Double-Stranded RNA-Dependent Protein Kinase Links Pathogen Sensing with Stress and Metabolic Homeostasis

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SUMMARY

As chronic inflammation is a hallmark of obesity, pathways that integrate nutrient- and pathogen sensing pathways are of great interest in understanding the mechanisms of insulin resistance, type 2 diabetes, and other chronic metabolic pathologies. Here, we provide evidence that double-stranded RNA-dependent protein kinase (PKR) can respond to nutrient signals as well as endoplasmic reticulum (ER) stress and coordinate the activity of other critical inflammatory kinases such as the c-Jun N-terminal kinase (JNK) to regulate insulin action and metabolism. PKR also directly targets and modifies insulin receptor substrate and hence integrates nutrients and insulin action with a defined pathogen response system. Dietary and genetic obesity features marked activation of PKR in adipose and liver tissues and absence of PKR alleviates metabolic deterioration due to nutrient or energy excess in mice. These findings demonstrate PKR as a critical component of an inflammatory complex that responds to nutrients and organelle dysfunction.

INTRODUCTION

Metabolic diseases appear as clusters including obesity, insulin resistance, type 2 diabetes, and cardiovascular disease and constitute a major global health problem with limited treatment options. In the past decade, it has been realized that the emergence of this cluster has strong inflammatory underpinnings (Hotamisligil, 2006). During the course of obesity, a broad array of inflammatory and stress responses are evoked in metabolic tissues, leading to chronic, low-grade local inflammation that plays a central role in the inhibition of insulin receptor signaling and disruption of systemic metabolic homeostasis. This atypical state, which we refer to as metaflammation (Hotamisligil, 2006), involves immune and nonimmune cells and the engagement of immune response pathways with nutrients and metabolites. However, the mechanistic basis of these extensive functional links and molecules that coordinate this network of responses remains to be understood.

If the nutrient and pathogen response systems were truly integrated, the involvement of pathogen sensors in metabolic regulation, especially during exposure to excess nutrients, would be anticipated. Such anticipation has stimulated the pursuit of pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) for a role in metabolism (Shi et al., 2006). Other than PRRs, there are only a few molecules that can assume such a role to carry the potential ability for direct recognition of pathogens and possess catalytic activity to directly couple to metabolic pathways. One such molecule is the double-stranded RNA-dependent protein kinase (PKR), which has been originally identified as a pathogen sensor and a proposed regulator of the innate immune response against viral infections in higher eukaryotes (Samuel, 1993). Virus-derived double-stranded RNA molecules are recognized and bound by PKR through the N-terminal double-stranded RNA-binding motifs, resulting in autophosphorylation through the activation of the intramolecular kinase domain (Garcia et al., 2006; Williams, 2001). Interestingly, in the context of infections, PKR can regulate or act in conjunction with major inflammatory signaling pathways that are implicated in metabolic homeostasis, including the c-Jun N-terminal kinase (JNK) and IκB kinase (IKK) (Bonnet et al., 2000; Goh et al., 2000; Takada et al., 2007). In metabolic disease, it is unclear how these and other inflammatory signaling molecules are coordinately regulated to disrupt metabolism. However, it is feasible to consider a model where these multiple signaling pathways act in concert by forming a response node or acting in complexes that yield to metabolic surplus.

Interestingly, among the very few substrates identified for the kinase activity of PKR, the major one is the eukaryotic initiation factor 2α (eIF2α), which regulates general protein synthesis (Holcik and Sonenberg, 2005; Ron and Walter, 2007). It has been postulated that PKR-mediated phosphorylation of eIF2α is a strategy to inhibit viral protein synthesis in host cells (Holcik and Sonenberg, 2005). However, in other contexts, inhibition of general translation through the same eIF2α phosphorylation...
represents a major arm controlling endoplasmic reticulum (ER) homeostasis (Ron and Walter, 2007). ER stress also plays an important role in the development of insulin resistance and diabetes, at least in part, by triggering JNK activity (Gregor and Hotamisligil, 2007; Ozcan et al., 2004). In both mouse and human, there is a consistent and marked increase in phosphorylation of eIF2α and JNK activity in obese metabolic tissues (Gregor et al., 2009). Hence, coordination of protein synthesis through eIF2α and stress signaling through JNK likely represent a relevant mechanism in the integration of inflammatory signals with metabolic outcomes, especially in the context of nutrient and energy surplus. PKR features properties to coordinate pathogen responses, endoplasmic reticulum function, inflammatory signaling, and translational regulation. Therefore, we postulate that PKR may represent a core component of a putative “metabolic inflammasome,” in other words, a mechanism integrating pathogen response and metabolic pathways that plays a critical role in metabolic homeostasis by controlling the action of major players such as JNK, IKK, and/or other mediators.

