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REVIEW

Chronic alcohol consumption potentiates the development of diabetes through pancreatic β-cell dysfunction

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Abstract

Chronic ethanol consumption is well established as a major risk factor for type-2 diabetes (T2D), which is evidenced by impaired glucose metabolism and insulin resistance. However, the relationships between alcohol consumption and the development of T2D remain controversial. In particular, the direct effects of ethanol consumption on proliferation of pancreatic β-cell and the exact mechanisms associated with ethanol-mediated β-cell dysfunction and apoptosis remain elusive. Although alcoholism and alcohol consumption are prevalent and represent crucial public health problems worldwide, many people believe that low-to-moderate ethanol consumption may protect against T2D and cardiovascular diseases. However, the J- or U-shaped curves obtained from cross-sectional and large prospective studies have not fully explained the relationship between alcohol consumption and T2D. This review provides evidence for the harmful effects of chronic ethanol consumption on the progressive development of T2D, particularly with respect to pancreatic β-cell mass and function in association with insulin synthesis and secretion. This review also discusses a conceptual framework for how ethanol-produced peroxynitrite contributes to pancreatic β-cell dysfunction and metabolic syndrome.

Key words: Ethanol consumption; Type 2 diabetes; Pancreatic β-cells; Glucokinase; Metabolic syndrome

Core tip: Chronic ethanol consumption induces Glucokinase (GCK) downregulation and inactivation through Tyr nitration, resulting in pancreatic β-cell apoptosis and dysfunction. Additionally, GCK proteins nitrated following ethanol treatment may be more susceptible to ubiquitination and consequent degradation than are native proteins of control cells. Peroxynitrite-mediated GCK downregulation or inactivation may perturb glucose metabolism and cellular antioxidant defense mechanisms, increasing susceptibility to insulin resistance and type-2 diabetes. Furthermore, peroxynitrite-generated activating transcription factor 3 may play as a major upstream regulator of GCK

downregulation in β-cell dysfunction and apoptosis.

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INTRODUCTION

Chronic alcohol consumption is associated with the increase of mortality, liver injuries, pancreatitis, hypertension, cardiomyopathy, and cerebrovascular diseases[1,2]. Although alcohol abuse is considered as a detrimental risk factor for human public health, there are evidences that low-to-moderate alcohol intake may exhibit certain beneficial effects, particularly regarding cardiovascular events, insulin resistance, and type-2 diabetes (T2D) through an augmentation of glucose-stimulated insulin secretion (GSIS) and insulin sensitivity[3,4]. Recently, some study also suggested that moderate drinking of wine drinking could decrease the incidence of nonalcoholic fatty liver disease, which is closely related to metabolic diseases including insulin resistance and obesity[5,6]. Most of these suggestions are based on epidemiological and controlled clinical data showing a J- or U-shaped relationship between alcohol consumption and the development of T2D[6,7], but they have still not fully explained the relationship between ethanol consumption and T2D. Furthermore, the mechanisms involved in the amelioration of insulin sensitivity by modest alcohol consumption ameliorates insulin sensitivity are not clearly understood. Recent studies demonstrated that drinking alcohol was associated with a loss of body weight and an increase of adiponectin secretion[8-10]. The enhancing effects of alcohol consumption on the release of plasma adiponectin may be very useful for the prevention of metabolic syndrome[6,11-13], since it was well known that adiponectin has a role to improve insulin sensitivity and fatty acid oxidation in various tissues. However, a recent study suggested that the administration of moderate amounts of ethanol to people or rodents with metabolic disorders, such as obesity or impaired glucose metabolism, could induce a higher risk of complications than was observed in healthy people[14].

Since alcohol is primarily metabolized by the enzyme alcohol dehydrogenase (ADH) in hepatocytes, the major functional cells that make up 70%-85% of the liver's cytoplasmic mass, the liver is the most susceptible organ for alcohol-induced damages[15-17]. A number of molecular mechanisms have been associated with the development and progression of alcohol-induced liver disease (ALD), which are determined by direct toxic effects of ethanol and indirect effects *via* various tissues and organs such as brain, heart, lung, and the neutrophils and macrophages of the immune system[15,18,19]. There is no question that ALD is considered as one of the major causing factors for increasing of morbidity and mortality *via* hepatic complications worldwide[20,21]. The "two-hit" hypothesis that is contributed to the progression from nonalcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis (NASH) can also be reflected to the development of alcoholic fatty liver disease (AFLD) to alcoholic steatohepatitis (ASH)[20,22,23]. An individual who has primary insult (fatty liver) by alcohol exposure may be predisposed to secondary insults such as steatohepatitis, liver inflammation and cirrhosis. A major stimulus for excessive cytokine production seen in the progression of AFLD to ASH is gut-derived endotoxin, lipopolysaccharide (LPS), a complex molecule found in the cell wall of many bacteria. Another stimulus contributing to ASH development is the generation of reactive oxygen species (ROS), as a by-product of alcohol metabolism in the liver[24]. Elevated levels of ROS are closely related to the development of ASH and NASH, which are regulated by the peroxidation of lipids, proteins, or DNA and mitochondrial damage. In general, the generation of ROS as a consequence of alcohol metabolism are caused by the alteration of mitochondrial oxidative chain *via* two major enzymes, cytochrome P450 2E1 (CYP2E1) and catalase that can metabolize alcohol to acetaldehyde[20,25,26]. ASH and NASH have a similar pathogenesis and histopathology but a distinct etiology and epidemiology[27]. Although ASH and NASH represent advanced stages of AFLD and NAFLD, respectively, the conditions responsible for the progression of uncomplicated liver steatosis to ASH or NASH are presently unknown. AFLD/ASH and NAFLD/NASH are increasingly relevant public health issues because of their close association with the worldwide epidemic of diabetes and obesity. These chronic liver diseases are common and are expected to substantially affect healthcare expenditures in future years.

