INTRODUCTION

Heightened insulin-mediated activation of the vasoconstrictor/pro-atherogenic mitogen-activated protein kinase (MAPK)-endothelin-1 (ET-1) signaling pathway is a distinct feature of endothelial dysfunction and is thought to represent a principal contributor to the pathogenesis of micro- and macrovascular disease associated with obesity and type 2 diabetes (1, 2). As such, a deepened understanding of the precipitating factors and mechanisms underlying the defects in vascular insulin signaling, and in particular the up-regulation of the ET-1 pathway, is an area of intense research and critical for the development of therapeutic strategies aimed at prevention of cardiovascular diseases (3).

Herein, we examine the potential role of endoplasmic reticulum (ER) stress in mediating impaired vascular insulin signaling. The ER is an organelle specialized in the synthesis, folding, assembly and modification of proteins (4, 5). ER homeostasis is strictly monitored and maintained in the face of a broad spectrum of potential stressors (4-7). However, prolonged periods of cell stress can lead to an accumulation of unfolded and/or misfolded proteins in the ER lumen that, upon exceeding its protein folding capacity, triggers an adaptive signaling cascade; i.e., the unfolded protein response (4-7). While initially a compensatory attempt to maintain cellular homeostasis, sustained ER stress and thus activation of the unfolded protein response is now thought to be the basis of a number of chronic diseases including metabolic and cardiovascular disease (4, 8-14). Indeed, emerging evidence suggests that ER stress is a key link between obesity, type 2 diabetes and the development of whole-body insulin resistance and cardiovascular disease (4, 10, 12-20). In addition, current data demonstrate that persistent ER stress in endothelial cells is associated with a pro-atherogenic endothelial cell phenotype, suggesting an involvement of vascular ER stress in mediating vascular disease (10, 21-26). In the present study we tested the hypothesis that experimental induction of vascular ER stress impairs insulin-stimulated vasomotor reactivity. We report that experimental induction of ER stress in isolated aortic rings with tunicamycin (20 µg/mL), a well-established inducer of ER stress, resulted in insulin-stimulated vascular contraction (–78±21% at 1000 µIU/mL; –100±27% at 10,000 µIU/mL; all p<0.05) rather than relaxation (+23±7% at 1000 µIU/mL; +43±8% at 10,000 µIU/mL; all p<0.05). Importantly, we found that insulin-stimulated vascular contraction as a result of ER stress was largely eliminated in the presence of tezosentan (3 µM), a nonselective endothelin-1 (ET-1) receptor blocker (+1±14% at 1000 µIU/mL; +8±17% at 10,000 µIU/mL). Similarly, inhibition of ET-1 receptors fully restored the impairment of acetylcholine-mediated relaxation induced by ER stress (maximal relaxation: control = 94±2%, tunicamycin = 76±5%, tunicamycin + tezosentan = 90±3). Furthermore, we demonstrate that ER stress caused a ~20-fold greater release of ET-1 from aortic endothelial cells under basal conditions as well as a ~15-fold increase under insulin-stimulated conditions (p<0.05). This ER stress-mediated up-regulation in ET-1 release from endothelial cells was accompanied by a ~3-fold increase in phosphorylation of p44/22 MAPK (p<0.05), a known pathway by which insulin signaling activates ET-1. Together, these findings support the hypothesis that vascular ER stress-mediated activation of ET-1 may be an underlying cause of impaired vasomotor responsiveness to insulin and endothelial dysfunction.

Key words: unfolded protein response, endothelial function, vascular insulin resistance, endothelin-1, endoplasmic reticulum stress, tunicamycin

MATERIALS AND METHODS

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri.
A total of 31 young adult, female Wistar rats (weight ~250 g) were used for the present study. Out of the 31 animals, 6 animals were used to examine in vitro effects of tunicamycin on insulin, acetylcholine, and sodium nitroprusside (SNP)-mediated aortic relaxation with and without endothelium denudation; 10 animals were used to examine in vitro effects of tunicamycin on insulin and acetylcholine-mediated aortic relaxation in the presence or absence of ET-1 receptor blocker tezosentan; 7 animals were used to examine in vitro effects of tunicamycin on phenylephrine and ET-1-mediated aortic contraction; and 8 animals were used to examine in vitro effects of tunicamycin on endothelial mRNA expression of ER stress markers. Detailed procedures are described below. All rats were housed in the College of Veterinary Medicine’s animal Care Facility. Rats were maintained in a temperature-controlled (21°C) environment on a 12:12-h light-dark cycle (7 AM to 7 PM). All animals were given ad libitum access to standard chow diet. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (100 mg/kg). Aortas were then harvested and animals were euthanized by removal of the heart in full compliance with the American Veterinary Medical Association Guidelines on Euthanasia.

