The FGF-2-triggered protection of cardiac subsarcolemmal mitochondria from calcium overload is mitochondrial connexin 43-dependent

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Aims

Fibroblast growth factor 2 (FGF-2) protects the heart from ischaemia- and reperfusion-induced cell death by a mechanism linked to protein kinase C (PKC)1-mediated connexin 43 (Cx43) phosphorylation. Cx43 localizes predominantly to gap junctions, but has also been detected at subsarcolemmal (SSM), but not interfibrillar (IFM), mitochondria, where it is considered important for cardioprotection. We have now examined the effect of FGF-2 administration to the heart on resistance to calcium-induced permeability transition (mPTP) of isolated SSM vs. IFM suspensions, in relation to mitochondrial PKC/Cx43 levels, phosphorylation, and the presence of peptide Gap27, a Cx43 channel blocker.

Methods and results

FGF-2 perfusion increased resistance to calcium-induced mPTP in SSM and IFM suspensions by 2.9- and 1.7-fold, respectively, compared with their counterparts from vehicle-perfused hearts, assessed spectrophotometrically as cyclosporine A-inhibitable swelling. The salutary effect of FGF-2 was lost in SSM, but not in IFM, in the presence of Gap27. FGF-2 perfusion increased relative levels of PKCe, phospho(p) PKCe, and Tom-20 translocase in SSM and IFM, and of Cx43 in SSM. Phospho-serine (pS) 262- and pS368-Cx43 showed a 30- and 8-fold increase, respectively, in SSM from FGF-2-treated, compared with untreated, hearts. Stimulation of control SSM with phorbol 12-myristate 13-acetate (PMA), a PKC activator, increased both calcium tolerance and mitochondrial Cx43 phosphorylation at S262 and S368. The PMA-induced phosphorylation of mitochondrial Cx43 was prevented by εV1-2, a PKCe-inhibiting peptide.

Conclusions

SSM are more responsive than IFM to FGF-2-triggered protection from calcium-induced mPTP, by a mitochondrial Cx43 channel-mediated pathway, associated with mitochondrial Cx43 phosphorylation at PKCe target sites.

Keywords

FGF-2 cardioprotection • Subsarcolemmal mitochondria • Interfibrillar mitochondria • Mitochondrial connexin 43 • Permeability transition

1. Introduction

Connexin 43 (Cx43) is a membrane phosphoprotein and the major constituent of cardiac intercellular channels (gap junctions) as well as hemichannels.1 In addition, Cx43 translocates to and is present at subsarcolemmal (SSM) cardiac mitochondria.2–4 Cx43 is absent or, compared with SSM, present at very low levels in cardiac interfibrillar mitochondria (IFM).4 Cx43 translocation to mitochondria is dependent on its interaction with heat shock protein 90 and the translocase of the outer membrane (TOM) complex, via one of its component proteins, Tom-20.5 There is evidence linking mitochondrial (mito-) Cx43 to pro-survival pathways in cardiomyocytes. Mito-Cx43 is involved in a signal transduction pathway that can prevent mitochondrial permeability transition pore (mPTP) formation.6 The mPTP is a large non-specific conductance channel that forms at the inner mitochondrial membrane under conditions of calcium overload/oxidative stress.6 Once formed the mPTP results in mitochondrial swelling, rupture of the outer mitochondrial membrane, release of apoptogenic mitochondrial contents to the cytosol, and cell death. Signals preventing mPTP formation during reperfusion after ischaemia promote cell survival and reduce myocardial damage.7 It is therefore important to define mechanisms and signals regulating mPTP formation.
We reported previously that several manipulations exerting protection against cardiac ischaemia and reperfusion (I/R) injury and tissue loss, including ischaemic preconditioning (IPC), administration of the 18-kDa fibroblast growth factor 2 (FGF-2), and treatment with the mitochondrial potassium-sensitive K<sub>ATP</sub> channel opener diazoxide, were characterized by robustly elevated levels of particular phosphorylation state(s) of connexin-43 (Cx43) and at protein kinase C (PKC)(ε) target sites including serine (S)262 and S368. Furthermore, we have shown that both FGF-2 and PKCε-mediated cardiomyocyte resistance to prolonged ischaemic injury in vitro were lost in cardiomyocytes expressing a mutant Cx43 that was unable to become phosphorylated at S262. An important role of Cx43 phosphorylation at S262 in cytoprotection was since confirmed in a different model. Our previous studies, however, did not examine the mitochondrial pool of Cx43. There is as yet no information as to whether FGF-2 administration affects cardiac SSM and/or IFM resistance to calcium overload, or mito-Cx43 phosphorylation in SSM.

