FGF-2 protects cardiomyocytes from doxorubicin damage via protein kinase C-dependent effects on efflux transporters

Jie Wang1, Mark W. Nachtigal2, Elissavet Kardami3,4, and Peter A. Cattini1*

1Department of Physiology, University of Manitoba, 745 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 3J7; 2Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Manitoba, Canada R3E 3J7; 3Department of Human Anatomy and Cell Sciences, University of Manitoba, Winnipeg, Manitoba, Canada R3E 3J7; and 4Institute of Cardiovascular Science, St. Boniface Hospital Research Centre, University of Manitoba, Winnipeg, Manitoba, Canada R2H 2A6

Received 26 August 2012; revised 20 December 2012; accepted 10 January 2013; online publish-ahead-of-print 22 January 2013

Aims
The anti-cancer anthracycline doxorubicin (DOX) increases the risk of cardiac damage, indicating a need to protect the heart and still allow the benefits of drug treatment. Fibroblast growth factor-2 (FGF-2) is cardioprotective against ischaemia–reperfusion injury. Our aim is to investigate: (i) the ability of FGF-2 to protect against DOX-induced cardiomyocyte damage and (ii) the contribution of efflux drug transport to any increase in injury-resistance.

Methods and results
Neonatal rat cardiomyocyte damage was assessed by measuring cell death markers and lactate dehydrogenase (LDH) activity in the culture medium. LDH activity was increased significantly after incubation with 0.5 μM DOX for 24 h in the absence but not presence of 10 nM FGF-2; this beneficial effect of FGF-2 was blocked by tyrosine kinase (FGF) receptor inhibition. An increase in efflux drug transporter RNA levels was also detected after FGF-2 treatment in the presence of DOX. The beneficial effect of FGF-2 against cell damage and increased transporter RNA levels were blunted with protein kinase C (PKC) inhibition. Finally, FGF-2 stimulated efflux transport of calcein and DOX, and treatment with efflux transporter inhibitors significantly attenuated the protective effect of FGF-2 from DOX-induced injury.

Conclusion
Administered FGF-2 increases resistance to DOX-induced cardiomyocyte damage, by a mechanism dependent on PKC as well as regulation of efflux transporter production and/or function.

Keywords
FGF-2 • Doxorubicin • Cardiac myocytes • Efflux transport • Drug resistance

1. Introduction
There are many causes of cardiac damage that can lead to heart failure including therapeutic agents used in the treatment of other diseases that can be cardiotoxic. Anti-cancer anthracyclines such as doxorubicin (DOX) are associated with an increased and cumulative risk of cardiac damage, thereby limiting their usefulness.1,3 Approximately one in 10 patients treated with DOX or its derivatives will develop cardiac complications up to 10 years after chemotherapy ends.1 Strategies are needed to protect the heart that will still allow the benefits of drug treatment. There are factors produced by the heart that are reported to exert cytoprotection and cardioprotection. Thus, it is possible that drugs like DOX might compromise this endogenous resistance to injury of heart cells and as a result contribute to the cardiotoxic effects observed. Understanding these endogenous factors, including how they function in an injury setting, may offer therapeutic agents, or targets, whose levels might be modulated to increase resistance to cardiac damage.3