In fact, pancreatic β-cells have a good compensatory response for increased insulin demand during the progression to insulin resistance and hyperglycemia[28]. However, if the cells were consistently overloaded, pancreatic β-cells fail to produce insulin, resulting in pancreatic β-cell dysfunction and subsequently, predisposes to the development of T2D[28]. Evidence from humans and rodents suggests that this condition may occur in part because of a decreased β-cell mass through its apoptosis[29,30]. Several reports also suggested that chronic ethanol consumption induced insulin resistance in insulin-sensitive tissues including liver and skeletal muscle by interfering with insulin signaling and causing

pancreatic β-cell apoptosis[31-33]. These effects are related to mitochondrial dysfunction and oxidative stress induced by acetaldehyde, the highly reactive metabolite of ethanol. Furthermore, it was well known that insulin-producing β-cells are very susceptible to changes in ER homeostasis[28,34,35]. ER stress excessively increased in pancreatic β-cells was associated with lipid accumulation and ROS production, lead to β-cell apoptosis and eventually, diabetes[36]. Therefore, pancreatic β-cells may be sensitive to ethanol-induced ER stress and oxidative stress, which represent the earliest events in glucose intolerance and are associated with mitochondrial dysfunction[37]. This reasoning suggests that the association of alcoholism with T2D is based on β-cell damage caused by ethanol consumption, but it remains controversial whether ethanol consumption inhibits insulin secretion by β-cells. In addition, the mechanisms underlying ethanol-mediated pancreatic β-cell dysfunction and apoptosis are not fully defined.

In the present review, we will also discuss the relationship between chronic ethanol consumption and metabolic disorders and suggest underlying mechanisms with a special focus on alcoholic fatty liver and pancreatic β-cell dysfunction and apoptosis *via* glucokinase nitration and its downregulation. As well, this review proposes potential therapeutic strategies to combat metabolic syndrome associated with pancreatic β-cell dysfunction and apoptosis induced by chronic exposure to ethanol.

ALCOHOL CONSUMPTION AND LIVER DISEASES

Alcohol consumption is recognized as leading life style risk factors that are associated with the development of metabolic syndrome including T2D as well as mental disability[38]. In particular, it has been known for many years that chronic alcohol consumption causes the development of hepatic steatosis, alcoholic hepatitis, and ultimately cirrhosis[39,40]. The pathogenesis of liver injury resulting from acute or chronic ethanol consumption may be different depends on various etiological characteristics[41]. Many other cell types, ranging from the innate immune cells to the liver parenchymal cells, *i.e.*, hepatocytes are involved in alcohol-induced liver injury[42]. Alcohol dehydrogenease (ADH) is the major pathway involved in oxidative ethanol metabolism in the liver and produces acetaldehyde, a highly reactive toxic molecule. Acetaldehyde produced from alcohol oxidation is typically metabolized rapidly to acetate, which is catalyzed by aldehyde dehydrogenase (ALDH)[43,44]. Alcohol oxidation induced by ADH and ALDH is accompanied by the conversion of NAD^+ to the reduced NADH, increasing the cellular NADH/NAD^+ ratio[15,45]. Additionally, ethanol-inducible CYP2E1, one of cytochrome P450 isozymes, is also predominantly involved in ethanol oxidation to acetaldehyde in the liver[15,46]. As like previously described, CYP2E1-medaited alcohol metabolism highly produces ROS and lipid peroxidation products, which lead to tissue damage. Another enzyme catalase located in peroxisomes can also oxidize ethanol by hydrogen peroxide (H_2O_2)-dependent pathway; however, this is considered as a minor pathway for alcohol metabolism[18]. It was well known that the oxidation of alcohol and produced acetaldehyde induced the changes of NADH/NAD^+ ratio in reduction-oxidative state of both the cytosol and mitochondria of hepatocytes[47]. NADH generated in mitochondria is conversely oxidized to NAD^+ by the electron transport chain. Similarly, in the condition of chronic alcohol consumption, the alterations in a variety of metabolic pathways observed may be caused by the change of NADH/NAD^+ ratio in the redox state. In particular, the increases of NADH/NAD^+ ratio by chronic ethanol consumption are correlated with a loss of mitochondrial membrane potential through mitochondrial permeability transition (MPT) opening[47]. Moreover, several studies also demonstrated that NADH is a key reducing cofactor for reduction of glutathione and an essential cofactor for pro-oxidative enzymes that generate reactive oxygen and nitrogen species (ROS and RNS, respectively),which could alleviate oxidative stress[48,49]. In general, superoxide ($O_2^{\bullet-}$) radical was produced at mitochondrial respiratory chain of various cell types including hepatocytes and pancreatic β-cells[50-52]. Despite of an important role of $O_2^{\bullet-}$ in alcohol consumption-mediated liver injury, oxidative damage induced by alcohol consumption is not sufficiently explained by $O_2^{\bullet-}$ alone[53]. Indeed, reactive radical involved in the oxidative damage caused by alcohol consumption is hydroxyl free radical ($^{\bullet}OH$), which is dependently formed by $O_2^{\bullet-}$production; its formation is associated with damaging enzymes, membrane lipids, and nucleic acids[53-55].Unless the generated free radical is quickly disposed of, the lifetime of $O_2^{\bullet-}$generated in mitochondria respiratory chain was prolonged and subsequently, the increased $O_2^{\bullet-}$ is sufficient to lead toxic effects *via* the formation of more potent oxidant H_2O_2through the reduction of $O_2^{\bullet-}$ by using superoxide dismutase (SOD)[56,57]. And also, superoxide is formed to peroxynitrite ($ONOO^-$), a strong oxidizing and nitrating species, by reacting with $NO^{\bullet}$[53,58].

Kupffer cells considered as hepatic macrophages is believed to play an important role in early liver injury event by exposure of alcohol *via* the recognition of endotoxin/LPS in the portal circulation[59,60]. Of the many factors associated with the development of alcoholic liver diseases, endotoxin/LPS, which is derived *via* the activation of gram-negative bacteria in gut, functions a major role in the progressive regulation of hepatic steatosis, inflammation, and