RESULTS

PKR Activity in Obesity and Metabolic Stress

If PKR were involved in nutrient-induced inflammatory responses, it would be anticipated that its activity is altered in conditions of nutrient surplus. To explore whether PKR activation is altered in obesity and metabolic stress, we first examined a model of severe genetic obesity and insulin resistance resulting from leptin deficiency (ob/ob), also known as Lepob/ob). There was a striking increase in PKR activity, which was assessed by autophosphorylation level of PKR using 32P-γ-ATP, in both white adipose tissue (WAT) and liver of ob/ob mice compared to lean controls (Figure 1A). Similarly, there was a significant upregulation of PKR activity in both WAT and liver tissues of mice fed with a high-fat diet (Figure 1B). In both models, we also observed an increase in PKR protein and mRNA levels, consistent with the reported autoregulation of PKR expression upon stimulation of its activity (Figures 1A and 1B and Figures S1A and S1B available online) (Gusella et al., 1995; Samuel, 1993). However, there was only a minor regulation of skeletal muscle tissue PKR activity in the ob/ob model and no regulation was evident in dietary obesity (Figures S1C and S1D). In adipose tissue, the principle source of increased PKR expression in dietary obesity was found in mature adipocyte fraction instead of stromal–vascular (SV) fraction (Figure S1A). Interestingly, in addition to PKR itself, mRNA levels of some other interferon target genes were also increased in adipocytes of obese WAT and liver (Figures S1A and S1B). As these mice were maintained in a specific pathogen-free environment, activation of PKR likely involves metabolic signals associated with nutrient and energy excess.

To determine whether PKR activity is related to metabolic stress and triggered by a nutrient, we next used an in vivo lipid infusion system, which acutely increases circulating free fatty acids (FFA) and causes insulin resistance (Kim et al., 2004; Shi et al., 2006). In this setting, there was also a significant increase in PKR activity in lean mice following lipid exposure (Figure 1C). These results demonstrate that PKR is activated by nutrient excess and metabolic stress in vivo. Given that PKR activity is induced by lipid infusion, we next explored whether FFA itself can also activate PKR by treating primary-isolated mouse embryonic fibroblast cells (MEFs) with palmitic acid. As shown in Figure 1D, palmitic acid exposure resulted in PKR activation. Since TLR4 has been implicated in fatty acid response (Shi et al., 2006), we asked whether PKR activation by lipids involved this PRR. However, in TLR4-deficient primary MEFs, palmitic acid-induced PKR activation was also detected at levels similar to those in wild-type (WT) controls (Figure S1E).

These experiments demonstrated that PKR is activated by lipids in vitro and in vivo, and this activation is not dependent on TLR4.
Induction of palmitic acid- and thapsigargin-induced PKR activity requires intact RNA-binding domain of PKR. New protein synthesis, at least in part, appears to be required for the presence of translational inhibitor cycloheximide. Therefore, shown as the mean ± standard error of the mean (SEM), *p < 0.05.

PKR Controls JNK Activity in Response to Metabolic Stress

JNK activity is modulated by PKR under several stress conditions (Goh et al., 2000). As PKR activation is also related to fatty acid exposure and ER stress (Gao et al., 2004; Urano et al., 2000), we next examined the impact of PKR on fatty acid- and ER stress-mediated JNK activation. To assess effects of PKR deficiency in cells, we elected to use MEFs from mice with a targeted mutation lacking the PKR kinase domain (Abraham et al., 1999). Treatment of these PKR-deficient (Pkr−/−) and WT (Pkr+/+) MEFs control primary-isolated MEFs with palmitic acid demonstrated that this fatty acid failed to activate JNK in the absence of PKR (Figure 2A). In a similar fashion, treatment of Pkr−/− and Pkr−/− MEFs with thapsigargin, an agent that induced ER stress, resulted in prominent induction of JNK phosphorylation in Pkr−/− relative to Pkr−/− cells (Figure 2B). ER stress-induced PKR activation was decreased in the presence of translation inhibitor cycloheximide. Therefore, new protein synthesis, at least in part, appears to be required for PKR activation during ER stress (Figure S2A). Taken together, these results demonstrate that PKR is a required component for JNK activation in response to lipid exposure and ER stress.

One mechanism by which JNK negatively regulates insulin signaling is through induction of IRS1 serine phosphorylation in response to fatty acid exposure and ER stress (Gao et al., 2004; Ozcan et al., 2004). If PKR controls JNK activation, it is conceivable that PKR may, indirectly or directly, target IRS1 function, which is critical in insulin action. To address whether fatty acid- or ER stress-induced IRS1 phosphorylation is dependent on PKR, we examined the effect of palmitic acid or thapsigargin treatment on IRS1 serine phosphorylation in Pkr−/− and Pkr−/− MEFs. Both of these treatments resulted in induction of IRS1 serine 307 phosphorylation in Pkr−/− MEFs but not in Pkr−/− cells (Figures 2C and 2D). As these data suggest that activated PKR modulates IRS1 serine phosphorylation, we next assessed the effect of a direct PKR activator, a virus-derived double-stranded RNA mimetic, polyinosinic-polycytidylic acid (polyI:C) (Garcia et al., 2006; Williams, 2001), on...
IRS1 phosphorylation in Pkr+/+ and Pkr−/− MEFs. This treatment also induced IRS1 serine phosphorylation with a marked increase in PKR expression in Pkr+/+ MEFs. However, poly(C)-induced IRS1 phosphorylation was not observed in Pkr−/− MEFs (Figure S2B). We also reconstituted PKR expression in the Pkr−/− MEFs as increased PKR expression itself induces PKR activity thus allowing comparisons in the same cellular background without stimulation of other signaling pathways (Figure S2C). As shown in Figure 2E, serine phosphorylation of IRS1 was induced by reconstitution of PKR in Pkr-deficient cells. In these cells, the level of insulin-induced tyrosine phosphorylation of IRS1 and insulin-induced interaction between IRS1 and p85, a regulatory subunit of phosphoinositide 3-kinase (PI3K), were both significantly diminished in PKR-reconstituted cells compared to the Pkr−/− controls expressing the empty vector (Figure 2F). Hence, PKR can induce IRS serine phosphorylation and block insulin action.