FGF-2 exists as both low (18 kDa) and high molecular weight isoforms, and is produced by both heart fibroblasts and myocytes, as well as in other tissues.4 The 18 kDa FGF-2 can regulate myocardial and vascular cell growth and differentiation, normally and in response to injury, and is cardioprotective both in vitro and in vivo.5 FGF-2 increased resistance to H₂O₂-induced damage to cardiomyocytes in culture, as well as ischaemia–reperfusion injury in murine hearts isolated in situ and after coronary ligation in vivo.4–7 FGF-2 also offers potential post-ischaemic and reperfusion benefits through its mitogenic and angiogenic effects.4–8 FGF-2 signals through multiple pathways, but acute cytoprotection of cardiomyocytes requires binding to plasma membrane tyrosine kinase FGF receptor (FGFR) 1, and
protein kinase C (PKC) activation. Cardioprotection by FGF-2 and ischemic preconditioning are associated with similar signal transduction pathways, and particularly PKCe. On release, FGF-2 is retained by the extracellular matrix and used to support growth and confer resistance to cardiac damage; certainly FGF-2 deficient mice are associated with decreased endothelial proliferation and are more susceptible to injury. FGF-2 can modify the sensitivity of cells to anti-cancer drugs, resulting in increased drug resistance or sensitivity by affecting efflux ATP-binding cassette (ABC) drug transporters, including multidrug resistance protein 1 (MDR1/ABC1/P-glycoprotein) and multidrug resistance related protein 1 (MRP1/ABCC1). Efflux transporters are localized to the plasma membrane and help remove toxic agents, including antracyclines, out of the cell in an energy-dependent manner. In this context, about 50% of DOX is not metabolized and is transported out of the cell unchanged. While efflux transporter function and function can be obstacles to chemotherapeutic efficacy, they could be beneficial in terms of protecting non-cancer cells from xenobiotic attack. Cardiomyocytes produce MDR1, MDR2, MRP1, and low levels of MRP2. Furthermore, MRP1 is implicated in cardioprotection by regulating the efflux of toxic products from cardiomyocytes at both the level of the sarcolemma and mitochondria. There is no report, however, on the effect of exogenous FGF-2 on cardiomyocyte survival and efflux transporters after DOX treatment. Multiple mechanisms, including oxidative stress, lipid peroxidation, mitochondrial dysfunction, and intercalation of nucleic acids, have been proposed to explain anthracycline-induced damage, including disruption of cardiomyocyte cell membranes. The clinical effects of DOX, including transient electrocardiographic changes and cardiomyopathy, have been modelled in rat cardiac cells, including neonatal cardiomyocytes. Concentrations of 0.1–1.0 μM DOX cause neonatal rat cardiomyocyte mitochondrial swelling and disruption of the plasma membrane, which are features of injury leading to cell death. Detection of normally intracellular lactate dehydrogenase (LDH) activity in the culture medium is an indicator of disruption of the membrane and damage. Here, we have used neonatal rat cardiomyocyte cultures treated with 0.5 μM DOX to assess the ability of FGF-2 to protect cardiomyocytes from induced cell damage as measured by LDH release, as well as affect multi-drug resistance gene expression and efflux transporter function.

2. Materials

2.1 Cultures

All procedures involving animals, their tissues, and cells conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011), and were approved by the local animal Protocol Management Review Committee. One-day-old Sprague–Dawley rats (36–40) were euthanized by decapitation, and ventricular myocytes isolated by enzymatic digestion and fractionation on a HEPES-buffered Percoll gradient as described. Myocytes were plated (1.3 × 10⁶) on collagen type I-coated dishes in Ham’s F-10 supplemented with 10% fetal bovine serum(36x89), 10% horse serum, and antibiotics.27,28

2.2 Growth factor and DOX treatments

Cultures were maintained in defined medium consisting of Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12), supplemented with 0.05% FBS, 0.66% albumin, 1% antibiotics, 0.02% ascorbic acid, and 1% insulin–transferrin–selenium (GIBCO, Life Technologies, Burlington, Canada) for 24 h. Cultures were fed with fresh medium supplemented with either 0.5 μM DOX (Sigma-Aldrich, Oakville, Canada) or 0.2–20 nM recombinant rat FGF-2 for 6 or 24 h. For pre-treatment with FGF-2, cultures were refed and treated with 10 nM FGF-2 for 30 min followed by 0.5 μM DOX treatment for up to 24 h. For tyrosine kinase FGF receptor inhibition, 20 nM SU5402 (Tocris Biosciences, UK) was added before co-treatment with FGF-2 for 30 min and addition of DOX. To assess PKC signalling, cultures were pre-treated with 5 μM chelerythrine or 20 nM bisindolylmaleimide I (BIM-1) for 15 min before co-treatment with FGF-2 for 30 min and addition of DOX.

