α induced positive effects on Ca\(^{2+}\) handling and cell shortening (Fig. S3 B–E). Thus, TNF-α–mediated ROS/NO production via caspase-8 activation increased RyR2 S-nitrosylation and SR Ca\(^{2+}\) leak.

**Roles of Caspase-8 and RyR2 Leak in Myocardial Reperfusion Injury.**

To determine whether the TNF-α–induced SR Ca\(^{2+}\) leak via RyR2 channels contributes to reperfusion injury, we performed 30 min of ischemia followed by reperfusion in vivo rats. TNF-α plasma levels were detected at 1 h and returned to baseline 6 h after reperfusion (276 ± 48 pg/mL at 1 h, n = 6; Fig. 2A). In parallel, cardiac caspase-8 activity was also significantly increased by 1 h after reperfusion, peaked at 6 h, and returned to baseline by 24 h (Fig. 2B). In addition, Bid cleavage increased significantly after 24 h of reperfusion as shown by the increase of the truncated Bid isoform (Fig. S4). RyR2 S-nitrosylation and calstabin2 depletion were also observed 24 h after reperfusion (Fig. 2C) without any change in phosphorylation of RyR2 at ser2808 (Fig. S2). S107 (25 mg/100 mL in drinking water) treatment for 1 wk before surgery prevented calstabin2 depletion from the RyR2 complex but did not affect S-nitrosylation of the channel (Fig. 2C). In contrast, Q-LETGD-OPh treatment (1 mg/kg i.p.) 15 min before reperfusion inhibited both RyR2 S-nitrosylation and depletion of calstabin2 from the RyR2 complex (Fig. 2C).

**Role of Caspase-8 and RyR2 Leak in Left Ventricular Remodeling.**

We subsequently analyzed and compared the longer-term effects of either early caspase-8 inhibition or S107 treatment on left ventricular remodeling 15 d after myocardial reperfusion. Histological analyses of the left ventricle were performed by using Masson trichrome staining to detect collagen fibers. There was an increase in extracellular matrix (i.e., fibrosis) that was prevented when animals were treated with Q-LETGD-OPh (1 mg/kg i.p.), 15 min before the reperfusion or pretreated with S107 (25 mg/100 mL in the drinking water) 1 wk before ischemia and up to 72 h after reperfusion, compared with vehicle-treated animals (Fig. 3A). As an index of hypertrophy, HW/BW ratio was sig-
significant increase in DMSO-treated animals and unchanged in Q-LETD-OPh or S107-treated animals (Fig. 3C). Both of which were significantly improved in animals treated with Q-LETD-OPh or S107 (Fig. 3C).

The cardiac ventricular remodeling observed 2 wk after reperfusion was associated with RyR2 S-nitrosylation, calstabin2 depletion from the RyR2 complex, without any modification of RyR2 phosphorylation at ser2808 (Fig. 4A and Fig. S2), and with an increase in RyR2 channel Po measured under conditions corresponding to diastole (low activating [Ca$^{2+}$]) ×150 nM; Fig. 4B). These results are consistent with a diastolic SR Ca$^{2+}$ leak. Once again, S107 inhibited depletion of calstabin2 from the RyR2 complexes without affecting S-nitrosylation of RyR2, whereas Q-LETD-OPh normalized both. RyR2 channel Po measured at 150 nM cytosolic Ca$^{2+}$, was partially or totally reduced to that observed in control channels from animals treated with S107 or Q-LETD-OPh, respectively. At the cellular level, Ca$^{2+}$ transient amplitudes were decreased by $\pm$20% and the rising phases was significantly slower in vehicle-treated animals (Fig. S9A and B). These changes were accompanied by decreased SR Ca$^{2+}$ content and fractional cell shortening (Fig. S9C and D). Thus, S107 or Q-LETD-OPH treatment prevented altered Ca$^{2+}$ handling and impaired cell shortening.