**Vasomotor reactivity of isolated aortic rings in response to endoplasmic reticulum stress**

The thoracic aortas were harvested, trimmed of perivascular fat and connective tissue, and sectioned into 2 mm rings in cold Krebs. Rings were then mounted on wire feet connected to isometric force transducers and submerged in 15 mL water-jacketed organ culture baths (made of glass) containing physiological Krebs solution (described below) maintained at 37°C for 1 h to allow for equilibration. Aortic rings were stretched to optimal length which was equivalent to a resting tension of ~3.75 g. Aortic rings were incubated with ER stress inducer tunicamycin (20 µg/mL) (27), or vehicle control (DMSO, 0.1%), for 60 min prior to vasomotor function assessments. Tunicamycin is an inhibitor of N-linked glycosylation and a well-established chemical to induce ER stress in vitro and in vivo (25, 27-32). Vasoreactivity was assessed with cumulative concentration-response curves of insulin (10 to 10,000 µU/mL), acetylcholine (10⁻⁶ to 10⁻⁴ M), SNP (10⁻⁶ to 10⁻⁴ M), ET-1 (10⁻⁶ to 10⁻³ M), and phenylephrine (PE, 10⁻⁹ to 10⁻⁴ M). A submaximal concentration of phenylephrine (3×10⁻⁴ M) was used to preconstrict all vessels prior to acetylcholine, insulin and SNP relaxation curves. To assess the extent to which the vasomotor effects of insulin and acetylcholine, with and without tunicamycin, were endothelium-dependent, the endothelium from designated aortic rings was removed by gently rubbing the luminal surface with a fine-tipped forceps. This is a well-established technique to examine the role of the endothelium (33-35). The contribution of ET-1 in altering insulin and acetylcholine-stimulated relaxation was assessed by incubating the rings with tezosentan (3 µM for 60 min), a nonselective ET-1 receptor blocker. Tezosentan was added into the bath at the same time as tunicamycin and vehicle controls. Between concentration-response curves, aortic rings were washed with Krebs every 15 min until resting tension was back to initial resting tension (i.e., ~3.75 g). For insulin, acetylcholine, and SNP curves, relaxation at each concentration was measured and expressed as percent maximum relaxation, where 100% is equivalent to loss of all tension developed in response to phenylephrine (36). The physiological Krebs buffer solution contained (in mM) 131.5 NaCl, 5.0 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 11.2 glucose, 20.8 NaHCO₃, 0.003 propanolol, and 0.025 EDTA. The solution was aerated with 95% O₂-5% CO₂ (pH 7.4) and maintained at 37°C.

Endothelium mRNA expression of endoplasmic reticulum stress markers in response to tunicamycin treatment

We confirmed that treatment of aortic rings with tunicamycin induces ER stress in endothelial cells. For this proof-of-concept experiment, we divided aortas from 8 rats into two aortic segments of ~8 mm of length each. After incubation of aortic segments with tunicamycin (20 µg/mL) or vehicle control (DMSO) for 60 min, reproducing same conditions as those in functional experiments, we scraped endothelial cells from the aortic segments and measured mRNA expression of GRP78 and CHOP (two classic ER stress-related genes). Briefly, artery segments were opened longitudinally and pinned down with the vessel lumen faced up. Trizol was applied on the endothelial surface, and, using a blade, the endothelial cell layer was gently scraped as described previously (37). This method of scraping the luminal surface yields an endothelial-cell enriched sample. Extraction of total mRNA and assessment of mRNA levels was performed via real time PCR as previously described (38-40). Primer sequences were designed using the NCBI Primer Design tool. The primer sequences for 18S were as follows: sense 5'-GCCGCTAGAGGTGAAATCTGG-3'; antisense 5’-CATTCTGGCAAATGCTTTCG-3’. The primer sequences for GRP78 were as follows: sense 5’-GCAGTGTCTACGTTGGTC-3’; antisense 5'-TCCAAGGTGAACACCACACC-3’. The primer sequences for CHOP were as follows: sense 5’-AGAGCCAAATAACACGCGGGA-3’; antisense 5’-ACCGTTTCTCTGGTACGT-3’. mRNA expression values are presented as 2⁻ΔCT whereby ΔCT = 18S CT - gene of interest CT (38-40).

