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Deregulated unfolded protein response in chronic wounds of diabetic *ob*/*ob* mice: A potential connection to inflammatory and angiogenic disorders in diabetes-impaired wound healing

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ABSTRACT

Type-2 diabetes mellitus (T2D) represents an important metabolic disorder, firmly connected to obesity and low level of chronic inflammation caused by deregulation of fat metabolism. The convergence of chronic inflammatory signals and nutrient overloading at the endoplasmic reticulum (ER) leads to activation of ER-specific stress responses, the unfolded protein response (UPR). As obesity and T2D are often associated with impaired wound healing, we investigated the role of UPR in the pathologic of diabetic-impaired cutaneuos wound healing. We determined the expression patterns of the three UPR branches during normal and diabetes-impaired skin repair. In healthy and diabetic mice, injury led to a strong induction of BiP (BiP/Grp78), C/EBP homologous protein (CHOP) and splicing of X-box-binding protein (XBP)1. Diabetic-impaired wounds showed gross and sustained induction of UPR associated with increased expression of the pro-inflammatory chemokine macrophage inflammatory protein (MIP)2 as compared to normal healing wounds. *In vitro*, treatment of RAW264.7 macrophages with tunicamycin, and subsequently stimulation with lipopolysaccharide (LPS) and interferon (IFN)-γ enhances MIP2 mRNA und protein expression compared to proinflammatory stimulation alone. However, LPS/IFNγ induced vascular endothelial growth factor (VEGF) production was blunted by tunicamycin induced-ER stress.

Hence, UPR is activated following skin injury, and functionally connected to the production of proinflammatory mediators. In addition, prolongation of UPR in diabetic non-healing wounds aggravates ER stress and weakens the angiogenic phenotype of wound macrophages.

1. Introduction

The unfolded protein response (UPR) is a response to cell stress caused by the accumulation of unfolded proteins within the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) due to hypoxia, glucose deprivation, inflammatory cytokines, toxins or due to loss of Ca^{2+} homeostasis as well as inadequate disulfide bond formation of nascent proteins. The UPR counters ER stress in several ways: attenuating protein translation to reduce ER workload, enhancing the degradation of misfolded proteins and increasing the capacity to accelerate protein folding in the ER [1,2].

UPR is achieved by three branches of intracellular signalling pathways which become activated by ER stress: inositol requiring enzyme (IRE)1, protein kinase-like endoplasmic reticulum kinase (PERK), and activating transcription factor (ATF)6 (Fig. 4). IRE1 cleaves with its endoribonuclease activity the mRNA of the transcription factor X-box-binding protein (XBP)1 which leads to translation of an activated form of XBP1 (spliced XBP1) responsible for upregulation of many UPR target genes like chaperones [3]. The expression of BiP/glucose regulated protein (Grp)78 is primarily regulated via ATF6 [4] and belongs to a pool of molecular chaperones. In the ER BiP is involved in folding and assembling of proteins, prevents the formation of aggregates and assist to refold misfolded proteins [5,6]. Transcription of C/EBP homologous protein (CHOP/GADD153) is primarily regulated via ATF6 and ATF4, which is in turn regulated by the PERK-eukaryotic initiation factor (eIF2α) branch [7]. Whereas IRE1 and ATF6 activities are attenuated during persistent ER stress, PERK signalling and hence CHOP expression are maintained and associated with cell death [8,9].

The last two decades have seen an explosive global increase in people diagnosed with adipositas and diabetes mellitus [10]. Low grade inflammation of adipose tissue due to accumulation of

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inflammatory macrophages has been implicated in the progression of diabetes mellitus [11]. In rodent tumour necrosis factor (TNF)α was suggested to be responsible for the development of insulin resistance [12].

Diabetic skin ulcerations represent a medical problem of clinical importance that still remains unsolved on a therapeutic basis [13]. To date, the impact of UPR on diabetes-associated wound healing disorders is still unknown.

Here we investigated activation of the UPR during normal and metabolically disturbed wound healing conditions and macrophage (Mϕ) specific production of inflammatory and angiogenic cytokines under ER stress.