2.3 Staining and fluorescence-activated cell sorting

Programmed cell death was assessed by fluorescence-activated cell sorting (FACS) using the Apoptosis & Necrosis Quantification Kit (Biotium Inc., CA, USA). Cells (1 × 10⁶) were co-stained with FITC-annexin V (for apoptotic cells), ethidium homodimer III (for necrotic cells), and Hoechst 33342 (for ‘all’ nuclei/cells); see Supplementary material online, Figure S1. Efflux transport of fluorescent calcine was assessed using the Multi-Drug Resistance Assay Kit (Calcein AM; ayman Chemical Company, Ann Arbor, USA). Briefly, cultures were treated with DOX and/or FGF-2, or 20 μM cytosine A (CsA), 2 μM verapamil, and 1 μM XR9576 as a positive control for efflux inhibition, before FACS analysis (MoFloXD, Beckman Coulter, Mississauga, Canada) within 1 h of staining. Data were analysed using the Summit v.5.2 program (Beckman) with ‘gating’ based on determinations for untreated cells, as well as annexin V, ethidium homodimer III, and calcein AM positive cells. Effects on intracellular DOX concentration in DOX and/or FGF-2-treated cardiomyocytes were also assessed by FACS as described.

2.4 LDH assay

Quantitative kinetic determination of LDH activity in conditioned cardiomyocyte medium was assessed using the In Vitro Toxicology Assay Kit (Lactic Dehydrogenase based) according to the manufacturer’s instructions (Sigma-Aldrich).

2.5 RNA isolation and real-time reverse transcriptase polymerase chain reaction

RNA extraction and qPCR was done using specific primers (Table 1) as described (see Supplementary material online, Figure S2). Minus RT reactions were performed as controls for the presence of genomic DNA. RNA levels in each sample (absolute quantification) was calculated from the standard curve and normalized to mouse beta-2 microglobulin (B2M) expression as appropriate. Tests were normally run in duplicate on three independent samples.

2.6 Statistical analysis

For single comparisons, paired t-tests were applied, and two-way analysis of variance (ANOVA) with a post-hoc Bonferroni test or one-way ANOVA with a post-hoc Tukey test were used for multiple (treatments and time) and single group (treatments) analyses, respectively. Mean values were considered significantly different if P < 0.05. Unless
stated otherwise, all studies were done in triplicate. In figures, comparisons made relative to a ‘control’ and arbitrarily set to 1.0 or 100%, are represented as *P<0.05, **P<0.01, and ***P<0.001. For comparisons made between treatment groups (and not arbitrarily set to 1.0 or 100%), these are represented as #P<0.05, ##P<0.01, and ###P<0.001.

Table 1 Primer sequences (5'-3') used for qPCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1a</td>
<td>AGCGGTCAGTGCTCACA</td>
<td>CTTGGCATATATGTCTGTAGCA</td>
</tr>
<tr>
<td>MDR1b</td>
<td>GAATAATGCTTATGAATCCAAAG</td>
<td>GGTATTGCTGCTGCTTCTGTA</td>
</tr>
<tr>
<td>MDR2</td>
<td>AGAGATTTGAGTGGAGCTAAGTA</td>
<td>TGGTTTCACATCCACCCTAT</td>
</tr>
<tr>
<td>MRP1</td>
<td>AAGGAGTCAGTCCTCAGG</td>
<td>AGAGTGACTGCTCTTACAGG</td>
</tr>
<tr>
<td>MRP2</td>
<td>GAAGGCTTGACGACCTATCT</td>
<td>CCACGTGAATCTCATCAGT</td>
</tr>
<tr>
<td>B2M</td>
<td>GACCGTGATTTTGCTGCTT</td>
<td>TCCCAATTCCGGTGA</td>
</tr>
<tr>
<td>FGF-2</td>
<td>TCTTCTGCGCATCCATCCAGA</td>
<td>CAGTGCCACATACCACTGAG</td>
</tr>
</tbody>
</table>

Figure 1 (A) Cardiomyocytes were treated with 10 nM FGF-2 or 0.5 μM DOX, or pre-treated with FGF-2 for 30 min before DOX addition for 24 h. Cells were harvested, stained with programmed cell death (PCD) markers (annexin V and ethidium homodimer III), and the proportion of PCD positive (+) cells assessed by FACS. Results are expressed as the mean plus or minus standard error of the mean (+SEM), relative to the control (untreated) value, which is arbitrarily set to 1. Data were assessed by one-way ANOVA with the Tukey–Kramer post-test. (B) Cultures were treated with FGF-2 or DOX, or pre-treated with FGF-2 for 30 min before DOX addition for 1–24 h. Culture medium was assessed for LDH activity. (C) Cultures were treated with FGF-2 or DOX, or pre-treated with FGF-2 for 30 min before DOX, or pre-treated with SU5204 or DMSO vehicle, for 30 min before FGF-2 and DOX. Culture medium was assessed for LDH activity. (D) Cultures were treated with 0.2–20 nM FGF-2 before DOX, and LDH assessment. Dose-dependent curves were drawn and analysed with Graphpad Prism. The results are expressed as the mean ± SEM, relative to the control (untreated) value, which is arbitrarily set to 1. Data were assessed by two-way ANOVA with the Bonferroni post-test. A value of P<0.05 is considered statistically significant: *P<0.05; **P<0.01; ***P<0.001.
3. Results

