Disease Models & Mechanisms 5, 259-269 (2012) doi:10.1242/dmm.008110

Metformin differentially activates ER stress signaling pathways without inducing apoptosis

Thomas Quentin[1,*], Michael Steinmetz[1], Andrea Poppe[1] and Sven Thoms[2,*]

SUMMARY

Endoplasmic reticulum stress signaling (ERSS) plays an important role in the pathogenesis of diabetes and heart disease. The latter is a common comorbidity of diabetes and worsens patient outcome. Results from clinical studies suggest beneficial effects of metformin – a widely used oral drug for the treatment of type 2 diabetes – on the heart of diabetic patients with heart failure. We therefore analyzed the effect of metformin on ERSS in primary rat cardiomyocytes. We found that metformin activates the PERK-ATF4 but not the ATF6 or IRE1-XBP1 branch in ERSS and leads to a strong upregulation of *CHOP* mRNA and protein. Surprisingly, long-term induction of CHOP by metformin is not accompanied by apoptosis even though CHOP is regarded to be a mediator of ER-stress-induced apoptosis. In conclusion, metformin induces distinct ER stress pathways in cardiomyocytes and our results indicate that CHOP is not necessarily a mediator of apoptosis. Metformin might exert its cardioprotective effect through selective activation of ERSS pathways in the cardiomyocyte.

INTRODUCTION

Metformin is a commonly used drug in the treatment of type 2 diabetes. Metformin suppresses endogenous glucose output and increases peripheral insulin sensitivity (Stumvoll et al., 1995; Fery et al., 1997). Diabetes is often associated with heart disease and worsens the prognosis of patients with heart conditions. Until recently it was unclear whether metformin was a suitable therapeutic in heart patients because unstable or acute congestive heart failure was reported to be associated with an increased risk of lactic acidosis with metformin (Khurana and Malik, 2010). A recent study, however, suggested that metformin is not contraindicated in conditions of heart failure. Results from a recent meta-study show that it might in fact be the most suitable drug to reduce mortality in diabetic patients with heart conditions (Eurich et al., 2007). For example, one study showed that diabetic patients with heart failure have a lower risk of readmission for heart failure if they are treated with metformin than do patients treated without or with other insulin sensitizers (Masoudi et al., 2005). The mechanisms of this metformin effect are unknown, and the question arises: does this cardioprotective effect directly manifest in the cardiomyocyte?

The ER is the major hub for protein sorting and folding in the cell. Protein overload of the ER can lead to activation of the unfolded protein response (UPR). UPR pathways are crucial in the pathogenesis of many human disorders, including neurophysiological diseases, metabolic disorders such as insulin resistance and type 2 diabetes, and cardiovascular diseases such as cardiac hypertrophy, heart failure, atherosclerosis and ischemic heart disease (Araki et al., 2003; Kim et al., 2008; Minamino and Kitakaze, 2010; Thoms et al., 2009).

Three signaling branches have been described whereby unfolded proteins can be sensed and UPR activated (Ron and Walter, 2007; Rutkowski and Hegde, 2010). Protein kinase RNA (PKR)-like ER kinase (PERK) initiates one of these signaling pathways. PERK is an ER-resident transmembrane protein that couples ER stress signals to translation inhibition. PERK phosphorylates the eukaryotic initiation factor 2 α (eIF2α) and itself at its cytoplasmic kinase domain, which leads to reduction of global protein biosynthesis, one of the feedback loops to reduce protein stress in the ER. eIF2α phosphorylation promotes the expression of activating transcription factors (ATFs) such as ATF4 and ATF5. Inositol-requiring enzyme 1 (IRE1) is the ER-bound sensor of another UPR signaling pathway. IRE1 is a conserved transmembrane protein with an ER-luminal sensor for misfolded proteins. Activation of IRE1 initiates the non-conventional splicing of the transcription factor X-box-binding protein 1 (XBP1), which is responsible for the activation of a larger number of ER-stress responsive genes, including chaperones of the ER. The third ER sensor in UPR pathways is the membrane-bound transcription factor ATF6. Upon activation, ATF6 is cleaved and releases its cytoplasmic domain, which enters the nucleus and activates ER stress response element (ERSE)-dependent gene products, including the ER-luminal chaperone binding immunoglobulin protein [Bip; also referred to as 78-kDa glucose-regulated protein (GRP78)]. Unfolded proteins in the ER are not the only trigger for UPR signaling. Energy deprivation, hypoxia, disturbed ER Ca^{2+} levels, pathogens, drugs and secondary metabolites can also induce UPR signaling. Accordingly, the UPR response can be termed endoplasmic reticulum stress signaling (ERSS).

ER stress that is prolonged or severe can lead to apoptosis. Apoptotic cell death of cardiomyocytes is involved in several cardiac disorders. All three ERSS branches have been associated

[1]Department of Pediatric Cardiology and Pediatric Intensive Care Medicine, and [2]Department of Pediatrics and Pediatric Neurology, University Medical Center, University of Göttingen, Göttingen 37099, Germany
*Authors for correspondence (thomas.quentin@med.uni-goettingen.de; sven.thoms@med.uni-goettingen.de)

Received 21 April 2011; Accepted 10 October 2011

with proapoptotic signals, but, depending on the circumstances and the tissues involved, ERSS can also lead to anti-apoptotic signaling. A downstream component of the pathways leading to apoptosis is the mitochondrial apoptosis machinery, which is able to integrate a number of stressors, including ER stress (Minamino and Kitakaze, 2010). Mitochondrial function and membrane integrity are regulated by the Bcl-2 protein superfamily, which consists of three groups, depending on the number of Bcl-2 homology (BH) domains: the anti-apoptotic members Bcl-2 and Bcl-x_L – containing four BH domains – interact and inhibit proapoptotic Bax and Bak, which contain three BH domains. Alternatively, Bcl-2 members heterodimerize with BH3-only proteins, including Bim and Puma, thereby releasing Bax and Bak, which in turn promote apoptosis. It is believed that CHOP [C/EBP homologous protein; also referred to as GADD153 (growth arrest and DNA damage 153)] mediates ER-stress-dependent apoptosis by downregulating Bcl-2 and elevating oxygen stress (McCullough et al., 2001; Minamino and Kitakaze, 2010).

The effect of metformin on ER stress in cardiomyocytes has not yet been investigated. Therefore, we wanted to study whether metformin is involved in ER stress induction in the heart, and, more specifically, which ERSS pathways are regulated by metformin. Using a rat cardiomyocyte system, we found that metformin differentially induces ER stress pathways but is not associated with apoptotic signaling – in spite of the strong and persistent induction of CHOP. These results might be important in understanding the effect of metformin on the heart, but they also shed light on the possible mode of action of the drug in diabetes.

RESULTS

Metformin-dependent CHOP induction in cardiomyocytes

Freshly isolated and cultivated neonatal rat cardiomyocytes provide a unique cell system to study drug effects on heart cells. To analyze the effect of metformin on cardiomyocytes, we treated freshly isolated neonatal rat cardiomyocytes with 2.5 mM metformin for 48 hours. Metformin significantly increased the mRNA (P<0.0001) and protein levels of CHOP, a prominent marker of several ER-initiated apoptotic signaling pathways (Fig. 1A). Next we tested the concentration and time dependency of CHOP induction (Fig. 1B,C). Starting at a concentration of 2.0 mM metformin, *CHOP* mRNA levels were significantly increased (P<0.0001; Fig. 1B). *CHOP* mRNA induction over control levels was significant (P=0.0002) after 24 hours incubation with 2.5 mM metformin (Fig. 1C). After 32 hours incubation, a plateau was reached and longer time of incubation did not result in a further enhancement of *CHOP* expression (Fig. 1C). *CHOP* mRNA induction was paralleled by induction of the CHOP protein (Fig. 1D).