2. Materials and methods

2.1. Animals

Female wild type C57BL/6J and C57BL/6J-*ob*/*ob* mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained under a 12-hour light/12-hour dark cycle at 22 °C.

2.2. Wounding of mice

Wounding of mice was performed as described previously [14]. Six full-thickness wounds (5 mm in diameter, 3–4 mm apart) were made on the back of each mouse by excising the skin and the underlying *panniculus carnosus*.

For each experimental time point tissue from 9 wounds isolated from 3 animals ($n = 3$, RNA analysis) and 6 wounds isolated from 3 animals ($n = 3$, protein analysis) were combined. Nonwounded back skin from 4 animals served as a control. All animal experiments were performed according to the guidelines and approval of the local Ethics Animal Review Board.

2.3. Cell culture

Murine RAW264.7 Mϕ were cultured in RPMI1640 supplemented with 10% endotoxinfree fetal calf serum. For stimulation experiments, 5×10^5 cells were seeded and grown for 24 h. Cell culture media were then replaced by fresh medium in the absence or presence of tunicamycin (2.5 μM, Tm) or thapsigargin (500 nM, Tg). After 1 h LPS (1 μg/ml, L) (*Escherischia coli* [055:B5], Sigma Aldrich, Taufkirchen, Germany) and IFNγ 20 ng/ml, I) (PeproTech, Hamburg, Deutschland) (LI) was added for indicated time periods.

2.4. RNA isolation and RNase protection analysis

RNA isolation and RNase protection assays were carried out as described previously [14,15]. All samples were quantified using PhosphoImager PSL counts per 15 μg of total wound RNA. 1000 cpm of the hybridization probe were used as size marker and hybridization against GAPDH was used as a loading control in all assays. The cDNA probes were cloned using RT-PCR. The probes corresponded to nt 425–709 (for XBP1, GenBank accession number BC029197), nt 573–800 (for CHOP, NM_007837), nt 1795–2080 (for BiP (Grp78), D78645.1) or nt 163–317 (for GAPDH, NM002046) of the published sequences.

2.5. Quantitative real-yime polymerase chain reaction (qRT-PCR) isolation

Predesigned primers to assess gene expression in wounds were purchased from Applied Biosystems (Darmstadt, Germany): Mm00436450_m1 (for MIP-2), Mm00437304_m1 (for VEGF) and 4352339E for GAPDH. qRT-PCR was performed as described previously [17].