3.1 FGF-2 pre-treatment increases resistance to DOX-induced injury

The effect of 10 nM FGF-2 pre-treatment on cell injury induced by 0.5 μM DOX in neonatal rat cardiomyocyte cultures was assessed at 24 h, using a combination of fluorescent markers for apoptosis and necrosis with FACS analysis (Figure 1A and see Supplementary material online, Figure S1). No effect of FGF-2 treatment alone was observed, and cells staining for either the apoptosis or necrosis marker accounted for <10% of the total cell population. There was, however, a consistent and significant increase in both ‘apoptosis’ and ‘necrosis’ positive cells with DOX treatment. The DOX effect was blunted by FGF-2 pre-treatment (Figure 1A). A significant decrease in total cell number, as measured by nuclear DNA staining, was also consistent with increased cell death induced by DOX, and the benefit of FGF-2 pre-treatment on cell survival (see Supplementary material online, Figure S3). The effect of 10 nM FGF-2 pre-treatment on plasma membrane integrity and cell damage was also assessed by measuring LDH activity in the culture medium of cardiomyocytes treated with or without 0.5 μM DOX for 1, 2, 6, and 24 h (Figure 1B). An increase in LDH activity over time in culture was observed. There were no significant effects of FGF-2 and/or DOX observed at 1 and 2 h treatment, but there were significant increases in LDH activity detected after DOX treatment at 6 and 24 h. These increases were not observed in cultures co-treated with DOX and FGF-2 (Figure 1B). This FGF-2-mediated decrease in LDH release was blocked with 20 μM SU5402, an FGF receptor inhibitor, at 24 h (Figure 1C). In addition, pre-treatment with increasing concentrations of FGF-2 with DOX indicated a dose-dependent effect on blocking LDH release and an EC50 of about 1.5 nM FGF-2 as tested (Figure 1D).

3.2 Resistance to DOX-induced damage seen with FGF-2 is sensitive to PKC inhibition

A role for PKC activation by FGF-2 was investigated by adding PKC inhibitors, either 5 μM chelerythrine (Figure 2A) or 20 nM BIM-1 (Figure 2B), before co-treatment with FGF-2, and subsequent addition of DOX. Cultures treated with DOX or no pre-treatment were included as controls. There was no effect of FGF-2 and either chelerythrine or BIM-1 when used alone at 24 h, however, there was a significant (2–6.5-fold) increase in LDH activity in the presence of DOX (Figure 2). As expected, a significant decrease in LDH activity was observed with FGF-2 pre-treatment in the presence of DOX at 24 h compared with DOX treatment alone. These decreases were blunted with either chelerythrine or BIM-1, such that levels were not significantly different from cultures treated with DOX alone.

3.3 MDR1, MRP1, and MRP2 RNA levels are increased by FGF-2 in the presence of DOX compared with DOX treatment alone

A possible effect of FGF-2 on efflux (drug) transporter RNA levels in cardiomyocytes was assessed by qPCR after 24 h (Figure 3). DOX administration induced a 10 and nine-fold increase in MDR1a and MDR1b RNA levels, respectively. In contrast, a 95% decrease in MDR2 RNA levels, respectively. In contrast, a 95% decrease in MDR2 RNA levels, respectively. In contrast, a 95% decrease in MDR1b RNA levels, respectively. In contrast, a 95% decrease in MDR1a and MRP1 transcripts. An attempt was also made to assess MRP2 RNA levels, however, although a low signal was detected with DOX treatment, the ‘untreated’ control was below the level of detection relative to MDR1, MDR2, and MRP1 transcripts. An increase in MDR1a RNA levels was suggested with FGF-2 treatment (significant by t-test) but, as with MDR1b and MRP1, the difference was not significant by ANOVA. A significant 50% decrease in MDR2 RNA levels was detected with FGF-2 administration. MDR1a, MDR1b, MRP1, and MRP2 RNA levels were all increased significantly with FGF-2 treatment in the presence of DOX, when compared with DOX alone: the increase in MDR2 RNA levels was ‘just not significant’ by ANOVA, but very significant by a t-test. To assess whether these increases were PKC-dependent, cultures were treated with or without chelerythrine prior to treatment with FGF-2 and DOX or DOX alone for 24 h.

