

# ER stress and the JNK pathway in insulin resistance

## Review Article

Hideaki Kaneto\*, Yoshihisa Nakatani, and Munehide Matsuhisa

Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

**\*Correspondence:** Hideaki Kaneto, MD, PhD, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; Tel. (81-6) 6879-3633; Fax (81-6) 6879-3639; e-mail: kaneto@medone.med.osaka-u.ac.jp

**Key words:** diabetes JNK pathway, ER stress, insulin resistance

**Abbreviations:** subunit of translation initiation factor 2, (eIF2); antisense ORP150 expressing adenovirus, (Ad-AS-ORP); c-Jun N-terminal kinase, (JNK); disappearance rate, (Rd); dominant-negative JNK expressing adenovirus, (Ad-DN-JNK); dominant-negative type, (DN); endoplasmic reticulum, (ER); fluorescein isothiocyanate, (FITC); GFP expressing control adenovirus, (Ad-GFP); glucose infusion rate, (GIR); glucose-6-phosphatase, (G6Pase); hepatic glucose production, (HGP); human immunodeficiency virus, (HIV-1); insulin receptor substrate-1, (IRS-1); intraperitoneal glucose tolerance test, (IPGTT); intraperitoneal insulin tolerance test, (IPITT); islet-brain-1, (IB-1); JNK-interacting protein-1, (JIP-1); mouse embryo fibroblasts, (MEFs); oxygen-regulated protein 150, (ORP150); pancreatic ER kinase, (PERK); phosphoenolpyruvate carboxykinase, (PEPCK); protein transduction domains, (PTDs); sense ORP150 expressing adenovirus, (Ad-S-ORP); wild type, (WT); X-box-binding protein-1, (XBP-1)

Received: 29 November 2004; Revised: 9 December 2004

Accepted: 14 December 2004; electronically published: January 2005

## Summary

The endoplasmic reticulum (ER) is an organelle which synthesizes various secretory and membrane proteins. These proteins are correctly folded and assembled by chaperones in the ER. During stressful conditions such as upon an increase in the misfolded protein level, the chaperones become overloaded and the ER fails to fold and export newly synthesized proteins, leading to ER stress. Under diabetic conditions ER stress is induced and the JNK pathway is subsequently activated, which is involved in the insulin resistance. Increase of ER stress and activation of the JNK pathway interferes with insulin action. In reverse, reduction of ER stress and suppression of the JNK pathway in obese diabetic mice markedly improve insulin resistance and ameliorate glucose tolerance. Taken together, increase of ER stress and subsequent activation of the JNK pathway play a crucial role in the progression of insulin resistance found in diabetes and thus could be a potential therapeutic target for diabetes.

## I. Involvement of ER stress in insulin resistance

Type 2 diabetes is the most prevalent and serious metabolic disease affecting people all over the world. The hallmark of the disease is insulin resistance as well as pancreatic  $\beta$ -cell dysfunction. Under diabetic conditions, various insulin target tissues such as liver, muscle, and fat become less responsive or resistance to insulin. This state is also often linked to other common diseases such as obesity, hyperlipidemia, hypertension, and atherosclerosis. The pathophysiology of insulin resistance involves a complex network of insulin signaling pathways. After insulin binds to insulin receptor on cell surface, insulin receptor and its substrates are phosphorylated, which leads to activation of various insulin signaling pathways. The endoplasmic reticulum (ER) is an organelle which synthesizes various secretory and membrane proteins.

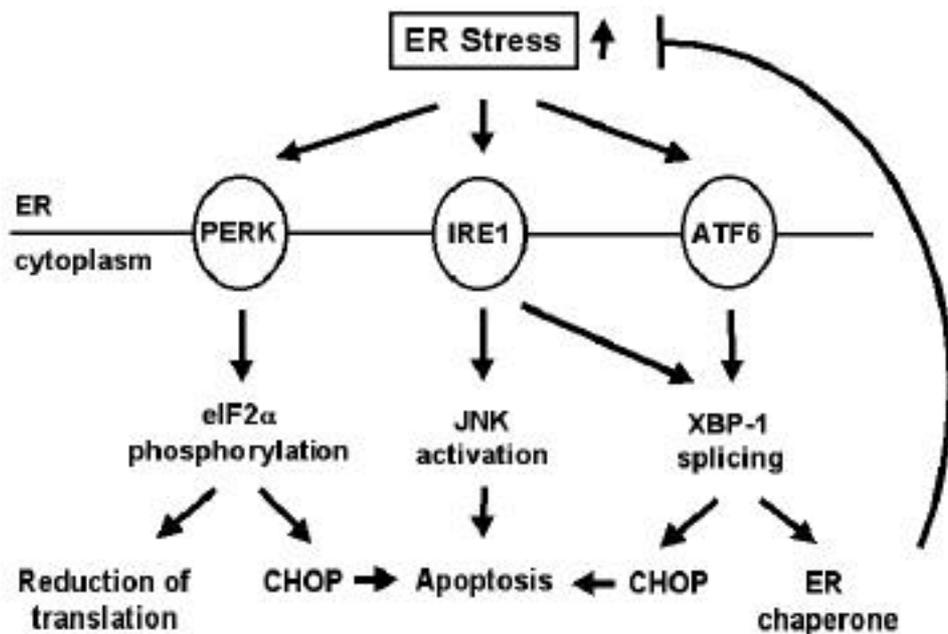
These proteins are correctly folded and assembled by chaperones in the ER. During stressful conditions such as upon an increase in the misfolded protein level, the chaperones become overloaded and the ER fails to fold and export newly synthesized proteins, leading to ER stress (Aridor et al, 1999; Harding et al, 1999; Ron et al, 2002; Tirasophon et al, 1998; Wang et al, 1998). Once ER stress is provoked in the cells, various pathways are activated (**Figure 1**). The pancreatic ER kinase (or PKR-like kinase) (PERK) is an ER transmembrane protein kinase that phosphorylates the subunit of translation initiation factor 2 (eIF2) in response to ER stress, and eIF2 phosphorylation leads to reduction of translation and induction of apoptosis (Shi et al, 1998; Harding et al, 1999; Shi et al, 2003). It is also known that ER stress activates the c-Jun N-terminal kinase (JNK) pathway, leading to induction of apoptosis in various cells (Urano et al, 2000). Furthermore, ER stress is known to trigger X-