We have now investigated the effects of FGF-2, administered to the ex vivo rat heart, on mitochondrial resistance to calcium-induced swelling (a measure of mPTP formation) in SSM and IFM suspensions, in the absence and presence of the Cx43 inhibitor peptide Gap27. SSM and IFM from control and FGF-2-treated hearts were analysed for their mito-PKCε, pPKCε, and Tom20-tri sacloase content, while SSM were also examined for Cx43 and phospho-serine (Ps)262-/pS368-Cx43. Additional studies have addressed the role of mitochondrial PKCε (mito-PKCε) on mito-Cx43 phosphorylation at S262 and S368. SSM were found to be more responsive, compared with IFM, to FGF-2-triggered protection from calcium-induced mPTP, by a mechanism dependent on mito-Cx43 function, and associated with mito-Cx43 phosphorylation at PKCε target sites.

2. Methods

This section describes key methods. A full description can be found in Supplementary material online, Materials and Methods.

2.1 Animals

Adult male Sprague–Dawley rats (250–280 g) were provided by the Central Animal Care Facility at the University of Manitoba and used as approved by the local Animal Care Committee of the National Research Council of Canada, according to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. Adult rats were deeply anaesthetized with ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight); once there was a complete lack of reflex response, hearts were excised and used for Langendorff perfusion.

2.2 Materials

Recombinant 18-kDa FGF-2 produced in Escherichia coli was purified to homogeneity as published previously, and used within 1 month of preparation. All Cx43 antibodies have been fully characterized and validated. A complete list of antibodies can be found in Supplemental material online. Table S1. A peptide inhibitor of PKCε (εV1-2) and its inactive scrambled control peptide (Scr) were from Calbiochem. Peptide Gap27 (SRPTEKTIFII) and a corresponding scrambled peptide (REKIIITSFIPT) were from AnaSpec. Protease inhibitor cocktail (PIC; #P8340) and phosphatase inhibitor cocktails 1 and 2 (PPIC1, # P2850 and PPIC2, #P5726) were from Sigma.

2.3 Isolated heart perfusion

Three perfused heart groups were studied; a schematic of experimental design is included in Supplementary material online, Figure S1. In the ‘control’ group, hearts were perfused with oxygenated Krebs–Henseleit solution (K–H; pH 7.4, 37°C) under non-recirculating conditions at a constant pressure of 80 mmHg, as we previously described. In the FGF-2 group, after a stabilization period of 20 min perfusion, FGF-2-supplemented K–H (10 μg FGF-2 in 12 mL) was infused with a peristaltic pump directly into the main line entering the heart, at 1 mL/min for 12 min, followed by an additional 8 min of perfusion with K–H. In the IPC group, hearts were subjected to three cycles of 3 min global ischaemia (complete interruption of flow), followed by 5 min reperfusion (complete re-establishment of flow).

2.4 Mitochondrial isolation

SSM and IFM were isolated from freshly obtained vehicle- or FGF-2-perfused hearts essentially, as previously described; a detailed protocol is included in Supplementary material online, Materials and Methods. Mitochondrial respiration was measured polarographically with a Clark-type electrode at 30°C in a 1-mL sealed chamber (Quibit Systems, ON, Canada), as previously described. Citrate synthase activity was determined using a ScienCell™ kit.

2.5 Removal of the outer mitochondrial membrane

Mitochondria can absorb and buffer calcium up to a point beyond which they start undergoing mPTP, which causes swelling and changes in light scattering properties of the suspension. This is measured as a decrease in optical density at 545 nm (A<sub>545</sub>). SSM or IFM were added to a 2-mL cuvette at 30°C and energized with 20 mM glutamate and 2 mM malate. ADP (75 μM) and oligomycin (2 μg/mL) were used in all experiments, unless otherwise stated. In some experiments, cyclosporine A (CsA; used at 0.5 μM) was added at the same time as ADP and oligomycin, prior to the addition of mitochondria. A<sub>545</sub> was measured continually, as calcium was added in stepwise increments of 125 μM per min, until no further change in absorbance was observed. At the end of experiment, maximum mitochondrial swelling (100%) was determined by adding alamethicin (15 μg/mL). In select experiments, mitochondrial suspensions were supplemented with Gap27, or scrambled Gap27, at 250 μM, prior to the addition of calcium.