Induction of the PERK-ATF4 pathway by metformin

To identify possible upstream effectors of CHOP, we analyzed the expression of PERK, which induces CHOP via ATF4. We determined PERK activation by western blotting through its phosphorylation status. We found induction of PERK phosphorylation with a corresponding increase in CHOP protein levels (Fig. 2A). Similar levels of PERK phosphorylation and CHOP induction were found in response to tunicamycin, a mixture of related nucleosides that block N-glycosylation and lead to the

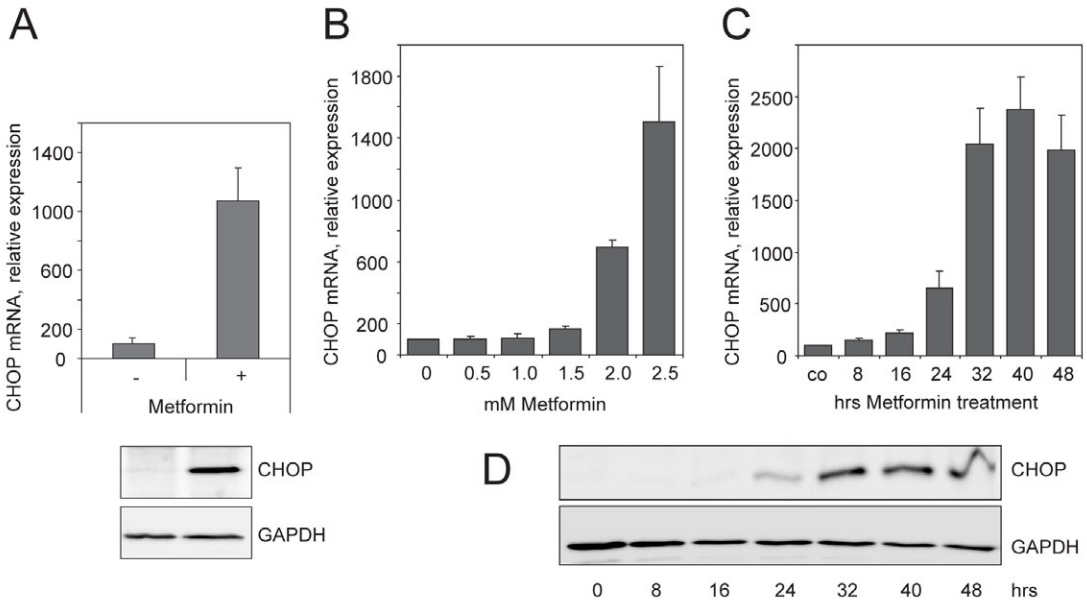

Fig. 1. Metformin leads to the induction of CHOP in rat cardiomyocytes. (A) Induction of *CHOP* mRNA and protein. Neonatal rat cardiomyocytes were cultured for 48 hours in the presence or absence of 2.5 mM metformin. mRNA was reverse transcribed and analyzed by quantitative PCR (qPCR). Measurements were normalized against the expression of the adiponectin receptor. CHOP levels in the absence of metformin were set to 100%. n=8, P>0.0001. Total protein were prepared from the same cultures and analyzed by SDS-PAGE and western blotting. (B) Concentration dependency of CHOP induction. Samples were treated for 48 hours with the indicated concentration of metformin and processed for qPCR as in A. n=2-4. (C) Time course of CHOP induction. Samples were treated for the indicated time span with 2.5 mM metformin and processed as in A. Co, 48 hour treatment in the absence of metformin. n=2. (D) Time course of CHOP protein induction in response to metformin. Total protein was harvested at the indicated time points and analyzed by SDS-PAGE and western blotting.

induction of ER stress (Fig. 2A). Furthermore, in a timecourse experiment, PERK phosphorylation was found to increase concomitantly with CHOP expression (Fig. 2B, Fig. 1D). The activation of the PERK-ATF4 pathway was further shown by quantitative PCR (qPCR) analysis of the PERK-dependent transcription factor ATF4, which was found to be upregulated by metformin in a time- and concentration-dependent manner (Fig. 2C-E). Similar to CHOP induction, saturation of *ATF4* induction was reached after 32 hours in the timecourse experiment (Fig. 2E).

To assess whether *CHOP* induction was indeed dependent on *ATF4*, we reduced *ATF4* levels by knockdown with siRNA. Treatment with *ATF4*-specific siRNA led to an 87% reduction of *ATF4* expression compared with treatment with control siRNA (Fig. 2F). With 13% *ATF4* present, metformin could induce *CHOP* only by a factor of 1.6 compared with a factor of 4.7 in the experiment with control siRNA (Fig. 2G). We noticed that the reduction in *CHOP* inducibility correlated with the efficiency of the *ATF4* knock down (Fig. 2H). These results show that *CHOP* induction is

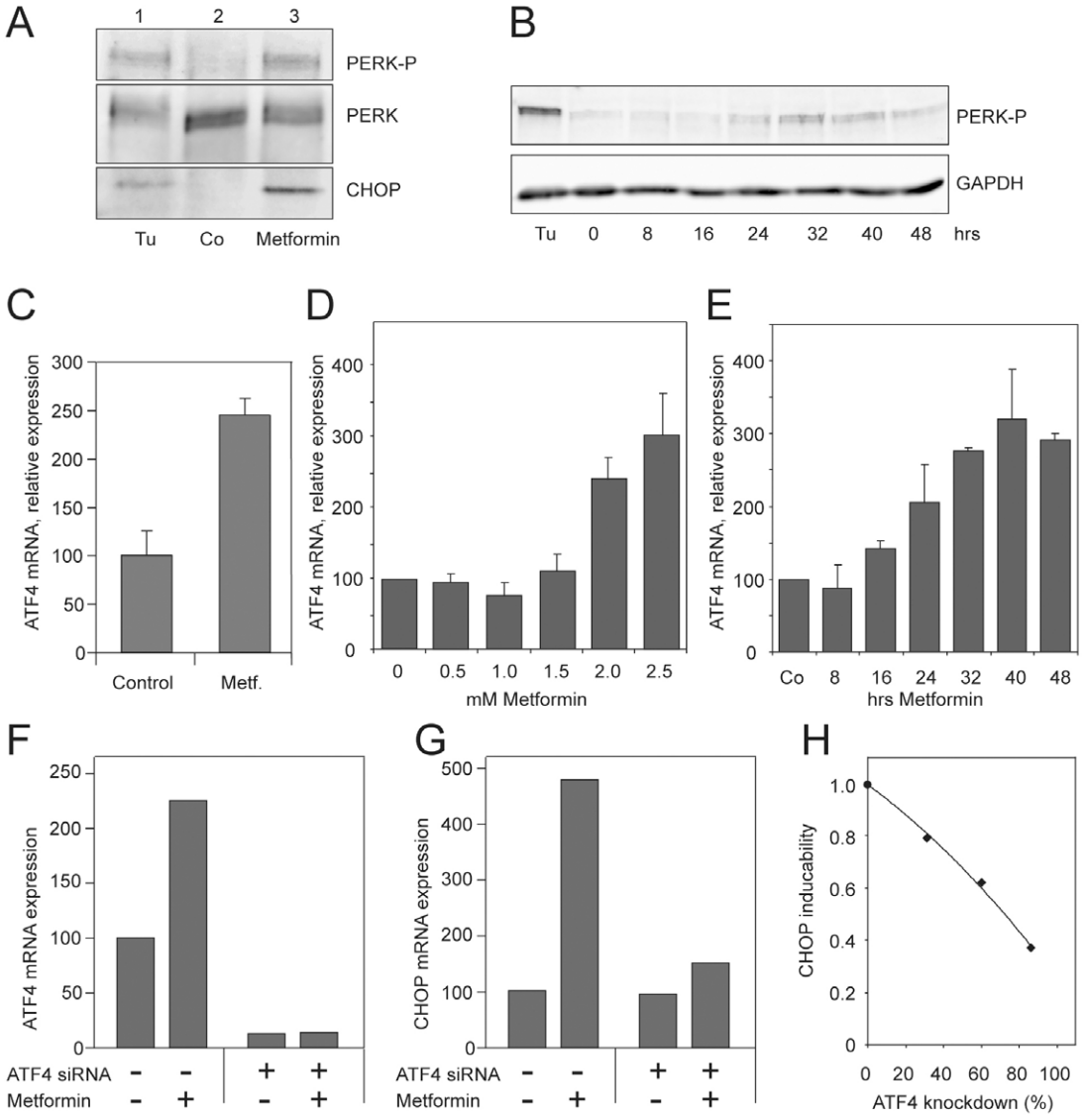

Fig. 2. Induction of the PERK-ATF4 pathway by metformin. (A) Phospho-PERK (PERK-P) is stimulated by metformin. Western blot analysis. Tu, Tunicamycin treatment for 6 hours; Co, 48-hour mock treatment; Metformin, 48-hour 2.5 mM treatment. (B) Time course of PERK phosphorylation in response to metformin. Samples were treated as in Fig. 1A. (C) *ATF4* mRNA induction by metformin. Conditions as in Fig. 1A, *n*=8. (D) Metformin-dependent induction of *ATF4*: concentration dependency after treatment for 48 hours. *n*=2-4. (E) Time course of *ATF4* induction during incubation with 2.5 mM metformin. co, 48-hour incubation in the absence of metformin. *n*=2. (F) Knockdown of *ATF4*. Cells were treated with siRNA directed against *ATF4* or with control siRNA. In both cases, *ATF4* mRNA levels were tested in the absence or presence of metformin. *ATF4* knockdown also blocks metformin-dependent upregulation. (G) Metformin-dependent *CHOP* induction is dependent on ATF4. In cells treated with *ATF4* siRNA, *CHOP* induction by metformin is suppressed. (H) Dependence of CHOP inducibility on *ATF4* knockdown. Degree of *CHOP* induction by metformin (*y*-axis: relative *CHOP* induction by metformin in the absence and presence of *ATF4* siRNA) is negatively correlated with *ATF4* knockdown. *n*=3.

dependent on *ATF4*. In conclusion, we found that metformin activates the PERK-ATF4-CHOP axis in ERSS.