box-binding protein-1 (XBP-1) splicing. XBP-1 is a transcription factor that modulates the ER stress response, and its spliced form is a key molecule in ER stress response through transcriptional regulation of various genes including molecular chaperones (**Figure 1**) (Yoshida et al, 2001; Iwawaki et al, 2003). It was previously reported that ER stress is involved in pancreatic  $\beta$ -cell apoptosis (**Figure 2**) (Inoue et al, 1998, Harding et al, 2001, 2002; Oyadomari et al, 2001, 2002). Oxygen-regulated protein 150 (ORP150), a molecular chaperone found in the ER, has been shown to protect cells from ER stress (Kuwabara et al, 1996; Tamatani et al, 2001). We recently reported that ORP150 overexpression markedly improved insulin resistance and ameliorated glucose tolerance in diabetic animals, indicating that ER stress plays a crucial role in insulin resistance (**Figure 2**) (Nakatani et al, 2004).

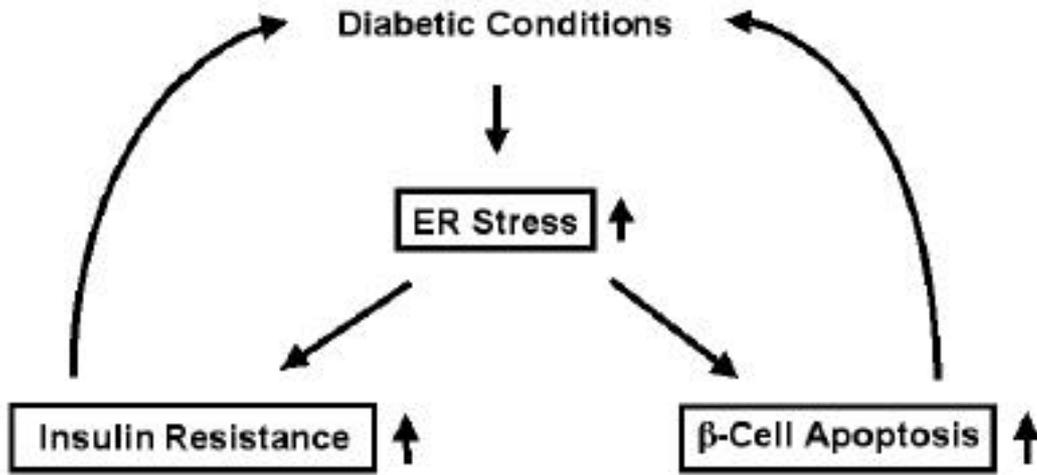
To examine whether ER stress is increased in the liver under diabetic conditions, we evaluated the ER stress level in the livers of 10 week-old obese diabetic C57BL/KsJ-db/db mice. Expression levels of KDEL and Bip, both of which are ER stress markers, were much higher in the obese diabetic mice compared to 10 week-old non-diabetic C57BL6 mice, indicating that ER stress is actually increased under diabetic conditions (**Figure 2**) (Nakatani et al, 2004). It was also reported that expression levels of several ER stress markers are increased in dietary (high-fat diet-induced) and genetic (ob/ob) models of obesity. PERK and eIF2 phosphorylation was increased in the liver of obese mice compared with lean controls. Furthermore, it was recently reported that increase of free

fatty acids, one of the contributory mechanisms for insulin resistance in obesity and type 2 diabetes, causes pancreatic  $\beta$ -cell apoptosis via ER stress (Kharroubi et al, 2004). Taken together, ER stress is induced in various tissues under diabetic conditions.