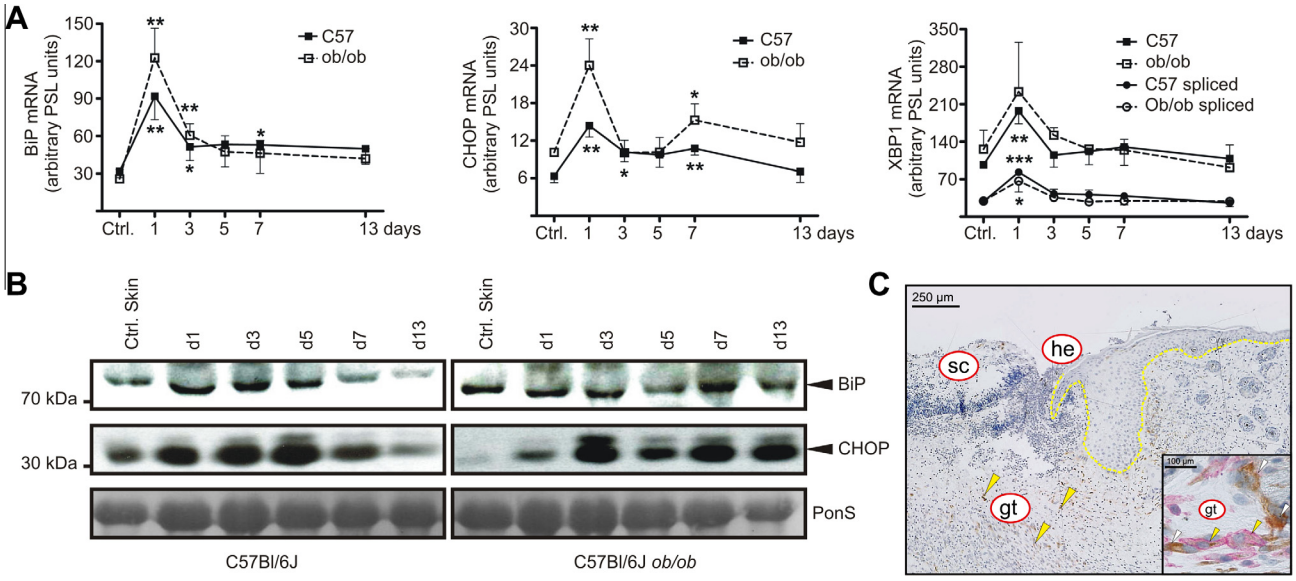

Fig. 1. Induction of UPR during dermal wound healing. (A) Expression of BiP, CHOP and XBP1 mRNA in nonwounded skin (Ctrl.) and wound tissue isolated from healthy C57BL/6J and diabetic *ob*/*ob* mice. Time after injury is indicated. A quantification of BiP, CHOP, XBP1 (spliced and unspliced) mRNA expression (PSL counts) as assed by RNase protection assay and PhosphoImager (fuji) analysis of the radioactive gels is shown. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to nonwounded skin. Each single time point depicts the mean ± SD obtained from 9 wounds ($n = 9$) isolated from 3 individual animals ($n = 3$). (B) Immunoblot analysis of BiP and CHOP protein in nonwounded skin (Ctrl. Skin) and wound tissue at indicated time points are shown for healthy C57BL/6J (B, left panel) or diabetic *ob*/*ob* mice (B, right panel). Each single time point depicts six wounds ($n = 6$) isolated from three individual animals. Equal loading was confirmed by Ponceau S (PonS) staining. (C) Immunohistochemical staining of BiP protein in 5-day sections isolated from C57BL/6J mice. Immunopositive signals (brown) are indicated by arrows. The epidermal boundary is indicated by a yellow line. Insert: 5-day sections were simultaneously stained for macrophage-specific F4/80 (red) and BiP (brown) protein. Double positive cells are indicated by yellow arrows, BiP positive F4/80 negative cells by white arrows. gt (granulation tissue); he (hyperproliferative epithelium); sc (scar).

2.6. Preparation of protein lysates and Western blot analysis

RAW264.7 Mϕ from cell culture experiments, skin and wound tissue biopsies were homogenized in lysis buffer as described previously [14]. Fifty micrograms of total protein from skin lysates were separated using SDS gel electrophoresis. After transfer to a PVDF membrane, specific proteins were detected using antisera raised against BiP, CHOP, p-eIF2a, total Akt, p-Akt(Ser473) (Cell Signaling, Frankfurt, Germany) and iNOS (Stressgene/Enzo Life Science, Lörrach, Germany). A secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence detection system was used to visualize the proteins. Phenylmethylsulfonyl fluoride, dithiothreitol, aprotinin, NaF, and Na_3VaO_4 were from Sigma. Leupeptin and ocadaic acid were from BioTrend (Köln, Germany). The enhanced chemiluminescence detection system was obtained from Amersham (Freiburg, Germany).

2.7. Immunohistochemistry

Skin biopsies were isolated from the back, fixed in formalin and embedded in paraffin. Four-micrometer sections were incubated over night at 4 °C with antisera raised against BiP (Cell Signaling, Frankfurt, Germany) or F4/80 (AbD Serotec, Düsseldorf, Germany). Specificity of staining was controlled for all primary antibodies by single incubation with the detection antibody alone. Sections were subsequently stained with the avidin-biotin-peroxidase complex system (Santa Cruz, Heidelberg, Germany) and 3,3-diaminobenzidine-tetra-hydrochloride (Sigma Aldrich, Taufkirchen, Germany) or Fast Red Substrate-Chromogen System (Dako, Hamburg, Germany) as chromogenic substrates. Finally, sections were counterstained with hematoxylin (AppliChem, Darmstadt, Germany) and mounted.