The IRE1-XBP1 and the ATF6 pathways are not induced by metformin

IRE1 catalyzes splicing of *XBP1* mRNA to *XBP1s* mRNA, which is 26 bp shorter than *XBP1* and encodes the basic-region leucine zipper (bZIP) transcription factor XBP1s. *XPB1* splicing is therefore a readout of the activation of the IRE1 arm in UPR signaling. In rodent full-length *XBP1*, a *Pst*I restriction site is destroyed during splicing. Treatment with of up to 2.5 mM metformin for 48 hours did not lead to an increase in *XBP1* splicing in cardiomyocytes (Fig. 3A, lanes 4-8). By contrast, tunicamycin applied for 6 or 3 hours initiated *XBP1* splicing (Fig. 3A, lanes 1 and 2). We quantified the response by expressing *XBP1* splicing as the percentage of the spliced form of *XBP1s* (Fig. 3B). We conclude that the highly conserved IRE1-XBP1 pathway is not induced by metformin.

mRNA expression of *ATF6*, the stress sensor of the third ERSS pathway, was not increased by metformin (Fig. 3C,D). Expression levels of *ATF6* were slightly reduced at lower concentrations or earlier time points only to reach baseline levels at the highest metformin concentration or the latest time points in the timecourse experiment (Fig. 3C,D). Upon activation, ATF6 protein is transported from the ER to the Golgi and cleaved by S1P and/or S2P proteases (Ye et al., 2000). The N-terminal fragment of ATF6 enters the nucleus and acts as a transcription factor for ER chaperones. Thus, *ATF6* mRNA levels are not a sufficient indicator of ATF6 activation. To determine whether metformin activates ATF6, we transfected cells with FLAG-ATF6. This construct allows detection of the N-terminal ATF6 domain by western blotting using

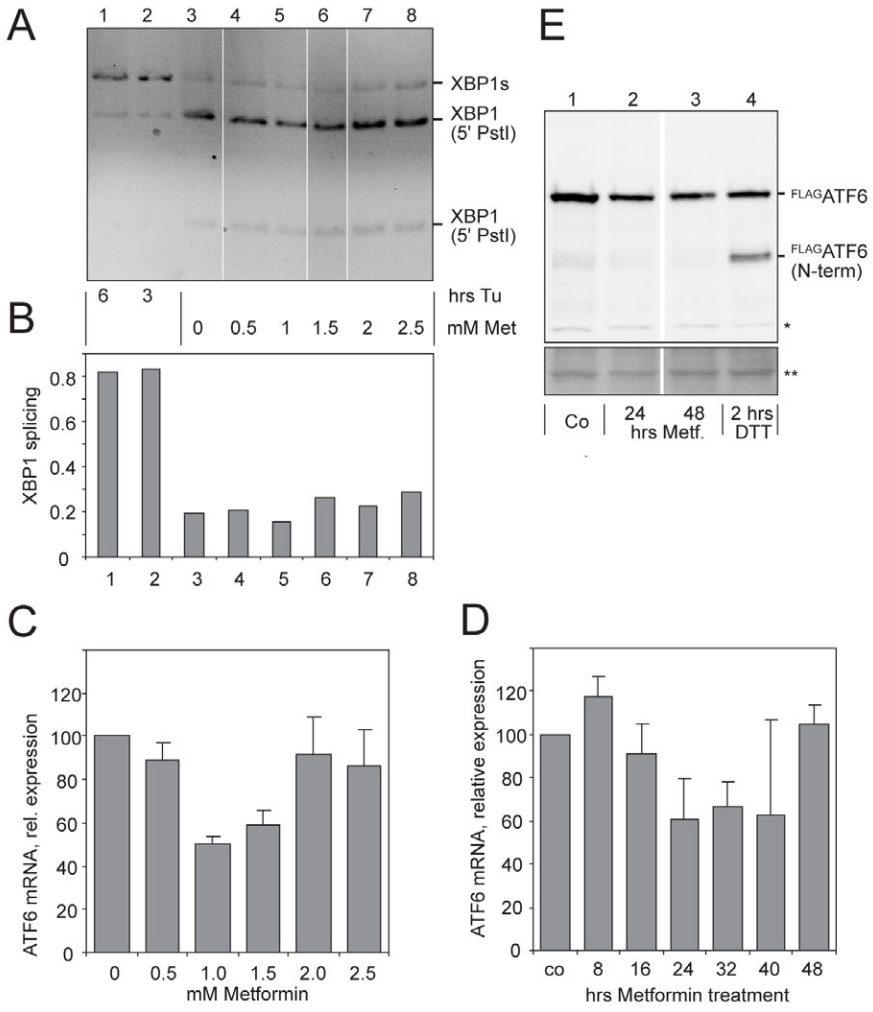

Fig. 3. The IRE1-XPB1 and the ATF6 pathways are not induced by metformin. (A) Metformin does not activate *XBP1* splicing. Splicing of *XBP1* mRNA was analyzed after restriction with *Pst*I. The unspliced form of *XBP1* can be cut by *Pst*I, but *XBP1s* (spliced) is not a *Pst*I substrate. *XBP1* splicing is strongly induced by tunicamycin (Tu). (B) Quantification of A. *XBP1* splicing is expressed as the relative proportion of the intensities of XBP1s and the sum of XBP1s and the larger form of the *Pst*I digest. (C,D) *ATF6* mRNA is not upregulated by metformin treatment. Concentration (C)- and time (D)-dependent analysis of mRNA expression. *n*=2. (E) ATF6 is not activated by metformin. Rat cardiomyocytes were transfected with *FLAG-ATF6*. ATF6 and activated N-terminal domain of ATF6 were detected by western blotting. 5 mM DTT treatment was used as a positive control. The band marked by one asterisk is the only band detected in the absence of transfection (not shown). Two asterisks: Poinceau-S-stained western blot as loading control.

anti-FLAG antibodies. The control experiment with 2 hours 5 mM DTT treatment indicated strong activation of ATF6 (Fig. 3E). By contrast, metformin could not activate ATF6 after 24 or 48 hours (Fig. 3E).

Metformin differentially affects UPR-associated factors

We then investigated the influence of metformin on the expression of other ERSS-associated factors. The luminal ER chaperone Bip is a mediator of ER stress that is intimately connected to all three branches of ERSS (Bertolotti et al., 2000; Shen et al., 2002). Bip is induced in conditions of heart failure and its induction has been associated with protection against ischemic injuries (Nakagawa et al., 2000; Nickson et al., 2007). Bip tended to be upregulated by metformin, but increases were not statistically significant (Fig. 4A) and, in the timecourse and dose-response experiments, the highest increase corresponded to the latest time points and the highest concentrations of metformin, respectively (Fig. 4B,C). Expression levels were found slightly depressed at 1 mM in the concentration curve (Fig. 4B) or after 8-24 hours metformin treatment (Fig. 4C). ATF3 belongs to the ATF family of bZIP transcription factors and has been implicated in a number a cellular stress responses (Thompson et al., 2009). Metformin, however, does not induce ATF3 expression (Fig. 5A,B).