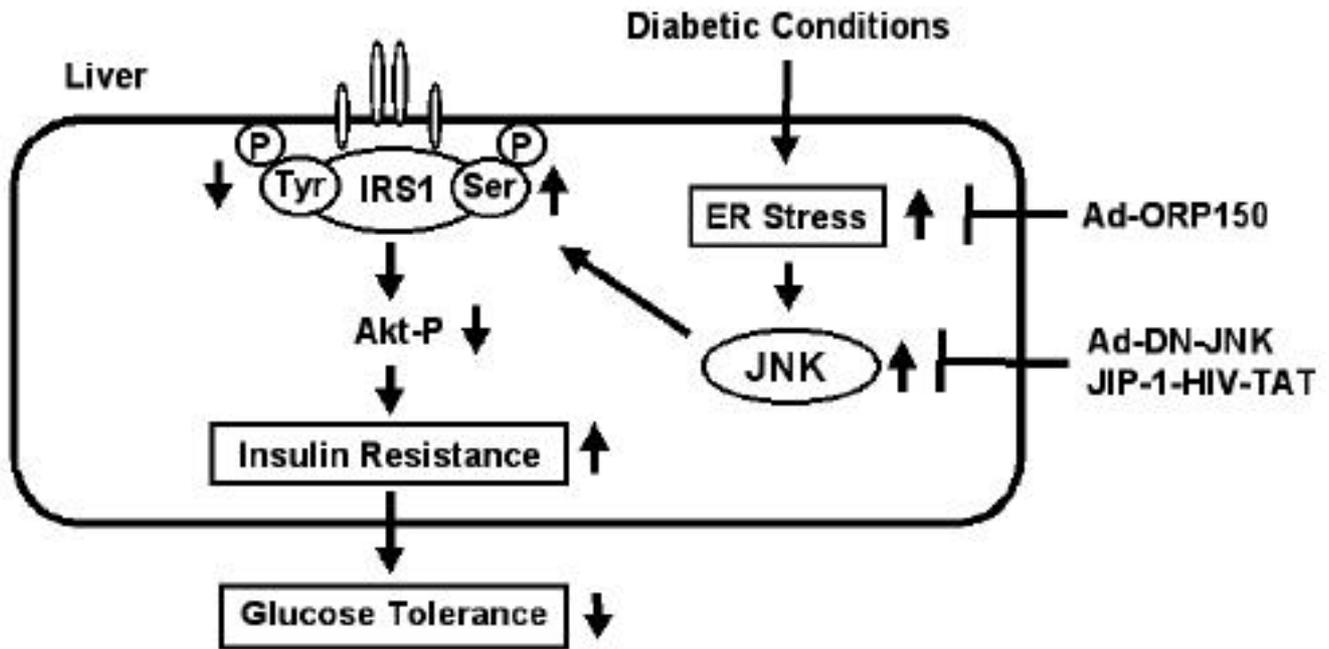
Consistent with earlier observations (Hirosumi et al, 2002), total JNK activity was also dramatically elevated in the obese mice (Ozcan et al, 2004). It was reported that when Fao liver cells were treated with tunicamycin or thapsigargin, agents commonly used to induce ER stress, insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) was significantly decreased. IRS-1 is a substrate for insulin receptor tyrosine kinase, and serine phosphorylation of IRS-1, particularly mediated by JNK, reduces insulin receptor signaling. Indeed, pretreatment of Fao cells with tunicamycin produced a significant increase in serine phosphorylation of IRS-1. Tunicamycin pretreatment also suppressed insulin-induced Akt phosphorylation (**Figure 3**) (Ozcan et al, 2004). Furthermore, inhibition of JNK activity with the synthetic inhibitor, SP600125, reversed the ER stress-induced serine phosphorylation of IRS-1. Pretreatment of Fao cells with a highly specific inhibitory peptide derived from the JNK-binding protein, JIP, also completely preserved insulin receptor signaling in cells exposed to tunicamycin. Similar results were obtained with the synthetic JNK inhibitor, SP600125. These results indicate that ER stress promotes a JNK-dependent serine phosphorylation of IRS-1, which in turn inhibits insulin receptor signaling (**Figure 3**) (Ozcan et al, 2004).



**Figure 1.** ER stress signaling. Once ER stress is induced in the cells, various pathways are activated. Induction of ER stress leads to eIF2 phosphorylation, JNK activation and XBP-1 splicing



**Figure 2.** Role of ER stress in diabetes. ER stress is induced under diabetes conditions, which is involved in insulin resistance and pancreatic  $\beta$ -cell apoptosis.



**Figure 3.** ER stress and the JNK pathway in insulin resistance. The JNK pathway is activated under diabetic conditions, which increases insulin resistance and worsens glucose tolerance

To examine a role of ER stress in insulin resistance *in vivo*, we prepared sense ORP150 expressing adenovirus (Ad-S-ORP), and a GFP expressing control adenovirus (Ad-GFP), and delivered each adenovirus to 8 week-old C57BL/KsJ-db/db obese diabetic mice from the cervical vein. We confirmed an increase in ORP150 expression in the liver upon adenovirus injection, but not in other tissues such as muscle and adipose tissue. In addition, expression levels of KDEL and Bip in Ad-S-ORP-treated mice were lower compared to those in Ad-GFP treated db/db mice, indicating that ORP150 is actually acting to decrease ER stress in the liver. There was no difference in body weight and food intake between Ad-S-ORP-treated- and Ad-GFP-

treated-db/db mice. When C57BL/KsJ-db/db mice were treated with Ad-S-ORP, nonfasting blood glucose levels were markedly reduced, whereas no such effects were observed in Ad-GFP-treated mice. Fasting blood glucose concentrations were also significantly lower in Ad-S-ORP-treated mice compared to Ad-GFP-treated mice. To examine the effects of ORP150 overexpression in the liver on insulin resistance, we performed the intraperitoneal insulin tolerance test (IPITT). The hypoglycemic response to insulin was larger in Ad-S-ORP-treated C57BL/KsJ-db/db mice compared to Ad-GFP-treated mice. To investigate this point further, we performed the euglycemic hyperinsulinemic clamp test. The GIR of Ad-

S-ORP-treated mice were significantly higher compared to Ad-GFP-treated mice, indicating that ORP150 overexpression in the liver reduces insulin resistance and thus ameliorates glucose tolerance in C57BL/KsJ-db/db mice. We also evaluated endogenous hepatic glucose production (HGP) in Ad-S-ORP-treated mice using tracer methods. HGP was significantly lower in Ad-S-ORP-treated mice compared to Ad-GFP-treated mice. These results indicate that the reduction of insulin resistance and amelioration of glucose tolerance by Ad-S-ORP overexpression are mainly due to the suppression of HGP (**Figure 3**) (Nakatani et al, 2004).