We next asked whether RNA-binding ability of PKR is required for palmitic acid- or ER stress-induced PKR activation. RNA-binding capacity of PKR is abolished by introducing a point mutation to its lysine 64 residue in the RNA-binding motif (McCormack et al., 1994; Wu and Kaufman, 1996). We reconstituted Pkr−/− MEFs with WT or RNA-binding defective (K64E) PKR by retrovirus-mediated gene transfer and examined PKR kinase activity after treatment with palmitic acid or thapsigargin. Wild-type PKR is activated by both palmitic acid and thapsigargin (Figures 2G and 2H). However, PKR with defective RNA binding (K64E) was not activated in palmitic acid- or thapsigargin-treated cells (Figures 2G and 2H). A mutation in the kinase domain of PKR abolished all activity and was used as a control (Figures 2G and 2H). These data demonstrate that PKR is a required component for JNK activation and IRS1 inhibition when induced by a nutrient, a pathogen component, or ER stress, and the RNA-binding domain of PKR is indispensable in both responses.

IRS1, a Direct Substrate of PKR

We next treated Pkr+/+ and Pkr−/− cells with TNF-α, which is well known to both induce PKR activity and block insulin action through IRS1 phosphorylation in WT cells (Hotamisligil et al., 1996). In these experiments, we noticed that PKR was detectable by western blot analysis following immunoprecipitation with an anti-IRS1 antibody, demonstrating a potential interaction between these proteins (Figure 3A). To verify the interaction between PKR and IRS1, we next performed the reciprocal experiment, where we immunoprecipitated PKR from protein extracts of TNF-α-treated Pkr+/+ MEFs and attempted to detect IRS1 by western blot analysis. PKR-deficient cells were used in the same setting as controls. These experiments also demonstrated the interaction between these two proteins (Figure 3B). To assess the specificity of PKR-IRS1 interaction, we also examined the relation between IRS1 and the PKR-like endoplasmic reticulum kinase (PERK), which is the closest homolog of PKR in mammals, and did not detect any interaction between these proteins with or without induction of ER stress (Figure S3).

We next performed an in vitro pull-down assay using recombinant IRS1 and PKR proteins. These experiments demonstrated that PKR directly interacts with IRS1 protein (Figure 3C). The robust interaction between PKR and IRS1 raised the possibility that PKR phosphorylates IRS1 directly. To address this possibility, we performed kinase assays using recombinant IRS1 and active PKR in vitro. As shown in Figure 3D, PKR directly phosphorylates IRS1 including the serine 307 residue. We next performed in vitro kinase assays after immunopurification of PKR from Pkr+/+ MEFs and used Pkr−/− cell extracts as a negative control. We found that IRS1 was phosphorylated by immunopurified PKR from TNF-α- or thapsigargin-treated Pkr+/+ MEFs (Figures 3E and 3F). More importantly, the increased serine phosphorylation of IRS1, which is detected by a phospho-specific antibody, was induced only by activated PKR (Figures 3E and 3F). Again, the induction of IRS1 phosphorylation was not observed in extracts prepared from TNF-α- or thapsigargin-treated Pkr−/− cells in control experiments (Figures 3E and 3F). We have previously shown that JNK1 plays a critical role in stress-induced IRS1 phosphorylation and insulin resistance (Hirosumi et al., 2002). To address whether JNK1 is required for PKR-mediated IRS1 phosphorylation, we next assessed effects of exogenous expression of PKR on IRS1 phosphorylation in primary Jnk1+/− and Jnk1−/− MEFs. By introducing PKR through adenovirus-mediated gene transfer, serine phosphorylation of IRS1 is dramatically increased in Jnk1+/− MEFs (Figure 3G). In Jnk1−/− MEFs, PKR still retained the ability to induce serine phosphorylation of IRS1 (Figure 3G), although this was significantly reduced in magnitude. These data suggest that PKR assumes a role in regulation of IRS1 phosphorylation in both a JNK1-dependent and -independent manner in cultured cells and illustrate the importance of functional interaction between these kinases to interfere with insulin action.

Metabolic Regulation and Insulin Action in Pkr−/− Mice

Given that PKR activity is strongly regulated in obesity and linked to signaling pathways interfering with metabolic homeostasis, we then used several different in vivo models to test the functional significance of PKR in the pathogenesis of obesity, insulin resistance, and type 2 diabetes. First, mice lacking PKR (Pkr−/−) and WT control (Pkr+/+) were placed on a high-fat diet (HFD) along with a control group of each genotype on regular diet (RD). The PKR-deficient model used in our study was generated by removing the kinase domain and hence retains no kinase activity (Abraham et al., 1999). On HFD, Pkr−/− mice developed obesity compared to mice kept on RD, while weight gain in Pkr+/+ mice was significantly lower, starting to become evident after 10 weeks of HFD (Figure 4A). Dual energy X-ray absorption (DEXA) analysis demonstrated reduced total body adipose mass in Pkr−/− mice (Figure 4B). In addition, consistent with reduced adiposity, serum leptin level in Pkr−/− mice was lower than that in Pkr+/+ mice (Figure 4C). Total serum adiponectin levels were not significantly different between genotypes (Figure 4D). In Pkr−/− mice, blood glucose levels were significantly lower than those in Pkr+/+ controls (Figure 4E). Examination of serum insulin levels revealed that the hyperinsulinemia observed in Pkr−/− mice was not evident in the Pkr+/− animals, indicating that these animals might exhibit enhanced insulin sensitivity (Figure 4F). To investigate systemic insulin sensitivity, we performed glucose tolerance tests (GTT) in Pkr−/− and Pkr+/+ mice at 6 and 14 weeks after the start of HFD. Since body weights of Pkr+/+ and Pkr−/−
mice did not exhibit significant differences until week 10–12, experiments performed at an early stage allowed evaluation of insulin sensitivity without changes in body weight. After 6 weeks of HFD, where the body weights were similar between genotypes, Pkr+/+ mice already showed significantly lower glucose levels after a glucose challenge compared to Pkr−/− mice (Figure 5B). On the other hand, Pkr+/+ mice did not exhibit significant differences until week 10–12, rates of oxygen consumption and carbon dioxide production of Pkr−/− mice were modestly but significantly higher than those of Pkr+/+ mice (Figures S4I and S4J), indicating that energy expenditure may be a potential mechanism for body weight reduction.