In summary, our experiments show that metformin differentially induces ERSS in cardiomyocytes, with a marked induction of CHOP and the PERK-ATF4 arm and no induction of the IRE-XBP1 or the ATF6 arm of the UPR. Bip is possibly induced, but *ATF3* mRNA was not upregulated. Because CHOP activation is associated with apoptotic signaling, we next tested the effect of metformin on the induction of pro- and anti-apoptotic factors.

Metformin treatment has a differential effect on Bcl-2 and BH domain protein regulation

The anti-apoptotic gene *Bcl-2* is repressed by CHOP, whereas the expression of the pro-apoptotic factor Bim and possibly also the pro-apoptotic and ER-stress-sensitive factor Puma are induced by CHOP (McCullough et al., 2001; Nickson et al., 2007; Puthalakath et al., 2007). We therefore analyzed the effect of metformin on the expression of these factors. *Bcl-2* expression was significantly reduced, whereas metformin did not effect the expression of *Bim* and *Puma* (Fig. 5C). By contrast, 3 or 6 hours tunicamycin treatment led to upregulation of *Bim* and *Puma* (Fig. 5D). This observation stresses the remarkable difference between metformin- and tunicamycin-induced ERSS: although both drugs affect the anti-apoptotic *Bcl-2* gene, only the canonical ER stressor tunicamycin affects the pro-apoptotic BH3-only proteins Bim and Puma.

Apoptosis is not induced by metformin treatment

Apoptotic ER stress has been suggested to be transmitted via activation of caspase 12 (Nakagawa et al., 2000), ultimately leading to the activation of caspase 3, the central executioner of apoptotic cell death. Treatment of cardiomyocytes with metformin did not lead to caspase 12 activation (Fig. 6A,B), whereas tunicamycin was a strong activator of caspase 12 (Fig. 6A,B). The apoptosis-inducer staurosporine is used to underline that caspase 12 activation is specific for ERSS-related apoptosis, because the staurosporine effect bypasses caspase 12 (Fig. 6B). Consequently, caspase 3 is also not activated by metformin, whereas staurosporine is a potent activator of caspase 3 (Fig. 6C).

Finally, we showed that the profound CHOP activation in response to metformin does not lead to apoptotic cell death. Terminal deoxynucleotidyl-transferase dUTP nick end labeling (TUNEL) of mock-, tunicamycin- or metformin-treated cardiomyocytes only showed apoptosis in the case of tunicamycin (Fig. 6D). In accordance with this observation, tunicamycin-treated cardiomyocytes assumed the granular appearance of apoptotic cells, whereas metformin-exposed cells maintained their smooth phenotype (Fig. 6E).

DISCUSSION

Cardiovascular disease is a common comorbidity in diabetic patients and is responsible for 80% of mortality in the diabetic population (Voulgari et al., 2010). In addition to an impairment of coronary vessels, pathophysiological changes in the metabolism of cardiomyocytes themselves are responsible for increased susceptibility of diabetic patients to heart failure leading to a diabetic form of cardiomyopathy and worsening the course of

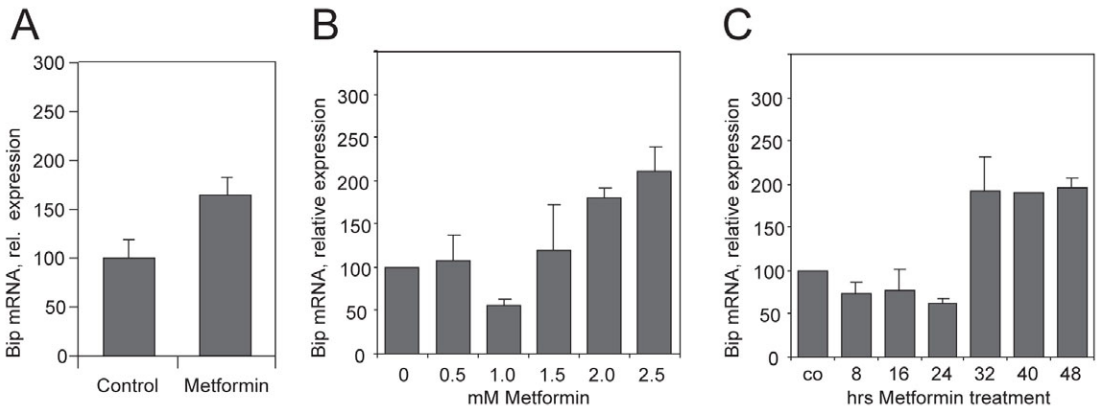

Fig. 4. Effect of metformin on the ER luminal chaperone Bip. (A) Induction of *Bip* (not significant: P=0.1, n=8). (B) Metformin-dependent induction of *Bip*: concentration dependency after treatment for 48 hours. n=2-4. (C) Time course of *ATF4* induction during incubation with 2.5 mM metformin. co, 48-hour incubation in the absence of metformin. n=2 (40 hours: n=1).

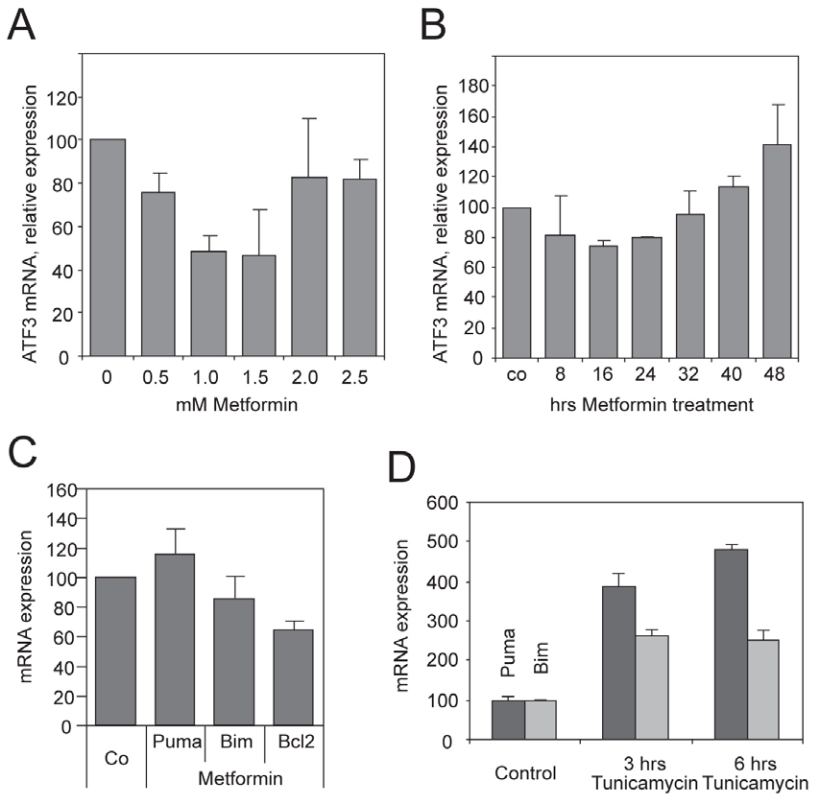

Fig. 5. Effect of metformin treatment on *ATF3* and UPR-to-apoptosis signaling. (A,B) *ATF3* expression is not upregulated upon metformin treatment. Concentration (A)- and time (B)-dependent analysis of mRNA expression. *n*=2. (C) *Puma* and *Bim* mRNA levels are not changed by metformin. *Bcl-2* expression is reduced by 40% ($P<0.02$, *n*=4). (D) Induction of *Puma* and *Bim* by tunicamycin.

ischemic heart insults (Barsotti et al., 2009; Voulgari et al., 2010). Metformin – the most common non-insulin anti-diabetic drug – has been shown to influence cardiac metabolism and its safety for cardiac disease patients is still under debate (Barsotti et al., 2009; Khurana and Malik, 2010). A large-scale clinical trail by the UK Prospective Diabetes Study (UKPDS) Group suggested that metformin reduces the risk of cardiovascular infarction that is associated with diabetes (UKPDS, 1998; Masoudi et al., 2005) and, in a meta-analysis, metformin has been reported to be the only anti-diabetic drug to be beneficial in patients with diabetes and heart conditions (Eurich et al., 2007).

Experiments conducted in vitro and in vivo have shown a beneficial effect of metformin on ischemic cardiomyocytes (Bhamra et al., 2008; Calvert et al., 2008; Solskov et al., 2008; Paiva et al., 2010). Independently, other researchers have shown that ER stress induces apoptosis in ischemic cardiomyocytes (Okada et al., 2004; Azfer et al., 2006; Szegezdi et al., 2006; Thuerauf et al., 2006). In the light of these results it became necessary to investigate the direct effect of metformin on ER stress pathways in order to gain more insight into the complex action of metformin on cardiomyocytes and to understand the molecular basis of its impact on the heart. We therefore investigated the influence of metformin on the ER stress pathways in cardiomyocytes using isolated neonatal rat cardiomyocytes.