Similarly, to examine the effects of antisense ORP150 expression in the liver on insulin sensitivity and glucose tolerance in non-diabetic animals, we prepared an antisense ORP150 expressing adenovirus (Ad-AS-ORP) and delivered each adenovirus to 8 week-old C57BL6 mice. The intraperitoneal glucose tolerance test (IPGTT) revealed that glucose tolerance is markedly worsened upon antisense ORP150 expression. Furthermore, in the euglycemic hyperinsulinemic clamp study, glucose infusion rate (GIR) of Ad-AS-ORP-treated C57BL6 mice were significantly lower compared to Ad-GFP-treated mice, indicating that ER stress in the liver reduces insulin sensitivity in C57BL6 mice. Furthermore, we evaluated HGP in Ad-AS-ORP-treated mice using tracer methods. HGP in Ad-AS-ORP-treated mice was significantly greater compared to Ad-GFP-treated mice. These results indicate that antisense ORP150 expression decreases insulin sensitivity at least in part by increasing HGP in non-diabetic mice (Nakatani et al, 2004).

To examine the molecular mechanisms involved in the alteration of insulin action by ER stress in our experiments, we evaluated the phosphorylation state of IRS-1 and Akt in the liver, which are key molecules for insulin signaling. IRS-1 tyrosine phosphorylation was markedly increased in Ad-S-ORP-treated C57BL/KsJ-db/db mice compared to Ad-GFP-treated mice. Concomitantly, an increase in Akt serine 473 phosphorylation was observed in Ad-S-ORP-treated C57BL/KsJ-db/db mice compared to Ad-GFP-treated mice (**Figure 3**). We next examined the expression levels of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), both of which are known to be regulated by insulin signaling. Both the expression of PEPCK and G6Pase was markedly decreased by Ad-S-ORP treatment in C57BL/KsJ-db/db mice. These results indicate that reduction of ER stress enhances insulin signaling which leads to a decrease in gluconeogenesis and amelioration of glucose tolerance (Nakatani et al, 2004). Taken together, sense ORP150 overexpression decreased insulin resistance and markedly improved glycemic control in diabetic model animals, and in contrast antisense ORP150 expression induced insulin resistance in nondiabetic control mice, indicating that ER stress plays a crucial role in the insulin resistance found in diabetes (**Figures 2, 3**).

Furthermore, it was reported that mice deficient in XBP-1, a transcription factor that modulates the ER stress response, develop insulin resistance. The spliced form of XBP-1 is a key molecule in ER stress response through

transcriptional regulation of various genes including molecular chaperones (**Figure 1**). In mouse embryo fibroblasts (MEFs) derived from XBP-1<sup>-/-</sup> mice, tunicamycin treatment resulted in increase of PERK phosphorylation. In these cells, there was also a rapid and robust activation of JNK in response to ER stress. When spliced XBP-1 expression was induced, there was a dramatic reduction in both PERK phosphorylation and JNK activation after tunicamycin treatment, indicating that XBP-1<sup>-/-</sup> cells are prone to ER stress. Thus, it is likely that alteration in the levels of spliced XBP-1 protein results in alteration in the ER stress responses. Furthermore, tunicamycin-induced IRS-1 serine phosphorylation was significantly reduced in fibroblasts exogenously expressing spliced XBP-1. The extent of IRS-1 tyrosine phosphorylation was significantly higher in cells overexpressing spliced XBP-1. In contrast, IRS-1 serine phosphorylation was strongly induced in XBP-1<sup>-/-</sup> MEFs compared with XBP-1<sup>+/+</sup> controls even at low doses of tunicamycin treatment. After insulin stimulation, the amount of IRS-1 tyrosine phosphorylation was significantly decreased in tunicamycin-treated XBP-1<sup>-/-</sup> cells compared with tunicamycin-treated wild-type controls (Ozcan et al, 2004).

Since complete XBP-1 deficiency results in embryonic lethality, BALB/c-XBP-1<sup>-/-</sup> mice with a null mutation in one XBP-1 allele were used in order to investigate the role of XBP-1 in insulin resistance and diabetes *in vivo*. XBP-1<sup>+/-</sup> mice treated with high fat diet developed continuous and progressive hyperinsulinemia. Blood glucose levels were also increased in the XBP-1<sup>+/-</sup> mice treated with high fat diet. During insulin tolerance test, the hypoglycemic response to insulin was also significantly lower in XBP-1<sup>+/-</sup> mice compared with XBP-1<sup>+/+</sup> littermates (Ozcan et al, 2004). PERK phosphorylation was increased in the liver of obese XBP-1<sup>+/-</sup> mice compared with wild-type controls treated with high fat diet. There was also a significant increase in JNK activity in XBP-1<sup>+/-</sup> mice compared with wild type controls. Consistently, Ser<sup>307</sup> phosphorylation of IRS-1 was increased in XBP-1<sup>+/-</sup> mice compared with wild-type controls. There was no detectable difference in any of the insulin receptor signaling components in liver and adipose tissues between genotypes taking regular diet. However, after treatment with high fat diet, major components of insulin receptor signaling in the liver, including IRS-1 tyrosine- and Akt serine-phosphorylation, were decreased in XBP-1<sup>+/-</sup> mice compared with wild type controls. A similar suppression of insulin receptor signaling was also evident in the adipose tissues of XBP-1<sup>+/-</sup> mice compared with XBP-1<sup>+/+</sup> mice (Ozcan et al, 2004). Taken together, induction of ER stress or reduction in the compensatory capacity through down-regulation of XBP-1 leads to suppression of insulin receptor signaling in intact cells via IRE-1 -dependent activation of the JNK pathway. Experiments with mouse models also yielded data consistent with the link between ER stress and systemic insulin action. Deletion of an XBP-1 allele in mice leads to enhanced ER stress, activation of the JNK pathway, reduced insulin receptor signaling, systemic insulin resistance, and type 2 diabetes. Therefore, ER stress is