2.6 Mitochondrial matrix swelling

Cytosolic calcium can absorb and buffer calcium up to a point beyond which they start undergoing mPTP, which causes swelling and changes in light scattering properties of the suspension. This is measured as a decrease in optical density at 545 nm (A<sub>545</sub>). Mitochondrial suspensions were supplemented with Gap27, or scrambled Gap27, at 250 μM, prior to the addition of calcium.

2.7 Cytochrome c release

Mitochondrial suspension. SSM (0.5 mg/mL) with 75 μM ADP were energized for 5 min with 20 mM glutamate, 2 mM malate, and 2 μg/mL of oligomycin before aliquoting (100 μL) into 1.5 mL tubes. Samples were subjected to increasing concentrations of added CaCl<sub>2</sub> (0–1000 μM) in the presence or absence of CsA at 30°C for 2 min. After a brief centrifugation, mitochondrial pellets and supernatants were processed for SDS–PAGE and western blotting for cytochrome c.

2.8 Phorbol 12-myristate 13-acetate stimulation

Mitochondria from non-stimulated hearts were suspended at a concentration of 4 mg/mL in suspension buffer (120 mM KCl, 10 mM HEPES pH 7.2, 10 mM succinate, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, containing 1: 100 dilution of PIC, PPIC1, and PPIC2) supplemented with 0.67 μM oligomycin and 50 μM ADP before dividing into four groups: (i) control + Scr (0.5 μM), (ii) control + εV1-2 (0.5 μM), (iii) stimulated with phorbol 12-myristate 13-acetate (PMA; 0.2 μM) + Scr (0.5 μM), and (iv) stimulated with PMA (0.2 μM) + εV1-2 (0.5 μM) for 2 min. Mitochondrial pellets were used for western blot analysis.

2.9 Electron microscopy

A cardiac mitochondrial suspension was fixed in a solution of 3% glutaraldehyde and 0.1 M phosphate. The mitochondria were pelleted and embedded in epon followed by sectioning. Sections were stained with
uranyl acetate and lead citrate, and viewed with a JEOL JEM 1230 Transmission Electron Microscope.

2.10 Immunelectron microscopy
As described previously, hearts were dissected and fixed overnight with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. SSM were fixed in the same solution. Ultrathin sections on nickel grids were blocked and incubated with primary mouse monoclonal antibody specific for Cx43 (610061, BD Transduction Laboratories), and subsequently with anti-mouse IgG antibody coupled with 5 nm gold particles (Sigma, ON, Canada). The grids were stained with uranyl acetate and viewed with a JEOL JEM 1230 Transmission Electron Microscope.

2.11 SDS–PAGE and western blotting
Total cardiac extracts were obtained from frozen tissue as described previously. Cardiac or mitochondrial lysates were resolved by polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride (PVDF) membrane (Roche) and antigen–antibody complexes detected by enhanced chemiluminescence (ECL® Plus, Amersham Biosciences).

2.12 Statistical analysis
Differences between two groups were compared using unpaired Student’s t-test analysis. One-way analysis of variance (ANOVA) was used when more than two groups (one treatment) were compared. Two-way ANOVA was also used, as needed, program Prism 6. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered significant, very significant, and extremely significant, respectively.

3. Results
3.1 Validation of SSM preparations
In order to address the role of mito-Cx43 and potential changes in mito-Cx43 phosphorylation or levels in response to FGF-2 treatment, it was important to establish the integrity and relative purity of the SSM preparations and to confirm the presence of Cx43 at SSM reported previously. As seen in Figure 1A, SSM preparations appeared structurally intact and free of contamination by other organelles or debris when examined by electron microscopy (EM). Western blot analysis (Figure 1B) showed enrichment of SSM for mitochondrial proteins (cytochrome c and adenine nucleotide transferase, ANT) compared with total heart lysates, and lack of contamination by markers of sarcosomal (Na+/K+ ATPase, caveolin 1, and N-cadherin), cytosolic (GAPDH), sarcoplasmic reticulum (SERCA2), nuclear (lamin B), Golgi (58K GgI), and lysosomal (Lamp1) subcellular compartments, while these markers were present in total heart lysates. Cx43 (43–45 kDa) was clearly detected in SSM. When SSM were subjected to digitonin to remove the outer mitochondrial membrane, the voltage-dependent anion channel (VDAC), an outer mitochondrial membrane protein, gradually disappeared while mito-Cx43 immunoreactivity remained unchanged, as did that of ANT, an inner mitochondrial membrane protein (Figure 1C). Immuno-EM of SSM, or rat heart ventricular sections, showed the presence of immunogold-labelled Cx43 at mitochondria, likely mitochondrial cristae, in both cases (Figure 1D and E). Incubation in the absence of the anti-Cx43 primary antibody did not elicit any immunogold-labelling (Figure 1F). State 3 and state 4 respiration and their ratio were similar to those published previously, indicative of well-coupled mitochondria (Supplementary material online, Table S2).