Biochemical and Molecular Alterations in Pkr−/− Mice

We next studied whether deficiency of PKR resulted in enhanced insulin action by examining in vivo insulin receptor-signaling capacity in WAT and liver tissues of mice on HFD. In intact animals, insulin-stimulated AKT phosphorylation on serine 473 was significantly increased in WAT extracts of Pkr−/− mice compared with those of Pkr+/+ controls (Figure 5A). There was also significantly increased insulin-stimulated AKT phosphorylation in liver tissue of Pkr−/− mice (Figure 5B). On the other hand,
we did not observe significant alterations in insulin signaling in the skeletal muscle of Pkr−/− mice (Figure 5A). These results demonstrate significant contribution of PKR to high-fat diet-induced insulin resistance. As eIF2α is a substrate of PKR, we determined the phosphorylation level of eIF2α on Ser52 using a specific antibody. These experiments demonstrate markedly decreased eIF2α phosphorylation in both adipose tissue and liver extracts of Pkr−/− mice compared with those of Pkr+/+ controls (Figures 5C and 5D). Dietary obesity is also characterized by increased JNK activity (Hirosumi et al., 2002), which is correlated with PKR activity (Figure 1). In liver and adipose tissues, JNK1 activity was significantly reduced in Pkr−/− mice compared with Pkr+/+ controls on HFD (Figures 5C and 5D and Figure S5B). These biochemical alterations were also reflected in the expression of inflammatory mediators induced by obesity in adipose tissue. In Pkr−/− mice, expression levels of several inflammatory cytokines such as Tnfa, Il6, and Il1b, and an anti-inflammatory cytokine Il10, a potential target of PKR (Chakrabarti et al., 2008), were significantly reduced in comparison to levels seen in Pkr+/+ controls on HFD (Figure 5E). In addition, expression of a macrophage marker F4/80 and an ER stress marker Grp78 was also significantly reduced in adipose tissue of Pkr−/− mice (Figure 5E). These changes in gene expression between genotypes were not observed in mice fed RD (Figure S5C).

Figure 5A. Glucose Metabolism and Insulin Sensitivity in Pkr−/− Mice
(A) Total body weight on regular (RD) or high-fat (HFD) diet. Obesity is induced by HFD starting immediately after weaning at 3 weeks of age. Data are shown as the mean ± SEM. *p < 0.05.
(B) Analysis of body fat by dual energy X-ray absorptiometry (DEXA). Data are shown as the mean ± SEM. *p < 0.05.
(C and D) Serum leptin (C) and adiponectin (D) levels after 6 hr daytime food withdrawal in Pkr+/+ (n = 5) and Pkr−/− (n = 6) mice on HFD for 15 weeks. Data are shown as the mean ± SEM.
(E) Blood glucose (E) and serum insulin (F) levels after 6 hr daytime food withdrawal in Pkr+/+ (n = 6) and Pkr−/− (n = 6) mice on HFD for 8 weeks. Data are shown as the mean ± SEM.
(F) Glucose tolerance tests were performed on Pkr+/+ (n = 6) and Pkr−/− mice (n = 6) on RD and HFD for 6 weeks. Data are shown as the mean ± SEM. *p < 0.05. See also Figure S4.

The Role of PKR in Lipid Infusion-Induced Acute Insulin Resistance
As shown in Figure 1, lipid exposure leads to PKR activation. To further explore the impact of PKR on insulin sensitivity in another setting and without the potential confounding effects of body weight or adiposity, we studied the effects of PKR activation on acute insulin resistance induced by lipids...
In this system, we administered lipid intravenously into the Pkr+/+ and Pkr−/− mice on RD and performed hyperinsulinemic-euglycemic clamp studies to examine the whole-body insulin sensitivity and glucose metabolism. Glucose infusion rates (GIR) during the clamp studies indicated that Pkr−/− mice required significantly higher levels of glucose infusion to maintain blood glucose consistent with increased insulin sensitivity (Figure 6A). In Pkr+/+ mice, GIR required to maintain euglycemia was reduced by 50.2% in comparison with saline-infused controls (Figure 6B). In contrast, lipid infusion exerted a significantly smaller effect on the GIR in Pkr−/− mice (Figure 6B).