Cytosolic Ca$^{2+}$ regulates the nuclear translocation of some transcription factors and the expression of Ca$^{2+}$-dependent genes known to contribute to ventricular remodeling (25). In that context, we hypothesized that the diastolic SR Ca$^{2+}$ leak via RyR2 channels after I/R might contribute to the cardiac remodeling process. The nuclear factor of activated T cells (NFAT) is a transcription factor involved in cardiac hypertrophy (26). Elevated cytosolic [Ca$^{2+}$]$^+$ activates the calmodulin-activated serine/threonine protein phosphatase calcineurin, which dephosphorylates NFATc resulting in nuclear translocation of NFAT and activation of hypertrophy genes (27) and immunologically important genes, such as TNF-α (28). We examined cytosolic to nuclear translocation of NFAT and observed increased NFAT after I/R (Fig. 4C). When the animals were treated with caspase-8 inhibitor (Q-LETD-OPh) or rycz (S107), NFAT was retained in the cytosol at levels similar to those observed in sham-operated animals and may contribute to the reduction in hypertrophy shown in Fig. 3B. Additionally, mRNA levels of the heart failure marker ANF were also significantly reduced with both treatments (Fig. 4D). In addition to lower levels of collagen (Fig. 3A), interstitial fibrosis as evidenced by increased levels of fibronectin mRNA was also prevented by caspase-8 inhibition or S107 inhibition of SR Ca$^{2+}$ leak (Fig. 4D). I/R also increased mRNA levels of molecules involved in TNF-α signaling including TNFR1, TNFR2, caspase-8, and TNF-α (Fig. 4E). This increase in mRNA levels was prevented by the caspase-8 inhibitor (Q-LETD-OPh) and by S107, suggesting a reduced inflammatory response. Taken together, these results indicate that an increase in ROS and NO production, via early caspase-8 activation, induces RyR2 S-nitrosylation and diastolic SR Ca$^{2+}$ leak, which contribute to I/R injury and long-term left ventricular remodeling.

Discussion

The therapeutic strategy of rapid reperfusion of ischemic myocardium is designed to preserve cardiac function. However, reperfusion itself has notable adverse effects including arrhythmias and cell death (29, 30). We now show that inhibiting RyR2-mediated diastolic SR Ca$^{2+}$ leak with a unique orally available drug called rycz (S107), which stabilizes the channel, or by caspase-8 inhibition, significantly reduces reperfusion injury, infarct extension, and left ventricular remodeling in the later phase of reperfusion (i.e., 15 d after reperfusion). LV remodeling after ischemia is caused by multiple factors including (i) ROS-mediated diastolic SR Ca$^{2+}$ leak; (ii) ROS production and inflammatory cytokines; (iii) structural changes of myocardium in response to mechanical stress; and (iv) myocardial fibrosis (31, 32). The present study reports the unique finding that inhibition of RyR2 mediated diastolic SR Ca$^{2+}$ leak before the reperfusion is sufficient to substantially reduce reperfusion injury, myocardial cell death, fibrosis, left ventricular remodeling, and inflammation.

In this study, we present a unique TNF-α-mediated signaling pathway wherein caspase-8 activation leads to S-nitrosylation of RyR2 and calstabin2 depletion from the channel complex. The subsequent increase in diastolic SR Ca$^{2+}$ leak contributes to reperfusion injury and left ventricular remodeling after acute I/R. TNFR1 is a death receptor that activates initiator caspases including caspase-8 (8). The resulting activation of caspase-8 is either sufficient to trigger the proteolytic activation of other caspases (i.e., caspase-3), or requires the proteolytic activation of proapoptotic proteins of the Bcl2 family in particular Bid, which triggers a loss of mitochondrial inner membrane potential $\Delta$ψ and ROS generation (10, 33–35). In cardiomyocytes, caspase-8 inhibition prevented TNF-α-induced loss of $\Delta$ψ and mitochondrial release of cytochrome c (10). Alternatively, the TNF/TNFFR1 complex is thought to regulate sphingolipid signaling pathways (36). After TNF-α binds to TNFR1 an early weak recruitment of FADD and stimulation of caspase-8 in the cell are sufficient to activate sphingomyelinase (21). Activation of sphingomyelinase initiates sphingolipid metabolism with ceramide, sphingosine, and sphingosine-1-phosphate formation and permits death-receptor oligomerization and caspase-8 activation (37). These bioactive sphingolipids induce cellular responses, such as mitochondrial ROS production (38) and NO synthesis (39). Hence, early inhibition of caspase-8 prevents TNF-α induced mitochondrial dysfunction and NO production (10) (Fig. 1). Concomitant ROS production and NO production would affect cellular signaling most likely through peroxynitrite formation and S-nitrosylation (13, 22). Oxidation of thiols on RyR2 may activate the channels...
resulting in a diastolic SR Ca\(^{2+}\) leak under pathological conditions (13, 15). Here, we show that S-nitrosylation and diastolic SR Ca\(^{2+}\) leak are associated with calstabin2 depletion from the channel complex, which is prevented by pharmacological inhibition of caspase-8 or with ryal S107 treatment. Of note, only caspase-8 inhibition prevented both calstabin2 depletion and S-nitrosylation. These results suggest that changes in the redox environment of the channel may lead to calstabin2 depletion and increased RyR2 leak under pathological conditions as shown in heart failure (15, 17).