Endothelin-1 release from cultured aortic endothelial cells

Rat aortic endothelial cells were maintained under standard culture conditions (37°C, 5% CO₂) with Rat Endothelial Cell Growth Medium (Cell Applications, San Diego, CA). At passage three, after reaching >95% confluency, endothelial cells were treated for 60 min with tunicamycin (20 µg/mL), or vehicle control (DMSO), in Dulbecco’s Modified Eagle Medium. Thereafter, media was removed and new media containing tunicamycin (20 µg/mL), or vehicle control, with and without insulin (10 to 10,000 µU/mL) was added for 15 min. Experiments were performed in 12-well plates and five replicates from independent experiments were obtained per condition. After treatments, the supernatant was collected for assessment of ET-1 concentration to determine the release of ET-1 from endothelial cells. Measurements of ET-1 concentration were performed using the ET-1 QuantiKine ELISA Kit (R&D Systems) as per the manufacture instructions. In addition, endothelial cells were lysed in Laemmli buffer and kept at ~80°C until immunoblot analysis for phosphorylation of MAPK.

Immunoblot analysis for phospho-specific and total p44/42-MAPK

Immunoblot analysis was performed as previously described (37, 41). Briefly, total protein content from each well of cultured endothelial cells was measured using the NanoOrange protein assay. Samples were then diluted with Laemmli buffer and 5 µg of protein from each sample was loaded onto polyacrylamide gels for separation by electrophoresis. Next, proteins were transferred to PVDF membranes and probed with the antibodies p44/42-MAPK (1:500; Cell Signaling), and phospho-specific p44/42-MAPK at Thr202/Tyr204 (1:250; Cell Signaling) (41). Total and phospho-specific densities were quantified, and the ratios of phospho-specific to total density were calculated.
Statistical analysis

Concentration-response curves from vasomotor function experiments, ET-1 concentration in supernatant of cultured endothelial cells, and phosphor-specific and total p44/42-MAPK protein expression were analyzed using a two-factor (condition × concentration) ANOVA. All data are presented as means ± S.E. For all statistical tests, the α level was set at 0.05. Statistical analyses were performed with SPSS 20.0. (SPSS, Chicago, IL).

RESULTS

No differences in aortic ring resting tension were observed among conditions before and after administration of tunicamycin and/or tezosentan (all p>0.05). In addition, no differences in aortic ring tension after preconstriction with phenylephrine were observed among conditions prior to dose-response curves (all p>0.05). As depicted in Fig. 1A, aortic rings treated with tunicamycin (ER stress inducer) exhibited insulin-stimulated constriction (−78±21% at 1000 µIU/mL; −100±27% at 10,000 µIU/mL; all p<0.05), whereas control aortic rings exhibited relaxation (+23±7% at 1000 µIU/mL; +43±8% at 10,000 µIU/mL; all p<0.05). Furthermore, acetylcholine-induced relaxation in tunicamycin-treated aortic rings was reduced compared to control rings (maximal relaxation: tunicamycin = 76±5% vs. control = 94±2%, p<0.05; Fig. 1B). In contrast, tunicamycin did not influence SNP-induced relaxation of aortic rings (maximal relaxation: tunicamycin = 109±6% vs. control = 111±9%, p>0.05; Fig. 1C). Removal of the endothelium from the aortic rings abolished the insulin (Fig. 1D) and acetylcholine-mediated (Fig. 1E) vasomotor effects in both tunicamycin and control-treated rings. Of note, application of tunicamycin into the bath for 60 min did not affect resting tension of aortic rings (p>0.05). As shown in Fig. 1F, treatment of aortic rings with tunicamycin for 60 min induced expression of GRP78 and CHOP mRNAs in endothelial scrapes (p<0.05), two classic markers of ER stress.