2.8. Enzyme-linked immunosorbent assay

Quantification of murine MIP2 and vascular endothelial growth factor $(VEGF)_{165}$ protein was performed using the respective murine Quantikine® enzyme-linked immunosorbent assay kits (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

2.9. Statistical analysis

Data are shown as means ± SD. Statistical comparison of two groups was carried out by using the non-parametric unpaired Student's *t* test with raw data.

3. Results

3.1. Skin wounding induces components of the UPR

Accumulation of misfolded proteins triggers the cell fade [8] as well as cell response to proinflammatory cytokines [16]. As literature was limited dealing with the activation of the UPR during tissue regeneration we investigated activation of the three branches of the UPR during normal and diabetes-impaired wound healing (Fig. 1A). UPR induction after wounding was measured by mRNA expression analysis of BiP, CHOP and XBP1. In wild type and diabetic *ob*/*ob* mice mRNA expression of BiP, CHOP and splicing of XBP1 was strongly induced at day 1 after wounding and declined at day 3. In diabetic mice a high expression of CHOP mRNA and CHOP and BIP protein recurred at day 7 after wounding (Fig. 1A and B). Moreover CHOP mRNA stays higher at d7 and d13 in comparison to wt mice.

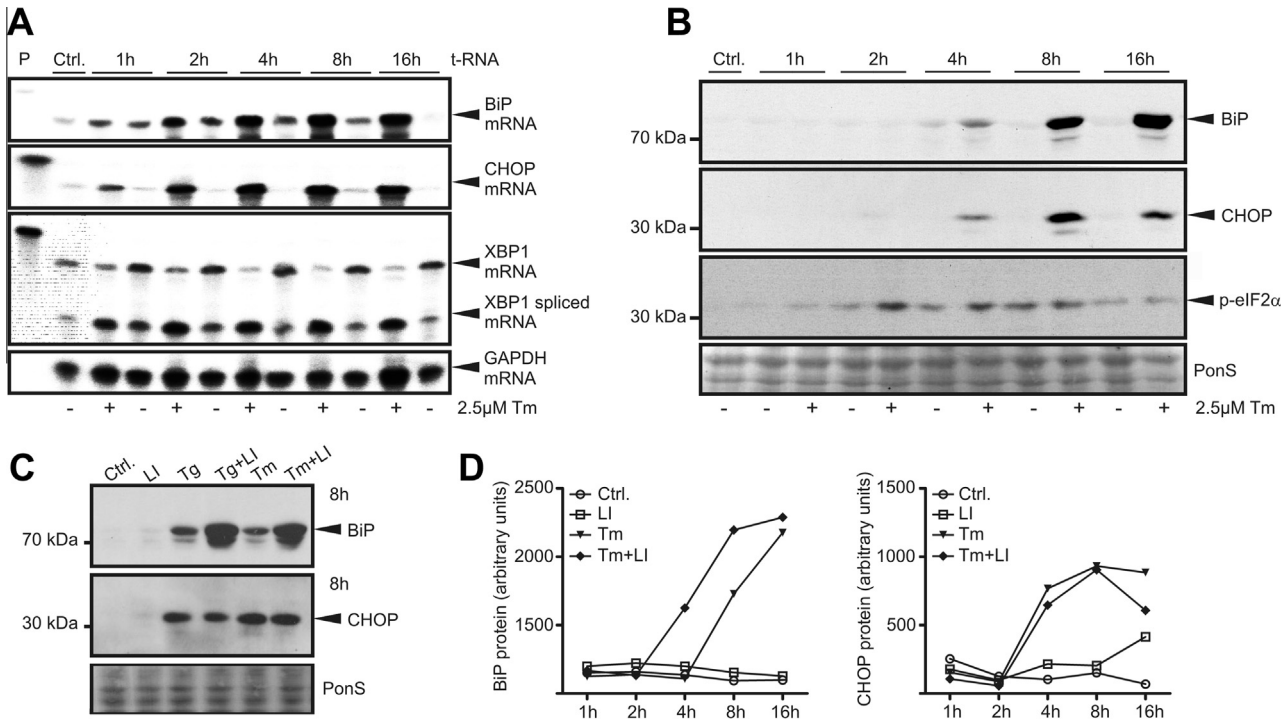

Fig. 2. Proinflammatory modulation of the UPR in macrophages. (A) Expression of BiP, CHOP and XBP1 (spliced and unspliced) mRNA in non-stimulated (Ctrl.) and Tm (2.5 μM) treated RAW264.7 Mϕ. Total cellular RNA (20 μg) was analyzed by RNase protection assay at the indicated time points. (B) Immunoblot showing BiP, CHOP and p-eIF2α protein of Tm (2.5 μM) treated RAW264.7 Mϕ at indicated time points. Equal loading was confirmed by Ponceau S (PonS) staining. (C) Total protein (50 μg) from RAW264.7 Mϕ treated with LI (LPS, 1 μg/ml; IFNγ 20 ng/ml), Tg (500 nM), Tm (2.5 μM), LI + Tg (LPS, 1 μg/ml; IFNγ 20 ng/ml; Tg 500 nM) or LI + Tm (LPS, 1 μg/ml; IFNγ 20 ng/ml; Tm 2.5 μM) were analyzed by immunoblotting for the presence of BiP and CHOP as indicated. Equal loading was confirmed by Ponceau S (PonS) staining. A quantification of BiP and CHOP protein (immunoblot INT counts) from datapoints of the respective immunoblot gel is shown in (D).