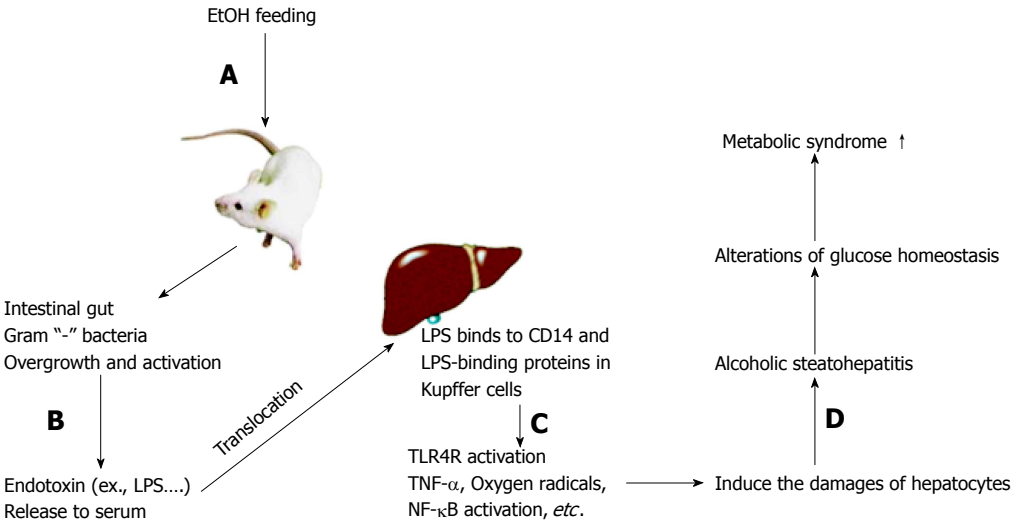

Figure 1 Proposed model by which ethanol feeding induces hepatocyte damage. A: Male 6-wk-old C57BL/6J mice (*n* = 8 in each group) were fed an ethanol diet for 8 wk. Individually caged mice were placed on a Lieber-Decarli regular liquid diet (Dyets; control diet: #710027, or ethanol diet: #710260). Mice were pair-fed with the control *vs* the 5% (v/v) ethanol diet for 8 wk; B: Alcohol intake induces the overgrowth and activation of intestinal gram-negative bacteria and subsequently increases the production of endotoxin (lipopolysaccharide, LPS) and its release into the blood; C: Released LPS activates toll-like receptor 4 (TLR4) on Kupffer cells, which are predominantly distributed in the lumen of hepatic sinusoids and exhibit endocytic activity against blood-borne materials entering the liver; D: Activated TLR4 drives Kupffer cells to produce inflammatory cytokines, chemokines, and reactive oxygen species *via* NF-kB activation, initiating an inflammatory cascade and hepatocyte damage.

fibrosis[61]. Recent experimental and clinical data also support that the stimulation of innate immune system is important to the progressive development of ALD[62]. The concept that the alter-regulation of innate immune response is a sufficient to induce ASH and NASH is supported by findings that alcohol consumption or high-fat diet intake increases the release of gut-derived endotoxin to the portal circulation, activating the hepatic immune Kupffer cells to produce pro-inflammatory cytokines, TNF-α and interleukin (IL)-6, and oxygen radicals[63-65].

There are several lines of evidence showing the role of gut-derived endotoxin on the development of both ASH and NASH (Figure 1). First, the intake of alcohol induces the damage the gastrointestinal barrier and subsequently diffuses the luminal bacterial products into the portal blood[66-68]. Second, acute and chronic alcohol feeding increased and/or activated intestinal gram-negative bacteria, subsequently elevating the production of endotoxin/LPS and its releasing into the blood. The production and release of endotoxin are observed in both human subjects and animal models of NAFLD/NASH. The serum levels of endotoxin released from alcohol-activated gram-negative bacteria were increased 5- to 20-fold in ASH patients, 3-fold in high-fat diet-fed healthy individuals, and 6- to 20-fold in NAFLD patients compared with normal subjects; the levels were also high compared with those of patients with non-alcoholic cirrhosis[63]. Third, LPS released from the gut microflora activates toll-like receptor 4 (TLR4) on Kupffer cells predominantly distributed in the lumen of hepatic sinusoids[69-71]. Fourth, activated TLR4 in Kupffer cells enhances the production of inflammatory cytokines, chemokines, and reactive oxygen species *via* NF-kB activation and increases the initiation of an inflammatory cascade and hepatocyte damage[72]. In general, oxidative stress induced by alcohol consumption causes the modification of hepatic proteins, which trigger immune reactions and consequent damage to hepatocytes. Actually, chronic ethanol consumption-mediated liver injury is concordantly associated with an increase in TNF-α, correlated with an increase in serum endotoxin levels[73]. In fact, macrophages release numerous cytokines when stimulated with endotoxin. The increased release of proinflammatory mediators (such as TNF-α, IL-1, IL-6, and ROS) and the infiltration of other inflammatory cells (such as neutrophils) eventually drive liver injury[66,74,75]. Accumulating evidence from experiments using knockout mice of these genes or their receptors suggests central roles for these inflammatory cytokines in the pathogenesis of ALD[76,77]. In fact, when monocytes and Kupffer cells are exposed to ethanol consumption, the expression and activities of endogenous signaling molecules and transcription factors are alternatively regulated[76,78]. Kupffer cells were activated by low concentrations of endotoxin (pg/mL to ng/mL), which is dependently regulated by CD14 receptor[66,79]. And also, when LPS binds to TLR4, the cooperation of LPS co-receptors, CD14and LPS-binding protein (LBP) are required to the interaction of LPS with TLR-4[59,79]. Since LBP, an acute phase reactant produced by hepatocytes, directly binds LPS and facilitates the association between LPS and CD14, low levels of endotoxin can be recognized by CD14 in the presence of LBP[59,80]. TLR4 deficiency prevented the development of both

ASH and NASH in association with the decrease inflammatory cytokines[63,81]. TLR4 can activate two distinct pathways, a MyD88-dependent and a MyD88-independent pathway[63,82]. Several studies using mouse models deficient in MyD88 or TLR4 supported that the pathogenic effect of TLR4 signaling in ASH is mediated *via* MyD88-independent pathway[63,82]. In a while, TLR4 signaling in the pathogenesis of NASH, including NF-kB activation and the upregulation of inflammatory cytokines is mediated by MyD88-dependent signaling[63,83-85]. In contrast to ASH, the development of NASH is closely associated with insulin resistance, which is correlated with endocrine interregulation between adipose tissue and the liver. Interestingly, the activation of TLR4 by MyD88-dependent pathway in macrophages was inhibited by adiponectin secreted from adipose tissue[63,82,86]. However, several studies show that there is no relationship between adiponectin and ASH. Despite of the differential regulation of adiponectin on both NAFLD/NASH and ASH, we cannot exclude the possibility that adiponectin may contribute to the pathogenesis of ASH and NASH by another signaling molecules or pathways.