Consistent with this result, the whole-body glucose disposal rates (Rd) observed in Pkr−/− mice during the clamp were significantly higher than those in Pkr+/+ controls (Figure 6C). A similar trend was also observed in hepatic glucose production (HGP) levels, although this did not reach statistical significance (Figure 6D). Examination of insulin-stimulated tissue glucose uptake revealed significant increase in muscle tissue of Pkr−/− mice compared to Pkr+/+ controls (Figure 6E). Although WAT glucose uptake was not significantly different between genotypes, a similar trend was also observed (Figure 6F). Under these conditions, lipid infusion did not result in differential activation of proapoptotic pathways between genotypes, indicating that the reported apoptotic activity of PKR was not an underlying contributor to the phenotype seen in the Pkr−/− mice (Figure S6). Taken together these data clearly show that lipid-induced PKR

Figure 5. Biochemical and Molecular Alterations in Pkr−/− Tissues
(A and B) Phosphorylation level of Akt on serine 473 in WAT (A) and liver (B) of Pkr+/+ and Pkr−/− mice on HFD for 20 weeks. The graphs on the right of each blot show the quantification of the results. Data are shown as the mean ± SEM. *p < 0.05, **p < 0.01. AU: arbitrary unit.
(C and D) Phosphorylation level of eIF2α on serine 52 and JNK1 kinase activity, which was detected by a kinase assay using immunopurified JNK1, 32P-γ-ATP, and recombinant c-Jun protein as substrate in WAT (C) and liver (D) of Pkr+/+ and Pkr−/− mice on HFD for 20 weeks. β-tubulin is shown as a control.
(E) Gene expression in WAT including proinflammatory cytokine levels in Pkr+/+ and Pkr−/− mice on HFD for 20 weeks. Data are shown as the mean ± SEM.
(p < 0.05, **p < 0.01.
(F and G) Haematoxylin and eosin staining of WAT (F) and liver (G) sections of Pkr+/+ and Pkr−/− mice, respectively. Scale bar, 200 μm.
(H) Triglyceride contents in liver of Pkr+/+ (n = 6) and Pkr−/− (n = 6) mice on HFD for 20 weeks. Data are shown as the mean ± SEM.
(I) Serum alanine aminotransferase level after 6 hr daytime food withdrawal in Pkr+/+ (n = 6) and Pkr−/− (n = 6) mice on HFD for 15 weeks. Data are shown as the mean ± SEM.
See also Figure S5.
activation is an important negative regulator of insulin action and that PKR directly regulates insulin sensitivity in multiple settings.

**DISCUSSION**

Although the integration of nutrient and pathogen response systems has been put forward as an attractive model to explain the inflammatory origin of metabolic disease, identification of specific molecules and mechanisms directly coordinating these extensive links has been a major challenge (Hotamisligil and Erbay, 2008). In this study, we uncovered a link between an established pathogen-sensing pathway mediated by PKR and metabolic homeostasis through regulation of JNK activity and insulin action (Figure 7). This raises an intriguing possibility that PKR may act as a central integrator in the inflammatory component of metabolic control by linking nutrient- and pathogen-sensing pathways.

One of the remaining important questions regarding the relationships between inflammatory pathways and insulin action is how the action of many molecules with similar biological functions are coordinated. Previous studies have shown direct interactions of PKR with the IKKβ-NF-κB pathway (Bonnet et al., 2000). In this study, we have demonstrated PKR’s direct interaction and modulation of IRS, a critical molecule in insulin action, and the major regulatory role over JNK activation. Taken together, these findings make PKR a very attractive molecule at the core of a complex that features major inflammatory and stress roads that intersect with metabolism. As activation of several stress signaling pathways produces similar outcomes in metabolic regulation, the possibility that these molecules work as part of a complex or a signaling node assembled and coordinated by a molecule of innate immunity with direct nutrient recognition potential is of critical importance. Such a role is highlighted for PKR in the integration of nutrient and pathogen sensing in relation to metabolic homeostasis (Figure 7). In this scenario, it is possible that the activity of one component is highly regulated by the other, and thus amplified feedback mechanisms may be in place. For example, the interaction and function of JNK or IKK may also influence PKR and PKR activation, in turn contributing to organelle stress. Hence, we suggest that PKR may be critical in the assembly and activation of a putative metabolic inflammasome (or metaflammasome). These interesting possibilities merit further investigation.

It is noteworthy that PKR responds to pathogens, nutrients, and organelle stress. Then, PKR plays a role in the mounting of adaptive and survival responses including suppression of general protein translation through phosphorylation of eIF2α and inhibition of anabolic effects of insulin action. The marked increase in PKR activity observed in multiple models of obesity featuring energy and nutrient excess may represent an adaptive attempt to interfere with synthetic pathways that would further accumulate energy. This is consistent with the late-onset effects...
PKR senses and responds to obesity, ER stress, and pathogen-related stress in concert with JNK, leading to metabolic disease under diverse physiological and pathological conditions. In this capacity, PKR not only integrates immune and metabolic response systems but also links endoplasmic reticulum homeostasis and unfolded protein response (UPR) to translational regulation through eIF2α and insulin signaling through IRS1. Finally, the kinases IRS1 and eIF2α may represent a “metabolic inflammasome” complex assembled and regulated by PKR.

The function of PKR activation may be limited to coordinated launching of an inflammatory response together with kinases such as JNK and IKK, as supported by the evidence provided in this study, which results in alterations in insulin action and possibly other metabolic outcomes. The most attractive implication of our observation is that PKR can directly sense nutrients or other metabolic products including most attractive implication of our observation is that PKR can act in conjunction with major inflammatory kinases and directly interact with a critical insulin signaling component that PKR can act in conjunction with major inflammatory kinases and directly interact with a critical insulin signaling component lead us to suggest PKR as a core component of a putative “metabolic inflammasome” that consists of major elements in inflammatory signaling and insulin action (Figure 7). This PKR-coordinated sensing and signaling complex may represent a central mechanism for the integration of molecular response and innate immunity with insulin action and metabolic pathways that are critical in chronic metabolic diseases. If small molecules can modulate in vivo PKR activity, therapeutic opportunities may arise from such efforts.