3. Results

3.1 FGF-2 pre-treatment increases resistance to DOX-induced injury

The effect of 10 nM FGF-2 pre-treatment on cell injury induced by 0.5 μM DOX in neonatal rat cardiomyocyte cultures was assessed at 24 h, using a combination of fluorescent markers for apoptosis and necrosis with FACS analysis (Figure 1A and see Supplementary material online, Figure S1). No effect of FGF-2 treatment alone was observed, and cells staining for either the apoptosis or necrosis marker accounted for <10% of the total cell population. There was, however, a consistent and significant increase in both ‘apoptosis’ and ‘necrosis’ positive cells with DOX treatment. The DOX effect was blunted by FGF-2 pre-treatment (Figure 1A). A significant decrease in total cell number, as measured by nuclear DNA staining, was also consistent with increased cell death induced by DOX, and the benefit of FGF-2 pre-treatment on cell survival (see Supplementary material online, Figure S3). The effect of 10 nM FGF-2 pre-treatment on plasma membrane integrity and cell damage was also assessed by measuring LDH activity in the culture medium of cardiomyocytes treated with or without 0.5 μM DOX for 1, 2, 6, and 24 h (Figure 1B). An increase in LDH activity over time in culture was observed. There were no significant effects of FGF-2 and/or DOX observed at 1 and 2 h treatment, but there were significant increases in LDH activity detected after DOX treatment at 6 and 24 h. These increases were not observed in cultures co-treated with DOX and FGF-2 (Figure 1B). This FGF-2-mediated decrease in LDH release was blocked with 20 μM SU5402, an FGF receptor inhibitor, at 24 h (Figure 1C). In addition, pre-treatment with increasing concentrations of FGF-2 with DOX indicated a dose-dependent effect on blocking LDH release and a EC50 of about 1.5 nM FGF-2 as tested (Figure 1D).

3.2 Resistance to DOX-induced damage seen with FGF-2 is sensitive to PKC inhibition

A role for PKC activation by FGF-2 was investigated by adding PKC inhibitors, either 5 μM chelerythrine (Figure 2A) or 20 nM BIM-1 (Figure 2B), before co-treatment with FGF-2, and subsequent addition of DOX. Cultures treated with DOX or no pre-treatment were included as controls. There was no effect of FGF-2 and either chelerythrine or BIM-1 when used alone at 24 h, however, there was a significant (2–6.5-fold) increase in LDH activity in the presence of DOX (Figure 2). As expected, a significant decrease in LDH activity was observed with FGF-2 pre-treatment in the presence of DOX at 24 h compared with DOX treatment alone. These decreases were blunted with either chelerythrine or BIM-1, such that levels were not significantly different from cultures treated with DOX alone.

3.3 MDR1, MRP1, and MRP2 RNA levels are increased by FGF-2 in the presence of DOX compared with DOX treatment alone

A possible effect of FGF-2 on efflux (drug) transporter RNA levels in cardiomyocytes was assessed by qPCR after 24 h (Figure 3). DOX administration induced a 10 and nine-fold increase in MDR1a and MDR1b RNA levels, respectively. In contrast, a 95% decrease in MDR1b RNA levels, respectively. In contrast, a 95% decrease in MDR1a and MRP1 transcripts. An increase in MDR1a RNA levels was suggested with FGF-2 treatment (significant by t-test) but, as with MDR1b and MRP1, the difference was not significant by ANOVA. A significant 50% decrease in MDR2 RNA levels was detected with FGF-2 administration. MDR1a, MDR1b, MRP1, and MRP2 RNA levels were all increased significantly with FGF-2 treatment in the presence of DOX, when compared with DOX alone: the increase in MDR2 RNA levels was ‘just not significant’ by ANOVA, but very significant by a t-test. To assess whether these increases were PKC-dependent, cultures were treated with or without chelerythrine prior to treatment with FGF-2 and DOX or DOX alone for 24 h.
Relative MDR1b, MDR2, MRP1, and MRP2 RNA levels were decreased significantly, while MDR1a transcript levels were unaffected by PKC inhibition (Figure 4A). There was also no effect of chelerythrine alone on MDR1a and 1b RNA levels, while those of MDR2 and MRP1 were decreased (Figure 4B). Furthermore, FGF-2 RNA levels were reduced significantly \( \approx 50\% \) after DOX treatment (see Supplementary material online, Figure S4).