3.2. The UPR is activated in Mϕ and non-immune cells

Wound healing is dependent on tightly controlled Mϕ which contribute to normal or disturbed wound inflammation, angiogenesis and contractive processes [17,18].

Besides non-immune cells Mϕ could be determined as cellular source of high BiP protein expression and thereby of an activated UPR (Fig. 1C). The double staining against the macrophage marker F4/80 and BiP identified BiP positive macrophages and BiP positive, F4/80 negative cells in the granulation tissue of wild type mice (Fig. 1C).

3.3. UPR activation in RAW264.7 Mϕ

Addition of Tm to RAW264.7 Mϕ resulted in a robust phosphorylation of eIF2α (Fig. 2B). This eIF2α activation was followed by induction of BiP and CHOP mRNA expression as revealed by RNase protection analysis (Fig. 2A). Tm treatment promotes the splicing of 26 nt out of the mature XBP1 mRNA to give rise to an activated, spliced XBP1 variant. Furthermore, expression of BiP and CHOP protein was markedly increased after Tm treatment (Fig. 2B).

3.4. Proinflammatory stimuli potentiate UPR activation

To investigate the influence of strong proinflammatory signals on UPR activation Tm or Tg treated RAW264.7 Mϕ were further stimulated with LI (Fig. 2C and D). LI stimulation synergistically enhances the expression of BiP protein at 4 and 8 h (Fig. 2C and D).

ER stress enhances MIP2 expression in response to proinflammatory stimuli. We could show that Tm leads to an induction of MIP2 mRNA and protein expression compared to unstimulated RAW264.7 cells (Fig. 3A and B). Additionally, subsequent stimulation with LI further enhances MIP2 mRNA and protein expression (Fig. 3A and B).

3.5. ER stress reduces VEGF expression in response to proinflammatory stimuli

It has been shown that the UPR branches: IRE1α, PERK, and ATF6 modulate VEGF mRNA expression in non-hematopoietic cells [19]. Similarly, RAW264.7 macrophages treated with Tm showed increased expression of VEGF mRNA and protein as measured by real-time PCR or ELISA, respectively (Fig. 3C and D). Further