3.2 SSM: effect of FGF-2 perfusion on calcium-induced swelling or cytochrome c release
SSM from hearts perfused with vehicle (control), FGF-2-supplemented solution (FGF-2), or subjected to an IPC-inducing protocol (three cycles of 5 min global ischaemia followed by reperfusion, as in ref.) were compared regarding their calcium capacity (tolerance), defined here as the total amount of CaCl2 needed to be added to the suspensions before a drop in A545 (an indicator of swelling) was observed spectrophotometrically. The IPC group was used as a positive control for our experimental system, as mitochondria from hearts subjected to IPC have increased calcium capacity. Measurements were obtained in the absence or presence of ADP and also in the absence or presence of CsA, a potent inhibitor of mPTP. Results are summarized in Table 1, and some representative tracings are included in Figure 2A and B. Regardless of the absence or presence of ADP, SSM from FGF-2-treated hearts had a significantly higher calcium capacity compared with those from vehicle-treated hearts. As expected, ADP itself raised calcium tolerance of both control- and FGF-2-heart-derived SSM preparations. Calcium tolerance of SSM from IPC hearts was also significantly higher compared with those from control hearts. In the presence of CsA, CaCl2 failed to induce swelling (drop in A545) in either control, FGF-2, or IPC mitochondria, unless it was added in excessive amounts (Table 1). These studies, by showing the expected increase in calcium tolerance of IPC mitochondria, validated our experimental system; in addition, they showed that FGF-2 treatment raised SSM resistance to calcium-induced mPTP.

To further validate our findings on the effect of FGF-2 on SSM by a different assay, we examined the effect of increasing CaCl2 on cytochrome c release. Opening of mPTP is accompanied by release of cytochrome c to the extramitochondrial space. In vitro, released cytochrome c is present in the supernatant fraction obtained after centrifugation of mitochondrial suspensions. In control SSM, increasing calcium (starting at 300 μM) caused cytochrome c release to the supernatant and a corresponding decrease in the pellet (Figure 2C). Cytochrome c release was completely prevented by CsA, indicating that it was the result of mPTP. SSM from FGF-2 hearts did not show any cytochrome c release, indicating that they were resistant to calcium-induced mPTP under our experimental conditions (Figure 2C). The mitochondria swelling approach was used in all subsequent experiments assessing resistance to calcium-induced mPTP.

3.3 The effect of FGF-2 perfusion on calcium-induced swelling of IFM vs. SSM suspensions, ± Gap27
To examine whether the protective effect of FGF-2 on mitochondria was SSM-specific, and to determine the role of a mito-Cx43 channel function, a second series of experiments compared calcium tolerance (swelling) of IFM and SSM suspensions from vehicle- or FGF-2-perfused hearts, in the presence of either Gap27 peptide or its inactive scrambled peptide. Individual SSM and IFM preparations were obtained from the same heart, and tested immediately after isolation. IFM from control- or FGF-2-perfused hearts had negligible levels of Cx43, see Supplementary material online, Figure S2A, were devoid of plasma membrane contamination, and were enriched in mitochondrial markers such as VDAC, ANT1, cytochrome c, and cyclophilin D at similar levels as SSM (Supplementary material online, Figure S2B). The scrambled Gap27 peptide had no effect on calcium tolerance of either
FGF-2 protects cardiac mitochondria from damage

SSM or IFM preparations, under any conditions (see Supplementary material online, Figure S3).