To determine the responsiveness to endotoxins, including LPS, in ASH, we used LPS/D-galactosamine (GalN)- or ConA-induced hepatitis liver injury models showing the pathology similar to human liver disorders[87]. Liver histological evidences such as T cell infiltration, necrosis, and apoptosis, are rapidly induced within 8-24 h[88]. Despite of extensive studies, the exact molecular mechanisms involved in Con A- or LPS/GalN-induced hepatitis remain elusive. Several studies demonstrated that activated cytokines and their downstream signals, Janus kinase-signal transducer and activator of transcription factor (JAK-STAT), are important to T cell-mediated liver injury by regulating various cellular functions[89-93]. In general, binding of these cytokines enhanced the phosphorylation of the receptor-associated JAKs, which, in turn, activate the STATs, including STAT1, 2, 3, 4, 5, and 6[94]. Phosphorylated STATs form dimers and translocate into the nucleus to activate the transcription of many target genes, including the SOCS (suppressor of cytokine signaling) family of inhibitory proteins[95,96], which then turn off the JAK-STAT signaling pathway. Our previous data show that the infiltrating granulocytes, massive inflammation and necrosis observed in these hepatitis models are regulated by the IFN-γ-activated STAT1 pathway, which is correlated with T cell activation, as endotoxin-induced hepatitis liver injury is significantly abolished in STAT1$^{-/-}$ or IFN-γ$^{-/-}$ mice[97]. In addition, serum transaminase levels, hepatic inflammation, and necrosis increased in ethanol-fed mice are strongly potentiated by a low-dose injection of ConA[98].

ALD is induced by the direct toxic effects of alcohol and its metabolite, acetaldehyde, in various cell types in the liver[99]. In particular, alcoholic steatosis is considered as a key initial metabolic alteration in the progressive development of ALD. Lipid droplets in hepatocyte cytoplasm may inhibit cellular functions and/or make hepatocytes more susceptible to toxic or stress factors. The pathogenesis of alcoholic steatosis are caused by various mechanisms including the induction of fatty acid synthesis, inhibition of fatty acid oxidation, and reduction of very low-density lipoprotein (VLDL) secretion into the blood[100]. Furthermore, increasing evidence also suggests that adipose tissue dysfunction may impact hepatic lipid metabolism. Therefore, lipid homeostasis *via* interregulation of the liver-adipose tissue axis is important to regulate whole-body energy homeostasis[101,102]. White adipose tissue (WAT) is the major organ used to store excess energy for maintaining the status of energy homeostasis, whereas when the energy is required to other organs, WAT enhances the lipolysis and then produced free fatty acids (FFAs) is released to other organs[101-103]. When excess fatty acids produced by a fat storage function disordered in WAT are released and taken up by the liver, hepatic steatosis is easily developed[104]. Indeed, the mechanisms involved in hepatic fat accumulation *via* the influx of FFAs into the liver are well established in the "two-hit" model of non-alcoholic fatty liver disease (NAFLD) progression. Similarly, several clinical studies have demonstrated that alcoholic steatohepatitis (ASH) is also associated with the loss of fat mass and higher liver fat[105]. Ethanol consumption promotes the lipolysis of adipose tissues and the production of excess FFAs, followed by the accumulation of a large amount of fat droplets in the cytoplasm of hepatocytes, causing hepatic steatosis. These developments are also correlated with increases in lipogenesis and/or gluconeogenesis induced by elevated fatty acid synthase (FAS) and/or PEPCK expression, respectively.

Different from Western populations, Southeast Asian populations, including that of Korean, have many lean patients with T2D or metabolic disorders that may be caused by alcohol consumption[106]. Although ASH and NASH exhibits similar characteristics in histological pathology and progressive pattern to advanced liver disease[63], a number of comparable pathogenic mechanisms such as immune signaling and its pathological mechanisms in both ASH and NASH are still not clearly understood. Additional studies to divide the differences in the pathogenesis in ASH and NASH are required.

ALCOHOL CONSUMPTION AND PANCREATIC β-CELL DYSFUNCTION

Insulin resistance (IR) and pancreatic β-cell dysfunction are considered as two major independent-risk

factor for the onset of T2D[107,108], despite of some differences of these factors in each individual. The impaired glucose homeostasis and insulin insensitivity are generally used for the clinical diagnosis of T2D[109], but the pathophysiology underlying in peripheral organs such as muscle, liver, and adipose tissues are various in each individual. Therefore, to exactly identify which risk factors are involved in the development of each case of T2D patient is very important. Several previous reports have commonly suggested that IR was also closely associated with classic risk factors of T2D, including the enhancement of adiposity, hypertension, and hyperlipidemia[107,110]. A number of studies have also examined risk factors associated with β-cell dysfunction, but these studies rarely included multivariable adjustment. In the patients with T2D, IR increased in peripheral tissues with major metabolic functions, is accompanied with the reduction of insulin secretion induced by pancreatic β-cell dysfunction and apoptosis[111,112]. Furthermore, it is now well accepted that the failure of β-cells to compensate for insulin resistance is a prerequisite for T2D development. Although risk factors involved in the onset of T2D in individuals are differ according to various etiological phenomenons of IR and β-cell dysfunction[107,113], this discrepancy in IR and β-cell dysfunction may be caused by differing extents and durations of exposure to life style-related risk factors, such as high fat diet, alcohol consumption, smoking, and a lack of physical activity.