involved in progression of insulin resistance and thus could be a potential therapeutic target for diabetes (Figures 2, 3).

## II. Involvement of the JNK pathway in insulin resistance

The JNK pathway (Hibi et al, 1993; Derijard et al, 1994; Davis et al, 2000; Chang et al, 2001) is known to be activated by ER stress (Urano et al, 2000) and thus is possibly involved in the progression of insulin resistance. We have recently examined the effects of modulation of the JNK pathway in the liver on insulin resistance and glucose tolerance (Nakatani et al, 2004). Overexpression of dominant-negative type (DN) JNK in the liver of obese diabetic mice dramatically improved insulin resistance and markedly decreased blood glucose levels. When C57BL/KsJ-db/db mice were treated with Ad-DN-JNK, nonfasting blood glucose levels were markedly reduced, whereas no such effect was observed in Ad-GFP-treated mice. IPITT, the hypoglycemic response to insulin was larger in Ad-DN-JNK-treated C57BL/KsJ-db/db mice compared to Ad-GFP-treated mice. To investigate this point further, we performed the euglycemic hyperinsulinemic clamp test. GIR in Ad-DN-JNK-treated mice was higher than that in Ad-GFP-treated mice, indicating that suppression of the JNK pathway in the liver reduces insulin resistance and thus ameliorates glucose tolerance in C57BL/KsJ-db/db mice. Furthermore, HGP was significantly lower in Ad-DN-JNK-treated mice. In contrast, there was no difference in the glucose disappearance rate (Rd) between these two groups. These results indicate that reduction of insulin resistance and amelioration of glucose tolerance by DN-JNK overexpression are mainly due to suppression of HGP (Figure 3) (Nakatani et al, 2004).

It has been reported that serine phosphorylation of IRS-1 inhibits insulin-stimulated tyrosine phosphorylation of IRS-1, leading to an increase in insulin resistance (Aguirre et al, 2000). IRS-1 serine 307 phosphorylation was markedly decreased in Ad-DN-JNK-treated mice. We also found an increase in IRS-1 tyrosine phosphorylation in Ad-DN-JNK-treated mice compared to control mice. Reduction of Akt serine 473 phosphorylation was observed in Ad-DN-JNK-treated C57BL/KsJ-db/db mice (Nakatani et al. 2004). Therefore, an increase in IRS-1 serine phosphorylation may be closely associated with the development of insulin resistance induced by JNK overexpression (Figure 3). Next, we examined the expression levels of the key gluconeogenic enzymes, PEPCK and glucose-6-phosphatase (G6Pase), both of which are known to be regulated by insulin signaling. Expression levels of both enzymes were markedly decreased by Ad-DN-JNK treatment in C57BL/KsJ-db/db mice (Nakatani et al, 2004). These results indicate that suppression of the JNK pathway enhances insulin signaling which leads to a decrease in gluconeogenesis and amelioration of glucose tolerance. Similar effects were observed in high-fat / high-sucrose diet-induced diabetic mice. Conversely, expression of wild type JNK in the liver of normal mice decreased insulin sensitivity. Taken

together, these findings suggest that suppression of the JNK pathway in the liver exerts greatly beneficial effects on insulin resistance status and glucose tolerance in both genetic and dietary models of diabetes (Figure 3) (Nakatani et al, 2004).

It has been also reported recently that JNK activity is abnormally elevated in the liver, muscle and adipose tissues in obese type 2 diabetic mouse models and that insulin resistance is substantially reduced in mice homozygous for a targeted mutation in the JNK1 gene (JNK-KO mice) (Hirosumi et al, 2002). When the JNK-KO mice were placed on a high-fat / high-caloric diet, obese wild type mice developed mild hyperglycemia compared to lean wild type control mice. In contrast, blood glucose levels in obese JNK-KO mice was significantly lower compared to those in obese wild type mice. In addition, serum insulin levels in obese JNK-KO mice were significantly lower compared to those in obese wild type mice. IPITT showed that hypoglycemic response to insulin in obese wild type mice was lower compared to that in obese JNK-KO mice. Also, IPGTT revealed a higher degree of hyperglycemia in obese wild type mice than in obese JNK-KO mice (Hirosumi et al, 2002). These results indicate that the JNK-KO mice are protected from the development of dietary obesity-induced insulin resistance. Furthermore, targeted mutations in JNK were introduced in genetically obese mice (ob/ob). Blood glucose levels in ob/ob-JNK-KO mice were lower compared to those in ob/ob wild type mice, and the ob/ob wild type mice displayed a severe and progressive hyperinsulinemia. Thus, JNK deficiency can provide partial resistance against obesity, hyperglycemia and hyperinsulinemia in both genetic and dietary models of diabetes. Taken together, obese type 2 diabetes is associated with activation of the JNK pathway, and the absence of JNK results in substantial protection from obesity-induced insulin resistance. These results strongly suggest that activation of the JNK pathway plays a crucial role in progression of insulin resistance found in type 2 diabetes (Figure 3).