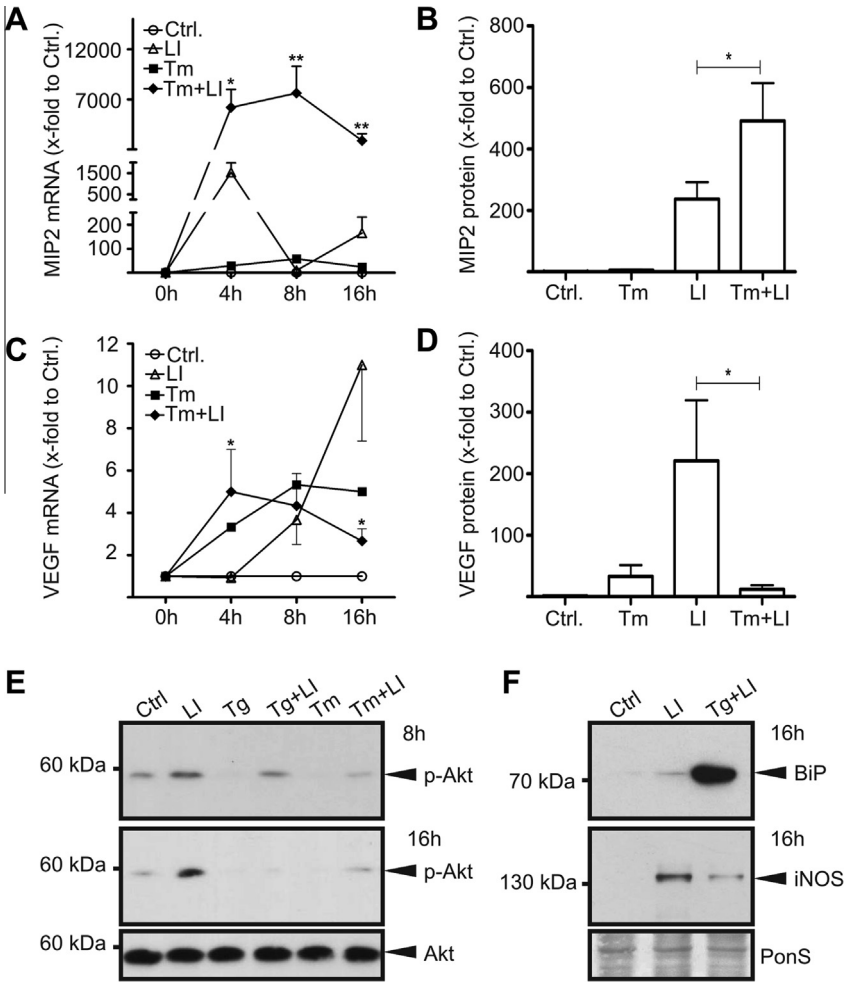

Fig. 3. UPR dependant modulation of wound healing relevant cytokines of proinflammatory activated RAW264.7 Mϕ. (A) MIP2 and (C) VEGF mRNA expression in RAW264.7 Mϕ treated with Tm (2.5 μM), LI (LPS, 1 μg/ml, IFNγ 20 ng/m) or LI + Tm (LPS, 1 μg/ml; IFNγ 20 ng/ml; Tm 2.5 μM). Non-treated cells serve as a control (ctrl). Total cellular RNA was analyzed by real-time PCR at indicated time points. $^{*}P < 0.05$; $^{**}P < 0.01$ compared to LI. Data represent mean ± SD obtained from 3 individual experiments ($n = 3$). ELISA analysis for MIP2 (B) and VEGF (D) protein expression in supernatants of RAW264.7 Mϕ treated with Tm (2.5 μM), LI (LPS, 1 μg/ml, IFNγ 20 ng/m) or LI + Tm (LPS, 1 μg/ml; IFNγ 20 ng/ml; Tm 2.5 μM). $^{*}P < 0.05$ compared to LI treatment. Data represent mean ± SD obtained from 3 individual experiments ($n = 3$). RAW264.7 Mϕ treated with LI (LPS, 1 μg/ml; IFNγ 20 ng/ml), Tg (500 nM), Tm (2.5 μM), LI + Tg (LPS, 1 μg/ml; IFNγ 20 ng/ml; Tg 500 nM) or LI + Tm (LPS, 1 μg/ml; IFNγ 20 ng/ml; Tm 2.5 μM) were analyzed by immunoblotting for the presence of *Akt-Serine 473 Phosphorylation* (E), or expression of BiP or iNOS protein (F). Non-treated cells serve as a control (ctrl). Total Akt (E) or PonS staining (F) served as a control for protein loading.

stimulation of RAW264.7 Mϕ with LI inhibited VEGF mRNA expression at 16 h compared to LI stimulation alone (Fig. 3C). LI induced VEGF protein expression was abolished after Tm pre-treatment (Fig. 3D).