Indeed, previous studies suggested that chronic ethanol consumption is generally considered as a potent causing factor for chronic pancreatitis and is associated with 50%-70% of chronic pancreatitis patients[114]. However, chronic alcohol consumption alone does not cause chronic pancreatitis because a little heavy or chronic alcohol drinkers clinically develop to chronic pancreatitis[115,116]. The roles and involved mechanisms of alcohol consumption in the development of chronic pancreatitis are complex and poorly understood. Histological examination of pancreatic tissue from patients with chronic pancreatitis reveals inflammatory cells and extensive fibrosis. Although some studies have suggested that chronic alcoholics progress to chronic pancreatitis through the recruitment of inflammatory cells[117], present studies about the relationship between alcohol consumption and pancreatitis have not provide sufficiently evidence for explaining the roles and mechanisms involved in ethanol consumption-mediated pancreas damage, particularly, with respect to pancreatic β-cell dysfunction and apoptosis. The acute effects of ethanol consumption on insulin secretion by pancreatic β-cells remain controversial, but alcohol consumption is generally considered an independent risk factor for T2D[118,119]. Recently, chronic ethanol feeding in rodents was reported to cause pancreatic β-cell dysfunction and to decrease β-cell mass *via* apoptosis[34]. It is expected that the association of alcoholism with T2Dis based on this ethanol consumption-induced damage to β-cells, but the effects of ethanol consumption on β-cell dysfunction characterized by the inhibition of insulin secretion remain controversial, as both inhibition and enhancement of insulin secretion by ethanol consumption have been reported. Although acute ethanol consumption enhanced insulin secretion form pancreatic β-cells and thus increased insulin sensitivity in peripheral tissues, the exact regulatory mechanisms involved in these events are clearly unknown. These events may be an early defense mechanism to compensate for ethanol-inhibited basal insulin secretion. However, several studies demonstrated negative effects of ethanol on the function of β-cells, such as insulin synthesis and insulin secretion[120]. Excessive ROS production may be a mechanism involved in ethanol consumption-induced pancreatic β-cell dysfunction, thus triggering the development of T2D. Increased ROS may be one of the earliest events associated with mitochondrial dysfunction-mediated glucose intolerance, and may play an important sensitizer for the progressing of β-cell dysfunction and apoptosis[34]. Particularly, contrast to hepatocytes, pancreatic β-cells are characterized by their very low levels of the H_2O_2-inactivating enzymes catalase and glutathione peroxidase despite adequate expression of the superoxide ($O_2^{\bullet-}$)-inactivating SOD. Therefore, pancreatic β-cells have a high susceptibility to the toxicity of H_2O_2 and other produced ROS. As well, ROS produced *via* mitochondrial dysfunction are closely associated with increased endoplasmic reticulum (ER) stress. ER, localized in cellular cytoplasm, is responsible for the synthesis, packaging, and assembly of secretory and membrane proteins[34,121]. In particular, insulin-producing β-cells are very sensitive to ER stress *via* the changes of ER homeostasis since excessive ER stress remarkably inhibited insulin synthesis. ER stress is caused or characterized by the accumulation of unfolded response proteins and the increase of ER stress activators[122]. Excessive ER stress triggers lipid accumulation and ROS production in various cell types including β-cells and hepatocytes and, consequently, leads to diabetes mellitus *via* apoptotic cell death[34,123]. These conclusions have been supported by several intervention studies using genetic modifications or chemical chaperones[33]. The administration of moderate amounts of ethanol to rodents with metabolic disorders may increase the risk of complications in metabolic diseases or diabetes[124], as a result of the increased ER stress and ROS production with mitochondrial dysfunction. In addition, several studies demonstrated that the reduction in insulin content caused by ethanol was associated with reduced insulin synthesis, which has been attributed to ER stress. Despite convincing

evidence that chronic ethanol consumption acts as a risk factor for T2D[125,126], the direct effects of ethanol on the β-cell mass and its function and the exact mechanisms involved in β-cell dysfunction and apoptosis by chronic ethanol consumption are little known. Furthermore, as the pleiotropic effects of ethanol could be mediated by altered gene expression or epigenetic modifications[127,128], a comprehensive assessment about the impacts of ethanol on the expression or modification of genes related to the regulation of β-cell function should be considered. As well, we could not exclude the physiological roles of uncoupling protein 2 (UCP2), an isoform expressed in insulin-secreting cells, on the impairment of β-cell function (GSIS) and apoptosis by chronic ethanol consumption[129]. UCP2 exerts as a mitochondrial anion carrier protein and as antioxidant to reduce the generation of mitochondrial ROS by uncoupling of the proton motive force, possible tuning of redox homeostasis[130,131]. It has been reported that a β-cell-specific knock out of UCP2 was associated with increased mitochondrial membrane potential, ATP content and, subsequently, with elevated glucose-induced insulin secretion[132]. Conversely, UCP2 overepxression strongly attenuated GSIS, thereby indicating that uncoupling of the proton influx by UCP2 has a negative effect on insulin secretory responsiveness. As well, pancreatic β-cells isolated from UCP2 knock-out mice had chronically higher ROS compared to wild type mice[131,132]. Although the relationship between ethanol consumption and physiological roles of UCP2 did not clearly understood, several studies suggested that the impairment of redox homeostasis and energy metabolism induced by chronic ethanol consumption may be caused by the alteration of uncoupling mechanisms by UCP2, thereby results in pancreatic β-cell dysfunction and apoptosis[133].

CHRONIC ETHANOL CONSUMPTION AND THE DOWNREGULATION OF GLUCOKINASE

Glucokinase (GCK) plays an important role in the maintenance of blood glucose homeostasis, acting both as a metabolic sensor for GSIS in pancreatic β-cells and as the major regulator of glucose uptake in hepatocytes[134,135]. Previously, we demonstrated that chronic heperglycemia-induced β-cell dysfunction and apoptosis were closely related to GCK downregulation, which resulted in ROS production and AMPK hyperactivation[134,136]. However, the relevant upstream regulators of GCK downregulation remain elusive. One of major transcription factor essential to the maintenance of β-cell function is pancreatic duodenal homeobox-1 (PDX-1), which regulates the expression of genes such as insulin, islet amyloid polypeptide, GCK, and Glut2, which are[137]. Another transcription factor that regulates β-cell function is sterol regulatory element-binding protein-1c (SREBP-1c) and it binds directly to the SRE elements of GCK promoter, increasing GCK expression and enhancing the action of insulin on GCK transcription[137,138]. Recently, although the precise mechanisms remain unclear, we reported that lipotoxicity promoted the activity of activating transcription factor (ATF)-3, a member of the ATF/cAMP-responsive element binding protein subfamily, which was also associated with the inhibition of PDX-1-induced GCK promoter activity[139-141]. *ATF3*is an early-responsive gene to a variety of stresses and functions as a stress-inducible transcriptional repressor or activator[142,143]. Given its frequent induction by various cellular stressors, ectopic expression of ATF3 in heart, liver, and pancreatic β-cells causes cardiac enlargement, liver or pancreatic β-cell dysfunction and apoptosis, and impaired glucose metabolism and diabetes, and is associated with the induction of signals involved in pancreatic β-cell dysfunction, such as proinflammatory cytokines, nitric oxide, and high concentrations of glucose and free fatty acids[34,144]. Indeed, ethanol-induced oxidative stress may be associated with the induction of ATF3.