We found that metformin treatment leads to a strong and persistent induction of CHOP. Consistently with CHOP activation, we found a pronounced activation of the PERK pathway (Fig. 7). Interestingly, however, the induction of the PERK-CHOP axis was not associated with increased levels of apoptosis. This is an unexpected finding because inducers of CHOP are known to lead to the induction of apoptosis. For cardiomyocytes, it was shown that CHOP induction by ischemia or mechanical stress results in apoptosis (Szegezdi et al., 2006; Cheng et al., 2009). We therefore investigated possible downstream targets in CHOP-mediated apoptosis in order to investigate their activation status after metformin treatment.

Factors mediating CHOP-induced apoptosis

Members of the Bcl-2 family are a possible CHOP target in apoptotic signaling. Pro-apoptotic Bcl-2 family members can trigger apoptosis by mitochondrial membrane permeabilization. For this to occur, the relative proportion of the anti-apoptotic and pro-apoptotic members is essential (Wong and Puthalakath, 2008; Weston and Puthalakath, 2010). Bcl-2 and the pro-apoptotic proteins Bim and Puma are transcriptionally regulated in response to cell stress (Wong and Puthalakath, 2008). The BH3-only family proteins Puma, Noxa, Bid and Bim have been described to mediate apoptosis after ER stress activation (Li et al., 2006; Puthalakath et al., 2007; Upton et al., 2008). A CHOP-dependent upregulation of the BH3-only protein Bim by ER stress, induced by thapsigargin, has been shown for fibroblast cell lines and thymocytes (Puthalakath et al., 2007). Puma has been shown to be responsive to chemical ER stressors and hypoxia and/or reoxygenation in cardiomyocytes (Toth et al., 2006; Nickson et al., 2007). We found that metformin leads to downregulation of *Bcl-2* in cultured neonatal rat cardiomyocytes. This might be a response to CHOP induction, because CHOP has been shown to decrease Bcl-2 expression in HeLa cells and fibroblasts (McCullough et al., 2001). By contrast, metformin failed to upregulate *Bim*, providing a possible explanation for the absence of apoptosis. Similarly, we

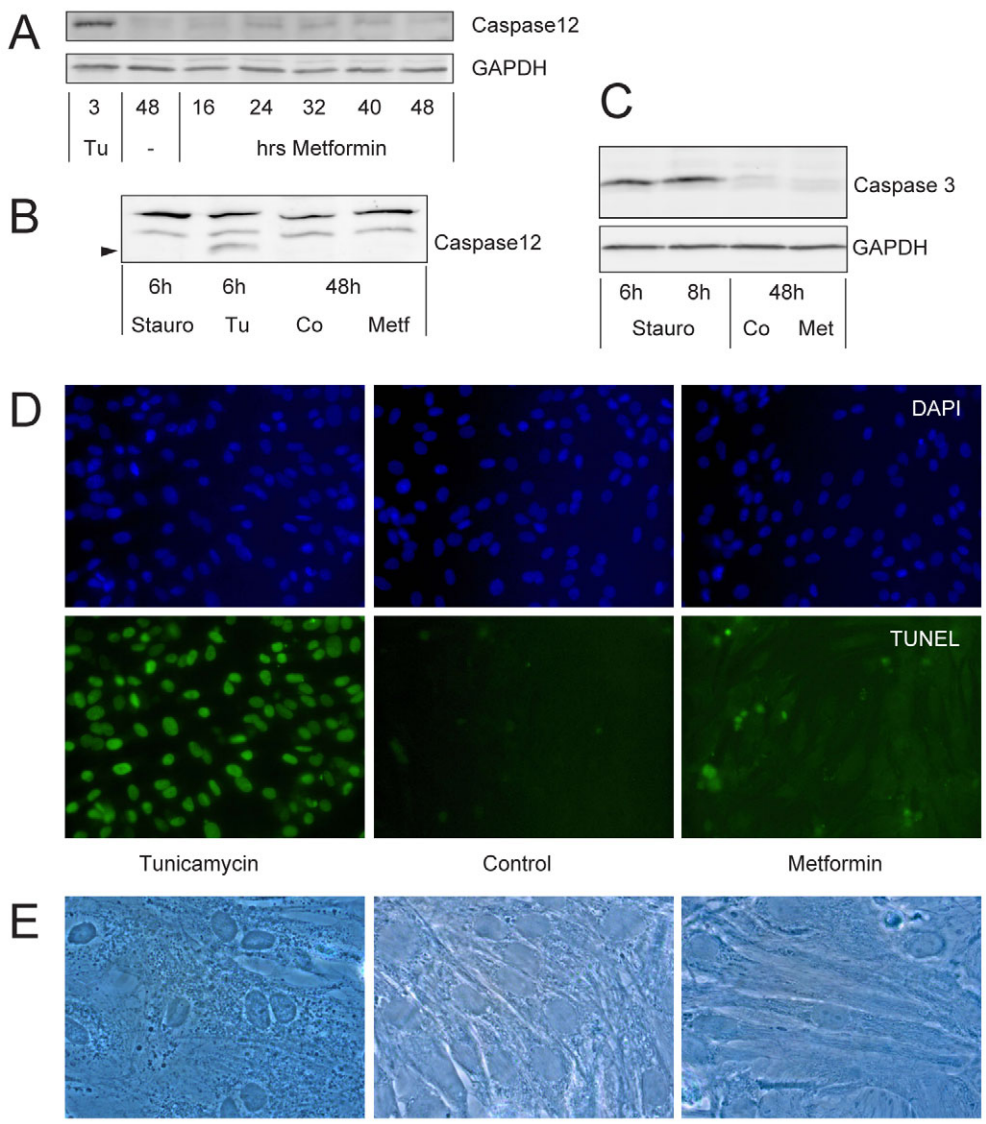

Fig. 6. Metformin treatment does not induce apoptosis. (A,B) Caspase 12 is not induced by metformin. (B) Caspase 12 is specifically induced by tunicamycin (Tu), but not by staurosporine. Co, control. (C) Induction of caspase 3 by staurosporine, but not by metformin. (D) Tunicamycin, but not metformin, causes apoptosis in neonatal rat cardiomyocytes: TUNEL (48 hours of 2.5 mM metformin; 6 hours of tunicamycin; mock treatment of 48 hours). (E) Phase-contrast bright-field microscopy: metformin does not induce apoptotic stress granules in cardiomyocytes. Cells were treated as in D.

observed that the pro-apoptotic BH3-only protein Puma was upregulated by tunicamycin-induced ER stress but not by metformin (Fig. 7).

The results of a recent publication support our finding that CHOP induction does not necessarily result in the onset of apoptosis. Neuronal cultures from *CHOP*$^{-/-}$ mice were found to be less sensitive to thapsigargin, and overexpression of CHOP protects neuronal cells against hypoxic injury (Halterman et al., 2010). Together with our results, these data challenge the common notion of CHOP as a pro-apoptotic factor.

Caspase 3 and 12 and the execution of apoptosis

Caspase 3 is the central executioner protein of apoptosis. The role of caspase 12 in ER-dependent apoptosis is still under debate, because functional caspase 12 has not been found in humans (Fischer et al., 2002). In neonatal rat cardiomyocytes, however, caspase 12 is activated when CHOP is induced by tunicamycin or thapsigargin (Szegezdi et al., 2006; Thuerauf et al., 2006). If CHOP is induced by hypoxia, some authors find caspase 12 activation (Terai et al., 2005; Szegezdi et al., 2006), whereas other authors do not find an involvement of caspase 12 (Thuerauf et al., 2006). In addition, knock down of caspase 12 has been shown to reduce hypoxia-induced apoptosis (Terai et al., 2005). It can be concluded that caspase 12 is involved in ER-stress-induced apoptosis in rodent cardiomyocytes. It is possible that caspase 12 is representative of another pathway or amplifies the Bcl-2 pathway. Our experiments show that metformin does not activate caspase 3 or caspase 12 and, accordingly, does not induce apoptosis in rat cardiomyocytes (Fig. 7). It has recently been suggested that metformin might exert its cardioprotective effect by enhancing the integrity of the mitochondrial membrane (Bhamra et al., 2008).