3.6. ER stress reduces Akt(Ser473) phosphorylation in response to proinflammatory stimuli

Phosphoinositol 3-kinase (PI3K)/Akt(Ser473) pathway is central to VEGF biosynthesis [20]. Accordingly, RAW264.7 Mϕ showed a slight increase of Akt(Ser473) phosphorylation following induction of ER stress via Tm or Tg (data not shown). LI-induced phosphorylation of Akt(Ser473) (8 and 16 h) was abolished after Tm or Tg pre-treatment (Fig. 3E) and fit to reduced VEGF protein expression during ER stress and LI stimulation (Fig. 3D).

Moreover Xiong et al. could show that inhibition of iNOS with N^G-monomethyl-L-arginin blocks LI mediated VEGF production in RAW264.7 Mϕ [21]. Here we could show that ER stress reduced LI induced iNOS protein expression (Fig. 3F).

4. Discussion

It has been shown that obesity plays a major role to initiation and progression of diabetes mellitus [22]. Up to now pharmacologic approaches to amiliorate diabetes-related wound healing disorders are still limited.

Within obese tissue organelle dysfunction of metabolic cells was found to contribute to metabolic abnormalities and inflammatory response. Three critical steps link ER stress to major inflammatory networks: activation of c-Jun N-terminal kinase (JNK)-activator protein (AP)1, IκB kinase nuclear factor κB (IKK)-NFκB pathways (Fig. 4) and the production of reactive oxygen species [23–25]. Although disturbance of inflammatory response has been implicated in diabetes-associated wound healing disorders [26] and inflammation and ER stress are closely connected, ER stress has not been analysed in the context of metabolic associated wound healing defects.

As macrophages are an important cellular source for proinflammatory cytokines and showed BiP expression after wounding, we questioned whether proinflammatory stimuli aggravate ER stress. An inflammatory challenge of RAW264.7 Mϕ pretreated with Tm or Tg led to a synergistically activated UPR response as indicated by BiP protein expression. This possibly accommodates the additive stress on the protein folding machinery due to proinflammatory stimulation. Similarly, prolonged inflammatory response in diabetic *ob*/*ob* mice following skin injury [27] may accounts for prolonged BiP and CHOP protein expression in *ob*/*ob* mice as found in this study. It should be noted here that ER stress has been implicated in enhancement of inflammation [24]. Especially a long lasting CHOP expression is functionally connected to programmed cell death [9,28,29]. Furthermore, wounds of non-obese diabetic mice show increased apoptosis throughout the healing process compared to wounds of non-diabetic animals [30].

In addition to a deregulated inflammatory response, *ob*/*ob* mice show a delay in VEGF expression during wound healing which is accompanied by an impaired angiogenesis [20,27,31]. It is known that keratinocytes of the hyperproliferative epithelium at the wound margins as well as macrophages contribute to VEGF expression after wounding [20]. *In vitro* we could show that ER stress induced VEGF expression in Mϕ. These data are in accordance to studies with non-immune cells where Ghosh et al. showed that the IRE1α-XBP1, PERK-ATF4, and ATF6 pathways independently regulate VEGFA mRNA expression [19]. Nevertheless, VEGF expression stimulated by ER stress mediators like Tm is marginal in comparison to VEGF expression mediated by proinflammatory stimuli.