FGF-2 administration to the heart resulted in significantly increased resistance to calcium-induced swelling in both IFM and SSM isolated from these hearts compared with, respectively, IFM and SSM from control hearts (Figure 3). The FGF-2-induced increase in calcium tolerance of SSM was, at 2.9-fold (± 0.33 SD, n = 6), significantly more pronounced than that in calcium tolerance of IFM, by 1.7-fold (± 0.12 SD, n = 6). We conclude that FGF-2 administration protects both types of cardiac mitochondrial populations from calcium overload, but SSM are more responsive to the protective effect.

In SSM from control hearts, incubation with Gap27 peptide significantly decreased calcium capacity, by 44%, compared with those incubated with scrambled peptide. In SSM from FGF-2-treated hearts, Gap27 decreased calcium capacity by 77%, eliminating the FGF-2-induced increased calcium tolerance (Figure 3). In the presence of Gap27, calcium capacity was minimal, and similar between SSM from FGF-2- or control-perfused hearts. In contrast, Gap27 had no effect on calcium capacity of IFM from either control or FGF-2 hearts (Figure 3). IFM from FGF-2 hearts retained higher calcium tolerance compared with IFM from control hearts regardless of Gap27. Some representative tracings are included in Supplementary material online, Figure S4. Our data indicate that mito-Cx43 channel function is mediating both baseline (control)- and FGF-2-induced resistance to calcium overload in SSM. The absence of an effect of Gap27 on IFM is consistent with negligible levels of Cx43 in this mitochondrial population.

Control heart-derived IFM displayed significantly higher calcium capacity, by 2.2-fold, compared with control heart-derived SSM (Figure 3). FGF-2-heart-derived IFM also displayed higher calcium capacity than FGF-2-heart-derived SSM, but the magnitude of the difference (at 1.3-fold) was less pronounced than in the control mitochondrial populations.

Figure 1 Characterization of SSM preparations. (A) Electron microscopy image of SSM. (B) Western blot analysis of total cardiac lysates (heart), and mitochondrial lysates (SSM) for Cx43 and for proteins present in: sarcolemma (Na+/K+ ATPase, caveolin 1, and N-Cadherin), cytosol (GAPDH), sarcoplasmic reticulum (SERCA2), nucleus (Lamin B), Golgi (58K Glg), and mitochondria (ANT, cytochrome c), as indicated. (C) SSM were treated with digitonin for 0, 15 and 30 min, followed by centrifugation to obtain the pellet fraction; pellets were probed for proteins present at the inner mitochondrial membrane (ANT) or the outer mitochondrial membrane (VDAC), as well as for Cx43, as indicated. (D–F) Immunoelectron microscopy images; in (D), SSM incubated with anti-Cx43 antibodies show immunoreactivity (immunogold dots, arrows) in mitochondria cristae (Mc); in (E), heart sections incubated with anti-Cx43 antibodies show immunoreactivity (immunogold dots, arrows) within mitochondria (M) but not myofibrils (MF); in (F), heart sections incubated in the absence of primary antibody (control) show the lack of immunogold staining. Size bars in (D, E) or (F) correspond to 100 or 500 nm.
3.4 The effect of FGF-2 perfusion on mito-PKCe, Cx43, and Tom-20

Previous studies have shown that IPC-induced cardioprotection is associated with increased levels of PKCe and/or Cx43 in cardiac mitochondria, due to translocation via the TOM translocase complex. To determine whether FGF-2 exerted similar effects, we determined relative levels of mito-PKCe, mito-pPKCe, and Tom-20 in SSM and IFM from control and FGF-2-treated hearts. Total mito-Cx43 and pS262- and pS368-Cx43 relative levels were also determined in SSM. As shown in Figure 4, Cx43, PKCe, pPKCe, and Tom-20 were clearly present in SSM from both control and FGF-2 hearts, assessed by western blotting and specific antibodies. The FGF-2 group displayed significant, 1.4-, 1.6-, 1.7-, and 2.0-fold increases, respectively, in total mito-Cx43, PKCe, pPKCe, and Tom-20 compared with the control group. In addition, while immunoreactive signals for pS262- and pS368-Cx43 were barely above background in SSM from control hearts, significant, 30- and 8-fold increases were detected in SSM from FGF-2 hearts.