Furthermore, activation of the JNK pathway is involved in pancreatic  $\beta$ -cell dysfunction as well as insulin resistance. Indeed, it was reported that activation of the JNK pathway leads to reduction of insulin gene expression and that suppression of the JNK pathway can protect  $\beta$ -cells from oxidative stress and some of the toxic effects of hyperglycemia (Kaneto et al, 2002; Kawamori et al, 2003). When isolated rat islets were exposed to oxidative stress, JNK, p38 MAPK, and PKC pathways were activated, preceding the decrease of insulin gene expression. Adenovirus-mediated overexpression of DN-JNK, but not the p38 MAPK inhibitor SB203580 nor the PKC inhibitor GF109203X, protected insulin gene expression and secretion from oxidative stress. Moreover, wild type (WT) JNK overexpression suppressed both insulin gene expression and secretion (Kaneto et al, 2002). These results were correlated with changes in the binding of the important transcription factor PDX-1 to the insulin promoter; adenoviral overexpression of DN-JNK preserved PDX-1 DNA binding activity in the face of oxidative stress, while WT-JNK overexpression decreased

PDX-1 DNA binding activity. Thus, it is likely that JNK-mediated suppression of PDX-1 DNA binding activity accounts for some of the suppression of insulin gene transcription and of  $\beta$ -cell function, which fits with the phenomenon that PDX-1 expression DNA binding activity is decreased in association with reduction of insulin gene transcription after chronic exposure to a high glucose concentration. Thus, it is likely that activation of JNK pathway leads to decreased PDX-1 activity and subsequent suppression of insulin gene transcription in the diabetic state (Kaneto et al, 2002).

To examine whether DN-JNK can protect  $\beta$ -cells from the toxic effects of hyperglycemia and to explore the potential therapeutic application for islet transplantation, we performed islet transplantation into diabetic mice. Isolated rat islets were infected with Ad-DN-JNK or Ad-GFP and cultured for 2 days; then 500 islets were transplanted under kidney capsules of STZ-induced diabetic Swiss nude mice. Blood glucose levels were not sufficiently decreased by transplantation of islets infected with Ad-GFP, which was probably due to toxic effects of hyperglycemia upon a marginal islet number, but were markedly decreased by Ad-DN-JNK. Four weeks after transplantation of islets infected with Ad-GFP, insulin mRNA levels in islet grafts were clearly decreased compared with those before transplantation, but relatively preserved by DN-JNK overexpression (Kaneto et al, 2002). These results suggest that DN-JNK can protect  $\beta$ -cells from some of the toxic effects of hyperglycemia during this transplant period, providing new insights into the mechanism through which oxidative stress suppresses insulin gene transcription in  $\beta$ -cells.

### III. The JNK pathway as a therapeutic target for diabetes

Protein transduction domains (PTDs) such as the small PTD from the TAT protein of human immunodeficiency virus (HIV-1), the VP22 protein of Herpes simplex virus, and the third  $\alpha$ -helix of the homeodomain of Antennapedia, a *Drosophila* transcription factor, are known to allow various proteins and peptides to be efficiently delivered into cells through the plasma membrane, and thus there has been increasing interest in their potential usefulness for the delivery of bioactive proteins and peptides into cells (Elliott et al, 1997; Frankel et al, 1988; Nagahara et al, 1998; Schwarze et al, 1999; Rothbard et al, 2000; Noguchi et al, 2003, 2004). We have recently evaluated the potential usefulness of a JNK inhibitory peptide in the treatment of type 2 diabetes and found that the cell permeable JNK inhibitory peptide (amino acid sequence: GRK KRR QRR RPP RPK RPT TLN LFP QVP RSQ DT) is very effective. This peptide is derived from the JNK binding domain of JNK-interacting protein-1 (JIP-1), also known as islet-brain-1 (IB-1), and has been reported to function as a dominant inhibitor of the JNK pathway (Bonny et al, 2001). To convert the minimal JNK-binding domain into a bioactive cell-permeable compound, a 20-amino acid sequence derived from the JNK-binding domain of JIP-1 (RPK RPT TLN LFP QVP RSQ DT) was covalently linked to a 10-amino

acid carrier peptide derived from the HIV-TAT sequence (GRK KRR QRR R); then to monitor peptide delivery, this JIP-1-HIV-TAT peptide was further conjugated with fluorescein isothiocyanate (FITC). First, to examine the effectiveness of the JNK inhibitory peptide *in vivo*, C57BL/KsJ-db/db obese diabetic mice were injected intraperitoneally with the JIP-1-HIV-TAT-FITC peptide. The FITC-conjugated peptide showed fluorescence signals in insulin target organs (liver, fat, muscle) and in insulin secreting tissue (pancreatic islets). Next, we examined whether the JNK pathway is inhibited after the treatment with JIP-1-HIV-TAT-FITC. In various tissues (liver, fat, and muscle), the JNK activity was actually suppressed by JIP-1-HIV-TAT-FITC in a dose-dependent manner (Kaneto et al, 2004).