### 3.4 FGF-2 has a positive effect on efflux transport

Retention of calcein was assessed to measure the effect of FGF-2 and DOX on efflux transporter activity. As positive controls, cardiomyocytes were pre-treated with 20 \( \mu \text{M} \) CsA, an inhibitor of MDR1 and MRP2 transporters,\(^30\) 2 \( \mu \text{M} \) verapamil, which blocks MDR1 in neonatal rat cardiomyocytes,\(^31\) and 1 \( \mu \text{M} \) XR9576, which is reported to inhibit MDR1 but not MRPs.\(^32\) Treatment with CsA, verapamil, and XR9576 resulted in significant increases in calcein in the presence and absence of DOX (Figure 5). In contrast, a significant decrease in calcein (evidence of increased efflux/removal) was observed with FGF-2 treatment in the presence and absence of DOX at 24 h (Figure 5A and see Supplementary material online, Figure S5). A direct effect on DOX levels was assessed by detecting DOX autofluorescence in cardiomyocytes by FACS. A significant increase in fluorescence was seen with DOX treatment, while levels were decreased with FGF-2 pre-treatment and DOX addition when compared with DOX treatment alone (Figure 5B and see Supplementary material online, Figure S6).

A possible relationship between the stimulation in efflux transport and increase in resistance to DOX-induced cell injury by FGF-2, as measured by LDH release, was examined by adding the transport inhibitors CsA, verapamil, or XR9576 to cultures before treatment with or without FGF-2 and/or DOX. There was no effect of CsA, verapamil, or XR9576 alone on LDH activity, but when each one was used in combination with DOX, there was a significant increase in LDH relative to DOX treatment alone (Figure 6A–C). This is consistent with retention of DOX and presumably increased cardiomyocyte membrane damage and LDH release resulting from the higher intracellular DOX concentration. As expected, FGF-2

---

**Figure 3** MDR1, MDR2, and MRP1 RNA levels are increased by FGF-2 in the presence of DOX. Cultures were treated with 10 nM FGF-2, 0.5 \( \mu \text{M} \) DOX, or pre-treated with FGF-2 for 30 min before treatment with DOX for 24 h. Isolated RNA was assessed by qPCR. The data for each of the RNAs (indicated) are expressed as described (for PCD+ cell number) in Figure 1A, except for MRP2, where a t-test was used.

**Figure 4** Decreases in MDR/MRP RNA levels are seen with chelerythrine pre-treatment, in the presence of FGF-2 and DOX. (A) Cultures were treated with 10 nM FGF-2 and 0.5 \( \mu \text{M} \) DOX without or with 5 \( \mu \text{M} \) chelerythrine (Chel) pre-treatment, as well as (B) Chel treatment alone as a control. RNA was assessed by qPCR at 24 h. The results are expressed as the mean \( \pm \) SEM, relative to the FGF-2 and DOX treatment values (FGF-2 + DOX), which are arbitrarily set to 100%. Data were assessed by t-test: \* \( P < 0.05 \); \** \( P < 0.01 \); \*** \( P < 0.001 \).
FGF-2 increases resistance to doxorubicin damage

Pre-treatment with FGF-2 interfered with LDH release indicating a significant effect on maintaining plasma membrane integrity. This increased resistance to damage was blunted with both chelerythrine and BIM-1, implicating PKC activation in the beneficial effect of FGF-2. More specifically, treatment with FGF-2 and DOX was shown to increase efflux transport and relative MDR/MDR RNA levels, when compared with DOX treatment alone, and the effect on all but MDR1a was sensitive to chelerythrine. Finally, the cytoprotective effect of FGF-2 was reduced or eliminated when cultures were treated with the efflux transport inhibitors CsA, XR9576, or verapamil, prior to FGF-2 and DOX treatment. This beneficial effect of exogenous FGF-2 may work in part by counteracting a reduction in endogenous FGF-2 synthesis, suggested by the decrease in RNA levels in the presence of DOX (see Supplementary material online, Figure S4). These observations implicate for the first time regulation of efflux drug transporter production and function in the cytoprotective properties of cardiomyocytes by FGF-2.