Cardioprotection through Bip

Beyond those proteins that are involved in specific ERSS pathways, the ER luminal chaperone Bip is one of the most general markers for a general induction of ER stress. Bip is induced in conditions of heart failure and its induction has been associated with protection against ischemic injuries and cardioprotection in general

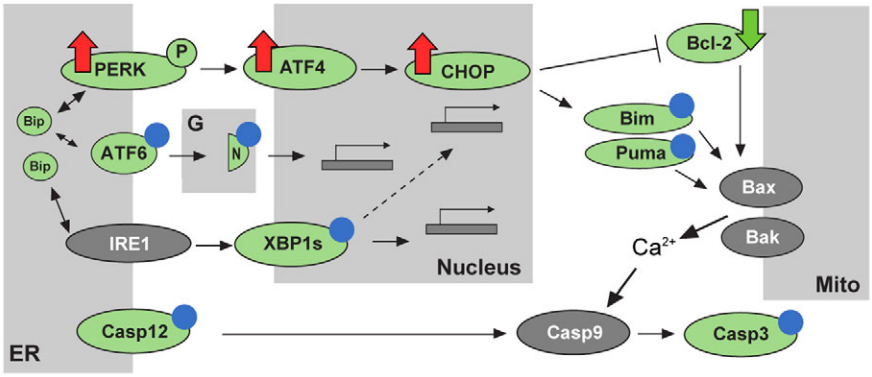

Fig. 7. Model: metformin triggers specific ERSS pathways without inducing apoptosis. Schematic integration of our results into the ERSS and apoptosis pathways. Factors investigated in this study are highlighted in green and symbols nearby indicate the effect of metformin on these factors: red and green arrows indicate upregulation and downregulation, respectively; gene products marked with a blue circle remain unchanged. Metformin leads to activation and phosphorylation of PERK, and, in turn, the upregulation of the transcription factor ATF4, which is one of the activators of the transcription factor CHOP. Chemical activation of ERSS (e.g. by tunicamycin) shifts the stoichiometric relation of Bcl-2 family members towards apoptosis by upregulation of proapoptotic BH3-only proteins (including Bim and Puma) and by downregulation of anti-apoptotic members such as Bcl-2. These changes are believed to be mediated by CHOP. By contrast, metformin leads to downregulation of Bcl-2, but the expression levels of Bim and Puma remain unchanged. Specific chemical activation of ERSS also leads to the activation of the ER-located caspase 12, and to splicing and activation of *XBP1*. By contrast, metformin neither activates *ATF6* proteolytic processing nor the IRE1-XBP1 arm of ERSS. In consequence, metformin does not activate the mitochondrial- and caspase-12-dependent ER stress pathways that eventually lead to the activation of caspase 3. G, Golgi compartment.

(Terai et al., 2005; Shintani-Ishida et al., 2006; Thuerauf et al., 2006; Groenendyk et al., 2010). Part of the Bip pool is found on the cell surface and reported to protect cells thereby (Hardy and Raiter, 2010). We found an approximate twofold induction of *Bip* upon metformin treatment, supporting the hypothesis that metformin induces ER stress in a manner that promotes the survival of cardiomyocytes.

Specific activation of the PERK-ATF4-CHOP axis

In our experiments, *Bip* activation was, however, weak in comparison to classical activators of ER stress. With respect to the downstream effects of PERK-ATF4 activation, this might indicate that we have to look for another protective ER stress factor to explain the cardioprotective role of metformin. With respect to the upstream factors of the PERK-ATF4 axis, this might explain the specific activation of only one of the three ER stress pathways, if the ER stress sensors respond to Bip recruitment with different threshold levels. The late induction of PERK by metformin is consistent with the idea that PERK is not directly activated by metformin. We speculate that the effect of metformin effect on the energy status of the cell causes the PERK-specific induction of UPR signaling.

Conclusion

Our data show that metformin triggers the UPR without inducing apoptosis. We speculate that metformin-dependent UPR induction capitalizes on the beneficial sites of ERSS, such as chaperone induction, but keeps the stress response below a threshold level that would induce apoptosis. CHOP induction alone is not sufficient for induction of apoptosis in cardiomyocytes and might require the induction of secondary pathways. It will now be interesting to understand how metformin leads to the induction of ERSS and whether activation of the well-known metformin target, AMPK, is involved in this process. The model presented in this study can help to understand which parameters trigger UPR-mediated protection and under which circumstances UPR contributes to apoptosis and tissue damage.

METHODS

Cardiomyocyte preparation and cell culture

For preparation of cardiomyocyte cultures, neonatal Wistar rats were sacrificed on day 1. Hearts were excised quickly, rinsed in PBS and cut into small pieces. Subsequently, tissue pulp was dissolved in a solution of 0.1% collagenase B (Cell Systems) with 0.2% trypsin (Biochrom) in PBS (Invitrogen) at 37°C. Cells were collected by centrifugation at 300 ***g*** for 5 minutes and resuspended in DMEM:F12 medium (Invitrogen) containing 10% FCS (Biochrom) with 1% penicillin/streptomycin (Invitrogen). Cells were plated for 90 minutes in 87-mm-diameter culture dishes at 37°C and 5% CO_2 to enrich the cardiomyocyte fraction and to remove fibroblasts. Under these conditions, fibroblasts adhere preferentially to the bottom of the culture dish. The non-adherent cells in the supernatant were collected by centrifugation as described above and plated on gelatine-coated (Sigma) 36-mm culture dishes (Nunc) at a density of approximately 100,000 cells per cm^2. At 1 day after isolation, the medium was renewed. At day 2 the cells formed a confluent spontaneously beating monolayer. After 48 hours, the medium was changed to the experimental medium containing 33% DMEM:F12 medium and 67% DMEM (no glucose, no sodium pyruvate; Invitrogen), and 1% FCS, 100 µM 5-bromo-2-deoxyuridine (Sigma) and 1% penicillin/streptomycin. All procedures involving experimental animals were performed in compliance with local animal welfare laws, guidelines and policies.

Where indicated, metformin (Sigma; stock solution: 300 mM in water), tunicamycin (Sigma; stock solution: 10 mg/ml in DMSO, final concentration 100 ng/µl) and staurosporine (Sigma; stock

solution 1 mM in DMSO) were added to the medium as specified in the Results section.

Transfection and siRNA experiments

Knock down of *ATF4* was carried out at day 2 by transfection with siRNA. A pool of four different siRNAs (ON-TARGETplus SMARTpool Rat ATF4; Thermo Scientific Dharmacon) directed against rat *ATF4* mRNA was used at 50 nM final concentration. A total of 6 µl DharmaFECT 4 transfection reagent (Thermo Scientific Dharmacon) were used per 36-mm culture dish. In each experiment, control cultures were performed using non-targeting siRNA (ON-TARGETplus non-targeting siRNA#1; Thermo Scientific Dharmacon). Cells were incubated with the transfection medium (DMEM:F12, 10% FCS, 100 µM 5-bromo-2-deoxyuridine, without penicillin/streptomycin). After 48 hours, transfection medium was changed to the experimental medium with or without metformin for another 24 hours.

In order to assay *ATF6* processing, cells were transfected at day 2 with the p3×*FLAG-ATF6* plasmid (Addgene plasmid 11975) described previously (Chen et al., 2002). Effectene Transfection Reagent (Qiagen) was used for transfection. 0.4 µg plasmid DNA dissolved in 100 µl EC buffer containing 3.2 µl Enhancer and 4 µl Effectene were used per 36-mm culture dish. Cells were incubated with transfection medium (DMEM:F12, 10% FCS, 100 µM 5-bromo-2-deoxyuridine, without penicillin/streptomycin). After 24 hours the transfection medium was changed to the experimental medium for a further 48 hours and metformin or dithiothreitol (Invitrogen) were added at the indicated time points. Transfection efficiency was determined by double immunofluorescence staining of the transfected cells to visualize the FLAG-taq and cardiomyocyte-specific desmin. Transfection efficiency was approximately 5% (p3×*FLAG-ATF6* plasmid). More than 80% of the FLAG-taq-positive cells were also stained for desmin, demonstrating that cardiac fibroblasts (ca. 20% of the cultured cells) were not preferentially transfected.