To investigate whether suppression of the JNK pathway exerts beneficial effects on diabetes, we treated C57BL/KsJ-db/db mice with the intraperitoneal injection of the JNK inhibitory peptide, JIP-1-HIV-TAT-FITC. There was no difference in body weight and food intake between the JIP-1-HIV-TAT-FITC-treated and untreated mice. Glucose tolerance test performed showed that glucose tolerance in JIP-1-HIV-TAT-FITC-treated mice was significantly ameliorated compared to untreated or the scramble peptide-treated mice. These data indicate that the JNK pathway is involved in the exacerbation of diabetes and that suppression of the JNK pathway could be a therapeutic target for diabetes (Kaneto et al, 2004). To investigate the possible effects of the JNK inhibitory peptide on insulin action, we performed insulin tolerance test. Reduction of blood glucose levels in response to injected insulin was much larger in JIP-HIV-TAT-FITC-treated mice compared to untreated mice, indicating that the peptide treatment improves the insulin sensitivity. To further investigate the effect of the peptide on insulin resistance, we performed the euglycemic hyperinsulinemic clamp test. The steady-state GIR in JIP-1-HIV-TAT-FITC-treated mice was significantly higher than that in untreated mice, indicating that JIP-1-HIV-TAT-FITC reduces insulin resistance in C57BL/KsJ-db/db mice (Kaneto et al, 2004). Furthermore, we evaluated endogenous HGP and glucose Rd in the JNK inhibitory peptide-treated mice. It is noted that Rd reflects glucose utilization in the peripheral tissues. HGP in JIP-1-HIV-TAT-FITC-treated mice was significantly lower than that in untreated mice. In addition, Rd in JIP-1-HIV-TAT-FITC-treated mice was significantly higher than that in untreated mice (Kaneto et al, 2004). These results indicate that JIP-1-HIV-TAT-FITC treatment reduces insulin resistance through decreasing HGP and increasing Rd. These data provide strong evidence that JNK is indeed a crucial component of the biochemical pathway responsible for insulin resistance *in vivo*. Furthermore, IRS-1 serine 307 phosphorylation was decreased in JIP-1-HIV-TAT-FITC-treated mice compared to control mice. We also found the increase of IRS-1 tyrosine phosphorylation in the peptide-treated mice compared to control mice. Concomitantly, increase of Akt serine 473 and threonine 308 phosphorylation both of which are known to be important for activation of the Akt pathway was observed in JIP-1-HIV-TAT-FITC-treated mice (Kaneto et al,

2004). In addition, to examine the effect of JIP-1-HIV-TAT-FITC treatment on insulin biosynthesis, we measured insulin mRNA level and content in pancreata of C57BL/KsJ-db/db mice which had been treated with the peptide. Insulin mRNA level and insulin content were significantly higher in the peptide-treated mice. Thus, we assume that the JNK inhibitory peptide exerted some beneficial effects on the pancreatic islets (Kaneto et al, 2004). Taken together, the cell-permeable JNK inhibitory peptide, JIP-1-HIV-TAT-FITC, improves insulin resistance and ameliorates glucose intolerance, indicating the critical involvement of the JNK pathway in diabetes and the usefulness of the cell-permeable JNK inhibitory peptide as a novel therapeutic agent for diabetes.

#### IV. Conclusion

Under diabetic conditions ER stress is induced and the JNK pathway is subsequently activated, which is involved in the insulin resistance. Increase of ER stress and activation of the JNK pathway interfere with insulin action. In reverse, reduction of ER stress and suppression of the JNK pathway in obese diabetic mice markedly improve insulin resistance and ameliorate glucose tolerance. Taken together, increase of ER stress and subsequent activation of the JNK pathway play a crucial role in the progression of insulin resistance found in diabetes and thus could be a potential therapeutic target for diabetes.