Apoptosis and necrosis are common mechanisms of acute and chronic cardiomyocyte loss from DOX-induced injury.134 Both annexin-V and ethidium homodimer III stain cells after severe injury and in the late stage of cell death regardless of an apoptotic or necrotic path. Thus, cells staining with both markers were included as injured in this analysis. Features of necrosis include swelling of cytoplasm and mitochondria, loss of membrane integrity, and cell lysis resulting in LDH release. We consistently observed significant decreases in LDH activity with 0.5 μM DOX treatment of our cardiomyocyte cultures (P < 0.001, n = 6), but some variation in the degree of response was noted between preparations. This presumably reflects the status of cells (and their membranes) following isolation, as illustrated by the 2.6-fold increase in LDH activity observed in an untreated ‘control’ culture over 24 h.

CsA is an effective immunosuppressant but is also reported to be cardioprotective in the isolated rat heart in a dose-dependent manner.135 We saw no effect of 20 μM CsA on cell damage/LDH release, but LDH activity doubled when CsA was used in combination with DOX. This is consistent with the reported role of CsA as an inhibitor and modulator of MDR1 and MRP2 efflux transporter function.30,36 and suggests increased retention and sensitivity to the damaging effects of DOX. Thus, the ability of CsA pre-treatment to interfere with the cytoprotective effect of FGF-2 in our cultures is consistent with a mechanism involving efflux transport. This was supported when the beneficial effect of FGF-2, defined as a significant reduction in LDH release compared with DOX treatment alone, was suppressed with the MDR1/P-glycoprotein inhibitors verapamil and XR9576. There was, however, a partial rescue by FGF-2 of the negative effect of CsA and XR9576 on LDH release in the presence of DOX. Thus, FGF-2 activity is not completely blocked by CsA and XR9576, whereas verapamil abrogated the beneficial effects of FGF-2. Further studies to evaluate the biological activities of verapamil, including blocking L-type calcium channels, may provide further insight into the mechanism of cardiomyocyte protection by FGF-2.

FGF-2 is known to affect sensitivity to anti-cancer drugs including DOX37–39 and cardiomyocytes express efflux transporters.19 Thus, the possibility existed that FGF-2 might also affect efflux transporter gene expression. MRP2 transcripts were, as expected, relatively low in our cultures, and only detected after DOX treatment;19 a further modest increase in MRP2 RNA was seen in the presence of FGF-2.

4. Discussion

Here we show for the first time that exogenous FGF-2 increases resistance to DOX-induced damage in neonatal rat cardiomyocytes.

Figure 5 FGF-2 has a positive effect on efflux transport in cardiomyocytes on (A) calcine and (B) DOX levels. Cardiomyocytes were pre-treated with 10 nM FGF-2, 0.5 μM DOX, or FGF-2 and DOX for 24 h. As a positive control, cardiomyocytes were pre-treated with efflux transport inhibitors, including 20 μM cyclosporine A (CsA), 2 μM verapamil, and/or 1 μM XR9576, with and without DOX. The results in (A) are expressed relative to the control values (untreated, or DOX alone as appropriate), and assessed as described (for PCD+ cell number) in Figure 1A. For (B), DOX concentration was calculated from a standard curve (see Supplementary material online, Figure S6B).