RNA isolation, reverse transcription and mRNA quantification

RNA was extracted from samples using Trizol reagent (PEQLAB) according to the instructions of the manufacturer. The RNA-containing phase was purified using RNeasy kit columns (Qiagen) following the manufacturer's protocol. RNA samples were stored at –80°C.

1 µg total RNA was transcribed into cDNA using random hexamer primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol in an i-Cycler thermocycler (Bio-Rad Laboratories). mRNA expression was determined using SYBR Green (Bio-Rad Laboratories). All measurements were performed as duplicates. For each gene, primer pairs spanning at least one intron (MWG Biotech and Metabion) were used in a final concentration of 0.2 µM (Table 1). Cycling conditions were as follows: in an initial step, Taq polymerase was activated at a temperature of 95°C for 15 minutes. Subsequently, amplification cycle conditions were 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds. Amplification cycles were repeated 40 times. After each run a melting curve analysis was performed to ensure amplification of the correct products. The expression of adiponectin receptor 1 (*adipor1*) mRNA was used to normalize the expression of the respective mRNA. Results were presented as percentage of change compared with the control group, the value of which was set arbitrarily at 100%. Statistical analyses were performed by comparing the normalized *C*t-values by a paired *t*-test or one-way ANOVA using Prism software (Graph Pad). Error bars indicate the standard error of the mean (s.e.m.). CHOP inducibility (*I*; Fig. 2H) was calculated as follows: $I=(2^{\Delta\Delta Ct}[CHOP_{ATF4\ siRNA\ with\ metformin}]/2^{\Delta\Delta Ct}[CHOP_{ATF4\ siRNA}])/(2^{\Delta\Delta Ct}[CHOP_{contr.\ siRNA\ with\ metformin}]/2^{\Delta\Delta Ct}[CHOP_{contr.\ siRNA}])$ and plotted against *ATF4* knockdown efficiency ($2^{\Delta\Delta Ct}[ATF_{contr.\ siRNA}]/2^{\Delta\Delta Ct}[ATF4_{ATF4.\ siRNA}]$).

Protein isolation, western blotting and SDS-PAGE

For western blotting analysis, proteins were extracted from cultured cardiomyocytes using Trizol reagent. Two volumes of isopropanol were added to the phenol phase after removal of the aqueous phase containing the RNA and the interphase containing the DNA. After incubation at room temperature for 10 minutes, proteins were sedimented by centrifugation (10,000 ***g***, 10 minutes, 4°C). The supernatant was discarded and the protein pellet was washed three times with ethanol containing 0.3 mM guanidine hydrochloride (20 minute incubation at room temperature, 20 minute centrifugation at 10,000 ***g***, 4°C). After a further wash step with ethanol the protein pellet was dried in a vacuum centrifuge. The protein pellet was dissolved in 1% SDS containing Complete protease inhibitors and Phosstop phosphatase inhibitors (Roche) by incubation at 75°C for 30 minutes under agitation. Protein concentration was determined using the BCA Protein Assay Kit (Thermo).

A total of 50 µg cellular protein extract per lane was subjected to SDS-PAGE (10-14% polyacrylamide; Roth). Proteins were

Table 1. Primers used for quantitative PCR

Gene	Acc. code	Forward primer (5′-3′)	Reverse primer (5′-3′)
CHOP	NM 001109986	GGAAGTGCATCTTCATACACCACC (OST567)	TGACTGGAATCTGGAGAGCGAGGGC (OST568)
ATF4	NM 024403	AGAATGGCTGGCTATGGATG	GCCAATTGGGTTCACTGTCT
ATF6	NM 001107196	AAGTGAAGAACCATTACTTTATATC (OST571)	TTTCTGCTGGCTATTTGT (OST572)
Bip/GRP78	NM 013083	TACTCGAATTCCAAAGATTCAG (OST565)	TCAAGCAGAACCAGGTC (OST566)
ATF3	NM 012912	TGGAGTCAGTCACCATCAA (OST563)	CATTCACACTCTCCAGTT (OST564)
Puma	NM 173837	CGTGTGGAGGAGGAGGAGT (OST589)	GGAGGAGTCCCATGAAGAGA (OST590)
Bim	NM 171988	AGTCTCAGGAGGAACCTGAAGATCT (OST600)	TCCGATCCTCCGCAGCT (OST601)
Bcl-2	NM 016993	GGAGGATTGTGGCCTTCTT	TGAGCAGCGTCTTCAGAGAC
Adipor1	NM 207587	CCACCATGCCATGGAGAAG	ATATTTGGTCTGAGCATGGTCAAG

TRANSLATIONAL IMPACT

Clinical issue

Type 2 diabetes is often associated with heart disease, which worsens patient prognosis. Recently, clinical studies have suggested that metformin – the most common non-insulin drug used to treat type 2 diabetes – reduces the mortality of diabetic patients that suffer from cardiac complications. Metformin functions as an insulin sensitizer that suppresses hepatic gluconeogenesis, and increases glucose uptake in skeletal and cardiac muscle. However, the cellular targets of metformin and its effect on cardiomyocytes are incompletely understood.

Results

Accumulating evidence indicates a causative role of endoplasmic reticulum stress signaling (ERSS) in both diabetes and heart disease. Here, the authors use metformin-treated rat cardiomyocytes as a model to assess the effects of metformin on this important target tissue, focusing on the role of ERSS. They find that only one of the three branches of ERSS, the PERK-ATF4 pathway, is upregulated following metformin treatment, leading to a strong induction of CHOP. Although CHOP is usually associated with the induction of apoptosis, metformin-induced CHOP induction did not lead to apoptosis or apoptotic signaling in cardiomyocytes.

Implications and future directions

On the basis of these data, the authors speculate that the clinically well-appreciated cardioprotective effect of metformin is associated with the specific induction of the PERK-ATF4-CHOP branch of ERSS, and that this pathway might also be important for the therapeutic effect of metformin in type 2 diabetes. The data also suggest that CHOP activation is not sufficient to initiate apoptosis in cardiomyocytes. Future translational studies should consider the interplay between insulin sensitizers and ER stress pathways in diabetes and heart disease.

transferred to nitrocellulose membrane (Schleicher & Schuell) and the membrane was blocked in TBS buffer containing 5% BSA for 2 hours or, in the case of caspase 12, over night. Proteins were identified by incubation at 4°C over night, or, in the case of caspase 12, at room temperature for 2 hours, in TBS buffer containing 1% Tween and 5% BSA together with the specific antibody. Antibody dilutions were: monoclonal mouse anti-CHOP, clone L63F7 (Cell Signaling), 1:1000; monoclonal rabbit anti-PERK, clone C33E10 (Cell Signaling), 1:1000; monoclonal rabbit anti-phospho(Thr980)-PERK, clone 16F8 (Cell Signaling), 1:1000; polyclonal rabbit anti-caspase-12 (Biovision), 1:1000; monoclonal rabbit anti-GAPDH, clone 14C10 (Cell Signaling), 1:4000; and anti-FLAG tag M2 (Stratagene), 1:1000. HRP-conjugated secondary antibodies were used in TBS buffer containing 1% Tween, 5% BSA for 2 hours at room temperature: goat anti-mouse Ig (Pierce), 1:2000, and goat anti-rabbit Ig (BD Pharmingen), 1:2000. Chemiluminescence was detected using Super Signal West Femto reagent (Thermo) on an X-ray film (Fuji).

TUNEL staining

Isolated neonatal rat cardiomyocytes were plated on a glass slide coated with laminin (Sigma). Staining procedure was performed using TUNEL Enzyme and TUNEL Label Mix (Roche) according to the manufacturer's instructions.

ACKNOWLEDGEMENTS

We are grateful to Ron Prywes for providing p3×*FLAG-ATF6* through Addgene. We thank Noa Lipstein, Elke Oetjen and Ralph Krätzner for comments on the manuscript.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

T.Q. and S.T. conceived the project, planned the experiments, analyzed and discussed the data, and wrote the paper. T.Q. and A.P. performed the experiments. M.S. initiated work on AMPK and on neonatal rat cardiomyocytes.

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

REFERENCES

Araki, E., Oyadomari, S. and Mori, M. (2003). Endoplasmic reticulum stress and diabetes mellitus. *Intern. Med.* **42**, 7-14.

Azfer, A., Niu, J., Rogers, L. M., Adamski, F. M. and Kolattukudy, P. E. (2006). Activation of endoplasmic reticulum stress response during the development of ischemic heart disease. *Am. J. Physiol. Heart Circ. Physiol.* **291**, H1411-H1420.