Another protein implicated in protecting mitochondria from mPTP, Table 1: Effect of FGF-2 perfusion on calcium capacity/tolerance of SSM suspensions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Calcium capacity (μM Ca^{2+}/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>Control group</td>
</tr>
<tr>
<td>No ADP</td>
<td>183 ± 28</td>
</tr>
<tr>
<td>+ ADP</td>
<td>342 ± 44</td>
</tr>
<tr>
<td>+ ADP, + CsA</td>
<td>6875 ± 361</td>
</tr>
</tbody>
</table>

Calcium capacity or tolerance was defined as the amount of added CaCl₂ capable of eliciting a drop in A₅₄₁ and was assessed in mitochondria from control (vehicle-treated), FGF-2-perfused hearts, or hearts subjected to IPC (positive control), incubated with or without ADP, and in the absence or presence of CsA, as indicated.

aSignificant difference (P < 0.05, one-way ANOVA) compared with corresponding controls; n = 5.
glycogen synthase kinase (GSK)-3β was however present in IFM from control hearts (Figure 5). IFM from FGF-2-treated hearts displayed the presence of PKC₁, pPKC₁, and Tom-20. In addition, IFM from FGF-2-treated hearts showed a significant increase in mito-GSK-3β and pS9-GSK-3β (Figure 5B).

3.5 The effect of PMA on isolated SSM

In the normal adult heart, Cx43 is constitutively phosphorylated at multiple sites; several kinases, including ERK and PKC, are known to phosphorylate Cx43 at specific sites.²⁵ Previously, we demonstrated that the FGF-2 or PMA-induced Cx43 phosphorylation at S262, which occurs in addition to the constitutive phosphorylation pattern, is mediated by PKCε but not the ERK pathway.²⁶–²⁸ To determine whether mito-PKCε is involved in mito-Cx43 phosphorylation at S262, and/or S368, SSM from control hearts were stimulated with the PKC activator, PMA, in the presence of either a PKCε inhibitor peptide, εV1-2, or its inactive control (scrambled, Scr) peptide. These reagents have been used successfully to inhibit mito-PKCε activity by others.²⁹ In the presence of the scrambled peptide, PMA elicited a very significant increase in pS262-Cx43 (14-fold) compared with control mitochondria, showing that Cx43 can be phosphorylated at S262 within the mitochondrial environment (Figure 6A and B). The PMA-induced increase in mito-Cx43 phosphorylation at S262 was prevented in the presence of the PKCε inhibitor peptide, indicating that mito-PKCε is responsible for phosphorylating mito-Cx43 at S262 in isolated SSM (Figure 6A and B). PMA also increased mito-pS368-Cx43 by 4-fold (Figure 6A and C), and the effect was blunted by εV1-2, and Figure 6A, C, and D shows that PMA significantly increased mitochondrial (SSM) calcium capacity. Although we did not test the effect of εV1-2 on calcium capacity, previous studies have shown that the PMA-induced increase in calcium capacity in isolated mitochondria is mediated by mito-PKCε.²⁹,³⁰

Figure 4 Effect of FGF-2 perfusion on select SSM-associated proteins. SSM from control (Con) or FGF-2-perfused hearts (FGF-2) were analysed by western blotting, n = 3 (independent experiments) for each group. (A) Representative western blot images of SSM lysates probed for total Cx43, pS262-Cx43, pS368-Cx43, and cyclophilin D (Cyp-D), used as loading control. Corresponding quantitative data are included in (B) for total Cx43, (C) for pS262-Cx43, and (D) for pS368-Cx43. (E) and (F). Representative western blot images of SSM lysates probed for Tom-20 or PKCε, as well as pPKCε, as indicated; corresponding quantitative data are included within each panel. Data were analysed using the Student’s t-test.
4. Discussion

Several novel findings are presented in this work. FGF-2 administration to the heart was shown to: (i) increase SSM and IFM resistance to calcium-induced mPTP, an effect that is more pronounced in SSM; (ii) increase relative mito-PKC\(_{1}\), pPKC\(_{1}\), and Tom-20 levels in both mitochondrial populations. In addition, (iii) the mechanism of FGF-2-triggered protection of SSM requires mito-Cx43 channel function and is associated with increased mito-Cx43 levels, as well as mito-Cx43 phosphorylation at the PKC\(_{1}\) target sites S262 and S368.