promoted a significant decrease in DOX-induced LDH release, approaching ‘control’ levels. This beneficial effect of FGF-2 was not observed with CsA, verapamil, or XR9576 pre-treatment. There was, however, a significant decrease in LDH release when FGF-2 was added in combination with CsA or XR9576, but not verapamil, in the presence of DOX. This partial rescue of the effect of CsA and XR9576 on increased retention and sensitivity to DOX did not go beyond DOX treatment alone and approach ‘control’ levels of LDH activity.
In contrast, MDR1a and 1b, as well as MDR2 and MRP1 transcripts appear to be relatively more abundant than MRP2, and RNA levels were increased by FGF-2 in the presence of DOX, providing further support for possible participation of CsA-sensitive MDR1 and MRP2. Additional evidence comes from our observations that the cytoprotection by FGF-2 is PKC-dependent, and that the increase in MDR1b, MDR2, and MRP2 RNA levels seen with FGF-2 and DOX vs. DOX treatment alone, were also sensitive to PKC inhibition; however, MDR1a levels were not affected by PKC inhibition. MDR-1b and MDR-2 are expressed at relatively high levels (>90% among MDRs) in heart tissue, compared with only 8% for MDR-1a. Thus, inhibition of MDR-1b and MDR-2 (as well as MRPs) by PKC inhibitors appears to be sufficient to block the beneficial effects of FGF-2 in relation to DOX-induced damage. This does not rule out possible participation of other efflux transporter proteins, or that additional signalling pathways may not affect levels/activity. Efflux transporter MDR-1a, 1b, and MRP-2 RNA levels were increased by DOX treatment, suggesting a positive effect on DOX efflux. This must be balanced with a decrease in MDR-2 transcripts, which suggests retention or a negative effect on DOX efflux. FGF-2, however, increased all efflux transporter RNAs examined, consistent with increased efflux or less retention of DOX depending on the specific transporter RNA. As such, all transporters may participate in FGF-2 protection against DOX. A decrease in calcein efflux or transporter function was seen with FGF-2 but not DOX alone. DOX (or FGF-2) can, however, increase MDR-1a and 1b but decreases MDR-2 RNA levels. Presumably the lack of significant changes in calcein levels would reflect the sum of effects of DOX (vs. control) on all transporters, including MDRs and MRPs.

The potential involvement of multiple signalling pathways and efflux drug transporters, raises the possibility that conditions offering different or varying degrees of effects on cardiomyocytes vs. tumour cells can be identified. This might be important, as similar effects on tumour cells would, perhaps, offset the possible benefit of FGF-2 to decrease the vulnerability of cardiomyocytes to DOX-induced injury. Support for differential effects on cardiomyocytes vs. tumour cells comes from data suggesting that FGF-2 can sensitize tumour cells, including those of breast and ovarian origin, to anti-cancer platinum compounds like cisplatin as well as, but to a much lesser degree, DOX. In this context, and although not explored, the number and type of FGF receptors on the tumour cells might also affect the response. Taken together, these observations are consistent with involvement of multiple signalling pathways that can result in chemo-resistance and/or chemo-sensitization, triggered in turn by factors influenced by dose and/or time of exposure to FGF-2.

In addition to its cardioprotective effects, FGF-2 is a potent angiogenic factor and as such may contribute to myocardial repair following DOX-induced damage, as it does following ischaemic injury in vivo. The beneficial effects of FGF-2 on efflux transport may extend beyond cardiomyocytes and include endothelial cells, which overexpress P-glycoprotein in response to DOX. This might increase available endothelial cells and thus indirectly benefit a subsequent angiogenic effect, in addition to increasing cardiomyocyte survival.

In summary, FGF-2 increases resistance to injury in cardiac cells in vitro and in the myocardium in vivo, and positive effects on mitochondrial function, programmed cell death and cell–cell communications have been implicated in these events via FGFR and PKC signalling. Increased removal of cytotoxic agents can now be added as a potential mechanism of cardiomyocyte protection, and specifically increased resistance to DOX-induced damage via an increase in efflux transporter production and function. This property would also be consistent with the concept of a role for FGF-2 in ‘self-protection’ of cardiomyocytes (and myocardium), and the observation that FGF-2-deficient mice are more susceptible to injury.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank J.-J. Santiago, B.E. Nickel, and R. Fandrich for preparation of FGF-2 and Dr M. Chan for expert technical assistance with fluorescent-activated cell sorting.
FGF-2 increases resistance to doxorubicin damage

Conflict of interest: none declared.

Funding

This work was supported by a grant from the Heart and Stroke Foundation of Canada (P.A.C.).

References

32. Stewart A, Steiner J, Mellows G, Laguda B, Bevan P. Phase I trial of XR9576 (Mdr1a, Mdr1b, Mdr2) in mice. Cardiovascular Therapeutics: translating molecular mechanism into preventative medicine.