**EXPERIMENTAL PROCEDURES**

**Mice**

Animal care and experimental procedures were performed with approval from animal care committees of Harvard University. Two different types of targeted mutations of PKR have been established and reported in mice, RNA-binding domain-defective and kinase-domain-defective models. Mutations of PKR have been established and reported in mice, RNA-binding domain-defective and kinase-domain-defective models (Abraham et al., 1998; Baltzis et al., 2002; Yang et al., 1995). In this study, the kinase-domain-defective PKR-deficient mice have been used. Male Pkr+/mice and Pkr−/− mice in the 129Sv x BALB/C mixed background (Abraham et al., 1999) were kept on a 12 hr light/12 hr dark cycle and were placed on a HFD (D12492: 60% kcal% fat; Research Diets), beginning at 3 weeks of age ad libitum. After 6 and 14 weeks on HFD, GTTs were performed by intraperitoneal glucose injection (1.5 g/kg) following an overnight food withdrawal. After 16 weeks on HFD, ITTs were performed by intraperitoneal insulin injection (1 IU/kg) following 6 hr daytime food withdrawal. After 20 weeks, these mice were sacrificed and tissues were collected for further analysis. Total body fat mass was assessed by dual energy X-ray absorptiometry (DEXA; Piximus). For metabolic measurements, mice were placed in an indirect open circuit calorimeter (Columbus Instruments). Serum insulin, leptin, and adiponectin levels were measured with ELISA (Alpco). Liver triglycerides were determined with a colorimetric system (Sigma-Aldrich) adapted for microtiter plate assays (Furuhashi et al., 2007). Serum alanine aminotransferase level was measured with Piccolo-lipid panel plus (Abaxis).

**Hyperinsulinemnic-Euglycemic Clamp Studies**

Hyperinsulinemnic-euglycemic clamps were conducted as previously described (Furuhashi et al., 2007). For the lipid-induced acute insulin resistance mouse model, 6-month-old male Pkr+/mice and Pkr−/− mice were anesthetized and the right jugular vein was catheterized with a PE-10 polyethylene tube filled with heparin solution (100 U/ml) (United States Pharmacopeia). After a 3 day recovery, overnight-fasted mice were preinfused with lipid (5 ml/kg/hr; Intralipid; Baxter Healthcare Corporation) or saline for 3 hr followed by further infusion with HPLC purified [3H]-glucose (0.05 µCi/min; Perkin Elmer) for 2 hr basal period. The Intralipid we used contains 20% Soybean oil. The Soybean oil is a refined natural product consisting of a mixture of neutral triglycerides of predominantly linoleic, oleic, palmitic, linolenic, and stearic acids. After the basal period, a 120 min hyperinsulinemnic-euglycemic clamp was conducted with a primed-continuous infusion of human insulin (Novolin; Novo Nordisk) at a rate of 12.5 mU/kg/min. Blood samples were collected at 20 min intervals for the immediate measurement of plasma glucose concentration, and 25% glucose was infused at variable rates to maintain plasma glucose at basal concentrations. Insulin-stimulated whole-body glucose disposal was estimated with a continuous infusion of [3H]-glucose throughout the clamps (0.1 µCi/min). To estimate insulin-stimulated glucose uptake in individual tissues, 2-[3H]-deoxyglucose
Portal Vein Insulin Infusion and Protein Extraction from Tissues
Following 6 hr food withdrawal, mice were anesthetized and insulin (2 IU/kg) or PBS was injected into mice through the portal vein. Three minutes after injection, tissues were removed, frozen in liquid nitrogen, and kept at −80 °C until processing. For protein extraction, tissues were placed in a cold lysis buffer (25 mM Tris-HCl [pH 7.4], 1 mM EGTA, 1 mM EDTA, 10 mM Na4P2O7, 10 mM NaF, 2 mM Na3VO4, 1% NP-40, 1 mM PMSF, 1% protease inhibitor cocktail [Sigma-Aldrich]). After homogenization on ice, the tissue lysates were centrifuged, and the supernatants were used for western blot analysis.

MEF Culture and Analysis
Primary Pkr+/+ and Pkr−/− MEFs were used to assess phosphorylated JNK level or JNK activity under palmitic acid, TNF-α, thapsigargin, or polyinosino-polycytidylic acid stimulation. Both Pkr+/+ and Pkr−/− cell lines were established using the standard 3T3 immortalization protocol and used to assess phosphorylation level of IRS1 and effects of PKR reconstitution on insulin action. At 70%–80% confluency, cells were serum depleted for 3 hr or overnight prior to the stimuli. Sodium palmitate was dissolved in water at 65 °C and prepared as 20 mM solution. In cell culture experiments, the 20 mM palmitic acid preparation was diluted with 0.5% BSA containing DMEM to obtain the 0.5 mM palmitic acid concentration. Reagents and recombinant cytokines were gently added to the culture dishes in the incubator. For long-term treatment, the cells were incubated overnight with the viral supernatant, supplemented with 8 μg/ml polybrene. Expression vectors of adenovirus were constructed by cloning flag-tagged human PKR in adenovirus vector pAD/CMV/V5-DEST, and the viruses were produced as described in Viral vector adenovirus system (Invitrogen). Amplified-virus was used to infect primary Jnk1+/+ and Jnk1−/− MEFs.