In addition, we suggested that ATF3 may play a critical role in ethanol-induced susceptibility to β-cell dysfunction and apoptosis, as well as hepatotoxicity, which can be triggered by an enhancement in peroxynitrite generation[141,145]. Peroxynitrite can stably react with cysteine and tyrosine residues on biological target proteins[146,147]. Similarly, NO generated by iNOS induction can directly induce the modification of target proteins through S-nitrosylation and nitration on cysteine and Tyr residues, respectively[148]. These modifications can also be associated with the changes of pathophysiological processes in organ tissues, and thus develops to diseases. A recent study demonstrated that insulin-induced neuronal nitric oxide synthase (nNOS) leads to S-nitrosylation of GCK, which helps to prevent the impaired glucose response and apoptosis of pancreatic β-cells[149]. However, other studies have demonstrated that peroxynitrite generated by cytokines or endotoxins can also induce impaired glucose homeostasis and pancreatic β-cell dysfunction and apoptosis[150,151].

Although the deleterious effects of chronic heavy alcohol consumption on the development of impaired fasting glucose or T2D are well known, the exact molecular mechanisms involved in chronic ethanol consumption-induced impaired glucose homeostasis and pancreatic β-cell dysfunction and apoptosis remain unclear. Recently, we demonstrated that chronic ethanol consumption-mediated pancreatic β-cell dysfunction and apoptosis are correlatively associated with peroxynitrite-mediated GCK down-

regulation and ATF3 induction[34]. As shown in Figure 2, pancreatic β-cell dysfunction and apoptosis were remarkably increased in chronic ethanol-fed mice. These processes were correlated with reductions in β-cell mass and insulin expression. Additionally, ethanol consumption inhibits the production of ATP and the synthesis or secretion of insulin in pancreatic β-cells. In contrast to previous studies showing that ethanol consumption is related to a decreased risk of T2D and cardiovascular disease, our data show that chronic ethanol consumption for 8 or 10 wk significantly impaired tolerance to both glucose and insulin, potentially as a result of reduced insulin synthesis or secretion *via* pancreatic β-cell dysfunction and apoptosis. However, we have also shown that the reduction in insulin synthesis detected in the β-cells isolated from ethanol-fed mice might be associated with the inhibition of insulin action and gluconeogenesis in the liver, correlated with hepatocellular damage caused by elevated plasma levels of alanine aminotransferase (ALT). These results suggest that the reduced insulin action upon glucose disposal in ethanol-fed mice was due to the reduction in insulin synthesis or secretion in pancreatic β-cells. Consistent with these results, Ser-307 phosphorylation of insulin receptor substrate 1 (IRS1) (inactive form) was strongly increased in the livers of ethanol-treated mice, whereas the activating Tyr-941 phosphorylation of IRS1 and Akt phosphorylation were significantly suppressed under the same conditions, suggesting reduced insulin action in the liver. Also, the increase of impaired glucose tolerance and the reduction of insulin sensitivity in peripheral tissues of ethanol-fed mice were strongly correlated with pancreatic β-cell apoptosis, characterized by increased levels of caspase-3 or PARP cleavage and the Bax/Bcl-2 ratio. The impaired glucose tolerance and pancreatic β-cell dysfunction of ethanol-fed mice are correlated with reductions in GCK protein expression and in those of glucose transporter GLUT2 and insulin. In contrast, the stress-inducible transcription factor ATF3 was significantly increased in pancreatic tissues and islet cells of ethanol-fed mice. These events were confirmed *in vitro* using the MIN6N8 pancreatic insulinoma cell line. In MIN6N8 cells, ethanol treatment strongly increased GCK expression at an early time point (6 h); expression remained high at 12 h but decreased thereafter, reaching baseline levels after 24 h, whereas ATF3 was significantly increased and predominantly localized in the nucleus. The early enhancement of GCK expression (6-12 h) following ethanol treatment might help to explain previous studies demonstrating that light-to-moderate ethanol consumption is associated with a lower risk of T2D. Indeed, several previous clinical studies demonstrated that low-to-moderate alcohol intake in obese participants or those with T2D was associated with low fasting glucose levels, which might subsequently lead to a diagnosis of non-T2D[6,8,10,151]. However, participants exposed to heavy alcohol consumption were more vulnerable to the development of impaired fasting glucose or T2D.

One explanation for these discrepancies might be associated with the amount and duration of ethanol exposure. Indeed, our studies show that chronic alcohol consumption for 8 or 10 wk significantly increased the development of impaired fasting glucose and β-cell apoptosis, followed by reductions in insulin and ATP production[34]. These results suggest that chronic ethanol consumption can completely abolish the early defense mechanism observed at an early time point of ethanol exposure in pancreatic β-cells and thus reduced the synthesis or secretion of insulin. Recently, we demonstrated that ATF3 inhibits the function of PDX-1 and decreases the synthesis of insulin in pancreatic β-cells, subsequently, leads to pancreatic β-cell dysfunction and apoptosis[152]. Similarly, our previous studies have also found that ATF3, especially, its C-terminal domain, is a potent upstream regulator for GCK downregulation and apoptosis induced by ethanol exposure in pancreatic β-cells[34]. Although the exact regulatory factors involved in the ethanol-induced susceptibility to pancreatic β-cell dysfunction and apoptosis remain clearly unknown, a number of evidences indicate that oxidative stress induced by ethanol exposure is a key cellular process to the development of organic toxicity, which can be triggered by enhancing peroxynitrite generation. Based on this knowledge, ethanol-induced ATF3 can be regulated by oxidative stress generated through CYP2E1 metabolism and may act as a critical regulator of the GCK downregulation induced by ethanol-generated oxidative stress in pancreatic β-cells. Several lines of evidence suggest that both ethanol-mediated GCK downregulation and ATF3 induction require ethanol metabolism (Figure 2). First, blocking ethanol metabolism with 4-MP attenuated the ethanol-induced reductions in GCK, insulin, and PDX-1 expression and the increase in ATF3. However, various effects of 4-MP on GCK downregulation were able to be mediated by ethanol-induced endogenous ATF3 or exogenously overexpressed ATF3 because 4-MP did not inhibit transfected ATF3 and/or ethanol-induced GCK downregulation. Second, acetaldehyde-induced GCK downregulation and β-cell apoptosis were inhibited by ATF3 depletion but not by 4-MP, suggesting that ATF3 may be a downstream regulator of ethanol metabolism and serve as an executive effecter of ethanol-mediated GCK downregulation. Our data also show that peroxynitrite-mediated GCK downregulation and apoptosis were attenuated by the peroxynitrite scavengers L-NMMA and uric acid (UA). In contrast to the potent effects of ATF3 on ethanol-induced GCK downregulation, ATF3 did not affect