As illustrated in Fig. 2A, insulin-stimulated contraction of aortic rings treated with tunicamycin was abolished (p<0.05) after incubation of aortic rings with tezosentan (a non-selective inhibitor of ET-1 receptors), whereas insulin-stimulated relaxation in control aortic rings was unaffected by tezosentan (p>0.05). Similarly, tunicamycin-induced impairment in acetylcholine-

Fig. 1. Effect of tunicamycin, an endoplasmic reticulum stress inducer, on insulin (panel A, n=15/condition), acetylcholine (panel B, n=16/condition), and SNP-mediated (panel C, n=6/condition) vasoreactivity of aortic rings. Panels D (n=6/condition) and E (n=6/condition) illustrate the effect of endothelial denudation on insulin and acetylcholine-mediated vasoreactivity of aortic rings treated with and without tunicamycin. Panel F depicts induction of mRNA GRP78 and CHOP in endothelial scrapes from aortic rings treated with and without tunicamycin for 60 min (n=8/condition). *denotes an effect of tunicamycin (p<0.05).
mediated relaxation was rescued (p<0.05) after incubation of aortic rings with tezosentan, whereas acetylcholine-induced relaxation in control rings was unaffected by tezosentan (p>0.05) (Fig. 2B). Of note, application of tezosentan into the bath for 60 min did not affect resting tension of aortic rings (p>0.05).

As depicted in Fig. 3A, aortic rings treated with tunicamycin exhibited reduced constriction to ET-1 compared to untreated rings (p<0.05). In contrast, tunicamycin did not significantly influence phenylephrine-induced constriction of aortic rings (Fig. 3B; p>0.05). ET-1 release from cultured endothelial cells was increased in cells treated with tunicamycin for 60 min (p<0.05). The effect of tunicamycin on ET-1 release was persistent after stimulating endothelial cells with insulin for 15 min. Under tunicamycin-treated conditions, insulin-stimulated release of ET-1 was significant at 100 and 1000 µU/mL of insulin (p<0.05), whereas under control conditions, insulin-stimulated release of ET-1 was significant at 1000 and 10,000 µU/mL of insulin (p<0.05; Fig. 4A). Furthermore, tunicamycin produced an increase in phosphorylation of p44/42 MAPK, relative to total p44/42 MAPK, in cultured endothelial cells under basal conditions and during stimulation with insulin for 15 min (p<0.05; Fig. 4B).

DISCUSSION

The primary finding of the present study is that vascular ER stress impairs insulin-stimulated vasomotor relaxation and that this impairment is largely mediated by ET-1 activation. In particular, we found that experimental induction of ER stress in isolated aortic rings resulted in insulin-stimulated vascular contraction which was abolished in the presence of an ET-1 receptor blocker. Further substantiating this finding, we provide evidence that ER stress increases the release of ET-1 from cultured aortic endothelial cells under basal conditions and upon insulin stimulation. Together, these data support the hypothesis that vascular ER stress-mediated activation of ET-1 may be an underlying cause of impaired vasomotor responsiveness to insulin.

Impaired vascular insulin signaling is proposed as an important contributor to the pathogenesis of micro- and macrovascular disease caused by obesity and type 2 diabetes (1, 2). With the rates of metabolic disorders on the rise (42, 43), understanding the factors and mechanisms contributing to impaired vascular insulin signaling is crucial for development of
therapeutic strategies aimed at prevention and treatment of cardiovascular disease. ER stress associated with obesity and type 2 diabetes contributes to whole body insulin resistance and emerging evidence indicates that sustained ER stress may be involved in the instigation of endothelial dysfunction and development of cardiovascular disease (4, 10, 12-14, 16-20). Herein we provide direct evidence that vascular ER stress is sufficient to impair vasomotor responses to insulin. That is, we found that experimental induction of ER stress in isolated aortic rings with tunicamycin, a well-established ER stress inducer, resulted in insulin-stimulated vascular contraction rather than mild relaxation. It is well established that insulin-stimulated relaxation is endothelium dependent and nitric oxide mediated (44-46). The fact that insulin-stimulated vascular contraction induced by ER stress was abolished after mechanical denudation of the endothelium strongly suggested that the signaling molecule mediating the increased contraction was also endothelial-derived. As ET-1 is a potent endothelium-derived, insulin responsive vasoconstrictor (46), we next examined the contribution of ET-1 action to the constriction effect of ER stress. Indeed, we found that insulin-stimulated vascular contraction as a result of ER stress was largely eliminated in the presence of a nonselective ET-1 receptor blocker. We then evaluated whether insulin-stimulated vascular contraction caused by ER stress was due to enhanced vascular sensitivity to ET-1 and/or increased production of ET-1 from endothelial cells. Of interest, we found that aortic rings treated with the ER stress inducer exhibited a blunted contraction to exogenous ET-1, compared to control aortic rings, suggesting that sensitivity to ET-1 was in fact reduced upon ER stress induction. Based on this observation we deduced that insulin-induced vascular contraction resulting from ER stress was stimulated by ET-1 production from endothelial cells. Further experiments carried out in cultured rat aortic endothelial cells indicated that ER stress caused a robust increase in p44/42 MAPK phosphorylation and release of ET-1 from endothelial cells under both basal and insulin-stimulated conditions. Collectively, these findings support the idea that ER stress is sufficient to impair insulin-stimulated vasorelaxation and that this impairment is likely due to an engagement of the endothelial MAPK/ET-1 pathway, as opposed to increased sensitivity to ET-1.