Kinase Assays
Tissue or cell lysates containing 100–300 μg of protein were mixed with agarose-conjugated PKR antibody (Santa Cruz). The mixture was agitated at 4 °C, pelleted by centrifugation, and washed with lysis buffer followed by additional washes with PKR kinase buffer (15 mM HEPES [pH 7.4], 10 mM MgCl₂, 40 mM KCl, 2 mM DTT) for equilibration. The beads were incubated in kinase buffer containing 10 μCi 32P-γ-ATP (PerkinElmer) at 30 °C for 20 min followed by SDS-PAGE. To assess the phosphorylation of IRS1 by PKR, in vitro kinase assays were performed with anti-PKR immunoprecipitates from lysates of Pkr+/+ and Pkr−/− MEFs, which were treated by TNF-α or thapsigargin. The anti-PKR immunoprecipitates were mixed with IRS1, which was immunopurified with anti-IRS1 antibody (Upstate Biotechnology) from serum-starved WT-IMEFs. Further procedures are provided in the PKR Kinase Assay with Recombinant Protein section of the Extended Experimental Procedures.

Quantitative Real-Time PCR Analysis
Total RNA was isolated using Trizol reagent (Invitrogen). For reverse transcription, total RNA was converted to first strand cDNA using a high capacity cDNA reverse transcription system (Applied Biosystems). Quantitative real-time PCR analysis was performed using SYBR Green in a real-time PCR machine (7300 Real Time PCR system; Applied Biosystems). Primers are listed in Table S1. To normalize expression data of WAT and liver, 36B4 and GAPDH mRNAs were used as an internal control gene, respectively.

Statistical Analysis
The mean values for biochemical data from each group were compared by Student’s t test. Comparisons between multiple time points were analyzed using repeated-measures analysis of variance, ANOVA. In all tests, p < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.01.001.

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REFERENCES


Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

Materials
Anti-IRS1 and anti-phospho-IRS1 (Ser307) were from Upstate Biotechnology (Lake Placid, NY). Antibody against PKR, JNK1, Akt, phospho-Akt, insulin receptor α subunit, β-tubulin, Fatty acid synthase (FAS), and Actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-eIF2α (Ser52) antibody was purchased from Invitrogen (Carlsbad, CA). Anti-phospho-insulin receptor (Tyr1162/1163) was purchased from Calbiochem (Gibbstown, NJ). Anti-phospho-JNK (Thr183/Tyr185) antibody was purchased from Cell Signaling Technology (Danvers, MA). Recombinant IRS1, agarose-conjugated PKR, and anti-phospho PKR antibody were purchased from Millipore (Billerica, MA). Active PKR recombinant protein was purchased from SignalChem (Richmond, BC, Canada).

Adipocyte and Stromal-Vascular (SV) Fractionation
Epididymal fat pads were isolated from 11-week-old mice fed either RD or HFD for 8 weeks. Following mincing in albumin containing Krebs-Ringer phosphate (KRP) buffer, 1 mg/ml Collagenase II (Sigma) and 0.2 mg/ml DNase I (Sigma) were added and incubated at 37°C for 20 min on a shaking platform. The cell suspensions were filtrated through 250 μm nylon mesh to remove undigested tissues. Adipose tissues were then separated by their ability to float after low-speed centrifugation. To obtain total SV fractions, the cells below the adipocyte layer were collected and centrifuged. The pellets were washed in albumin containing KRP buffer.

Determination of Metabolic Parameters in Hyperinsulinemic-Euglycemic Clamp Study
For the determination of plasma 3H-glucose and 2-14C-DG concentrations, plasma was deproteinized with ZnSO4 and Ba(OH)2, dried to remove 3H2O, resuspended in water and counted in scintillation fluid for detection of 3H and 14C. The plasma concentration of 3H2O was determined by the difference between 3H counts with and without drying. Tissue 2-14C-DG-6-phosphate content was determined in homogenized samples that were subjected to an ion-exchange column to separate 2-14C-DG-6-phosphate from 2-14C-DG. Rates of basal hepatic glucose production and insulin-stimulated whole-body glucose uptake were determined as the ratio of the 3H-glucose infusion rate to the specific activity of plasma glucose at the end of the basal period and during the final 30 min of clamps, respectively. Hepatic glucose production during the hyperinsulinemic-euglycemic clamps was determined by subtracting the glucose infusion rate from the whole-body glucose uptake. Glucose uptake in individual tissues was calculated from the plasma 2-14C-DG profile, which was fitted with an exponential curve, and tissue 2-14C-DG-6-phosphate content.

PKR Pull-Down Assay
An agarose-conjugated PKR (Millipore) was mixed with recombinant full-length IRS1 protein (Millipore) in interaction buffer (5 mM Tris-HCl [pH 7.4], 25 mM KCl, 1 mM MgCl2, 0.25% Triton X) and then agitated at 4°C. After the agitation, the agarose-conjugated PKR was pelleted by centrifugation and washed with the interaction buffer followed by SDS-PAGE. One part of supernatant was kept to detect unbound IRS1.

PKR Kinase Assay with Recombinant Protein
For kinase assay with recombinant protein, full-length IRS1 (Millipore) was dephosphorylated by λ protein phosphatase (New England BioLabs) and used as a substrate. The kinase assay was performed with an active PKR (SignalChem) and the dephosphorylated IRS1 in kinase buffer (5 mM Tris-HCl [pH 7.4], 25 mM KCl, 1 mM MgCl2, 0.25% Triton X, 10 mM ATP, 10 μCi 32P-γ-ATP) followed by SDS-PAGE.