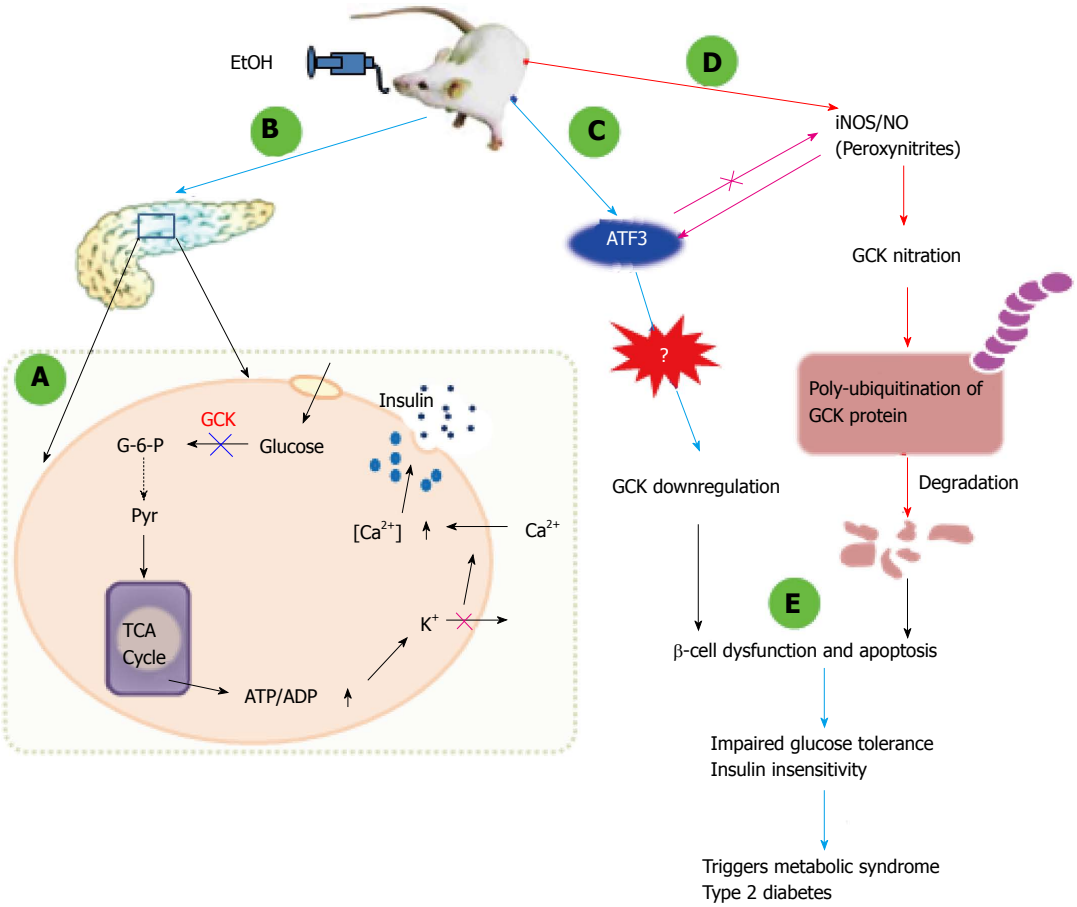

Figure 2 Proposed model by which peroxynitrite-mediated activating transcription factor 3 and glucokinase nitration in ethanol-fed mice trigger metabolic syndrome through pancreatic β-cell dysfunction and apoptosis. A: Under normal conditions, when glucose is taken up by pancreatic β-cells, it is converted to glucose-6-phosphate by glucokinase. Pyruvate transferred into the mitochondria activates the TCA cycle, which produces ATP. Increased ATP/ADP ratios prevent potassium efflux *via* inhibiting potassium channels and opening calcium channels. Increased intracellular calcium levels activate insulin maturation and its secretion; B: In mice fed with a 5% (v/v) ethanol diet for 8 wk, glucokinase (GCK) protein expression was significantly decreased in pancreatic tissues and isolated islet cells; C: Ethanol-fed mice showed high expression of activating transcription factor (ATF) 3. ATF3 is regulated by the ethanol metabolism pathway because ethanol-induced ATF3 was significantly decreased by 4-methylpyrazole, an inhibitor of cytochrome P450 2E1. ATF3 expression is regulated by the peroxynitrite-dependent pathway, but ATF3 did not affect the ethanol-mediated production of peroxynitrite. Despite this discrepancy, the C-terminal domain of ATF3 is involved in the downregulation of GCK. However, the role of ATF3 and the exact regulatory mechanisms involved in GCK downregulation are still not fully understood; D: Ethanol-produced peroxynitrite significantly increased GCK nitration on tyrosine residues; this effect was abolished by an iNOS inhibitor, L-NMMA, or the peroxynitrite scavengers, uric acid and deferoxamine. Interestingly, the nitrated GCK was more susceptible to ubiquitination than were the native proteins of control cells, making it vulnerable to degradation; E: Cells with downregulated GCK expression lack the ability to synthesize insulin and eventually progress to apoptosis. Mice lacking the ability to produce insulin may easily develop metabolic syndrome with impaired glucose tolerance and insulin insensitivity, resulting in type 2 diabetes.