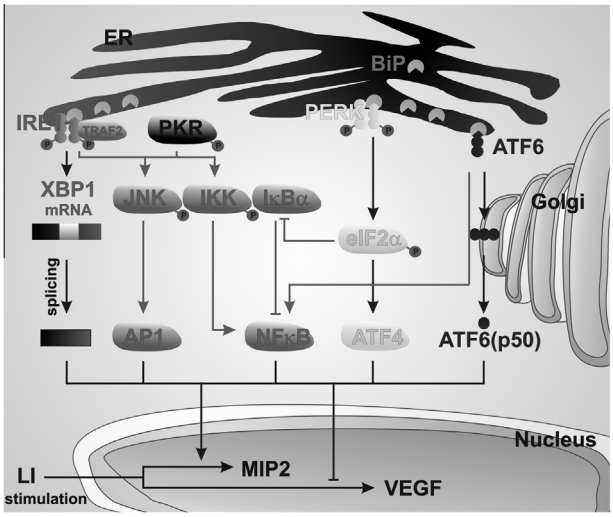

Fig. 4. UPR pathways and its impact on MIP2 and VEGF production. ER stress leads to binding of the ER chaperon BiP to un- or misfolded proteins. The interaction of BiP with ER stress sensors like IRE1, PERK and ATF6 is decreased which results in the activation of UPR and inflammatory response. IRE1 autophosphorylation leads to activation of IRE1 and removal of a 26-base intron from XBP1 mRNA. Spliced XBP1 mRNA is a transcriptional activator of the UPR whereas unspliced XBP1 mRNA functions as an inhibitor [35]. Homodimerization and autophosporylation of PERK result in phosphorylation of the translation initiation factor eIF2α, leading to overall reduction of the initiation of mRNA translation. On the other hand, phosphorylated eIF2α promotes the translation of ATF4. The release of BiP from ATF6 allows ATF6 translocation to the Golgi apparatus, where it is cleaved into an ATF6 p50 fragment. Association of activated IRE1 and TRAF2 leads to the activation of JNK, IKK and their downstream targets AP1 and NFκB, respectively. Obesity derived ER stress activates protein kinase R (PKR) which contributes to JNK and IKK activation [36]. In addition, the PERK/eIF2α pathway forces NFκB activation *via* repression of IκBα mRNA translation because IκBα has a shorter halftime compared to NFκB. Transcriptional activity of NFκB is further increased by ATF6 [37]. Compared to pro-inflammatory stimulation alone, LI induced MIP2 production is further enhanced by induction of UPR whereas VEGF production is blunted. AP1 (activator protein 1); ATF4/6 (activating transcription factor 4/6); BiP (BiP/glucose regulated protein (Grp)78); eIF2a (eukaryotic translation initiation factor 2α); IκBα (inhibitor of nuclear factor κBα) IKK (inhibitor of κ kinase); IRE1 (inositol-requiring enzyme 1); JNK (c-Jun N-terminal kinase); LI (LPS/IFNγ); MIP2 (macrophage inflammatory protein 2); NFκB (nuclear factor κB); PERK (PKR-like eukaryotic initiation factor 2α kinase); PKR (protein kinase R); TRAF2 (TNF receptor-associated factor 2); VEGF (vascular endothelial growth factor); XBP1 (X-box-binding protein 1).

More important, we could show that ER stress blunted inflammation induced VEGF expression in Mϕ and reduced Akt phosphorylation at Serin 473. Park et al. could show that LPS stimulation of macrophages transiently caused Akt phosphorylation, and inhibition of Akt activation blocks the production of VEGF and cyclooxygenase 2 expression in response to LPS [32].

In agreement with the observed role of nitric oxide to enhance VEGF translation in human keratinocytes, murine macrophages and during cutaneous wound healing [14,21], we could show that ER stress reduces LI-mediated iNOS expression and Akt(Ser473) phosphorylation in Mϕ which is in turn functionally connected to VEGF expression.

Interestingly, Ozawa et al. could show that local administration of the ER chaperone oxygen-regulated protein 150 in the wound bed, increases VEGF expression which in turn accelerates neovascularization and wound repair [34]. Finally, as administration of chemical chaperones has been shown to reduce ER stress and improve insulin sensitivity in *ob*/*ob* mice [33], improving ER homeostasis may be a promising tool to treat diabetes-associated wound healing disorders.

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