4.1 Calcium tolerance of SSM and IFM under baseline conditions

Under baseline conditions, calcium capacity was lower in SSM compared with IFM (Figure 3), in agreement with previous reports.\(^{17,31,32}\) The molecular basis for the difference is not known. It is intriguing that the higher calcium capacity of IFM was associated with the relative lack of proteins generally considered to strengthen resistance of mitochondria to calcium overload-induced mPTP, such as mito-Cx43,\(^{33}\) PKC\(_{1}\),\(^{24}\) as well as Tom-20\(^{34}\) (Figure 5); conversely, the lower calcium capacity of SSM (compared with IFM) was associated with the presence of these proteins (Figure 4). One would be tempted to suggest that, if anything, the presence of Cx43/PKC\(_{1}\) in mitochondria has a negative effect on calcium tolerance under baseline conditions. The situation is however more complex, as indicated by experiments conducted in the presence of Gap27, and by examining mitochondrial populations from FGF-2 treated hearts.

Incubation with the Gap27 peptide that blocks Cx43 channel and hemichannel function\(^{35}\) decreased calcium tolerance of SSM even further, indicating that mito-Cx43 exerts a protective effect mediated by its hemichannel function, and that the effect is specific for SSM (Figure 5). Gap27 had no effect on calcium tolerance of IFM, supporting the conclusion that baseline vulnerability to calcium is regulated by distinct, mito-Cx43 channel-dependent or -independent mechanisms in SSM vs. IFM.

Relative levels of pS262- or pS368-Cx43 in control SSM (Figure 4) were relatively low, mirroring findings for total cardiac pS262- or pS368-Cx43.\(^{3} \) Thus, it can be argued that mito-Cx43 phosphorylation at these specific sites, which occurs in addition to the constitutive phosphorylation of mito-Cx43, may not be required, or may play a minor role, in mito-Cx43 functionality under baseline conditions.

4.2 Calcium tolerance of SSM and IFM from FGF-2-treated hearts

FGF-2 administration to the heart increased resistance to calcium overload-induced mPTP in both SSM and IFM (Figure 3). It is logical to...
suggest that protective effects at the mitochondrial level mediate the well-established FGF-2-induced cardioprotection from 1–R-induced myocardial damage and dysfunction. Because our studies required isolation of mitochondrial populations immediately after FGF-2 perfusion, it was not possible to confirm the protective effect of FGF-2 if these specific hearts were exposed to 1–R. By necessity, we would have needed to examine the effects of FGF-2 in a separate group of hearts. This requirement has been met in several studies from our laboratory documenting that perfusion of the rat heart with an FGF-2-supplemented solution, as done in the present study results in preservation of energy stores, reduced cardiac damage and cell death, and improved recovery of contractile function after 30 min of global ischaemia and 60 min of reperfusion.11–19

The relative magnitude of the protective effect of FGF-2 was higher in SSM (by 70%, Figure 3), providing further evidence for differences between the IFM and SSM mitochondria populations. Increased responsiveness to a protective manipulation has been noted previously for SSM: SSM are more sensitive, compared with IFM, to diazoxide-mediated protection from calcium injury.20 Diazoxide-induced protection of the whole heart is mediated by the opening of the mito-KATP channel; it remains to be determined whether the protective effects of FGF-2 perfusion on SSM vs. IFM are mediated by differential effects on the mito-KATP channel and downstream signals.

The protective effect of FGF-2 on SSM required the hemichannel function of mito-Cx43, as it was eliminated in the presence of Gap27 (Figure 3). Thus, hemichannel functionality is mediating both baseline and FGF-2-induced calcium tolerance of SSM. Our findings are in agreement with a previous study, reporting that inhibition of rat brain mito-Cx43 with the channel blocker carbonoxolone increased vulnerability to calcium-induced mPTP.21 Cx43 mimetic peptides have been used by other groups to show that mito-Cx43 is stimulating ADP-dependent complex I respiration by a mechanism requiring hemichannel functionality.22

Several reports have pointed to a positive relationship between mito-Cx43 levels and cytoprotection.3,4,23–28 This would suggest that the FGF-induced increase in mito-Cx43 levels (Figure 4) is required to induce increased calcium tolerance in SSM. If that were the case, it should not be possible to stimulate protection in SSM in vitro, where mito-Cx43 levels remained constant. This is not what we found: PMA stimulation of control SSM increased calcium tolerance (Figure 6); thus, an increase in mito-Cx43 is not necessary for a protective effect to manifest in normal mitochondria. What may be required instead of, or in parallel to, increased mito-Cx43 levels, is an increase in mito-Cx43 with the channel blocker carbenoxolone increased vulnerability with a previous study, reporting that inhibition of rat brain Cx43 from interacting with Kir 6.1, a constituent of the plasma membrane KATP channel.53

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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