Our observation that ER stress impairs acetylcholine-induced vascular relaxation supports recent data in rodent models demonstrating that the impairment of aortic endothelial function associated with hypertension (27) and type 1 diabetes (47) was largely rescued with intraperitoneal injections of tauroursodeoxycholic acid, a chemical chaperone that inhibits ER stress. Similarly, Zhou et al. (48) reported in a rat model of
chronic kidney disease that impaired insulin-stimulated relaxation in aortic rings was improved with systemic alleviation of ER stress. Furthermore, our findings are consistent with existing evidence supporting the involvement of ER stress and the consequent unfolded protein response in the regulation of MAP kinases (49). For example, it has been shown that induction of ER stress with thapsigargin, a sarco(endoplasmic reticulum Ca\(^{2+}\) ATPase inhibitor, increases phosphorylation of p44/22 MAPK in cultured smooth muscles cells within 15 min (50). Our study supports and extends these previous findings by providing direct evidence that experimentally induced vascular ER stress produces an impairment in insulin-stimulated vasorelaxation through an up-regulation of the ET-1 pathway.

The finding that vascular induction of ER stress markedly up-regulates the ET-1 pathway is relevant in light of the growing body of literature indicating that excess ET-1 signaling contributes to the pathogenesis of endothelial dysfunction (51). Along these lines, here we show that the ER stress-mediated impairment of acetylcholine-induced vascular relaxation was resolved with ET-1 receptor blockade, suggesting a contribution of ET-1 action to the blunting of endothelium-dependent relaxation in the context of ER stress. Apart from its direct vasoactive activity, ET-1 has been implicated in pro-atherogenic processes within the vascular wall (51). Specifically, ET-1 activates macrophages, resulting in the release of pro-inflammatory and chemotactic mediators, enhances endothelial expression of vascular adhesion molecules, and increases formation of reactive oxygen species (51-53). Notably, current evidence derived from studies in the placenta indicates that ET-1 can also induce ER stress (54). Although to our knowledge whether ET-1 can cause ER stress in vascular cells has not been examined, it is tempting to speculate that ER stress-mediated production of ET-1 may further compromise vascular ER homeostasis, thus creating a self-perpetuating vicious cycle. In the context of obesity and diabetes, several factors could signal vascular induction of ER stress including hyperglycemia (55), increased levels of circulating pro-inflammatory cytokines (56), and free fatty acids (57, 58).

In summary, we provide evidence that induction of ER stress in isolated aortic rings resulted in insulin-stimulated vascular contraction which was nearly eradicated in the presence of an ET-1 receptor blocker. In this regard, we also demonstrate that ER stress is associated with an increased release of ET-1 from endothelial cells under basal conditions and during insulin stimulation. Collectively, these findings support the idea that vascular ER stress-mediated activation of ET-1 may be an underlying cause of impaired vasmotor responsiveness to insulin and endothelial dysfunction. It should be noted that young female rats were used in the present study. Additional research should examine whether induction of ER stress in arteries from different groups of animals (e.g., female vs. male, young vs. old, lean vs. obese) would produce distinct vasmotor responses to insulin and other endothelium-dependent vasodilators.

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