ethanol-mediated iNOS or NO production, indicating that ATF3 may be a downstream regulator of the ethanol metabolism-mediated iNOS/NO pathway, which associates GCK downregulation with β-cell apoptosis. Mice lacking hepatic glucokinase show typical signs of maturity-onset type 2 diabetes of the young (MODY2), which results from impaired glucose tolerance caused by the failure of glucokinase activity to facilitate hepatic glucose utilization and glycogen synthesis[135,153]. We have demonstrated that ethanol-generated peroxynitrite can directly regulate GCK levels and activity. Similar to NO generated by iNOS, exogenously administrated NO can also directly change the structure of target proteins *via* nitration and S-nitrosylation on Tyr and cysteine residues, respectively, thereby altering pathophysiological processes[154-156]. Several previous studies about the regulation of GCK activity have been focused to the role of protein interactions with the GCK regulator (GCKR) or glucose[48,157]. However, the precise control mechanisms involved in the regulation of cellular GCK activity or its expression at the posttranslational or posttranscriptional level, especially in β-cells, still remain unclear. In pancreatic β-cells, insulin was recently shown to activate neuronal-type nitric oxide synthase (nNOS), which forms a complex with GCK on the surface of secretory granules, leading to the S-nitrosylation of GCK following the dissociation of GCK from granules[158]. Furthermore, several previous studies have demonstrated that NO and endothelial nitric oxide synthase (eNOS) generated by insulin-like growth factor-1 (IGF-1) and insulin prevent serum starvation-induced RINm5F β-cell apoptosis[159]. However, in contrast to the protective

effect of insulin or IGF-1-induced iNOS and NO on GCK S-nitrosylation, which prevents pancreatic β-cell dysfunction, peroxynitrite generated through various stresses, such as cytokines or endotoxins, also serves as a vital mechanism that causes impaired glucose metabolism and apoptosis in rat, mouse, and human pancreatic β-cells[160]. Similar to previous reports showing that peroxynitrite can directly damage proteins, DNA, and lipids[161], our study also shows that ethanol metabolism-generated peroxynitrite increases Tyr nitration of GCK, which is correlated with GCK downregulation. Similarly, recombinant GST-GCK was effectively nitrated on its Tyr residues after incubation with peroxynitrite. However, ATF3 did not directly affect GCK nitration, although ATF3 plays a critical role in regulating GCK expression. Ethanol-induced GCK nitration and iNOS induction were not altered by C-terminal domain-deleted ATF3(ΔC) or ATF3 siRNA. In contrast, ATF3 strongly inhibited GCK nitration in ethanol-cotreated cells even though ethanol-induced iNOS expression was not affected by ATF3, possibly due to the nearly complete reduction in GCK protein expression elicited by the combination of ethanol and ATF3. Previous studies have demonstrated that nitration modification is associated with the functional loss of target proteins due to the inhibition of Tyr residues important to the activity of protein[161,162]. The activity of GCK in converting glucose to glucose-6-phosphate was also inhibited by ethanol and SIN-1, but reversibly restored by the peroxynitrite scavengers L-NMMA, UA, and DFO. However, the GCK inactivation induced by ethanol or SIN-1 was not implicated in the S-nitrosylation of GCK because DTT, an S-nitrosylation inhibitor, had little effect on the inhibition of GCK activity induced by ethanol or peroxynitrite. In addition, the interaction of immunoprecipitated GCK with pBad and the GCK translocation into the mitochondrial fraction were determined by peroxynitrite-mediated GCK nitration.

Protein nitration can also induce the functional loss of target proteins *via* rapid degradation[48,161,162]. Our data also show that the ethanol-mediated nitrated GCK may be more susceptible to ubiquitination than are the native proteins of control cells, resulting in degradation[34]. The levels of GCK nitration and ubiquitination were concurrently increased in ethanol-treated cells and correlated with the reduced GCK protein levels, suggesting that nitrated proteins can be more easily ubiquitinated and degraded. Our data also show that GCK downregulation and the reduction of its activity by ethanol exposure are predominantly regulated by the modification *via* tyrosine nitration. However, the exact mechanism involved in the GCK modification on Tyr residues by ethanol-generated peroxynitrite remains unclear. Further studies involving ESI-MA are needed to confirm which Tyr residues are involved in the peroxynitrite-induced GCK downregulation and inactivation. In addition, because antioxidant enzymes are sensitive to the oxidative toxic effects of peroxynitrite[150], the inactivation of cellular antioxidant defense enzymes observed in ethanol-treated mice or cells is caused by excessive production of peroxynitrite, subsequently, potentiates the harmful cellular or organ defects. Indeed, our data showed that peroxynitrite-mediated GCK downregulation was reversely improved by the treatment of antioxidants such as NAC and L-NMMA. Additionally, excessive ROS is involved in the induction of protein damage by enhancing the proteolytic susceptibility to unfolded proteins and increasing the accessibility of proteases to the peptide bonds of proteins[162]. Previously, we have demonstrated that chronic exposure to high glucose significantly reduces GCK translocation to the mitochondrial membrane, accompanied with the reduction of GCK protein expression, and then conversely, increases the interaction of Bax with a voltage-dependent anion chain (VDAC) located in mitochondrial membrane, resulting in β-cell apoptosis[136], which were correlated with the induction of oxidative stress and mitochondrial dysfunction[134]. Thus, it is possible that GCK inactivation by peroxynitrite-mediated nitration is, partially associated with the inhibition of cellular reduction-oxidation status and the increase of oxidative damage observed during ethanol consumption. In addition, despite the lack of an effect of ATF3 on ethanol-mediated GCK nitration at the posttranslational level *via* the induction of iNOS and NO production, ATF3 may still play as a key regulator in GCK downregulation and pancreatic β-cell dysfunction and apoptosis. Therefore, we are now examining whether ATF3 plays a critical role in the transcriptional regulation of GCK. We have identified five potential binding sites for ATF/CREB in the GCK promoter using the TRANSFAC transcription factor binding database. Moreover, we have found that ATF3 can directly interact with specific binding sites in the 5′-flanking region of the GCK promoter, and then confirmed these interactions using mutated versions of the constructs.

CONCLUSION

Increasing evidence supports the idea that chronic alcohol consumption may trigger the progression or development of type 2 diabetes through impaired glucose metabolism and pancreatic β-cell dysfunction and apoptosis. Moreover, low-to-moderate alcohol consumption can also induce structural changes in the GCK protein *via* tyrosine nitration; reducing the activity of the nitrated enzyme. Here, we show for the first time that chronic ethanol consumption induces GCK downregulation and inactivation *via* the nitration at the Tyr residues of GCK, followed by pancreatic β-cell dysfunction and apoptosis. Our results strongly suggest that GCK proteins are nitrated following ethanol treatment, making

them more susceptible to ubiquitination (compared with the native proteins of control cells) and thus vulnerable to degradation. Peroxynitrite-mediated GCK downregulation or inactivation may also perturb glucose metabolism and cellular antioxidant defense mechanisms, increasing susceptibility to insulin resistance and T2D. Furthermore, peroxynitrite-generated ATF3 may play as a potent upstream regulator of GCK downregulation and β-cell dysfunction and apoptosis.

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