Barsotti, A., Giannoni, A., Di Napoli, P. and Emdin, M. (2009). Energy metabolism in the normal and in the diabetic heart. *Curr. Pharm. Des.* **15**, 836-840.

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P. and Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* **2**, 326-332.

Bhamra, G. S., Hausenloy, D. J., Davidson, S. M., Carr, R. D., Paiva, M., Wynne, A. M., Mocanu, M. M. and Yellon, D. M. (2008). Metformin protects the ischemic heart by the Akt-mediated inhibition of mitochondrial permeability transition pore opening. *Basic Res. Cardiol.* **103**, 274-284.

Calvert, J. W., Gundewar, S., Jha, S., Greer, J. J., Bestermann, W. H., Tian, R. and Lefer, D. J. (2008). Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes* **57**, 696-705.

Chen, X., Shen, J. and Prywes, R. (2002). The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *J. Biol. Chem.* **277**, 13045-13052.

Cheng, W. P., Wang, B. W. and Shyu, K. G. (2009). Regulation of GADD153 induced by mechanical stress in cardiomyocytes. *Eur. J. Clin. Invest.* **39**, 960-971.

Eurich, D. T., McAlister, F. A., Blackburn, D. F., Majumdar, S. R., Tsuyuki, R. T., Varney, J. and Johnson, J. A. (2007). Benefits and harms of antidiabetic agents in patients with diabetes and heart failure: systematic review. *BMJ* **335**, 497.

Fery, F., Plat, L. and Balasse, E. O. (1997). Effects of metformin on the pathways of glucose utilization after oral glucose in non-insulin-dependent diabetes mellitus patients. *Metabolism* **46**, 227-233.

Fischer, H., Koenig, U., Eckhart, L. and Tschachler, E. (2002). Human caspase 12 has acquired deleterious mutations. *Biochem. Biophys. Res. Commun.* **293**, 722-726.

Groenendyk, J., Sreenivasaiah, P. K., Kim, Do H., Agellon, L. B. and Michalak, M. (2010). Biology of endoplasmic reticulum stress in the heart. *Circ. Res.* **107**, 1185-1197.

Halterman, M. W., Gill, M., DeJesus, C., Ogihara, M., Schor, N. F. and Federoff, H. J. (2010). The endoplasmic reticulum stress response factor CHOP-10 protects against hypoxia-induced neuronal death. *J. Biol. Chem.* **285**, 21329-21340.

Hardy, B. and Raiter, A. (2010). Peptide-binding heat shock protein GRP78 protects cardiomyocytes from hypoxia-induced apoptosis. *J. Mol. Med.* **88**, 1157-1167.

Khurana, R. and Malik, I. S. (2010). Metformin: safety in cardiac patients. *Heart* **96**, 99-102.

Kim, I., Xu, W. and Reed, J. C. (2008). Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat. Rev. Drug Discov.* **7**, 1013-1030.

Li, J., Lee, B. and Lee, A. S. (2006). Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. *J. Biol. Chem.* **281**, 7260-7270.

Masoudi, F. A., Inzucchi, S. E., Wang, Y., Havranek, E. P., Foody, J. M. and Krumholz, H. M. (2005). Thiazolidinediones, metformin, and outcomes in older patients with diabetes and heart failure: an observational study. *Circulation* **111**, 583-590.

McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y. and Holbrook, N. J. (2001). Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol. Cell. Biol.* **21**, 1249-1259.

Minamino, T. and Kitakaze, M. (2010). ER stress in cardiovascular disease. *J. Mol. Cell. Cardiol.* **48**, 1105-1110.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A. and Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403**, 98-103.

Nickson, P., Toth, A. and Erhardt, P. (2007). PUMA is critical for neonatal cardiomyocyte apoptosis induced by endoplasmic reticulum stress. *Cardiovasc. Res.* **73**, 48-56.

Okada, K., Minamino, T., Tsukamoto, Y., Liao, Y., Tsukamoto, O., Takashima, S., Hirata, A., Fujita, M., Nagamachi, Y., Nakatani, T. et al. (2004). Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic

constriction: possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis. *Circulation* **110**, 705-712.

Paiva, M. A., Goncalves, L. M., Providencia, L. A., Davidson, S. M., Yellon, D. M. and Mocanu, M. M. (2010). Transitory activation of AMPK at reperfusion protects the ischaemic-reperfused rat myocardium against infarction. *Cardiovasc. Drugs Ther.* **24**, 25-32.

Puthalakath, H., O'Reilly, L. A., Gunn, P., Lee, L., Kelly, P. N., Huntington, N. D., Hughes, P. D., Michalak, E. M., McKimm-Breschkin, J., Motoyama, N. et al. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* **129**, 1337-1349.

Ron, D. and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **8**, 519-529.

Rutkowski, D. T. and Hegde, R. S. (2010). Regulation of basal cellular physiology by the homeostatic unfolded protein response. *J. Cell Biol.* **189**, 783-794.

Shen, J., Chen, X., Hendershot, L. and Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev. Cell* **3**, 99-111.

Shintani-Ishida, K., Nakajima, M., Uemura, K. and Yoshida, K. (2006). Ischemic preconditioning protects cardiomyocytes against ischemic injury by inducing GRP78. *Biochem. Biophys. Res. Commun.* **345**, 1600-1605.

Solskov, L., Lofgren, B., Kristiansen, S. B., Jessen, N., Pold, R., Nielsen, T. T., Botker, H. E., Schmitz, O. and Lund, S. (2008). Metformin induces cardioprotection against ischaemia/reperfusion injury in the rat heart 24 hours after administration. *Basic Clin. Pharmacol. Toxicol.* **103**, 82-87.

Stumvoll, M., Nurjhan, N., Perriello, G., Dailey, G. and Gerich, J. E. (1995). Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **333**, 550-554.

Szegezdi, E., Duffy, A., O'Mahoney, M. E., Logue, S. E., Mylotte, L. A., O'Brien, T. and Samali, A. (2006). ER stress contributes to ischemia-induced cardiomyocyte apoptosis. *Biochem. Biophys. Res. Commun.* **349**, 1406-1411.

Terai, K., Hiramoto, Y., Masaki, M., Sugiyama, S., Kuroda, T., Hori, M., Kawase, I. and Hirota, H. (2005). AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. *Mol. Cell. Biol.* **25**, 9554-9575.

Thompson, M. R., Xu, D. and Williams, B. R. (2009). ATF3 transcription factor and its emerging roles in immunity and cancer. *J. Mol. Med.* **87**, 1053-1060.

Thoms, S., Gronborg, S. and Gartner, J. (2009). Organelle interplay in peroxisomal disorders. *Trends Mol. Med.* **15**, 293-302.

Thuerauf, D. J., Marcinko, M., Gude, N., Rubio, M., Sussman, M. A. and Glembotski, C. C. (2006). Activation of the unfolded protein response in infarcted mouse heart and hypoxic cultured cardiac myocytes. *Circ. Res.* **99**, 275-282.

Toth, A., Jeffers, J. R., Nickson, P., Min, J. Y., Morgan, J. P., Zambetti, G. P. and Erhardt, P. (2006). Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. *Am. J. Physiol. Heart Circ. Physiol.* **291**, H52-H60.

UKPDS. (1998). Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study Group. *Lancet* **352**, 854-865.

Upton, J. P., Austgen, K., Nishino, M., Coakley, K. M., Hagen, A., Han, D., Papa, F. R. and Oakes, S. A. (2008). Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. *Mol. Cell. Biol.* **28**, 3943-3951.

Voulgari, C., Papadogiannis, D. and Tentolouris, N. (2010). Diabetic cardiomyopathy: from the pathophysiology of the cardiac myocytes to current diagnosis and management strategies. *Vasc. Health Risk Manag.* **6**, 883-903.

Weston, R. T. and Puthalakath, H. (2010). Endoplasmic reticulum stress and BCL-2 family members. *Adv. Exp. Med. Biol.* **687**, 65-77.

Wong, W. W. and Puthalakath, H. (2008). Bcl-2 family proteins: the sentinels of the mitochondrial apoptosis pathway. *IUBMB Life* **60**, 390-397.

Ye, J., Rawson, R. B., Komuro, R., Chen,X., Davé, U. P., Prywes, R., Brown, M. S. and Goldstein, J. L. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell* **6**, 1355-1364.