JNK Kinase Assay
For JNK kinase assay, tissue lysates containing 500 μg of protein were mixed with JNK1 antibody (Santa Cruz) and protein G sepharose beads. The mixture was agitated at 4°C, pelleted by centrifugation, and washed with the lysis buffer followed by additional washes with JNK kinase buffer (25 mM HEPES [pH 7.4], 20 mM MgCl2, 20 mM β-glycerophosphate, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na3VO4, 1 mM PMSF) for equilibration. The beads were incubated in kinase buffer containing 10 μCi 32P-γ-ATP and c-Jun protein at 30°C for 20 min followed by SDS-PAGE.
Figure S1. Related to Figure 1

(A and B) Expression of PKR and other interferon α/β target genes in adipocytes, stromal vascular (SV) fraction (A), and liver (B) of WT mice fed high-fat diet for 8 weeks. Adipocytes and SV fraction were isolated from white adipose tissues. Data are shown as the mean ± SEM. *p < 0.05.

(C and D) Regulation of PKR activity and expression in skeletal muscle. Genetic (ob/ob) (C) and dietary (D) mouse models of obesity were used to examine PKR activity by a kinase assay using immunopurified PKR and 32P-γATP in skeletal muscle with age- and sex-matched lean controls. β-tubulin protein is shown as controls. The graphs show the quantification of the data. *p < 0.05.

(E) Palmitic acid-induced PKR activation in Toll-like receptor 4 (TLR4)-deficient MEFs. Primary Tlr4+/+ and Tlr4−/− MEFs were treated with 0.5 mM palmitic acid for 90 min. Phosphorylation level of PKR was examined with anti-phospho-PKR (Thr451) antibody. PKR activity was assessed by autophosphorylation level of PKR using 32P-γATP.
Figure S2. Related to Figure 2

(A) Thapsigargin-induced PKR expression and activation in the presence of cycloheximide (CHX). Wild-type MFEs were pretreated with 20 μg/ml CHX for 30 min before addition of 100 nM thapsigargin for 1 hr. Cell lysates were analyzed by western blot analyses with antibodies as indicated. Phosphorylation level of PKR was examined with anti-phospho-PKR (Thr451) antibody.

(B) Polyinosinic-polycytidylic acid (polyI•polyC)-induced IRS1 phosphorylation in PKR-dependent manner. Induction of IRS1 phosphorylation after 100 μg/ml polyinosinic-polycytidylic acid (polyI•polyC) treatment for 2 hr in Pkr+/+ and Pkr−/− MEFs. Phosphorylation level of IRS1 on serine 307 was examined with anti-phospho-IRS1 antibody.

(C) Expression level of retrovirus-mediated Flag-tagged PKR in Pkr−/− MEFs. Flag-tagged PKR was introduced by retrovirus-mediated gene transfer in Pkr−/− MEFs. After puromycin selection, the cell lysates were analyzed by western blot analyses with antibodies as indicated.
Figure S3. Related to Figure 3
Association of IRS1 with PKR but not with PERK.
(A) Cell lysates were prepared from 300 nM thapsigargin-treated or nontreated MEFs for 3 hr followed by immunoprecipitation with anti-IRS1 antibody and western blot analyses with anti-PKR or anti-PERK antibodies.
(B and C) Cell lysates were prepared from 100 nM thapsigargin-treated or nontreated MEFs for 1 hr followed by immunoprecipitation with anti-IRS1 antibody and western blot analyses with anti-PKR or anti-PERK antibodies.
Figure S4. Related to Figure 4

(A–D) Glucose/insulin tolerance tests in Pkr+/+ and Pkr−/- mice on RD and HFD. Glucose tolerance tests were performed in Pkr+/+ (n = 5) and Pkr−/- mice (n = 6) on HFD (A) or RD (C) for 14 weeks. Insulin tolerance tests were performed in Pkr+/+ (n = 5) and Pkr−/- mice (n = 6) on HFD (B) or RD (D) for 16 weeks. Data are shown as the mean ± SEM. *p < 0.05.

(E–J) Metabolic studies in Pkr+/+ and Pkr−/- mice on HFD. Metabolic studies were performed in Pkr+/+ (n = 6) and Pkr−/- (n = 7) mice on HFD for 10 weeks. (E) Heat production, (F) Daily food intake, (G and H) Physical activity determined by automatic sensors on x (G) and z axes (H), (I and J) rates of oxygen consumption (I) and carbon dioxide production (J). Data are shown as the mean ± SEM.
Figure S5. Related to Figure 5

(A and B) PKR expression and activation in skeletal muscle in obese mouse models. (A) Insulin-stimulated IRβ tyrosine 1162/1163 and Akt serine 473 phosphorylation in skeletal muscle of Pkr+/+ and Pkr−/− mice on HFD. Tissue lysates were analyzed by western blot with antibodies as indicated. (B) JNK1 activity was detected by a kinase assay using immunopurified JNK1, 32P-γ-ATP, and recombinant c-Jun protein as substrate. β-tubulin is shown as a control.

(C) Gene expression in WAT including proinflammatory cytokine levels in Pkr+/+ and Pkr−/− mice on RD. Data are shown as the mean ± SEM.
Figure S6. Related to Figure 6
Apoptotic gene expression after lipid infusion in liver of Pkr+/+ and Pkr^−/− mice. Tissue lysates were analyzed by western blot analyses with antibodies as indicated. Tunicamycin (TM) treated liver sample was used as a control for induction of liver injury.