#### References

- Aguirre V, Davis R and White MF (2000) The c-Jun NH<sub>2</sub>-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser<sup>307</sup>. **J Biol Chem** 275, 9047-9054.
- Aridor M and Balch WE (1999) Integration of endoplasmic reticulum signaling in health and disease. **Nature Med** 5, 745-751.
- Bonny C, Oberson A, Negri S, Sause C and Schorderet DF (2001) Cell-permeable peptide inhibitors of JNK: novel blockers of  $\beta$ -cell death. **Diabetes** 50, 77-82.
- Chang L and Karin M (2001) Mammalian MAP kinase signalling cascades. **Nature** 410, 37-40.
- Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. **Cell** 103, 239-252.
- Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M and Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. **Cell** 76, 1025-1037.
- Elliott G and O'Hare P (1997) Intracellular trafficking and protein delivery by a herpesvirus structure protein. **Cell** 88, 223-233.
- Frankel AD and Pabo CO (1988) Cellular uptake of the tat protein from human immunodeficiency virus. **Cell** 55, 1189-1193.
- Harding HP and Ron D (2002) Endoplasmic reticulum stress and the development of diabetes: a review. **Diabetes** 51, S455-461.
- Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD and Ron D (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk<sup>-/-</sup> mice reveals a role for translational control in secretory cell survival. **Mol Cell** 7, 1153-1163.
- Harding HP, Zhang Y and Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. **Nature** 397, 271-274.
- Hibi M, Lin A and Karin M (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. **Genes Dev** 7, 2135-2148.
- Hirosumi J, Tuncman G, Chang L, Karin M and Hotamisligil GS (2002) A central role for JNK in obesity and insulin resistance. **Nature** 420, 333-336.
- Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, Mueckler M, Marshall H, Donis-Keller H, Crock P, Rogers D, Mikuni M, Kumashiro H, Higashi K, Sobue G, Oka Y and Permutt MA (1998) A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). **Nature Genet** 20, 143-148.
- Iwawaki T, Akai R, Kohno K, Miura M (2004) A transgenic mouse model for monitoring endoplasmic reticulum stress. **Nature Med** 10, 98-102.
- Kaneto H, Nakatani Y, Miyatsuka T, Kawamori D, Matsuoka T, Matsuhisa M, Kajimoto Y, Ichijo H, Yamasaki Y and Hori M (2004) Possible novel therapy for diabetes with cell-permeable JNK inhibitory peptide. **Nature Med** 10, 1128-1132.
- Kaneto H, Xu G, Fujii N, Kim S, Bonner-Weir S and Weir GC (2002) Involvement of c-Jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression. **J Biol Chem** 277, 30010-30018.
- Kawamori D, Kajimoto Y, Kaneto H, Umayahara Y, Fujitani Y, Miyatsuka T, Watada H, Leibiger IB, Yamasaki Y and Hori M (2003) Oxidative stress induces nucleo-cytoplasmic translocation of pancreatic transcription factor PDX-1 through activation of c-Jun N-terminal kinase. **Diabetes** 52, 2896-2904.
- Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL (2004) Free fatty acids and cytokines induce pancreatic  $\beta$ -cell apoptosis by different mechanisms: role of nuclear factor- $\kappa$ B and endoplasmic reticulum stress. **Endocrinology**, 145, 5087-96.
- Kuwabara K, Matsumoto M, Ikeda J, Hori O, Ogawa S, Maeda Y, Kitagawa K, Imuta N, Kinoshita T, Stern DM, Yanagi H and Kamada T (1996) Purification and characterization of a novel stress protein, the 150-kDa oxygen-regulated protein (ORP150), from cultured rat astrocytes and its expression in ischemic mouse brain. **J Biol Chem** 271, 5025-5032.
- Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham D.G, Lissy NA, Becker-Hapak M, Ezhevsky SA and Dowdy SF (1998) Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p-27<sup>Kip1</sup> induces cell migration. **Nature Med** 4, 1449-1452.
- Nakatani Y, Kaneto H, Kawamori D, Hatazaki M, Miyatsuka T, Matsuoka T, Kajimoto Y, Matsuhisa M, Yamasaki Y and Hori M (2004) Modulation of the JNK pathway in liver affects insulin resistance status. **J Biol Chem** 279, 45803-45809.
- Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M, Matsuoka T, Ozawa K, Ogawa T, Hori M, Yamasaki Y and Matsuhisa M (2004) Involvement of ER stress in insulin resistance and diabetes. **J Biol Chem** (in press).
- Noguchi H, Kaneto H, Weir GC and Bonner-Weir S (2003) PDX-1 protein containing its own Antennapedia-like protein transduction domain can transduce pancreatic duct and islet cells. **Diabetes** 52, 1732-1737.
- Noguchi H, Matsushita M, Okitsu T, Moriwaki A, Tomizawa K, Kang S, Li ST, Kobayashi N, Matsumoto S, Tanaka K, Tanaka N and Matsui H (2004) A new cell-permeable

- peptide allows successful allogeneic islet transplantation in mice. **Nature Med** 10, 305-309.
- Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E and Mori M (2002) Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. **J Clin Invest** 109, 525-532.
- Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E and Mori M (2001) Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. **Proc Natl Acad Sci USA** 98, 10845-10850.
- Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH and Hotamisligil GS (2004) Endoplasmic reticulum stress links obesity, insulin action and type 2 diabetes. **Science** 306: 457-461.
- Ron D (2002) Translational control in the endoplasmic reticulum stress response. **J Clin Invest** 110, 1383-1388.
- Rothbard JB, Garlington S, Lin Q, Kirschberg T, Kreider E, McGrane PL, Wender PA and Khavari PA (2000) Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. **Nature Med** 6, 1253-1257.
- Schwarze SR, Ho A, Vocero-Akbani AM and Dowdy SF (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. **Science** 285, 1569-1572.
- Shi Y, Taylor SI, Tan S.-L and Sonenberg N (2003) When Translation Meets Metabolism: Multiple Links to Diabetes. **Endocr Rev** 24, 91-101.
- Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L and Wek RC (1998) Identification and Characterization of Pancreatic Eukaryotic Initiation Factor 2 -Subunit Kinase, PEK, Involved in Translational Control. **Mol Cell Biol** 18, 7499-7509.
- Tamatani M, Matsuyama T, Yamaguchi A, Mitsuda N, Tsukamoto Y, Taniguchi M, Che YH, Ozawa K, Hori O, Nishimura H, Yamashita A, Okabe M, Yanagi H, Stern DM, Ogawa S and Tohyama M (2001) ORP150 protects against hypoxia/ischemia-induced neuronal death. **Nature Med** 7, 317-323.
- Tirasophon W, Welihinda AA and Kaufman RJ (1998) A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. **Genes Dev** 12, 1812-1824.
- Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP and Ron D (2000) Coupling of Stress in the ER to Activation of JNK Protein Kinases by Transmembrane Protein Kinase IRE1. **Science** 287, 664-666.
- Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M and Ron D (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. **EMBO J** 17, 5708-5717.
- Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. **Cell** 107, 881-91.



Hideaki Kaneto