SR Ca\(^{2+}\) leak is thought to trigger cellular damage after acute ischemia and reperfusion. Several studies have reported RyR2 dysfunction after I/R (24). Ca\(^{2+}\) overload has been reported to play a pathological role after reperfusion and ventricular arrhythmias (40, 41). Moreover, reperfusion is associated with the recovery of ATP phosphorylation potential, which restores SR Ca\(^{2+}\)-ATPase activity and increases Ca\(^{2+}\) sequestration into the SR (24). SR Ca\(^{2+}\) overload can cause oscillations of cytosolic Ca\(^{2+}\). Short-term oscillations in cytosolic Ca\(^{2+}\) have been implicated in the genesis of reperfusion arrhythmias (23). Indeed, caspase-8 inhibition, which prevents early RyR2 dysfunction, has a profound impact on reperfusion arrhythmias (Fig. 2). In addition, these results suggest a connection between circulating TNF-α levels and arrhythmias in acute ischemia (42, 43).

During I/R, aberrant intracellular Ca\(^{2+}\) leak is taken up by the mitochondria (44). Ca\(^{2+}\) accumulation in the mitochondria leads to activation of mitochondrial permeability transition pore (MPTP) (45). Immediately after MPTP activation, mitochondria swell and release apoptogenic factors like cytochrome c and AIF, which activates caspase-dependent and independent execution of apoptosis. This study points out a potential involvement of a dual detection mechanisms sensitizing large-scale MPTP opening and mitochondrial membrane permeabilization (46–48). Accordingly, this sensitizing mechanism suggests that caspase-8−induced mitochondrial depolarization alone, without SR Ca\(^{2+}\) leak, will not be sufficient to trigger cell death but would require a commitment [Ca\(^{2+}\)]\(_{\text{mito}}\) oscillations. Discrete modification of the SR Ca\(^{2+}\) leak may thus be sufficient to prevent large scale swelling and allowing functional recovery of the mitochondria (46–48). The present study suggests that leaky RyR2 may contribute to mitochondrial Ca\(^{2+}\) accumulation during I/R and to an amplification loop leading to reperfusion injury (9) given that inhibiting calstabin2 dissociation from the RyR2 complex reduces RyR2-mediated SR Ca\(^{2+}\) leak and is protective. Improving SR Ca\(^{2+}\) handling thus appears to be a potential unique target for reducing reperfusion injury, independently of reducing arrhythmia occurrence per se (24, 49).

In conclusion, the present study highlights the pathophysiological roles of TNF-α-induced caspase-8 activation and ROS/NO production, in the control of RyR2 function after acute myocardial infarction. Early reperfusion induces S-nitrosylation of RyR2 and calstabin2 depletion from the channel complex.
Caspase-8 activation also participates in reperfusion injury. Thus, both caspase-8 inhibition and RyR2/calstabin2 normalization are potential targets for the prevention of the effects of reperfusion, including myocardial cell death, arrhythmias, and late left ventricular remodeling after acute myocardial infarction.

**Materials and Methods**

See SI Materials and Methods for detailed descriptions.

**Animal Model and Cell Dissection.** Eight-week-old Wistar Kyoto rats (Janvier) were used. The investigations conformed to the guidelines for the Care and Use of Laboratory Animals (National Institutes of Health No. 85–23, revised 1996) and European directives (96/669/EEC). For IPR protocol, the left coronary artery was ligated and aspirated 30 min (rats) and 1 h (mice). After 15 min of ligation, animals were randomly given vehicle (10% DMSO in 0.9% saline), Q-LETD-OPh (i.p., 1 mg/kg in 10% DMSO), or Q-LETD-OPh (i.p., 1 mg/kg in 10% DMSO). In some experiments, animals were pretreated with S107 (25 mg/100 mL in the drinking water) 1 wk before ischemia and up to 72 h after reperfusion). Single cardiomyocytes were enzymatically isolated from the left ventricles as described before (50).

**Statistics.** Data are presented as mean ± SEM. Statistical significance was assessed by using the Student’s t test (for paired or unpaired samples) or when three or more groups were compared, one-way analysis of variance (ANOVA) with a Newman–Keuls post hoc test. P < 0.05 was considered significant.

**ACKNOWLEDGMENTS.** We thank C. Garraud-Moquereau, for technical assistance; N. Brunel, Plateforme Technologique Phénotypage du Petit Animal et Microdossages, IFR65/US15, Paris, for technical assistance with the TNF-α assay; and Prof. J. Morel, Montpellier Hospital, for providing Etanercept. This work was supported by the Institut de la Santé et de la Recherche Médicale, Région Languedoc Roussillon, and the Leducq Foundation, CAERUS Transatlantic Network of Excellence.


